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Evaluation of *in vitro* methods of anthelmintic efficacy testing against *Ascaridia galli*

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Research Paper

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Abstract

To investigate methods for *in vitro* assessment of anthelmintic efficacy against the chicken nematode *Ascaridia galli* this study firstly evaluated sample preparation methods including recovery of eggs from excreta using different flotation fluids and induced larval hatching by the deshelling–centrifugation method and the glass-bead method with or without bile. It then evaluated two *in vitro* assays, the *in-ovo* larval development assay (LDA) and larval migration inhibition assay (LMIA), for anthelmintic efficacy testing against *A. galli* using fresh eggs and artificially hatched larvae, respectively. Four anthelmintics, thiabendazole (TBZ), fenbendazole (FBZ), levamisole (LEV) and piperazine (PIP) were employed using an *A. galli* isolate of known susceptibility. The results suggested that the LDA and LMIA could successfully be used to generate concentration response curves for the tested drugs. The LDA provided EC_{50} values for inhibition of egg embryonation of 0.084 and 0.071 µg/ml for TBZ and FBZ, respectively. In the LMIA, the values of effective concentration (EC_{50}) of TBZ, FBZ, LEV and PIP were 105.9, 6.32, 349.9 and 6.78×10^7 nM, respectively. For such *in vitro* studies, a saturated sugar solution showed high egg recovery efficiency (67.8%) and yielded eggs of the highest morphological quality (98.1%) and subsequent developmental ability (93.3%). The larval hatching assays evaluated did not differ in hatching efficiency but the deshelling–centrifugation method yielded larvae that had slightly better survival rates. For final standardization of these tests and establishment of EC_{50} reference values, tests using isolates of *A. galli* of defined resistance status need to be performed.

Introduction

Current chemical strategies to control gastrointestinal nematodes in animal agriculture are heavily dependent on regular treatment with broad-spectrum anthelmintic drugs. Similarly, in poultry, anthelmintics have been the mainstay for the control of helminth infections worldwide (Ruff, 1999; Gauly *et al.*, 2001). Relatively few anthelmintics have been registered and approved to control parasitic nematodes of economic importance in poultry (Permin & Hansen, 1998; McDougald, 2020). Heavy reliance on a limited number of drugs combined with application of sub-therapeutic doses during mass application are likely to increase selection pressure for anthelmintic resistance (AR) in poultry nematodes (Knapp-Lawitzke *et al.*, 2015; Tarbiat *et al.*, 2016). At present, evidence of AR in chicken nematodes is scarce although the expansion of the free-range production system usually involving regular application of anthelmintics may further increase the risk of resistance (Tarbiat *et al.*, 2016; Collins *et al.*, 2021; Soudkolaei *et al.*, 2021; Feyera *et al.*, 2021a) *et al.* In order to delay the onset and spread of AR and ensure optimal use of the available anthelmintics, regular monitoring of the efficacy of anthelmintics is essential (Coles *et al.*, 2006; Demeler *et al.*, 2010).

In poultry, anthelmintic efficacy assessment is mainly based on the worm count reduction test using standardized procedures as detailed by Yazwinski *et al.* (2003). This *in vivo* method is expensive in terms of labour and cost of maintaining experimental birds in different treatment groups. If available, non-invasive and reliable *in vitro* assays comparable with *in vivo* assays offer substantial savings and welfare benefits (Dobson *et al.*, 1986). Furthermore, it is also suggested that *in vivo* AR testing assays be supported by complementary *in vitro* tests in gastrointestinal nematodes (Coles *et al.*, 1992; Demeler *et al.*, 2012). The egg hatch assay and larval development assay (LDA) are the most commonly used *in vitro* bioassays for monitoring AR in nematode species (Várady & Čorba, 1999; FAO, 2004; Albonico *et al.*, 2005). There are several modifications of these assays for the detection of AR to benzimidazoles (BZs), levamisole (LEV) and macrocyclic lactones largely in ruminant nematodes (Wagland *et al.*, 1992; Várady & Čorba, 1999; Taylor *et al.*, 2002; Demeler *et al.*, 2010). These assays mainly use the pre-parasitic stages (egg or larvae) and measure efficacy in the form of inhibition of larval development, hatch, or motility. Thus, such assays are useful mainly in

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nematode parasites where hatched larvae, not embryonated eggs, are the infective stage in the lifecycle.

Unfortunately, the life cycle of ascarid nematodes of poultry renders the commonly used egg hatch assay and LDA unusable for most classes of anthelmintics (Zhao *et al.*, 2017). Embryonated eggs do not hatch spontaneously outside the chicken host and there is no free-living larval stage (Hansen *et al.*, 1956; Rogers, 1961; Salih & Saleem, 1987), making it difficult to employ these methods. However, an *in ovo* LDA, a variant of LDA for ruminant nematodes, proved to be successful for detection of BZ resistance in *A. galli* (Tarbiat *et al.*, 2017). It measures the embryonic development of parasite eggs to third larval stage in the presence of increasing concentrations of anthelmintics. However, this assay cannot be used for anthelmintics that do not demonstrate ovicidal activity including LEV, piperazine (PIP) and macrocyclic lactones such as ivermectin.

Induced hatching of the embryonated eggs and *in vitro* maintenance of hatched larvae is a prerequisite for any *in vitro* larval assays developed for *A. galli*. Many factors, both chemical and physical, can induce hatching of infective ascarid eggs (Salih & Saleem, 1987; Han *et al.*, 2000). Several researchers have used artificial media for hatching *A. galli* eggs *in vitro* (Elliott, 1954; Hansen *et al.*, 1956; Chatterjee & Singh, 1968; Dick *et al.*, 1973; Feyera *et al.*, 2020)*et al.et al.et al.*. The established methods include incubation of embryonated eggs with deshelling reagents including sodium hydroxide (NaOH) and sodium hypochlorite (NaOCl) followed by centrifugation to free the larvae. With these methods, the thick egg shell is digested leaving the contents enclosed in a thin membrane which is disrupted on centrifugation freeing the enclosed larvae (Hansen *et al.*, 1956; Dick *et al.*, 1973; Feyera *et al.*, 2020). The existing larval hatching assays for *A. galli* need improvement in terms of hatching time, percentage yield and reduction of procedure-induced larval mortality. Reliable and time saving methods have been described for other ascarid nematodes that could be of value to overcome these drawbacks. The glass-bead hatching assay is one such method that employs mechanical shell disruption using glass beads coupled with magnetic stirring with or without stimulating agents such as bile, carbon dioxide (CO_2), sodium chloride (NaCl), sulphur dioxide, and sodium bicarbonate (Han *et al.*, 2000).

Even though artificial larval hatching assays have been described for *A. galli*, the possibility of using artificially hatched *A. galli* larvae for anthelmintic sensitivity studies has not been investigated. Zhao *et al.* (2017) reported a larval migration assay using artificially hatched larvae of *A. suum* which allowed accurate estimation of effective concentration (EC_{50}) values of BZ, tetrahydropyrimidin and imidazothiazole anthelmintics. This approach appears promising for the use of artificially hatched larvae of *A. galli* for *in vitro* efficacy evaluation of multiple classes of anthelmintics with different modes of action. The three commonly used anthelmintics classes in poultry, BZs, LEV and PIP exhibit different pharmacological effects on *A. galli*. The BZs disrupt energy metabolism whereas LEV and PIP cause worm paralysis (Lacey, 1988).

In vitro parasite assays require sufficient numbers of viable parasite stages especially eggs. For assays involving *A. galli,* eggs are most commonly obtained from mature worms either by disruption of the worm's uterus (Permin *et al.*, 1997; Rahimian *et al.*, 2016) or by *in vitro* culturing of female worms in artificial media and recovering eggs shed into the media (Sharma *et al.*, 2017; 2018; Feyera *et al.*, 2020). These approaches are not always feasible given the requirement of chicken necropsy to recover mature worms (Feyera *et al.*, 2020). Excreta collected from chickens harbouring *A. galli*-specific infection can also serve as source of eggs (Rahimian *et al.*, 2016; Tarbiat *et al.*, 2018) but methods for recovery of clean eggs from excreta have not been optimized. While a number of experimental studies have used *A. galli* eggs recovered from host excreta by sequential sieving and washing through a series of sieves (with gradually reducing mesh sizes, ranging from 1 mm to 36 µm) followed by flotation–centrifugation procedures, to the best of our knowledge, the effect of different flotation solutions on recovery efficiency and subsequent egg viability has not been thoroughly investigated to date. The most commonly used egg flotation fluids for coprological samples are magnesium sulphate ($MgSO_4$), sucrose, NaCl, sodium nitrate, and zinc sulphate ($ZnSO_4$) (Ballweber *et al.*, 2014; Gibbons *et al.*, 2014). Most solutions are used at saturation or near-saturation and a specific gravity (SG) from 1.2 to 1.3 is best for separating most nematode eggs from faecal debris (Foreyt, 2013). Saturated salt solutions have a tendency to crystalize rapidly, and cause significant distortion of eggs particularly at high SGs (David & Lindquist, 1982; Ballweber *et al.*, 2014). Additionally, the integrity of some parasite elements will vary in different solutions (Zajac & Conboy, 2012). Sugar solution is recommended if the eggs are required for culturing as it is considered to have little effect on the egg viability (Gibbons *et al.*, 2014). A recent study (Daş *et al.*, 2020) reported that sugar solution increased *A. galli* egg recovery from excreta by approximately 10%, relative to the lower SG NaCl solution.

The purpose of the present study was, therefore, to adapt and evaluate *in vitro* drug exposure assays based on eggs or larvae of *A. galli* for testing the efficacy of different classes of anthelmintics. It also aimed to describe optimized non-invasive methods that would yield high numbers of minimally damaged parasite stages (eggs or larvae) for *in vitro* anthelmintic sensitivity assays or other uses. We hypothesized that *in vitro* drug sensitivity assays based on fresh excreta eggs or artificially hatched larvae would enable estimation of ECs (EC_{50}/EC_{99}) of different anthelmintics that could then be correlated with effective plasma drug concentrations or *in vivo* efficacy values. The specific propositions tested were: (i) use of saturated sugar solution as flotation fluid will allow recovery of higher numbers of morphologically normal and viable *A. galli* eggs from chicken excreta compared to saturated salt solutions; (ii) the *in-ovo* LDA will be a suitable test to estimate the EC_{50}/EC_{99} of ovicidal BZ anthelmintics against *A. galli* eggs; (iii) the glass-bead larval hatching method will be superior to the chemical deshelling–centrifugation method in terms of hatching percentage and post-hatch survival rate of *A. galli* larvae; and (iv) LEV, PIP and BZ anthelmintics will exhibit inhibitory effects on the migration behaviour of artificially hatched *A. galli* larvae in a concentration-dependent fashion but have different EC_{50}/EC_{99} values.

Materials and methods

Study design

This study consisted of two main parts. Part 1 involved optimization of methods that would yield high numbers of minimally damaged eggs or larvae for subsequent *in vitro* anthelmintic sensitivity assays. In Part 1a we evaluated different flotation solutions for recovery of *A. galli* eggs from excreta to determine the solution that would yield the highest numbers of morphologically normal and viable *A. galli* eggs for an *in ovo* LDA. In Part 1b we then

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compared two assays for artificially inducing egg hatching, a deshelling–centrifugation method (Dick *et al.*, 1973; 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 larval migration inhibition assay (LMIA). In Part 2, drug exposure assays were evaluated, using eggs or artificially hatched larvae of *A. galli*, to create concentration–response curves and determine the *in vitro* drug efficacy estimation values (EC_{50}/EC_{99}). In Part 2a an *in ovo* LDA was applied to fresh *A. galli* eggs recovered by an optimized technique in Part 1a following an assay procedure described earlier (Tarbiat *et al.*, 2017). In Part 2b a modification of a LMIA described for *Ascaridia suum* (Zhao *et al.*, 2017) was applied to *A. galli* larvae hatched by an optimized method as developed in Part 1b.

Parasite material and source

An *A. galli* isolate (UNE 2020-QLD-2), which was originally recovered from naturally infected laying hens on a commercial poultry farm in Queensland, Australia, with 'no history of application of anthelmintics', was employed. This isolate had undergone a single experimental passage in young cockerels (infected at day-old) in a previously reported experiment (Feyera *et al.*, 2021b). Eggs were obtained from fresh excreta samples collected from chickens in individual cages harbouring *A. galli* infection late in this experiment by placing paper sheets beneath the individual cages between approximately 5 pm in the afternoon and 10 am the next morning. The isolate had been shown in a separate study to have no evidence of resistance to LEV, PIP or flubendazole (Feyera *et al.*, 2021b).

Anthelmintics

The anthelmintics employed in this study were powder formulations of LEV, PIP, thiabendazole (TBZ), and fenbendazole (FBZ) (Sigma, St. Louis, USA). For both the LDA and LMIA assays, stock solutions of the drugs were prepared in 100% dimethyl sulfoxide (DMSO) while serial dilutions were carried out using 0.5 and 2% DMSO, respectively, for LDA and LMIA to achieve the final drug concentrations tested. The concentration ranges of the tested drugs 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). Accordingly, for the LDA only the ovicidal drugs TBZ and FBZ were used in two-fold serial dilution with final concentration ranges of 9.94×10^4–3.18×10^6 nM (0.02–0.64 µg/ml) for TBZ and 6.68×10^4–2.14×10^6 nM (0.02–0.64 µg/ml) for FBZ. For the LMIA all compounds were tested in a four-fold serial dilution providing final concentration ranges of 1.25–1280 nM (2.5×10^{-4}–0.258 ng/ml) for TBZ, 0.125–128 nM (3.74×10^{-5}–0.128 ng/ml) for FBZ, 2.5–2560 nM (5.1×10^{-4}–0.523 ng/ml) for LEV, and 7.82×10^4–8.0×10^7 nM (6.73–6891 ng/ml) for PIP. Distilled water containing 0.5% and 2% DMSO, respectively, were used as negative control samples for the LDA and LMIA.

Optimization of methods for efficient isolation of eggs or larvae

Recovery of A. galli eggs from excreta (Part 1a)

Fresh excreta samples were collected from chickens harbouring *A. galli* artificial infection and kept in individual cages, for a separate study approved by the animal ethics committee of the University of New England, Australia (approval number AEC19−070). Fresh droppings were collected by placing paper sheets beneath the individual cages overnight. The excreta materials were pooled and thoroughly mixed using a glass rod in a plastic bucket for 5 min to obtain a homogenized mixture. Three subsamples of 2.5 g were examined by a modified McMaster method using Whitlock egg counting chamber (Whitlock, 1948) with a diagnostic sensitivity of 40 egg per gram of excreta (EPG) to obtain an estimate of the initial excreta egg counts (EECs).

Eggs were extracted from fresh excreta using a consistent procedure involving sequential washing with water through a series of sieves followed by flotation and centrifugation to obtain a clean concentrated egg sample (Daş *et al.*, 2020). From the homogenized excreta sample of known initial EPG, 5 replicates of 22.5 g of excreta for each flotation solution were subjected to the recovery procedure detailed below. The saturated flotation solutions used in this experiment and their corresponding 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 ZnSO4 (SG 1.35).

Excreta samples were flushed with tap water and passed through a series of sieves with mesh aperture sizes of 1000, 500, 250 and 90 µm, with the eggs then collected on the final 36 µm sieve. Eggs were then washed off 36 µm sieve and cleaned and concentrated by flotation coupled with centrifugation. Briefly, the material retained on the 36 µm sieve was washed off and transferred to 50 ml conical centrifuge tubes. The tubes were then centrifuged at $1620 \times g$ for 1 min and the supernatant was discarded leaving approximately 5 ml of the sieve content in the tubes. The tubes were then topped up with flotation solution, the contents mixed and then no more than 5 min later centrifuged at $1619 \times g$ for 1 min. After centrifugation, the supernatant containing eggs was poured off through 36-µm sieve followed by several rinses with a large volume of deionized water. The washed eggs on the sieve were then recovered by rinsing and stored in water (boiled and cooled) at 4 °C for not more than two days before being used for subsequent steps. Recovery efficiency, morphological quality of eggs at the time of recovery and subsequent developmental ability of eggs were assessed as described below.

Recovery efficiency. For each flotation solution, egg recovery efficiency was calculated relative to the initial mean egg count in the excreta material. Total number of eggs recovered and concentrated from each sample was estimated by a modified McMaster method applying a diagnostic sensitivity of 40 EPG.

$$\text{Recovery efficiency (\%)} = \left(\frac{\text{total number of eggs recovered}}{\text{expected number of eggs}}\right) * 100$$

Morphological quality of eggs at the time of recovery. For each sample, 200–300 eggs were examined microscopically and classified as damaged or normal (intact) based on morphological keys described earlier (Feyera *et al.*, 2020) and shown in fig. 1 using an Olympus BX40 microscope equipped with a digital Olympus DP50 camera (Nikon Corporation, Tokyo, Japan).

Developmental ability of eggs. For each sample, an estimated 500 eggs were placed in 1.5 ml Eppendorf tubes, the tubes topped up

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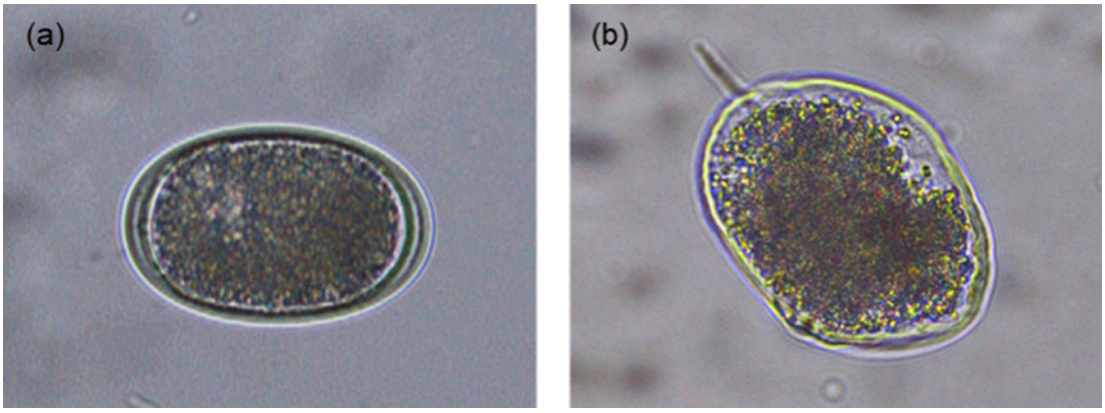

Fig. 1. Representative examples of the morphological quality of fresh *Ascaridia galli* eggs at the time of recovery from chicken excreta: (a) normal (intact); (b) damaged (undulating and irregular egg shell).

with 0.1 N sulphuric acid (H_2SO_4) and incubated aerobically at 26 °C in a digital incubator for 14 days. To ensure aerobic conditions during embryonation, tube lids were opened and samples were aerated manually for 5 min three times per week. After the incubation period, the percentage of embryonated eggs was assessed by microscopically examining the morphological characteristics of 100–200 eggs. The proportion of eggs at different stages of development (undeveloped, early development, vermiform and embryonated) or dead were recorded for each category as described previously (Feyera *et al.*, 2020) and shown in fig. 2.

Evaluation of artificial egg hatching methods (Part 1b)

In this part, a deshelling–centrifugation method and a glass-bead hatching method, were compared and where possible optimized to maximize hatching yield, hatched larval viability and temporal change in larval survivability over time. The eggs used were recovered from excreta as described above using the sugar flotation solution, then resuspended in 0.1 N H_2SO_4 and incubated aerobically at 26 °C for 6–8 weeks. To ensure aerobic conditions, samples were aerated manually by opening the lids and gentle agitation of tubes three occasions per week. Embryonation status was confirmed by light microscopy at 100× magnification prior to inclusion in the hatching assays.

For each hatching assay, five replicates of 2000 embryonated *A. galli* eggs suspended in hatching solutions in 5 ml Falcon tubes (2000 eggs per tube) were subjected to each of the egg hatching methods described below. Following hatching, live hatched larvae were separated from dead larvae and egg shell debris by active migration into sterile Hanks' buffered salt solution (HBSS) at 38 °C using a Baermann apparatus equipped with 20 µm mesh (Han *et al.*, 2000). The live larvae in each replicate tube were then washed, counted and suspended in larval culture media (Roswell Park Memorial Institute (RPMI) 1640 supplemented with 2 mm L-glutamine, 100 U/ml penicillin and 100 lg/ml streptomycin) and incubated at 37 °C. To assess temporal change in post-hatch larval survivability, at least 50 larvae per replicate were counted at different time points (0, 12, 24, 36, 48, 60, 72, 84 and 96 h) and their viability determined using the methylene blue (Fronine Pty Ltd, Melbourne, Australia) exclusion method. Hatched larvae in solution were mixed 1:1 with a 1:10,000 solution of methylene blue and examined under light microscopy at 100× magnification as described previously (Feyera *et al.*, 2020) and shown in fig. 3. Live larvae remained motile and unstained (fig. 3a), whereas dead larvae absorbed the methylene blue stain (fig. 3b) with uptake of dye indicating larval death and inactivation.

Method 1: deshelling–centrifugation method

A chemical–mechanical hatching assay originally developed for *A. galli* (Dick *et al.*, 1973) and recently described by Feyera *et al.* (2020) was used with slight modification. Briefly, eggs in 0.1 N H_2SO_4 were first washed thoroughly with 0.9% NaCl to remove the embryonation medium. Then, eggs were placed for 24 h at 26 °C in a solution containing equal parts of 4% NaOH and 4% NaOCl. After this treatment, the samples were incubated in 0.2% Tween-80 for 1 h and washed three times in distilled water by centrifugation. Liberation of the larvae was then achieved by centrifugation at $930 \times g$ for 10 min. Live larvae were then separated from unhatched eggs and debris by migration into sterile HBSS using a Baermann apparatus equipped with 20 µm mesh. The larvae were then washed in sterile phosphate buffered saline (PBS), counted and suspended in larval culture media (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 lg/mL streptomycin) and stored aerobically at 37 °C until evaluated for larval viability at various times as described above.

Method 2: glass-bead hatching method

The glass-bead hatching method described for *A. suum* eggs (Han *et al.*, 2000; Zhao *et al.*, 2017) was adopted with slight modification. Briefly, eggs were washed thoroughly with 0.9% NaCl to remove the embryonation medium (0.1 N H_2SO_4). The eggs were then suspended in hatching buffer (HBSS with or without 5% bovine bile (Sigma, St. Louis, USA)) and hatched by stirring with 2 mm glass beads using a magnetic stirrer for 30 min at 37 °C. Live larvae were then separated from unhatched eggs and debris by migration into sterile HBSS using a Baermann apparatus equipped with 20 µm mesh. The larvae were then washed in sterile PBS, counted and suspended in larval culture media (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 lg/mL streptomycin) and stored aerobically at 37 °C until evaluated for larval viability at various times as described above.

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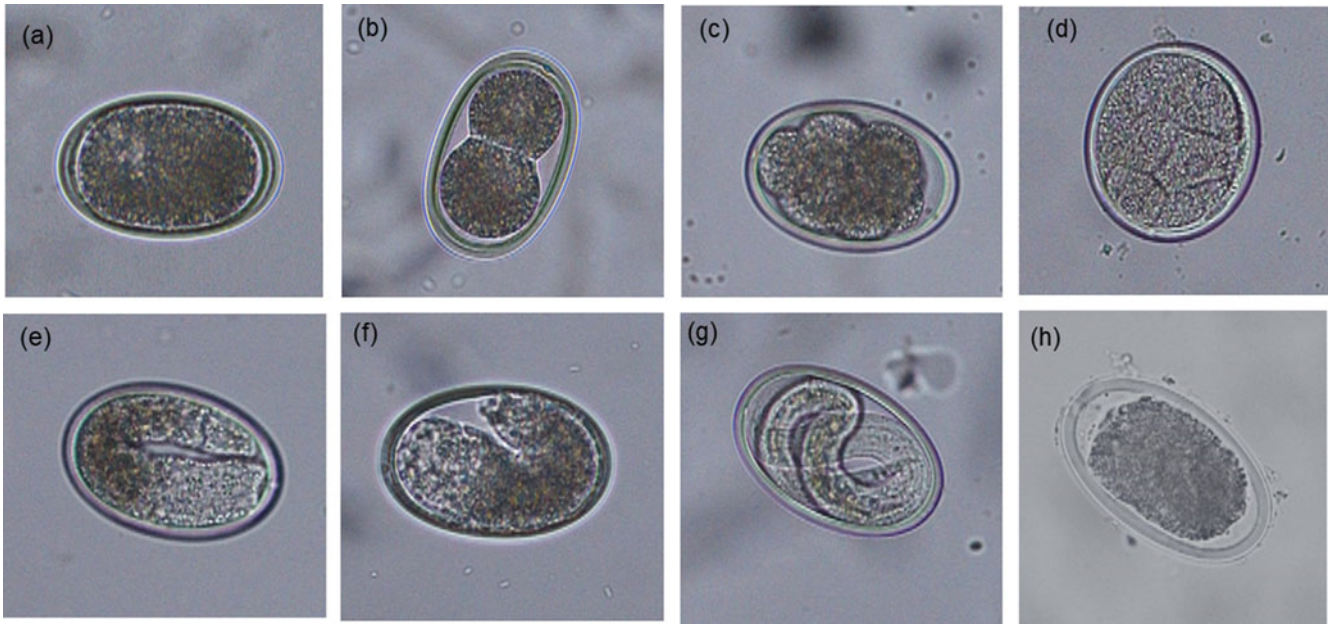

Fig. 2. Morphological characteristics of *Ascaridia galli* eggs at different developmental stages following 14 days of incubation at 26 °C (original magnification 200×): (a) undeveloped; (b–d) early development; (e and f) vermiform; (g) embryonated; and (h) dead.

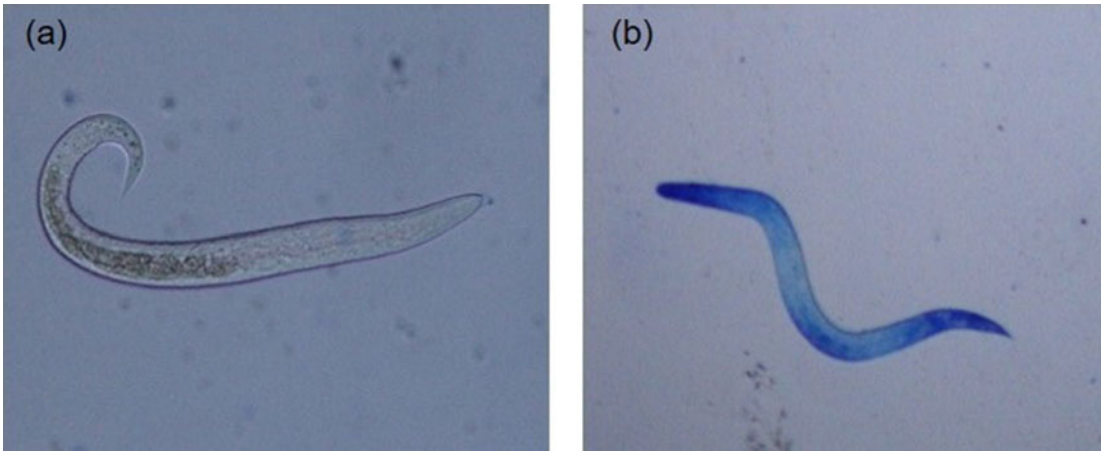

Fig. 3. Artificially hatched *Ascaridia galli* larvae stained with methylene blue (original magnification 200×): (a) live and active larvae demonstrating intact membrane and impermeability to methylene blue; and (b) dead larvae demonstrating uptake of methylene blue.

Evaluation of in vitro *drug exposure assays (Part 2)*

LDA (Part 2a)

The LDA was conducted essentially as described previously (Tarbiat *et al.*, 2017). Fresh *A. galli* eggs recovered from excreta using the sugar solution were used. Briefly, the *A. galli* eggs were exposed to a series of gradually increasing drug concentrations in micro-titre plates with 5 replicates per interval for each anthelmintic. To prevent dehydration, each plate was covered with a plate sealer which allows passage of air molecules and placed in a water bath. The sealed plates were then incubated at 26 °C for two weeks to achieve optimum egg development rate as described previously (Feyera *et al.*, 2020). At the end of the incubation period 10 µl of Lugol's iodine solution was added to each well and plates were examined under an inverted microscope. A minimum of 100 eggs per well were counted and the number of embryonated eggs at each drug concentration was determined as described above. Percentage inhibition of egg embryonation was calculated relative to the negative control using the formula (Wagland *et al.*, 1992):

$$\text{Percentage inhibition of egg embryonation} = 100 - \left(\frac{\text{number of embryonated eggs in test well}}{\text{mean number of embryonated eggs in control wells}}\right) * 100$$

LMIA (Part 2b)

The LMIA was conducted essentially as described previously for *A. suum* (Williams *et al.*, 2016, Zhao *et al.*, 2017), using *A. galli* larvae hatched by the deshelling–centrifugation method. Briefly, 100 larvae (in duplicate) were incubated with graded

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concentrations of anthelminthic drugs 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 for 15–18 h at 38 °C in an atmosphere containing 5% CO_2. Then, an equal amount of 1.5% agar solution (45 °C) was added to each well and mixed thoroughly. The agar was allowed to set prior to the addition of fresh culture media on top to cover the agar and the plates returned to the incubator overnight. The next day, the media containing larvae that had migrated from the setting agar was collected from each well and the number of larvae in the sample enumerated as described earlier (Williams *et al.*, 2016). Percentage inhibition of migration was calculated relative to the negative control using the formula (Wagland *et al.*, 1992):

$$\text{Percentage inhibition of migration} = 100 - \left(\frac{\text{number of larvae migrated in test well}}{\text{mean number of larvae migrated in control wells}}\right) * 100$$

Statistical analysis and determination of EC_{50} and EC_{99}

The JMP® software version 16.0 (SAS Institute Inc., Cary, USA) was used for data analysis and creation of concentration–response curves. Distributions of the data and model residuals were assessed for compliance with the assumptions of analysis of variance (ANOVA). No data transformations were required except log transformation of drug concentrations for creation of concentration–response curves and determining EC_{50} and EC_{99}. Data from egg recovery from excreta (including differences in egg recovery efficiency between different flotation solutions, morphological quality of eggs and subsequent developmental ability) and larval hatch assays (hatching efficiency) were analysed by one-way ANOVA using the fit least squares platform of JMP. Egg embryonation inhibition and larval migration inhibition were calculated relative to eggs or larvae recovered in control wells and expressed as percentage inhibition. Temporal changes in larval viability over time were analysed with repeated measures ANOVA using a mixed restricted maximum likelihood model with individual sample fitted as random factor and treatment and time and their interactions fitted as fixed effects. Nonlinear logistic regression (logistic 3P) was used to define the dose–response curve and calculate EC_{50}, EC_{99} with 95% confidence limits. Statistical significance was set at $P < 0.05$ throughout.

Results

Recovery of A. galli eggs from excreta (Part 1a)

The type of flotation solution significantly ($P = 0.003$) affected the number of eggs recovered relative to the expected number of eggs contained in the excreta material. $ZnSO_4$ (68.3%) and sucrose (67.8%) solutions provided the highest egg recovery efficiency followed by sucrose–NaCl solution (65.6%), while NaCl (58.3%) and $MgSO_4$ (57.9%) extracted the least (fig. 4). There was a strong positive association between recovery efficiency and SG of the flotation solution ($R^2 = 0.79$; $P = 0.03$).

The type of flotation solution also had a significant effect ($P = 0.004$) on the morphological quality of recovered eggs. The sugar solution yielded the highest proportion (98.1%) of morphologically normal eggs as assessed by morphological appearance whereas $ZnSO_4$ resulted in the highest percentage of damaged eggs (9.7%) at the time of recovery compared to others (table 1). There was no major difference between the remaining flotation solutions in terms of the quality of eggs recovered.

Furthermore, the type of flotation solution used to recover eggs from excreta had significant ($P = 0.0074$) effect on the subsequent developmental ability of *A. galli* eggs (table 1). Eggs isolated by sucrose solution had the highest embryonation capacity (93.3%) followed by those isolated by $MgSO_4$ (87.1%). A positive linear association between morphological quality at the time of recovery and developmental ability of eggs was observed ($n = 25$; $R^2 = 0.16$; $P = 0.047$).

Combining the effects of flotation solution on efficiency of recovery and embryonation capacity an estimated yield of 63, 50, 49.7, 57 and 56% embryonated eggs from excreta could be expected following use of sucrose, $MgSO_4$, NaCl, sucrose–NaCl and $ZnSO_4$ as flotation solutions, respectively.

Evaluation of in vitro A. galli larvae hatching assays (Part 1b)

The deshelling–centrifugation and the glass-bead with 5% bile or without bile, respectively, resulted in 97.4, 95.2 and 94.9% hatching of the embryonated eggs with no statistically significant difference. Repeated measured analysis of post-hatch larvae survival data following incubation in RPMI media revealed significant effects of hatching method ($P = 0.0053$) and incubation period ($P < 0.0001$) without significant interaction between these effects (fig. 5). Overall, larvae hatched by the deshelling–centrifugation method demonstrated higher percentage survivability ($P = 0.004$) than those hatched by the glass-bead hatching assays. Larval survival decreased with incubation time with percentage survival diminishing to as low as <10–15% after 96 h of incubation almost in all cases with poor motility. This observation, 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.

LDA (Part 2a)

The mean percentage development to embryonation of eggs in the control wells was 92.5%. There was a concentration dependent inhibition of *in ovo* larval development for the two BZ drugs tested (fig. 6). EC_{50} and EC_{99} estimates are shown in table 2. FBZ had a slightly lower EC_{50} value (0.071 µg/ml) than TBZ (0.084 µg/ml).

LMIA (Part 2b)

As expected, the anthelmintics caused a concentration-dependent inhibition of larval migration (fig. 7). EC_{50} and EC_{99} values are shown in table 3. The concentration–response curve revealed that FBZ exhibited the lowest EC_{50} value (6.32 nM) whereas PIP the highest EC_{50} value (6.78×10^7 nM) with the EC_{99} estimates also following a similar trend.

Discussion

The present study evaluated two *in vitro* drug exposure bioassays for anthelmintic efficacy testing against *A. galli* as well as the

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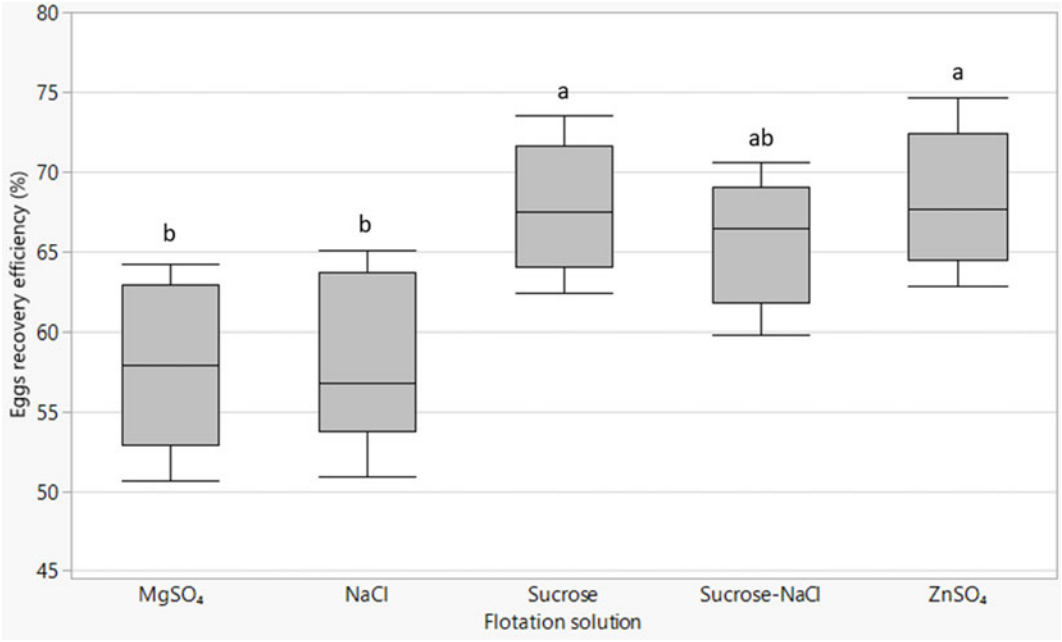

Fig. 4. Box plots of *Ascaridia galli* eggs recovery efficiency by different flotation fluids with lines from bottom to top representing the minimum, first quartile, median, third quartile and maximum values. Mean values of plots not sharing a common letter (a, b) differ significantly ($P < 0.05$).

Table 1. Morphological quality at the time of recovery and subsequent developmental ability of *Ascaridia galli* eggs recovered by different flotation solutions.

		Morphological quality at the time of recovery (%)		Development (%) following aerobic incubation at 26 °C for 14 days			
Flotation solution	Specific gravity (SG)	Intact	Damaged	Undeveloped	Early development	Vermiform	Embryonated
sucrose	1.27	98.1 ± 1.21^{a}	1.89 ± 0.54^{b}	0.00 ± 0.00	0.78 ± 0.48	0.45 ± 0.24	93.3 ± 0.73^{a}
sodium chloride (NaCl)	1.20	92.9 ± 1.61^{b}	$7.14 \pm 1.61^{a,b}$	0.00 ± 0.00	1.19 ± 0.59	0.78 ± 056	85.3 ± 2.25^{b}
NaCl–sucrose	1.28	$93.8 \pm 1.18^{a,b}$	$6.24 \pm 1.17^{a,b}$	0.19 ± 0.00	0.80 ± 0.37	0.99 ± 0.44	$86.7 \pm 2.18^{a,b}$
magnesium sulphate	1.20	$94.2 \pm 1.21^{a,b}$	5.77 ± 1.21^{a}	0.00 ± 0.12	0.99 ± 0.54	1.40 ± 0.51	$87.1 \pm 1.39^{a,b}$
Zinc sulphate	1.36	90.3 ± 1.34^{b}	9.71 ± 1.34^{a}	0.02 ± 0.20	2.01 ± 0.71	1.19 ± 0.37	82.1 ± 2.30^{b}

Values are mean ± SE; SE = standard error; SG = specific gravity. Means within columns not sharing a common letter (a, b) in the superscript differ significantly ($P < 0.05$) for each measurement and factor.

associated pre-assay sample preparation methods to maximize the yield of high-quality eggs and larvae for this purpose. It was found that the *in-ovo* LDA measuring inhibition of larval development in the egg shows considerable promise for the evaluation of efficacy of BZ anthelmintics against *A. galli*. This was evidenced by the clear concentration–response curves obtained enabling estimation of $EC_{50/99}$ values of FBZ and TBZ. While the LDA method is restricted to anthelmintics with ovicidal activity, the more complex LMIA method evaluated also showed promise as a method for the creation of concentration–response curves enabling quantification of anthelmintic activity for anthelmintics acting on development stages beyond the egg. Among the flotation solutions tested, the saturated sucrose solution provided excellent recovery (67.8%) of fresh *A. galli* eggs from excreta with highest morphological quality (98.1%) and embryonation capacity (93.1%). The larval hatching assays evaluated did not differ in hatching efficiency but the deshelling–centrifugation method yielded larvae that had a better survival rate. These findings are discussed in more detail and in light of the original propositions below.

The first proposition of this study that sugar solution would allow better recovery of morphologically normal and viable *A. galli* eggs from excreta compared to saturated salt solutions was supported by the findings. Sucrose solution yielded a significantly higher number of minimally damaged eggs with superior developmental ability compared to the other flotation solutions tested. Taking into account all of the effects of flotation solution, the yield of fully embryonated eggs following extraction from excreta using sucrose, $MgSO_4$, NaCl, sucrose–NaCl, and $ZnSO_4$ and incubation in 0.1 N H_2SO_4 aerobically at 26 °C for 14 days would be 63, 50, 49.7, 57 and 56%, respectively. The sucrose solution thus resulted in an embryonated egg yield that is 7–13 percentage units higher than the other flotation fluids. As observed in other studies, the recovery efficiency of a flotation solution may in part be attributed to the SG of the flotation solution used (Cringoli *et al.*, 2004; Vadlejch *et al.*, 2011; *et al.* 2017;

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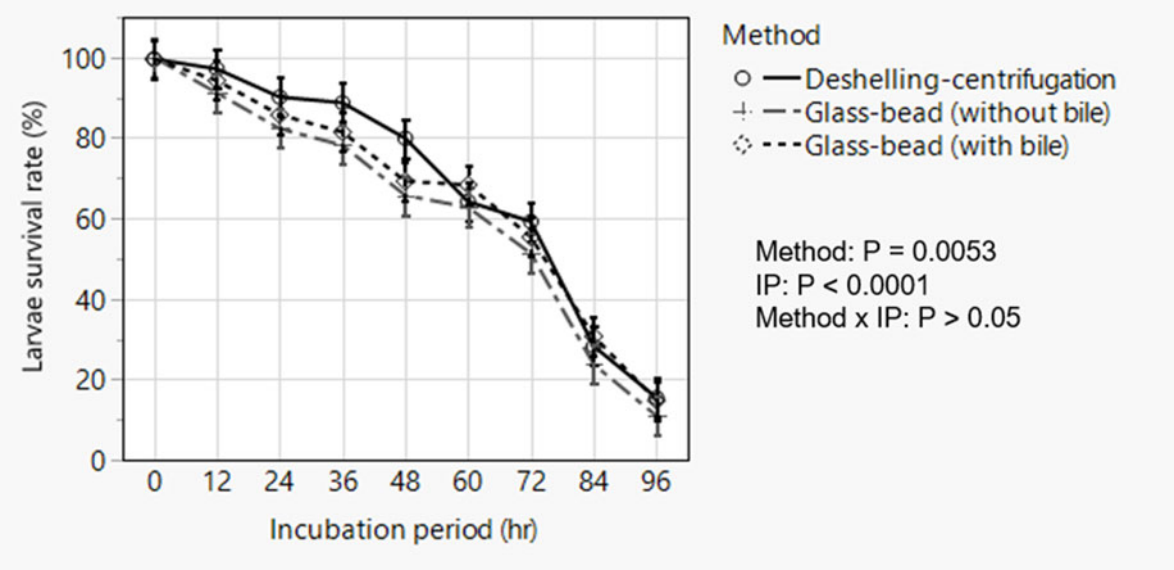

Fig. 5. Analysis of temporal change in post-hatch survivability of artificially hatched *Ascaridia galli* larvae showing overall effects of method of hatching and incubation period in Roswell Park Memorial Institute media. Data are presented as least squares means with standard errors following statistical analysis. IP = incubation period.

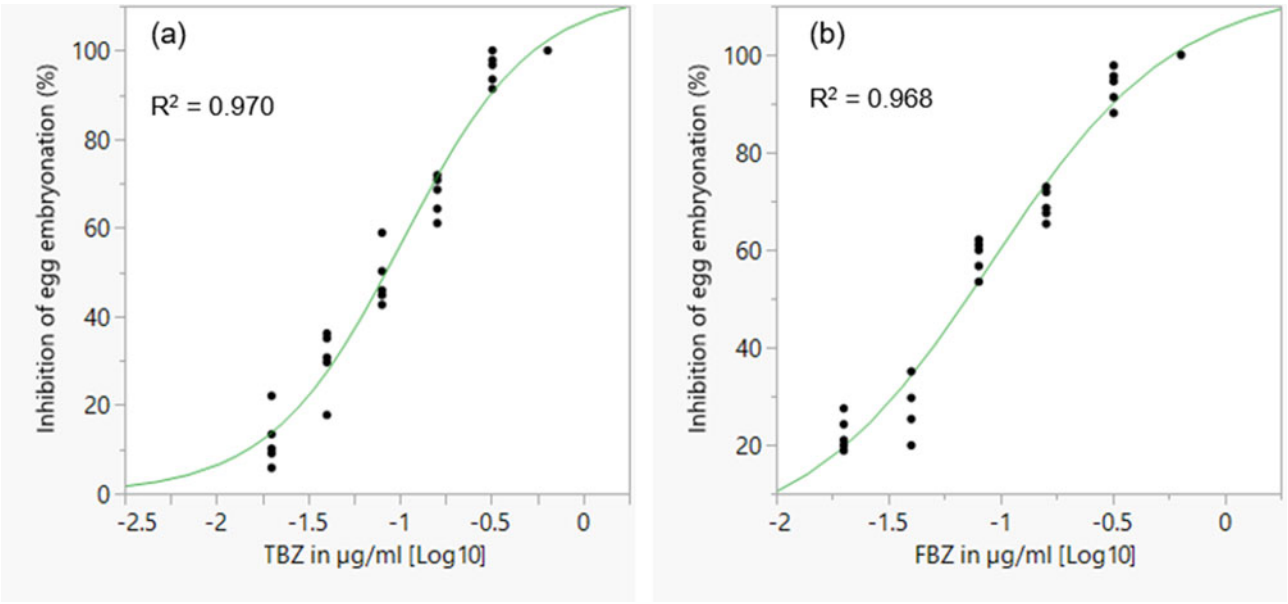

Fig. 6. Concentration–response curves for TBZ (a) and FBZ (b) anthelmintics in a larval development assay for assessing anthelmintic efficacy in *Ascaridia galli*. TBZ = thiabendazole; FBZ = fenbendazole. Individual points represent replicates and the curve is a logistic 3P fit.

Table 2. EC_{50} and EC_{99} estimates (± standard error for log estimates) for inhibition of egg development to the embryonated stage based on a larval development assay of an *Ascaridia galli* isolate with no recent history of exposure to anthelmintics and known susceptibility to flubendazole.

	EC_{50}			EC_{99}		
Anthelmintic	$Log_{10}EC_{50}$ (µg/ml)	EC_{50} (µg/ml)	EC_{50} (nM)	$log_{10}EC_{99}$ (µg/ml)	EC_{99} (µg/ml)	EC_{99} (nM)
TBZ	−1.074 ± 0.021	0.084	4.17×10^5	−0.298 ± 0.057	0.504	2.50×10^6
FBZ	−1.148 ± 0.022	0.071	2.37×10^5	−0.268 ± 0.061	0.539	1.80×10^6

TBZ = thiabendazole; FBZ = fenbendazole; EC = effective concentration.

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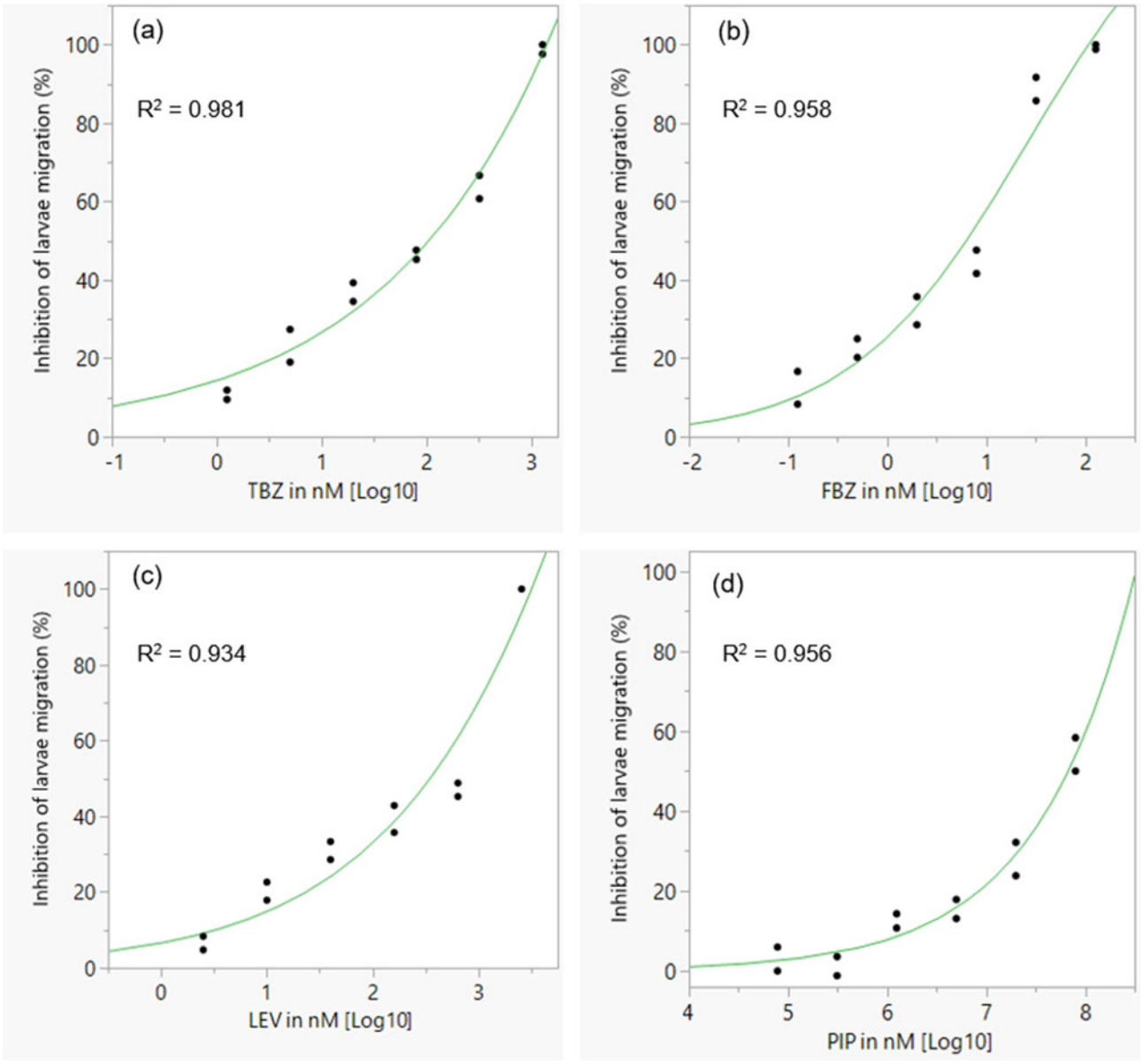

Fig. 7. Concentration–response curves for different classes of anthelmintics in a larval migration inhibition assay for assessing anthelmintic efficacy in *Ascaridia galli*. (a) TBZ; (b) FBZ; (c) LEV; (d) PIP. TBZ = thiabendazle; FBZ = fenbendazole; LEV = levamisole; PIP = piperazine; individual points represent replicates and the curve is a logistic 3P fit.

Table 3. EC_{50} and EC_{99} estimates (± standard error for log estimates) for inhibition of larval migration in a larval migration inhibition assay using an *Ascaridia galli* isolate with no recent history of exposure to anthelmintics and known susceptibility to LEV and PIP.

	EC_{50}			EC_{99}		
Anthelmintic	$\log_{10}EC_{50}$ (nM)	EC_{50} (nM)	EC_{50} (ng/ml)	$\log_{10}EC_{99}$ (nM)	EC_{99} (nM)	EC_{99} (ng/ml)
TBZ	2.025 ± 0.070	105.9	0.0213	3.131 ± 0.056	1352.1	0.272
FBZ	0.801 ± 0.095	6.32	0.0019	2.006 ± 0.119	101.4	0.0304
LEV	2.544 ± 0.121	349.9	0.0719	3.488 ± 0.115	3076.1	0.628
PIP	7.831 ± 0.052	6.78×10^7	5.84×10^3	8.502 ± 0.375	3.18×10^8	2.74×10^4

TBZ = thiabendazle; FBZ = fenbendazole; LEV = levamisole; PIP = piperazine; EC = effective concentration. For PIP, the EC_{99} values are considerably outside the range of concentrations evaluated.

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Daş *et al.*, 2020) consistent with the positive association observed between SG and recovery efficiency in the present study. As noted by Ballweber *et al.* (2014) the use of flotation solutions with high SG while favouring flotation of eggs, may simultaneously result in distortion of eggs. This was evident in the present study in the case of $ZnSO_4$ which yielded the highest recovery rate due to its high SG, but lowest morphological quality and developmental ability as has also been reported previously (Amoah *et al.*, 2017). Unlike the high SG salt solutions, the saturated sugar solution showed excellent recovery efficiency while also yielding eggs of highest morphological quality and subsequent developmental ability. This is consistent with literature that sugar solution should be used if the eggs are required for culturing as it is considered to have little effect on the egg viability (Gibbons *et al.*, 2014). While saturated NaCl is the most commonly used flotation solution in the laboratory, it yielded eggs of significantly inferior morphological quality as well as embryonation capacity compared to the sugar solution. In summary, the sugar solution can be recommended for recovering eggs from excreta for assays (such as LDA) when high numbers of fresh *A. galli* eggs with a high embryonation potential are required.

Our second proposition that the glass-bead method would result in a higher hatching percentage and post-hatch survival rate of *A. galli* larvae compared to the deshelling–centrifugation method was not supported by the findings. The three methods evaluated did not differ in hatching efficiency consistent with previous work reporting 96–98% for *A. galli* employing the deshelling–centrifugation method (Dick *et al.*, 1973; Feyera *et al.*, 2020) and 94–96% for *A. suum* using the glass-bead method (Han *et al.*, 2000). However, the deshelling–centrifugation method using Tween-80 in the deshelling solution yielded larvae with better post-hatch survival rates in RPMI media especially during the first 48 h post-hatch. The use of Tween-80 is considered advantageous not only to enable a high hatching rate, but also consistently high viability of larvae recovered (Dick *et al.*, 1973) although the mechanism of these effects is unknown. The disadvantage of this method is that it involves egg hatching in two steps, that is, deshelling followed by liberation of larvae which is time consuming in comparison to the glass-bead method. The glass-bead method is a shorter procedure in terms of time required for hatching and is potentially useful if optimized but it did not provide any advantage over the former despite addition of bovine bile which was reported to enhance hatching, migration and survival of *A. suum* eggs depending on the method of hatching used (Han *et al.*, 2000). In the glass-bead method, the physical stimulus of magnetic stirring with glass beads is thought to be responsible for inducing egg hatching of *A. suum*, but in the present study also appeared to damage a proportion of the larvae thereby reducing their viability and subsequent ability to migrate as also reported previously (Han *et al.*, 2000; Vejzagić *et al.*, 2015). 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 in enriched larval culture media at 38.5 °C (Dick *et al.*, 1973). Overall larval viability decreased by 0.87% every hour of incubation in artificial media at 37 °C indicating a complete loss of viability by around 120 h. This suggests that incubation in RPMI at 37 °C may not be ideal 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. It has been suggested that culture medium essential for the growth of several animal cell lines and containing most of the amino acids, vitamins and other metabolites are suited for *in vitro* maintenance of artificially hatched *Ascaris* larvae (Cleeland & Laurence, 1962). Regarding temperature, earlier studies involving *in vitro* cultivation of *A. galli* and *Ascaris lumbricoides* larvae used a culture temperature of 37–38 °C (Cleeland & Laurence, 1962; Dick *et al.*, 1973) with larvae undergoing active exsheathing and development. The influence of incubating *A. galli* larvae at 37 °C (4.5 °C below the 41.5 °C body temperature of chickens) is unknown but cultivation of *A. lumbricoides* larvae at temperatures as low as 20–25 °C maximized survival time up to 100 days presumably due to lower metabolic activity (Cleeland & Laurence, 1962).

The third proposition that the *in-ovo* LDA would be a suitable method to estimate the EC_{50} of BZ anthelmintics for efficacy testing in *A. galli* was supported by the data. The evaluated LDA appears to be suitable for the creation of reliable concentration–response curves and calculation of EC_{50} allowing for assessment of AR for anthelmintics with ovicidal activity. The current LDA is in part a validation of a previous report by Tarbiat *et al.* (2017) who also found that this assay could be successfully used to assess the sensitivity of *A. galli* eggs to inhibition of development by TBZ. This technique is known to have high sensitivity, reproducibility and ease of interpretation (Várady *et al.*, 2007). Given that the broad spectrum BZs will continue to be the most widely applied anthelmintics in the poultry industry worldwide and the LDA is a rather simple assay to perform, it should be considered as a method of choice for rapid *in vitro* AR testing in this class of anthelmintics compared to the more labour intensive LMIA. However, to estimate the resistance level in a worm population using this method, calibration of EC_{50} or EC_{99} results with gold standard measures of anthelmintic efficacy such as the worm count reduction test is required. As AR does not appear to be currently widespread in the poultry industry (Tarbiat *et al.*, 2017; Feyera *et al.*, 2021a, *et al.* b, *et al.* 2022), finding *A. galli* isolates of known resistance status for such calibration is a substantial challenge. The EC_{50} value for TBZ obtained in the current study was slightly lower (0.084 µg/ml) compared to the previously reported EC_{50} value of 0.101–0.106 µg/ml (Tarbiat *et al.*, 2017) using drug sensitive *A. galli* isolates. A possible reason might be that the isolates used by Tarbiat *et al.* (2017) had a history of repeated exposure to BZ anthelmintics. It should be noted that the *A. galli* isolate used in our study originated from a commercial poultry farm with no history of anthelmintic use and shown to be fully susceptible to LEV, PIP and flubendazole (Feyera *et al.*, 2021b). The EC_{99} estimates, however, were slightly higher compared to the EC_{99} values of 0.33–0.35 µg/ml for TBZ reported by Tarbiat *et al.et al.* (2017). Identifying and testing of various resistant and susceptible isolates of *A. galli* will be necessary in order to calibrate EC_{50} and EC_{99} values against gold standard tests of AR to the BZ anthelmintics such as the worm count reduction test (Yazwinski *et al.*, 2003). The applicability of the LDA for mixed nematode species samples, which commonly occur in the field, also requires further evaluation, but it is likely that the ovicidal effects of BZ anthelmintics will extend to the other important nematode infections of chickens.

The fourth proposition of this study that LEV, PIP and BZ anthelmintics would exhibit inhibitory effects on the migration behaviour of artificially hatched *A. galli* larvae in a concentration-dependent fashion but would have different EC_{50}/EC_{99} values was supported by the findings. Following exposure to anthelmintic drugs, the migration of *A. galli* larvae from agar gel was shown to be inhibited by anthelmintics in a dose-dependent manner. This

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is the first effort to develop such an assay using artificially hatched *A. galli* larvae and the concentration–response curves created for the tested anthelmintics showed that artificially hatched larvae of *A. galli* could be used for estimation of EC_{50} and thus *in vitro* AR testing using the LMIA provided calibration using strains of known AR status is done. This method is based on the nematode migration behaviour in the solidified agar gel, thereby allowing for counting of larvae that moved out of agar gel and quantifying the inhibitory effect of the nematode migration (Zhao *et al.*, 2017). Dead larvae or larvae whose migration is inhibited by anthelmintic drugs stay inside of the agar gel, only active larvae are able to migrate out of the agar gel (Williams *et al.*, 2014; Zhao *et al.*, 2017). This assay allowed for assessment of anthelmintic activity simply by the counting of all migrated larvae rather than relying on assessment of individual worms as either motile or non-motile. The values of EC_{50} of TBZ, FBZ and LEV estimated in the current study are within the concentration ranges reported for the same compounds using LMIA against artificially hatched larvae of drug sensitive *A. suum* isolates which ranged 74–150, 4.9–13.9 and 358–1150 nM, respectively (Zhao *et al.*, 2017). PIP exhibited the highest EC_{50} values 190–10,000 fold higher than the other anthelmintics in the current study. Against adult ascarids the therapeutic dose of PIP (100 mg/kg) is 4-fold higher than that of LEV (28 mg/kg) and up to 20-fold higher than that of BZ anthelmintics such as FBZ (5–10 mg/kg). However, PIP has been shown to have poor efficacy against immature *A. galli* (Leiper, 1954; Horton-Smith & Long, 1956; Alicata, 1958; Feyera *et al.*, 2021b, *et al.*, 2022) and this appears to extend to freshly hatched larvae in the LMIA as the likely reason for the very much higher EC_{50} values observed for this anthelmintic. In summary, the current study showed that the LMIA, using artificially hatched *A. galli* larvae, could potentially be used for anthelmintic sensitivity studies against *A. galli*. However, this assay is methodologically complex to implement compared to the LDA and may not offer sufficient advantages over the gold standard worm count reduction test to warrant optimization for routine diagnosis of AR in *A. galli*. Its applicability for evaluating AR to PIP also requires further investigation given the selective efficacy of PIP for adult *A. galli* and low sensitivity of larval stages to its effects.

Conclusions

The current study provided strong confirmatory evidence that a LDA based on inhibition of *A. galli* egg embryonation will generate reliable concentration–response curves for the ovicidal BZ anthelmintics TBZ and FBZ. The LDA thus shows considerable potential for *in vitro* anthelmintic efficacy testing for BZ drugs, provided EC_{50} values can be calibrated against gold standard measures of efficacy such as the worm count reduction test. The LDA is an easy test to implement, but will not work for the anthelmintics which do not exhibit ovicidal activity. The advantages of this test are that it could be deployed on excreta samples sent in from a farm, and would not require animal testing and sacrifice. However, the applicability of the LDA for mixed species samples, which commonly occur in the field, requires further evaluation. The present study found that there are reasonable prospects for developing *in vitro* anthelmintic efficacy tests based on a LMIA that would be effective for evaluating the full range of anthelmintics, but the method is far more complex to implement than LDA and may not offer sufficient advantages over the worm count reduction test to warrant optimization. Its applicability for predicting the efficacy of PIP against adult worms is also uncertain, given the high level of resistance to its effects shown by larvae in this study. For *in vitro* studies, a saturated sugar solution is most appropriate for recovering eggs from excreta prior to use in assays. Further work is needed to optimize larval hatching/freeing methods, together with optimal temperature and media for maintenance of newly hatched larvae. Whatever *in vitro* tests are developed, standardization of assays and development of EC_{50} or EC_{99} reference values linked to known levels of anthelmintic resistance would also need to be achieved.

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Conflicts of interest. None.

Ethical standards. None.

References

Albonico M, Wright V, Ramsan M, Haji HJ, Taylor M, Savioli L and Bickle Q (2005) Development of the egg hatch assay for detection of anthelminthic resistance in human hookworms. *International Journal for Parasitology* **35(7)**, 803–811.

Alicata JE (1958) Observations on the dosage and method of administration of piperazine citrate to chickens for the control of *Ascaridia galli*. *Poultry Science* **37(1)**, 89–96.

Amoah ID, Reddy P and Stenström TA (2017) Effect of reagents used during detection and quantification of *Ascaris suum* in environmental samples on egg viability. *Water Science and Technology* **76(9)**, 2389–2400.

Ballweber L, Beugnet F, Marchiondo A and Payne P (2014) American Association of Veterinary Parasitologists' review of veterinary fecal flotation methods and factors influencing their accuracy and use—Is there really one best technique? *Veterinary Parasitology* **204(1–2)**, 73–80.

Chatterjee R and Singh K (1968) *In vitro* culture and hatching of *Ascaridia galli* eggs. *Indian Journal of Veterinary Science* **38(3)**, 517–523.

Cleeland R and Laurence KA (1962) *In vitro* cultivation of *Ascaris lumbricoides* var. *suum* larvae. *The Journal of Parasitology* **48(1)**, 35–38.

Coles G, Bauer C, Borgsteede F, Geerts S, Klei T, Taylor M and Waller P (1992) World Association for the Advancement of Veterinary Parasitology (W. A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology* **44(1–2)**, 35–44.

Coles G, Jackson F, Pomroy W, Prichard R, von Samson-Himmelstjerna G, Silvestre A, Taylor M and Vercruysse J (2006) The detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology* **136 (3–4)**, 167–185.

Collins J, Jordan B, Bishop A and Kaplan RM (2021) Fenbendazole resistance in *Heterakis gallinarum*, the vector of *Histomonas meleagridis*, the causative agent of blackhead disease in poultry. *bioRxiv - Zoology*. doi:10.1101/2021.03.30.437780

Cringoli G, Rinaldi L, Veneziano V, Capelli G and Scala A (2004) The influence of flotation solution, sample dilution and the choice of McMaster slide area (volume) on the reliability of the McMaster technique in estimating the faecal egg counts of gastrointestinal strongyles and *Dicrocoelium dendriticum* in sheep. *Veterinary Parasitology* **123(1–2)**, 121–131.

Cringoli G, Maurelli MP, Levecke B, Bosco A, Vercruysse J, Utzinger J and Rinaldi L (2017) The Mini-FLOTAC technique for the diagnosis of helminth and protozoan infections in humans and animals. *Nature Protocols* **12(9)**, 1723–1732.

Daş G, Klauser S, Stehr M, Tuchscherer A and Metges CC (2020) Accuracy and precision of McMaster and Mini-FLOTAC egg counting techniques using egg-spiked faeces of chickens and two different flotation fluids. *Veterinary Parasitology* **283**, 109158.

David ED and Lindquist WD (1982) Determination of the specific gravity of certain helminth eggs using sucrose density gradient centrifugation. *The Journal of Parasitology* **68(5)**, 916–919.

https://doi.org/10.1017/S0022149X22000177 Published online by Cambridge University Press

Demeler J, Kleinschmidt N, Küttler U, Koopmann R and von Samson-Himmelstjerna G (2012) Evaluation of the egg hatch assay and the larval migration inhibition assay to detect anthelmintic resistance in cattle parasitic nematodes on farms. *Parasitology International* **61(4)**, 614–618.

Demeler J, Küttler U, El-Abdellati A, *et al.* (2010) Standardization of the larval migration inhibition test for the detection of resistance to ivermectin in gastro intestinal nematodes of ruminants. *Veterinary Parasitology* **174(1–2)**, 58–64.

Dick JW, Leland SE Jr and Hansen M (1973) Hatching and *in vitro* cultivation of the nematode *Ascaridia galli* to the third-stage larva. *Transactions of the American Microscopical Society* **92(2)**, 225–230.

Dobson R, Donald A, Waller P and Snowdon K (1986) An egg-hatch assay for resistance to levamisole in trichostrongyloid nematode parasites. *Veterinary Parasitology* **19(1–2)**, 77–84.

Elliott A (1954) Relationship of aging, food reserves, and infectivity of larvae of *Ascaridia galli*. *Experimental Parasitology* **3(4)**, 307–320.

FAO (2004) Helminths: Anthelmintic resistance: Diagnosis, management and prevention. *Guidelines resistance management and integrated parasite control in ruminants Module* **2**, 78–118.

Feyera T, Ruhnke I, Sharpe B, Elliott T, Campbell D and 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**, 1–9.

Feyera T, Ruhnke I, Sharpe B, Elliott T, Shifaw A and Walkden-Brown SW (2021a) Comparative therapeutic efficacies of oral and in-water administered levamisole, piperazine and fenbendazole against experimental *Ascaridia galli* infection in chickens. *Veterinary Parasitology* **298**, 1–9.

Feyera T, Sharpe B, Elliott T, Shifaw AY, Ruhnke I and Walkden-Brown SW (2021b) Anthelmintic efficacy evaluation against different developmental stages of *Ascaridia galli* following individual or group administration in artificially trickle-infected chickens. *Veterinary Parasitology* **301**, 1–11.

Feyera T, Shifaw A, Sharpe B, Elliott T, Ruhnke I and Walkden-Brown SW (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.

Foreyt WJ (2013) *Veterinary parasitology reference manual*. 5th ed. Ames, Iowa State University Press. p. 235.

Gauly M, Bauer C, Mertens C and Erhardt G (2001) Effect and repeatability of *Ascaridia galli* egg output in cockerels following a single low dose infection. *Veterinary Parasitology* **96(4)**, 301–307.

Gibbons LM, Dennis EJ, Mark TF and Hansen J (2014). The RVC/FAO Guide to Veterinary Diagnostic Parasitology. Available at https://www.rvc.ac.uk/review/Parasitology/EggCount/Principle.htm (accessed 22.09.2021).

Han Q, Eriksen L, Boes J and Nansen P (2000) Effects of bile on the *in vitro* hatching, exsheathment, and migration of *Ascaris suum* larvae. *Parasitology Research* **86(6)**, 630–633.

Hansen M, Terhaar C and Turner D (1956) Importance of the egg shell of *Ascaridia galli* to the infectivity of its larva. *The Journal of Parasitology* **42(2)**, 122–125.

Horton-Smith C and Long P (1956) The anthelmintic effect of three piperazine derivatives on *Ascaridia galli* (Schrank 1788). *Poultry Science* **35(3)**, 606–614.

Hu Y, Ellis BL, Yiu YY, Miller MM, Urban JF, Shi LZ and Aroian RV (2013) An extensive comparison of the effect of anthelmintic classes on diverse nematodes. *PLoS ONE* **8**, e70702.

Knapp-Lawitzke F, Krücken J, Ramünke S, von Samson-Himmelstjerna G and Demeler J (2015) Rapid selection for β-tubulin alleles in codon 200 conferring benzimidazole resistance in an *Ostertagia ostertagi* isolate on pasture. *Veterinary Parasitology* **209(1–2)**, 84–92.

Lacey E (1988) The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. *International Journal for Parasitology* **18(7)**, 885–936.

Leiper J (1954) The piperazine compound V. I9 for the removal of *Ascaris* and *Oesophagostomum* from the pig. *Veterinary Record* **66(40)**, 596–599.

McDougald LR (2020) Internal parasites. pp. 1157–1191. In Swayne DE, Boulianne M, Logue CM, *et al.* (Eds) *Diseases of poultry*. Hoboken, New Jersey, John Wiley & Sons, Inc.

Permin A and Hansen J (1998) Epidemiology, diagnosis and control of poultry parasites. *FAO Animal Health Manual* **4**, 24–29.

Permin A, Bojesen M, Nansen P, Bisgaard M, Frandsen F and Pearman M (1997) *Ascaridia galli* populations in chickens following single infections with different dose levels. *Parasitology Research* **83(6)**, 614–617.

Rahimian S, Gauly M and Daş G (2016) Embryonation ability of *Ascaridia galli* eggs isolated from worm uteri or host faeces. *Veterinary Parasitology* **215(1)**, 29–34.

Rogers WP (1961) The physiology of hatching of eggs of *Ascaridia galli*. *Journal of Helminthology* **35**(S1), 151–156.

Ruff MD (1999) Important parasites in poultry production systems. *Veterinary Parasitology* **84(3–4)**, 337–347.

Salih NE and Saleem KM (1987) *In vitro* hatching of the infective eggs of *Ascaridia galli* in tissue extracts. *Veterinary Parasitology* **24(3–4)**, 263–268.

Scare JA, Dini P, Norris JK, Steuer AE, Scoggin K, Gravatte HS, Howe DK, Slusarewicz P and Nielsen MK (2020) Ascarids exposed: A method for in vitro drug exposure and gene expression analysis of anthelmintic naïve Parascaris spp. *Parasitology* **147(6)**, 659–666.

Sharma N, Hunt P, Hine B, Sharma NK, Swick R and Ruhnke I (2017) *Ascaridia galli* challenge model in laying hens. *The Journal of Advances in Parasitology* **4**, 41–46.

Sharma N, Hunt PW, Hine BC, Sharma NK, Swick RA and Ruhnke I (2018) Detection of *Ascaridia galli* infection in free-range laying hens. *Veterinary Parasitology* **256**, 9–15.

Soudkolaei AS, Kalidari GA and Borji H (2021) Anthelmintic efficacy of fenbendazole and levamisole in native fowl in northern Iran. *Parasites & Vectors* **14**, 1–8.

Tarbiat B, Jansson D, Tydén E and Höglund J (2016) Comparison between anthelmintic treatment strategies against *Ascaridia galli* in commercial laying hens. *Veterinary Parasitology* **226**, 109–115.

Tarbiat B, Jansson D, Tydén E and Höglund J (2017) Evaluation of benzimidazole resistance status in *Ascaridia galli*. *Parasitology* **144**(10), 1338–1345.

Tarbiat B, Rahimian S, Jansson D, Halvarsson P and Höglund J (2018) Developmental capacity of *Ascaridia galli* eggs is preserved after anaerobic storage in faeces. *Veterinary Parasitology* **255**, 38–42.

Taylor M, Hunt K and Goodyear K (2002) Anthelmintic resistance detection methods. *Veterinary Parasitology* **103(3)**, 183–194.

Vadlejch J, Petrtýl M, Zaichenko I, Čadková Z, Jankovská I, Langrová I and Moravec M (2011) Which McMaster egg counting technique is the most reliable? *Parasitology Research* **109(5)**, 1387–1394.

Várady M and Čorba J (1999) Comparison of six *in vitro* tests in determining benzimidazole and levamisole resistance in *Haemonchus contortus* and *Ostertagia circumcincta* of sheep. *Veterinary Parasitology* **80(3)**, 239–249.

Várady M, Čudeková P and Čorba J (2007) *In vitro* detection of benzimidazole resistance in *Haemonchus contortus*: Egg hatch test versus larval development test. *Veterinary Parasitology* **149(1–2)**, 104–110.

Vejzagić N, Thamsborg SM, Kringel H, Roepstorff A, Bruun JM and Kapel CM (2015) *In vitro* hatching of *Trichuris suis* eggs. *Parasitology Research* **114(7)**, 2705–2714.

Wagland B, Jones W, Hribar L, Bendixsen T and Emery D (1992) A new simplified assay for larval migration inhibition. *International Journal for Parasitology* **22(8)**, 1183–1185.

Whitlock H (1948) Some modifications of the McMaster helminth egg-counting technique and apparatus. *Journal of the Council for Scientific and Industrial Research* **21**, 177–180.

Williams AR, Fryganas C, Ramsay A, Mueller-Harvey I and Thamsborg SM (2014) Direct anthelmintic effects of condensed tannins from diverse plant sources against *Ascaris suum*. *PLoS One* **9**, e97053.

Williams AR, Soelberg J and Jäger AK (2016) Anthelmintic properties of traditional African and Caribbean medicinal plants: Identification of extracts with potent activity against *Ascaris suum in vitro*. *Parasite* **23**, 24.

Yazwinski T, Chapman H, Davis R, Letonja T, Pote L, Maes L, Vercruysse J and Jacobs D (2003) World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for evaluating the effectiveness of anthelmintics in chickens and turkeys. *Veterinary Parasitology* **116**, 159–173.

Zajac AM and Conboy GA (2012) *Veterinary clinical parasitology*. 8th ed. Ames, Wiley-Blackwell. p. 354.

Zhao J, Williams AR, Hansen TVA, *et al.* (2017) An *in vitro* larval migration assay for assessing anthelmintic activity of different drug classes against *Ascaris suum*. *Veterinary Parasitology* **238**, 43–48.

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