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Veterinary Medicine and Animal Science

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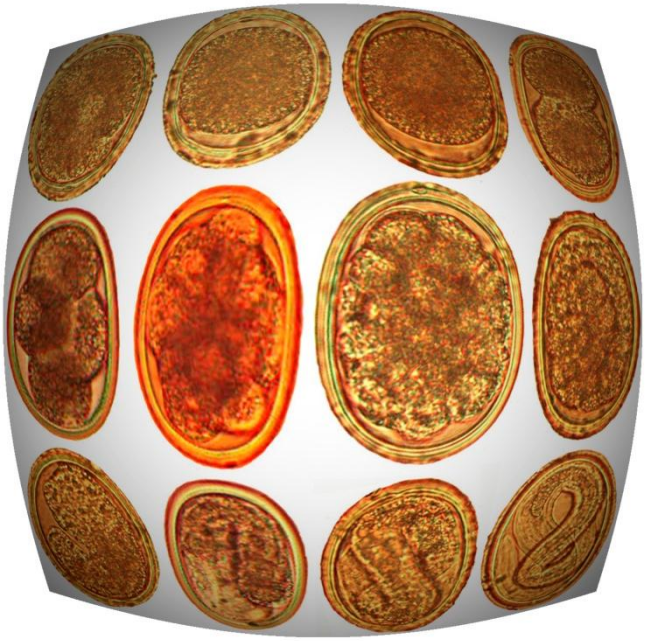

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1 Abstract

As the EU ban on conventional cages (1999 EU-directive) drew closer, most European countries moved towards alternative systems. Consequently, barn and free-range systems has become increasingly popular. Regardless of how welfare has improved, this major change in husbandry likely affects the parasite transportation. Accordingly, recent reports have unanimously shown that prevalence of round worm infection, in particular *Ascaridia galli* has increased dramatically. Although management practices have shown to be somehow efficient with regard to parasite control in caged system, at this time there is no effective way to prevent and eliminate the existing parasite in non-caged system. For better understanding the infection dynamics of *A. galli* a series of experiments was carried out to study the environmental factors (temperature, relative humidity, pH and oxygen) influencing the fate of *A. galli* eggs within the environment. In addition, two more experiments were run to evaluate the efficacy of thiabendazole and commercial broad spectrum disinfectant Chlorocresol (Interkokask). Survivability of the eggs with regard to the age of the parasite eggs was investigated as well.

The results presented herein suggest that there is a direct relationship between temperature and the percentage of viability which was increased proportionally with the temperature up to 30°C. A narrow range of temperature between 30-35°C considered as threshold temperature range. Moreover, high percentage of survival after exposure to -5°C indicates that this species is likely cold adapted. Our result demonstrated that oxygen is an essential requirement for *A. galli* egg development. No optimal pH at which maximum development could occur was determined. In fact it was found that intact eggs are highly adaptable to both very acidic and baric environments. Our results indicated that relative humidity has a significant effect on viability of *A. galli* eggs and that these eggs need highly saturated atmosphere to complete the development. Chlorocresol was found to be capable of killing *A. galli* eggs when presented at level of 1%. The LDA showed the inhibitory activity of thiabendazole on larval development with LD_{50} value of 0.052µg/ml. The results indicate that eggs of *A. galli* are capable of remaining viable and surviving throughout the year. Moreover, upon introduction of infective eggs to aviaries, development likely happens during all seasons. Therefore, it is essential to adopt alternative control strategies in order to lower infection risk in organic production systems which are gaining popularity.

2 List of figures

3 List of tables

4 Abbreviations used in the text

A. galli	**Ascaridia galli**
A. suum	Ascaris suum
BZs	Benzimidazoles
DMSO	Dimethyl sulfoxide
EU	The European Union
E. coli	Escherichia coli
h	Hour
H. contortus	Haemonchus contortus
H. gallinarum	Heterakis gallinarum
Inc.	Incorporation
KH_2po_4	Potassium dihydrogen phosphate
LC50	Lethal concentration
LD50	Lethal dose
Ltd.	Limited company
mg	Milligram
min	minute
ml	Milliliter
mm	Millimeter
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH_4OH	Ammonium hydroxide
OH^-	Hydroxide
RH	Relative humidity
rpm	Revolutions per minute
RTU	Ready to use
TBZ	Thiabendazole
T. colubriformis	Trichostrongylus colubriformis
v	Volume
WHO	World Health Organization
°C	Degree centigrade
µg	Microgram
µl	Microliter
µm	Micrometer

5 Introduction

5.1 General overview

Both domestic and wild animals are subject to a wide variety of parasites. In domesticated animals, parasite burden has become overwhelming because parasite dispersal and infective stages accumulate in the soil as a result of confinement of animals in pastures and/or pens. Nematodes are an important group of invertebrates including free-living, animal-parasitic and plant-parasitic species. They not only live in a wide variety of habitats, from moderate and cold environments to habitats in plants and animals, but many species also show an incredible ability to sustain severe adverse environmental conditions.

Among phylum Nematoda, the suborder Ascaridina comprises a number of important parasites of domestic animals and humans, namely in the superfamily Ascaridoidea and Heterakoidea such as *Ascaris suum* in pigs, *Ascaris lumbricoides* which affects human and *Ascaridia galli* and *Heterakis gallinarum* of gallinaceous domesticated birds. Most nematodes are more or less host-specific meaning they will have their life cycle by means of using only a single type of host animal.

Although the host animal is a primary environment for parasites, transmission stages such as eggs and larvae must also survive periods of time outside their hosts. Eggs of soil-transmitted intestinal nematodes require a latency period of development in the environment before they become infective and during such off-host stages they have to survive without energy and nutrient sources in a situation where locating a host may be problematic. Thus, as free-living nematodes they have to survive environmental fluctuations and need to withstand adverse conditions during their dispersal phase. Therefore, different species of nematodes have evolved different methods to ensure their survival. The epidemiology of ascaroid infections depend largely upon their eggs that are exposed to these abiotic and biotic variables, which may affect resistant stages of parasites. The prevalence of parasite infections varies with climatic conditions and husbandry conditions. Still, ascaroids are spread and present in diverse habitats all over the world. Studies at both macroepidemiology and microepidemiology levels are required for complete understanding of both parasite transmission and where they might be effectively controlled.

5.2 Statement of problem

Keeping poultry for commercial use has increased in recent decades. The most commonly kept poultry are chickens, ducks, geese and turkeys. Among these, domestic chickens (*Gallus domesticus*) are the most important. According to WATTAgNet.com (2009), worldwide poultry meat production has increased from 59.7 million tons in 1997 to 86.8 million tons in 2007. Of these, chicken meat productions impart 50.8 and 74.3 million tons in 1997 and 2007 respectively. Worldwide layer numbers have gone up slightly from 4,826 million in 1999 to approximately 6,200 million in 2010. World egg output expanded by 34% between 1997 and 2007. Production in Asia and Africa has increased by 47% and 37% within this period. In contrast, over the same period, world market share for Europe showed a noticeable reduction from 20% to less than 16%. The switch from conventional cages to less intensive production system is believed to be the reason for this drop in EU's contribution.

The modern commercialized intensive system (conventional battery cages and more recently alternative systems) and traditional extensive or rural scavenging system are the two main poultry production systems. In traditional system, the output in terms of both egg and meat production is low. Intensive commercial system is by contrast concentrated on few big farms with significantly higher flock size and sophisticated in terms of management, which consequently lead to higher output (both eggs per hen and weigh gain for broilers) (Permin *et al.* 1998).

Globally most laying hens are kept in conventional system. Back in 2008, 74.4% (69% conventional and 5.4% enriched) of all laying hens in commercial production were kept in conventional and enriched cages in the European Union (EUWEB 2011). However, increasing awareness of consumers, producers and animal experts regarding animal welfare and demand for naturally produced food products, has led to the EU Directive 99/74/EC to ban battery cages by January 2012. In Sweden, a ban on conventional battery cages has been in place since 1998; however, replacement did not happen until the end of a phase-out period in 2004. Nowadays in Sweden, furnished cages, litter-based housing systems indoors and free-range production shelter all Swedish laying hens (Tauson 2005; Jansson *et al.* 2010). Conventional battery cage usage declined to 29.3% in Europe by 2012 and free-range/organic system with 14.1% and barn with 19.8% showed an increase in their popularity respectively (EUWEB 2011).

This major change in husbandry likely influences parasite transmission. With the ban on battery cages, new non-cage systems have developed in which prevention of parasite infection has proved to be difficult. In free range and litter-based housing systems indoors where the bedding material and available pastures gives an increase in faecal contact, spread of parasitic disease seems inevitable.

None of the earlier studies mentioned high mortality rates in chickens due to parasite infection however, researches have proven that parasite infection may have a negative effect on both welfare of the animal and productivity as well as on mortality in some cases (Hemsley 1971; Kijlstra and Eijck 2006; Gauly *et al.* 2007; Phiri *et al.* 2007).

Detailed analysis of the cross-sectional prevalence study of gastrointestinal helminthes conducted on 268 adult chickens randomly selected from 16 farms in Denmark revealed that *Heterakis gallinarum* (72.5%), *Ascaridia galli* (63.8%) and *Capillaria obsignata* (53.6%) were the most commonly detected helminths in organic layer production, while prevalence figures in hens in conventional cages were lower (Permin *et al.* 1999). Moreover, Pennycott and Steel (2001) stated that a high proportion of free-range flocks in England carry parasitic worms, especially *A. galli* and *H. gallinarum.* They also mentioned that flocks were already infected with parasitic worms upon introduction to the laying sites. Investigation on prevalence of ascarid infections in Swedish commercial laying hens in 2004 and 2008 showed that the overall prevalence was significantly higher in 2008 compared with 2004 and that parasite infection in non-cage systems in both years was considerably higher than in caged flocks (Jansson *et al.* 2010). A later study in Germany on 144 laying hens from 11 free range farms (13 hens per farm, randomly selected) revealed that prevalence of infection with *A. galli*, *H. gallinarum* and *Capillaria* sp. was 66.6%, 84% and 75.1%, respectively (Kaufmann and Gauly 2009). Likewise, data from a study in Germany between 2007 and 2010 on 740 laying hens from 18 organic free range farms showed that almost all hens harbored at least one helminth species and the most prevalent species were *H. gallinarum* (98.0%) followed by *A. galli* (88.0%) and *Capillaria spp*. (75.3%) (Kaufmann *et al.* 2011). A study of infection dynamics of *A. galli* in laying hens in both organic and conventional farms in Sweden discovered that all flocks became infected following the arrival of the birds with infective eggs presented in the environment (Höglund and Jansson 2011).

Data from Africa obtained from four rural localities showed that *A. galli* with a prevalence range of 22.2-43.8% was one of the most common parasites after *Tetrameres americanus* and *Gongylonema ingluvicola* (Mukaratirwa and Khumalo 2010).

In commercial egg production systems, management practices essentially determine the extent of parasite infection. Stocking rate, removal of manure, biosecurity measures, disinfection procedures and production according to the "all in-all out" principle have apparently decreased the significance of parasitic infection (Permin *et al.* 1998; Jansson *et al.* 2010). Although management may control transmission of parasites with intermediate hosts in commercial free-range system (Permin *et al.* 1999), at this time there is no effective way to prevent and eliminate the existing parasitic infections in non-cage production systems.

5.3 Objectives of study

The aim of this project was to investigate how environmental factors, such as temperature, pH, humidity, oxygen, as well as a disinfectant and the anthelmintic thiabendazole affect the development and survival of *A. galli* eggs. A series of laboratory experiments was conducted to study the intra capsular development of *A. galli* under laboratory conditions. It was hypothesized that data from this study may assist in developing more efficient control measures for *A. galli*, considering both cleaning procedures and properly timed anthelmintic treatments.

6 Literature review

6.1 Morphology

Ascaridia galli is a parasitic roundworm that belongs to the superfamily Ascaroidea within the phylum Nematoda and it occurs worldwide in galliform birds of all ages. The adult worms live in the lumen of the intestine, but are occasionally also found in the crop, gizzard and rarely in the oviduct or body cavity. The body is semitransparent, cylindrical and has a creamy-white color. Like all other nematodes, *A. galli* is diecious with distinct sexual dimorphism. Detailed aspects of *A. galli* morphology have been described by Ramadan and Znada (1992).

Females are longer than males with a length of 72-116 mm and a straight posterior terminal, whereas males are around 51-76 mm and possess a curved posterior terminal (Ashour 1994). In the anterior end, both sexes have a prominent mouth with three distinct lips, bearing teeth-like denticles on their edges (Hassanain *et al.* 2009). The entire body is covered with a thick cuticle, which is striated transversely throughout the length of the body.

6.2 Life cycle

The life cycle of *A. galli* is direct and involves a single host. Sexually mature worms live in the lumen of the small intestine, whereas eggs containing infective stage larvae (L_3) develop in the environment (PERMIN 1997). The eggs are oval and surrounded by three layers: the inner permeable layer called the vitelline membrane, a thick resistant shell and a thin albuminous layer (Ackert 1931; Hansen *et al.* 1956). These layers are a key factor for its resistance against desiccation and its long-term persistence in the environment. Larvae do not hatch in the environment; instead, they moult inside the eggs until they become infective (L_3).

At optimal conditions (temperature and humidity) most of the fertile eggs within 24 hours, start dividing into the two-cell stage (Ramadan and Znada 1992). In the next 24 hours, the second division takes place and gives rise to the three-cell stage. The four-cell stage is normally seen within three days in most of the eggs. After 3 days, a morula with blastomeres is formed, which is completed by the end of the fifth day. After 8 days, the so called “tad pole” stage develops and after two additional days a vermiform embryo is developed. Within the next three to four days, this transforms into the coiled and fully mature infective L_3 larva (Ramadan and Znada 1992). The whole process may take between 7 to 20 days or longer depending on the temperature and relative humidity (Reid 1960; Permin *et al.* 1998).

The life cycle is completed when new hosts ingest the infective eggs. After ingestion, the infective eggs are mechanically transported to the proventricalus and gizzard and further down to the duodenum where they hatch within the first 24 hours. Triggering factors that signal the larvae to hatch are believed to be temperature, carbon dioxide level and pH levels (Dick *et al.* 1973; Salih and Saleem 1987). Following hatching, the larvae burrow into the mucosal layer of the small intestine to enter the histotrophic phase (Ackert 1931). The duration of the histotrophic phase is 3 to 54 days before the larvae return to the intestinal lumen where they reach final maturity (Permin *et al.* 1998). However, this period is dose-dependent and probably very much related to the phenomenon of arrested development (Ikeme 1971; Herd and McNaught 1975). After the histotrophic phase, the mature worms settle down in the lumen of duodenum where they live and feed on ingesta and produce huge number of eggs that are passed with the faeces into the external environment where the life cycle continues (Ramadan and Znada 1992). The prepatent period varies from 5-8 weeks (Pankavich *et al.* 1974; Permin *et al.* 1998).

6.3 Pathogenesis and clinical symptoms

Young birds seem to be more susceptible to *A. galli* infection than adults and manifest greater degree of damage. Penetration of the parasite into the duodenal or jejunal mucosa may cause haemorrhagic enteritis, anaemia often associated with severe diarrhea as well as loss of appetite, weakness, decrease activity, ruffled feathers and dirty cloacal region (Ikeme 1971; Adang *et al.* 2010). Established larvae in some cases cause destruction of the glandular epithelium (PERMIN 1997). Moreover, adhesion of the mucosal villi may occur due to proliferation of secretory cells. Not only the larvae can cause pathological lesions, also adult worm can cause damage to the epithelium in the form of pressure atrophy upon villi (Ikeme 1971).

In addition to reported pathological signs in chicken, Adang *et al.* (2010) reported that liver of the infected pigeons had fatty degeneration with coagulation necrosis of the hepatic cells. The authors also found necrotized tissues in the lungs, heart and kidneys of the infested birds.

What is more, a number of studies have been carried out to investigate the effect of combined infections caused by helminthes, bacteria and virus and their effect on production parameters. Earlier studies on the effect of *A. galli* on the immune system in chickens led to further investigations studying the influence of *A. galli* on subsequent *E. coli* infections. Accordingly it was suggested that combined infections has a significant impact on weight

gain and more severe pathological manifestation in group with combined infections (Permin *et al.* 2006). Following the same theme, the effect of *A .galli* on subsequent *pasteurella multocida* infections was shown to be predominantly on weight gain and egg production (Dahl *et al.* 2002). These studies indicate that interactions between parasitic, bacterial and viral diseases exist.

In addition, from welfare standpoint, it has been reported that infected birds manifested behavioral changes, for instance, infested chickens showed a higher food intake and lower activity as well as changes in ground pecking and nesting activity during the both prepatent and patent periods (Gauly *et al.* 2007). Let us add by also saying that *A. galli* can negatively affect the table egg quality. One such case is when the adult worms is occasionally seen in the chicken`s egg. Parasite can migrate up the oviduct through cloaca and participate in the egg-formation process (Akinyemi *et al.* 1980; Omran 1982; Höglund and Jansson 2011). Although presence of parasite worm in hen's egg is not considered as hazard for public health, it can cause potential consumer complaint. However contaminated eggs can be easily identified during candling process.

7 Materials and methods

7.1 Preparation of eggs

Egg suspensions were prepared from fresh fecal material as needed to prevent the negative effect of aging. Two different batches of faeces obtained from two different commercial farms in Sweden were used during this project. The first batch originated from a laying hen flock housed on litter indoors and the second batch came from an organic laying hen flock. Fresh faeces (<24 hours old) were collected from litter belts (2011/06/17). Faeces from the first batch mixed in a small plastic container with cold water to prevent egg development during preparation. The material was then washed through a series of standard mesh stainless steel sieves with the final sieve having a mesh size of 150 µm to remove larger particles. At the end of the process, eggs were collected by filtration using a cylindrical sieve with an opening size of 70 µm and washed again before being placed in 50 ml screw-cap (Falcon) centrifuge tubes. The tubes were then centrifuged on IEC CL30 centrifuge (TECHTUM lab®) at 3000 rpm for 3 min to settle the debris including eggs. The material at the bottom of the tubes was collected after removing the supernatant water with a water aspirator. After shaking the tube thoroughly, saturated NaCl was added to fill the tube and the tubes were then centrifuged at 1500 rpm for 5 min. The thin layer of supernatant water containing the eggs was collected by an electronic pipette and added to new tubes filled with distilled water, which then underwent through series of centrifugation steps to obtain clean eggs. The volume was adjusted to provide a concentration of approximately 10,000 eggs per ml distilled water.

The second egg suspension was prepared from the second batch of faeces, which was received (2011/07/20). Egg suspensions were prepared as described above except that combination of both 70 µm and 110 µm filters were used to ensure complete egg recovery. Materials from both sieves were collected in 50 ml screw-cap centrifuge tubes.

7.2 Temperature assay

7.2.1 Preparation of the media

Eight rectangular plastic flasks (50 ml) were prepared. Each flask was labeled with date, temperature and approximate age of the eggs. The eggs were supplied for the assay in distilled water without any added antifungal or antibacterial agents. Each flask contained 10 ml distilled water and 400 µm of the previously prepared egg suspensions. The total number of eggs was calculated to be around 6,800 eggs in each flask.

7.2.2 Methods

During this experiment, the eggs were exposed to different temperatures ranging from 5°-35°C for 42 days. Temperatures and time of exposure were chosen because they were within the minimum and maximum range reported previously for the development of *A. galli* eggs (Reed O. Christenson 1942; Reid 1960; Cruthers *et al.* 1974; Gazal 2009; Onyirioha 2011). Each flask was dedicated to a specific temperature and was then exposed to a constant temperature and 100% relative humidity for 42 days. Loss of solution by evaporation was adjusted through the addition of distilled water as required. Samples were taken once a week and at least 200 eggs per bottle were examined and photo documented at each sampling point. To prevent temperature fluctuations, samples were taken directly from the flask within the incubator. A few drops of the egg suspension were obtained and studied under the microscope with 20 and 40X magnification. In order to study the effect of aging on development of the eggs, the same procedure was repeated with one-month latency following the first trial.

7.3 pH assay

7.3.1 Preparation of the media

For the sake of accuracy, all the solutions were sterilized (filtered 0.2 µm) and made based on their suitability for biological assays with a molarity of 0.1 as shown in table 1.

Table 1: *Combination of buffers in order to obtain desired pH levels.*

pH ranges	Buffer
2.5	Glycine/hydrochloric acid
3.5-5.5	citric acid/sodium citrate
6.5-7.5	KH2PO4/NaOH
8.5	Tris (hydroxylmethyl) aminomethane/hydrochloric acid
9.5-10.5	Glycine/Sodium hydroxide
11.5	Sodium hydrogen orthophosphate/ Sodium hydroxide
12.5	Potassium chloride/Sodium hydroxide

7.3.2 Methods

Two 24-well plates were prepared and labeled with date and pH. In each well 30 µl of egg suspension plus 1.970 µl of previously prepared buffer were added except for the control wells, which only contained the egg suspension and distilled water. Plates were sealed with Parafilm to prevent excessive evaporation. To provide the eggs with required oxygen during the assay, two small holes were made into the Parafilm. All the solutions and the controls had three replicates and were incubated at 25°C until the control group developed into the coiled embryo stage.

7.4 Humidity assay

7.4.1 Preparation of the media

In order to create media with different humidity levels, it was decided to use glycerol/water solutions (table 2) due to simplicity of use.

Table 2: *Relative humidity levels obtained by different glycerol/water solutions.*

Desired RH (%)	Mixture of glycerol/water	
	Glycerol (ml)	Water (ml)
50	79	21
70	64	36
90	33	67

7.4.2 Methods

This experiment was designed to study the effect of relative humidity on the development of *A. galli* eggs. To do so, twenty Petri dishes were divided into four groups and each group was dedicated to a specific RH level (0%, 50%, 70% and 90%). The glycerol-water mixture was prepared according to the above formulas and 30 ml of the mixture was added to each Petri dish. A few drops of the egg suspension containing approximately 200 eggs were placed on a microscope slide and the slide was placed on top of the mixture in each Petri dish. To avoid air exchange between the contents of the Petri dishes and the outside environment, all the Petri dishes were tightly sealed using Parafilm and incubated at 25°C for a period of two weeks. Every 4 days, one dish from each group was removed from the incubator, and the slide was studied under the microscope after adding a few drops of water.

7.5 Anthelmintic assay

7.5.1 Preparation of different thiabendazole (TBZ) dilutions

To create dilutions in a range of 0.01 to 0.1µg/ml, 50 mg of thiabendazole (SIGMA-ALDRICH, Inc.) was weighed into a weighing boat and carefully transferred into a 100 ml volumetric flask. Powder remnants were then washed into the flask using 25 ml DMSO, The solution was then mixed thoroughly and the total volume was made up to 50 ml with distilled water. This gave us a stock solution of 1mg/ml, which was used to prepare a suitable range of working TBZ solution from 0.01 to 0.1µg/ml as shown in the table 3.

Table 3: *Preparation of working solutions by diluting stock solutions in distilled water (Total volume was adjusted to be 10ml).*

Stock solution µl	Distilled water µl	Working dilutions µg/ml
25	9975	2.5
50	9950	5
75	925	7.5
100	9900	10
125	9875	12.5
150	9850	15
175	9825	17.5
200	9800	20

7.5.2 Methods

Two 24-well plates were prepared. In each well 10 µl of egg suspension (containing 150-200 eggs) and 10 µl of a working solution was added (except the control wells which only contained the egg suspension and distilled water). Finally, distilled water was added in two steps to make a final volume of 2 ml in each well. The plates were sealed with Parafilm to prevent excessive evaporation. To provide the eggs with required oxygen during the assay, two small holes were into the Parafilm.

All concentrations and controls had three replicates and were incubated for 14 days at 25°C until the control group fully developed to mature L_3 larvae. At the end of the incubation period plates were studied under the microscope to calculate the number of developed and undeveloped eggs. Collected data were analyzed and a drug response curve was drawn by GraphPad Prism software.

7.6 Disinfectant

7.6.1 Preparation of the media

The aim of this assay was to evaluate the efficacy of the industrial livestock housing broad-spectrum disinfectant chlorocresol (Interkokask®RTU) against *A. galli* eggs. Two concentrations were tested, i.e. 1% and 2% solution. A 2% solution is recommended for use in barns. To do so, the solutions were diluted with water in the proportions of 1:99 and 2:98 ratio (v/v).

7.6.2 Methods

Due to several cleaning steps in this experiment, large numbers of eggs were used to ensure that at the end of the cleaning processes enough eggs would remain for microscopy. Accordingly, 100 µl of the egg suspension in 50 ml flacon tubes in three replicates were incubated with 1% and 2% solution of disinfectant along with the control group containing no disinfectant for 4 h, 24 h, 4 days and 7 days. At the end of the incubation period, eggs were washed free of the disinfectant by repeated centrifugation in distilled water at 1000 rpm for 3 min. The cleaned eggs were then incubated for two more weeks to study the development of the eggs post exposure. At the end of the incubation period, at least 150 eggs were obtained for microscopic observation and the percentage of embryonated/ non-embryonated eggs was calculated.

7.7 Oxygen

7.7.1 Preparation of the media

In this experiment the role of anaerobic conditions on the development of *A. galli* eggs as well as survival of fully developed larvae within the eggs were studied. Distilled water was used as medium and to reduce the dissolved oxygen level, molecular activity of the water was increased by boiling the water for at least one hour.

7.7.2 Method

To generate an anaerobic atmosphere (<1% oxygen), AnaeroGen™ sachets were used in order to avoid the need for evacuation/ replacement or the production of hydrogen. Two groups, one with preincubated eggs containing larvae and one with undeveloped eggs were set up. Each group included four 24-well plates and in each plate, three replicates. Plates were

filled with cooled boiled water and approximately 200 developed eggs or undeveloped eggs were added to the wells. The plates were then placed inside the plastic bags provided by the AnaeroGen kit. Prior to sealing, an AnaeroGen sachet was removed from its protective foil packet and placed into anaerobic plastic bags. Control plates with access to oxygen along with plates deprived of oxygen were incubated at 25°C for 16 days. One bag from each group was taken out every four days and after breaking the seal, plates were studied under the microscope to observe developmental changes. Plates were then incubated for another two weeks and the percentage of developed eggs was calculated at the end of the incubation period. The status of the larvae was assessed based on their motility.

7.8 Freezing and thawing

7.8.1 Methods

The aim of the experiment was to study the influence of temperature fluctuations between 5°C and -5°C on the development of the *A. galli* eggs. A climatic cabinet (LabRum Klimat Ltd.) was programmed to imitate the rise and fall of the temperature that may occur during night and daytime. The cabinet was adjusted to perform the following program;

Decrease of 3°C per hour until the temperature reached 5°C and this temperature was maintained for 5 hours and then further decrease of 3°C per hour till it reached -5°C and stayed at this temperature for 24 hours. Twelve Eppendorf tubes were set up and labeled with exposure time and then 600 µl of the egg suspension was pipette into the tubes and then placed in the cabinet. Samples were taken every 30´, 60´, 3 h, 6 h, 12 h and 24 h upon the initiation of the program. For each sampling point were two replicates. Samples were thawed at room temperature and incubated at 25°C for two weeks afterward. At the end of the incubation period, the percentages of developed and undeveloped eggs were calculated.

8 Morphological classification of the embryonic stages of *A. galli*

In order to simplify the identification and classification of the different stages of the *A. galli* eggs during the development course, the whole process was divided into five groups. 1) Both mature infertile eggs and fertile eggs that had not gone through division process were counted as undeveloped. 2) Eggs in cleavage stage were divided to early morula stage if eggs contained 2-16 cells and late morula stage if eggs were between early morula and initiation of differentiation. 3) Vermiform stage was referred to those eggs in which internal mass started differentiating and those contained early vermiform embryos. 4) Those eggs that completed the developing process and contained apparent coiled embryos counted as L_3 stage. 5) Any other egg with unusual intra capsular mass or destructed eggshell reckoned as dead.

9 Results

9.1 Temperature

In this experiment, effects of desiccation and direct sunlight were eliminated as contributing factors and consequently, temperature was the principal element in the causation of the obtained results. No development was observed by inverted microscopy in eggs incubated at 5°C and 10°C during the 42 days of incubation. Normal development did not occur at 15°C until after 42 days when they gradually started to develop into vermiform stage larvae and they completed the development in approximately three months. Development occurred rapidly at 30°C and was completed within 7 days however, 83% of the eggs reached the L_3 stage at this point. As the temperature was lowered, development time increased as follows: 25°C, 14-days; 20°C, 21-days and 15°C, 90-days. The lowest temperature at which developmental stages could be observed was 15°C. At 25°C, more than 95% of the eggs developed into L_3 larvae stage in 14 days.

At 33°C, development of the eggs was markedly decreased as few of the eggs reached the vermiform stage after nine days and 82.5% of the eggs were still in the early to late morula stage. Only 17.5% developed to L_3 stage larvae after 16 days and roughly, 25.5% of the eggs were counted as fully embryonated after 31 days at 33°C.

At 35°C, the inhibitory effect of high temperature was prominent. The development was limited to the 6 to 8-cell (early morula) stage after nine days. After 16 days, 97.6% of eggs were in developing stage, and 2.3% reached the L_3 stage and 3.5% were counted as fully developed eggs after 31 days of incubation.

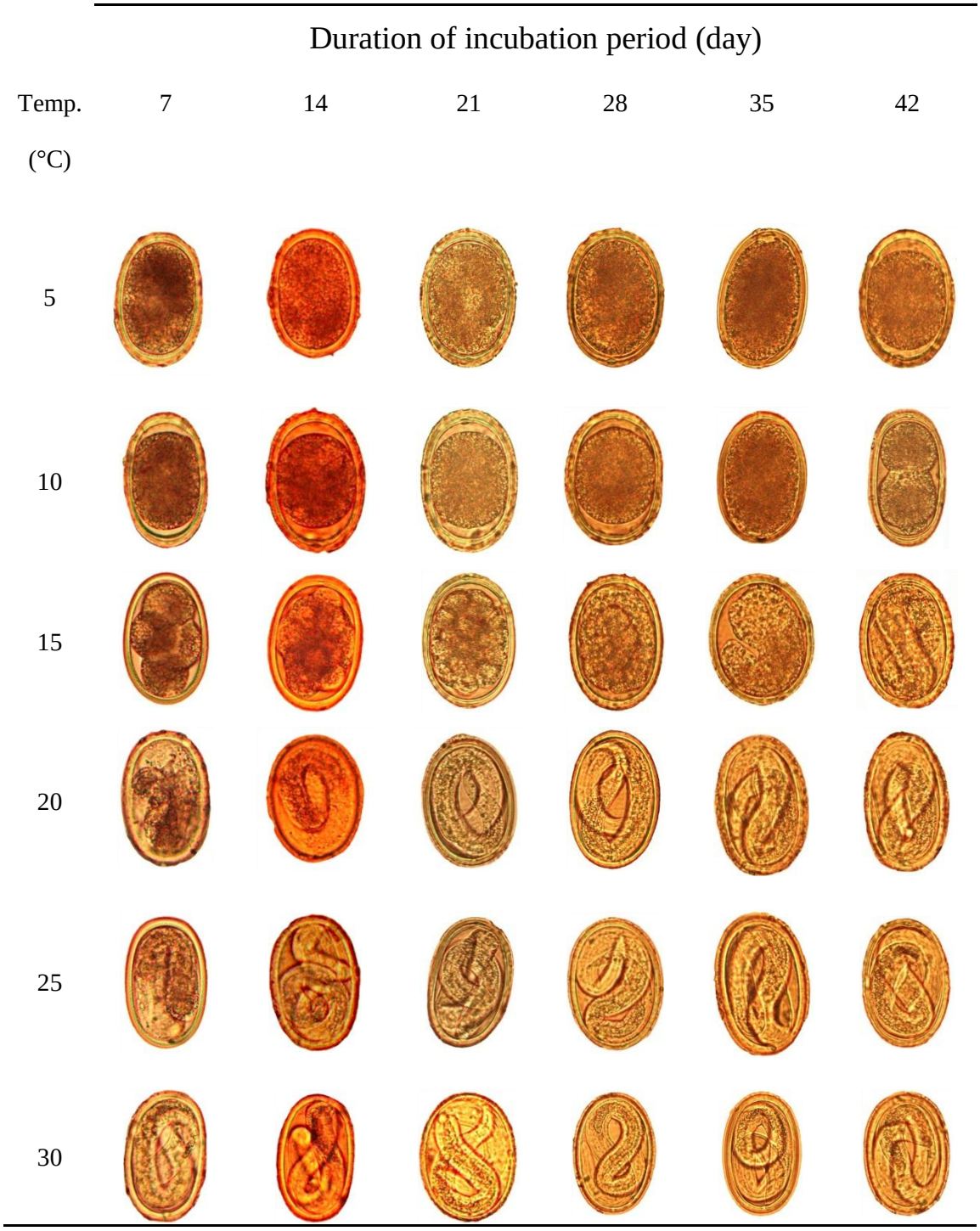

Figure 1: *An overview of A. galli eggs development in accordance to different temperature and exposure time.*

9.2 Aging

Results from the second trial were matched with our hypothesis regarding the negative effect of aging on development of *A. galli* eggs. As with the trial with fresh eggs, normal development from the one-celled stage to fully infective larvae did not occur at 5°C or 10°C in all sampling points in the month-old eggs. Similarly, none of the samples showed development to L_3 larvae before 7 days. Full development was delayed in aging eggs until 14 days at 30°C. Eggs incubated at 25°C were not able to reach the vermiform stage in 7 days in contrast to the first trial. The development of the eggs to L_3 stage was not observed at 20°C after 21 days. A very low percentage of the eggs developed to almost L_3 after 42 days in the second trial, in contrast to the fresh eggs of which almost 90% developed in the first trial. In the second trial, the eggs that were incubated at 15°C showed the following results: 34.4% developing, 39.4% vermiform stage and 26.1% fully developed larvae after 74 days.

9.3 pH

Rapid exposure of the parasite to the wide range of acidic and basic environmental conditions (from pH 2.5 to 12.5) showed that during the two weeks of incubation at 25°C a majority of the eggs in all three replicates, developed into fully mature L_3 larvae as shown in table 4.

Table 4: *Mean percentage of development of all three replicates in each sample containing different PH Value.*

pH	Con	2.5	3.5	4.5	5.5	6.5	7.5	8.5	9.5	10.5	11.5	12.5
Mean development of three replicates to L_3 stage larvae (%)	84.2	84	78.5	78.5	79.4	80.7	81.5	80.6	82.6	84.3	77.5	84

9.4 Humidity

Following are the results according to each sampling point.

First sampling point (after 4 days):

Almost 95% of the eggs incubated in 90% RH, developed to the early vermiform stage within 4 days of incubation and during this period, three eggs were seen with motile larvae inside. Although development was found to be surprisingly rapid at 90% RH in comparison to temperature assay, it was not in fact faster than that of the eggs cultured in water with access to oxygen in control group. Exposure to 70% RH allowed the eggs to develop to late morula stage (8-16 cells). No egg with mature larvae was seen. In the Petri dish with 50% RH, less than 10% of the eggs were in early morula stage with 2-8 cells. Under complete drought condition, no eggs survived. The control eggs developed readily with more than 95% reaching the early vermiform stage.

Second sampling point (after 8 days):

The figures remained constant for the eggs incubated in 90% RH. Altogether, 95% developed to the late vermiform stage. Likewise, under 70% RH the eggs were still in the early morula stage. Only three eggs were seen with non-motile vermiform larva inside and only one egg with a motile larva was seen. In the case of 0 and 50% RH, all the eggs were dead after eight days.

Third sampling point (after 16 days):

Under 90% RH condition, 97% of the eggs had completed development (L_3 stage) and remained motile. Few eggs survived in 70% RH after 16 days and none of the eggs were able to develop further than the early morula stage. The remaining eggs were dead. Eggs incubated in both 0% and 50% RH were dead.

Fourth sampling point (after 20 days):

No differences were observed regarding further development under 90% RH. Eggs in all other groups were dead.

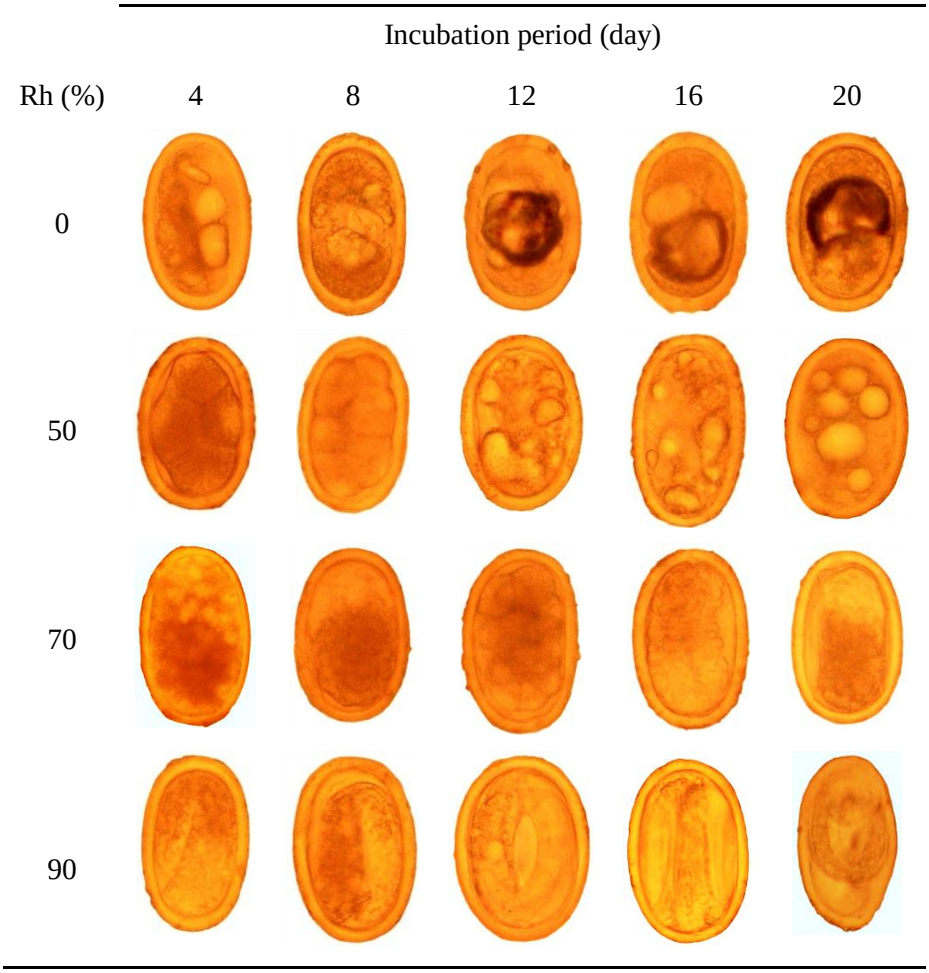

Figure 2: *An overview of A. galli eggs development with regard to different relative humidity and exposure time.*

9.5 Anthelmintic

Data from the larval developmental assay (LDA) were analyzed to determine the LD_{50}, concentration of thiabendazole required to inhibit 50% of the eggs from developing to the infective third larval stage (L_3). The percentage of the eggs failing to develop to L_3 at each drug concentration were transformed to GraphPad Prism® and plotted against the $\log_{10}$ of the concentration of anthelmintic and a dose response carve was then developed as illustrated in figure 3. The LD_{50} value for TBZ was recorded as 0.052 µg TBZ.

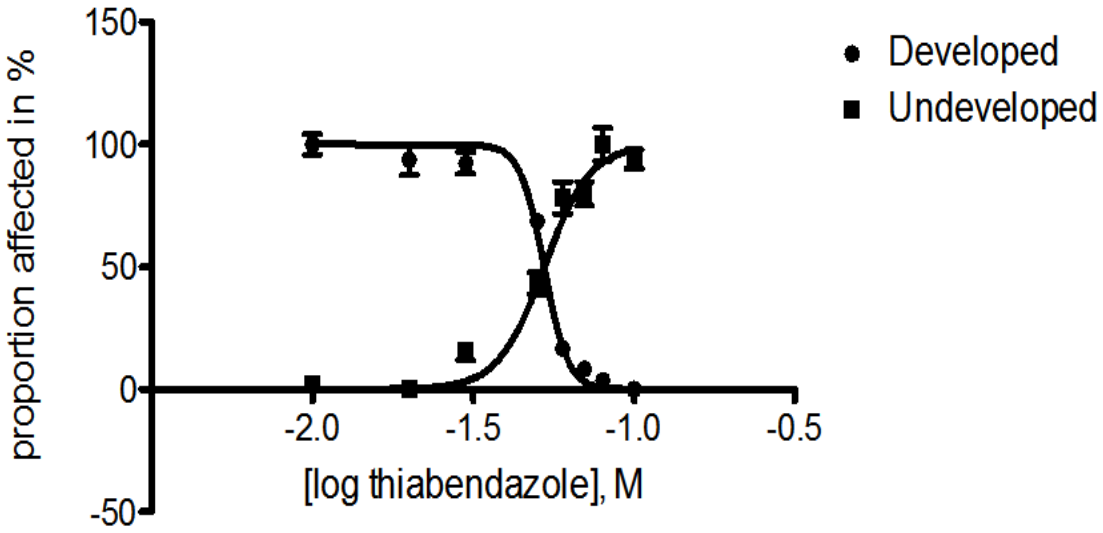

Figure 3: the effect of thiabendazole on larval development in Ascaridia galli.

9.6 Disinfectant

The control group showed an 83% embryonation rate. The lowest exposure time of 4 hours produced zero percentage development at both concentrations. In fact, the disinfectant had a destructive effect on the intra-capsular cell mass (figure 4). All longer exposure times stopped all development at both concentrations.

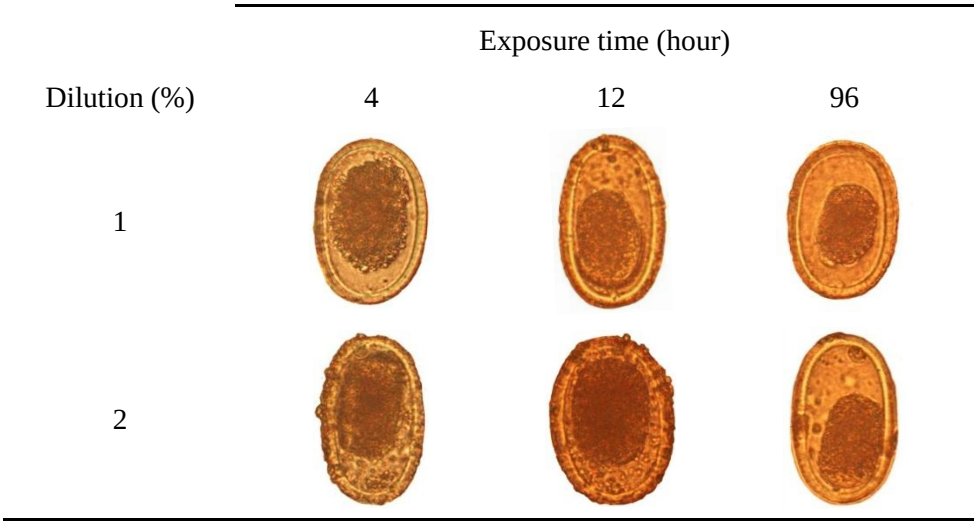

Figure 4: *Illustration of internal-cell mass changes caused by 1 and 2% dilution of Chlorocresol at different sampling points.*

9.7 Oxygen

Observation of undeveloped eggs and eggs containing mature larvae after each exposure time revealed the following results. Except the fact that less than 5% of the eggs were in the early morula stage when the experiment begun, the rest of the eggs in all groups did not go through the development process while they were exposed to anaerobic condition. Morphologically, all the eggs with L_3 larvae appeared normal but none of them were motile at the end of the experiment. In contrast to this, the control group contained undeveloped eggs cultured in saturated water at 25°C readily started the normal development process indicating no lack of vitality. Moreover, the control group containing eggs with mature larvae remained motile throughout the experiment.

After removing eggs from anaerobic conditions and incubating both groups of eggs for a period of an additional period of two weeks, the following results were obtained at the end of the incubation period. In the first group that had been exposed to anaerobic environment for four days, 88.8% of the eggs on average developed to fully mature larvae. In the second group after being exposed to anaerobic condition for 8 days, 89.8% of the eggs reached the L_3 stage. In the third group with the exposure time of 12 days, 86.6% of the eggs were counted as fully developed larvae. Likewise, 86.6% of the parasite eggs in the fourth group that had been exposed to anaerobic condition for 16 days reached the L_3 larvae stage at the end of the incubation period.

9.8 Freezing and thawing

Due to an unknown reason, the egg suspensions in the Eppendorf tubes did not freeze despite the fact that the cabinet showed a temperature of -5°C during the experiment. In total 78.7% of the parasite eggs that had been exposed to sub-zero temperature for 24 hours developed into active embryos upon removal from -5ºC and subsequent incubation at 25°C. As the exposure time reduced development rate increased slightly as follows, 80.9% after 12 hours, 79.3% at 6 hours exposure, 80.0% upon 3 hours, 83.2% after 60 min. the 83.5% Value obtained at 30 minute exposure time was still lower than control group. The control group cultivated at 25ºC showed 85.6% development to the L_3 stage.

10 Discussion

10.1 Temperature

Despite the fact that morphological classification of the embryonic stages of *A. galli* eggs is not absolutely defined and collecting eggs of same stage from fecal material was impossible, development was relatively uniform. The experiments uncovered the following results. Firstly, the infective larvae of *A. galli* develop in the egg between temperature 15°C and 30°C. Within this range, increasing the temperature hastened the development and reduced the time required for complete development. Moreover, at temperatures higher than 30°C, an inversed relationship was observed between temperature and viability of the eggs.

Secondly, these results revealed a narrow range of temperatures at which development was either slow or ceased completely. This range from 30-35°C could be considered a threshold temperature range. Finally, unlike the previously reported 16.7 to 19°C (Aaron 1950; Reid 1960; Gazal 2009; Onyirioha 2011) as the minimum temperature at which development occurs, our result suggests that *A. galli* eggs are able to develop to L_3 larval stage at temperatures as low as 15°C. This result is in agreement with recent data provided by Onyirioha (2011). It is noteworthy to mention that more than 90% of the eggs at this temperature developed to mature larvae. Therefore, it is likely that *A. galli* eggs may develop at temperatures even lower than 15°C, although longer time is required.

Different embryonation temperatures ranging between 17 and 32°C have been published by a number of researchers as the optimum temperature at which rapid development as well as the highest percentage of fully developed larvae is accomplished (Cram 1924 ; Aaron 1950; Hass and Todd 1962; Arene 1986; Barnard *et al.* 1987; Fleming 1987). Observation in the present work indicates the optimum temperature for embryonation of *A. galli* eggs to be 25°C which is more or less similar to those obtained by Gazal (2009) and Onyirioha (2011) who stated 25°C and 28°C, respectively for this species.

The shortest embryonation time was achieved upon incubation at 30°C. However, infectivity and longevity was not assessed in this study. As a matter of fact Arene (1986) found that maximum larval viability and ability to penetrate tissues *in vitro* happened at a temperature which was 10°C lower than optimum temperature achieved in the same study for *A. suum* eggs. Thus, he suggested that the optimal temperatures for development and larval viability and survivability differ. In a recently conducted field study in Sweden it was shown that the mean temperature in six different barns barely reached 22°C between May 2009 and august

2010 and the coldest temperature documented during this period was 4°C (unpublished data). It would be of a great value to see if the infectivity of *A. galli* eggs is temperature dependent. According to Arene (1986) and the fact that in Sweden within-barn temperatures often do not reach 25°C for long periods of the year, this might be an important factor to consider when studying the epidemiology of *A. galli*.

According to Geenen *et al.* (1999), *A. suum* eggs reached the motile larval stage after 17 days of cultivation, but apparently did not become infective for mice until after further 6 weeks of cultivation. This might indicate that formation of the coiled larvae inside the eggs does not necessarily reflect the infectivity of the larvae. By the same token, the production of microscopically mature larvae does not necessarily represent the percentage of the infective larvae and eggs may need an additional period of incubation following the appearance of the motile larvae to reach maximum infectivity.

Our experiments show that temperatures above 33°C were unfavorable to development of the eggs. Heat resistance of *A. galli* eggs was not studied in this paper, yet earlier studies reported that thermal death point of *A. galli* ranged between 48 to 58°C depending on exposure time (Ackert 1931; Reed O. Christenson 1942). However, investigations on Swedish farms clearly showed that despite using high-pressure (50-80°C) and/or watersteam cleaning, this did not effectively control the spread of the infection (Höglund and Jansson 2011).

It is a well-known fact that free-living stages of parasites are exposed to environments where climatic factors exhibit daily cycles. Therefore, data from studies under constant laboratory conditions cannot be applied directly to field conditions. In this respect, it was shown that daily fluctuations in temperature could accelerate parasite development rate (Saunders *et al.* 2000; Saunders *et al.* 2002).

10.2 Aging

The question of aging as a factor that may affect the virulence of parasite eggs has not been thoroughly discussed. It is a well-known fact that free-living stages of some parasites have to spend periods of time outside their host. Within these variable environments, except the influence of environmental factors (temperature, humidity and pH) on survival of the parasite eggs, the age of these eggs as another important factor worth consideration.

This study showed that egg mortality was an age-dependent phenomenon. However, it is worth mentioning that within the environment, eggs may exist in different developmental stages from two-cell stage to fully developed mature L_3 larvae. Our laboratory results indicate that undeveloped eggs lose their viability relatively fast. Large discrepancy between the percentages of the month-old eggs that reached the L_3 stage and fresh eggs in this study is however controversial. In this respect, field studies have shown that this particular parasite eggs can survive environmental conditions and remain viable for a long period of time. We also found that aging phenomenon decelerates the development process which means that these eggs require longer time to become mature and infective. Infectivity of the eggs was not covered in this study however, TODD (1950) stated that virulence is inversely proportional to aging of the infective *A. galli* eggs. Nonetheless, the difficulty with this argument is that production of thousands of eggs by adult worms provides enough infective eggs to infest the host with relativity high number.

10.3 pH

Based on the collected data in this study no optimal pH for egg development could be determined. *A. galli* is highly adaptable to the different environmental pH values. Our results support the data provided by Pecson *et al.* (2007) who stated that at 20°C no pH effect was seen over 80 days of incubation. Likewise, observation of Nelson and Darby (2001) pointed out that neither acid nor diethyl ether alone caused any decrease in *Ascaris* egg viability. In the study carried out by Nordin *et al.* (2009) to monitor inactivation of uterus-derived *A. suum* eggs at high pH level (>13) at 24°C no significant inactivation was detected. As suggested by the same authors this phenomenon can partly be explained by the fact that charged molecules, in this case OH^- require active trans-membrane transport, resulting in higher resistance to pH alone.

On the other hand, according to Maya *et al.* (2010), pH of 12.5 and 80% humidity for a duration of 8 months is needed to completely inactivate any genera of non-larval helminthes eggs. Despite the fact that development continued at all different pH levels, Lazdina and Grinberga (1978) noted that prolonged preservation of the *A. galli* eggs in acid media considerably inhibits the establishment of the *A. galli* in the chicken. Along the same theme, Owen (1984) reported that acid pH levels inhibited normal development of *A. suum* eggs at all temperatures. However the above statements are in contrast to our results.

Other researchers approached the issue from a sanitation and sewage treatment perspective. According to Ghiglietti *et al.* (1995), a combination of alkalization with NH_4OH and a temperature of 30°C inactivated ascaris eggs. Moreover, in a more detailed study Pecson and Nelson (2005) stated that the combination of ammonia , high temperature and pH needed to achieve 99% inactivation. Explanations could be found in the work of Barrett (1976) who believed that the resistant lipid membrane of the egg begins to lose its integrity upon long exposure to heat, leaving the egg vulnerable to osmotic effects of presented chemicals. For that reason, heat may increase the permeability of the membrane leading to more easily penetration of ammonia and consequently inactivation of the egg. According to our result, lime-wash as suggested by some researchers as part of a general control principle may not be an effective approach to control the *A. galli* infection.

10.4 Humidity

No work has been previously reported concerning the moisture requirements of developing *A. galli* eggs. However due to similarity in the egg's shell of the intestinal parasites, relatively similar results to those obtained from other nematodes were expected.

Glycerol has three hydroxyl groups that are responsible for its solubility in water and its hygroscopic nature. On one hand, the ability to attract moisture from the air and hold it is one of the most valuable properties of glycerol that makes it a good candidate for this experiment. On the other hand, contrary to utilizing saturated salt solutions, all the media in this way are made from the same substances. What is more, as long as relative humidity is constant, a temperature change within normal atmospheric limits has little effect on the equilibrium concentration.

Our results show that under laboratory conditions RH has a pronounced effect on viability of *A. galli* eggs and that *A. galli* eggs are highly susceptible to desiccation. Eggs of this parasite

failed to develop and died relatively fast (within 4 days) under conditions where the atmosphere was partially saturated (50%). In contrast to this, the eggs died more slowly in the more saturated environment (70%) indicating that the range in humidity is directly correlated with egg development. When compared to the control group with eggs cultivated at 90% RH, development time remained constant irrespective of humidity level. The exact minimum moisture requirement of this species is not covered in this paper. However, an approximate range was assessed to be 70% to 100% RH. This study shows that *A. galli* eggs require a highly saturated atmosphere before they could finish the development process at 25ºC. As it is mentioned in earlier section (P.22), *A. galli* eggs are able to develop at 30ºC in 7 days. Due to the fact that these eggs could survive for almost 8 to 9 days in 70% RH, it can be concluded that *A. galli* eggs might be able to complete the development process and survive dehydration provided that development can proceed to the L3 stage during drying, and before the embryo loses a critical amount of water at higher temperature.

Other researchers confirmed the minimum moisture requirements for Ascaris egg to be 80% at 22 ºC (Otto 1929; Wharton 1979). A study conducted by Aaron (1950) revealed that *A. suum* eggs spread on glass slides required 100% humidity to complete development. By contrast, Gaasenbeek and Borgsteede (1998) found the survival of *A. suum* eggs, which were kept at a relative humidity of 100% and 77.5% to be 96% and 62% respectively after 12 weeks. Waller and Donald (1970) demonstrated that eggs of *Haemonchus contortus* and *Trichostrongylus colubriformis* can survive dehydration and complete the development process. Hence, these results suggest significant inter-species differences. The differences between species may be explained by basic differences in the: 1) egg size and their surface-volume ratio; 2) different time requirement to complete the development; 3) the eggshell structure and component.

When exposed to desiccation, the lipid layer is the main permeability barrier of the eggshell to water loss (Wharton 1980). In an earlier study Wharton (1979) observed that Ascaris eggs do not survive long-term desiccation despite the fact that they lose water very slowly relative to their surface-volume ratio. This was because the rate of water loss of unhatched larvae increased as the temperature was raised. According to Waller (1971), the differences between the inner layer of the eggshell of *H. contortus* and *T. colubriformis* may be the key factor for explanation of the fact that the eggshell of *H. contortus* is more permeable to water loss than that of *T. colubriformis*.

Clarke and Perry (1980) explained the role of trehalose which is present in the perivitelline fluid surrounding the unhatched larvae in alteration of the eggshell permeability. Moreover, it is found that the saccharide trehalose is involved in desiccation protection of the unhatched larvae (Behm 1997). It is suggested that the osmotic stress induced by trehalose on the unhatched larvae causes the reduction in larval water content to levels where locomotion is inhibited and as a result utilization of energy reduces and thus a dormant phase is induced (Clarke and Perry 1980). Furthermore, it is suggested by Crowe *et al.* (1992) that during the dehydration process, trehalose possibly protects the cell membrane from damage by conserving the structure and functional integrity. From the above statements, it is reasonable to conclude that: 1) survival of *A. galli* eggs positively correlated with an increase in humidity. 2) The eggshell and perivitelline fluid components of the egg may play an important role in protecting the unhatched infective larvae against desiccation.

Because infection with *A. galli* persisted in the environment throughout an earlier study (Höglund and Jansson 2011) despite the fact that mean RH (68.7%) was below minimum moisture requirement of the eggs, it would be interesting to study whether *A. galli* eggs can recover from desiccation and if so, to what extend the damage can be repaired or the normal physiology can be restored. Speculatively, moisture levels may differ within barns and pastures, leading to the possibility of infective hot-spots.

10.5 Anthelmintic

The most widely used method to control parasite infection in many circumstances is treatment with anthelmintic. Although due to their cost, interfere with natural host immunity mechanisms and increase in anthelmintic resistance prevalence, preventive measures are considered more favorable by farmers. Benzimidazoles in this respect are one of the most commonly used drugs in animals with broad spectrum activity (Campbell 1990; Cook 1990). They are effective against nematodes, some trematodes and adult castrates. It is well known fact that tubulin is the essential protein of microtubules and the latter play crucial rule in many functions in eukaryotic cells (Dustin 1979; Prescott 1988). BZs exert their effects by binding tubulin (polymerization of the tubulin molecules to microtubules) (Lubega and Prichard 1991; Permin *et al.* 1998).

Ovicidal activity of anthelmintic drug has been shown against many different nematode eggs, yet reports regarding effectiveness of anthelmintic drugs against developing stages of *A. galli* eggs are scarce. Ovicidal activity of anthelmintic is usually measured as the proportion of

eggs that are either not capable of hatching in egg-hatch assay or not being able to develop in larval development assay in vitro.

In the present study egg development was largely arrested at the one-cell stage by TBZ treatment at the highest concentration (0.1µg/ml). However, with reduction in drug concentration, regular cell division was observed in the cultured eggs resulting in detection of different stages from two-cell to L_3 stage throughout the experiment. Lubega and Prichard (1991) found that egg supernatants bound more drug due to egg cells containing more tubulin compared with the cells of larvae and adult worms. The authors also stated that tubulin content (per mg protein) decreases from egg through larva to adult stage suggesting that developing stages (eggs or larvae) may be more susceptible to BZs than adult worms. However studies suggested that the effects of BZs in vitro may not necessarily represent their efficacy in vivo (Lacey *et al.* 1987).

Regarding the LD50 value obtained in this study, there is no reference for TBZ in this respect to compare our result with. However, the LD50 value of TBZ shows high-affinity binding since it is far below the drug concentration in the gastrointestinal tract. Infectivity of the eggs was not assessed in this experiment so there is no confirmation regarding the infectivity of those eggs in which the development appeared to be normal.

Laying hens in alternative systems are at a higher risk for infections with parasitic worms than those kept in a cage system. The fact remains that by adopting new housing system the poultry roundworm, specifically *A. galli* started reappearing in the egg production industry and become problematic in non-caged laying hens (Hoglund *et al.* 2012). With newly emerging parasitic disease and unsuccessful preventive measures under intensive management system it is likely that most worm control strategies will soon rely heavily on the use of anthelmintic. The issue of anthelmintic resistance is much discussed in the world today and developing resistant populations is therefore of great concern.

To the best of my knowledge, there is no report concerning anthelmintic resistance in poultry. Currently the only available and approved anthelmintic drug for poultry sector in Sweden is flubendazole (SID PO Verminator®) which has been shown to be effective. It is claimed, however that the risk of re-infection after deworming is high. With regard to latter issue, Höglund and Jansson (2011) confirmed that egg reduction was only temporary following anthelmintic treatment and that parasite eggs re-appeared by 2-4 weeks post deworming. The probable reason for such outcome was explained by the authors to be either suboptimal administration of the anthelmintic or resistance of parasites in their histotrophic phase (tissue

stage). According to data presented in this paper and recent report regarding effectiveness of anthelmintic on *A. galli*, adopting a different approach to the way anthelmintic are currently used deserves consideration.

10.6 Disinfectant

Ascaris eggs are resistant to wide range of relatively toxic solutions such as 14% hydrochloric acid, 0.4% nitric acid, 0.5% sodium hydroxide and 4% Formaldehyde. It is believed that this characteristic is mainly due to impermeable lipoid inner membrane of the shell. However, chloroform, ethyl ether, phenols and cresols have been proved able to alter this lipoid membrane (WHO 2004).

Ovicidal efficiency of Chlorocresol "industrial livestock housing broad-spectrum disinfectant" (Interkokask® RTU) was proved. Even though manufacturer stated in their product manual that 2% dilution of disinfectant for two hours is required for complete elimination of worm eggs, our observation showed that even 1% dilution of Chlorocresol disinfectant killed all the eggs in our laboratory experiment. It is claimed, however, that disinfectants are generally not fully effective against parasite eggs (Permin *et al.* 1998). In this respect work of Höglund and Jansson (2011) provided sufficient evidence of above statement. Despite using a disinfectant or in some cases combination of different disinfectants, all flocks became infected post placement with residual infective eggs. This might indicate that although disinfectants were proved to be lethal to parasite eggs under laboratory condition, the way these substances are currently used within the field is not beneficial.

10.7 Oxygen

It is often said that the overall metabolism of many nematodes is suppressed in the environments where not enough oxygen is provided. Within the environments where oxygen concentration is low, development of the infective egg stage may become slow or even cease (WHO 2004). Our results support the above hypotheses. What should be established at the very outset is that developing *A. galli* eggs are obligate aerobes. However, this study confirms that unembryonated eggs will survive for at least 16 days at 25ºC in anaerobic condition yet development will not take place. These results are in agreement with the work of Saunders *et al.* (2000) who reported that undeveloped *H. gallinarum* eggs remained viable even after 60 days of exposure to very low oxygen conditions. However, unlike our findings, it was

mentioned that the embryonation rate of such egg was reduced and the length of time to the initiation of embryonation, once introduced to aerobic condition was shortened. According to our data, exposure time (in this case as long as 16 days) had no effect on either development rate or the time needed at this temperature for complete development. As for the eggs with mature larvae, exposure to oxygen-free environment apparently induces a dormant phase with no visible motility. However, the difficulty with this argument is that further viability and infectivity of the larvae cannot be checked microscopically. Others have also reported that the development of *Ascaris* eggs is suppressed by a low oxygen concentration (Gaasenbeek and Borgsteede 1998). Adaptation of the nematode parasites to adverse environmental conditions is well documented. It is believed that, by entering the dormant state that alters their metabolic rate, they improve their chance of survival. Several species of nematodes are capable of anoxybiosis, which is defined as a dormant state associated with an undetectable metabolic rate, but this seems to be reversible (Mizgajska-Wiktor 2006).

As for *A. suum* eggs, it is believed that glycogen and especially triacylglycerols stored in the egg power the development process and that all the enzymes involve in tricarboxylic acid cycle and β-oxidation are present in this process and metabolism is aerobic (Marr and Muller 1995). It seems that activity of enzymes associated with aerobic metabolism is hindered in *A. galli* eggs, and an anaerobic pathway is likely the only answer to survival of the eggs when exposed to anaerobic condition. From another viewpoint, dependence on aerobic pathways not only waste the limited amount of energy and nutrients in the eggs, but also results in concentration of acidic end products as a result of the impermeability of the eggshell to these compounds.

10.8 Freezing-thawing

Free-living stages of nematodes are exposed to climatic changes during their development process in different environments and within these habitats; they are more likely being exposed to sub-zero temperatures for varying periods of time. Low temperatures may bring several problems for organisms such as changes in the viscosity and organization of membrane with a corresponding loss of function as well as changes in the structure and function of proteins and reduction in metabolic activities. An ability to survive cold is for that reason and important part of their ecology and life cycle in a temperate climate. Some nematodes have a physical barrier such as eggshell or sheath, which protects them against inoculative freezing and allows their body fluids to supercool to temperature as low as -38ºC (Devine 2010). In addition to the physical barrier, nematodes may survive sub-zero temperatures by a number of cold tolerance strategies. Freeze avoidance and freezing tolerance are the main two strategies (Wharton 1999). Freezing-tolerant parasite survive the freezing of at least part of their body fluids and freeze-avoiding parasite survive freezing temperature by removing or masking ice nucleators.

In our experiment, survival of 78.7% of *A. galli* eggs after exposure of the sample to sub-zero temperature indicates that this species is likely cold adapted. Earlier cryobiologic study conducted by Cruthers *et al.* (1974) pointed out that more *A. galli* eggs survived freezing to -22ºC than to -93ºC without any egg being seen with a ruptured or fractured shell. A possible explanation for this could be that when freezing of the egg's surroundings occurs at a high sub-zero temperature due to the difference in vapor pressure between water and ice at the same time, the parasite eggs lose water to its surroundings, dehydrate rather than freeze, and survive by cryoprotective dehydration. However, since in our experiment egg suspension in Eppendorf tube did not freeze, it is hard to support this hypothesis.

The 10% difference between development rate of the control group and study group in our experiment suggests that exposure to freezing temperature may to some extent reduce the development rate of *A. galli* eggs. This finding is in agreement with Dziekonska-Rynko and Jablonowski (2004) who stated that storage at low temperature slowed down the *A. suum* eggs development and reduced the number of eggs successfully completed the embryogenesis. WICKWARE (1940) showed that unembryonated eggs of *H. gallinae* can withstand freezing temperature (-4°C) for a period of 172 days and such exposed eggs were capable of causing infection of young white Leghorn chicks. What is more, *T. colubriformis* survived repeated freezing thawing at -10ºC whereas freezing and thawing of infective larvae at -28ºC killed all

larvae after the second cycle. It is also shown that embryonated eggs were more resistant than unembryonated eggs (Andersen *et al.* 1966). Likewise, a later study by Wharton and Allan (1989) on the same species revealed that all free-living stages of *T. colubriformis* survived exposure to freezing temperature and that the third-stage juvenile (J_3) was the most resistant stage. They suggested that in water J_3 uses a mixture of freezing-avoiding and freeze-tolerant strategies.

Sweden's climate varies from north to south, but in general has cold winters and cool to mild spring and autumn. During the wintertime in moist soil the presence of large volumes of water, seem to favor freezing at a high sub-zero temperature and hence favoring cryoprotective dehydration. There is no sufficient information about animal nematodes to state which cold tolerance strategy they use. It would also be interesting to see how many freezing-thawing cycles parasites may withstand. Since exposure to sub-zero temperature did not affect the viability of the eggs, contaminated outdoor areas in free-range system could be a potential risk in the spring in Sweden assuming that embryonated eggs may survive periods of desiccation

11 Conclusion

Based on the results of the present study along with previously reported data regarding environmental effects on development of *A. galli* eggs as well as other more or less closely related species of nematodes and overall seasonal changes in temperature in Sweden, one could conclude that eggs of *A. galli* are capable of remaining viable and surviving throughout the year. However, developmental time may vary depending on seasonal conditions. Between April and May eggs that had been deposited earlier, slowly begin to develop. During June till September normal development happens (1-3 weeks), however, fluctuation in temperature during this period when the outdoor mean high temperature varies between 19°C and 24°C and the mean low temperature ranges from 9°C to 14°C may hasten the development rate. In this respect, it is more likely that infection in free-range flocks originates from outdoor area during this period. Moreover, during the cold season between October till mid-May the temperature barely goes bellow -20°C yet cold enough to prevent the development, indicating that eggs are more likely preserved within the environment and infection re-emerge as suitable temperature for development is provided. Wintergardens during the cold seasons may provide shade/wet circumstances, which may favor slow development of the eggs. Mean relative humidity in Sweden for an average year is recorded as 77.8% and on a monthly basis ranges from 65% in May to 88% in November. As mentioned earlier, unhatched larvae may survive desiccation to some extent and if it is the case, embryonated *A. galli* eggs probably survive in deep litter and soil. In this respect, it is worth mentioning that various soil types have different water retention capacities, which provide the passage of gasses and moisture within the soil profile. The reserved water that has accumulated in forgoing wet winter permits survival of most organisms over dry summers. This phenomenon may play a role in providing enough moisture for developing eggs even when atmospheric humidity is below their minimum moisture requirement.

Since environmental data from field study indicated that the mean in-barn temperature was always above the development threshold, upon introduction of infective eggs to aviaries, development likely happens during all seasons. During the warm seasons in litter-based system, temperature may increase to the point that hasten the development process and spread the infective larvae all over the pens. Consequently, developed and infective eggs will probably remain viable within the environment.

Although in vitro efficacy of Chlorocresol disinfectant was proved even at lower concentration than it was originally suggested by the manufacturer, in-field effectiveness of such product remains controversial (Höglund and Jansson 2011). Better litter managements to remove accumulated eggs may be effective however, due to the slow, but continuing cold season egg development in barns, a more regular cleaning routine may be required to assist in control.

12 Future research and development

To the best of our knowledge most of the available information regarding Ascaridia galli are either outdated or focused mainly on the adult stages of parasite within the definitive hosts. There is an obvious lack of recent and up to date knowledge regarding survival and fate of free-living stages of the Parasite in the external environment. Within this project, undeveloped egg response to both physical and chemical factors affecting parasite egg survival was investigated. However, how embryonated eggs containing mature larvae respond to climatic factors remains unexplained. For instance, it would be interesting to see how many Freezing-thawing cycles parasite egg may withstand. Moreover, it would be useful to examine the metabolic pathways that take place during changes in surrounding environment with regard to oxygen availability. And finally to see if the Parasite eggs can recover from desiccation and to what extent damage can be repaired and normal physiology can be restored. These call for further research.

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