Sustainable worm control with improved understanding, methods and information availability

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A report for Australian Eggs Limited

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Foreword

This project was conducted to address the increasing importance of gastrointestinal worm infection in chickens under free range management systems. Specific objectives were to:

- Identify the current prevalence and magnitude of infection with key worm species in the free range sector of the layer industry, their perceived importance and the current methods used to control them.
- Develop improved methods for diagnosing flock infection levels.
- Evaluate the level of anthelmintic resistance in Australian isolates of key worm species.
- Optimise methods for maintaining parasite worm eggs for R&D and develop challenge protocols to facilitate critical experimentation.
- Develop and maintain a live collection of Australian worm isolates of known anthelmintic resistance status to be made available to other researchers or industry.
- Develop an online portal for integrated worm management in poultry.

This project was funded from industry revenue, which is matched by funds provided by the Australian Government. It was also supported by funding provided by Invetus Pty Ltd and the University of New England in the form of support for project staff and students assisting with project implementation.

This report is an addition to Australian Eggs Limited's range of peer reviewed research publications and an output of our R&D program, which aims to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

Most of our publications are available for viewing or downloading through our website:

www.australianeggs.org.au

Printed copies of this report are available for a nominal postage and handling fee and can be requested by phoning (02) 9409 6999 or emailing research@australianeggs.org.au.

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We are very grateful to the egg producers who gave their valuable time to answer our survey and particularly those who provided access to their flocks for the intestinal worm prevalence study.

Australian Eggs Limited provided the operating funds that supported this project while the University of New England and Invetus Pty Ltd provided the academic researcher staffing costs. Two postgraduate students at UNE, Teka Feyera Dewo and Anwar Shifaw Yesuf provided major support for the project and in turn were supported by UNE, which provided IPRA postgraduate scholarships.

About the Authors

Prof. Steve Walkden-Brown is a veterinarian and Professor in Animal Science at the University of New England. He has a strong research interest in managing gastrointestinal nematode infections in ruminants, while on the poultry side his main research interest to date has been in the diagnosis and control of endemic viral diseases in broilers. Steve provided overall direction and management of the project, and is principal supervisor of Teka Feyera.

Teka Feyera Dewo is a veterinarian and academic at the College of Veterinary Medicine, Jigjiga University, Ethiopia who is currently undertaking a PhD at UNE under the supervision of the academic team on this project. Teka has played a leading role in the design and implementation of most of the studies in this project, including the online farmer survey, studies on the maintenance and amplification of nematode stocks, and the anthelmintic resistance studies.

Anwar Shifaw Yesuf is a veterinarian from the University of Gondar, Ethiopia who is currently undertaking a M.Rur.Sci. at UNE under the supervision of the academic team on this project. Anwar has played a leading role in the design and implementation of the field prevalence studies, diagnostic methods comparison and a large experiment on optimising methods for *in vitro* maintenance of *Ascaridia galli* stocks.

Dr Brendan Sharpe is a research veterinarian for Invetus Ltd a veterinary health research service provider. Brendan has a long history of interest and involvement in poultry including a PhD at UNE on disease investigation and control in broiler chickens and a stint with Baiada Poultry as a field veterinarian in Tamworth. Brendan was central to the development of the project and took the leading role in development of the web site resource by the project and in the design of the anthelmintic resistance studies.

Dr Isabelle Ruhnke is a veterinarian and Senior Lecturer in Animal Science at the University of New England. She has a background in poultry nutrition with a strong research interest in free range production systems and their optimisation. Isabelle is the principal supervisor of Anwar Yesuf and took a leading role in developing the online survey and organising the field prevalence and methods comparisons studies. She was also a major contributor to the web site materials.

Dr Tim Elliott is a parasitologist who was formerly with Invetus Pty Ltd but has moved to the University of New England where he is currently the operations manager at the Centre for Animal Research and Teaching. Tim has contributed to all aspects of the project, with important roles in the methods comparison work and the anthelmintic resistance testing.

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Abbreviations

AEC	Animal ethics committee
AEL	Australian Eggs Limited
AM	Arithmetic mean
ANOVA	Analysis of Variance
APVMA	Australian Pesticides and Veterinary Medicines Authority
AR	Anthelmintic resistance
BZ	Benzimidazole
CART	Centre for Animal Research and Teaching
CI	Confidence interval
CL	Confidence level
CRC	Cooperative Research Centre
CVMP	Committee for veterinary medicinal products
CY	Cyclophosphamide
DEC	Day of egg collection
Dex	Dexamethasone
DMSO	Dimethyl sulfoxide
DO	Day-old
EC	Effective concentration
ECS	Excreta consistency score
EE	Extraction efficiency
EEC	Excreta egg count
EECR	Excreta egg count reduction
EHA	Egg hatch assay
EPG	Eggs per gram
EU	European Union
FBZ	Fenbendazole
FEC	Faecal egg count
FECR	Faecal egg count reduction
FECRT	Faecal egg count reduction test
FLBZ	Flubendazole
FS	Flotation solution
GABA	Gamma-aminobutyric acid
GIT	Gastrointestinal tract
GM	Geometric mean
h	Hour
ha	Hectare
HBSS	Hanks' Buffered Salt Solution
IP	Incubation period
IPRA	International Postgraduate Research Award
IS	Isolate
LCL	Lower confidence level
LDT	Larval development test
LEV	Levamisole
LHA	Larval hatching assay

LMIA	Larval migration inhibition assay
LMIT	Larval migration inhibition test
LSM	Least-squares means
m	Metre
MF	Mini-flotac
MM	Modified McMaster
mm	Millimetre
NA	Not applicable
PBS	Phosphate-buffered saline
PIP	Piperazine
R&D	Research and development
RPMI	Roswell Park Memorial Institute
SD	Standard Deviation
SEM	Standard error of the mean
SG	Specific gravity
SP	Storage period
TBZ	Thiabendazole
UCL	Upper confidence level
UNE	University of New England
WAAVP	World Association for the Advancement of Veterinary Parasitology
WC	Worm count
WCR	Worm count reduction
WCRT	Worm count reduction test
WPI	Weeks post-infection

Executive Summary

Free range layers have an increased exposure to gastrointestinal worm infections due to the management system, and incidences of high prevalence and worm burdens have been observed in free range flocks by project team members and industry personnel. The registered medicants/anthelmintics available to control infection at the outset of this project were many decades old, and their efficacy had not been recently evaluated in Australia. The methods available for researching nematodes and tapeworms in poultry lag far behind those of other species such as ruminant livestock.

This project was therefore established to: (a) identify the current prevalence and magnitude of infection with key worm species in the free range sector of the layer industry, their perceived importance and the current methods used to control them; (b) develop improved methods for diagnosing flock infection levels; (c) evaluate the level of anthelmintic resistance in Australian isolates of key worm species; (d) optimise methods for maintaining parasite worm egg stocks and develop challenge protocols to facilitate critical experimentation; (e) develop and maintain a live collection of Australian worm (egg) isolates of known anthelmintic resistance status to be made available to other researchers or industry; and (f) develop an online portal for information on worms and worm management in poultry.

To achieve these objectives the project initially undertook an online survey of free range egg producers to determine their attitudes to worms and worm control methods. With some delays due to COVID-19 this was followed up by evaluating the prevalence and worm burden of different worm species on five free range layer farms by slaughter and worm count of approximately 100 birds per flock. Worms from these farms were then tested to see if they had developed resistance to the anthelmintics currently being used to control worms in Australia. Towards the end of the project, faecal samples (10 intestinal and 10 caecal droppings per flock) from 16 flocks of different ages were submitted for faecal egg count. Between these activities, many experiments were conducted to investigate the best methods for counting eggs in chicken faeces, sampling strategies for diagnosis of worm burdens, preserving worm eggs for use in further testing, optimising infection protocols in chickens and the potential to conduct anthelmintic resistance tests in the laboratory, rather than in chickens. The team also worked to prepare content for web pages on worms and worm control in layer chickens.

The online survey revealed that worm infection was common, but only of moderate concern to free range farmers. There was greater concern about tapeworms than the roundworm nematode species. Most producers regularly monitored and treated for worm infection. The on-farm prevalence survey revealed a high prevalence of *A. galli* and *H. gallinarum* and moderate prevalence of *Capillaria* spp. on four of five farms and high tapeworm burdens on two of five farms. Of the 16 flocks submitting intestinal and caecal droppings, 100% had putatively *A. galli* eggs in intestinal droppings with a mean within-flock prevalence of 71% and mean FEC of 440 EPG. Similarly, 100% of flocks exhibited *Capillaria* spp. infection with mean prevalence of 37.3% and FEC of 18 EPG. In the caecal droppings 15 of the 16 flocks had eggs putatively of *H. gallinarum* with mean prevalence of 78% and FEC of 404 EPG.

No resistance to levamisole, piperazine, fenbendazole or flubendazole was detected in worm isolates from the on-farm prevalence study. For some medications, mass application in water reduced effectiveness relative to individual bird dosing. Piperazine only demonstrated adequate efficacy against adult large roundworm, and gave inadequate control of other nematodes or immature stages of infection. In the one study when a fenbendazole product not registered for use in poultry was administered in drinking water, efficacy was reduced below 90%. Care should be taken when

administering these non-water-soluble (suspension) medications in the water system. The new in-feed formulation of Flubendazole was highly effective against nematodes and tapeworms.

With regard to diagnostic methods, the traditional modified McMaster faecal egg counting method (Whitlock 1948) was found to be superior to the newer MiniFlotac method (Cringoli et al. 2013) for routine examination of faeces for chicken eggs and submission of fresh faeces from farms was found to be the most suitable diagnostic sample for simple and cost-effective evaluation of worm burdens. Methods to enable storage of worm eggs for up to 40 weeks were developed but attempts to freeze eggs for longer term storage were not successful. A chick infection model for efficient multiplication of worms was developed, as were strategies to most efficiently multiply stocks of worms. Preliminary studies into laboratory methods (not involving chickens) for testing anthelmintic efficacy against chicken nematodes indicated significant potential in this area. Five isolates of large roundworm and one isolate of the caecal worm have been characterised and maintained at UNE by a mixture of egg storage and reinfection of birds. Web page content has been developed and awaits implementation with Australian Eggs Limited (AEL).

The project has shown that nematodes and tapeworms can be common on free range farms with burdens in some cases likely leading to some production loss. Farmer awareness of worm prevalence was mostly good, with perhaps an overestimation of the importance of tapeworms. No evidence of a decline in efficacy of the anthelmintic was available to control worms was detected, despite a very long (> 50 years) history of usage of levamisole and piperazine. Despite this, piperazine should not be recommended as a dewormer of choice to control worm infections due to its poor efficacy against caecal and hair worms, and immature stages of all of the worm species. The recently approved (2020 in Australia) in-feed drug flubendazole proved to be highly efficacious against both roundworm and tapeworm. Care should be taken with the off-label prescribed anthelmintics presenting as suspensions, such as fenbendazole or other benzimidazole anthelmintics, when administered in drinking water as they are not water soluble.

Major advances were made in the understanding and methods available to work with defined stocks of chicken nematode species, and six characterised worm isolates are available as a result. However, due to the inability to store isolates indefinitely by freezing, there are significant costs associated with the ongoing maintenance of specified worm stocks. This work will be of greater importance, should anthelmintic resistance emerge in Australian commercial layers.

Consideration should be given to investigate production responses to the proper control of tapeworm infections, given the presence now of a highly effective anthelmintic (Flubenol®) for this purpose in the marketplace and producer concerns about these worms. These would need to be farm scale investigations as tapeworms are not easily investigated in controlled infection studies due to the indirect lifecycle and involvement of intermediate hosts. Consideration should also be given to regular monitoring of anthelmintic efficacy in the sector at 5–10 year intervals.

Overall Conclusions

1. Poultry nematodes and tapeworms are common on free range farms with burdens potentially leading to production loss if not adequately controlled.

2. Surveyed free range egg farmers were more concerned about tapeworm infection than roundworm infections. This probably reflects the previous lack of registered anthelmintics for their control and frequency of visual detection. With the registration for poultry use in 2020 of flubendazole (Flubenol®), which is highly effective against both tapeworms and roundworms, a field study could be conducted to investigate the production benefits of proper control of roundworms, tapeworms or both.

3. No anthelmintic resistance was detected in worms from five different farms suggesting that it is not a major problem at this time. Despite this, the studies revealed two areas of concern:
    a. Poor efficacy of piperazine against any nematodes other than adult *Ascaridia galli.* It should not be used as an anthelmintic of choice.
    b. Reduced efficacy of off-label fenbendazole when administered at a low dose in water.

4. The newly registered anthelmintic Flubenol® administered in-feed for seven days proved to be highly effective for controlling both tapeworm and roundworm.

5. Excreta egg counts by the relatively simple modified McMaster method were found to be far less time consuming and more accurate than the MiniFlotac method, in agreement with other recent studies. It is the method of choice for field monitoring of faecal egg counts. The MiniFlotac method has good sensitivity and may be useful where detection of infection with low burdens is important.

6. A strong association between faecal egg count and adult *A. galli* worm burdens was found during the project. The association was weaker when mixed infections with other species were found. Nevertheless, faecal egg counts can be a useful measure of worm burdens. Usefulness is improved if:
    a. The distinctive eggs of *Capillaria* spp. and *Trichostrongylus* spp. are always counted separately from other nematode eggs (eggs of *A. galli* and *H. gallinarum* are difficult to differentiate).
    b. Intestinal droppings (containing mostly eggs from *A. galli* and *Capillaria* spp.) are evaluated separately from caecal droppings (containing mostly eggs from *H. gallinarum*).

7. Prospects for developing *in vitro* anthelmintic efficacy tests based on a larval development test are good for the ovicidal benzimidazole anthelmintics. The larval development test (LDT) is an easy test to implement, but will not work for the other anthelmintics. The advantages of such tests are that they could be deployed on faecal samples sent in from a farm, and would not require animal testing and culling of birds.

8. There are reasonable prospects for developing *in vitro* anthelmintic efficacy tests based on a larval migration inhibition assay (LMIA) that would be effective for evaluating the full range of anthelmintics, but the method is far more complex to implement than LDT and may not offer sufficient advantages over the WCRT to warrant optimisation.

9. For *in vitro* studies, a saturated sugar solution is most appropriate for extracting eggs from faeces prior to use in assays or infection studies.

10. Eggs for experimentation can be effectively obtained from faeces or from worms collected from infected chickens. In both cases, egg viability of 90% or above can be achieved. Collection from faeces involves more laboratory work, and unless the chickens are infected with a single species of worm, it should not be used to produce eggs of a single species. Moderate levels of eggs (approx. 6,000/worm) can be obtained from adult female *A. galli*, incubated at 37˚C for three days in a relatively simple process that will produce eggs of high quality.

11. Freezing of eggs for prolonged storage does not appear to be feasible with the thick egg wall probably being disrupted during the freezing process despite the addition of DMSO (dimethyl sulfoxide) as a cryoprotectant (Anchordoguy et al. 1987). Our studies, however, were not exhaustive in this area. There is probably greater potential to cryopreserve hatched larvae, now that good hatching protocols are available. This could be explored for the storage of very valuable defined isolates.

12. Long-term preservation of eggs in the unembryonated state requires the combination of low temperatures (4°C) and absence of air. Storage in 0.1 N H_2SO_4 provides the optimum maintenance of viability. Long-term preservation of eggs in the embryonated state is also possible, but requires the combination of warm temperatures (26°C), presence of air and the presence of an inhibitor of microbial growth, in our study ideally 0.1 N H_2SO_4.

13. Using either of the optimised methods identified at 12 above, loss of viability of approximately 2%/week for 20 weeks can be expected during storage and if this linear rate of decline is maintained, storage for up to 40 weeks (10% remaining viability) can be expected, prior to needing to passage the eggs back through chickens.

14. Chickens as young as day old are suitable for use in an infection model to amplify stocks of *A. galli.* In such chickens *A. galli* caused a modest dose-dependent decline in growth.

15. Immunosuppression in most cases removed the negative effect of infection on growth and in the case of dexamethasone, increased worm burden and egg counts. The interaction between immunosuppression and worm burden effects on growth may indicate that the cost of mounting a host immune response in the gut is implicated in the production loss associated with *A. galli* infection.

16. Amplification of *A. galli* stocks by waiting until worm maturity, then harvesting the worms and obtaining eggs from them is a relatively inefficient means of multiplication of stocks. Instead, periodic total faecal collection and egg separation from the faeces would be more efficient given the high fecundity of the *A. galli* parasite.

17. Long-term storage of eggs appears to delay development of the parasite in the host and this needs to be taken into account when amplifying stocks (longer duration required for worm maturation).

18. Maintenance of infections in caged mature birds is risky as they tend to terminate the infections in the absence of ongoing infection by ingestion of eggs. This may be overcome by constant reinfection with higher doses and greater frequency than attempted in our studies. Reinfection can be with eggs extracted from the chicken's own faeces.

19. UNE has successfully characterised and maintained five isolates of *A. galli* and one of *H. gallinarum*, which are available for others to use until the end of the project. None of the isolates exhibit anthelmintic resistance characteristics.

1 Background to the project

1.1 Background

Poultry are susceptible to a wide spectrum of gastrointestinal nematode (roundworm) infections. More than 50 nematode species have been described in poultry and the majority inflict pathological damage to the organ they infest (Permin & Hansen 1998). Most nematodes of poultry affect the digestive tract; few others affect the trachea, lung or eyes. Nematodes of the genera *Ascaridia* (large roundworms), *Heterakis* (caecal worms) and *Capillaria* (capillary worms or threadworms) are generally the most common nematodes encountered in commercial poultry operations (Ruff 1999).

Cestodes (tapeworms) are commonly encountered in poultry reared under free range or backyard conditions. About 1,400 tapeworm species have been described in domesticated poultry and wild birds, and the pathogenicity of the majority of these tapeworms remains unknown. It has been suggested that a great number of tapeworms are either harmless or have a mild pathogenicity, while few species such as *Raillietina echinobothrida* and *Davainea proglottina* can cause severe infection in the host (Permin & Hansen 1998; Macklin 2013). All cestodes of poultry have indirect life cycles and require intermediate hosts such as insects (beetles, flies, ants or grasshoppers), crustaceans, earthworms and snails or slugs, depending on the species of tapeworm (Ruff 1999). The intermediate hosts are essential to perpetuate the life cycle, and infections are therefore rare in caged poultry systems. Birds acquire tapeworm infections by ingestion of the intermediate hosts that live in contaminated environmental material such as litter or the range environment (Permin & Hansen 1998; Ruff 1999; Taylor et al. 2007).

A high incidence of nematode infection has been observed prior to the project in layers by project team members, as has a high incidence of tapeworm infection. Infections are facilitated by increased faecal oral cycling and in some cases by the availability of vectors such as earthworms and other invertebrates. The industry trend towards barn and free range systems therefore increases the likelihood of severe worm infections and associated potential problems of diarrhoea, reduced feed efficiency, immunosuppression, other disease (e.g. blackhead) and overall mortality. Heavy infections, mostly with *A. galli*, have been observed on several farms by project team members during post-mortem examination of layers. Worms historically have been typically controlled in extensively housed production systems using one of only two registered anthelmintics, piperazine (PIP) and levamisole (LEV). Both of these anthelmintics have been in use since the 1960s and there is concern that there may be emerging resistance to these ageing anthelmintics and their possible removal from use by the regulatory authorities. Neither of these chemical medications control tapeworms, which are in part controlled by controlling access to intermediate hosts, but which can be cleared from the host by the newer generation benzimidazole (BZ) anthelmintics. such as albendazole or fenbendazole. As a consequence of these factors, off-label use (prescribed by a veterinarian) of BZ anthelmintics to control worms in chickens occurs. Off-label prescription requires understanding and compliance with the maximum residue limits and withholding periods for eggs for the product being described. With the recent (2020 in Australia) registration of flubendazole as an in-feed medication for control of round and tapeworms in chickens, the level of off-label use may be declining but there may be resistance to the in-feed for 7 days mode of application of flubendazole. In the EU, where the benzimidazole class of anthelmintics are the only anthelmintics available, anthelmintic resistance to fenbendazole has been reported in both *A. galli* and *H. gallinarum* in layers. A free range industry survey by the Poultry CRC in 2014 found that 32% of poultry producers reported presence of both internal and external parasites while the remaining 68% of respondents had either never checked or could not see any signs of parasites (Singh et al. 2017). Up to 50% of the respondents were neither satisfied with the control options for preventing and treating internal and external parasites. A subsequent CRC project on *A. galli* provided improvements in diagnosis and methodology but was

restricted to this single worm species, did not look at anthelmintic resistance, and did not establish a live collection of parasites for future research. This current project aimed to build on the earlier work to reduce the costs to industry associated with ineffective or unwarranted treatments, helping to manage diarrhoea, immunosuppression, blackhead and mortality associated with poorly controlled worm infections. Project findings, together with practical information on worms and their control in poultry, will be incorporated in a new web site providing information and advice on worm infection and control in chickens.

2 Online survey of free range egg producers

2.1 Introduction

A prior survey of the demographics and practices of semi-intensive free range farming systems in Australia by (Singh et al. 2017) comprised a total of 79 questions related to nutrition, pasture management, welfare and health, animal housing, environmental impact and economics. There were 41 respondents and, while intestinal worms were not identified as a major cause of mortality or problems, 32% of poultry producers reported the presence of both internal and external parasites while the remaining 68% of respondents had either never checked or could not see any signs of parasites. To more clearly identify producer experiences and attitudes to worms and their current worm control practices, this project undertook a further more narrowly scoped survey of free range egg producers. The survey aimed to identify worm control perceptions, problems, and practices so that the industry can control internal parasites more effectively.

2.2 Methods

A survey questionnaire comprising 37 questions was designed in consultation with AEL and the Project Steering Committee. It comprised 6 sections, these being: 1) participant information; 2) farm and flock characteristics; 3) perceived worm importance and monitoring practices; 4) deworming and anthelmintic use practices; 5) husbandry-based practices for gastrointestinal worm control; and 6) other information, including willingness to participate in on-farm surveys of worm prevalence. The survey and associated documentation were approved by the UNE Human Ethics Committee (Approval HE19-207).

The online survey was implemented using Survey Monkey with AEL emailing their egg producers with a covering letter and link to the survey. Links were also provided in the Eggstra! AEL magazine. The survey opened on 20/12/2019 with an initial email message followed by a reminder email on 29/1/2020 before the survey closed on 29/3/2020. The overall number of responses (16) was disappointing. Based on approximately 200 free range egg producers this represents a response rate of 8%. There were good responses to the first email introducing the survey (6) and the reminder email (8) but the absence of a final reminder email prior to survey closure seemed to limit the response rate. There was no response at all a reminder included in the Eggstra! publication. One final respondent was obtained via a phone call.

The survey questionnaire and tabulated findings on each question findings can at Appendices 1 and 2 to this report.

2.3 Results

While detailed results and graphs can be found at Appendix 2 some of the main findings are summarised below.

2.3.1 Participant information

The 16 participants were drawn from all states of Australia with highest representation from Victoria (5), NSW (4) and Queensland (3). The majority of the farmers (38%) had 30–40 years of poultry farm experience, while a slightly lower proportion (31%) had less than 10 years of experience.

2.3.2 Farm and flock characteristics

Most respondents appeared to run a mixture of enterprises with 10/12 respondents to this question (85%) running free range, 8% free range organic, 54% barn and 77% cage operations in their enterprises. The highest proportion of respondents (38%) had been running free range enterprises for 30–40 years, while 31% had been running them for less than 10 years. Most sites were multi-aged (62%) and reared pullets (77%), while 23% purchased started pullets. The most common flock size per shed was 11,000–20,000 (24%), with smaller flocks of 10,000 or less reported by 31% of respondents. Most farms (62%) stocked at 8–10 hens/m^2 in the shed and 1,500/ha on the range (50%). The total bird number on the respondent enterprises was most commonly between 10,000 and 100,000 (30%) but 25% of respondents had bird numbers of 0.25–0.5 million, and a single respondent had over 1m birds. A wide range of housing features was employed by the respondents, with very high levels of use of perches (92%) and high use of slatted floors and manure belts (both 62%).

2.3.3 Perceived worm importance and monitoring practices

Most respondents (77%) felt that they had a good understanding of the lifecycle of gastrointestinal worms of chickens, with 100% correctly identifying that infection can occur due to ingestion of worm eggs, and 92% indicating correctly that some infections occur due to ingestion of intermediate hosts such as earthworms and other invertebrates. There was a high level of agreement (85–100%) that worm infections caused weight loss, reduced egg production, poor feed conversion, behavioural change and increased mortality. Only 23% of respondents felt that worm infection had no effect or promoted healthier more natural birds.

Regular monitoring of birds for worm infection was practised by 61% of respondents and occasional monitoring by 31%, with only a single respondent (8%) indicating that they never monitor for worm infection. Monitoring ranged widely from weekly/ongoing monitoring of cull and dead birds by necropsy (33%) to approximately annual testing for worms (8%). The most common method of monitoring of parasites was by necropsy and physical detection in the gut (83%), while 25% used faecal egg counts and 8% examined faeces for the presence of worms. The examination for worms was mostly (92%) conducted by farm or company staff at various levels, with only 8% using laboratory diagnosis exclusively. Use of veterinarians and/or laboratories for monitoring was reported by 42% of respondents.

A high proportion of respondents (77%) reported detection of large roundworm (*Ascaridia*) in the previous 12 months, with similar proportions reporting detection of tapeworms (69%) and caecal worms (*Heterakis*) but much lower proportion reporting presence of hair worms (*Capillaria*) (23%). Most respondents (46%) felt that there was no seasonal variation in the worm burden observed in their chickens. When asked about their level of concern regarding worm infection, most respondents reported a 'medium' level of concern about worm infection in general (42% selected this level of concern) and *Ascaridia* in particular (46%), while 38% expressed a 'high' level of concern about tapeworm infection and 50% a 'low' level of concern about caecal worms.

2.3.4 Deworming and anthelmintic use practices

The majority of respondents (54%) deworm their flocks regularly based on a set schedule while 31% treat occasionally or irregularly, with the remainder not treating or unsure if their flocks are treated. Most respondents treating for worms do so in a 'tactical' fashion, in response to seeing high worm burdens or egg counts (64%) while the remaining 36% treat more strategically on a regular or fixed schedule. The anthelmintic most used was levamisole (73% of respondents) followed by piperazine (45%) fenbendazole (e.g. Panacur®) (36%) and mebendazole (e.g. Benzicare®) (18%). Only one respondent reported the use of natural therapies to control worms. Only 18% of respondents withheld

feed or water prior to administration of anthelmintics. The majority of respondents (73%) monitored the success of deworming treatment, mostly by the methods covered in Section 2.3.3 for worm monitoring, i.e. primarily by necropsy and inspection of gut contents (73%) but also examination of faeces for expelled worms (36%). All respondents indicated that they used a veterinarian as a source of advice for deworming treatments, with 73% also indicating that common sense and experience were important in decision making and 27% using advice from fellow farmers. With regard to the efficacy of dewormers, most respondents (64%) felt that efficacy had not changed over time and only two respondents (18%) felt that there had been a decline in efficacy. The inverse proportions suspected that they had dewormer resistance in their flock. The great majority (81%) of respondents would like to see more registered deworming products made available.

2.3.5 Husbandry-based practices that may influence gastrointestinal worm control

A minority of respondents (31%) reported that they practise a range rotation system although none identified parasite control as a reason for this. Range rotation was exclusively practised to maintain pasture cover and range area quality. Only 7 respondents used a litter system and of these, 6 practised complete litter replacement and one added fresh litter on top of old litter. Two respondents reported occasional use of old litter. When asked if practices other than use of dewormers were used to control worms, 38% responded in the affirmative. Practices that were thought by respondents to reduce the risk of worm infection cycling in their free range chickens included cleaning permanent facilities between batches (100%), using chemical disinfectants between flocks (85%), routine biosecurity measures (62%), and range treatment (46%). Other practices such as keeping the pasture on the range short, harrowing the range, and natural therapies were thought to be effective by 23–31% of respondents.

2.4 Discussion, conclusions and implications

The disappointing response rate was likely due to survey fatigue, which is common in the current climate with online surveys proliferating due to the ease of reaching out to respondents and collection and collation of survey data. It may also be attributed in some cases to aversion to filling in surveys online, although no requests were made to obtain a hard copy version of the survey, an option offered to survey participants. There was a clear response to each email message about the survey and indeed all but one response was in response to an email message. Given this, consideration should be given in future to increasing the number of reminder email messages from the single reminder used in this survey.

Despite the poor response rate, the specific nature of the survey and the geographic and farm size and system diversity of the respondents suggests that the nature of the responses is likely to be broadly in line with industry perception and practices. The findings as a whole suggest that the majority of the industry perceives worm infection as a moderate rather than a major problem and feels that the current dewormers work effectively, although they would like to see additional products made available. Presumably the latter response is due to the lack of a registered product to control tapeworm infection at the time of the survey. This has been rectified by the subsequent registration of Flubenol® an in-feed preparation of flubendazole for this purpose, although this mode of delivery and the long duration of treatment (7 days) may not stop the use of off-label BZ treatments administered in water. The level of understanding of worms, monitoring practices and control practices did not reveal any major deficiencies of concern. The greater level of concern about the effects of tapeworms than roundworms may reflect their ongoing presence despite LEV and Piperazine (PIP) treatments which are ineffective against tapeworms, or their perceived negative effects on the host. Given the difficulty of conducting controlled experiments with tapeworm infections, the only way to practically assess their impact is to compare performance in treated and

untreated flocks in a large field study, which would require a sound experimental design.

3 Physical survey of worm prevalence and magnitude of infection

3.1 Introduction

In addition to obtaining producer perspectives on the worm problems in their flocks in the online survey, the project aimed to assess the actual prevalence and magnitude of helminth infections in free range flocks in Australia. A systematic review and meta-analysis of previous surveys on the prevalence of helminth infection worldwide has recently been published by the project team (Shifaw et al. 2021), but there have been no detailed studies from Australia apart from a 1942 report in Queensland in which 91.7% of 348 birds sampled from throughout Queensland were found to be infected with internal parasites (Broadbent 1942). In that study, 5 nematode and 7 cestode species were identified, with prevalence of some of the major species being 76.7% for *Heterakis gallinarum*, 39.6% for *Ascaridia galli*, 35.9% for *Raillietina tetragona*. Knowing the major species present and the levels of infection in modern flocks will help inform advice and control strategies for industry.

3.2 Methods

Two different approaches were taken to achieving this objective:

- Detailed culling, necropsies and worm count studies on 5 flocks of collaborating farms within driving distance of UNE (worm count studies).
- Request mail in of intestinal and caecal dropping samples from a further 16 flocks from free range layer farms located at more distant parts of Australia (excreta submission studies).

Ethical approval for the studies was obtained under UNE AEC approval 19-082.

3.2.1 Worm count studies

A cross-sectional prevalence study was conducted on five selected free range layer flocks in Queensland and NSW. Birds were assessed for helminth burdens at the time of normal depopulation of the flock at the end of their productive life. Farm selection and flock recruitment were based on the following pre-defined criteria:

- Free range producer.
- No anthelmintic treatment applied to the target flock in the three months prior to sampling (apart from Flock 1).
- Agreement to provide 100 randomly selected hens at the time of scheduled depopulation.
- Within convenient driving distance from Armidale NSW (approx. 500 km radius).

Details of the five selected farms are provided in Table 3-1. A target sample size of 100 chickens was used, based on an assumed prevalence of 50% with a desired absolute precision of 10% and a 95% level of confidence (Thrusfield 2007). The selected birds (507 in total) were subjected to detailed post-mortem examination for worm count in accordance with the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Yazwinski et al. 2003) to determine prevalence, spectrum and burden of worm species circulating on the farms.

For post-mortem examination, birds were euthanised by cervical dislocation at each farm. Immediately after killing, gastrointestinal tracts were removed from the carcass, packed in zip lock

plastic bag, ice cooled (4°C) and transported to the parasitology laboratory at the University of New England, and either stored at 4°C for examination within 48 h, or frozen (-20°C) for later examination. In the laboratory, the gastrointestinal tracts from oesophagus to rectum were then dissected longitudinally using Mayo scissors and contents flushed with tap water through a 100 µm mesh sieve. The contents of the sieve were then transferred to a Petri dish containing physiological saline. Following counting of all visible parasites in the lumen contents the scraped mucosa was examined under a stereomicroscope at 40x magnification. Helminth species differentiation was carried out based on the morphological characteristics (i.e. helminthological keys) as described by Permin and Hansen 1998, Yazwinski and Tucker 2008, and McDougald 2020.

Table 3-1 Details of participating farms in the worm count studies

Information	Farm number				
	1	2	3	4	5
Farm location	QLD	QLD	Coastal NSW	Northern NSW	Sydney
Hen strain	Lohmann Brown Classic	Hy-Line Brown	Isa Brown	Isa Brown	Isa Brown
Date of sampling:	23-25/07/2019	4/8/2020	27/10/2020	4/12/2020	9/12/2020
Hen age at the day of sampling (week)	74	80	78	104	75
Production type	Free range	Organic free range	Organic free range	Free range	Free range
Flock size	40,000	3,416	8,000	4,000	15,000
Indoor stocking density (hens/m^2)	9	9	4	N/A	1
Range stocking density (hens/ha)	1,500	900	615	1,250	10,000
Time since last treatment for internal parasites	27 days	Never treated	Never treated	2.5 months	62 weeks
What product was used?	Levamisole	None	None	Piperazine	Mebendazole

During tract examination, digesta samples were taken from the terminal end of the large intestine contents of each chicken for faecal egg count (FEC) analysis and correlation with worm counts. Faecal egg counts were determined by the modified McMaster (MM) technique employing the basic principle described by (Whitlock 1948), providing a limit of detection of 40 EPG. Two and a half grams of each faecal sample was diluted in 47.5 ml of saturated salt solution (SG 1.20), thoroughly homogenised, sieved and a 0.5 ml aliquot loaded into a chamber on a Whitlock universal slide, and examined under 40x magnification power. Eggs counted were multiplied by 40 to provide FEC in EPG in faeces units.

3.2.2 Excreta submission studies

Based on the findings of the method optimisation studies (Section 5) it was concluded that conducting FEC using the MM method on 20 samples per flock would provide a good estimate of flock infection levels and provide a practical field diagnostic test for helminth infection. In order to collect information on burdens of *A. galli* and *H. gallinarum*, which have similar eggs, it was decided to request submission of 10 intestinal droppings (containing predominantly *A. galli* eggs and the easily identifiable *Capillaria* spp. eggs) and 10 caecal droppings containing predominantly *H. gallinarum* eggs (Thapa et al. 2015a). Accordingly free range producers who had expressed interest in collaborating with the project were contacted and asked to submit samples in a kit supplied to them including collection instructions and materials, a lab submission form and pre-paid addressed return mail pouch to submit the samples in.

Samples from a total of 18 flocks from seven farms were submitted for faecal egg count analysis. Samples from two flocks were from young chicks 3–4 weeks of age before the prepatent period of the worms, and were negative so are not included in the report. Details of the 16 flocks with meaningful data are provided in Table 3-2.

Table 3-2 Details of participating farms in the excreta submission studies

Flock	State	Farm No	Farm type	Age (wks)	Breed	Last deworming	Birds/flock
1	Victoria	1	Free range	34	Hyline Brown	at 8 weeks	NA
2	Victoria	1	Free range	55	Hyline Brown	at 8 weeks	NA
3	Victoria	2	Barn	5	Hyline Brown	No	27,000
4	Victoria	2	Barn	6	Hyline Brown	No	27,000
5	Victoria	3	Barn	11.5	Hyline Brown	No	34,000
6	Victoria	3	Barn	10.5	Hyline Brown	No	34,000
7	Victoria	4	Barn	11.5	Hyline Brown	No	13,000
8	Victoria	4	Barn	9.5	Hyline Brown	No	13,000
9	Tasmania	5	Free range	42	Hyline Brown	No	15,000
10	Tasmania	5	Free range	53	Hyline Brown	No	19,000
11	Tasmania	5	Free range	29	Hyline Brown	No	19,000
12	Tasmania	5	Free range	69	Hyline Brown	No	15,000
13	Victoria	6	Barn	10	Hyline Brown	No	27,000
14	Victoria	6	Barn	11	Hyline Brown	No	27,000
15	Victoria	7	Barn	12.5	Hyline Brown	No	17,000
16	Victoria	7	Barn	14	Hyline Brown	No	13,000

~~FR – free range, DL.~~

3.2.3 Statistical analysis

All descriptive statistics were performed using JMP 14 (SAS Institute Inc., Cary, NC, USA). Prevalence and worm counts (worm load/burden) were carried out for 507 hens from 5 farms. Excreta egg counts were analysed from faecal samples taken from the rectum of chickens during post-mortem examination. Both worm count and egg count data had skewed distributions with most chickens having low burdens. Untransformed means are presented, as this is industry standard, but medians and ranges are also presented to better represent the underlying distributions. The prevalence of individual helminth species was calculated as the proportion of the host population examined that was infected with a specific parasite during the study. Worm prevalence was analysed using contingency table analysis in JMP 14 software. Mean worm burden per hen was calculated to determine the magnitude or intensity of infection. The association between parasitological parameters (nematode worm burden, nematode EPG, and female worm count of *Heterakis gallinarum* and *Ascaridia galli*) was assessed using Pearson's correlation analysis.

3.3 Results

3.3.1 Worm count studies: prevalence and worm count

The prevalence and worm count of helminth parasites based on post-mortem examination are presented in Table 3-3. Out of 507 hens, 84.6% were infected with one or more helminth parasites.

The most prevalent species of nematodes were *Heterakis gallinarum* (86.7%), followed by *Ascaridia galli* (71.0%) and *Capillaria* spp. (34.9%), with considerable variation between farms. The overall prevalence of cestodes was 18.7%. *H. gallinarum* had the highest worm burden with a mean of 45.5 worms per hen. Mean worm counts for *A. galli*, *Capillaria* spp., and cestodes (tapeworms) were 17.9, 2.72 and 0.63 worms per hen, respectively (Table 3-3). Overall, the hens harboured an average of 57.2 worms of which 56.5 were nematodes. There was variation in prevalence, worm count and parasite composition across farms, particularly for Farm 1, where an anthelmintic treatment had been given 4 weeks prior to the worm count. The farm-specific mean worm counts for *A. galli* and *H. gallinarum* ranged from 0.69–44.9 and 5.95–79.5 worms per hen, respectively. The sex ratio (female:male worms) was 1.38:1 for *A. galli*, and 1.77:1 for *H. gallinarum* (Table 3-3).

The results of the faecal egg counts conducted on the contents of the terminal large intestine are shown in Table 3-4. Overall, 86% of birds on Farms 2–5 had nematode eggs in their faeces (range 66–100%). The overall mean untransformed FEC was 522 EPG (range 89–1022), with every farm different from every other farm when the FEC data were analysed on the cube root transformed scale Table 3-4.

The association between different parasitological measurements on individual chickens across the farms is shown in Table 3-5. There were strong positive associations between most variables. With regard to the association between worm counts and faecal egg counts, an overall correlation of 0.67 was found, however, the association between *A. galli* female worm count and FEC was 0.89. Associations of *H. gallinarum* and *Capillaria* spp. worm counts and FEC were much lower than observed for *A. galli.*

Table 3-3 Prevalence and worm counts (WC, mean, range and median) per hen in five free range flocks at the end of production

Parasites	Farm 1* (QLD)				Farm 2 (QLD)				Farm 3 (Coastal NSW)				Farm 4 (Northern NSW)				Farm 5 (Greater Sydney Region)				Total prev. %	Total WC
	Prev.%	WC	Range	Median	Prev.%	WC	Range	Median	Prev.%	WC	Range	Median	Prev.%	WC	Range	Median	Prev.%	WC	Range	Median		
A. galli	28	0.7	0-7	0	71	11.40	0-102	5	98	29.4	0-220	21	58	3.21	0-13	1.5	100	WC	4-353	31	71	17.9
Sex ratio (F:M)	NA	NA	NA	NA	-	1.36	0±3.5	1.33	-	1.34	0.38-3	1.29	-	1.58	0-4	1.6	-	44.9	0.66-2.5	1.23	-	1.38
H. gallinarum	NA	NA	NA	NA	81.3	32.6	0-215	20	99	64.9	0-412	41.5	67	5.95	0-23	4	100	1.4	12-287	60	86.7	45.5
Sex ratio (F:M)	NA	NA	NA	NA	-	2.27	1.5-5.3	2.0	-	1.50	0.72-4	1.4	-	2.16	0-5	1.8	-	79.5	0.7-2.75	1.33	-	1.77
Capillaria spp.	NA	NA	NA	NA	33.6	1.06	0-7	0	51	3.76	0-32	2	19	1.00	0-11	0	36	1.37	0-41	0	34.9	2.72
Total nematodes	NA	NA	NA	NA	93.5	45.0	0-234	35	100	98.1	3-476	71.5	73	10.0	0-36	8.5	100	5.18	21-541	102	79.1	56.5
Raillietina tetragona	NA	NA	NA	NA	2.80	0.09	0-4	0	0	0	0	0	0	0	0	0	16	129.6	0-6	0	4.70	0.17
Raillietina echinobothrida	NA	NA	NA	NA	2.80	0.06	0-3	0	0	0	0	0	0	0	0	0	10	0.60	0-5	0	3.19	0.11
Raillietina cesticillus	NA	NA	NA	NA	1.87	0.05	0-3	0	2.00	0.06	0-5	0	0	0	0	0	17.2	0.36	0-12	0	5.20	0.20
Choanotaenia infundibulum	NA	NA	NA	NA	0	0	0	0	0	0	0	0		0	0	0	18	0.69	0-11	0	4.40	0.22
Hymenolepis cantaniana	NA	NA	NA	NA	0	0	0	0	0	0	0	0	0	0	0	0	18	0.89	0-5	0	4.40	0.22
Total Cestodes	45	NA	NA	NA	7.5	0.20	0-4	0	2	0.06	0-5	0	0	0	0	0	39	0.42	0-21	0	18.7	0.63
All worms	54	0.7	0-7		95.3	45.2	0-234	35	100	98.1	3-476	71.5	73	10.0	0-36	8.5	100	2.96	21-541	105	84.6	57.2

* This farm was surveyed very early on in the project prior to the development of the full protocol. WC was only performed for A. galli and presence or absence of tapeworms was recorded.

Table 3-4 Faecal egg counts in contents of terminal large intestine

Farm	Prevalence of nematode FEC positive samples (%)	Median FEC	Arithmetic mean FEC (EPG)	Back-transformed from cube root		
				Mean FEC (EPG)	Lower 95%	Upper 95%
2	81%	120	279	131[c]	95	175
3	98%	520	714	564[b]	462	680
4	66%	80	89	35[d]	20	55
5	100%	740	1022	817[a]	685	964
Overall	86%	280	522	273	241	308

[abc] Means not sharing a common letter in the superscript are significantly different (P < 0.05).

Table 3-5 Pearson correlation coefficients between parasitological variables (worm counts – WC and faecal egg counts – FEC in EPG) in nematode infected free range chickens

Parasitological variable	Total nematode WC	*A. galli* WC (♀)	*H. gallinarum* WC (♀)	*A. galli* WC (♀+♂)	*H. gallinarum* WC (♀+♂)	*A galli* + *H. gallinarum* WC (♀)	*Capillaria* spp. WC
A. galli WC (♀)	0.71*						
H. gallinarum WC (♀)	0.87*	0.39*					
A. galli WC (♀+♂)	0.71*	0.99*	0.39*				
H. gallinarum WC (♀+♂)	0.89*	0.40*	0.98*	0.40*			
A. galli + *H. gallinarum* WC (♀)	0.98*	0.70*	0.90*	0.70*	0.89*		
Capillaria spp. WC	0.04*	0.01¥	0.01¥	0.01¥	0. 02¥	0.02¥	
Nematode FEC (EPG)	0.67*	0.89*	0.38*	0.88*	0.39*	0.65*	0.02¥

¥ P < 0.05.
* P < 0.0001.

3.3.2 Excreta submission study: excreta egg counts in intestinal and caecal droppings

On all farms, egg counts of *Capillaria* spp. and putatively *A. galli* (henceforth referred to as *A. galli* in this section) in intestinal droppings and putatively *H. gallinarum* (henceforth referred to as *H. gallinarum* in this section) in caecal droppings were recorded (Table 3-6). Very similar prevalence of infection was recorded for *A. galli* (overall 71% range 30–95%) and *H. gallinarum* (overall 78% range 50–90%). Mean egg counts were also very similar (*A. galli* 407 range 0–6600, *H. gallinarum* 404, range 0–6480). Due to the skewed nature of the data, median values were much lower, being 120 and 200 respectively. *Capillaria* spp., although detected on all farms, had a low prevalence (27%, range 9–50%) with low egg counts (mean 18, range 0–160, median 0) (Table 3-6).

When FEC was analysed on the transformed scale, the 6 hen flocks had significantly higher burdens of *H. gallinarum* than the 10 pullet flocks (raw means 599 and 276 EPG respectively; P = 0.03). There was a reverse, but non-significant trend for *A. galli* with higher counts in pullets (440 EPG) than hens (345 EPG) (P = 0.49).

Table 3-6 Nematode worm egg counts in intestinal and caecal droppings from 16 flocks submitted by free range and barn producers

Flock	Farm	Intestinal dropping FEC (EPG)								Caecal dropping FEC (EPG)			
		Putatively *A. galli* FEC (EPG)				*Capillaria* spp. FEC (EPG)				Putatively *H. gallinarum* FEC (EPG)			
		Prevalence	Mean	Median	Range	Prevalence	Mean	Median	Range	Prevalence	Mean	Median	Range
1	1	8/10, 80%	664	160	0 - 5280	2/10, 20%	12	0	0 - 80	9/10, 90%	976	140	0 - 4280
2	1	8/10, 80%	500	240	0 - 2880	3/10, 30%	16	0	0 - 80	9/10, 90%	1068	220	0 - 6480
3	2	8/10, 80%	120	120	0 - 280	3/10, 30%	16	0	0 - 80	8/10, 80%	184	160	0 - 440
4	2	8/10, 80%	172	160	0 - 360	3/10, 30%	16	0	0 - 80	9/10, 90%	196	180	0 - 520
5	3	8/10, 80%	504	420	0 - 1360	5/10, 50%	32	20	0 - 80	9/10, 90%	404	300	0 - 1240
6	3	9/10, 90%	456	460	0 - 1080	2/10, 20%	24	0	0 - 160	8/10, 80%	524	280	0 - 2080
7	4	9/10, 90%	316	280	0 - 880	2/10, 20%	12	0	0 - 80	8/9, 89%	373	360	0 - 840
8*	4	20/21, 95.2%	1503	840	0 - 6600	7/21, 33%	30	0	0 - 160				
9	5	6/10, 60%	76	40	0 - 280	4/10, 40%	36	0	0 - 160	8/9, 89%	347	360	0 - 560
10	5	7/10, 70%	188	100	0 - 960	3/10, 30%	16	0	0 - 80	7/10, 70%	428	260	0 - 1040
11	5	5/10, 50%	116	40	0 - 440	2/10, 20%	12	0	0 - 80	7/10, 70%	228	180	0 - 640
12	5	8/10, 80%	528	220	0 - 1520	4/10, 40%	24	0	0 - 80	7/10, 70%	524	500	0 - 1360
13	6	3/10, 30%	24	0	0 - 120	2/10, 20%	8	0	0 - 40	5/10, 50%	112	40	0 - 360
14	6	2/10, 20%	28	0	0 - 200	1/10, 10%	4	0	0 - 40	6/10, 60%	184	160	0 - 440
15	7	6/11, 54.5%	55	40	0 - 160	1/11, 9.1%	4	0	0 - 40	8/11, 72.7%	222	120	0 - 880
16	7	7/10, 70%	88	80	0 - 280	3/10, 30%	12	0	0 - 40	8/10, 80%	296	260	0 - 840
Overall		**116/149, 71%**	**407**	**120**	**0 - 6600**	**47/172, 27%**	**18**	**0**	**0 - 160**	**122/172, 78%**	**404**	**200**	**0 - 6480**

See Table 3-2 for details of each flock. * Due to a misunderstanding only intestinal droppings were submitted from Farm 8.

3.4 Discussion, conclusions and implications

3.4.1 Nematode ('roundworm') infection

Infection with the 2 major nematode species *Ascaridia galli* and *Heterakis gallinarum* was detected in all farms where they were investigated (20 and 19 farms respectively) in both the worm study and excreta submission studies, as was the case for *Capillaria* spp. (20 farms). In the worm count study, the prevalence of infection the former two species was high (58–100%) apart from Farm 1 (28% for *A. galli*), which was sampled less than a month after anthelmintic treatment. Interestingly if the anthelmintic treatment had been fully effective against all stages of the lifecycle, Farm 1 should have been negative. Anthelmintic resistance testing of the *A. galli* isolate from that farm was undertaken and is reported in Section 4. The prevalence of the *Heterakis gallinarum* observed in the current study (86.7% in the worm count study and 78% in the excreta study) is broadly consistent with the 76.7% prevalence found in the study of (Broadbent 1942) in Queensland some 60 years ago, but the prevalence of *A. galli* (39.6 %) and *Capillaria* spp. (~7%) in that study was considerably lower than in the current studies. In the current studies, the mean *A. galli* prevalence was 71% in both the worm count study and the excreta submission study, and the respective values for *Capillaria* spp. were 34.9 and 27%. The nematode prevalence results in the current study are also consistent with the responses to the online survey (Section 2) in which 77% and 62% of respondents indicated that they had detected *A. galli* and *H. gallinarum* respectively in the past 12 months on their farms. The prevalence is higher in the present study than that reported worldwide in the meta-analysis of (Shifaw et al. 2021) with median prevalence values of ~38% for *A. galli* and ~30% for *H. gallinarum* in that report. 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. 2021) found a mean prevalence of helminth infection of 78% and 74% in free range and backyard flocks compared to 43.4% for barn and 20.8% for caged systems respectively.

The mean worm burdens of *A. Galli* (17.9) and *H. gallinarum* (45.5) detected in the current study were higher than those reported by (Broadbent 1942), the equivalent values being 8.4 and 30.4 respectively in that study. For *Capillaria* spp. The reverse was true, with the earlier study reporting 14.7 worms per bird compared to 2.7 worms per bird in the present experiment. Total nematode counts were understandably very low (< 1/bird) on Farm 1, which had received recent Levamisole treatment and lower on Farm 4 (10/bird), which had received piperazine treatment some 9 weeks earlier, than on Farms 2 and 3, which had never had anthelmintic treatment (45 and 98/bird respectively) and Farm 5, which had been treated with Benzicare® 62 weeks prior to sampling (130/bird).

It is difficult to predict the level of production loss associated with these levels of roundworm nematode infection as clear associations between level of infection and production loss vary in the literature. However, most reports indicate that higher infection rates with A. *galli* can have a negative effect on weight gain in chickens (Ackert 1931; Reid & Carmon 1958; Permin et al. 1998). Other authors, however, suggested that the magnitude of growth depression ranges from zero, or even growth stimulation (Todd & Hansen 1951), to significant reduction (Chubb & Wakelin 1963). Nevertheless, the literature generally indicates that 'high' worm burdens, particularly with *A. galli*, cause damage to the intestinal lining, blood loss, increased susceptibility to bacterial infections, nutrient depletion, obstruction of the intestine and potentially death. Most infected birds will not show clinical signs, but as the burdens rise sluggishness, ruffled feathers, drooped wings, depression, loss of appetite and bodyweight, altered hormone levels, retarded development, anorexia (severely reduced bodyweight), and increased mortality (death rates) are observed (Ramadan & Znada 1991). *A. galli* appears to reduce egg performance only at high infestation levels (Sharma et al. 2019), although complication with secondary bacterial infections may greatly exacerbate this. Infection with *Heterakis gallinarum* has less direct negative impact than *A. galli*, and clinical signs of infection are

again usually not obvious. However, due to larval migration through the wall of the blind sacs, wall thickening, nodules and inflammation can be observed in severe cases. In severe infection, egg production of the laying hens can be reduced. In some cases, the larvae can also migrate to the liver and cause damage visible as small nodules (McDougald 2020). An outbreak of blackhead disease, caused by *Histomonas meleagridis,* is a possible complication of *Heterakis* infection. *H. meleagridis* is a protozoan parasite carried by *Heterakis* eggs. Blackhead disease can be associated with significant morbidity in chickens, with signs including depression, reduced growth, diarrhoea, reduced egg production and increased mortality and culling (Permin & Hansen 1998; McDougald 2020).

3.4.2 Cestode (tapeworm) infection

A high prevalence of tapeworm infection was only detected on two farms in the worm count study, Farm 1 (45%) and Farm 5 (39%). On Farm 4 situated on the northern tablelands of NSW, no tapeworms were observed at all for reasons that are not clear. On the two remaining farms (2 and 3), a low prevalence of tapeworms was found (7.5% and 2% respectively). Where tapeworm speciation was carried out (Farms 2, 3 and 5) *Raillietina* spp. Were most common, ranging in prevalence from 1.9–17.2% by species. On Farm 5, two additional species were observed at moderate prevalence, *Choanotaenia infundibulum* and *Hymenolepis cantaniana* (both 18% prevalence). In the very early Queensland study of Broadbent (1942) overall prevalence of tapeworm infection is 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, than in the present study. The comparatively low prevalence of tapeworm infection in the present study also contrasts somewhat with the 69% of survey respondents who claim to have detected tapeworms in the past 12 months and the 38% of respondents who had a 'high' level of concern about tapeworm infection (higher than the 25% for both *A. galli and H. gallinarum*).

Cestodes (tapeworms) are commonly encountered in poultry reared under free range or backyard conditions (Permin & Hansen 1998). About 1,400 tapeworm species have been described in domesticated poultry and wild birds, and the pathogenicity of the majority of these tapeworms remains unknown. It has been suggested that a great number of tapeworms are either harmless or have a mild pathogenicity, while few species such as *Raillietina echinobothrida* can cause severe infection in the host (Macklin 2013; Permin & Hansen 1998). All cestodes of poultry have indirect life cycles and require intermediate hosts such as insects (beetles, flies, ants or grasshoppers), crustaceans, earthworms and snails depending on the species of tapeworm (Ruff 1999). The intermediate hosts are essential to perpetuate the life cycle and infections are therefore rare in caged systems. Birds acquire tapeworm infection by ingestion of the intermediate hosts that breed in contaminated litter (Permin & Hansen 1998; Ruff 1999; Taylor et al. 2007). Because of their complex lifecycles tapeworms are difficult to work with experimentally, as the eggs shed from the mature worms in chicken faeces are not infective for chickens as is the case with nematode eggs. Culture thus requires identification of the intermediate host and then maintenance of both hosts to propagate the parasite. They are also mostly long and fragile organisms, which are difficult to enumerate and identify accurately in the gut unless great care is taken in dissection. Further research is required to assess the effects of tapeworm infection particularly of individual species.

4 Anthelmintic efficacy/resistance testing

Each of the experiments in this section has been published before (Feyera et al. 2021) or following the preparation of this report (Feyera et al. 2022a,b,c). The experiments in this area their organisation for this report and the papers arising from them are:

1. Study 1. Initial experiment evaluating resistance in a suspected LEV resistant *A. galli* isolate comparing oral and water administration of 4 anthelmintics or mixtures and evaluation of faecal egg count reduction (FECR) tests as an alternative to traditional worm count reduction (WCR) test for assessing anthelmintic efficacy (Feyera et al. 2021).

   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**, 109514. doi:https://doi.org/10.1016/j.vetpar.2021.109514

2. Study 2. Assessment of anthelmintic efficacy in two flocks of layers naturally infected with different nematode species (individual and mass water treatment) (Feyera et al. 2022a) 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*, 100723. doi:https://doi.org/10.1016/j.vprsr.2022.100723

3. Study 3. Assessment of anthelmintic efficacy in two additional isolates of *A. galli* in artificially infected chicks with trickle infection to assess efficacy against both mature and immature stages of the parasite (Feyera et al. 2022b)

   Feyera, T., Sharpe, B., Elliott, T., Shifaw, A. Y., Ruhnke, I., & Walkden-Brown, S. W. (2022). Anthelmintic efficacy evaluation against different developmental stages of Ascaridia galli following individual or group administration in artificially trickle-infected chickens. *Veterinary Parasitology*, **301**, 109636. doi:https://doi.org/10.1016/j.vetpar.2021.109636

4. Study 4. Evaluation of *in vitro* methods of evaluating anthelmintic resistance, coupled with optimisation of methods to harvest eggs from faeces for this purpose (Feyera et al. 2022c).

   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**. doi:10.1017/S0022149X22000177

4.1 Introduction

The chemical control of helminth infections in poultry is dependent on a limited number of anthelmintic products worldwide (Ruff 1999; Tarbiat et al. 2017). The available chemicals are mainly applied to control parasitic nematodes of economic importance such as *A. galli* (Permin & Hansen 1998; McDougald 2020). There have been no new products registered specifically for control of gastrointestinal worms in chickens in many years. In Australia, levamisole (LEV) and piperazine (PIP), two of the historically most common water-administered anthelmintics for poultry, have been the only registered chemicals to treat nematode infections until a very recently registered product based on the benzimidazole anthelmintic, flubendazole. The two registered products have been used for more than half a century with no recent appraisal of their efficacy status. While both of these products have a nil egg withholding period, levamisole is widely used in commercial layer flocks whereas piperazine tends to be used in young birds and breeder flocks (Ruhnke 2015). Two benzimidazole (BZ) compounds, fenbendazole (FBZ) and mebendazole, are also sometimes used by the industry in specific scenarios and under veterinary prescription, particularly to control tapeworm infections. An additional BZ anthelmintic, flubendazole (FLBZ, Flubenol®) with broad-spectrum activity against chicken tapeworms as well as nematodes, was registered for in-feed use in Australia in 2020 and was able to be used only in the later studies in this series.

LEV and PIP produce antiparasitic effects against chicken nematodes via different mechanisms. LEV acts as a cholinergic receptor agonist at the parasite neuromuscular system. It selectively activates the excitatory nicotinic acetylcholine receptors on nematode muscle cells and elicits spastic paralysis of susceptible nematode (Martin & Robertson 2010). PIP is a selective gamma-aminobutyric acid (GABA) agonist that activates and gates GABA receptors on nematode muscle. It causes hyperpolarisation of muscle cells and nerve membranes at the neuromuscular junction leading to parasite immobilisation by flaccid paralysis and consequent removal from predilection site (Del Castillo et al. 1964; Riviere & Papich 2018). They bind to tubulin and inhibit the polymerisation of tubulin to microtubules. This interferes with all the functions ascribed to microtubule systems at the cellular level and leads to parasite death (Lacey 1988).

Control programs are often based on repeated anthelmintic treatments with the same compound (Agneessens 2006; Tarbiat et al. 2016b) and was also seen in response to our industry survey (Section 2). This, combined with potential for sub-therapeutic doses received by some birds during mass medication provides significant selection pressure for anthelmintic resistance (Martin & Robertson 2010; Knapp-Lawitzke et al. 2015). In the US, reduced efficacy against ascarids of extensively used anthelmintics such as FBZ and LEV has been documented in laying hens and turkeys (Yazwinski et al. 2009; Yazwinski et al. 2013). Information on the current efficacy status of available anthelmintics is needed to allow for evidence-based recommendations for optimal use of these anthelmintics or to provide an incentive for the registration of new products.

In poultry, anthelmintics can be administered either individually or as a flock treatment. Individual administration has the advantage of delivering the correct dose, but this mode of application is not economically feasible on large scale poultry farms. For flock treatment, application via drinking water is the most favoured method of administration (Esmail 1996) (Survey results, Section 2). However, anthelmintic dose uptake will not be homogeneous in all the targeted birds as individual water intake can be influenced by several factors such as bodyweight, age, environmental temperature and other management factors (Landoni & Albarellos 2015). Furthermore, poor anthelmintic palatability can lead to potential rejection or patchy delivery of the target dose, as voluntary consumption is a key factor for feed or water administered anthelmintics (Schmid et al. 2010; Aleo et al. 2018). These can predispose birds to consume sub-therapeutic dose levels, which not only will affect efficacy but may provide the basis for selection of anthelmintic resistance.

Ongoing monitoring of the efficacy of available anthelmintics is important for sustainable worm control in animal agriculture (Demeler et al. 2012). Only one test is currently widely available for evaluating anthelmintic resistance in poultry; the worm count reduction (WCR) test based on controlled slaughter trial (Yazwinski et al. 2003). In order to be considered as efficacious, at least 90% reduction of the targeted worm population is required (Vercruysse et al. 2001; Yazwinski et al. 2003). In other animal industries, the faecal egg count reduction (FECR) test is the most widely used method for detecting anthelminthic resistance (Coles et al. 1992). Resistance should be declared if the percentage FECR is below 95% for ruminants (Coles et al. 1992), however, lower cut off values (90%) have been used in horses and pigs (Coles et al. 2006; Kaplan & Vidyashankar 2012; Vidyashankar et al. 2012). Guidelines for the use of FECR test, hereafter called faecal egg count reduction (FECR) test, have not been established for poultry, and faecal egg counts (FEC) are only considered as supportive data (Yazwinski et al. 2003). Poultry FEC data are frequently characterised by a low mean, high variability, and frequent zero count observations (Demeler et al. 2012). To the best of our knowledge no attempts have been made to evaluate different methods of FECR estimations in parallel with WCR test for anthelmintic efficacy assessment in poultry.

Furthermore, as more cost effective alternatives, various *in vitro* tests or bioassays have been developed for the detection of anthelmintic resistance (Vaarst & Sørensen 2009). These assays measure the effects of anthelmintics on physiological processes such as development, growth and movement (Várady & Čorba 1999). However, the majority is almost exclusively used for research purposes and usually require maintenance of standard laboratory strains, both anthelmintic susceptible and resistant, for comparative purposes (FAO 2004). The egg hatch assay (EHA) and larval development test (LDT) are the most commonly used *in vitro* bioassays for monitoring anthelmintic resistance (Várady & Čorba 1999; FAO 2004; Albonico et al. 2005). The LDT allows the detection of resistance against a wide range of anthelmintics, irrespective of their mode of action (Várady & Čorba 1999; FAO 2004), but is challenging for species, such as *A. galli* where egg hatch occurs within the host. The concentration of anthelmintic required to block development is related to an anticipated *in vivo* efficacy (FAO 2004). Several modifications of these assays have been published for the detection of resistance to benzimidazoles, levamisole and macrocyclic lactones (Wagland et al. 1992; Várady & Čorba 1999; Demeler et al. 2010). Some of these tests include egg hatch paralysis assay, larval paralysis

assay, a larval paralysis assay with physostigmine, larval micromotility assay and larval migration inhibition assays (Várady & Čorba 1999). However, most of these methods have disadvantages in terms of applicability and interpretation or reproducibility of findings and inadequacy for use in the field (Coles et al. 1992; Várady & Čorba 1999).

With this in mind a series of experiments was conducted on the project with the following objectives:

- to evaluate the resistance status of current isolates of poultry nematodes against LEV, PIP, FBZ or FLBZ, and a levamisole-piperazine combination (LEV-PIP);
- to compare the efficacy of calibrated oral administration to individual chickens against mass administration in water or feed;
- to compare the FECR and WCR tests for determining anthelmintic efficacy in chickens; and
- to evaluate the feasibility of *in vitro* methods based on eggs obtained from faeces, to determine anthelmintic efficacy against poultry nematodes.

As noted above, the approach to achieving the objectives was to undertake a series of experiments grouped into 4 studies as follows:

1. Study 1. Anthelmintic efficacy against a suspected LEV resistant isolate comparing oral and water administration of anthelmintics and WCR and FECR tests.
2. Study 2. Anthelmintic efficacy in naturally infected layers with different nematode infections.
3. Study 3. Anthelmintic efficacy against two additional isolates of *A. galli* in trickle infected chicks, including efficacy against immature stages of the parasite.
4. Study 4. Evaluation of *in vitro* methods of evaluating anthelmintic efficacy.

Ethical approval for the studies was provided by the UNE Animal Ethics Committee (approvals AEC19-070 and AEC20-082). Each study is reported separately below.

4.2 Study 1 – Anthelmintic efficacy against a suspected LEV resistant isolate

4.2.1 Methods – Study 1

The study comprised a controlled efficacy trial conducted following the standard guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) for anthelmintic efficacy testing in chickens and turkeys (Yazwinski et al. 2003).

Experimental birds

A total of 120 one-day-old Isa Brown layer cockerel chickens were purchased from a commercial hatchery (Tamworth, NSW, Australia). Chicks were kept for six weeks in floor pens before being moved to enriched individual layer cages in which the experimental treatments occurred.

413. ***galli*** **isolate and artificial infection**

The *A. galli* field strain (UNE-QLD1-2019) used in this study was originally isolated from naturally infected free range laying hens obtained from a commercial poultry farm with a history of regular application of LEV. This is Farm 1 in our on-farm prevalence survey (Section 3.2, Table 3-1). Adult female worms collected from fresh intestines of infected hens killed 27 days after flock deworming with LEV were used as the source of eggs for artificial infection. We therefore defined this isolate as ‘a case of suspected resistance to LEV’. This isolate underwent a single experimental passage for amplification in *A. gall* free layer chickens before being used in this experiment. The adult *A. galli*

female worms were collected from the commercial hens according to standard parasitological techniques (Permin et al. 1997b; Yazwinski et al. 2003). In order to do so, the hens were humanely killed, dissected, and the jejunum and ileum opened longitudinally and mature *A. galli* worms identified and washed into Petri dishes using sterile phosphate-buffered saline.

To isolate eggs, the *A. galli* worms were transferred into RPMI media (with 0.1% 100 units/mL penicillin, 100 µg/mL of streptomycin, 250 ng/mL amphotericin B (Sigma-Aldrich Pty Ltd, St Louis, USA) in a glass jar to a volume that covered the worms (Sharma et al. 2017 Feyera et al. 2020). The *A. galli* worms were then cultured for three days at 37°C, changing the media every 24 hours. Every 24 hours, for 72 hours, the media containing parasite eggs was collected then centrifuged at 425 x g for 1 min, and eggs concentrated at the bottom of the media were collected using transfer pipettes. Eggs were subsequently resuspended in 0.1 N H_2SO_4 and incubated aerobically at 25°C for 6 weeks. Full embryonation was confirmed microscopically. Only eggs demonstrating a coiled larval stage were classed and counted as viable/embryonated as described by Feyera et al. (2020). For inoculation, embryonated eggs were diluted with tap water to generate the desired concentration (eggs/ml) containing the infection dose to be given to each bird. The number of eggs/ml suspension was determined with a modified McMaster method using Whitlock universal egg counting chamber (Whitlock 1948). All the chicks (n = 120) were inoculated via crop gavage needles with an infection dose of 450 eggs in 3 split doses (150 eggs at a time) over one week starting from the day of arrival.

To monitor infection development and the onset of egg shedding in the infected birds, faecal samples were collected from individual birds starting from 6 weeks post-infection. Fresh droppings were collected by placing paper sheets beneath the individual cages during daylight hours when egg production is maximal (Wongrak et al. 2015). At 8 weeks of age (also 8 weeks post-infection), 108 birds that had a pre-treatment FEC of at least 100 EPG (Yazwinski et al. 2003) were admitted to the trial. All cockerels were weighed one day before their scheduled treatment. The cockerels were then randomised on the basis of FEC and bodyweight into one of nine experimental groups of 12 birds each, compatible with WAAVP guidelines requiring a minimum of 6 infected birds per treatment group on the day of treatment (Yazwinski et al. 2003). Each anthelmintic treatment was applied to a group either by oral bolus or in drinking water so there were eight treatment groups and one untreated control group (Table 4-1). On the day of treatment (d 0), individual bird faeces were collected from all birds to assess pre-treatment FEC.

Experimental outline and anthelmintic administration

The study involved two registered commercial anthelmintics for poultry in Australia namely LEV (CCD Levamisole®, 850 mg/g LEV as levamisole dihydrochloride) and PIP (CCD Piperazine®, 530 mg/g PIP as piperazine hydrochloride) both water soluble powders (CCD Animal Health, Tamworth, NSW, Australia) plus their combination (LEV-PIP), and FBZ (Panacur 25®, Intervet Australia Pty Ltd, East Bendigo, Victoria, Australia, 25 mg FBZ/ml) as a potential off-label prescription product. Details on experimental outline, treatment groups and anthelmintic regimens are presented in Table 4-1.

Table 4-1 Study 1 – Experimental groups and dosage regimens for the tested products

Anthelmintic	N	Mode of application	Dose	Application details
LEV	12	Individual oral	28 mg/kg	Bolus dose
	12	Group drinking water	28 mg/kg	0.8 mg LEV/ml of drinking water over 8 hrs
PIP	12	Individual oral	100 mg/kg	Bolus dose
	12	Group drinking water	100 mg/kg	2.5 mg PIP/ml of drinking water for 8 hrs each day over 2 days
FBZ	12	Individual oral	10 mg/kg	Bolus dose
	12	Group drinking water	5 mg/kg	0.023 mg FBZ/ml of drinking water for 8 hrs each day over 5 consecutive days (off-label use only, dose based on (CVMP, 2014))
LEV-PIP	12	Individual oral	28 mg/kg LEV + 100 mg/kg PIP	Bolus dose
	12	Group drinking water	28 mg/kg LEV + 100 mg/kg PIP	0.8 mg LEV/ml of drinking water for 8 hrs; 2.5 mg PIP/ml of drinking water for 8 hrs each day over 2 days
Untreated control	12	NA	NA	NA

LEV: Levamisole.
PIP: Piperazine.
FBZ: Fenbendazole.
LEV-PIP: Levamisole-piperazine combination.
NA: N*ot applicable.*

LEV and PIP were applied as per the manufacturer's recommendation for each product. The LEV-PIP regimen involved both compounds co-administered at their full recommended individual dose rates. FBZ was tested as an off-label prescription, which is marketed as an oral suspension for cattle and sheep in Australia. The dosage regimen for this formulation was adopted from recommendations for a similar product (Panacur AquaSol 200 mg/ml suspension) used against *A. galli* in drinking water in Europe (CVMP 2014). For individual oral treatment, anthelmintics were delivered into the crop via gavage needles individually as a bolus using a dose rate calculated based on the bodyweight of the individual bird. For birds treated via drinking water, water consumption for each group was measured over three consecutive days, consumption levels averaged, and each anthelmintic dose was offered in 60% of normal daily water consumption for a period of 8 daylight hours. The medication was administered via nipple drinkers connected to a medication tank for each group, with dose calculation based on total bodyweight of all birds in the specific group. Birds were withheld from drinking water overnight and anthelmintics applied the following morning for 8 hours in drinking water. At the end of the 8-hour treatment period, when the total amount of medicated water offered had been consumed, medication tanks were turned off and untreated drinking water provided to the experimental birds. In all cases of reconstitution in water, mixtures were stirred until a homogeneous solution or suspension was formed. Control birds were left unmedicated.

Necropsy and parasitological measurements

Ten days post-anthelmintic treatment (d 10) on the same day as necropsy, individual faecal samples were collected from each cockerel from paper sheets placed under each cage to estimate post-treatment individual FEC. For post-mortem examination, birds were humanely killed by cervical dislocation and their intact intestines from the gizzard to the vent retracted, cleaned of all mesentery and then opened longitudinally. Worm recovery and burdens were determined according to standard laboratory methods consistent with guidelines published by the WAAVP (Yazwinski et al. 2003). The

digesta obtained from the small intestine was flushed with tap water through a 100 µm mesh sieve and examined for the presence of adult and immature *A. galli*. Adult worms were collected and enumerated then the sample was examined under a microscope to enumerate immature worms. The FECs were determined using the modified McMaster method with a minimum detection level of 40 EPG using universal Whitlock slides (Whitlock 1948). Briefly, 2.5 g faeces was made up to 50 ml in saturated NaCl solution, homogenised and loaded into the 0.5 ml slide chambers and eggs counted.

Statistical analysis and efficacy calculations

Data were statistically analysed using JMP® software version 14.3.0 (SAS Institute Inc., Cary, NC, USA). Statistical tests were performed on the worm count data (primary parameter) and the egg count data (secondary parameter) using ANOVA. Each individual bird was considered as one experimental unit with fixed effects being anthelmintic treatment and route of administration. Prior to statistical analysis, worm counts and FEC were logarithmically transformed for compliance with the assumptions of ANOVA. A significance level of $P \leq 0.05$ was used throughout the analysis.

Anthelmintic efficacy was calculated as WCR% (gold standard) using geometric mean worm counts according to WAAVP guidelines for testing effectiveness of anthelmintics in poultry, and efficacies below 90% were considered ineffective (Yazwinski et al. 2003).

$$\% \text{ Efficacy} = 100 \times \left(\frac{\text{Mean number of worms in control} - \text{Mean number of worms in treated}}{\text{Mean number of worms in control}}\right)$$

As supportive data and for comparison with WCR%, FECR% was calculated using two different methods:

FECR1: This method is recommended by WAAVP (Coles et al. 1992). It employed post-treatment arithmetic mean FECs of both the treated and control groups.

$$\text{FECR\%} = 100 \times \left(\frac{\text{AM post treatment control FEC} - \text{AM post treatment FEC}}{\text{AM post treatment control FEC}}\right)$$

Confidence levels of 95% were calculated using an Excel spreadsheet originally created based on RESO computer program for analysis of FECRTs in sheep which allows entry of test results (raw worm egg count data) from up to 20 animals per group. The calculations were based on those published in 'Anthelmintic Resistance': Report of the Working Party for the Animal Health Committee of the Standing Committee on Agriculture (Waller et al. 1989) and interval estimations for proportions as described elsewhere (Brown et al. 2001).

FECR2: This was adopted from a method described by Pook et al. (2002) without modification. The FECR proportions of individual birds were calculated (Pre-treatment FEC – Post-treatment FEC/Pre-treatment FEC) the data transformed (arcsine) and group means calculated (FECR% = 100 × (sin (transformed group mean))^2). Confidence levels at the level of 95% were calculated using the standard deviation of the mean of the data generated in Microsoft Excel prior to retransformation (Pook et al. 2002).

Because the cut-off values for FECR tests have not yet been harmonised for poultry, a threshold of 90% similar to the WCR test, and as also used in horses and pigs (Coles et al. 2006; Kaplan & Vidyashankar 2012; Vidyashankar et al. 2012), was considered for both FECR methods.

4.2.2 Results – Study 1

Infection, worm count reduction and anthelmintic efficacy

Out of the 120 birds initially infected, 110 (91.7%) were found to be shedding worm eggs at the onset of treatment (8 weeks post-infection) but only 108 (90%) achieved a minimum of 100 EPG before treatment and thus were recruited for this study. All birds (100%) in the untreated control group harboured *A. galli* infection at necropsy 10 days after treatment. For each anthelmintic, a higher proportion of birds treated via drinking water harboured worm infection than their corresponding individual oral administration groups. Worm count data and percentage anthelmintic efficacies are presented in Table 4-2.

All the untreated control birds harboured at least 2 worms with a mean worm count of 7.67±0.91 per bird. The untreated control birds had a significantly higher ($P < 0.0001$) worm counts compared to the treatment groups irrespective of mode of anthelmintic application. Mode of anthelmintic application had a significant effect ($P = 0.03$) on the worm count at necropsy, with birds receiving individual oral administration having lower counts than those receiving medication in drinking water. Treatment with LEV, PIP and LEV-PIP either by individual or water administration significantly depressed worm counts and attained the desired efficacy value ≥ 90%. However, LEV-PIP combination was the most effective although it did not differ significantly in efficacy from LEV or PIP alone (Table 4-3). FBZ (10 mg/kg) as single oral bolus achieved optimum WCR (97.2%), however, FBZ delivered via drinking water (at the rate of 1 mg/kg over 5 days) demonstrated the lowest anthelmintic efficacy (88.7%) and the highest intestinal worm load (1.42±0.59 *A. galli* worms/bird).

Table 4-2 Study 1 – Proportion of birds harbouring infection, number of worms recovered at necropsy and the anthelmintic efficacies for each treatment group

Treatment	Proportion of birds harbouring infection (%)	Total worm count/bird				Efficacy (%)
		Range	AM	Ln [count + 1] ±SE	GM	
Untreated control	12/12 (100)	2-14	7.67	2.09±0.13[a]	7.08	NA
LEV						
Individual oral	1/12 (8.3)	0-1	0.08	0.06±0.13[bc]	0.06	99.1
Group drinking water	2/12 (16.7)	0-3	0.33	0.17±0.13[bc]	0.19	96.4
PIP						
Individual oral	3/12 (25.0)	0-3	0.42	0.23±0.13[bc]	0.26	96.3
Group drinking water	4/12 (33.3)	0-6	0.83	0.34±0.13[bc]	0.45	93.7
FBZ						
Individual oral	2/12 (16.7)	0-1	0.17	0.12±0.13[bc]	0.13	97.2
Group drinking water	5/12 (41.7)	0-6	1.42	0.59±0.13[b]	0.81	**88.7**
LEV-PIP						
Individual oral	0	0	0.00	0±0.13[c]	0	100
Group drinking water	2/12 (16.7)	0-2	0.25	0.15±0.13[bc]	0.16	97.7

AM: Arithmetic mean.
SE: Standard error.
GM: Geometric mean.
NA: NI applicable.
LEV: Levamisole.
PIP: Piperazine.
FBZ: Fenbendazole.
LEV-PIP Levamisole-piperazine combination.
Mean values in the same column not sharing a common letter in the superscript (a, b, c) are statistically different ($P < 0.05$).
Statistical analyses were based on transformed data (Ln [count+1]).
Value in bold indicates efficacy < 90%.

Faecal egg count reduction

The mean pre- and post-treatment FEC estimated by two methods are shown in Table 4-3. All experimental groups had similar (P > 0.05) pre-treatment (d 0) mean FECs. However, post-treatment (d 10) FECs were significantly (P < 0.0001) reduced in all treatment groups compared to the untreated control group. A positive linear association existed between post-treatment individual FEC (EPG) and worm count (Figure 4-1). The relationship indicates that a single worm contributes on average about 57 eggs per gram of faeces when sampled. For each anthelmintic treatment, individual oral administration caused a greater reduction (P = 0.031) in FEC compared to medication in drinking water. When an FECR cut-off value similar to the WCR (≥ 90%) was applied, the two FECR methods agreed in 8/8 (100%) of treatment groups, but FECR1 generally provided lower estimated efficacies than FECR2 possibly due to the influence of the control group on the calculation method. With both FECR methods, only FBZ delivered via drinking water (at the rate of 1 mg/kg over 5 days) demonstrated a reduction below the required minimum cut-off value (< 90%).

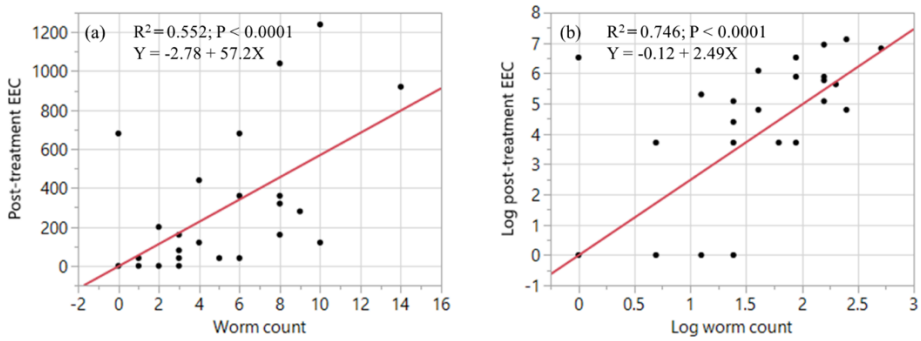


Figure 4-1 Study 1 – Linear regression plot showing the association between post-treatment individual FEC (EPG) and worm count

(a) FEC against worm count.
(b) Log FEC (Ln [count+1]) against log worm count (Ln [count+1]).
FEC: Faecal egg count.
EPG: Eggs per gram of faeces.

FECR1 was more strongly correlated with WCR (P = 0.0004) than FECR2 (P = 0.0019), probably due to the later slightly overestimating the efficacy values or indicating the advantage of using control group in FECR1. Although the FECR calculation methods appeared to agree with the WCR values, the two tests did slightly differ in the magnitude of efficacy estimated.

Table 4-3 Study 1 – Mean pre-/post-treatment faecal egg counts and percentage egg count reductions as estimated by two methods

Treatment	Pre-treatment FEC		Post-treatment FEC			FECR% (LCL, UCL)	
	Number of birds with worm eggs detected (%)	AM	Number of birds with worm eggs detected (%)	AM	Ln [count+1]±SE	FECR1	FECR2
Untreated control	12/12 (100)	310	11/12 (91.7)	440	5.25±0.45[a]	NA	NA
LEV	12/12 (100)						
Individual oral	12/12 (100)	323	0	0	0.00±0.45[b]	100	100
Group drinking water	12/12 (100)	486	1/12 (83.3)	13.3	0.43±0.45[b]	97 (73, 100)	99 (95, 100)
PIP	12/12 (100)						
Individual oral	12/12 (100)	440	1/12 (83.3)	6.67	0.37±0.45[b]	98 (87, 100)	99 (82, 100)
Group drinking water	12/12 (100)	264	2/12 (16.7)	33.3	0.80±0.45[b]	92 (46, 99)	97 (65, 99)
FBZ	12/12 (100)						
Individual oral	11/12 (91.7)	563	1/12 (83.3)	3.33	0.31±0.45[b]	99 (93, 100)	99 (99, 100)
Group drinking water	12/12 (100)	347	4/12 (33.3)	100	1.67±0.45[b]	**77** (2, 95)	**81** (68, 97)
LEV-PIP	12/12 (100)						
Individual oral	12/12 (100)	360	0	0	0.00±0.45[b]	100	100
Group drinking water	12/12 (100)	290	1/12 (83.3)	16.7	0.44±0.45[b]	95 (67, 100)	99 (94, 100)

FEC: Faecal egg count.
AM: Arithmetic mean.
SE: Standard error.
UCL: Upper confidence level.
LCL: Lower confidence level.
NA: Not applicable.
LEV: Levamisole.
PIP: Piperazine.
FBZ: Fenbendazole.
LEV-PIP: Levamisole-piperazine combination.
FECR as calculated by WAAVP method (FECR1) and AM of arcsine-transformed individual bird FECR% (FECR2).
Means (Ln [count+1]) along the same column with unlike superscripts (a, b) are different ($P < 0.05$).
Values in bold indicate efficacy < 90%.

4.2.3 Discussion and conclusions – Study 1

This is the first formal report on the efficacy of commercial anthelmintics against chicken ascaridiasis in Australia. For all products tested, individual oral administration provided an overall better anthelmintic efficacy compared to group medication in drinking water. Worm count reduction was the primary efficacy variable assessed but two measures of faecal egg count reduction were also evaluated and found to be in agreement with the WCR data. Contrary to our suspicion, there was no evidence of resistance of the test *A. galli* isolate to both LEV and PIP. LEV in combination with PIP also provided excellent efficacy when administered by either method as did FBZ administered as a single oral bolus (10 mg/kg). However, FBZ delivered via drinking water at a lower dose rate over 5 days demonstrated the lowest anthelmintic efficacy (88.7%).

Birds treated via drinking water had relatively higher mean worm and egg counts but also higher prevalence of infection post-treatment than those treated individually. This clearly illustrates the risk of less uniform delivery when using mass administration methods. In this case, poor or uneven delivery of the target anthelmintic dose in drinking water could be due to multiple factors. The main reason may be low palatability of the anthelmintics. For instance, LEV and BZ formulations are well known for their bitter taste (Riviere & Papich 2018), which may affect voluntary uptake by birds when applied via the drinking water system. Likewise, anthelmintic solubility problems may also impede water administered anthelmintics from reaching therapeutic levels (Dorrestein et al. 1986). While LEV and PIP are water soluble (Riviere & Papich 2018), the FBZ oral suspension is characterised by poor solubility in water (Sander & Schwartz 1994). No attempt was made during the experiment to maintain the suspension in the medication tanks by continuous mixing, and some settling of FBZ out of suspension may have occurred during each 8-hour administration period. This would have been exacerbated by the low dose rate used, based on the cumulative action of BZ anthelmintics in their nematode targets (Lacey 1988; Riviere & Papich 2018). In this regard it is pertinent to note that the recent registration of the BZ anthelmintic flubendazole for chickens in Australia is for administration in feed, not in water.

Despite the lower overall efficacy of water administration, it should be noted that LEV and PIP alone, or their combination provided adequate efficacy (as assessed by WCR) by both modes of administration. It was only for FBZ that a major discrepancy in efficacy between the two administration modes was observed for likely reasons covered in the discussion above.

The percentage efficacies derived from FECR%, by both FECR1 and FECR2, were in agreement (100%) with their corresponding WCR% and there were strong positive correlations between the efficacy estimates generated by all the three methods, suggesting that either FECR method is likely to result in similar efficacy values with the WCR using the current infection model and mean FEC values exceeding 300 EPG. The current study employed a mature *A. galli* infection model with no continuing infection and/or larval development, and anthelmintic treatment instituted after the maximum pre-patent period (8 weeks post-infection) where worm load and worm egg output are expected to be positively correlated (Daş et al. 2017). These results are in accordance with comparative FECR studies in ruminants for the case where there is no continuing larval development (Lyndal-Murphy et al. 2014). Efficacies obtained by FECR1 appeared to show a slightly stronger association with the corresponding WCR than FECR2, probably indicating the advantages of using the control group in efficacy calculation as in the WCR test. However, FECR1 produced wider CIs indicating more variability and this may lead to issues with declarations of resistance if CL is considered in the calculation as has been recommended in other species. FECR2 has the advantage that birds were compared with themselves reducing variation in efficacy calculations and therefore increases the power of the statistical test (Pook et al. 2002), but it does not account for changes in worm egg count during the 10-day test period due to factors other than the anthelmintic treatment. FECR guidelines have not been readily adopted for measuring anthelmintic resistance in poultry partly because the cost of

conducting WCR is low relative to most other species, and also because the association between worm burdens and egg counts may be influenced by a number of factors. In chickens FECs are recognised as being highly variable and as such the gold standard method for quantifying efficacy is by direct worm counts at necropsy (Yazwinski et al. 2003). However, using the experimental model of the present experiment based on chicks infected with *A. galli* at day old and protected against additional infection or cross infection with other parasites, the agreement between WCR and FECR was very good.

Contrary to our suspicion, the results of the current study did not give any evidence of reduced susceptibility of the test *A. galli* isolate to LEV or PIP. This isolate originated from a farm where LEV had been used regularly and the isolate came from worms that had survived a mass water treatment with LEV. Given that this product has been in the market for half a century in Australia, induction of resistance in the resident ascarid population could be expected in farms where this anthelmintic has been used extensively. Widespread resistance of LEV in sheep (Barton 1983; Overend et al. 1994; Besier & Love 2003) and cattle (Rendell 2010; Cotter et al. 2015) nematodes occurs in Australia. Since our study involved only a single isolate, more controlled trials are needed to ascertain the current performance of this product in different poultry facilities across Australia. In the US, where LEV was highly effective (100%) at the time of introduction for use in poultry (Kates et al. 1969; Cruthers 1975), depressed activity (< 90%) against ascarids in turkeys (*A. dissimilis*) has been documented (Yazwinski et al. 2009), however, we failed to uncover a single report of LEV resistance in *A. galli* or other chicken nematodes. This could be considered as positive for the poultry industry given that anthelmintic resistance is prevalent and of grave concern in the ruminant production sector.

Like LEV, PIP also exhibited optimal efficacy (≥ 90%) against the test isolate irrespective of mode of administration although it showed slightly lower efficacy than LEV. Historically, PIP has also been extensively used to control nematode infection in breeder flocks in Australia (Ruhnke 2015). The good efficacy demonstrated by PIP in the current study is supported by its therapeutic spectrum of activity that it is effective mainly against adult *A. galli* (Del Castillo et al. 1964) and may not eliminate immature parasites adequately at the recommended dose (Presson & Yazwinski 1983; Nilsson & Alderin 1988). The current infection was treated at 8 weeks post-infection and immature worms were very rarely encountered during worm recovery. Furthermore, the test isolate had no clear history of recent exposure to PIP and the observed efficacy shows significant susceptibility of the test isolate to the dose rate used in the current study (100 mg/kg), which is recommended by the manufacturer and routinely used in the field.

Anthelmintic combination is an established strategy for sustainable parasite control in ruminant grazing systems (Anderson et al. 1991; Little et al. 2011). Combinations enable control of nematodes with anthelmintic resistance (Anderson et al. 1991; Little et al. 2011) or slow the development of resistance to the component anthelmintic classes (Bartram et al. 2012; Smith 2014). In the current study, LEV-PIP combination provided excellent anthelmintic efficacy, which was generally similar to LEV. Given the relatively lower efficacy following PIP treatment, it is likely that LEV contributed predominantly to the efficacy of the combination. However, an additive or synergistic pharmacodynamic effect between these two anthelmintics against *A. galli* could not be discounted.

In the current study, FBZ was tested as an off-label product, primarily prescribed by industry veterinarians due to its additional action on tapeworms. As a single oral treatment (10 mg/kg), it achieved an efficacy above the minimum threshold (≥ 90%) (Vercruysse et al. 2001; Yazwinski et al. 2003). However, its application in drinking water (at the rate of 1 mg/kg over 5 days) resulted in sub-optimal efficacy (88.7%). The optimum efficacy demonstrated by oral regimen indicates a significant susceptibility of the test isolate to FBZ. But, because this was an off-label dose rate and the tested product was an oral formulation for ruminants, caution should be exhibited in drawing conclusions from these data except where they reflect the actual field practice. The dose rate used in drinking water has been standard for chickens in various European countries (Panacur AquaSol) with

percentage efficacies of up to 100% against *A. galli* (CVMP 2014). This document mentions that formulation of FBZ as Panacur AquaSol increases its solubility and oral bioavailability to some extent. The significant discrepancy in efficacy of FBZ between the oral regimen and in drinking water can be ascribed to different factors. First, because BZ anthelmintics as a chemical class have limited water solubility and are generally administered as micronised suspensions it is likely that this low efficacy could be due to resultant settling of the active ingredient out of suspension leading to poor dose delivery (Vermeulen et al. 2002). Sedimentation of water suspensible formulations of FBZ has also been reported to account for lower therapeutic efficacy elsewhere (Sander & Schwartz 1994). Second, although this product was administered over 5 days in drinking water, the discontinuous nature of application over 8 hrs per day may have been insufficient to achieve the cumulative binding effect at the parasite target site lowering its pharmacological activity. In conclusion, this sub-optimal efficacy could increase the risk for development of resistance to FBZ in the case of frequent repeated use. The development of nematode resistance to BZs has become a significant problem in other animal industries, but so far not in the poultry sector.

Conclusions

This controlled efficacy study showed that individual oral administration resulted in higher anthelmintic efficacy compared to group medication in drinking water. There was no evidence of loss of susceptibility of the test *A. galli* isolate to both LEV and PIP contrary to our hypothesis. The combination of these products also exhibited excellent efficacy and provides an option for control of chicken ascaridiasis in the future should resistance to one or both anthelmintics emerge as a problem. FBZ applied in drinking water at the dose rate used had inadequate efficacy and may potentially predispose to selection for BZ resistance in *A. galli* if used widely in the field. Using the current artificial infection model with anthelmintic treatment instituted after the prepatent period, FECR provided very good agreement with WCR and can potentially be used to screen for anthelmintic resistance in *A. galli*. Additional controlled anthelmintic efficacy studies are needed using worm isolates sourced from different poultry facilities across Australia.

4.3 Study 2 – Anthelmintic efficacy in naturally infected layers with different nematode infections

The overall objective of this study was to provide additional data on the current efficacy status of commonly used anthelmintics in the Australian poultry sector using a different model to that of Study 1, one based on using mature chickens with natural infections.

4.3.1 Methods – Study 2

Study farms and experimental birds

This study employed naturally infected laying hens obtained from two free range egg farms in Australia (Farms 3 and 5 from Section 3). The farms had different histories of anthelmintic use in the recent past (Table 3-1). These farms were selected based on the on-farm parasite survey where both farms had high prevalence of infection with *A. galli* and *H. gallinarum (*98–100%, Table 3-3). From each farm, 110–120 birds were transported to UNE poultry research facilities and housed in individual cages for the anthelmintic efficacy trial under UNE animal ethics approval number AEC20-082.

- **Farm 3 (F3)** is an organic free range farm with no recent history of anthelmintic use. A total of 110 laying hens at the age of scheduled depopulation (78 weeks old) were selected from a flock harbouring *A. galli*, *H. gallinarum* and *Capillaria* spp. with a corresponding prevalence of 98, 99 and 51% respectively.

- **Farm 5 (F5)** is a free range farm with a recent history of use of mebendazole in drinking water (birds treated at 13 weeks of age). A total of 120 laying hens (75 weeks old) naturally infected with ranges of helminth parasites were recruited from a flock having 97, 100, 27 and 35% prevalence of *A. galli*, *H. gallinarum*, *Capillaria* spp. and Cestodes respectively.

Experimental outline and anthelmintic administration

A commercial layer ration and water were offered *ad libitum* over the experimental period. After a 1-week acclimatisation period and screening for nematode eggs in the faeces, the hens from each farm were then stratified on the basis of FEC into experimental groups of 22 birds (F3) or 20 birds (F5) with approximately similar FEC. This exceeds the WAAVP guidelines requiring a minimum of 6 infected birds per treatment group on the day of treatment. Details on experimental outline, treatment groups and anthelmintic regimens for each farm are presented in Table 4-4.

Table 4-4 Study 2 – Experimental groups and dosage regimens for the tested products

Farm	Number of birds	Anthelminthic	Mode of application	Dose	Dosage regimen
F3	22	Levamisole	Oral inoculation	28mg/kg	Bolus dose
	22	Piperazine	Oral inoculation	100 mg/kg	Bolus dose
	22	LEV-PIP	Oral inoculation	28 mg/kg LEV + 100 mg/kg PIP	Bolus dose
	22	Fenbendazole	Oral inoculation	10mg/kg	Bolus dose
	22	Untreated	NA	NA	NA
F5	20	Levamisole	Oral inoculation	28mg/kg	Bolus dose
	20	Piperazine	Oral inoculation	100 mg/kg	Bolus dose
	20	LEV-PIP	Oral inoculation	28 mg/kg LEV + 100 mg/kg PIP	Bolus dose
	20	Flubendazole-N	In feed	30 ppm	Over 7 days
	20	Flubendazole-C	In feed	60 ppm	Over 7 days
	19	Untreated	NA	NA	NA

Farm numbers and descriptions are from Table 3-1, and the descriptions are retained throughout the report for consistency and ease of cross referencing.
NA: Not applicable.
LEV: Levamisole.
PIP: Piperazine.
LEV-PIP: Levamisole-piperazine combination.
Flubendazole-N: Flubendazole nematode dose (30ppm).
Flubendazole-C (60ppm): Flubendazole cestode dose.

Excreta egg count

On the day of treatment (d 0), individual bird faeces were collected from all treatment groups to assess pre-treatment FEC. Individual faeces materials were also collected from each bird right before necropsy to estimate post-treatment individual FECs using the MM methods outlined at 4.2.1.

Worm recovery and count

Ten days post-anthelmintic treatment (d 10), all experimental birds were humanely killed to recover worms from the intestines for assessment of total worm count. Both adult and immature stages of nematode worms were recovered and enumerated based on standard parasitological procedures described elsewhere (Yazwinski et al. 2003; Tucker et al. 2007; Tarbiat et al. 2016a).

Data analysis

Data were statistically analysed using JMP® software version 14.3.0 (SAS Institute Inc., Cary, USA). Statistical tests were performed using a one-way ANOVA. Prior to statistical analysis, worm counts and FEC were logarithmically transformed (ln [count + 1]) for compliance with the assumptions of ANOVA. Anthelmintic efficacy was calculated using standard WAAVP formula for both WCR% and FECR% (Coles et al. 1992; Yazwinski et al. 2003). A 90% cut-off value was considered for both WCR% and FECR%.

4.3.2 Results – Study 2

Worm count data from both farms are presented in Table 4-5 (F3) and Table 4-6 (F5). On Farm 3 the prevalence of infection with *A. galli, H. gallinarum* and *Capillaria* spp. in control birds was 100%, 95.5% and 50% respectively in the untreated control group, with average adult worm counts of 22.8, 58.2 and 7.3 respectively. A high proportion of birds (> 90%) also carried immature worms at somewhat lower counts (Table 4-5). On Farm 5 the prevalence of infection with *A. galli, H. gallinarum* and *Capillaria* spp. in control birds was 94.7%, 94.7% and 30% respectively in the untreated control group, with average adult worm counts of 37.4, 61.8 and 5.9 respectively. A high proportion of birds (> 94%) also carried immature worms of *A. galli* and *H. gallinarum* at somewhat lower counts (Table 4-6).

The level If infection on both farms was therefore ideal for assessment of anthelmintic activity against *A. galli* and *H. gallinarum* but somewhat less so for *Capillaria* spp. Unlike Farm 3, Farm 5 also had significant tapeworm infections (26.3% prevalence and mean burden on 0.84 in controls), allowing evaluation of the efficacy of anthelmintics against these parasites.

Significant reductions in all parasites targeted by relevant chemicals were observed. Arithmetic mean counts are shown in Table 4-5 and Table 4-6 for Farms 3 and 5 respectively and geometric mean counts with statistical differences are shown for both farms in Table 4-7. Percentage efficacies for the anthelmintics based on treatment group geometric means (WCR%) are shown in Table 4-8. These show that levamisole and LEV+PIP treatments had good efficacy against both mature and immature stages of all 3 nematode species on both farms, with no effect on tapeworm counts as expected. On the other hand, piperazine was only fully effective against adult *A. galli* on both farms, as also observed in Study 1. It has reduced efficacy (82%) against immature *A. galli*, and poor efficacy on both farms against adult and immature stages of *H. gallinarum* and *Capillaria* spp. (25–66%). Fenbendazole administered orally provided good control of all 3 roundworm species (94–99%). Flubendazole at the nematode control level of inclusion in feed for 7 days (30 ppm) allowed for the control of roundworms of all species (> 99%) but less consistent control of tapeworms (91.7%). At the cestode (tapeworm) control level of (60 ppm), 100% control of all species was achieved. There is a slight risk of selecting for anthelmintic resistance in the tapeworm population if the lower dose is used, so if tapeworms are present, it would be recommended to use the higher dose.

The relationship between worm numbers and egg counts, differs widely for different sized nematodes, with egg counts tending to be dominated by the larger more prolific egg producing species when there are mixed infections as is the case in this study. This is evident in the increasing strength of association of FEC with worm counts as worm counts progress from all species and stages, to just adult *A. galli* (Figure 4-2). The FECR test did detect the sub-optimal efficacy of piperazine on Farm 1, but not Farm 2 while confirming the efficacy of the other anthelmintic treatments (Table 4-9). Because of the lack of specificity and accuracy of the FECR method in mixed infections it cannot be recommended as other than a crude measure of anthelmintic efficacy.

Table 4-5 Study 2 Farm 3 – Nematode counts at necropsy (arithmetic mean) by treatment group

Nematodes	Parameter	Treatment groups				
		Untreated	Levamisole	Piperazine	LEV+PIP	Fenbendazole
A. galli						
Adult	Mean[1]±SD	22.8±18.1	0.00±0.00	1.30±0.00	0.00±0.00	0.91±1.90
	Range	3-60	0	0-8	0	0-6
	Percentage infected	100	0	22.7	0	18.2
Immature	Mean±SD	6.8±7.69	0.59±1.98	2.05±3.55	0.00±0.00	0.64±1.40
	Range	0-31	0-5	0-10	0	0-5
	Percentage infected	95.5	13.6	31.8	0	22.7
H. gallinarum						
Adult	Mean±SD	58.2±12.3	1.68±3.47	15.5±12.3	0.00±0.00	2.86±5.18
	Range	0-270	0-8	0-37	0	0-12
	Percentage infected	95.5	22.7	86.4	0	27.3
Immature	Mean±SD	16.2±16.6	1.27±2.62	8.09±6.74	0.36±1.22	1.05±2.59
	Range	0-69	0-9	0-24	0-4	0-3
	Percentage infected	90.9	22.7	86.4	9.1	18.1
Capillaria spp.						
	Mean±SD	7.27±9.65	0.41±1.14	5.82±1.14	0.14±0.47	0.27±0.77
	Range	0-31	0-4	0-27	0-1	0-2
	Percentage infected	50	13.6	45.4	4.5	9
All nematodes						
	Mean±SD	116.8±91.2	4.18±5.37	33.1±21.2	0.55±1.37	5.59±7.71
	Range	9-362	0-14	3-69	0-4	0-6
	Percentage infected	100	45.5	100	18.1	54.5

[1] Means are the arithmetic mean numbers of helminths per bird for the treatment group.
LEV-PIP: Levamisole-piperazine combination.

Table 4-6 Study 2 Farm 5 – Nematode counts at necropsy (arithmetic mean) by treatment group

Nematodes	Parameter	Treatment groups					
		Untreated	Levamisole	Piperazine	LEV+PIP	Flubendazole-N	Flubendazole-C
A. galli							
Adult	Mean[1]±SD	37.4±29.1	0.60±1.43	1.10±1.97	0.00±0.00	0.00±0.00	0.00±0.00
	Range	0-108	0-4	0-7	0	0	0
	Percentage infected	94.7	10	25	0	0	0
Immature	Mean±SD	8.10±4.87	0.65±1.39	2.25 ±3.79	0.00±0.00	0.00±0.00	0.00±0.00
	Range	0-17	0-5	0-7	0	0	0
	Percentage infected	94.7	10	30	0	0	0
H. gallinarum							
Adult	Mean±SD	61.8±60.7	2.75±5.16	31.9±26.3	1.05±2.35	0.40±0.99	0.00±0.00
	Range	0-258	0-12	0-21	0-8	0-3	0
	Percentage infected	94.7	25	75	20	10	0
Immature	Mean±SD	33.4±21.8	3.15±5.45	24.6±18.7	1.50±3.12	0.45±1.23	0±0.00
	Range	0-114	0-12	0-49	0-6	0-3	0
	Percentage infected	94.7	20	80	20	10	0
Capillaria spp.							
	Mean±SD	5.94±11.7	0.15±0.67	2.00±4.05	0.00±.00	0.00±0.00	0.00±0.00
	Range	0-43	0-3	0-14	0	0	0
	Percentage infected	30	5	25	0	0	0
All nematodes							
	Mean±SD	145.1±93.3	7.20±7.91	61.8±42.3	2.85±5.04	0.80±1.74	0.00±0.00
	Range	39-445	0-20	7-145	0-9	0-3	0
	Percentage infected	100	45	100	35	15	0
Cestodes							
	Mean±SD	0.84±1.71	0.85±1.66	0.75±1.59	0.90±1.59	0.05±0.22	0.00±0.00
	Range	0-6	0-6	0-5	0-7	0-1	0
	Percentage infected	26.3	30	20	30	50	

[1] Means are the arithmetic mean numbers of helminths per bird for the treatment group.
LEV-PIP: Levamisole-piperazine combination.
Flubendazole-N: Flubendazole nematode dose (30ppm).
Flubendazole-C (60ppm): Flubendazole cestode dose.

Table 4-7 Study 2 – Geometric means of nematode counts by farm and treatment group

Farm	Nematode	Treatment groups						
		Untreated	Levamisole	Piperazine	LEV+PIP	Fenbendazole	Flubendazole-N	Flubendazole-C
F3	*A. galli*							
	Adult	16.1[a]	0[b]	0.50[b]	0[b]	0.42[b]	-	-
	Immature	4.27[a]	0. 19[b]	0.77[b]	0[b]	0.20[b]	-	-
	H. gallinarum							
	Adult	29.2[a]	0.59[c]	9.82[b]	0[c]	0.92[c]	-	-
	Immature	2.61[a]	0.48[c]	5.61[b]	0.14[c]	0.31[c]	-	-
	Capillaria spp.	2.60[a]	0.20[b]	1.93[a]	0.08[b]	0.16[b]	-	-
	All nematodes	60.7[a]	1.90[c]	18.7[b]	0.27[c]	2.56[c]	-	-
F5	*A. galli*							
	Adult	21.9[a]	0.26[b]	0.55[b]	0[b]	-	0[b]	0[b]
	Immature	6.37[a]	0.27[b]	0.94[b]	0[b]	-	0[b]	0[b]
	H. gallinarum							
	Adult	38.1[a]	0.99[c]	14.0[b]	0.45[c]	-	0.21[c]	0[c]
	Immature	24.9[a]	0.80[c]	12.5[b]	0.59[c]	-	0.22[c]	0[c]
	Capillaria spp.	1.24[a]	0.07[b]	0.69[ab]	0[b]	-	0[b]	0[b]
	All nematodes	118.2[a]	3.29[c]	45.8[b]	1.13[c]	-	0.37[c]	0[c]
	Cestodes	0.43[a]	0.45[a]	0.35[a]	0.49[a]		0.04[a]	0[a]

[a,b,c] Means on the same row with not sharing the same superscripts are different ($P < 0.05$).
- Not tested.
LEV-PIP: Levamisole-piperazine combination.
Flubendazole-N: Flubendazole nematode dose (30ppm).
Flubendazole-C (60ppm): Flubendazole cestode dose.

Table 4-8 Study 2 – Percentage efficacies for anthelmintics based on treatment group geometric means

Farm	Nematode	Efficacy by treatment group					
		Levamisole	**Piperazine**	**LEV+PIP**	**Fenbendazole**	**Flubendazole-N**	**Flubendazole-C**
F3	*A. galli*						
	Adult	100	96.8	100	97.4	-	-
	Immature	95.3	**81.8**	100	95.2	-	-
	Overall	99.1	93.7	100	94.5	-	-
	H. gallinarum						
	Adult	97.9	**66.4**	100	98.9	-	-
	Immature	95.0	**42.0**	98.5	96.7	-	-
	Overall	96.8	**60.7**	99.6	97.	-	-
	Capillaria spp.	92.1	**25.1**	96.7	93.9	-	-
	All nematodes	96.8	**69.2**	99.6	95.7	-	-
F5	*A. galli*						
	Adult	98.8	97.5	100	-	100	100
	Immature	95.9	**85.2**	100	-	100	100
	Overall	96.0	91.0	100	-	100	100
	H. gallinarum						
	Adult	97.4	**63.2**	98.8	-	99.4	100
	Immature	96.8	**49.6**	97.6	-	99.1	100
	Overall	96.2	**47.2**	98.9	-	99.3	100
	Capillaria spp.	94.2	**44.3**	100	-	100	100
	All nematodes	96.2	**61.2**	99.0	-	99.7	100
	Cestodes	**-5.72**	**17.1**	**-15.0**	-	91.7	100

Values in bold represent efficacies < 90%.
- Not tested.
LEV-PIP: Levamisole-piperazine combination.
Flubendazole-N: Flubendazole nematode dose (30ppm).
Flubendazole-C (60ppm): Flubendazole cestode dose.

Table 4-9 Study 2 – Mean pre-/post-treatment faecal egg counts and percentage worm egg count reductions by treatment group

Farm	Treatment group	Faecal egg count*		FECR%
F3	Untreated	1160.0±234.7[a]	1065.5±89.6[a]	NA
	Levamisole	1130.9±234.7[a]	1.82±89.6[b]	99.8
	Piperazine	1229.1±234.7[a]	121.8±89.6[b]	**88.6**
	LEV+PIP	954.5±234.7[a]	0.00±89.6[b]	100
	Fenbendazole	1085.5±234.7[a]	14.5±89.6[b]	99.7
F5	Untreated	821.0±214.9[a]	993.7±66.5a	NA
	Levamisole	758.0±214.9[a]	0.00±64.8[b]	100
	Piperazine	782.0±214.9[a]	36.00±64.8[b]	96.2
	LEV+PIP	830±214.9[a]	0.00±64.8[b]	100
	Flubendazole-N	914.0±214.9[a]	4.00±64.8[b]	99.6
	Flubendazole-C	722.0±220.5[a]	0.00±64.8[b]	100

* Values shown in the table are raw data (AM), but statistical analyses were based on transformed data (Ln [count+1]).
Values in bold represent FECR < 95%.
LEV-PIP: Levamisole-piperazine combination.
Flubendazole-N: Flubendazole nematode dose (30ppm).
Flubendazole-C (60ppm): Flubendazole cestode dose.
NA: Not applicable.
[a b] Values not sharing a common letter in the superscript are statistically different.

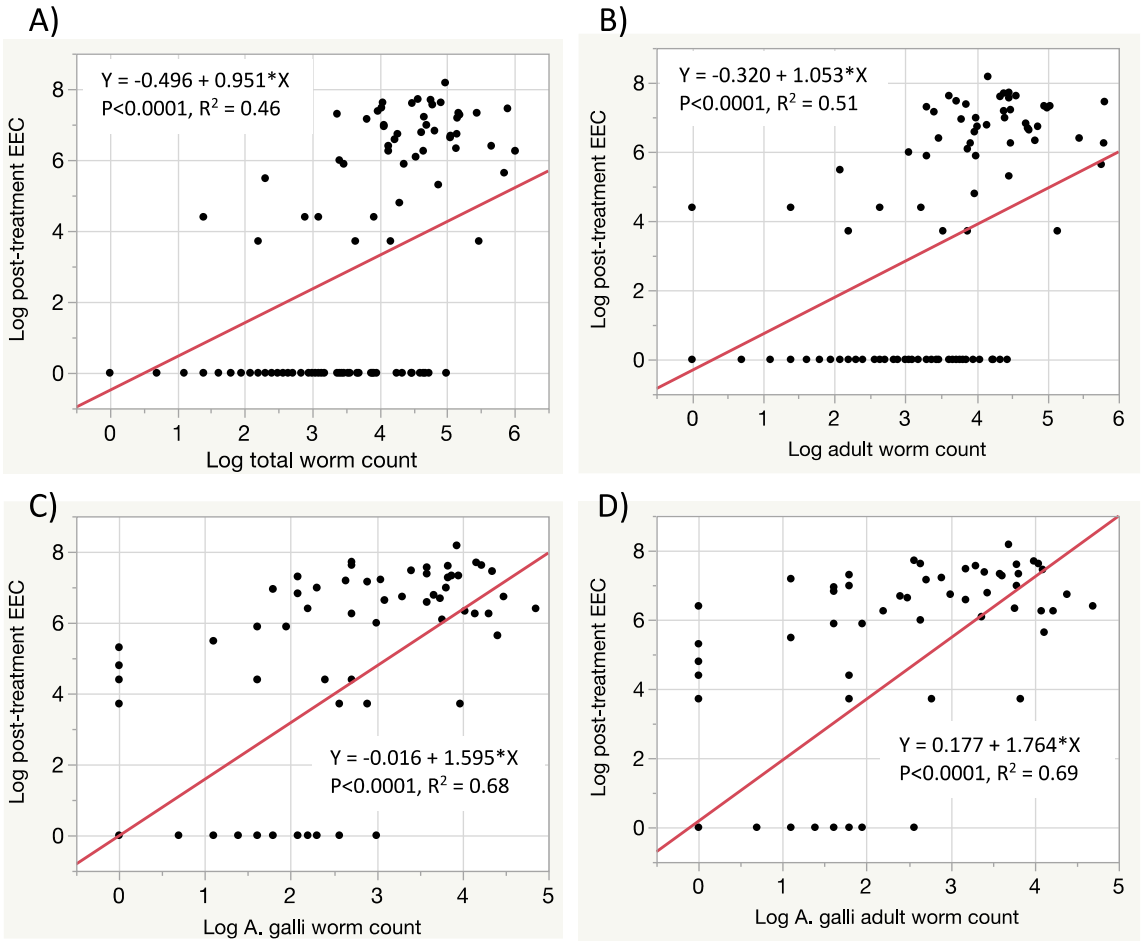


Figure 4-2 Study 2 – Linear regression plots showing the association between post-treatment individual nematode FEC (Log_n EPG) and A) Log_n total worm count, B) Log_n adult worm count, C) Log_n *A. galli* worm count and D) Log_n *A. galli* adult worm count showing the increasing strength of the association with each

4.3.3 Discussion and conclusions – Study 2

Conducting the test on birds with a high prevalence of natural mixed infections provided a much more detailed assessment of anthelmintic efficacy than obtained in Study 1, which evaluated efficacy against a single isolate of a single species, and only against adult worms of that species (*A. galli)*. The results of Study 2 are very clear in that they indicate good efficacy of levamisole, LEV+PIP, FBZ and FLBZ against the nematode species, and FLBZ against tapeworms (FBZ not tested against tapeworms). However, piperazine demonstrated adequate efficacy only against adult *A. galli*, with poor efficacy against immature *A. galli* and all stages of *H. gallinarum* and *Capillaria* spp. on both farms. Levamisole and piperazine, or their combination, were not efficacious against tape worms. Given these results, egg producers have better choices than piperazine for control of nematode infections in their chickens, and other registered products should be used in preference.

The results obtained for the efficacy of piperazine against *H. gallinarum*, *Capillaria* spp. and immature *A. galli* are in line with the label claim and general literature, and do not reflect the presence of emerging resistance. The manufacturer's claim is that it is effective mainly against susceptible strains of *A. galli*. Likewise, previous reports on the efficacy of different PIP compounds indicated poor efficacy against immature forms of *A. galli* and the nematode species *H. gallinarum* and *Capillaria* spp. For instance, Colglazier et al. (1960) tested piperazine citrate (capsules) at 100 mg for birds weighing 1.9–3.9 kg and observed an overall efficacy of 99, 45 and 35% against *A. galli*, *H. gallinarum* and *Capillaria* spp., respectively whereas at a double dose (200 mg), they recorded efficacy of 100, 66 and 29% respectively. When this compound was tested at 0.05 and 0.1% in drinking water, much lower efficacy was recorded for *H. gallinarum* (19–45%) and *Capillaria* spp. (3%). The authors, however, did not specify the developmental forms of the tested nematodes. Chege et al. (2017) also reported that PIP citrate delivered in water at 3 mg/kg produced around 59% efficacy against *H. gallinarum*. Similarly, Edgar et al. (1957) after testing several compounds of PIP at different dose rates and mode of administration recorded efficacy of 95–100% and 75–100% against mature and immature *A. galli* respectively, and 0–86% against *H. gallinarum* suggesting a dose dependent efficacy of the PIP compounds tested in their study. It can therefore be deduced that the observed efficacy results for PIP hydrochloride tested in the current study are in line with the label claim and the literature.

The efficacy testing in this study for all anthelmintics other than flubendazole was done by individual oral dosing, and mass medication was only tested with the in-feed flubendazole. However, the results of Study 1 suggest that efficacy from individual treatment reflects that of mass treatment, apart from the case of low dose fenbendazole administration in water. Therefore, the results of this study are strongly supportive on the ongoing efficacy of levamisole, fenbendazole and flubendazole against the nematode species, and flubendazole against tapeworms, while showing the limitations of piperazine as a broad spectrum nematicide.

While there was some association between the FECR results and the gold standard WCR results, the use of FECR cannot be recommended as a substitute for the WCR in mixed infections with worm species that have different levels of egg production per female worm. In the present study, the faecal egg counts appeared to best reflect the numbers of adult *A galli* worms, a large and highly fecund species. Because of the lack of specificity and accuracy of the FECR method in mixed infections it cannot be recommended as other than a crude measure of anthelmintic efficacy.

4.4 Study 3 – Anthelmintic efficacy against adult and immature worms of two additional isolates of *A. galli*

The overall objective of this study was to evaluate the anthelmintic efficacy status of two further isolates of *A. galli* as part of our investigation of anthelmintic resistance status of chicken nematodes in Australia, and characterisation of field isolates of key worm species. This study largely used the methods of Study 1 (artificial infection of young chickens and evaluation of both individual and mass treatment), with the following additional features:

- Trickle infection of the chickens to enable evaluation of efficacy against immature as well as mature stages.
- Inclusion of the newly registered flubendazole anthelmintic.
- Assessment of the effect of treatment on the viability of *A. galli* eggs after treatment (i.e. assessment of ovicidal effect of treatment).

4.4.1 Methods – Study 3

Experimental design

Two separate controlled experiments employing two *A. galli* isolates in artificially infected chickens were conducted according to the standard guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) for anthelmintic efficacy testing in chickens and turkeys (Yazwinski et al. 2003).

Experimental chickens

One-day-old layer cockerel chickens (Isa Brown) were purchased from a commercial hatchery (Tamworth, NSW, Australia). Chicks were kept in the same experimental room in floor pens (0.09 m^2/bird) with wood shavings as bedding material for 6 weeks, and then moved to enriched individual layer cages with wire flooring (0.37 m^2) up to the end of the experiment. A commercial layer ration and water were offered *ad libitum* over the experimental period.

Ascaridia galli isolates

Two *A. galli* isolates (Isolate UNE 2020-QLD-2 and Isolate UNE 2019-UNE-1) were employed in these experiments. Isolates and their origins are detailed in Table 7-2.

- UNE 2020-QLD-2 : Originally isolated from naturally infected free range (organic) laying hens in a private commercial poultry farm in Australia with no recent history of exposure to anthelmintics. This is Farm 2 of the on-farm worm prevalence survey (Table 3-1).
- UNE 2019-UNE-1: Originally isolated from naturally infected free range hens used for a behavioural study at the UNE poultry research facility. The hens from which this isolate was recovered at necropsy arrived at the facility at day old and were never treated with anthelmintics. The isolate reflects resident parasite worm populations from preceding experiments in the free range facility involving *A. galli*. Therefore, this isolate had no clear history of recent exposure to anthelmintics. This isolate underwent 3 experimental passages for maintenance in worm-free layer chickens before being used in this experiment.

Artificial infection protocol

Adult female worms collected from fresh intestines of infected hens killed at the time of scheduled depopulation (IS1) or termination of *A. galli* propagation experiment (IS2) were used as the source of eggs for artificial infection. All the chicks were inoculated via crop gavage needles with an infection dose of 600 eggs in 6 split doses (100 eggs at a time) starting from the day of arrival. Our inoculation approach was conducted in such a way that three different populations of *A. galli* developmental stages could be obtained. Initially, the birds were trickle infected with 300 eggs over 1 week starting from day 0 (day old). This was followed by a second trickle infection (reinfection) with a total of 300 eggs over 2 weeks, which was conducted at weeks 11 and 6 post-last trickle infection respectively for IS1 and IS2. Anthelmintic treatment was instituted 5–7 days after the final dose of larvae (Yazwinski et al. 2003; Ferdushy et al. 2012; Ferdushy et al. 2014).

Experimental outline and anthelmintic administration

At 8–13 weeks of age (equivalent to 8–13 weeks post-initial infection), 192 birds (96 birds per isolate), which had a pre-treatment FEC of at least 100 EPG (Yazwinski et al. 2003), were admitted to the trial. The birds were then stratified on the basis of FEC and bodyweight into one of eight experimental groups of 12 birds each. This is compatible with WAAVP guidelines requiring a minimum of 6 infected birds per treatment group on the day of treatment. Details on experimental outline, treatment groups and anthelmintic regimens are presented in Table 4-10.

Table 4-10 Study 3 – Experimental groups and dosage regimens for the tested products

Anthelmintic	Route	Dose	Dosage regimen
Piperazine	Oral inoculation	100 mg/kg	Bolus dose
	Drinking water	100 mg/kg	2.5 mg PIP/ml of drinking water for 8 hrs each day over 2 days
Levamisole	Oral inoculation	28 mg/kg	Bolus dose
	Drinking water	28 mg/kg	0.8 mg LEV/ml of drinking water over 8 hrs
Levamisole-piperazine combination (LEV-PIP)	Oral inoculation	28 mg/kg LEV + 100 mg/kg PIP	Bolus dose
	Drinking water	28 mg/kg LEV+ 100 mg/kg PIP	0.8 mg LEV/ml of drinking water for 8 hrs; 2.5 mg PIP/ml of drinking water for 8 hrs each day over 2 days
Flubendazole	Oral premix	30 ppm	Over 7 days
Untreated control	NA	NA	NA

NA: Not applicable.
LEV-PIP: Levamisole-piperazine combination.

Excreta egg count

On the day of treatment (d 0), individual bird faeces were collected from all treatment groups to assess pre-treatment FEC. Individual faecal material was also collected from each bird just prior to necropsy to estimate post-treatment individual FECs using the Modified McMaster method described previously (Section 4.2.1).

Worm recovery and count

Ten days post-anthelmintic treatment (d 10), all experimental birds were euthanised to recover worms from the intestine for assessment of total worm count. After euthanasia, the intestines were cut open and intestinal contents from the small intestine were sieved (mesh size 100 µm) and then rinsed several times with tap water. Adult worms and luminal larvae were recovered and enumerated as described elsewhere (Yazwinski et al. 2003; Taylor et al. 2007; Tarbiat et al. 2016a). The histotrophic larvae in the intestinal mucosa were recovered with pepsin–HCl digestion method as described earlier (Ferdushy et al. 2012; Ferdushy et al. 2014) with some modifications.

Egg recovery from expelled worms and embryonation

The adult *A. galli* worms that were expelled with the faeces following treatment were collected manually on papers placed under each individual cage. At least 3 intact female worms per treatment (irrespective of mode of application) were collected 24–120 hrs post-treatment. For the untreated groups, eggs were recovered from worms collected at necropsy. The worms were cleaned with PBS and dissected to recover eggs for embryonation according to established parasitological procedure (Daş et al. 2010; Rahimian et al. 2016). Eggs were cultured in 5 replications (500 eggs/replicate) for 14 days and developmental status recorded as described previously (Feyera et al. 2020).

Data analysis

Data were statistically analysed using JMP® software version 14.3.0 (SAS Institute Inc., Cary, USA). Statistical tests were performed using a one-way ANOVA. Prior to statistical analysis, worm counts and FEC were logarithmically transformed for compliance with the assumptions of ANOVA. Anthelmintic efficacy was calculated using standard WAAVP formula for both WCR% and FECR%. A 90% cut-off values were considered for WCR% and FECR% respectively.

4.4.2 Results – Study 3

Infection and worm recovery rate

All birds in the untreated control group harboured *A. galli* infection at necropsy. Untreated controls of IS1 presented generally lower worm load than those of IS2 infection (Table 4-11). Worm count data for both isolates (IS1 and IS2) are presented in Table 4-11 portraying mean counts, range and percentage of birds harbouring a specific *A. galli* development stage encountered at necropsy. The proportions of untreated control chickens harbouring adult worms were 91 and 100% for IS1 and IS2 groups respectively, with 75% in both groups harbouring larvae in the gut lumen and 66.7% in both groups having larvae in the gut mucosa. Thus, the experiment was set up to test the efficacy of the test compounds on both adult and larval stages of *A. galli*.

Anthelmintic efficacy

Geometric mean worm counts for the different treatment groups, significant differences between them and the corresponding anthelmintic efficacy (WCR%) data are presented in Table 4-12. LEV administered orally to individual chickens had adequate efficacy (90–100%) against all stages of *A. galli* of both isolates. When administered in drinking water adequate efficacy was maintained against adults and luminal larvae, but sub-optimal control of histotrophic larvae (88.2 and 87.7%) was achieved. For both isolates PIP provided adequate control of adult worms (92–97%) by both routes of administration but exhibited sub-optimal efficacy against luminal larvae (80–84%) and histotrophic larvae (61–73%). LEV + PIP in combination provided adequate control (> 90%) of all stages for both

routes of administration. Flubendazole in the feed at the nematode control inclusion rate (30 ppm) provided 100% efficacy against all stages.

Anthelmintic efficacies based on FECR% are summarised in Table 4-13. Eggs were detected in the faeces of 10/12 (83.3%) of untreated control chickens for each isolate, with arithmetic mean worm counts of 177 and 207 EPG for the two isolates respectively. FEC reductions of > 90% were observed for all treatments, with all treatments achieving 100% reduction apart from LEV in drinking water, and PIP by either route for Isolate UNE 2020-QLD-2 and by drinking water application for Isolate UNE 2019-UNE-1.

Table 4-11 Study 3 – Levels of *A. galli* count at necropsy (arithmetic mean) by treatment groups

A. galli isolate	Treatment	Mode of application	Adult			Luminal larvae			Histotrophic larvae		
			Mean[1]±SD	Range	Infected (%)	Mean[1]±SD	Range	Infected (%)	Mean[1]±SD	Range	Infected (%)
UNE 2020-QLD-2	Untreated	NA	4.33±3.47	0-13	91	2.93±3.03	0-10	75	3.92±4.66	0-14	66.7
	LEV	Oral inoculation	0.00±0.00	0	0	0.00±0.00	0	0	0.17±0.57	0-2	8.3
		Drinking water	0.25±0.62	0-2	8.3	0.17±0.58	0-2	8.3	0.33±0.89	0-3	16.7
	PIP	Oral inoculation	0.33±0.89	0-3	16.7	0.58±1.24	0-4	25	0.99±1.65	0-5	33.3
		Drinking water	0.41±0.90	0-3	25	0.50±1.00	0-3	16.7	1.00±1.59	0-6	41.7
	LEV-PIP	Oral inoculation	0.00±0.00	0	0	0.00±0.00	0	0	0.00±0.00	0	0
		Drinking water	0.17±0.58	0-2	8.3	0.00±0.00	0	0	0.17±0.59	0-2	8.3
	FLBZ	Oral premix	0.00±0.0	0	0	0.0±0.00	0	0	0.00±0.00	0	0
UNE 2019-UNE-1	Untreated	NA	6.10±6.33	2-23	100	3.05±3.31	0-12	75	3.25±2.99	0-8	66.7
	LEV	Oral inoculation	0.25±0.62	0-2	16.7	0.00±0.00	0	0	0.33±0.77	0-2	16.7
		Drinking water	0.33±0.65	0-3	16.7	0.17±0.58	0-2	8.3	0.41±0.90	0-3	25
	PIP	Oral inoculation	0.00±0.00	0	0	0.50±1.01	0-4	16.7	1.25±2.01	0-6	41.7
		Drinking water	0.33±0.89	0-3	16.7	0.58±1.24	0-3	25	1.17±2.20	0-7	33.3
	LEV-PIP	Oral inoculation	0.00±0.00	0	0	0.00±0.00	0	0	0.00±0.00	0	0
		Drinking water	0.17±0.56	0-2	8.3	0.08±0.38	0-1	8.3	0.25±0.62	0-2	16.7
	FLBZ	Oral premix	0.00±0.00	0	0	0.00±0.00	0	0	0.00±0.00	00	

[1] Means are the arithmetic mean numbers of worms per bird for the treatment group.

SD: Standard deviation.
IS1: Isolate 1.
IS2: Isolate 2.
LEV: Levamisole.
PIP: Piperazine.
LEV-PIP: Levamisole-piperazine combination.
FLBZ: Flubendazole.
NA: Not applicable.

Table 4-12 Study 3 – Geometric means of different developmental stages and corresponding anthelmintic efficacy (%) by treatment group

A. galli isolate	Treatment	Mode of application	Adult		Luminal larvae		Histotrophic larvae		Overall	
			GM*	Efficacy	GM*	Efficacy	GM*	Efficacy	GM	Efficacy
UNE 2020-QLD-2	Untreated	NA	3.32[a]	NA	1.55[a]	NA	1.60[a]	NA	7.90[a]	NA
	LEV	Oral inoculation	0.00[b]	100	0.00[b]	100	0.09[b]	93.0	0.10[b]	98.7
		Drinking water	0.16[b]	95.2	0.09[b]	93.8	0.19[b]	**88.2**	0.43[b]	94.6
	PIP	Oral inoculation	0.17[b]	95.1	0.33[b]	**78.8**	0.57[b]	**64.8**	1.10[b]	**86.6**
		Drinking water	0.26[b]	92.2	0.30[b]	**80.4**	0.62[b]	**61.4**	1.31[b]	**83.4**
	LEV-PIP	Oral inoculation	0.00[b]	100	0.00[b]	100	0.00[b]	100	0.00[b]	100
		Drinking water	0.10[b]	97.1	0.00[b]	100	0.09[b]	94.0	0.20[b]	97.5
	FLBZ	Oral premix	0.00[b]	100	0.00[b]	100	0.00[b]	100	0.00[b]	100
UNE 2019-UNE-1	Untreated	NA	4.51[a]	NA	1.89[a]	NA	2.11[a]	NA	9.99[a]	NA
	LEV	Oral inoculation	0.12[b]	97.3	0.00[b]	100	0.20[b]	**90.5**	0.33[b]	96.7
		Drinking water	0.19[b]	95.8	0.10[b]	95.0	0.26[b]	**87.7**	0.57[b]	94.3
	PIP	Oral inoculation	0.00[b]	100	0.30[b]	**84.0**	0.70[b]	**67.1**	1.13[b]	**88.6**
		Drinking water	0.18[b]	96.1	0.33[b]	**82.6**	0.60[b]	**72.6**	1.10[b]	**89.2**
	LEV-PIP	Oral inoculation	0.00[b]	100	0.00[b]	100	0.00[b]	100	0.00[b]	100
		Drinking water	0.09[b]	97.9	0.06[b]	96.8	0.16[b]	92.4	0.25[b]	97.5
	FLBZ	Oral premix	0.00[b]	100	0.00[b]	100	0.00[b]	100	0.00[b]	100

GM: Geometric mean.
Worm counts were logarithmically transformed (ln [count + 1]), averaged and then back-transformed to approximate the GMs.
Statistical analysis was performed using transformed data (ln [count + 1]).
Values in bold represent efficacy < 90%.
[a, b] Means on the same row with unlike superscripts are different ($P < 0.05$).
LEV: Levamisole.
PIP: Piperazine.
LEV-PIP: Levamisole-piperazine combination.
FLBZ: Flubendazole.
NA: Not applicable.

Table 4-13 Study 3 – Mean pre-/post-treatment faecal egg counts and percentage worm egg count reductions by treatment group

A. galli isolate	Treatment	Mode of application	Pre-treatment FEC		Post-treatment FEC		FECR%
			Number of birds with eggs detected (%)	AM±SD	Number of birds with eggs detected (%)	AM±SD	(UCL, LCL)
UNE 2020-QLD-2	Untreated	NA	10/12 (83.3)	176.7±228.5[a]	10/12 (100)	163.3±143.2[a]	NA
	LEV	Oral inoculation	10/12 (83.3)	150.0±125.5[a]	0/12 (0)	0.00±0.00[b]	100
		Drinking water	10/12 (83.3)	210.0±211.0[a]	1/12 (8.3)	3.33±11.6[b]	97.9 (100, 86)
	PIP	Oral inoculation	10/12 (83.3)	160.0±144.7[a]	1/12 (8.3)	3.33±11.6[b]	97.9 (100, 86)
		Drinking water	11/12 (91.7)	233.3±189.0[a]	1/12 (8.3)	6.67±23.1[b]	95.8 (99,83)
	LEV-PIP	Oral inoculation	10/12 (83.3)	196.7±175.2[a]	0/12 (100)	0.00±0.00[b]	100
		Drinking water	11/12 (91.7)	166.7±111.6[a]	0/12 (100)	0.00±0.00[b]	100
	FLBZ	Oral premix	11/12 (91.7)	183.3±177.6[a]	0/12 (100)	0.00±0.00[b]	100
UNE 2019-UNE-1	Untreated	Water	10/12 (83.3)	206.7±166.9[a]	8/12 (66.7)	234.3±0.00[a]	NA
	LEV	Oral inoculation	11/12 (91.7)	163.3±170.1[a]	0/12 (100)	0.00±0.00[b]	100
		Drinking water	11/12 (91.7)	206.7±186.7[a]	1/12 (8.3)	6.67±23.1[b]	97.1(100, 88)
	PIP	Oral inoculation	11/12 (91.7)	230.0±140.7[a]	0/12 (100)	0.00±0.00[b]	100
		Drinking water	10/12 (83.30	186.7±150.9[a]	1/12 (8.3)	3.33±11.5[b]	98.6 (100, 90)
	LEV-PIP	Oral inoculation	10/12 (83.3)	220.0±235.4[a]	0/12 (100)	0.00±0.00[b]	100
		Drinking water	10/12 (83.3)	183.3±141.1[a]	0/12 (100)	0.00±0.00[b]	100
	FLBZ	Oral premix	10/12 (83.3)	210.0±226.9[a]	0/12 (100)	0.00±0.00[b]	100

Values shown in the table are raw data (AM), but statistical analyses were based on transformed data (Ln [count)+1]).
AM: Arithmetic mean.
LEV: Levamisole.
PIP: Piperazine.
LEV-PIP: Levamisole-piperazine combination.
FLBZ: Flubendazole.
NA: Not applicable.
[a b] Values in the same column not sharing a common letter in the superscript are statistically different.

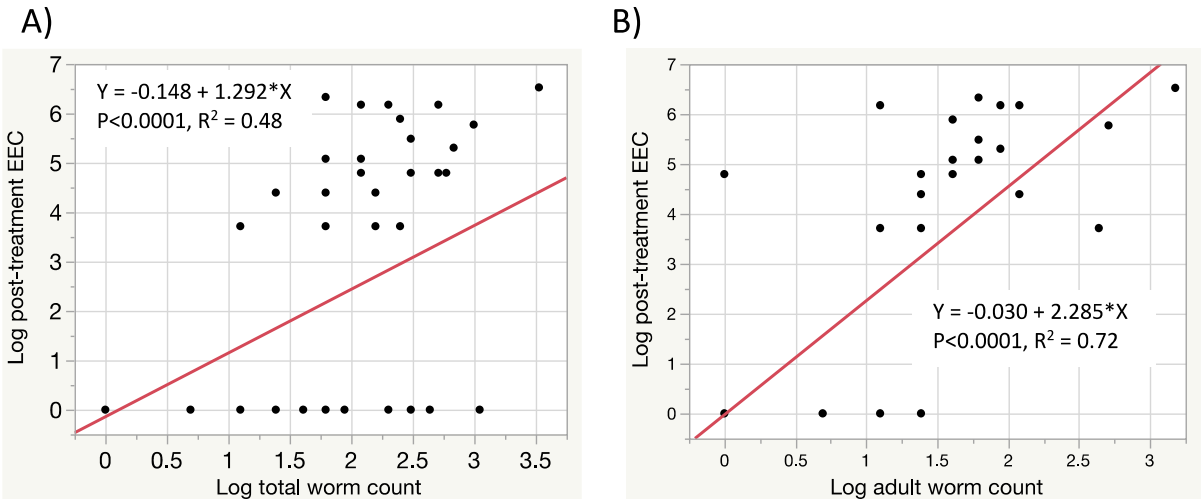


Figure 4-3 Study 3 – Linear regression plots showing the association between post-treatment individual FEC (Log_n EPG) and A) Log_n total worm count or B) Log_n adult worm count

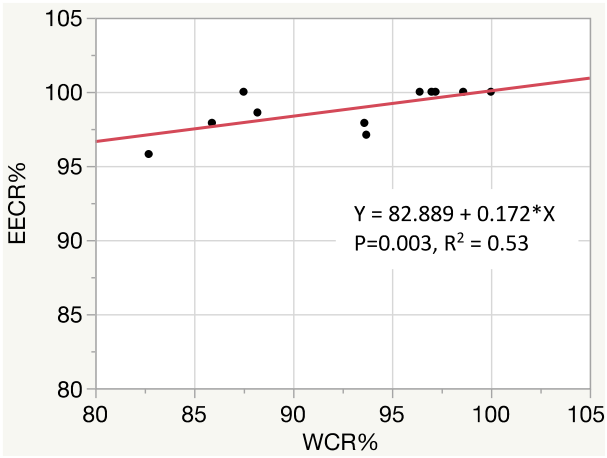


Figure 4-4 Study 3 – Linear regression plot showing the association between anthelmintic efficacies of the anthelmintics as calculated by WCR% and FECR%

WCR: Worm count reduction.
FEC: Faecal egg count reduction.

Embryonation ability of eggs recovered from expelled worms

The developmental profile of eggs recovered from worms expelled by treated birds is shown in Figure 4-5. There was no significant difference ($P < 0.05$) in developmental and/or mortality profiles of eggs recovered from the two isolates. Egg embryonation appeared normal and similar ($P > 0.05$) in samples collected from LEV (46.4–54.3%), PIP (43.3–52.2%) or LEV-PIP (44.6–48.2%) treated groups and was similar to the embryonation rate from eggs from the untreated controls (49.9–50.7%). A very negligible proportion of eggs (< 1%) from all these treatments remained at early developmental stages. In contrast, egg development was completely absent (79.5–82.9%) or largely arrested at the early development stage (15.2–19.1%) in eggs collected from FLBZ treated birds, and only a small proportion (1.37–2.12%) were able to develop to the fully coiled larval stage. The cumulative percentage of dead/abnormal eggs was highest ($P < 0.0001$) in those recovered from FLBZ treated birds (79.5–82.9%) compared to other sources including the untreated control. The percentage dead/abnormal eggs was similar ($P > 0.05$) for LEV (44.7–52.4%), PIP (47.3–56.3%), LEV-PIP (51.3–54.6%) and the untreated sources (48.3–50.1%).

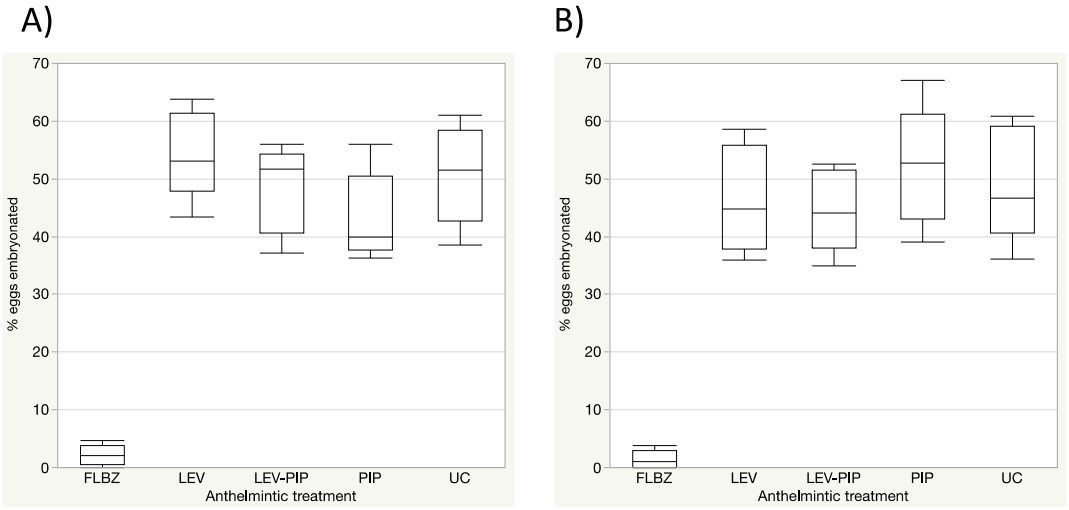


Figure 4-5 Study 3 – Box plots showing the degree of embryonation after 2 weeks incubation in 0.1 N H2SO4 of *A. galli* eggs isolated from worms expelled by chickens following treatment with levamisole, piperazine, levamisole-piperazine combination and flubendazole for isolates UNE 2020-QLD-2 (A) and UNE 2019-UNE-1 (B)

LEV: Levamisole.
PIP: Piperazine.
LEV-PIP: Levamisole-piperazine combination.
FLBZ: Flubendazole.
UC: Untreated control.

4.4.3 Discussion and conclusions – Study 3

There was no evidence of resistance to the anthelmintics in these two isolates of *A. galli.* Only PIP failed to attain the desired efficacy level (> 90%) against both isolates and this was due to the previously observed (Study 2) poor efficacy against immature stages, which is a characteristic of the anthelmintic, rather than a sign of developing resistance. PIP provided excellent efficacy against adult worms of both UNE 2020-QLD-2 and UNE 2019-UNE-1 isolates but poor efficacy against immature stages with poorest performance against histotrophic larvae in the gut mucosa. Both isolates, irrespective of their developmental stages, were highly susceptible to FLBZ. LEV exhibited excellent efficacy to adult and luminal larvae but comparatively lower efficacy to histotrophic stage (generally

around the 90% cut-off value and even lower when applied in water). Group administration of the anthelmintics via drinking water resulted in lower efficacy values compared to the individual oral treatment supporting the earlier observation in Study 1. However, water treatment was generally effective (> 90% efficacy) and can continue to be used with confidence as individual bird application is impractical in the field. PIP only provides adequate control of adult nematodes and the poorer control of immature stages will result in earlier onset of egg production following treatment and thus greater levels of environmental contamination with worm eggs.

Egg counts basically reflect the adult female *A. galli* population and as such do not reflect anthelmintic effects on non-reproducing larval stages of the lifecycle. These stages are typically more resistant to the effects of anthelmintics as seen in Study 2 as well and thus FECR% will generally overestimate anthelmintic efficacy as compared to the gold standard WCR%. This was observed in this study and FECR% should therefore only be used as an indicator of anthelmintic efficacy against adult *A. galli.*

Eggs recovered from worms expelled after treatment with LEV, PIP or LEV-PIP had similar ability to embryonate as those from untreated birds and this may provide an option for recovering eggs for *A. galli* propagation experiments without having to sacrifice birds. Further research is needed to confirm the infectivity of such eggs but if they have embryonated, it is highly likely that they will be infective. In contrast, only a negligible proportion of eggs recovered from worms expelled post-FLBZ treatment were able to embryonate indicating the *in utero* ovicidal potential of FLBZ *in vivo*. This is in keeping with the consistently observed ovicidal action of the BZ class of anthelmintics in other species including ascarid worms of pigs (Boes et al. 1998). The difference in ovicidal action of the anthelmintics observed in this study may have significant epidemiological consequences and is another factor to be taken into account in selecting an appropriate anthelmintic to use.

4.5 Study 4 – Evaluation of *in vitro* methods of evaluating anthelmintic efficacy

This study aimed at evaluating *in vitro* anthelmintic exposure assays based on eggs or larval stages of *A. galli* for testing the efficacy of different classes of anthelmintics. It also describes optimised non-invasive methods that would yield a high number of minimally damaged parasite stages (eggs or larvae) for *in* vitro anthelmintic sensitivity assays. The general hypothesis under test was that *in vitro* anthelmintic sensitivity assays based on fresh faecal eggs or artificially hatched larvae would enable estimation of effective concentrations (EC_{50}/EC_{99}) of different anthelmintics that can be correlated with effective plasma anthelmintic concentrations or *in vivo* efficacy values.

4.5.1 Methods – Study 4

Study design

This study consisted of two main parts:

- Part 1 involved optimisation of methods that would yield a high number of minimally damaged eggs or larvae for subsequent *in vitro* anthelmintic sensitivity assays. This part evaluated different flotation solutions for extraction of *A. galli* eggs from faeces to determine the best solution that would yield high concentration of morphologically normal and viable *A. galli* eggs for an *in ovo* LDT and then compared two larval hatch assays, a deshelling-centrifugation method (Feyera et al. 2020) and a glass-bead hatching method with or without bile (Han et al. 2000) to select the best method for subsequent LMIA.
- In part 2, anthelmintic exposure assays were evaluated, using eggs or artificially hatched larvae of *A. galli*, to determine the *in vitro* anthelmintic efficacy values (EC_{50}/EC_{99}). These included:

i) An *in ovo* LDT using fresh *A. galli* eggs recovered by an optimised technique from Part 1 and following an assay procedure described elsewhere (Tarbiat et al. 2017); ii) A modification of a LMIA described for *A. suum* elsewhere (Zhao et al. 2017) using *A. galli* larvae hatched by an optimised method from part 1.

Parasite worm material and source

The *A. galli* isolate used was UNE 2020-QLD-2 (Table 7-2) originally recovered from naturally infected laying hens in an organic private commercial poultry farm in Queensland, Australia with no recent history of application of anthelmintics (Farm 2 in Table 3-1). This isolate underwent a single experimental passage in young cockerels in an experiment approved by the animal ethics committee of the University of New England (AEC19-091).

Anthelmintics

The anthelmintics employed in this study were water soluble formulations of LEV, and PIP and powdered suspensions of thiabendazole (TBZ), and fenbendazole (FBZ) (Sigma). The concentration ranges of the test anthelmintics were chosen based on plasma concentration values (including C_{max}) reported for poultry or concentration ranges used in determining EC_{50} values of these compounds against other ascarid species (Hu et al. 2013; Tarbiat et al. 2017; Zhao et al. 2017; Scare et al. 2020).

4.5.1.1 Part 1: Optimisation of methods for efficient isolation of eggs and obtaining larvae

Extraction of *A. galli* eggs from faeces

Five flotation solutions were used to extract eggs from faeces using a consistent procedure involving sequential sieving and washing through a series of sieves (with gradually reducing mesh sizes, 1 mm to 36 µm) followed by flotation-centrifugation steps as described elsewhere (Daş et al. 2020). The saturated flotation solutions used in this experiment and their corresponding specific gravity (SG) were sucrose solution (Sheather's solution, SG 1.27), sucrose-NaCl solution (SG 1.28), NaCl (SG 1.2), MgSO4 (Epsom salts, SG 1.2) and Zn SO4 (SG 1.35).

Parasitological measurements

Extraction efficiency (EE), and the morphological quality of eggs at the time of recovery and subsequent developmental ability of eggs were assessed as described in Section 6 and shown in Figure 4-6 and Figure 4-7.

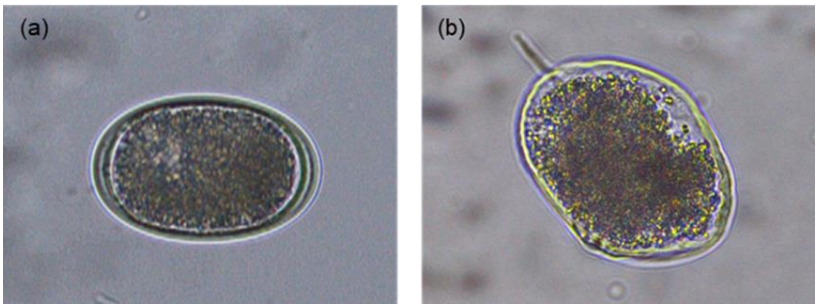


Figure 4-6 Morphological quality of fresh *A. galli* eggs at the time of recovery from chicken excreta: (a) normal (intact); damaged (b)

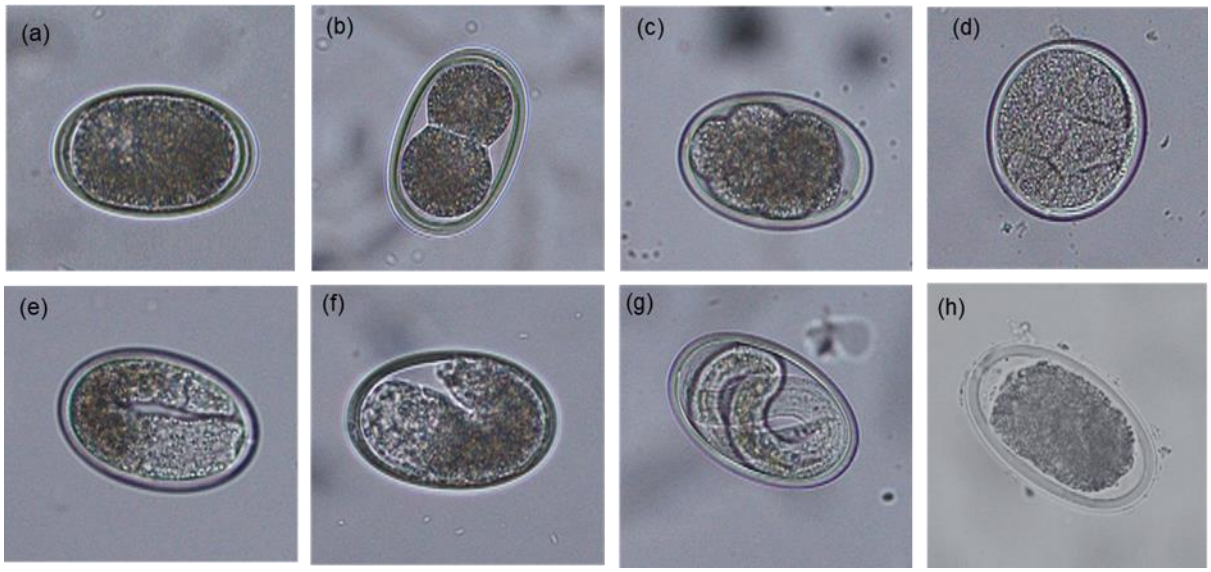


Figure 4-7 Morphological characteristics of *A. galli* eggs at different developmental stages (original magnification 200×): undeveloped (a); early development (b-d); vermiform (e and f); embryonated (g) and dead (h)

Evaluation of sample preparation for *in vitro A. galli* larval assays

Two established procedures, a deshelling-centrifugation method (Feyera et al. 2020) and a glass-bead hatching method with or without bile (Han et al. 2000; Zhao et al. 2017), were compared and where possible optimised to identify a convenient hatch assay in terms of hatching yield, hatched larval viability and temporal change in larval survivability over time. For both hatching assays, embryonated *A. galli* eggs suspended in hatching solutions in falcon tubes in 5 replicates were subjected to hatching as per the relevant method. To assess temporal change in post-hatch larval survivability, larvae were incubated in RPMI media and at least 50 larvae per replicate were counted at different time points (0, 12, 24, 36, 48, 60, 72, 84 and 96 hours), and their viability recorded under light microscopy as described in Section 6.1.

4.5.1.2 Part 2: Evaluation of in vitro anthelmintic exposure assays

***In ovo* larval development test (LDT)**

The LDT was conducted essentially as described previously (Tarbiat et al. 2017). Fresh *A. galli* eggs isolated from faeces using sugar solution as flotation fluid were used for this assay. Briefly, the *A. galli* eggs were exposed to a series of gradually increasing anthelmintic concentrations in micro-titre plates with 5 replicates per interval for each anthelmintic. The plates were incubated at 25°C for 2 weeks to achieve optimum egg development rate. At the end of the incubation period, a minimum of 100 eggs per well were counted and the number of embryonated eggs at each anthelmintic concentration was determined according to (Feyera et al. 2020).

Larval migration inhibition assay

The larval migration inhibition assay was conducted essentially as described previously for *A. suum* (Williams et al. 2016; Zhao et al. 2017). Briefly, 110 larvae (in duplicate) were incubated with graded concentrations of anthelminthic in 96 well plates with larval culture medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 lg/mL streptomycin) in each well. The plates were incubated overnight at 37°C and an equal amount of 1.5% agar solution (45°C) was

added to each well and mixed thoroughly. The next day, the media was collected from each well and the number of larvae that had migrated from the setting agar was enumerated by microscopy. Percentage inhibition of migration was calculated relative to the negative control using the formula:

$$–00 - (\frac{\text{number of larvae migrated in test well}}{\text{number of larvae migrated in control wells}}) * 100$$

Nonlinear logistic regression (logistic 4P) was used to calculate EC_{50}, EC_{99} and the 95% confidence limits using log transformed drug concentration data.

4.5.2 Results – Study 4

4.5.2.1 Extraction of A. galli eggs from faeces

Extraction efficiency. The type of flotation solution used significantly (P = 0.0032) affected the number of eggs isolated relative to the expected number of eggs contained in the faeces material. ZnSO4 (68.3% recovery) and sucrose solution (67.8 %) provided the best egg extraction efficiency, followed by sucrose-NaCl solution (65.6 %) while NaCl (58.3 %) and MgSO4 (57.9 %) extracted the fewest eggs (Figure 4-8). There was a positive linear association between extraction efficiency and the SG of the flotation solution (R^2 = 0.79).

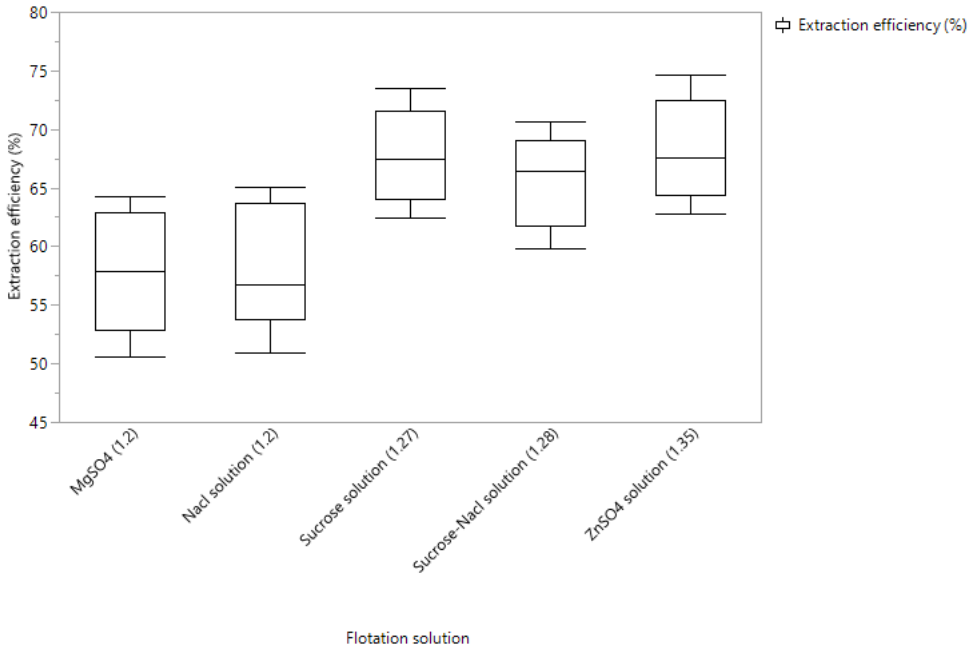


Figure 4-8 Egg recovery efficiency by different flotation fluids

Morphological quality of eggs at the time of recovery

The type of flotation fluid also had a significant effect (P = 0.0039) on the morphological quality of recovered eggs. The sugar solution yielded the highest proportion of morphologically normal eggs (98.1%) as assessed by morphological appearance (intact vs damaged), whereas $ZnSO_4$ resulted in the highest percentage of damaged eggs (9.7%) at the time of recovery compared to others (Table 4-14). There was no major difference between the remaining flotation fluids in terms of the quality of eggs recovered.

Developmental ability of eggs. The type of flotation fluid used to recover eggs from faeces had significant ($P < 0.0074$) effect on the subsequent developmental ability of *A. galli* eggs (Table 4-14). Eggs isolated by sucrose solution had the highest embryonation rate (93.3%) followed by those isolated by MgSO4 (87.1%). A positive correlation existed between morphological quality at the time of recovery and developmental ability of eggs ($R^2 = 0.16$).

Table 4-14 Morphological quality at the time of recovery and subsequent developmental ability of *A. galli* eggs extracted by different flotation solutions

Flotation fluid solution	SG	Morphological quality at the time of recovery (%)		Developmental ability (%) following aerobic incubation at 26°C			
		Intact	Damaged	Undeveloped	Early development	Vermiform	Embryonated
Sugar	1.27	98.1±1.2^{a}	1.9±1.2^{b}	0.00±0.12	0.78±0.54	0.45±0.44	93.3±1.87^{a}
Salt	1.2	92.9±1.2^{b}	7.1±1.1^{ab}	0.00±0.12	1.19±0.54	0.78±0.44	85.3±1.87^{b}
Salt-sugar	1.28	93.8±1.2^{ab}	6.2±1.2^{ab}	0.19±0.12	0.80±0.54	0.99±0.44	86.7±1.87^{ab}
MgSO4	1.2	94.2±1.2^{ab}	5.8±1.2^{a}	0.00±0.12	0.99±0.54	1.40±0.44	87.1±1.87^{ab}
ZnSO4	1.36	90.3±1.2^{b}	9.7±1.2^{a}	0.02±0.12	2.01±0.54	1.19±0.44	82.1±1.87^{b}

Images of these morphological classifications can be found in in Figure 4-6 and Figure 4-7.

4.5.2.2 Evaluation of in vitro A. galli larvae hatching assays

***Hatching efficiency*.** The deshelling-centrifugation method and the glass-bead with 5% bile or without bile methods respectively resulted in 97.4, 95.2 and 94.9% hatching of the embryonated eggs with no statistically significant difference.

Post-hatch survivability of larvae. Larvae hatched by the deshelling-centrifugation method demonstrated higher percentage viability than glass-bead hatching assays at least for the first 48 hours. Later on, larval survival rate is similar for all of the methods without major differences overall (Figure 4-9). Larva survival decreased with incubation time, with percentage viability diminishing to as low as < 10–15% after 96 hrs of incubation in RPMI media almost in all cases with poor motility (Figure 4-9). This method, however, employed a viability dye exclusion method and did not formally assess temporal change in the migratory behaviour (change in motility) of larvae over time.

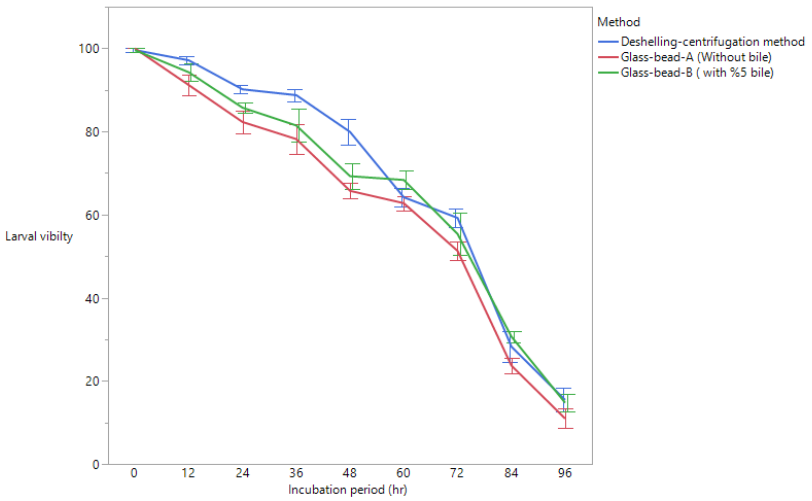


Figure 4-9 Temporal change in survivability of *A. galli* larvae artificially hatched by different methods and subsequently incubated in RPMI media

4.5.2.3 Evaluation of in vitro anthelmintic exposure assays

In ovo larval development test (LDT)

Eggs isolated by sucrose solution were used for this assay, which tested two the BZ anthelmintics thiabendazole (TBZ) and fenbendazole (FBZ) as BZ anthelmintics are known to be ovicidal. There was a concentration-dependent inhibition of *in ovo* larval development both anthelmintics (Figure 4-10). The EC_{50} and EC_{99} estimates for TBZ and FBZ were very similar (
Table ***4-15***).

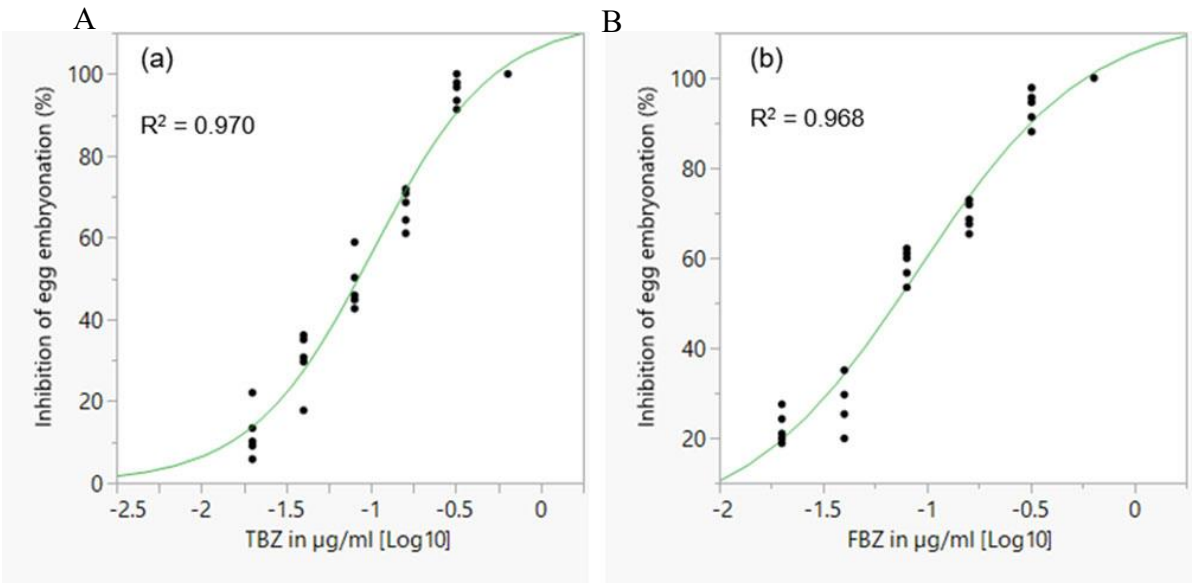


Figure 4-10 Concentration-response curves for TBZ (A) and FBZ (B) anthelmintics in LDT for assessing anthelmintic resistance in *A. galli*

Table 4-15 EC_{50} and EC EC_{99} estimates of LDT using an *A. galli* isolate with no recent history of exposure to anthelmintics

Anthelmintic	EC_{50}		EC_{99}	
	Log EC_{50}	EC_{50} (µg/ml)	LogEC_{99}	EC EC_{99} (µg/ml)
TBZ	-1.118	0.076	-0.303	0.498
FBZ	-1.195	0.064	-0.263	0.546

TBZ: Thiabendazole.
FBZ: Fenbendazole.
EC: Effective concentration.

Larval migration inhibition assay (LMIA)

As expected, the anthelmintics caused a concentration-dependent inhibition of larval migration (Figure 4-11). FBZ exhibited the lowest EC_{50} and EC_{99} values whereas LEV exhibited the highest values (Table 4-16)

Table 4-16 EC_{50} and EC_{99} estimates of LMIA using an *A. galli* isolate with no recent history of exposure to anthelmintics

Anthelmintic	EC_{50}		EC_{99}	
	$LogEC_{50}$	EC_{50} (nM)	$LogC_{99}$	EC_{99} (nM)
TBZ	1.976	94.5	3.146	1400.9
FBZ	0.898	7.91	1.960	91.2
LEV	2.641	437.0	3.430	2691.8
PIP*	1.819	65.9	2.507	321.1

TBZ: Thiabendazole.
FBZ: Fenbendazole.
LEV: Levamisole.
PIP: Piperazine.
EC: Effective concentration.
* corresponding concentration is mM.

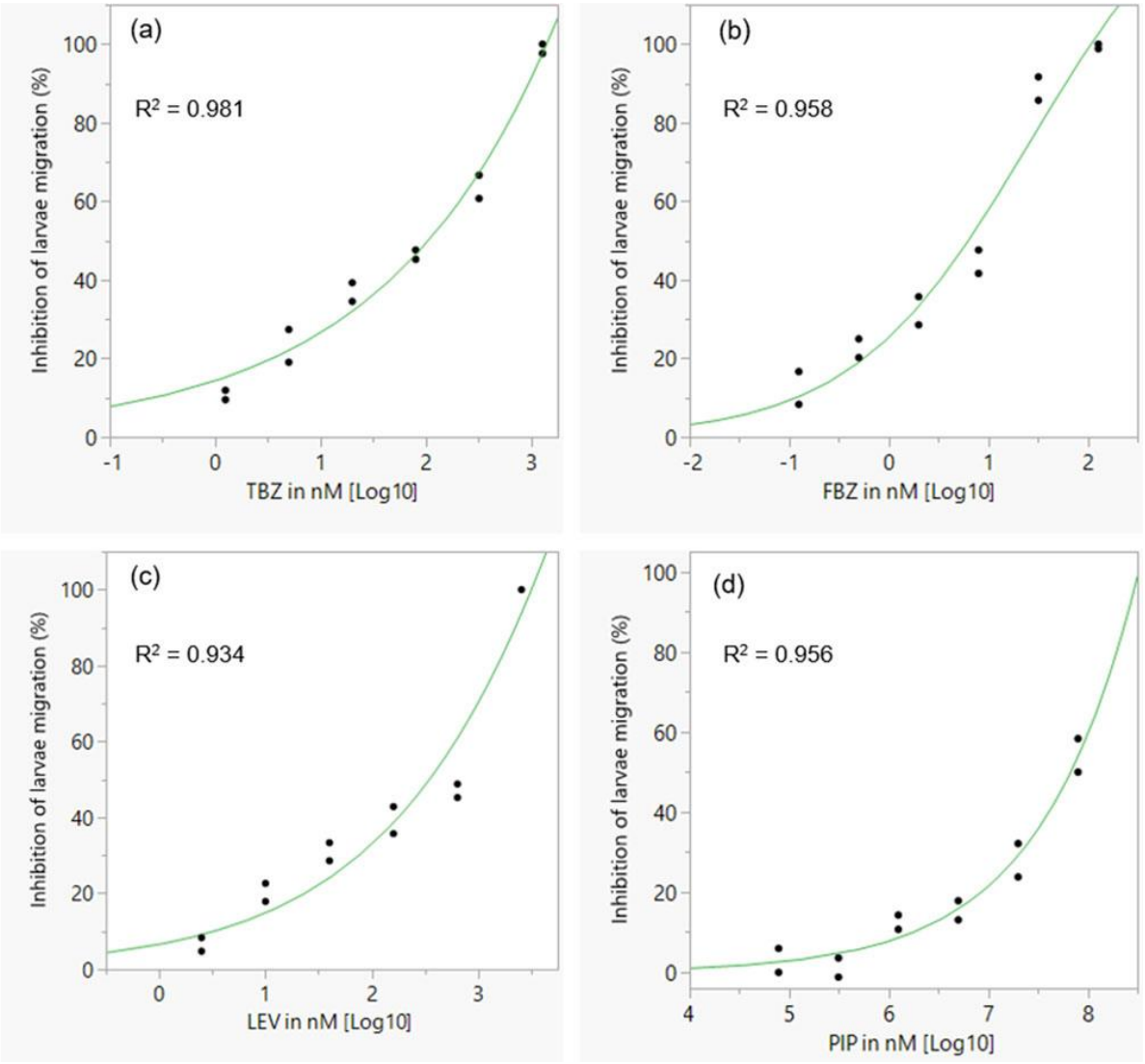


Figure 4-11 Concentration-response curves for different classes of anthelmintics in LMIA for

assessing anthelmintic efficacy in *A. galli* – (a) TBZ; (b) FBZ; (c) LEV; (d) PIP

LMIA: Larval migration inhibition assay.
TBZ: Thiabendazole.
FBZ: Fenbendazole.
LEV: Levamisole.
PIP: Piperazine.

4.5.3 Discussion and conclusions – Study 4

Egg extraction from faeces. Sugar solution yielded the highest number of minimally damaged eggs with excellent developmental ability compared to other solutions. Hence the sugar solution is most appropriate for extracting eggs from faeces for assays (such as LDT) requiring a high number of fresh *A. galli* eggs with high embryonation potential.

Larval hatching assays. Even though all assays tested had similar hatching efficiency, the deshelling centrifugation method seems to yield larvae that have a relatively better post-hatch survival rate especially in the first 48 hrs of incubation in RPMI media. Overall larval viability decreased by 0.87% every hour of incubation in artificial media indicating a complete loss of viability by around 120 hrs. The glass-bead method is a shorter procedure in terms of time required for hatching and is potentially useful if optimised. The post-hatch survival rate of newly hatched larvae observed in the current study is very low compared to earlier reports indicating larval survival up to 8–10 days in an ordinary culture media and as long as 112 days with *in vitro* development into adult in enriched larval culture media (Dick et al. 1973). This suggests that RPMI may not be an ideal culture media for maintaining longevity of newly hatched larvae and requires that the larvae be used within 2–3 days of hatching for tests such as LMIA. Future study should determine the preferred *in vitro* conditions to maintain maximum viability of newly hatched *A. galli* larvae.

Larval development test. The *in ovo* LDT tested here appears to suit creation of concentration-response curves and calculation of EC_{50} allowing for assessment of AR for anthelmintics with ovicidal activity. Given that the all-purpose benzimidazoles are likely to be globally the most widely applied anthelmintics in the poultry industry in the future and LDT is a straightforward assay, it could be the method of choice for rapid *in vitro* AR testing in this class of anthelmintics, rather than the more labour intensive LMIA. For this to to occur, ideally the relationship between *in vitro* EC_{50} values and *in vivo* anthelmintic resistance needs to be defined so that the latter can be predicted from the former. This is lacking at present.

LMIA. The concentration-response curves created for the tested anthelmintics appear to show that artificially hatched larvae of *A. galli* could be used for estimation of EC_{50} and thus *in vitro* AR testing. However, this assay is methodologically complex and labour intensive, and may not be a method of choice for routine diagnosis of AR in *A. galli* given that LDT or other egg-based assays can provide a good option for the BZ anthelmintics and the gold standard WCRT is still feasible in poultry due to the relatively low value of individual chickens.

4.6 Anthelmintic efficacy/resistance studies – overall findings and implications

The series of experiments carried out in this section have produced some clear outcomes and implications for industry. These are summarised below:

1. No evidence of anthelmintic resistance was detected. The resistance studies involved worms from 5 free range farms (5 for *A. galli*, 2 for *H. gallinarum* and *Capillaria* spp.). These data reinforce the perception of producers in the online survey, that current treatments are working, and suggest that anthelmintic resistance is currently not a major threat to the industry. While development of resistance cannot be discounted in the future, the prolonged persistence of eggs in the environment and relatively low frequency of treatment in free range systems likely means that a significant parasite population is "in refugia" at the time of any one treatment. This likely has contributed to the apparently slow development of resistance to date.

2. PIP has inadequate efficacy against immature larvae of all species, and adults of *H. gallinarum* and *Capillaria* spp. It is only fully efficacious against adult *A. galli* worms. This is a feature of the anthelmintic itself rather than being evidence of emerging resistance. There appears to be little reason to use this chemical when none of the other available anthelmintics suffer from this inadequacy.

3. Mass application of anthelmintics in water reduces efficacy slightly for the water-soluble anthelmintics LEV and PIP but is the only practical way off application and in the case of LEV, but not PIP efficacy in water against immature stages is maintained. There was a greater reduction in efficacy of the non-soluble anthelmintic FBZ (micronised suspension) when administered in water in Study 1 at a comparatively low dose rate without agitation of the solution.

4. The newly registered anthelmintic flubendazole (Flubenol®) administered in feed over 7 days proved to be highly effective against all stages of roundworm infection, and cestode infection.

5. Excreta egg count reduction provides a reasonable alternative for assessing anthelmintic efficacy against adult *A. galli* worms in monospecific artificial infections but overestimates anthelmintic efficacy in studies involving multiple species or immature stages of infection. Because the latter is an important component of anthelmintic efficacy given the comparatively long prepatent period of nematodes such as *A. galli*, FECRT should not be considered as a sensible alternative to the gold standard WCRT.

6. Prospects for developing *in vitro* anthelmintic efficacy tests based on a larval development test are good for the ovicidal BZ anthelmintics. The LDT is an easy test to implement, but will not work for the other anthelmintics. The advantages of such tests are that they could be deployed on faecal samples sent in from a farm, and would not require animal testing and sacrifice.

7. There are reasonable prospects for developing *in vitro* anthelmintic efficacy tests based on a larval migration inhibition test (LMIT) that would be effective for evaluating the full range of anthelmintics, but the method is far more complex to implement than LDT and may not offer sufficient advantages over the WCRT to warrant optimisation.

8. For *in vitro* studies, a saturated sugar solution is most appropriate for extracting eggs from faeces prior to use in assays. Further work is needed to optimise larval hatching/freeing methods and media for maintenance of larvae. Standardisation of assays and EC_{50} values and association with development of resistance would also need to be achieved.

5 Diagnostic method optimisation

The main focus of this work was a systematic comparison of the modified McMaster and mini Flotac® methods of estimating faecal egg counts and an evaluation of what is the most practical sample to send in from the field for estimation of flock worm burden. This work has been written up as a scientific paper and published as shown below:

Shifaw, A, Feyera, T, Elliott, T, Sharpe, B, Walkden-Brown, SW, Ruhnke, I (2021) Comparison of the Modified McMaster and Mini-FLOTAC methods for the enumeration of nematode eggs in chicken faecal samples with known and unknown egg numbers and environmental samples collected from free range farms. *Veterinary Parasitology* 299: 109582.

5.1 Introduction

Diagnosis of nematode infection and regular monitoring of 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 ranged from direct smear (Beaver 1950) to simple flotation and centrifugal flotation techniques (Gordon & Whitlock 1939; MAFF 1986; Cringoli et al. 2010; Cringoli et al. 2017), and recently advanced automated egg-counting techniques using computational (Mes et al. 2001; Mes et al. 2007) and smartphone-based image analysis systems (Slusarewicz et al. 2016; Scare et al. 2017). Detection of nematode eggs in poultry faeces using flotation is a reliable method for confirmation of the presence of gastrointestinal nematodes (Macklin 2013). Flotation methods involve separating eggs from faecal 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; Lester & Matthews 2014). Excreta egg counting techniques that determine parasite eggs per gram of faeces and use flotation are based on the microscopic examination of an aliquot of faeces suspension from a known volume of an faecal sample (Nicholls & Obendorf 1994; Cringoli et al. 2004).

Clear identification of the most practical, sensitive, accurate, reliable and precise faeces based diagnostic tools is needed to facilitate uptake of regular detection and monitoring of nematode parasite infection in the poultry industry. The usefulness of any faeces egg counting technique is influenced by the sensitivity, accuracy and precision of the method. The McMaster method was developed and improved at the University of Sydney, McMaster laboratory (Gordon & Whitlock 1939; Whitlock 1948), and it is still the most widely used egg counting technique for the detection of parasite infection in animal species. As described by various authors (Pereckienė et al. 2007; Vadlejch et al. 2011; Ballweber et al. 2014; Nápravníková et al. 2019), several McMaster modifications have been published over time to improve the diagnostic performance of the method that vary the amount of flotation solution used, the density of the flotation solution, the weight of the faeces and aliquot examined, the flotation time, the absence or presence of centrifugation, the number of counting chambers or slide area, and multiplication factors, which all affect the sensitivity, accuracy, and precision of the technique (Dunn & 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 at low EPG counts (Mes et al. 2001; Daş et al. 2020) and high variability (Daş et al. 2011; Daş et al. 2020), it is the most popular method due to its simple protocol, re-use of equipment, reasonably cheap cost and low labour time (Daş et al. 2020). The diagnostic performance and reliability of the McMaster technique greatly improves with increasing magnitude of infection (Nápravníková et al. 2019). As discussed by several authors (Cringoli et al. 2004; Pereckiene et al. 2010; Bosco et al. 2014), the sensitivity, precision and accuracy of the McMaster technique depends on the volume of faeces suspension under the slide area to be examined. For

instance, Cringoli et al. (2004) showed that 1.0 mL McMaster slide area (volume) was the most reliable and gold standard for estimating gastrointestinal strongyle EPG in sheep, whereas the smaller sample volumes (0.15, 0.3 and 0.5 mL) produced unreliable overestimates.

The FLOTAC and its derivative, the Mini-FLOTAC methods 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 incorporates a centrifugal flotation system in a chambered device sensitive to one EPG (Cringoli 2006; Cringoli et al. 2010). The FLOTAC technique outperformed egg counting technique in terms of diagnostic performance for detecting parasite infection (Cringoli et al. 2010). However, the main drawback of this technique is its complexity and the need of a special centrifuge device. In addition, it has been reported that the FLOTAC is time and labour-intensive, and requires several steps to process each sample. 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 be easily carried out, transported to any laboratories, and used on-farm (Cringoli et al. 2017; Bosco et al. 2018). However, the processing time of individual faecal sample using Mini-FLOTAC has been reported as 12 min (Cringoli et al. 2017), which is two-fold higher than the sample processing time reported (6 min) for the McMaster method (Noel et al. 2017; Daş et al. 2020).

The diagnostic performance of the Mini-FLOTAC has been assessed and compared to the McMaster technique in several mammalian host-parasite studies indicating that the Mini-FLOTAC showed higher sensitivity, accuracy, and precision. However, there are only two published studies comparing 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). The latter and more recent study evaluated MM and MF for the recovery rate of chicken nematode eggs in egg spiked faecal samples using salt or sugar flotation solutions (Daş et al. 2020).

For further optimising the methods for evaluation of nematode infections in chickens, 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 both spiked samples containing a wider range of EPG without and with using multiple operators, and with additional inclusion of faeces and environmental 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 faecal samples will be the most suitable sample to collect from free range layer flocks to indicate the infection level.

5.2 Methods

To meet the aims of this study, three related experiments were conducted. Experiments 1 and 2 compared the MM and MF using faecal samples containing known numbers of eggs without and with operator effects, respectively. Experiment 3 evaluated the two methods using individual caged laying chicken faeces and environmental samples obtained from naturally infected free range laying chicken farms. This research was approved by the Animal Ethics Committee of the University of New England with approval number AEC19-082.

5.2.1 Experimental overview

Experiment 1. Method comparison with egg spiked samples without operator effect

MM and MF were compared for the enumeration of *A. galli* eggs in chicken faecal samples spiked with known numbers of *A. galli* eggs. Faeces were collected from caged chickens free of gastrointestinal nematode infection confirmed by necropsy. A 2 x 6 factorial arrangement was employed to test two faecal egg 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 the time spent on sample processing (sample preparation from faeces weighing to slide reading and egg counting) for each sample replicate recorded. Eggs for spiking the faecal samples were harvested from faeces of chickens with mono-specific *A. galli* infections as described previously (Rahimian et al. 2016; Daş et al. 2020). In brief, the prepared faeces slurry was flushed under tap water and passed through a series of 6 sieves (PluriSelect Life Science, Leipzig, Germany) with mesh apertures of 750, 500, 200, 150, 90 and 75 µm in order to separate the eggs from the larger faecal particles, and the eggs were then collected on a 30-µm sieve. Eggs retained on the sieve were washed off using tap water into a 50 mL tube. This tube was centrifuged at 1620 x g for 1 min allowing for concentration of the parasite eggs at the bottom of the tube and then supernatant was removed leaving a volume of 5 mL in the tube. Then 45 mL of saturated sodium chloride; specific gravity (S.G.) = 1.2 was added and the tubes again centrifuged at 1620 x g for 1 min. After centrifugation, the 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 before being used to spike the egg-free faeces as detailed below. The samples were then subjected to faecal egg count (FEC) on the same day using the MM and MF methods.

Experiment 2. Method comparison with egg spiked samples with different operators

The effect of the operator on the diagnostic performance of the MM and MF methods was assessed using chicken faeces spiked with *Ascaridia galli* eggs in a 2 x 2 x 3 x 4 factorial arrangement testing the effects of egg counting techniques (MM and MF), the person preparing the samples (preparers A and B), EPG levels (5, 50, 500 EPG) and the persons counting the eggs (counters or 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 (reading of slides) were recorded from faeces weighing to slide reading. 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). Worm egg-free faeces were collected from individual chickens raised to be free of nematode infection, with worm free status confirmed by both repeated negative FEC and subsequent necropsy examination and examination of gut contents for presence of worms.

Experiment 3. Method comparison with samples from natural infection in the field

The final experiment compared the two egg counting techniques (MM and MF) for assessing FEC in faeces and environmental samples obtained from commercial free range layer flocks harbouring natural nematode infections from the field. This experiment employed a 2 x 2 x 9 factorial arrangement testing the effect of two egg counting techniques (MM and MF), farms (A and B, equivalent to Farms 3 and 5 respectively in Table 3-1) and sample types (n = 9).

5.2.2 Detailed methods

Preparation of faecal samples of known EPG count (Experiments 1 and 2)

To prepare faecal samples of known EPG count, spiking of known numbers of *A. galli* eggs into worm egg-free chicken faecal samples was performed. Egg count per ml of a stock solution was determined using the MM technique based on the arithmetic mean counts of 3 aliquots of 0.15 ml. The required number of spiked eggs and volume containing them was then calculated for each level of egg concentration (i.e. EPG level). The egg solutions (0.45–2 ml) were then added to the weighed (50–100 g) egg free faeces for each EPG level and thoroughly mixed with a spatula to achieve the known EPG counts (Experiment 1 = 5, 50, 200, 500, 1000, and 1500 EPG; Experiment 2 = 5, 50 and 500 EPG).

Collection of Individual fresh chicken faeces and environmental samples (Experiment 3)

Two hundred and thirty randomly selected free range laying chickens from two commercial free range farms (A and B) were transported to the University of New England and placed into individual cages. After a one-week adaptation period, fresh individual faecal samples were collected on paper sheets placed beneath each cage within an hour of paper placement, and subjected to FEC by the MM and MF methods performed on the same day. Individual caged chicken faecal samples were mixed by stirring with a spatula and two sub-samples (2.5 g for MM and 5 g for MF) were taken for faecal egg count analysis, resulting in a total of 230 counts by each method.

The environmental sample types included fresh floor faecal samples from the poultry house floor (individual = 20; pooled = 1 per farm), shed hard faeces from the poultry house floor (individual = 5; pooled = 1 per farm), shed floor litter material from the poultry house (individual = 5; pooled = 1 per farm), and hard faeces from the range area (individual = 5; pooled = 1 per farm). Pooled samples were obtained by pooling samples from five different indoor and outdoor locations. Pooled faecal samples were analysed in five replicates for each method.

Laboratory procedure for faecal egg counting

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 faeces collection, homogenisation and filtration through a 250 µm filter located under the lid of the Fill-FLOTAC. In brief, 5 g of faeces was weighed into the Fill-FLOTAC container, 45 ml of saturated sodium chloride salt solution (SG 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 40x magnification power with eggs counts multiplied by 5 to provide the FEC 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 faecal sample was diluted in 47.5 ml of saturated salt solution (SG 1.20), thoroughly homogenised, sieved and a 0.5 ml aliquot loaded into a chamber on a Whitlock universal slide, and examined under 40x magnification power. Eggs counted were multiplied by 40 to provide an FEC in EPG units.

5.2.3 Statistical analysis

All statistical analyses were performed in JMP 14 (SAS Institute Inc., Cary, NC, USA). FEC values 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 FECs 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 FEC 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, FEC were subjected to analysis of variance fitting up to three effects, these being farm, sample type and egg counting technique, and interactions up to three-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 for significant difference between the mean measurement variation and accuracy of the methods. Linear regression analysis was used to evaluate linear associations between measured variables. The sensitivity of MM and MF techniques to detect the presence of infection was determined within each EPG level, and calculated as Sensitivity (%) = [True Positives/(True Positives +False Negatives)]∗100. Precision of the two methods was done by comparing 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 individual observer mean EPG count for each EPG level and method. Percentage accuracy was calculated with a modification of the formula described previously (Bosco et al. 2018) to ensure the numerator represents the absolute difference between true and observed values [100-(Abs(true FEC-observed FEC) /true FEC)∗100]. Statistical significance was considered at $P < 0.05$ for all analyses.

5.3 Results

5.3.1 Experiment 1 – Sensitivity, accuracy, and precision of MM and MF

Test Accuracy. A total of 144 counts were analysed by both method in egg spiking Experiment 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 5-3. The mean egg counts obtained by the MM method were 21% higher ($P < 0.0001$) than by the MF method. 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 5-1. This revealed that both methods underestimated the true EPG counts, but as EPG level increased the MF method underestimated the true EPG level to a greater extent (31%) than the MM method (8%) as shown in **Error! Reference source not found.**. Because of this, MM was significantly more accurate for ≥ 50 EPG levels (82.1%) than MF (67.8%) ($P = 0.024$; Table 5-1), while they did not differ in accuracy overall.

Test sensitivity. 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).

Table 5-1 Experiment 1 – Mean faecal egg count, sensitivity, accuracy, and coefficient of variation (CV) between replicates (n = 12) for FEC determined by MM and MF methods in faecal samples containing different known concentrations of *A. galli* nematode eggs

Test attribute	ECT	Known concentration of eggs (EPG) or range included in overall estimates							
		5	50	200	500	1000	1500	5-1500	≥ 50
Arithmetic mean FEC	MM	0.0^{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}
Accuracy %	MM	0	60.0	78.4	90.7	89.7	91.9	68.4^{a}	82.1^{a}
	MF	60	70.0	65.5	67.4	66.4	70.0	66.5^{a}	67.8^{b}
CV %	MM	NA	60.3	7.7	3.6	3.6	1.8	NA	15.8^{a}
	MF	88.2	5.1	2.5	2.2	1.8	0.6	16.7	2.4^{a}

CV: Coefficient of variation.
NA: Not applicable.
MM: Modified McMaster.
MF: Mini-FLOTAC.
EPG: Eggs per gram of faeces.
FEC: Faecal egg count.
ECT: Faecal egg counting technique.
$^{a\,b\,c}$ Different superscripts indicating a significant difference ($P < 0.05$) are based on cube root transformed P-value.

Test coefficient of variation (CV). 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 did not differ significantly (15.8 and 2.4% respectively).

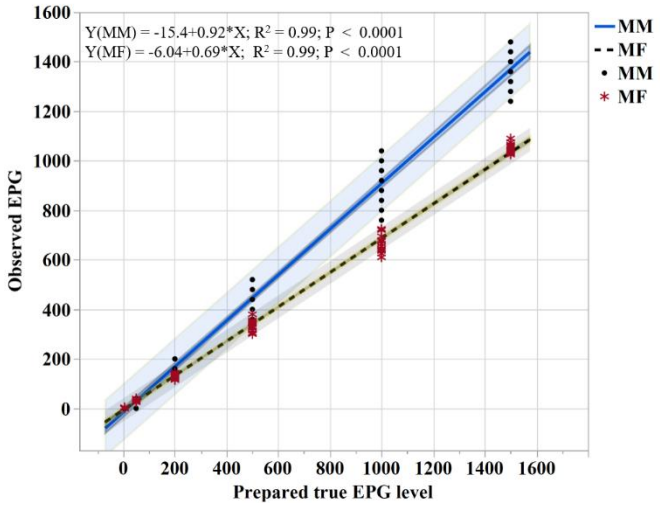


Figure 5-1 Experiment 1 – Linear regression showing the linear association between FEC determined by the Modified McMaster (MM) and Mini-FLOTAC (MF) methods in egg spiked chicken faecal samples at increasing EPG level for each egg counting technique

The association between observed EPG and true EPG level is shown by the fitted solid blue and dotted black line for MM and MF, respectively with their confidence regions.
Each point represents the individual count for replicates of each spiked EPG level (n = 12).

5.3.2 Experiment 2 – Effect of operator factors on sensitivity, accuracy and precision of MM and MF

Test accuracy. A total of 144 counts were analysed by both methods in egg spiking Experiment 2. Accuracy of MM and MF with are presented in Table 5-2. Analysis of faecal 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 (30 EPG) than MF (25 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 = 0.360–0.998). When the accuracy of counts relative to known EPG was considered, MM was more accurate for ≥ 50 EPG levels (53.4%) than MF (33.6%; Table 5-2). Both methods underestimated true egg count in the hands of 4 observers.

Table 5-2 Experiment 2 – Mean egg counts, sensitivity, percentage accuracy, and coefficient of variation of MM and MF based on observer counts for each EPG level

Test attribute	EET	Known concentration of eggs (EPG) or overall estimates				
		5	50	500	5-500	≥ 50
Arithmetic mean EPG (n = 24)	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)	MM	33.3[a]	45.8[b]	100a	59.7[b]	72.9[b]
	MF	37.5[a]	100[a]	100a	79.2[a]	100[a]
Accuracy % (n = 24)	MM	0.00	43.2	63.7	35.6[a]	53.4[a]
	MF	38.0	27.6	39.6	35.1[a]	33.6[a]
Inter-observer variation % (n = 24)	MM	37.6	38.1	6.6	27.4[a]	22.4[a]
	MF	52.5	11.5	2.8	22.2[a]	7.2[a]

CV: Coefficient of variation.
FEC: Faecal egg count.
EPG: Eggs per gram of faeces.
MM: Modified McMaster.
MF: Mini-FLOTAC.
ECT: Faecal egg counting technique.
[a b c] The superscripts indicating the significant difference (P < 0.05) for methods are based on cube root transformed P-value.
It should be noted that accuracy of methods at 5–500 EPG, and ≥ 50 were calculated based on the formula provided at 5.2.3 Statistical analysis where CV of methods at 5–500 and ≥ 50 was calculated based on average CV of EPG levels.

Test sensitivity. Sensitivity of MM and MF with operator factors are presented in Table 5-2. MF had higher overall sensitivity than MM (79.2 and 59.7%, respectively; P < 0.05).

Test coefficient of variation (CV). Coefficient of variation of MM and MF based on observer counts are presented in Table 5-2. Inter-observer coefficient of variation declined with increasing EPG level for both methods which did not differ in overall CV, being 22.2 and 27.4% respectively for MF and MM. Intra-observer was a major source of variation, particularly at low EPG levels but there was no statistically significant difference detected between methods.

Table 5-3 Summary of analysis of variance of faecal egg count (FEC) in Experiments 1 and 2 showing significance and least squares means

Experiment/factor/level	Cube root (CR) Least Square Means EPG ±SEM	CR back transformed LSM EPG (95% CI)	Arithmetic mean EPG	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}	1,014±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^{aa}	180±1.0^{a}	667±1.4^{a}
Method*EPG Level	P < 0.0001			P = 0.03	P < 0.0001	P < 0.0001
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}
Egg counters (Observers)	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}
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

LSM: Least square means; SEM: Standard error of the mean; CI: Confidence interval; EPG: Eggs per gram of faeces; CR: Cube root; MM: Modified McMaster; MF: Mini-FLOTAC.

$^{a\,b\,c}$ Different superscripts indicate a significant difference (P < 0.05) for each factor.

Preparation and counting time are presented in seconds.

5.3.3 Experiment 3 – Method comparison in samples from natural infection

The results of analysis of variance for factors affecting faecal egg count in Experiment 3 is presented in

Table ***5-4***. Of the 230 fresh individual faecal samples analysed, 91.7 and 96.5% were positively identified for nematode infection for MM and MF respectively (P = 0.023). The analysis of FEC data from these samples revealed significant effects of farm and egg counting methods 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.

The comparison analysis of samples collected from the environment and individual caged chicken faeces revealed significant effects of farm, egg counting method, and sample type. Interactions between these effects were only significant for farm x sample type (P = 0.002) (

Table ***5-4***). The MM method resulted in significantly higher EPG counts than MF (264 and 161 EPG, respectively). There were no significant differences between pooled fresh floor faeces, individual caged fresh chicken faeces and individual fresh floor faeces, indicating that fresh floor faeces (pooled or individual) may predict the true population FEC. The significant interaction between the effects of sample type and farm is shown in

Table **5-5** and revealed that pooled fresh floor faecal samples (787 EPG), and individual fresh floor faecal samples (484 EPG) and pooled shed hard faeces (396 EPG) yielded significantly higher EPG counts than both pooled and individual shed floor litter material and hard range faeces, demonstrating that these environmental samples significantly underestimated the quantity of FECs.

5.3.4 Effect of faecal 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 are presented in Table 5-2. The MF method took significantly longer in both preparation time and counting time per sample in both experiments, resulting in a total time per sample that was 3.94 and 4.43-fold higher in Experiments 1 and 2, respectively. 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 5-4 Experiment 3 – Least Squares Means of faecal egg count across fixed effect and interaction in naturally infected individual fresh faeces and environmental samples

Experiment/factor/level	Cube root LSM EPG±SEM	CR back transformed LSM EPG (95% CI)	Arithmetic mean EPG
A) Individual caged faecal sample			
Overall Mean	8.1±0.2	529 (467-596)	850
Farms	P = 0.002		
A	8.6±0.2[a]	638 (538-750)	1001
B	7.5±0.2[b]	433 (359-516)	699
Methods	P = 0.029		
MM	8.5±0.2[a]	604 (509-709)	955
MF	7.7±0.2[b]	460 (382-549)	745
B) Comparison of environmental samples and individual caged chicken faeces			
Overall Mean	5.9±0.2	205(166-256)	708
Farm	P = 0.023		
A	5.4±0.3[b]	161 (113-220)	784
B	6.4±0.3[a]	264 (197-347)	637
Method	P = 0.034		
MM	6.4±0.3[a]	261(194-342)	808
MF	5.4±0.3[b]	164 (115-224)	608
Sample types	P < 0.0001		
Pooled fresh floor faeces	9.2±0.7[a]	787 (477-1210)	795
Individual fresh faeces (caged chickens)	8.1±0.2[a]	529 (473-589)	844
Individual fresh floor faeces	7.8±0.4[ab]	484 (364-626)	677
Pooled hard shed faeces	7.3±0.7[abc]	396 (207-673)	428
Individual hard shed faeces	6.4±0.7[bc]	267 (126-486)	315
Pooled shed floor litter	5.4±0.7[cd]	159 (64-320)	176
Individual shed floor litter	4.3±0.7[d]	84 (24-195)	123
Individual hard range faeces	3.7±0.7[d]	53 (13-140)	125
Pooled hard range faeces	0.8±0.7[e]	0.65 (-0.2-12)	8
Significant interactions (P-value)			
Farm*Sample type	P = 0.002		

LSM: Least square means.
SEM: Standard error means.
CI: Confidence interval.
EPG: Egg per gram of faeces.
DF: Degree of freedom.
CR: Cube root.
MM: Modified McMaster.
MF: Mini-FLOTAC.
[a b c d] The superscripts indicate the significant difference (P < 0.05) for each sample type and method based on P-values obtained from cube root transformed FEC.

Table 5-5 Experiment 3 – Excreta egg count and proportion of positive samples for MM and MF in different faecal samples collected from naturally infected individual caged chickens or their environment

	Farm A		Farm B		Overall proportion of positive samples (%) ~~regardless of farm type~~	
Sample types combined	**CRBT LSM EPG (95% CI)**	**Arithmetic Mean EPG**	**CRBT LSM EPG (95% CI)**	**Arithmetic Mean EPG**	**MM**	**MF**
Pooled fresh floor faeces	750 (354-1366)^{a}	760	827 (401-1480)^{a}	831	100	100
Individual fresh faeces (caged)	638 (547-738)^{a}	1001	433 (366-507)^{a}	699	91.7^{b}	96.5^{a}
Individual fresh floor faeces	323 (202-484)^{ab}	538	691 (481-954)^{a}	816	95	95
Pooled shed hard faeces	300 (103-661)^{abc}	343	509 (213-999)^{ab}	382	100	100
Individual shed hard faeces	206 (59-496)^{abcd}	249	339 (122-726)^{ab}	514	100	100
Pooled shed floor litter	122 (26-338)^{abcd}	141	204 (58-492)^{abc}	211	100	100
Individual shed floor litter	31 (2-138) ^{bcd}	49	176 (46-441)^{abc}	197	100	100
Individual hard range faeces	49 (5-183)^{bcd}	150	58 (6-203)^{bcd}	101	70	70
Pooled hard range faeces	0^{d}	0	5 (-0.02-53)^{cd}	16	30	33

This table represents a significant interaction between farm and combined sample type.
CRBT: Cube root back transformed.
EPG: Egg per gram of faeces.
CI: Confidence interval.
$^{a\,b\,c\,d}$ The superscripts indicating the significant difference ($P < 0.05$) for each sample type and farms are based on cube root transformed P-value.
Values without superscript under modified McMaster (MM) and Mini-FLOTAC (MF) column indicate no significance difference between methods ($P > 0.05$).

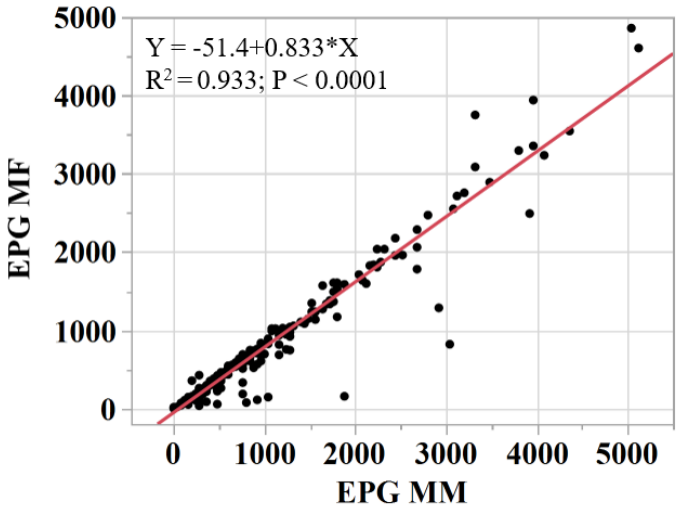


Figure 5-2 Linear regression showing the linear association between FEC determined by the Modified Mc Master (MM) and Mini-FLOTAC (MF) methods in naturally infected individual fresh faecal samples at increasing EPG (egg per gram of faeces) values for each egg counting technique. Each dot point represents an faecal sample from a single chicken

5.4 Discussion, conclusions and implications

The most important finding of this study was that while both the MM and MF underestimated the true FEC, the underestimation by MF was far greater and increased with increasing FEC. This underestimation was not only observed in both spiking studies but also in Experiment 3 with samples of unknown EPG where the FECs for the MF method were significantly lower than those detected by the MM method. This low accuracy of detection by the MF method in the FEC range coupled with the much 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 FEC makes it a more appropriate test when determining freedom from infection is required. With regard to sample type to submit from the field, our data showed that fresh faecal samples collected from the floor, either individual or pooled, had the best prediction of the true flock FEC as determined by individual counts on 110120 chickens per farm. Egg counts in hard range faeces and floor litter significantly underestimated the population FEC.

The egg spiking results showed that the MM method recovered more nematode eggs > 50 EPG level compared to MF with the divergence in accuracy 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 in the range of FEC where the MF method undersestimates actual FEC. The accuracy of MM methods increased as EPG or infection levels were increasing whereas MF continued to underestimate the true population of FEC, 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 FEC 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 are at least 10-fold higher than a minimum detection limit of 50 EPG (Thapa et al. 2015a; Daş et al. 2020). The large amount of time required to process samples by 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 should be the preferred method for field evaluation of FEC in poultry flocks where treatment decisions are required. However, the greater sensitivity of MF at very low EPG levels which is a feature of the method and has been reported in other studies (Noel et al. 2017; Scare et al. 2017; Bosco et al. 2018; Amadesi et al. 2020; Daş et al. 2020) make it a preferred method for diagnosing very low nematode burdens, or testing to certify freedom from infection.

Operator factors did not have a significant effect on mean egg counts in the present study, indicating that both methods are able to be implemented effectively by different operators. However, the reliability and the accuracy of all egg counting techniques relies on having experienced and skilful operators (Ballweber et al. 2014).

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 analytical sensitivity by design (Lester & Matthews 2014; Daş et al. 2020). The sensitivity of egg counting techniques relies on the amount of faecal sample to be examined, the true EPG level (concentration or density of eggs) and the multiplication factor of egg counting methods (Ballweber et al. 2014; Lester & 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 FEC is important.

In this study, MF had a relatively lower coefficient of variation or a higher overall precision than MM. This is likely due to the combined effect of Fill-FLOTAC homogenising and filtering device, and the two counting chambers allowing for the examination of a larger amount of faecal 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 variation was greater for MM than in MF overall. The precision and sensitivity of egg counting techniques can be generally improved by examining larger volumes of faeces suspension by increasing the number of chambers counted, performing multiple faecal egg counts, or repeating of slide reads from the same chambers (Cringoli et al. 2004; Vidyashankar et al. 2012; Ballweber et al. 2014; Lester & 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 proposition that pooled fresh floor faecal samples will be the most suitable sample to collect from a free range flock to indicate infection level was supported by the findings of Experiment 3. The analysis of samples collected from the environment revealed that pooled fresh floor faeces (787 EPG) and individual fresh floor faeces (484 EPG) yielded significantly higher EPG counts than shed floor material and hard range faeces types ($P < 0.0001$).

In conclusion, the higher accuracy and the much shorter sample processing time of the MM method make it far more suitable for field evaluation of FEC in chickens than 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 FEC 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 faecal samples collected from the floor of the poultry house and counted individually, or pooled, provided a good estimate of the population FEC. Flock FEC was significantly underestimated in dried range faeces and litter material.

6 Nematode egg recovery and storage

A fundamental issue of working with chicken nematodes is the difficulty of maintaining defined strains of the parasites that have been characterised and would be useful for use in research studies (e.g. strains or isolates with known levels of anthelmintic resistance). For most ruminant nematodes the infective stage of the life cycle is an infective larva on pasture, and these are amenable to long-term storage in liquid nitrogen following exsheathment (Van Wyk & Gerber 1980). For the chicken nematodes, the infective stage is usually a thick walled embryonated egg, which is difficult to freeze without injury, and which has a limited storage life (several months) at refrigerator or room temperatures. Another important component of attempts to develop and maintain strains for experimental studies is the source of nematode eggs to begin or maintain the process. Should they be sourced from mature worms (requiring extraction of worms from the chicken host) or from faeces? To address these issues four studies were carried out, three led by Teka Feyera Dewo and the last by Anwar Shifaw Yesuf. One of the studies, on extraction of *A. galli* eggs from faeces is reported in an earlier section (4.5.2.1) and revealed extraction rates of 58–68% of eggs from faeces, with a high extraction rate (67.8%) and the best egg viability following extraction (98.1%) achieved using a saturated sucrose solution. The remaining 3 studies are reported in this section and comprise:

1. Viability and developmental capacity of *A. galli* eggs recovered from mature worms in artificial media then subjected to different storage and incubation conditions (Teka Feyera).

   Published as (Feyera et al. 2020).

   Feyera, T., Ruhnke, I., Sharpe, B., Elliott, T., Campbell, D. L. M., & Walkden-Brown, S. W. (2020). Viability and development of Ascaridia galli eggs recovered in artificial media followed by storage under different conditions. Journal of Helminthology, 94, e199. doi:10.1017/S0022149X2000084X

2. Survival of *A. galli* eggs exposed to low temperatures and cryoprotectant (Teka Feyera).
3. Optimisation of methods for prolonged laboratory storage of viable *A. galli* eggs (Anwar Shifaw).

   Publsihed as Shifaw et al. (2022).

   Shifaw, A., Feyera, T., Elliott, T., Sharpe, B., Ruhnke, I., & Walkden-Brown, S. W. (2022). Method optimisation for prolonged laboratory storage of Ascaridia galli eggs. Veterinary Parasitology, 309, 109758. doi:https://doi.org/10.1016/j.vetpar.2022.109758

6.1 Study 1 – Viability and developmental capacity of *Ascaridia galli* eggs recovered from mature worms in artificial media then subjected to different storage and incubation conditions

6.1.1 Introduction – Study 1

Among the helminths affecting chickens, the large round worm *Ascaridia galli* (*A. galli*) is the most important in free range laying hens (Katoch et al. 2012; Thapa et al. 2015a). *A. galli* belongs to the genus *Ascaridia* within the family Ascaridiidae and occurs worldwide as a small intestinal parasite of galliform birds (Soulsby 1982). It has a direct life cycle, which includes two principal stages: the sexually active adult worms in the intestine of the host and a free-living stage (eggs) in the environment. Nematode egg shedding in the faeces starts approximately 5–8 weeks after infection. This is followed by the *in ovo* development of eggs to infective larvae (L3) in the external environment. The life cycle is

completed when a new host ingests infective eggs, which hatch (L3) in the lumen of the small intestine. The L3 then embed into the mucosa where they moult to become L4, which finally develop into adults (Ackert 1931; Permin & Hansen 1998; Taylor et al. 2007).

Experimental infections are important to better understand this parasite and its effects. Low infection rates and burdens after artificial infection have been reported (Permin et al. 1997a; Kaufmann et al. 2011; Sharma et al. 2018) thus reducing the power to detect the effects of experimental factors. The *in vivo* propagation of ascarids depends on the source of eggs, which must incubate and embryonate outside the host (Elliott 1954). Thus, variable artificial infection rates may be due not only to host factors such as individual variation in host susceptibility (Gauly et al. 2005; Kaufmann et al. 2011) but also to the viability and embryonation status of the infective material used (Elliott 1954). Early studies showed that the infectivity (Elliott 1954) and severity of infection (Todd et al. 1952) of *A. galli* larvae diminished with age of embryonated eggs. Eggs used to induce *A. galli* infection can be obtained from several sources: the host faeces (Luna-Olivares et al. 2012; Ferdushy et al. 2013), physical removal from, or disruption of the worm's uterus (Gauly et al. 2002; Daş et al. 2010) or by *in vitro* culturing of female worms in artificial media and recovering eggs shed into the media typically across 3–5 days (Dick et al. 1973; Salih & Saleem 1987; Ruhnke et al. 2017; Sharma et al. 2017).

A. galli eggs are highly adaptable to wide ranges of environmental conditions primarily due to the inherent characteristics of their thick eggshells (Cruthers et al. 1974; Thapa et al. 2017). However, their long-term survival and developmental capacity are affected by several factors. The reported optimum embryonation temperature for *A. galli* ranges between 25 and 30°C (Christenson et al. 1942; Ramadan & Abouznada 1992; Mero & Gazal 2008; Tarbiat et al. 2015). As storage temperature increases, egg development gradually reduces and may totally cease beyond 35°C (Ackert 1931; Reid 1960). *A. galli* eggs can develop into the L3 larval stage at temperatures as low as 15°C, while at sub-zero temperatures (down to -5°C) viability may be preserved depending on the duration of exposure but no development occurs (Tarbiat et al. 2015). This may suggest a strategy for cold tolerance in *A. galli* like other nematodes (Wharton 1980). Furthermore, a relative humidity of approximately 85–90% and continuous oxygen supply are necessary for *A. galli* egg embryonation (Hansen et al. 1953; Tarbiat et al. 2015). Several options for the embryonation of *A. galli* eggs under laboratory conditions have been identified (Permin et al. 1997c; Katakam et al. 2014), allowing development of embryonated infective stages within a few days or weeks depending on the method being used (Permin & Hansen 1998; Onyirioha 2011). However, the developmental potential of eggs can be significantly affected by the pre-embryonation storage conditions (Tarbiat et al. 2018), the embryonation media (Permin et al. 1997c), as well as the maturation level of eggs during egg recovery (Rahimian et al. 2016).

A recent study found that less than 40% of eggs manually removed from worm uteri embryonate, whereas approximately 90% of eggs obtained from poultry faeces do so (Rahimian et al. 2016). It has been suggested that not all eggs obtained *in uteri* are mature and able to complete embryonation (Tiersch et al. 2013) whereas adult female worms appear to shed only mature eggs (Kim et al. 2012). In line with this, a number of *A. galli* studies have used eggs oviposited by mature female worms under *in vitro* incubation in artificial media such as physiological saline (0.85% NaCl) (Dick et al. 1973; Salih & Saleem 1987) and Roswell Park Memorial Institute (RPMI) media (Ruhnke et al. 2017; Sharma et al. 2017). This approach can be considered the most feasible, efficient and established method of recovering mature *A. galli* eggs for experimental purposes. However, while it has been proven that *A. galli* eggs can successfully be recovered by incubating mature female worms in artificial media, information on the number, viability and embryonation profile of eggs recovered at different times post-incubation of mature females is lacking. Likewise, the viability of larvae within the embryonated eggs is rarely assessed before use for experimental infections and the duration for which eggs are

stored and incubated varies between studies (Katakam et al. 2014).

Therefore, the objectives of the present study were to define the rate and quality of egg production by mature *A. galli* worms cultured in RPMI media as well as temporal changes in egg viability and embryonation capacity under different storage and incubation conditions. The general hypotheses in this experiment were: i) rate of egg production and quality of eggs harvested will decline with time of incubation of mature worms; ii) overall viability of stored eggs will decline at different rates following storage without embryonation at 4°C and after embryonation at 26°C; and iii) larvae liberated from morphologically normal vermiform eggs will have high viability as assessed by motility and vital staining.

6.1.2 Methods – Study 1

Ascaridia galli eggs

Mature female *A. galli* worms were collected from the intestines of naturally infected free range Isa Brown laying hens (n = 50) during post-mortem examination in an experiment approved by the animal ethics committee of the University of New England (approval AEC17-092). For the worm harvest, the hens were humanely killed, dissected, and the intestines repositioned. The jejunum and ileum were opened longitudinally and mature *A. galli* worms identified, collected into Petri dishes and washed in sterile phosphate-buffered saline (PBS). A total of 223 mature females were transferred into a glass jar with RPMI media (Sigma-Aldrich Pty Ltd, St Louis, USA) including 0.1% 100 units/mL penicillin, 100 µg/mL of streptomycin, 250 ng/mL amphotericin B, to a volume that covered the worms (Sharma et al. 2017). The worms were then cultured for three days at 37°C, changing the total media every 24 hours. After every 24 hours, the media containing parasite eggs was collected into 50 ml screw cap falcon tubes by rinsing the jar with fresh RPMI media. The egg suspension was then centrifuged (Beckman Coulter Inc, Brea, California, USA) at 425 g for 1 min, and eggs concentrated at the bottom of the media were collected using transfer pipettes. Eggs recovered over three different collection days were kept separately in 50 ml falcon tubes. To estimate the mean daily as well as total *A. galli* egg production under this *in vitro* worm incubation system, a 20 ml egg suspension in distilled water was made for each collection day and egg counts performed in triplicates using a modified McMaster egg count technique (Whitlock 1948). For all the egg collection days, 200–300 eggs were examined microscopically at 100× magnification in triplicates to estimate the proportions of intact (undamaged) eggs at the time of recovery. The recovered eggs were then stored in boiled and cooled sterile water at 4°C for later use.

Experimental design of egg storage component

The experimental subjects of this study were *A. galli* eggs stored at 4°C for 1 week. Eggs separately recovered on 3 consecutive days (days 1, 2 and 3) were subjected to either: (1) a 3 x 3 factorial arrangement of storage in water at 4°C (1, 4 or 8 weeks) followed by aerobic incubation in 0.1 N H_2SO_4 at 26°C (2, 4 or 6 weeks); or (2) prolonged storage in water at 4°C for 14 weeks; a factorial arrangement of day of egg collection (3) x storage period (8) (Figure 6-1). There were 3 replicates of each treatment combination. An egg suspension in sterile distilled water (boiled and cooled) was prepared from the sample of each collection day. A total of 162 Eppendorf tubes (1.5 ml) were filled with 1 ml of an *A. galli* egg suspension containing approximately 500 eggs and then the tube lids sealed. These samples were then subjected to each condition and after the relevant treatment periods, eggs were monitored for viability and development as detailed below. To ensure aerobic conditions during embryonation, tube lids were left open and samples were aerated manually for 5 minutes three times

per week.

Monitoring of egg development and viability

The development of eggs was evaluated by examining egg morphology under a compound binocular microscope equipped with a digital Nikon H550S camera (Nikon Corporation, Tokyo, Japan). A minimum of 100 eggs per Eppendorf tube were examined and the proportion of eggs at different stages of development (undeveloped, early development, vermiform and embryonated) or damaged/dead were recorded for each category using the morphological classifications described elsewhere (Tarbiat et al. 2015; Thapa et al. 2017) and shown in Figure 6-2. Unembryonated eggs contained a single cell, which almost completely filled the eggshell and appeared granulated. Eggs undergoing mitosis (2 cells and above) without signs of differentiation were classified as early development stages. The vermiform stage was characterised by a non-motile (tadpole-like) embryo, which almost filled the entire capsular space with high terminal opacity. Embryonated eggs that had completed development contained coiled slender motile larvae. Eggs with an abnormal intra-capsular mass, disrupted eggshell and a shrunken internal embryonic mass were considered dead (Figure 6-2).

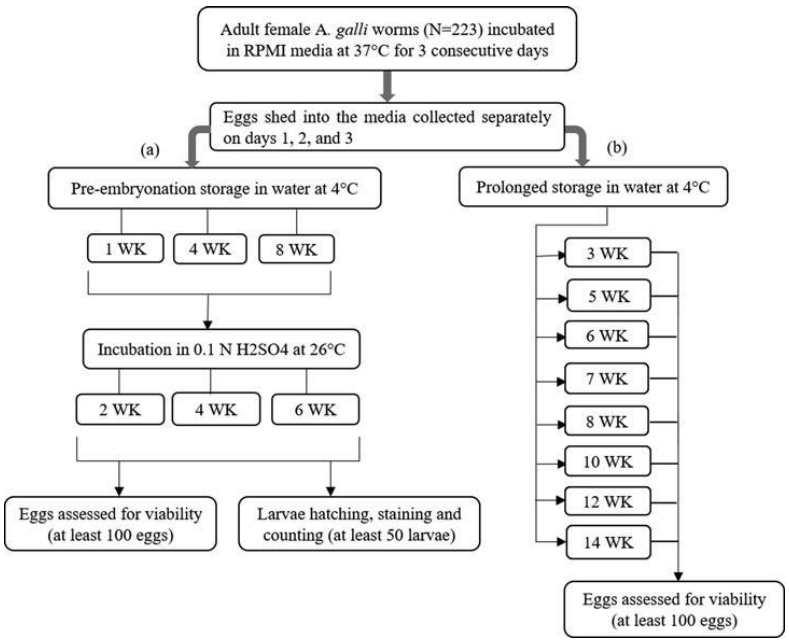


Figure 6-1 Experimental design

A. galli eggs recovered after 1, 2 or 3 days of *in vitro* culture of adult female worms were subjected to: (1) storage in water at 4°C (1, 4 or 8 weeks) followed by incubation in 0.1 N H2SO4 at 26°C (2, 4 or 6 weeks); or (2) prolonged storage at 4°C (up to 14 weeks). Egg development and viability were assessed by microscopic examination of at least 100 eggs. At the end of each incubation period at 26°C, embryonated eggs were subjected to induced larvae hatch followed by vital staining and microscopic examination of at least 50 larvae for viability assessment.

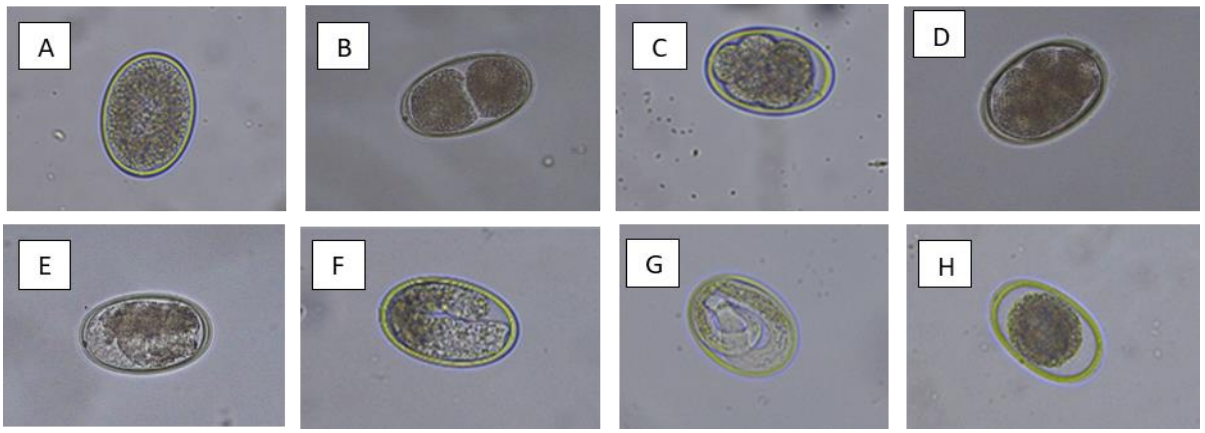


Figure 6-2 ***Morphological characteristics of*** **Ascaridia galli** ***eggs at different developmental stages (original magnification 200x): unembryonated (A); early development (B, C and D); vermiform (E and F); embryonated (G) and dead (H)***

In vitro larvae hatch and viability test

At the end of each incubation period at 26°C, embryonated eggs were subjected to *in vitro* hatching, liberating the larvae using a modification of the method developed by Dick et al. (1973). *A. galli* eggs were placed in a solution containing equal parts of 4% NaOH (Chemsupply Pty Ltd, Gillman, Australia) and 4% NaClO (Pental Limited, Shepparton, Australia) for 24 hours at 25°C. After this treatment, the samples were incubated in 0.2% Tween-80 (Redox Pty Ltd, Auburn, Australia) for 1 hour and washed three times in distilled water followed by centrifugation at 425 g for 3 minutes and removal of the supernatant. Liberation of the larvae was enforced by centrifugation at a speed of 930 g for 10 minutes. The liberated larvae were tested for viability using the methylene blue (Fronine Pty Ltd, Melbourne, Australia) exclusion method, where uptake of dye indicates cell death and inactivation as described earlier (Shafir et al. 2011). Hatched larvae in solution were mixed 1:1 with a 1:10,000 dilution of methylene blue. Viable larvae remained motile and unstained (Figure 6-3 a b), whereas nonviable larvae absorbed the methylene blue stain (Figure 6-3 c d). At least 50 liberated larvae were counted per sample and their viability recorded by microscopic examination at 100× magnification.

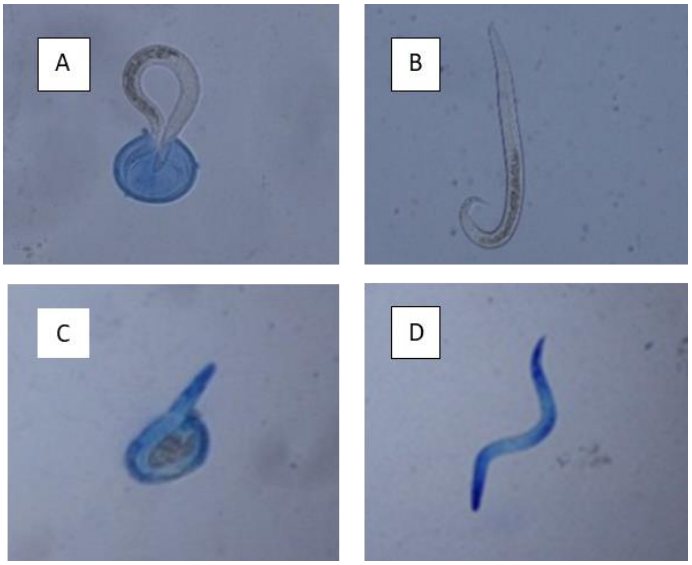


Figure 6-3 Hatched *A. galli* larvae stained with methylene blue (original magnification 200x)

(A) Viable larvae demonstrating intact membrane and impermeability to methylene blue with stained remnant of eggshell debris.
(B) Fully liberated viable motile larvae demonstrating intact membrane and impermeability to methylene blue.
(C) Nonviable larvae demonstrating uptake of methylene blue with remnant of eggshell debris.
(D) Fully liberated nonviable larvae demonstrating uptake of methylene blue.

Statistical analysis

Data were statistically analysed using JMP® software version 14.3.0 (SAS Institute Inc., Cary, NC, USA). Distributions of the data and model residuals were assessed for compliance with the assumptions of ANOVA. No data transformations were required. Two analyses were carried out to evaluate effects of study factors on egg viability and embryonation capacity. In the first analysis, day of egg collection (1, 2, or 3), pre-embryonation storage period at 4°C (1, 4, or 8 weeks), and incubation period at 26°C (2, 4, or 6 weeks), and their interactions were fitted as fixed effects in a linear model. In the second analysis, the day of egg collection (1, 2, or 3), storage period at 4°C (3–14 weeks), and their interactions were fitted as fixed effects in a linear model. In order to evaluate overall changes in egg viability, the percentage viable egg stages were defined as the proportion of eggs in developmental stages other

than dead. Percentages were based on triplicate counts of 100 eggs, so a total of 300 observations. For larval measurements a one-way ANOVA was performed to test the effect of incubation period on the viability of hatched larvae. Percentages were based on triplicate counts of 50 eggs or 50 larvae, so a total of 150 observations. All post hoc comparisons were performed using Tukey's HSD test and data were presented using least-squares means (LSM) and standard errors (SE). Linear regression was used to test the association between egg/*in ovo* larvae viability and storage or incubation periods. The significance was set at $P < 0.05$ throughout the analysis.

6.1.3 Results – Study 1

Rate and quality of egg recovery by day of worm incubation

A total of 223 mature female worms were recovered from 50 Hy-Line Brown hens humanely killed on the same day and used to collect eggs for three consecutive days. The size of the worms was not measured but all were viable and structurally intact. A total of 1.35×10^6 eggs were collected with 49.2, 38.5 and 12.3% recovered on days 1, 2 and 3 of *in vitro* worm incubation, respectively. The mean egg yield per female worm per day was 2973 ± 125, 2326 ± 14, and 745 ± 69 on days 1, 2 and 3 of collection, respectively ($P < 0.0001$) and a single female worm produced 6,044 eggs on average. The percentage of intact (undamaged) eggs at the time of recovery was 99% with no difference between eggs collected on different days ($P = 0.69$).

Effects of experimental factors on egg viability and embryonation capacity

Table 6-1 summarises the effects of day of egg collection, storage period at 4°C and incubation period at 26°C on the percentage of *A. galli* eggs in different developmental stages. Comparison of overall egg viability status between egg collection days revealed small differences, with eggs from day 2 having the highest viability ($P = 0.044$). Pre-embryonation storage period at 4°C had a marked effect on egg viability. Eggs stored for 1 week had a significantly higher percentage of viable stages (91.8%) than eggs kept for 4 weeks (73.7%), which in turn had a higher percentage than those stored for 8 weeks (52.2%) ($P < 0.0001$). Percentage viable stages also declined significantly with incubation period. Eggs incubated for 2 weeks had the highest overall viability (76.9%) followed by those incubated for 4 (72.6%) or 6 (65.3%) weeks ($P < 0.0001$). There was a 1.95% ($y=80.4-1.95x$) loss in egg viability every week of aerobic incubation at 26°C. There were no significant interactions ($P > 0.05$) between treatment effects for total viable stages.

Irrespective the duration of the incubation period, the vast majority of eggs had either reached the coiled embryo stage (66.8%) or were dead (27.4%) when being stored at 26°C (Table 6-1). Treatment effects on percentage embryonation were thus similar to those for total viable stages presented above. Percentage embryonation showed a sharp decline with storage period at a rate of 5% ($y=88.5-5x$) per week ($P < 0.0001$) but not incubation period. The proportions of undeveloped, early development and vermiform stages diminished drastically ($P < 0.0001$) within the pre-embryonation storage period and incubation period. There were significant interactions between the effects of storage period and embryonation period on the early stages of development (Table 6-1). These were largely due to unusually high values for these stages in samples stored for 4 weeks and incubated for 2 weeks.

Viability of hatched larvae

The induced egg hatch resulted in liberation of 96.398.5% of larvae from the eggshell. Incubation period at 26°C had a highly significant effect on the percentage viability of liberated larvae (P = 0.0007). A significantly higher percentage of viable larvae were observed in eggs incubated for 2 (88.2%; P = 0.0002) or 4 (86.4%; P = 0.0095) than for 6 (82.6%) weeks. Larvae viability declined by 1.4% (y=91.3-1.4x) for every week of incubation at 26°C between 2 and 6 weeks.

Table 6-1 Percentages (LSM) of different *A. galli* egg developmental stages following pre-embryonation storage at 4°C (1–8weeks) and subsequent aerobic incubation at 26°C (1–6 weeks)

Parameter		Unembryonated	Early development	Vermiform	Embryonated	Viable stages	Dead
N		81	81	81	81	81	81
Overall mean ± SE		1.23±0.12	2.6±0.17	1.81±0.13	66.82±0.61	72.56±0.57	27.44±0.57
Effect and Level							
Day of egg collection (DEC)		P=0.04	P=0.32	P=0.67	P=0.003	P=0.022	P=0.022
	1	1.41±0.21	2.52±0.28	1.90±0.22	65.37±1.05	71.22±0.99	28.28±0.99
	2	0.78±0.21	2.51±0.28	1.64±0.22	69.94±1.05	74.88±0.99	25.12±0.99
	3	1.50±0.21	3.05±0.28	1.87±0.22	65.14±1.05	71.57±0.99	28.43±0.99
Storage period (SP)		P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001
(weeks)	1	1.30±0.21	2.22±0.28	3.14±0.23	85.12±1.05	91.80±0.99	8.20±0.99
	4	2.06±0.21	4.08±0.28	1.92±0.23	65.19±1.05	73.67±0.99	26.33±0.99
	8	0.32±0.21	1.78±0.28	0.35±0.23	49.74±1.05	52.21±0.99	47.79±0.99
Embryonation period		P<0.0001	P<0.0001	P<0.0001	P=0.013	P<0.0001	P<0.0001
(EP) (Weeks)	2	3.11±0.44	5.50±0.28	3.50±0.22	64.62±1.05	76.74±0.99	23.26±0.99
	4	0.36±0.44	1.24±0.28	1.18±0.22	69.20±1.05	72.00±0.99	28.00±0.99
	6	0.21±0.44	1.34±0.28	0.74±0.22	66.63±1.05	68.93±0.99	31.07±0.99
Interactions (P-values)							
	DEC*SP	0.04	0.29	0.19	0.60	0.27	0.27
	DEC*EP	0.24	0.90	0.73	0.36	0.45	0.45
	SP*EP	<0.0001	<0.0001	<0.0001	0.15	0.05	0.05
	DEC*SP*EP	0.39	0.61	0.20	0.93	0.93	0.93

DEC: Day of egg collection.
SP: Storage period.
EP: Embryonation period.

Table 6-2 Percentages (LSM) of different *A. galli* egg developmental stages following prolonged storage in water at 4°C

Parameter		Undeveloped	Early development	Vermiform	Viable stages	Dead
N		81	81	81	81	81
Overall mean ± SE		66.15±0.60	4.23±0.18	0.12±0.03	70.51±0.61	29.49±0.61
Effect and Level						
Day of egg collection (DEC)		P=0.39	P=0.009	P=0.70	P=0.86	P=0.86
	1	65.13±1.04	5.04±0.32	0.08±0.06	70.25±1.06	29.75±1.06
	2	67.17±1.04	3.66±0.32	0.15±0.06	70.97±1.06	29.03±1.06
	3	66.16±1.04	4.00±0.32	0.14±0.06	70.32±1.06	29.68±1.06
Storage period (weeks)		P<0.0001	P<0.0001	P=0.016	P<0.0001	P<0.0001
	3	92.56±1.76	2.44±0.53	0.00±0.10	95.00±1.79	5.00±1.06
	5	89.02±1.76	4.96±0.53	0.34±0.10	94.32±1.79	5.68±1.06
	6	77.94±1.76	4.32±0.53	0.00±0.10	82.27±1.79	17.72±1.06
	7	80.88±1.76	6.88±0.53	0.44±0.10	88.22±1.79	11.78±1.06
	8	65.70±1.76	4.02±0.53	0.11±0.10	69.82±1.79	30.18±1.06
	10	49.27±1.76	4.17±0.53	0.11±0.10	53.55±1.79	46.45±1.06
	12	40.26±1.76	3.41±0.53	0.00±0.10	43.67±1.79	56.33±1.06
	14	33.61±1.76	3.67±0.53	0.00±0.10	37.28±1.79	62.72±1.06
Interactions (P-values)	DEC*SP	0.96	0.15	0.62	0.83	0.83±1.06

DEC: Day of egg collection.
SP: Storage period.

Effect of prolonged storage at 4°C on egg developmental stages and mortality rate

Following storage at 4°C, the majority of eggs either remained undeveloped but viable (66.2%) or dead (29.5%) with the former decreasing and the latter increasing with storage time (Table 6-2). Only a small proportion (4.2%) entered the early development stage, a negligible proportion (0.12%) reached the vermiform stage and no egg was fully embryonated. Proportions of all egg developmental stages as well as egg mortality rate did not differ ($P > 0.05$) between the egg collection days. The percentage of viable eggs diminished significantly with refrigeration period by 6.2% per week ($y=119.9-6.2x$, $P < 0.0001$). In contrast, egg mortality sharply increased with storage period ($P < 0.0001$) reaching 62.7% after 14 weeks of storage. There was no significant interaction ($P > 0.05$) between day of egg collection and storage period on the observation of any of the egg developmental stages and mortality.

6.1.4 Discussion and conclusions – Study 1

These results showed that per capita egg yield declined with time of worm incubation but the morphological quality of eggs harvested did not differ over the first 3 days of egg collection. However, day of egg recovery did have minor effects on subsequent egg development and embryonation ability. Much larger effects on egg viability were induced by differences in the duration of storage at 4°C and following incubation at 26°C. Overall viability of eggs declined at different rates at these two temperatures. A high rate of loss of egg viability (6.2% per week) was observed after storage at 4°C, whereas a smaller but significant rate of decline in egg (2.0%) and *in ovo* larval (1.4%) viability (3.4% in aggregate) per week of incubation period at 26°C was observed.

Our first hypothesis that the rate of egg production and quality of egg harvested will decline with time of incubation of mature worms was supported by our findings in the case of egg production, but not egg quality. The mean egg yield per female worm per day significantly declined by the day of worm incubation. Overall, a single female worm produced about 6,044 eggs over three days with the highest proportion (49.2%) recovered on day 1 and the least (12.4%) on day 3. Given that *A. galli* is a large and highly fecund nematode, the mean (2,015) per capita egg production per day in the current *in vitro* system appeared to be substantially lower than estimates of *in vivo* egg production. While it is accepted that worm size, parasite density and host-parasite interaction can affect fecundity of *A. galli*, *in vivo* per capita egg production rates in faeces ranging from 9,000 (Daş et al. 2017) to as high as 40,000 (Wongrak et al. 2015) per day were reported. A likely reason for the lower egg yield *in vitro* is the artificial environment and lack of food resources such that by the third day of incubation the majority of the worms were immobile (but intact) indicating probable death. However, the morphological quality of *A. galli* eggs harvested by the current procedure was not affected by the duration of worm incubation in RPMI media. The proportion of morphologically abnormal eggs at the time of recovery was very low (≤ 1%) irrespective of the day of collection.

Subsequent egg development and embryonating ability was mainly affected by factors other than the day of egg collection. Our finding that a high proportion of harvested eggs embryonate following shorter storage and incubation periods (> 80%) affirms the observation that mature female worms lay mature eggs that can complete development (Kim et al. 2012). Overall, this method of egg collection generated a large number of eggs of high concentration and quality with minimal effort once worms were collected. It did not require separation from faeces or macerated worm tissues, nor did it suffer from a low or variable percentage of viable eggs. It can be concluded that the current egg collection approach can be considered as the most favourable for assays involving *A. galli* eggs where initial viability and embryonation ability is a priority.

The second hypothesis that the viability of stored eggs will decline at different rates following storage without embryonation at 4°C and after embryonation at 26°C was supported by the data with a higher rate of loss of viability observed during storage at 4°C. Storage of fresh unembryonated eggs at 4°C caused a 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. This was evident in both the short-term (up to 8 weeks, Table 6-1) and relatively prolonged (up to 14 weeks; Table 6-2) storage conditions of the current study. In the 14-week storage experiment without inducing embryonation, morphological assessment showed a linear decline in overall egg viability of 6.2% every week of storage at 4°C, with percentage viability reducing to only 37% after 14 weeks. As 4°C is a commonly used preservation temperature for laboratory storage of nematode eggs, our data indicate that refrigerated *A. galli* eggs may experience complete loss of viability by around 20 weeks of storage despite the fact that these eggs have resistant thick shells and have been shown to survive extreme low temperatures for shorter periods (Cruthers et al. 1974; Tarbiat et al. 2015). In the 8-week experiment, the rate of loss of total viable stages assessed following embryonation was somewhat lower at around 5.7% per week of storage at 4°C, and the ability to embryonate was reduced at a rate of 5% per week. In agreement with these observations, Tarbiat et al. (2018) observed that the embryonation ability of *A. galli* eggs declined linearly with the duration of aerobic refrigeration in water at a rate of approximately 4.4% per week over a 72-day storage period. However, when eggs were stored in water at 4°C under anaerobic conditions the embryonation capacity declined by only about 0.15% per week. These suggest that presence of oxygen during storage at 4°C had a major adverse effect on the viability of eggs. In the present experiment, anaerobic conditions were approximated by storing eggs in boiled and cooled water with lids sealed but this appears not to have been sufficient to exclude air totally.

As expected, egg development was very limited at 4°C with undeveloped viable eggs or dead eggs being dominant. The percentage of eggs that entered early development stages never exceeded 5% during storage at this temperature. This observation is in accordance with the report of Tarbiat et al. (2015) who recorded that *A. galli* egg development was highly limited at 5°C. This result is also similar to the reported inhibition of development of *Toxocara canis* eggs at 4°C (Gamboa 2005) and *Ascaris suum* eggs at 5°C (Kim et al. 2012).

The rate of decline in egg viability following incubation at 26°C for 2–6 weeks comprised two components. The first was a decline in morphological normality of eggs and the second a decline in the viability of larvae artificially hatched from morphologically normal eggs. The former showed a rate of decline of 2% per week during the six-week period at 26°C coupled with a decline in larval viability of 1.4% per week. The additive effects of these two rates of loss (3.4%) was suggestive of a slower rate of loss of viability during storage at 26°C than at 4°C (5.7–6.2%). These indicate that a storage period of at least 40 weeks at 26°C would elapse before a total loss of viability occurred compared to the estimated 20 weeks at 4°C. Findings from early studies support our observation that *A. galli* eggs can survive for prolonged period at embryonation temperatures. For instance, Elliot (1954) observed that embryonated *A. galli* eggs stored aerobically in water for 36 weeks at 28°C were infective to chickens. Similarly, Butler and Christenson (1942) reported that cultures of embryonated *A. galli* eggs remained viable for nearly 2 years as assessed by an *in ovo* larval motility testing method. However, some other researchers were of the opinion that infectivity may not be maintained in *A. galli* egg cultures stored at room temperature (26–30°C) beyond 8–10 months (Ackert et al. 1947; Todd et al. 1952; Elliott 1954). In the present study, antimicrobial agents were not used during incubation at 26°C and it is possible that reduction in egg viability over time may be attributed to microbial degradation. It has been shown *in vitro* that bacterial (Bottjer et al. 1985; Tian et al. 2007) and fungal (Terrill et al. 2004; Thapa et al. 2015b) activities can damage eggs of parasitic nematodes. Anecdotally, this was infrequently observed during microscopic observation of the samples in the current study, probably due to the embryonation medium (0.1 N H_2SO_4) limiting microbial growth. These findings raise the

possibility that aerobic storage of eggs at 26°C in the embryonated stage may prolong the effective storage time of *A. galli* eggs relative to storage in the unembryonated stage at 4°C. Aerobic conditions are important if storing at this temperature as Tarbiat et al. (2018) demonstrated rapid loss of viability of eggs stored at 26°C under anaerobic conditions. The combined results of the present study and that of Tarbiat et al. (2018) suggest that under anaerobic conditions, storage at 4°C maximises the duration of viability, whereas under aerobic conditions, storage at 26°C maximises the duration of viability.

Our third hypothesis that larvae liberated from morphologically normal vermiform eggs will have high viability as assessed by motility and vital staining was supported by the findings. The present effort is the first to assess viability of fully embryonated *A. galli* larvae by inducing *in vitro* larvae hatch followed by viability dye exclusion. The hatching assay adopted in this study was reported to have 97–98% hatch efficiency resulting in liberation of 92–93% viable larvae (Dick et al. 1973). In consonance with this report, in the current study, 96–98% of embryonated eggs were induced to hatch as observed by the vital staining coupled with microscopic observation immediately after hatching. The analysis result revealed that the viability of hatched larvae decreased by 1.4% for every week of incubation at 26°C up to 6 weeks, with values of 88.2, 86.4 and 82.6% following 2, 4 and 6 weeks of incubation respectively. Our results indicate that for optimum yield of embryonated eggs, a two-week incubation period is preferable to longer periods.

Previous studies have established that *A. galli* eggs recovered from worm uteri are not good sources of infective material because of maturity differences and low embryonation potential (Rahimian et al. 2016). Likewise, faecal eggs are also not always a safe source of infective material especially for inducing *A. galli* specific infections because of the morphological similarity with eggs of *H. gallinarum* (Thienpont et al. 1986). In the present study, the key finding is that eggs oviposited *in vitro* are shed in large numbers and have high and similar embryonation ability with fully matured faecal eggs reported in other studies (Rahimian et al. 2016; Tarbiat et al. 2018). However, using *A. galli* eggs recovered by the current approach is not always feasible given the absolute requirement of chicken necropsy. Other clear advantages of this method include less risk of egg damage, good mature egg yield, being less labour-intensive and resolving the problem of mixed infections. Future studies should compare the infectious capacity of *A. galli* eggs recovered from faeces and by the *in vitro* collection system evaluated herein.

In conclusion, this study showed that high quality *A. galli* eggs could be recovered from mature female worms incubated at 37°C in RPMI media for at least three consecutive days. Total egg production was 6,044 per female worm with 49.2, 38.5 and 12.3% recovered on days 1, 2 and 3 of incubation respectively. Egg viability and subsequent embryonation ability was minimally affected by the day of egg recovery but strongly affected by the storage and incubation conditions. Overall viability of eggs declined at different rates following pre-embryonation storage at 4°C and after incubation at 26°C being 5.7–6.2% and 3.4%, respectively. A novel combination of hatch assay followed by vital staining revealed that storage duration had significant but relatively small adverse effects on the viability of larvae hatched from morphologically normal embryonated eggs. From a practical point of view, the high rate of loss of viability during storage observed in this study has clear implications for work involving the maintenance of *A. galli* strains or experiments requiring sufficient numbers of viable eggs. In the absence of standardised methods for *in vitro* maintenance of viable *A. galli* eggs, our findings and those of others would indicate that under aerobic conditions storage at 26°C may be preferable to storage at 4°C whereas the converse appears to be true under anaerobic conditions. Further studies on optimisation of storage methods to maximise the duration of viability of *A. galli* eggs are warranted.

6.2 Study 2: Survival of *Ascaridia galli* eggs exposed to low temperatures and cryoprotectant

6.2.1 Introduction – Study 2

The objective of this study was to investigate the survivability of *A. galli* eggs exposed to low temperatures and cryoprotectant. This addresses the possibility to optimise methods for long-term storage and cryopreservation of viable infective stages of parasite stocks (defined strains) for future research. The main research question of this experiment was "Could worm eggs survive storage at low temperatures in the presence of the cryoprotectant dimethylsulphoxide (DMSO)?"

The experimental subjects of this study were *A. galli* eggs recovered from mature female worms incubated at 37°C in RPMI media (Study 1 of this chapter). Two egg developmental stages were defined and used for this preservation trial:

1. Unembryonated eggs stored at 4°C in distilled water (boiled and cooled) for about 4 weeks from the day of isolation.
2. Embryonated eggs incubated at 26°C in 0.1 N H2SO4 for 4 weeks from the day of isolation.

For both categories of eggs the percentage pre-storage damage and viability status was estimated by microscopic examination of 200–300 eggs.

6.2.2 Methods – Study 2

Experimental design and setup

This study employed a 2 x 3 x 3 x 4 factorial experimental design, with the following factors and levels with 3 replicates:

- Egg developmental stages: unembryonated, embryonated.
- Storage medium: Water, 5% DMSO, 10% DMSO.
- Storage temperature: 4°C, -20°C, -80°C.
- Storage period: 1, 2, 4, 8 weeks.

For all storage conditions, concentrated eggs were resuspended in the respective preservation medium (1 ml) and distributed in to 1.5 ml cryostorage vials with rubber lids in such a way that each vial contains 1000 eggs/ml. For freezing storage, all vials were incubated at room temperature for 30 minutes prior to storage to enable permeation of the cryoprotectant (DMSO) into the egg. A slow cooling rate was employed for eggs stored at -20°C and -80°C. Briefly, the cryovials were put into a portable cryomachine, which was set at a cooling rate of 1°C/min until it reached -20°C or -80°C. Then the cryovials were moved into the corresponding freezing temperatures for storage.

Monitoring viability

Separate approaches were used to assess viability of the two egg categories (unembryonated and embryonated) stored for specific period. To establish viability of unembryonated eggs, the storage medium was replaced with 0.1 N H2SO4 (recommended embryonation medium) and incubated at 26°C for 28 days under aerobic conditions. At the end of the incubation period at 26°C, at least 100 eggs from each sample were examined microscopically and the proportion of viable eggs at different stages of development (unembryonated, early development, vermiform and embryonated) or

damaged/dead were recorded for each category by adopting the morphological classifications described earlier. The viability of embryonated eggs preserved for different storage periods was assessed by inducing *in vitro* egg hatching to liberate larvae by an artificial method described earlier coupled with dye (methylene blue) exclusion method as in Study 1 of this section.

6.2.3 Results – Study 2

Survival of *Ascaridia galli* eggs after storage at low temperatures in the presence of a cryoprotectant

The cryoprotectant (DMSO) was unable to prevent the eggs from freezing damage at -20°C and -80°C. No egg, irrespective of their developmental stages, survived for even one week of storage at these two temperatures. However, egg viability was maintained but declined with storage period at 4°C irrespective of the storage medium (Figure 6-4). Storage period and storage temperature had a significant ($P < 0.0001$) main as well as interaction effects on the survivability of eggs during the observation period (Figure 6-4).

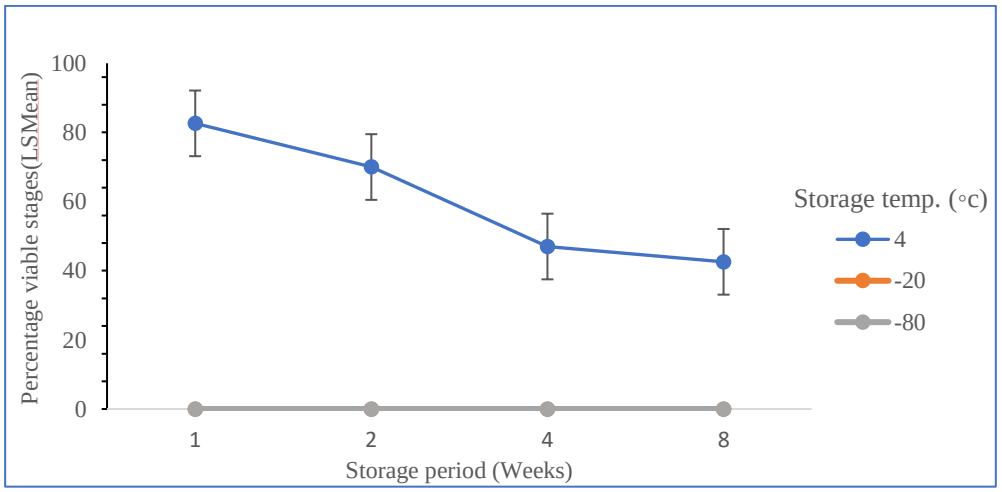


Figure 6-4 Study 2 – Viability of *A. galli* eggs stored at different temperatures

Developmental capacity of *Ascaridia galli* eggs was preserved after storage in DMSO

Table 6-3 **Percentages (LS means) of different A. galli egg developmental stages following pre-embryonation refrigeration (1–8 weeks) in DMSO or water and subsequent aerobic incubation at 26°C for 4 weeks**shows percentages (LS means) of different *A. galli* egg developmental stages following pre-embryonation refrigeration (1–8 weeks) in DMSO or water and subsequent aerobic incubation at 26°C for 4 weeks. The analysis revealed that the vast majority of eggs completed embryonation after 4 weeks of incubation period irrespective of the storage medium used during the pre-embryonation storage at 4°C. Only a very low proportion of eggs (< 4%) remained at early development or vermiform stages. The embryonation profile of eggs was significantly ($P < 0.0001$) affected by pre-embryonation storage period as observed also in Study 1. Eggs stored for only 1 week demonstrated the highest embryonation capacity relative to those refrigerated for 2 weeks or more. Eggs stored in water (82.3%) or 5% DMSO (80.9%) showed better ($P < 0.05$) percentage embryonation than in 10% DMSO (75.1%) (Table 6-3).

Unembryonated *Ascaridia galli* eggs survived refrigeration (4°C) better than embryonated eggs

Even though different viability assessment approaches were used, it was noted that unembryonated *A. galli* eggs survived refrigeration (4°C) better than embryonated eggs whether stored in DMSO or water. At the end of the 8 weeks observation period, percentage viability of unembryonated eggs stored in 10% DMSO, 5% DMSO and water was reduced to 35.6, 43.5, and 43.8% respectively (Figure 6-5). The corresponding values for the fully embryonated eggs (larvae) stored in 10% DMSO, 5% DMSO and water were 20.7, 22.9 and 23.7% respectively (Figure 6-6). In both cases, taken as a whole, eggs stored in water or 5% DMSO had similar and higher ($P < 0.05$) viability status compared to those stored in 10% DMSO. The rate of loss of embryonation capacity of eggs stored in water was 5.36%/week.

Table 6-3 Percentages (LS means) of different *A. galli* egg developmental stages following pre-embryonation refrigeration (1–8 weeks) in DMSO or water and subsequent aerobic incubation at 26°C for 4 weeks

Developmental stage	Storage media	Storage period (weeks)				P-values		
		1	2	4	8	SM	SP	SP x IP
Undeveloped	Water	0.00	0.00	0.00	0.00	0.41	0.38	0.45
	DMSO (5%)	0.00	0.00	0.00	0.00			
	DMSO (10%)	0.00	0.00	0.00	0.32			
Early development	Water	3.31	1.59	1.97	2.92	0.54	0.12	0.14
	DMSO (5%)	1.49	1.95	3.07	1.32			
	DMSO (10%)	2.58	1.66	3.52	1.30			
Vermiform stage	Water	0.74	0.78	0.33	0.64	0.38	0.80	0.87
	DMSO (5%)	0.38	0.31	0.00	0.35			
	DMSO (10%)	1.13	0.35	0.96	0.33			
Embryonated	Water	82.30	71.11	48.58	44.81	0.0001	0.0001	0.89
	DMSO (5%)	80.87	70.03	43.58	42.12			
	DMSO (10%)	75.06	62.21	38.10	33.64			
Dead	Water	13.63	26.51	49.12	51.62	0.0001	0.0001	0.73
	DMSO (5%)	17.26	27.70	53.34	56.23			
	DMSO (10%)	21.23	35.78	56.52	64.41			

SM: Storage media.
SP: Storage period.

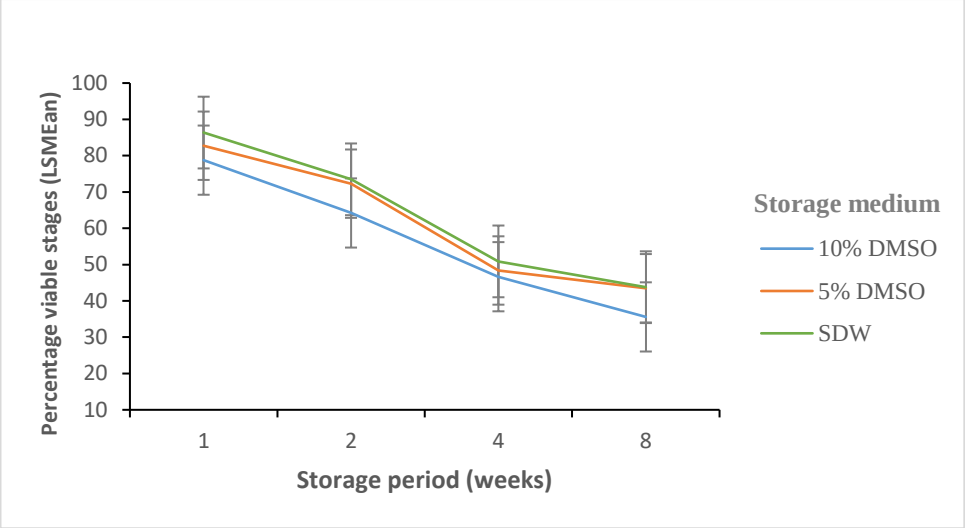


Figure 6-5 Temporal changes in percentage viability (LS Means) of *A. galli* eggs following pre-embryonation refrigeration in DMSO or water (1–8 weeks) and subsequent aerobic incubation in 0.1 N H2SO4 for 4 weeks

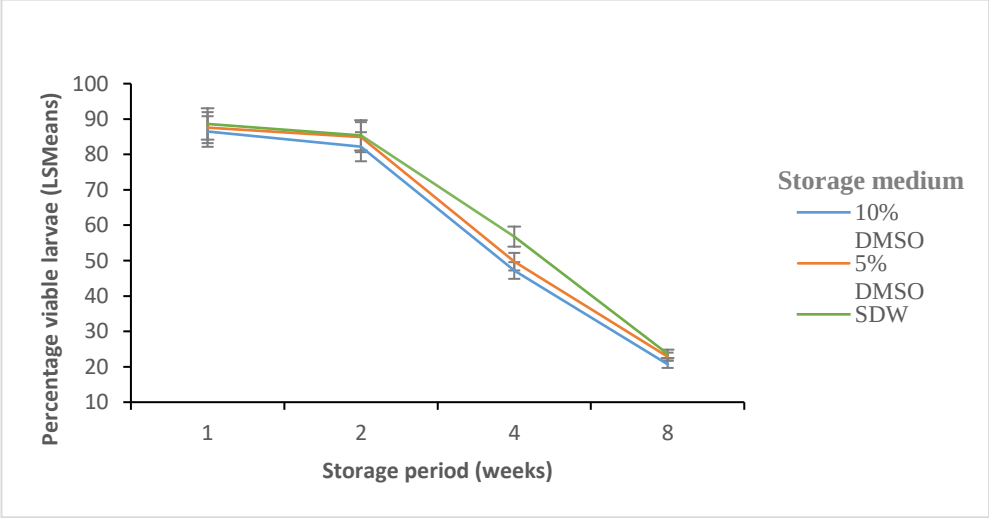


Figure 6-6 Temporal changes in viability of larvae liberated from fully embryonated *A. galli* eggs following refrigeration (4°C) in DMSO or water (1–8 weeks)

6.2.4 Discussion and conclusions – Study 2

The key finding of this study were:

- DMSO was unable to prevent the *A. galli* eggs from freezing damage at -20°C or -80°C.
- Inclusion of DMSO in the storage media caused a moderate reduction in egg viability with increasing storage time at 4°C.
- Egg viability and developmental capacity declined with storage period at 4°C at 5.36 %/week in water, comparable but slightly lower than the 6.2%/week decline observed in Study 1.
- Unembryonated *A. galli* eggs survived refrigeration (4°C) better than embryonated eggs.

From the observations of the current study, it appears that long-term preservation of intact *A. galli* eggs at freezing temperatures and penetrating cryoprotectants such as DMSO may not be possible.

Thus, further optimisation of methods of storage at temperatures above freezing is warranted to enable maintenance of viable infective stocks. In particular, the different effects of aerobic condition on the unembryonated and embryonated stages of development and their effects on long-term storage require further clarification and this is the basis for Study 3.

6.3 Study 3 – Optimisation of methods for prolonged laboratory storage of viable *Ascaridia galli* eggs

6.3.1 Introduction

In both Studies 1 and 2, the rates of decline in egg viability following incubation at 4°C or 26°C predicted a complete loss viability by 20 weeks and 40 weeks respectively. Findings from early studies support our observation that *A. galli* eggs can survive for a prolonged period at embryonation temperatures. For instance, Elliot (1954) observed that embryonated *A. galli* eggs stored aerobically in water for 36 weeks at 28°C were infective to chickens. Similarly, Butler and Christenson (1942) reported that cultures of embryonated *A. galli* eggs remained viable for nearly 2 years as assessed by an *in ovo* larval motility testing method. However, some other researchers were of the opinion that infectivity may not be maintained in *A. galli* egg cultures stored at room temperature (26–30°C) beyond 8–10 months (Ackert et al. 1947; Todd et al. 1952; Elliott 1954). Storage of embryonated eggs thus appears to be optimal under aerobic conditions, and oxygen is a requirement for embryonation to occur (Hansen et al. 1953; Tarbiat et al. 2015). On the other hand, Tarbiat et al. (2018) observed that the embryonation ability of *A. galli* eggs declined linearly at a rate of approximately 4.4% per week over a 72-day storage period (similar to our findings in Study 1) when stored under aerobic conditions in water, but under anaerobic conditions the embryonation capacity declined by only about 0.15% per week. These suggest that presence of oxygen during storage at 4°C had a major adverse effect on the viability of eggs. In our Study 1, the attempts at creating an anaerobic environment appear not to have been successful, as our rate of loss of viability of eggs was comparable with storage under aerobic conditions in the study of Tarbiat et al. (2018).

Given the clear evidence in the literature that unembryonated eggs at 4°C maintain viability best under anaerobic conditions, but that eggs require oxygen for embryonation to occur and appear to maintain viability best under aerobic conditions, it remains unclear as to which combination of oxygen availability and temperature will enable 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 comparing storage at both 4°C (unembryonated) and 26°C (embryonated) under both aerobic and anerobic conditions in a factorial experiment that included additives to prevent microbial growth when storage was at 26°C.

The basic hypotheses under test were: 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) additives to control microbial growth 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 control of microbial growth will provide the longest duration of viability of stored eggs.

6.3.2 Methods – Study 3

6.3.2.1 Ascaridia galli eggs

A. galli eggs were extracted from fresh faecal samples collected from chickens subjected to artificial infection with *A. galli* as part of the project. Fresh faeces were collected by placing papers beneath the birds that were housed in individual cages overnight. The faecal material was combined in a plastic bucket and thoroughly mixed using a glass stick for 10 minutes to homogenise the samples. The *A. galli* eggs were then extracted from the faeces as described previously (Rahimian et al. 2016; Tarbiat et al. 2018). Briefly, the prepared faecal slurry was flushed with tap water and passed through a series of sieves with mesh aperture sizes of 750, 500, 250, 90 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, the sieve was washed off and its content transferred to conical centrifuge tubes (50 mL). The tubes were then centrifuged at 1620 x g for 1 min and the supernatant was discarded, leaving 5 ml of the content in the tube. The tube containing the sieve contents was then filled to the volume of 50 mL with saturated salt solution (SG 1.2) and centrifuged again at 1620 x g for 1 min. After centrifugation, the supernatant containing eggs were passed through the 30 µm sieve with a large amount of deionised water and washed off the sieve onto tubes, and eggs were stored in water (previously boiled and cooled) at 4°C for not more than 24 hrs before being used for the storage steps outlined below.

6.3.2.2 Experimental design and setup

The survival, viability and embryonation ability of *A. galli* eggs was investigated using a 2 x 2 x 3 x 5 factorial arrangement to test the effect 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) with each combination replicated 3 times for the whole procedure, resulting in a total of 180 counts. The factorial arrangement was in such a way that eggs were stored under specific conditions for 4–20 weeks to allow repetitive measures in 4-weekly intervals. Briefly, clean *A. galli* eggs (≈500) suspended in each storage medium in 1.5 ml Eppendorf tubes filled to the top were subjected to each storage condition and, after the relevant treatment periods, eggs were monitored for viability and development as described below. A small pore of 2 mm diameter was created in the lids of all the sample tubes, allowing for a constant supply of oxygen in the aerobic treatment. To create anaerobic conditions, storage tubes with a tightly closed lid with a 2 mm pore was placed in racks (one for each sampling date) enclosed in vacuum seal plastic bags and vacuum sealed using a high-capacity vacuum sealer. Experimental samples were then stored at either temperature until removal and assessment at the defined storage period.

6.3.2.3 Monitoring of egg viability and development

Egg monitoring for viability was executed 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) all 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 statistical analysis to assess the viability of egg samples stored at both temperatures under different conditions at directly comparable times in a full factorial model.

After the incubation period, egg viability was evaluated by microscopically examining morphological characteristics of eggs 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 by (Feyera et al. 2020) and shown in Figure 6-7.

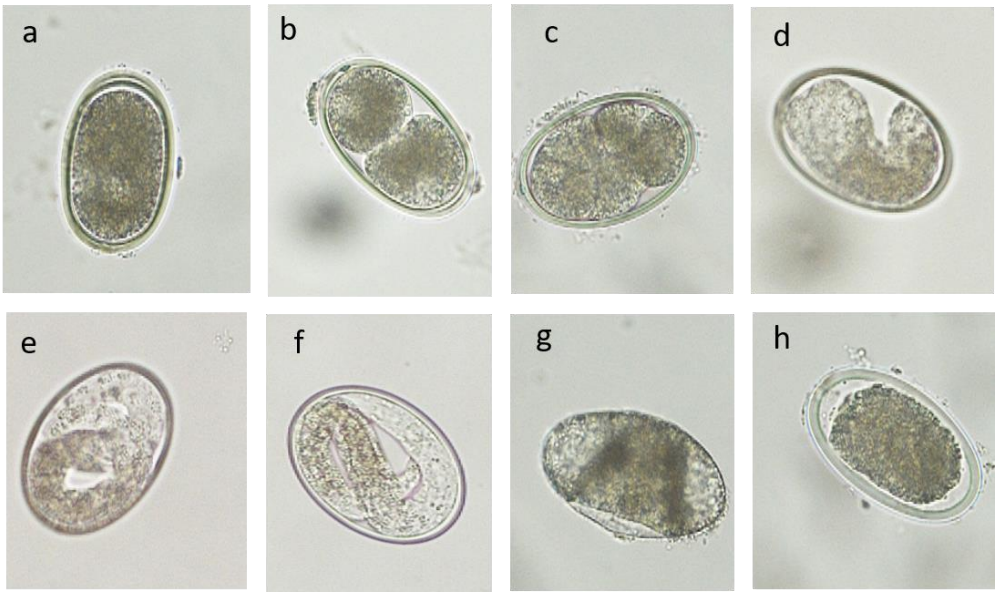


Figure 6-7 Morphological characteristics of different developmental stage of *A. galli* eggs: undeveloped (a); early developing without differentiations (b and c); vermiform (d); embryonated (e and f); dead (g and h)

6.3.2.4 Statistical analysis

Data analysis was performed using appropriate model in JMP® software version 14.3.0 (SAS Institute Inc., Cary, NC, USA). Data were tested for normality in line with the assumptions of analysis of variance (ANOVA) and transformation of data was not required. Full factorial analysis of variance (ANOVA) in linear model platform of JMP was used to analyse the different eggs developmental stages (undeveloped, developed, vermiform, embryonated and viable stages) fitting the fixed effects of storage medium (water, 2% formalin, or 0.1 N H_2SO_4), storage conditions (aerobic or anaerobic), storage temperature (4 or 26°C), 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 for triplicate counts of 100 eggs, resulting in a total of 300 counts per triplicate samples. Data were presented using least-squares means (LSM) and standard errors (SEM). Comparisons of the percentage of LSM were performed using Tukey's HSD test. Linear regression was used to test the association between egg viability and embryonation with storage medium, storage condition, storage temperature or periods. $P < 0.05$ was considered significant difference in all analysis.

6.3.3 Results – Study 3

The percentage of morphologically intact or normal (undamaged) eggs at the time of initial storage was 99.3%. The viability percentage of these eggs following incubation at 26°C aerobically for the 14 days was 90.5%, indicating a high level of initial viability of the eggs used in the study. The effects of fixed experimental factors and their interactions on the percentage of *A. galli* eggs in different developmental stages are presented in Table 6-4.

There was a very close association between the proportion of eggs embryonated at the end of each treatment period and the proportion of viable eggs, indicating that the vast majority of viable eggs

embryonated within 2 weeks of exposure to 26°C under aerobic conditions. For the purposes of discussion here, the percentage of eggs that were embryonated will be taken as the marker of full viability. As can be seen in Table 6-4 all main effects in the statistical model, and the majority of interactions were highly statistically significant, indicating complex relationships between the factors tested. These are best revealed in the 4-way interaction plots presented in Figure 6-8. These reveal the following basic findings in the results.

- **Aerobic conditions**. As predicted, viability was enhanced by proper anaerobic conditions at 4°C, and aerobic conditions at 26°C. However, the effects of having the inappropriate conditions regarding oxygen availability were much greater at 26°C with no viability retained after 12 weeks in any storage medium, and after 4 weeks when stored in water at this temperature under anaerobic conditions. The adverse effects of exposing unembryonated eggs to oxygen at 4°C were large also, but did not preclude retention of viability in some eggs out to 20 weeks.

- **Storage medium**. Storage in 0.1 N H_2SO_4 produced the best retention of viability under a wide range of conditions, both at 26°C (expected) and 4°C (unexpected). Water was a poor medium at 26°C and a medium of intermediate quality at 4°C. Conversely, 2% formalin provided outcomes almost equivalent to 0.1 N H_2SO_4 at 26°C, but at 4°C it resulted in the fastest loss of viability.

- **Temperature**. Good maintenance of viability could be attained at both temperatures, but under very different conditions. At 4°C strict anaerobic conditions are important providing 34–52% retained embryonation potential after 20 weeks of storage. Anaerobic storage at 4°C in 0.1 N H_2SO_4 provided excellent maintenance of viability (52% at 20 weeks, rate of loss of viability of 1.91%/week). Maintenance of viability was lower for the other storage media. At 26°C strict aerobic conditions 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 provided excellent maintenance of viability (47% at 20 weeks, rate of loss of viability of 2.18%/week). Having a preservative to inhibit microbial growth appears to be essential with a rapid decline in viability to complete loss by 20 weeks when stored at 26°C in water alone. 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 6-4 Study 3 – Results of analysis of variance – percentages (LSM±SEM) of different *A. galli* egg developmental stages in different storage mediums for aerobically and anaerobically refrigerated eggs either at 4°C or 26°C for 20 weeks prolonged storage period

Parameter	Undeveloped	Early development	Vermiform	Embryon-ated	Viable stages	Dead
Overall means ±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 medium (SM)	P = 0.2529	P = 0.9225	P = 0.1245	P < 0.0001	P < 0.0001	P < 0.0001
0.1%NH2SO4	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}
Storage condition (SC)	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	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	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	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	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	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	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	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*SC	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
SC*ST	P = 0.0017	P < 0.0001	P = 0.0455	P < 0.0001	P < 0.0001	P < 0.0001
SP*SC*ST	P = 0.3778	P = 0.1121	P = 0.0524	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
SC*SM	P = 0.3096	P = 0.7661	P = 0.5395	P = 0.8199	P = 0.7777	P = 0.7777
SP*SC*SM	P = 0.5824	P = 0.9775	P = 0.5790	P < 0.0001	P < 0.0001	P < 0.0001
ST*SM	P = 0.7338	P = 0.1768	P = 0.1060	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
SC*ST*SM	P = 0.2311	P = 0.1278	P = 0.1782	P = 0.0035	P = 0.0128	P = 0.0128
SP*SC*ST*SM	P = 0.4120	P = 0.1058	P = 0.9676	P < 0.0001	P < 0.0001	P < 0.0001

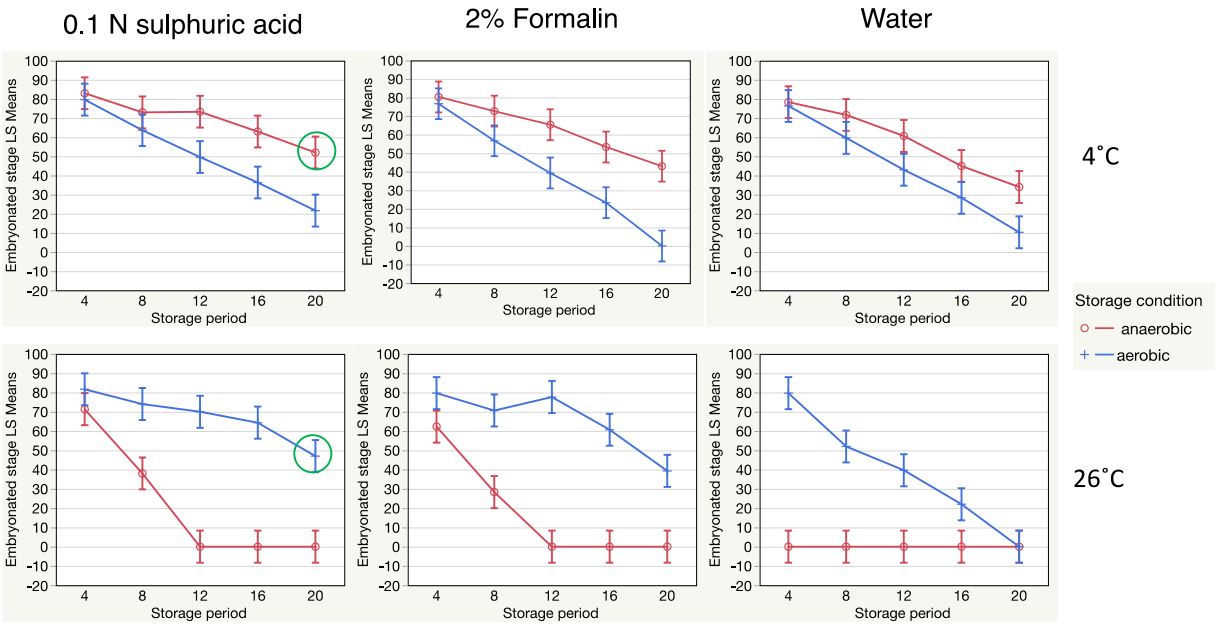


Figure 6-8 Interaction plot showing the highly significant 4-way interaction between the effects of aerobic status, storage temperature, storage medium and storage period in weeks on embryonation of eggs following storage

The top panel represents storage at 4˚C, the lower 26˚C, and the vertical panels represent the three storage media used. Red lines are for anaerobic storage, green for aerobic.
The green circles represent the treatment combinations providing the best maintenance of egg viability.

6.3.4 Discussion and conclusions – Study 3

The results and implications of this study are very clear and provide methods of storage at either 4° or 26°C that result in a loss of viability over time of only approximately 2%/week, a far lower rate of decline than observed in the previous two studies when storage conditions were not optimised. At this rate of decline, starting with egg viability of 90%, 50% viability is retained at 20 weeks and 10% at 40 weeks, when passage back into chickens would need to be planned to maintain stocks.

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. For many nematode species, egg development can be inhibited by lack of oxygen, and it appears that storage at 4°C helps maintain the unembryonated state while limiting the rate of egg inactivation. On the other hand, 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 a requirement to maintain viability of the egg in the embryonated state. However, storage medium had important effects within each storage temperature as discussed below.

Our third hypothesis that additives to control microbial growth will be required to achieve maintenance of viability at 26°C, but not 4°C was only partially accepted. At 26°C these additives had major effects by enhancing maintenance of viability under both aerobic and anaerobic conditions, presumably by inhibiting microbial growth and egg spoilage. However, at 4°C storage in 0.1 N H_2SO_4 but not 2% formalin, also improved maintenance of viability under both aerobic and anerobic conditions, by mechanisms which are not clear.

The fourth hypothesis that a combination of aerobic storage at 26°C with control of microbial growth will provide the longest duration of viability of stored eggs was only partly supported by the results. While excellent maintenance of viability was obtained at 26°C with aerobic storage in 0.1 N H_2SO_4 a similar but numerically higher level of maintenance of viability was obtained by anaerobic storage at 4°C, again in 0.1 N H_2SO_4 storage medium.

In conclusion, this study has clearly demonstrated the requirement for anaerobic storage conditions for unembryonated eggs at 4°C but aerobic storage conditions for storage of embryonated eggs at 26°C. Within this dependency, the study has identified storage protocols at either 4°C or 26°C that enable storage of eggs for 20 weeks reduction in viability from 90% to approximately 50% over that period. If the linear decline in viability is maintained beyond 20 weeks for these treatments, the projected storage time until egg viability is 10% and eggs need to be passaged back into chickens is approximately 40 weeks. While storage at 4°C may seem ideal, achieving anaerobic conditions in the absence of an effective vacuum sealer is difficult (as shown in Studies 1 and 2) so storage at 26°C or room temperature aerobically may be preferred for simplicity. Our previous study showed that storage at 4°C is lethal once eggs have been embryonated, so they should not be returned to this temperature after embryonation.

6.4 Nematode egg recovery and storage – overall findings and implications

The series of experiments reported in this area have produced some clear outcomes and implications for industry. These are summarised below.

1. Eggs for experimentation can be effectively obtained from faeces or from worms collected from infected chickens. In both cases, egg viability of 90% or above can be achieved. Collection from faeces involves more laboratory work, and unless the chickens are infected with a single species of worm, it should not be used to produce eggs of a single species. Saturated sugar as the flotation fluid for separation is recommended. High levels of eggs (approx. 6,000/worm) can be obtained from adult female *A. galli*, incubated at 37°C for 3 days in a relatively simple process that will produce eggs of high quality.

2. Freezing of eggs for prolonged storage does not appear to be feasible with the thick egg wall probably fracturing during the freezing process despite the addition of DMSO as a cryopreservative. Our studies, however, were not exhaustive in this area. There is probably greater potential to cryopreserve hatched larvae, now that good hatching protocols are available. This could be explored for the storage of very valuable defined isolates.

3. Study 3, arising out of the findings of Study 1, has provided great clarity on the factors affecting storage of eggs in the unembryonated and embryonated forms, highlighting the opposite requirements for oxygen during storage of these forms. Long-term preservation of eggs in the unembryonated state requires the combination of low temperatures (4°C) and absence of air (most likely oxygen). Storage in 0.1 N H_2SO_4 provides the optimum maintenance of viability. Long-term preservation of eggs in the embryonated state is also possible, but requires the combination of warm temperatures (26°C), presence of air (most likely oxygen) and the presence of an inhibitor of microbial growth, in our study ideally 0.1 N H_2SO_4.

4. Using either of the optimised methods identified at 3 above, loss of viability of approximately 2%/week for 20 weeks can be expected during storage and if this linear rate of decline is maintained, storage for up to 40 weeks (10% remaining viability) can be expected, prior to needing to passage the eggs back through chickens.

7 Multiplication and maintenance of parasite worm egg isolates in chickens

In Section 6 of this report, methods for recovery and maintenance of worm eggs outside the host were investigated. Despite identifying good methods for prolonging egg viability for many months, the ideal of indefinite storage by cryopreservation at freezing temperatures was not obtained. Therefore, the maintenance of any defined parasite stocks will always require passage through the host, between storage periods in the laboratory. The most efficient means of amplifying egg stocks in the host are therefore important, as is the case if starting out with a small amount of infective material from the field and needing to build up parasite stocks for experimentation. In this section experiments on amplification of worm egg stocks by passage in chick and adult hosts were investigated, as was the maintenance of specific parasite infections in mature hens for long periods. Three studies were carried out by Teka Feyera Dewo with assistance from Anwar Shifaw Yesuf. The studies are reported in this section and comprise:

1. Propagation of *A. galli* in adult layer chickens.
2. Propagation of *A. galli* in young chicks with and without immunosuppression.
3. Maintenance of defined nematode isolates at UNE.

As with the previous sections, each of these studies will be described in turn.

7.1 Study 1 – Propagation of *A. galli* in adult layer chickens

Objective: To amplify *A. galli* eggs in adult layer chickens for subsequent worm recovery. Many studies have used adult birds to passage *A. galli* and a wide range of egg dose rates (typically 500–1500 eggs/chicken) and dosing regimens (bolus or trickle infection) have been used. In this small study carried out in 2019, eggs of two *A. galli* isolates that were nearing the end of their storage life were amplified in some mature chickens left after a previous experiment by a different researcher.

7.1.1 Methods – Study 1

The experiment was conducted at the Laureldale caged layer facticity at UNE. Two *A. galli* isolates were amplified in this experiment. The first isolate (UNE 2019-QLD-1) was originally recovered from naturally infected laying hens in a private free range poultry farm in Queensland, Australia (Farm 1 in Table 3-1). The other isolate (UNE 2019-UNE-1) was recovered from laying hens experimentally used for a previous behavioural study (AEC17-092) at the UNE free range facility at Laureldale.

A total of 52 laying hens (24 weeks old) were used for this experiment. The birds were dewormed with levamisole (28 mg/kg) and piperazine (100 mg/kg) combination (co-administered at individual dose rates *per os*). Seven days post-deworming, each hen received approximately 300 viable eggs in two split doses four days apart. The eggs used had different storage histories at 26°C at the time of inoculation, these being 8 weeks post-incubation at 26°C for UNE 2019-QLD-1 (Isolate 1) and 15 weeks post-incubation at 26°C for UNE 2019-UNE-1 (Isolate 2). Isolate 1 was inoculated to 9 birds whereas Isolate 2 was inoculated to 42 birds. The birds were kept in enriched individual layer cages with wire flooring (0.37 m^2) up to the end of the experiment. The birds were killed 9 weeks post-infection to recover worms from the intestine. Excreta samples were also collected from the terminal large intestine to estimate individual excrete egg count (FEC) from a subset of the birds (n = 33).

Worms recovered from each bird were pooled (separately for each isolate) and incubated at 37°C in RPMI media for 4 consecutive days, changing the media every 24 hours. After every 24 hours, the media containing parasite eggs was collected into 50 ml screw cap falcon tubes by rinsing the jar with fresh RPMI media. Eggs recovered over four different collection days were kept separately to determine egg production/female worm/day.

The measurements made were total worm count, individual FEC (EPG) and egg production/female worm/day.

7.1.2 Results – Study 1

Of the 51 birds, 42 (84.3%) harboured *A. galli* infection (0–52 worms) at necropsy with a mean worm count of 10.8 per bird and a median of 7 worms/bird. A total of 549 *A. galli* worms (57 Isolate 1 + 492 Isolate 2) were recovered, of which the vast majority (395, 72.8%) surprisingly were immature worms of different sizes. A large number of small sized worms were particularly noticed in birds infected with Isolate 2, which had experienced a long period of storage (15 weeks) at 26°C. Mature males (n = 73) and females (n = 77) of varying sizes respectively constituted about 13.2 and 14% of the worm population. Thus, eggs were recovered from 77 (17 Isolate 1 + 60 Isolate 2) female worms.

Overall, a total of 245,890 eggs were recovered with 46.2, 34.9, 15.5 and 3.4% recovered on days 1, 2, 3 and 4 of *in vitro* worm incubation respectively (Figure 7-1). The mean egg yield per female worm per day was 1474 ± 9, 1114 ± 35, 486 ± 35 and 110 ± 5 on days 1, 2, 3 and 4 of collection, respectively ($P < 0.0001$) and a single female worm produced 3,184 eggs on average. Per capita egg production was higher for Isolate 1 (4,184) than the Isolate 2 (2,897) (Figure 7-1).

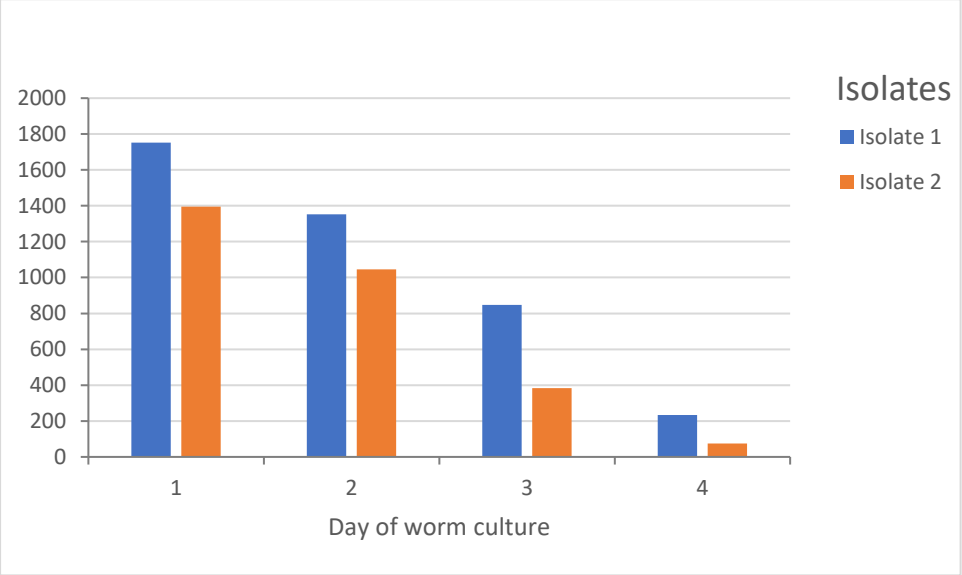


Figure 7-1 Per capita egg production per female worm per day from mature *A. galli* female worms incubated at 37°C in RPMI media for 4 consecutive days

Mean FEC was 367 EPG and there was positive linear association between total worm count and FEC, which was improved when plotted on the log scale (Figure 7-2).

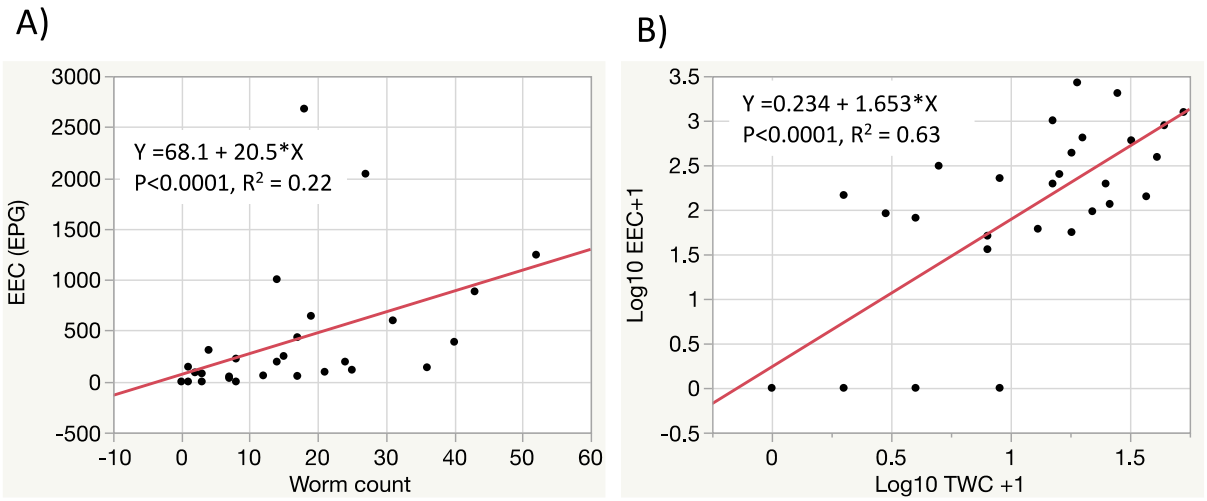


Figure 7-2 Linear relationship between total worm count and faecal egg output A) on a linear scale, B) on a log scale

7.1.3 Discussion and conclusions – Study 1

Per capita egg production was lower in the current experiment (3,184) than obtained in our previous study (6,044) (Feyera et al. 2020) (Section 6.1), possibly because of differences in worm sizes and stage of maturity. The most likely cause of this was the prolonged period of storage prior to infection in the present study relative to the earlier study. This was supported by the significant difference in the mean egg production per worm between the two isolates in this experiment being 4,184 and 2,897 for Isolates 1 and 2 respectively, with Isolate 2 having almost double the storage time at 26°C as Isolate 1. Differences in host immunity are unlikely to be the cause as the hens from the earlier study had been reared for months in a free range situation with constant exposure to worm challenge, whereas birds in the current experiment had been caged for quite a long period of time with an unknown level of infection.

Worm count and faecal egg output were positively correlated as would be expected in an experiment with *A. galli* as the only nematode involved. Interestingly 3 chickens had worms (1, 3 and 8 worms) but had negative egg counts. This may reflect the issues with late development an immaturity of the worms observed in this study.

The issue of the extent to which the duration of storage of eggs influences the rate of development of the nematodes in chickens infected with these eggs is an area that warrants further research as it has implications for the methodology and recommendations for maintaining or multiplying nematode stocks.

7.2 Study 2 – Propagation of *A. galli* in young chicks with and without immunosuppression

This study has been reported by Feyera at al., (2022d)

Feyera, T., Shifaw, A. Y., Ruhnke, I., Sharpe, B., Elliott, T., & Walkden-Brown, S. W. (2022). Ascaridia galli challenge model for worm propagation in young chickens with or without immunosuppression. Veterinary Parasitology, 301, 109624. doi:https://doi.org/10.1016/j.vetpar.2021.109624

7.2.1 Introduction and objectives – Study 2

An early challenge on this project was to maintain stocks of field strains of *A. galli* we had collected while the viability of eggs stored in water at 4°C was declining rapidly. This required us to determine the most efficient means of multiplying stocks for further experimentation and maintenance of the isolates. The literature is not clear on this, with experiments demonstrating that infection as early as day old have been successful, but with most studies involving infections of mature birds, as in Study 1 above. The basic premise of the present study was that layer male cockerel chicks, which are normally killed at the hatchery, could be an inexpensive resource for worm multiplication, with young birds requiring less space and feed for maintenance than older birds, and also lacking prior immunity to worms, thus resulting in high establishment and development rates and yields of both worms and eggs. In ruminant models of nematode egg production, immunosuppression has been used successfully to boost parasite numbers and nematode egg production in analogous situations, without adverse effects on the host. This is because, for some of these nematodes (most notably the small scour worm nematodes of sheep), it appears that many of the adverse effects of parasite infection are due to the host immune response to the parasite, and suppressing this response enables large burdens of worms to be carried without commensurate increases in pathology (Greer et al. 2005; Greer 2008; Greer et al. 2008; Dever et al. 2016).

The overall objective of this study then was to develop an *A. galli* challenge model for worm propagation and amplification in young chickens. Within this overarching objective we tested three factors, which may have a bearing on the efficiency of the model, namely:

- The effect of age of chicken at infection (day old or 2 weeks) on *A. galli* infection establishment, worm load and faecal egg output.
- The effect of immunosuppression on *A. galli* infection establishment, worm load and faecal egg output (none, dexamethasone, cyclophosphamide).
- The effect of dose of infective eggs (100, 300, 900) on *A. galli* infection establishment, worm load and faecal egg output.

7.2.2 Materials and methods – Study 2

The experiment was conducted at two UNE research facilities, the Centre for Animal Research and Teaching (CART) facilities at the on-campus animal house and Laureldale caged layer facility. The experiment had ethical approval (UNE AEC approval AEC19-070).

7.2.2.1 Experimental design

The experiment employed a complete factorial (2 x 3 x 4 x 2) design involving four factors as summarised below and shown in Table 7-1.

- Age at infection. 2 levels. Day old and two weeks of age at time of first treatment.
- Immunosuppression. 3 levels administered by intramuscular injection into breast muscle on

3 occasions, with each trickle infection sham (saline), dexamethasone (300 μg/kg), cyclophosphamide (75 mg/kg).

- Dose of infective *A. galli* eggs. 4 Levels. Total doses of 0, 100, 300, 900 eggs administered by gavage into the crop split into 3 applications over one week.
- Age at worm recovery. Week 8 or 10 post-initial infection.

Table 7-1 Experimental design, groups and number of chickens used in the experiment

Dose (eggs/bird)	Age at infection						Total
	Day old			2 weeks of age			
	None	Dex	CY	None	Dex	CY	
0	16	16	16	16	16	16	96
100	16	16	16	16	16	16	96
300	16	16	16	16	16	16	96
900	16	16	16	16	16	16	96
Total	64	64	64	64	64	64	384
Worm recovery Wk 8	32	32	32	32	32	32	192
Worm recovery Wk 10	32	32	32	32	32	32	192

7.2.2.2 Chickens

A total of 384 one-day-old layer cockerel chickens (Isa Brown) were purchased from a commercial hatchery (Tamworth, NSW, Australia). Chicks were kept in the same experimental room in floor pens (0.9 m²/bird) with wood shavings as bedding material for 6 weeks, and then moved to enriched individual layer cages with wire flooring (0.37 m²) up to the end of the experiment (Figure 7-3). A commercial layer grower ration (Riverina Pty Ltd, Queensland, Australia) and water were offered *ad libitum* over the experimental period. The chickens arrived at UNE CART facility in two batches; 192 first and the remaining 192 two weeks after the first batch. This gave rise to two age groups, which were able to be administered treatments on the same days.

Figure 7-3 Study 2 – Photographs of the cockerels in their individual cages at the Laureldale layer facility

7.2.2.3 Infection and immunosuppressant administration

The *A. galli* isolate (UNE 2018-UNE-1) used in this experiment was originally recovered from free range laying hens experimentally used for a behavioural study at UNE and had undergone a single experimental passage for amplification in worm free layer chickens (Study 1 of this section) before being used in this experiment. All the chicks were separately inoculated with four different doses of embryonated *A. galli* eggs; 0, 100, 300, 900 eggs in 3 split doses over 1 week. Dexamethasone, cyclophosphamide or sterile saline (sham) were administered concurrently with the split infection treatments. Of the 384 chickens in total, 192 commenced treatments on the day of arrival (2nd batch of chicks) whereas the remaining 192 commenced treatment concurrently at 2 weeks of age (1st batch of chickens).

7.2.2.4 Sampling and measurements

Bodyweight: Individual bird bodyweight was recorded on d0 (before treatment), d7 (end of treatment), and then every other week up to the end of the experiment (i.e. 2, 4, 6, 8, 10 weeks post-infection)

Excreta collection: Individual faecal samples were collected from 8 birds in each group at 3, 4, 5, 6, 7, 8 and 10 WPI. Sampling was in a repeated approach from the same birds individually marked and identified throughout the experiment. Excreta samples were collected by placing paper sheets beneath the individual cages during the daytime from early morning to around noon. Excreta consistency score (ECS) was recorded for each faecal sample at WPI 8 and 10 (1=normal; 2=medium; 3=diarrhoeic).

Blood sample: Blood samples were collected from 8 birds in each group (the same bids as for faecal sampling) in a repeated manner at 2, 4, 6, 8, and 10 WPIs and serum extracted for ELISA assay.

Worm recovery: Birds were sacrificed at 8 (n = 192) and 10 (n = 192) weeks post-infection for total worm (adult and immature) recovery and enumeration.

7.2.2.5 Data analysis

Data were statistically analysed using JMP® software version 14.3.0 (SAS Institute Inc., Cary, NC, USA). Distributions of the data and model residuals were assessed for compliance with the assumptions of ANOVA. Excreta egg count and worm count data were log transformed whereas infection establishment rate data (egg dose/worm count at necropsy * 100) was arcsine transformed (proportion). Bodyweight data were not transformed. For the analysis, age at infection collection (day old or 2 weeks old), immunosuppression (dexamethasone, cyclophosphamide or sham), and infective egg dose (0, 100, 300, or 900) and their interactions were fitted as fixed effects in a linear model. Temporal changes in bodyweight and faecal egg shedding pattern over time were analysed using repeated measures analysis with individual bird fitted as a random factor in a REML (restricted maximum likelihood) model with the other factors and interactions fitted as fixed effects.

7.2.3 Results – Study 2

7.2.3.1 Total worm count and worm establishment rate

The mean number of adult worms collected per bird was 5.07 and the mean number of immatures was 0.31, indicating that the great majority of worms had achieved maturity by the 8- and 10-week PI recovery times. Untransformed total worm counts in the different treatment groups are shown in Figure 7-4. Statistical analysis of the transformed total worm count data revealed that count was significantly affected by immunosuppression (P = 0.027) and infective egg dose (P = 0.004), whereas the effects of age at infection (P = 0.12) and day of worm recovery (P = 0.57) were not significant. There were no significant interactions (P > 0.05) between treatment effects for worm load. Slightly more worms were recovered after day old infection than 2 week old infection (P = 0.12), and birds immunosuppressed with dexamethasone had significantly higher counts than birds given the sham treatment, with the birds administered cyclophosphamide being intermediate between the two. Worm count did not differ between birds dosed with 300 or 900 eggs, but both groups had significantly higher counts than those administered 100 eggs (P = 0.004). These effects can be seen in the arithmetic mean data shown in Figure 7-5.

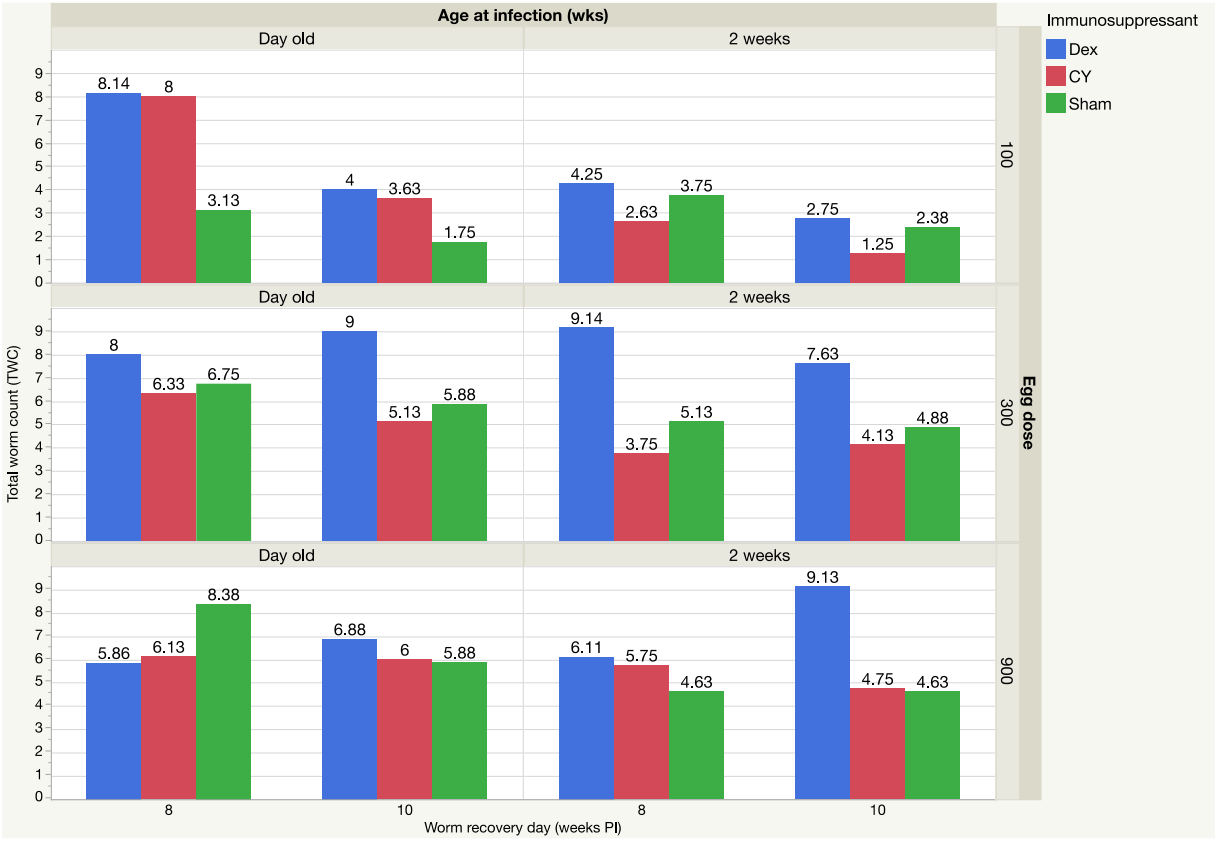


Figure 7-4 Study 2 – Arithmetic mean worm counts for all treatment groups

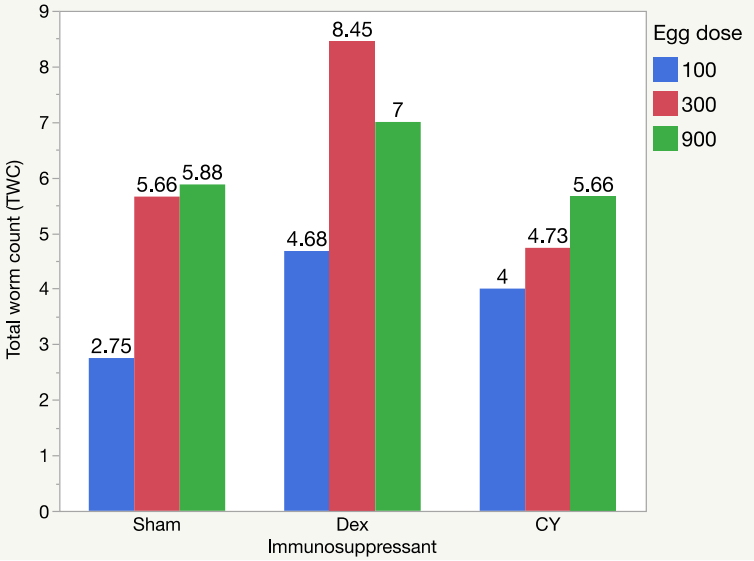
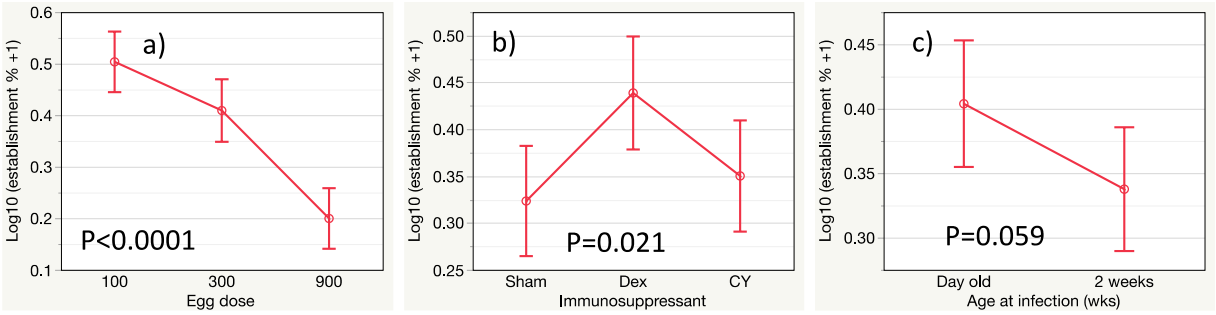


Figure 7-5 Study 2 – Arithmetic mean total *A. galli* worm counts showing the overall effects of immunosuppression and infective egg dose

The fact that worm burdens did not differ between chickens administered 300 or 900 eggs was suggestive of differences in the rate of establishment of worms from the inoculated eggs. Overall crude establishment rate was low (2.2%, range 0–28%, median 1% establishment in individual birds). Analysis of Log_{10} establishment rate revealed that it was significantly affected by infective egg dose ($P < 0.0001$), immunosuppression ($P = 0.021$) and age with a trend towards higher establishment rate in chicks infected at day old rather than 2 weeks of age ($P = 0.059$) (Figure 7-6c). An inverse relationship was noted between infective dose and establishment rate with the lowest dose (100) resulting in the highest establishment rate (Figure 7-6a). There were no significant interactions ($P > 0.05$) between treatment effects for establishment rate.



7-6 Study 2 – *A. galli* artificial infection establishment rate in chickens in relation to (a) age at infection, (b) immunosuppression and (c) infective egg dose

7.2.3.2 Excreta egg counts

Egg shedding started at week 5 and increased over time in all infected groups (Figure 7-7). Arithmetic mean faecal egg counts increased steadily from 33 EPG at week 5 to 478 EPG at week 10. Repeated measures analysis of cube root transformed FEC data revealed significant effects of immunosuppression ($P < 0.05$), infective egg dose ($P < 0.0001$) and weeks post-infection ($P < 0.0001$) but not age of infection ($P = 0.22$) or any of the interactions between effects. These effects can be seen in Figure 7-7, which presents the main effects of interest as both untransformed means and least squares means of cube root transformed FEC following analysis.

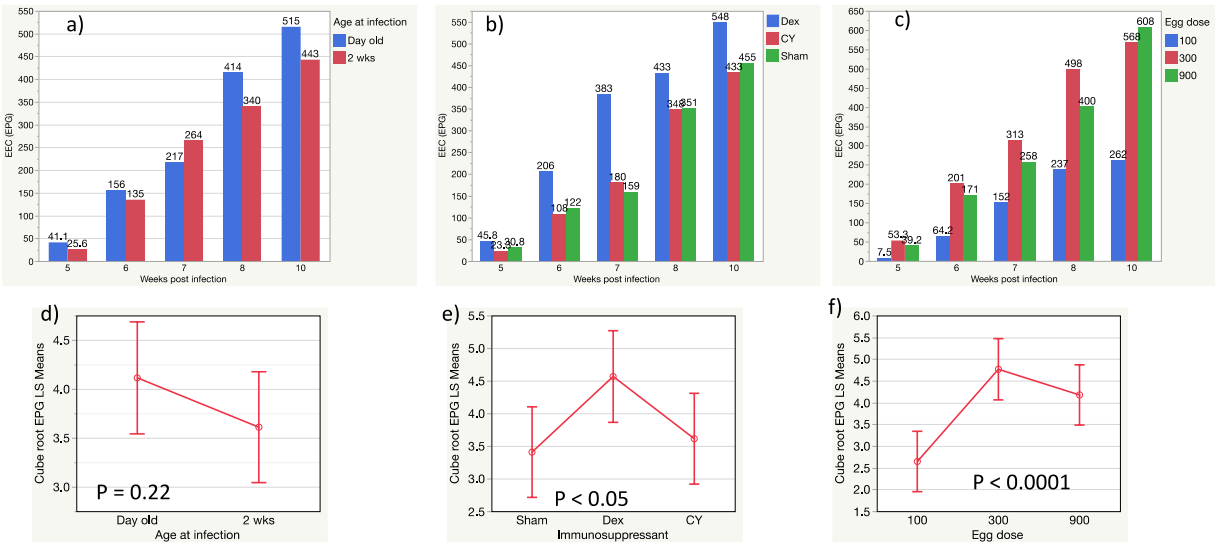


Fig. 7-7 Study 2 – Excreta egg counts showing the effects of key weeks post-infection, age at infection, immunosuppression treatment and infective egg dose expressed as simple arithmetic means (a–c) or least squares means of cube root transformed data following statistical analysis (d–f)

There was a slight trend (P = 0.22) towards higher egg counts in day old infected birds. As for total worm counts, birds immunosuppressed with dexamethasone had significantly higher FEC than sham controls, with those treated with cyclophosphamide intermediate (P < 0.05). Birds infected with 300 or 900 eggs had significantly higher (P < 0.0001) FEC throughout the monitoring period than those that received 100 infective eggs (P < 0.0001).

7.2.3.3 Bodyweight

Bodyweights for the two age groups were analysed separately, but repeated measures analysis of both age groups revealed complex interaction between the effects of immunosuppressant and egg dose rate. These are shown, with P values in Figure 7-8. What they reveal is that in sham immunosuppressed (i.e. control) birds, infection with *A. galli* caused a significant linear dose-dependent reduction in growth rate due to infection (Figure 7-8 c) and d)). In day old infected chicks, both immunosuppressants inhibited growth in normal birds, but reduced the negative effects of infection as infection dose increased. In birds infected at 2 weeks of age, the pattern was different with dexamethasone depressing growth with no amelioration of the effect of infecting dose, while for cyclophosphamide, the amelioration extended to causing an increase in bodyweight with increasing worm infection rate (Figure 7-8 c) and d)).

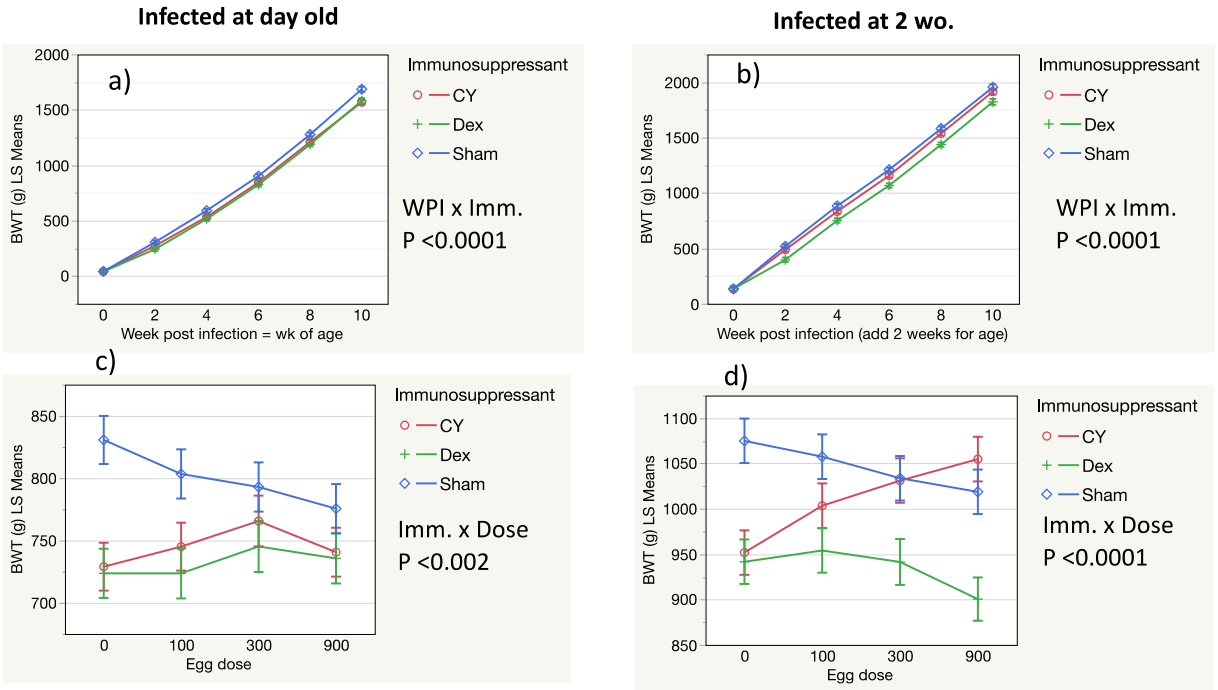


Figure 7-8 Study 2 – Analysis of bodyweight data showing significant interactions between the effects of egg infection dose, immunosuppression and time post-infection for birds infected at day old (left panels, a) and c)) or at 2 weeks of age ((right panels, b) and d))

7.2.4 Discussion, conclusions and implications – Study 2

Our results suggest that infection at day old with infective doses of 300 eggs coupled with immunosuppression with dexamethasone gives best results for efficient propagation of *A. galli* worms in young chickens. In deciding whether to include immunosuppression in the model, consideration of the added complexity of treatment and potential adverse effects should be taken into account although no negative health effects of the immunosuppression treatments were observed in this experiment apart from a small reduction in growth. It should be noted that the levels of infection observed in this study (mean of 5 worms/bird) tended to be lower than those seen in naturally infected free range hens in the study in Section 3 (Farm Survey) or in Study 1 above in adult hens. The 4 farms that did not have prior anthelmintic treatment in the on-farm studies had mean *A. galli* worm counts of 11.4, 29.4, 3.2, 44.9 per hen (Table 3-3), while in Study 1 above the mean worm count was 10.8. Whether this reflects the larger body size of the mature hens, or in the case of the field study, continuous exposure to infection, is not clear.

The interaction between the effects of immunosuppression and infection dose on bodyweight are interesting. The linear reduction in overall bodyweight with increasing worm dose observed in sham treated chickens revealed a modest negative dose-dependent negative effect of worm infection in these birds. This negative effect of increasing worm dose was not observed in birds treated with immunosuppressants (except perhaps for dexamethasone in 2 week old challenged birds). In sheep, the host immune response to infection is recognised as contributing to production loss caused by infection with certain gastrointestinal nematodes, most notably *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (Greer et al. 2005; Greer 2008; Greer et al. 2008; Dever et al. 2016). It is possible that a similar effect is being observed in the chickens in this experiment, however, the short period of immunosuppression (during the week of infection only) contrasts with the prolonged immunosuppression used in the sheep studies.

In practical terms this model of infection has the potential to yield significant levels of infectious material. For every chick infected with 300 *A. galli* eggs, one could expect to harvest the following yields of eggs by either collection of worms or faeces.

- **Collection from female *A. galli* worms**. Our previous studies in young chickens using a similar infection model established that *in vitro* egg production per adult female *A. galli* harvested was 6,044 depending on worm size and maturity level. Taking adult worm count per normal bird infected with 300 eggs (5.2 worms/bird, assume 50% female = 2.6 females/bird) the total egg yield at bird sacrifice at 8 weeks would be 2.6 * 6044 = 15,714 eggs or a 52-fold increase on the starting material. This is a rather modest rate of increase. The rate of increase is slightly higher with the 100 egg dose (2.7 worms/bird, assume 50% female = 1.35 females/bird * 6044 = 8159 eggs or a 81-fold increase) so lower doses can be used if the stock of infective egg material is low.
- **Collection from faeces**. Towards the end of the experiment, the birds would have been producing approximately 140 g/day each of faeces (Daş et al. 2017). The average FEC from the 300 EPG no immunosuppression, day old infected group at 8 weeks post-infection was 492 EPG. Total collection of faeces for a single day with an egg recovery rate of 68% (Section 4.5.2.1) would be 140 * 492 * 0.68 = 46,838 eggs in a single day or a 156-fold recovery of the original infective dose daily. Although this method requires significantly more laboratory time and effort than harvesting worms and incubating them, this would be a much more efficient means of multiplying stocks than sacrifice and worm harvest. The study reported in Section 4.5.2.1 demonstrated that egg viability of eggs extracted from faeces is equivalent to those harvested from cultured worms.

7.3 Study 3 – Maintenance of defined nematode isolates at UNE

7.3.1 Introduction, methods and current status – Study 3

Currently, at UNE 5 isolates of *A. galli* and one of *H. gallinarum* that have been tested for anthelmintic resistance are being maintained. Details are provided in Table 7-2. Their current status is that each isolate was inoculated in layer cockerel chicks on March 25, 2021. On this date, the first of 3 doses of 100 EPG given over one week was administered.

Table 7-2 List of chicken nematode isolates currently maintained at UNE

Species	**Isolate name**	**Farm of origin** (see Table 3-1)	**Year of isolation**	**Comment**
A. galli	UNE 2019-UNE-1	UNE	2019	Tested for anthelmintic resistance. See 4.4
A. galli	UNE 2019-QLD-1	1	2019	Tested for anthelmintic resistance. See 4.2
A. galli	UNE 2020-QLD-2	2	2020	Tested for anthelmintic resistance. See 4.4
A. galli	UNE 2020-NSW-1	3	2020	Tested for anthelmintic resistance. See 4.3
A. galli	UNE 2020-NSW-2	5	2020	Tested for anthelmintic resistance. See 4.3
H. gallinarum	UNE 2020-NSW-2	5	2020	Tested for anthelmintic resistance. See 4.3

The cockerels containing the isolates will be maintained at UNE until the end of the project. If AEL wishes to maintain the isolates, Invetus is prepared to do so. A quote for providing this service is appended to the project report.

One approach to maintaining isolates we have looked at is maintaining chronic infection in either cockerels or hens in individual cages. However, we have observed that over time, FEC and worm counts decline and many birds will completely throw off the infections. Thus, constant monitoring of FEC and probably more frequent trickle infections are required to maintain patent infections in chronically infected birds. An alternative, which was not tested, was to house infected chickens in groups on the ground, where constant reinfection should maintain infections, as occurs in the field. However, with this method there is an elevated risk of contamination with different species or isolates of the nematode strain being maintained.

Table 7-3 Infection history of the chicken nematode isolates maintained at UNE in chronically infected birds until the end of the project.

Species	Isolate	Initial infection	Bird type	No. of birds	Trickle infection months (mm-yy)**
A. galli	UNE 2019-UNE-1	17/08/20	Layer cockerel	10	08-20, 11-20, 01-21
A. galli	UNE 2019-QLD-1	05/11/20	Layer cockerel	10	08-20, 11-20, 01-21
A. galli	UNE 2020-QLD-2	05/11/20	Layer cockerel	10	08-20, 11-20, 01-21
A. galli	UNE 2020-NSW-1	27/10/20*	Layer hen	10	None
A. galli	UNE 2020-NSW-2	30/12/20*	Layer hen	9	12-20, 01-21
H. gallinarum	UNE 2020-NSW-2	30/12/20*	Layer hen	10	11-20

* Natural infections. Date indicates date of arrival of hens at UNE.
** Trickle infections are 300 eggs administered over 1–2 weeks.

Table 7-4 FEC and worm count in chronically infected cockerels and hens

Isolate	FEC 5/2/2021		WC 8/2/2021		FEC 11/3/2021		WC 12/3/2021	
	+/total	Mean (EPG)	+/total	Mean WC	+/total	Mean (EPG)	+/total	Mean WC
UNE 2019-UNE-1 (*Ag*)	1/10	8	2/6	0.8	0/4	0	0/4	0
UNE 2019-QLD-1 (*Ag*)	2/10	16	2/6	0.8	0/4	0	0/4	0
UNE 2020-QLD-2 (*Ag*)	2/10	12	3/6	1.2	0/4	0	0/4	0
UNE 2020-NSW-1 (*Ag*)	2/10	120	3¼4.2	1/4	720	0/3	0	
UNE 2020-NSW-2 (*Ag*)	2/9	13	2/5	1.4	0/5	0	0/5	0
UNE 2020-NSW-2 (*Hg*)	0/10	0	1/5	0.6	0/5	0	0/5	0

7.3.2 Discussion – Study 3

We have managed to maintain field isolates of chicken nematodes at UNE through a mixture of *in vitro* maintenance of eggs and passage in chickens. We had hoped that chronically infected chickens in cages would be a good way to maintain specific isolates, but for reasons that are not fully clear, when birds are mature they tend to throw off the infections over a period of 3–5 months, even with modest reinfection periodically. This may be overcome by more frequent challenge with higher doses of eggs collected and separated from the birds' own faeces. The alternative is to place birds in biosecure pens to reinfect themselves with the same isolate, but this runs the risk of cross infection.

Although we have six field isolates of potential value to researchers and industry, none exhibit anthelmintic resistance and so they do not have unique defining characteristics that would appear to warrant the costs of ongoing maintenance. This is a decision for AEL.

7.4 Multiplication and maintenance of parasite isolates in chickens – overall findings and implications

The main conclusions that can be drawn from the studies reported above are listed below.

1. Chickens as young as day old are suitable for use in an infection model to amplify stocks of *A. galli.*

2. In such chickens, *A. galli* caused a modest dose-dependent decline in growth.

3. Immunosuppression in most cases removed the negative effect of infection on growth and in the case of dexamethasone, increased worm burden and egg counts. The interaction between immunosuppression and worm burden effects on growth indicate that the cost of mounting a host immune response in the gut is implicated in the production loss associated with *A. galli* infection.

4. Amplification of stocks by waiting until worm maturity, then harvesting the worms and obtaining eggs from them is a relatively inefficient means of multiplication of stocks. Instead, periodic total faeces collection and egg separation from the faeces would be more efficient given the high fecundity of the *A. galli* parasite.

5. Long-term storage of eggs appears to delay development of the parasite in the host and this needs to be taken into account when amplifying stocks (longer duration required for worm maturation).

6. Maintenance of infections in caged mature birds is risky as they tend to terminate the infections. This may be overcome by constant reinfection with higher doses and greater frequency than attempted in our studies. Reinfection can be with eggs extracted from the chicken's own faeces.

7. UNE has successfully characterised and maintained 5 isolates of *A. galli* and one of *H. gallinarum*, which are available for others to use until the end of the project. None of the isolates exhibit anthelmintic resistance characteristics.

8 Plain English Summary

Project Title:	Sustainable worm control with improved understanding, methods and information availability
Australian Eggs Limited Project No	1BS003
Researchers Involved	S.W. Walkden-Brown, T. Feyera Dewo, A. Shifaw Yesuf, B. Sharpe I. Ruhnke, and T. Elliott
Organisations Involved	Animal Science, School of Environmental and Rural Science University of New England Armidale NSW 2351
Phone	02 6773 5152 0413 107 973
Fax	N/A
Email	swalkden@une.edu.au
Objectives	1. Identify the current prevalence and magnitude of infection with key worm species in the free range sector of the layer industry, their perceived importance and the current methods used to control them. 2. Develop improved methods for diagnosing flock infection levels. 3. Evaluate the level of anthelmintic resistance in Australian isolates of key worm species. 4. Optimise methods for maintaining parasite stocks and develop challenge protocols to facilitate critical experimentation. 5. Develop and maintain a live collection of Australian worm isolates of known anthelmintic resistance status to be made available to other researchers or industry. 6. Develop an online portal for integrated worm management in Poultry.
Background	Free range layers have increased exposure to gastrointestinal worm infections due to the management system, and prevalence of these is anecdotally high. The registered anthelmintics available to control infection are very old and their efficacy has not been recently evaluated in Australia. Methods available for researching nematodes of poultry lag far behind those of other species such as ruminant livestock.
Research	1. An online survey of free range egg producers was conducted to determine their attitudes to worms and worm control methods. Unfortunately a low response rate was obtained. 2. The prevalence and worm burden of different worm species was evaluated on 5 free range layer farms, and the prevalence and magnitude of nematode egg counts in intestinal and caecal droppings evaluated from a further 16 flocks.

	3. Worms from five farms were tested to see if they had developed resistance to the anthelmintics being used to control worms in Australia. 4. Many experiments were conducted to determine the best methods for counting eggs in chicken faeces, for preserving worm eggs for use in future experiments, for optimising infection protocols in chickens, and to evaluate the potential to conduct anthelmintic resistance tests in the laboratory, rather than in chickens. 5. Content for web pages on worms and worm control in chickens were prepared and will be displayed on the AEL web site.
Outcomes	1. The 16 respondents to the online survey revealed that worm infection was common, but only of moderate concern to free range farmers. There was greater concern about tapeworm than the large roundworm. Most producers regularly monitored and treated for worm infection. 2. The prevalence surveys found a high prevalence of nematode infections particularly for roundworm and caecal worm. The on-farm prevalence study found high tapeworm burdens on two of five farms, with lighter infections on the other farms. 3. No resistance to levamisole, piperazine, fenbendazole or flubendazole was detected in the 5 flocks tested. For some anthelmintics, mass application in water reduced effectiveness relative to individual bird dosing. Piperazine only demonstrated adequate efficacy against adult large roundworm and gave inadequate control of other roundworms or immature stages of infection. The new in-feed formulation of flubendazole was highly effective against all nematodes and tapeworms. 4. The traditional modified McMaster faecal egg counting method was found to be superior on most important counts to the newer MiniFlotac method for routine examination of faeces for chicken eggs. 5. Methods to enable storage of worm eggs for a predicted 40 weeks were developed, but attempts to freeze eggs were not successful. A chick infection model for efficient multiplication of worms was developed as were strategies to most efficiently multiply stocks. Preliminary studies into laboratory methods for testing anthelmintic efficacy against chicken nematodes indicated significant potential in this area. 6. Five isolates of large roundworm and one isolate of the caecal worm have been characterised and maintained at UNE by a mixture of egg storage and reinfection of birds. 7. Web page content has been developed and awaits implementation with AEL.

Implications	1. The project has shown that nematode infection is common on free range farms, with burdens in some cases possibly leading to some production loss. Tapeworm infection was less common and examined on fewer farms. 2. Farmer awareness of worm prevalence was mostly good, with perhaps an overestimation of the importance of tapeworms. 3. No evidence of a decline in efficacy of the anthelmintics available to control worms was detected in 5 flocks despite a very long (> 50 years) history of usage of levamisole and piperazine. Despite this, piperazine should not be recommended as an anthelmintic of choice to control worm infections due to its poor efficacy against caecal and hair worms, and immature stages of all of the worm species. The recently approved in-feed anthelmintic fenbendazole proved to be highly efficacious against both roundworm and tapeworm. In the one study when off-label fenbendazole was administered in drinking water, efficacy was reduced below recommended levels. Care should be taken when administering these non-water-soluble anthelmintics in the water system. 4. Advances were made in the understanding and methods available to work with defined stocks of chicken nematode species, and 6 characterised worm isolates are available as a result. However, due to the inability to store isolates indefinitely by freezing, there are significant costs associated with ongoing maintenance of specified worm stocks. This work will be of greater importance, should anthelmintic resistance emerge in Australian chicken nematode populations. 5. Consideration should be given to investigate production responses to proper control of tapeworm infections, given the presence now of a highly effective anthelmintic for this purpose in the marketplace. These would need to be farm scale investigations as tapeworms are not able to be readily investigated in controlled infection studies.
Key Words	Chicken, worm, roundworm tapeworm, nematode, cestode, anthelmintic, Ascaridia, Hetarakis, Capillaria, resistance, survey, prevalence, diagnosis
Publications	**Journal papers** Feyera, T, Ruhnke, I, Sharpe, B, Elliott, T, Campbell, DLM, Walkden-Brown, SW (2020) Viability and development of *Ascaridia galli* eggs recovered in artificial media followed by storage under different conditions. *Journal of Helminthology* **94**, e199. doi:10.1017/S0022149X2000084X Shifaw, A, Feyera, T, Walkden-Brown, SW, Sharpe, B, Elliott, T, Ruhnke, I (2021) Global and regional prevalence of helminth infection in chickens over time: a systematic review and meta-analysis. *Poultry Science* **100**, 101082. doi:https://doi.org/10.1016/j.psj.2021.101082 Feyera, T, Ruhnke, I, Sharpe, B, Elliott, T, Shifaw, A, Walkden-Brown, SW (2021) Comparative therapeutic efficacies of oral and in-water administered levamisole, piperazine and fenbendazole against experimental *Ascaridia galli* infection in chickens. *Veterinary Parasitology* **298**, 109514. doi:https://doi.org/10.1016/j.vetpar.2021.109514 Shifaw, A, Feyera, T, Elliott, T, Sharpe, B, Walkden-Brown, SW, Ruhnke, I (2021) Comparison of the Modified McMaster and Mini-FLOTAC methods for the

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