Ascaridia galli in laying hens: Adaptation of a targeted treatment strategy with attention to anthelmintic

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Abstract

Ascaridia galli is a parasitic roundworm of wild and domesticated fowl in the *Ascaridiidae* family within phylum Nematoda. The direct life cycle includes free-living eggs in the environment and larvae and adults worms in the small intestine of the host. Infection is associated with reduced health and production losses in laying hens. Since the EU ban on battery cages in 2012, the occurrence of *A. galli* has increased markedly in many European countries. Biosecurity measures, treatment with anthelmintic drugs, and cleaning and disinfection of barns between flocks are central to control the parasite. Treatment usually starts when parasite eggs are detected in faecal samples or when farmers observe worm expulsion or health impairment. At this late stage, worm infection levels and thus environmental contamination with parasite eggs are often already high.

This thesis therefore set out with the aim to gain more knowledge on optimal anthelmintic treatment strategies against *A. galli* by A) providing knowledge on the infection dynamics of *A. galli* in relation to deworming, B) validating a deworming strategy based on targeted treatment and C) applying tests for the detection and monitoring of anthelmintic resistance (AR).

We showed that flubendazole (FLBZ) was highly effective against all *A. galli* stages under field condition on two farms. However, the hens became re-infected within a week post treatment. We concluded that the way anthelmintics are used on farms needs to be refined. Comparison of the TT strategy and single treatment strategy (CT) with untreated control groups (UT) showed that barn contamination with parasite eggs, individual faecal egg counts (FEC) and worm burdens were lower in flocks where the TT strategy was applied. No significant difference was observed between the CT and UT flocks with regard to worm burdens and FEC. We concluded that a single treatment late during the production cycle is suboptimal and cannot prevent the build-up of infection and barn contamination. We recommended that the TT strategy should instead be considered as a better alternative to a single treatment late during production.

Investigation into mutations of the β-tubulin isotype 1 gene at codon 167, 189 and 200 showed no evidence of AR. Moreover, the results of the faecal egg count reduction test and the larval development test did not point at existence of AR on the investigated farm.

Keywords: poultry, ascaridiosis, control strategy, β-tubulin, benzimidazole, parasite egg, chicken

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Dedication

To Mom and Dad, You have been an outstanding inspiration to me, always being there for me during the good and the bad. You are the number one reason I am where I am today. Without your enduring love and support I could never have accomplished so much.

To my best friend, my soulmate, and my wife, Olga. Thanks for your constant support and love, willingness to listen and ability to deal with me at my best and at my worse.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Tarbiat, B., Jansson, D.S., Moreno, L., Lanusse, C., Nylund, M., Tydén, E., Höglund, J. (2016). The efficacy of flubendazole against different developmental stages of the poultry roundworm *Ascaridia galli* in laying hens. *Veterinary Parasitology*, 218, 66–72. doi: org/10.1016/j.vetpar.2016.01.012. (Year).

II Tarbiat, B., Jansson, D.S., Tydén, E., Höglund, J. (2016). Comparison between anthelmintic treatment strategies against *Ascaridia galli* in commercial laying hens. *Veterinary Parasitology*, 226, 109–115. doi: org/10.1016/j.vetpar.2016.07.006.

III Tarbiat, B., Jansson, D.S., Tydén, E., Höglund, J. (2017). Evaluation of benzimidazole resistance status in *Ascaridia galli*. *Parasitology*, 144, 1338–1345. doi: org/10.1017/S0031182017000531.

The contribution of Behdad Tarbiat to the papers included in this thesis was as follows:

I Performed the laboratory work in cooperation with co-authors. Mainly responsible for data interpretation in collaboration with main and co-supervisors and statistician. Drafted the manuscript and assisted the corresponding author.

II Determined field and laboratory design in cooperation with co-authors. Performed field work with some support from co-authors and colleagues. Performed the majority of the laboratory analyses including parasite egg and worm isolation. Mainly responsible for data interpretation in collaboration with main and co-supervisors and statistician. Drafted the manuscript and handled correspondence with the journal

III Determined field and laboratory design in cooperation with co-authors. Performed the majority of the experiment set ups and molecular lab work. Mainly responsible for data interpretation in collaboration with main and co-supervisors and statistician. Drafted the manuscript and was corresponding author.

Abbreviations

Ala	Alanine
AR	Anthelmintic resistance
bp	Base pair
BZ	Benzimidazole
CI	Confidence interval
CT	Conventional treatment
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EHT	Egg hatch test
EPG	Egg per gram faeces
EU	European union
FBZ	Fenbendazole
FEC	Faecal egg count
FECR	Faecal egg count reduction
FECRT	Faecal egg count reduction test
FLBZ	Flubendazole
GI	Gastrointestinal
Glu	Glutamic acid
H-FLBZ	Hydrolysed flubendazole
HPLC	High-performance liquid chromatography
HTS	High throughput sequencing
KJ	Kilojoule
LC50	Lethal concentration 50
LC90	Lethal concentration 90
LDT	Larval development test
LEV	Levamisole
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
Phe	Phenylalanine
R-FLBZ	Reduced flubendazole
SEK	Swedish krona
SNP	Single nucleotide polymorphism

SS	Stock solution
TBZ	Thiabendazole
TT	Targeted treatment
Tyr	Tyrosine
USA	United States of America
UT	Untreated
WAAVP	World Association for Advancement of Veterinary Parasitology
WPP	Weeks post placement
WS1	Working solution 1
WS2	Working solution 2

1 Background

Parasitic worms are common in all kinds of livestock and create major health and welfare problems worldwide, with economic impact due to production losses and disease. Parasites of poultry are no exception. The identification of *Ascaridia galli* (Schrank, 1788) previously known as *A. lineata* or *A. perspicillum* dates back to the 18th century. However, it was not until the early 20th century when systematic research started on this species. Since the introduction of conventional battery cages in Sweden in the end of 1950 (Erlandsson & Österlund, 2007), *A. galli* was rarely diagnosed in commercial caged flocks. The importance of *A. galli* became evident after the EU-wide ban (Council Directive 1999/74/EC) on conventional battery cages in 2012. In Sweden however, the law was already implemented since 1999 and by 2005 all conventional battery cages had been replaced by either enriched cages, aviaries or free-range systems. Over the past 20 years there has been a dramatic increase in Swedish laying hens raised in aviaries and free-range chickens including organic, from 11% in 1995 (Erlandsson & Österlund, 2007) to 87% in 2018 (Öberg, 2018). The problem with aviary system concerning *A. galli* is that it promotes faecal-oral contact which is the main route of microorganisms including nematodes transmission. This created a good opportunity for *A. galli* to become a centre of attention (Höglund & Jansson, 2011; Jansson *et al.*, 2010).

Biosecurity measures, cleaning and disinfection between consecutive flocks and anthelmintics are central to parasite control in poultry. However, current methods of roundworm control have proven to be insufficient in barn and free-range egg production, including organic farming (Höglund & Jansson, 2011). There is a growing body of literature suggesting that certain helminth eggs including those of *A. galli* are highly resilient to adverse conditions (Tarbiat *et al.*, 2015; Wharton, 1983). This makes it difficult to eradicate the infection upon its introduction to the barn environment. Questions have been raised about the use of plants with bioactive properties against *A. galli* (Abdel Aziz *et al.*, 2018; Abdelqader *et al.*, 2012). However, to our knowledge, this approach has not been

embraced by many farmers, perhaps due to lack of commercially available approved productions.

A main challenge faced by many egg producers is to avoid the spread and build-up of *A. galli* eggs. Today, many Swedish farmers deworm their laying hen flocks when parasitological analysis of faecal samples detect ascarid eggs or when they observe expelled worms or notice suboptimal performance or health impairment. Hence, deworming is sometimes initiated late during the production cycle. At this point worm loads may already be high and the barn environment is heavily contaminated with parasite eggs. Another concern is that the annual sale of BZs, which according to the Swedish Board of Agriculture, has increased dramatically since BZs were first introduced in 2009 (Grima, 2016). To date, few guidelines have been offered to producers on how to use anthelmintics within the context of control strategies.

This project therefore sought to obtain data which would help to combat the current *A. galli* problem in laying hen industry and it aimed to unravel some of the mysteries surrounding the risk for selection leading to the emergence of anthelmintic resistance (AR). The thesis begins by investigating the efficacy of FLBZ against *A. galli*. It will then go on to explore the significance of a targeted treatment (TT) strategy in laying hens. Last but not least, it will explore various techniques for the detection of AR in *A. galli*.

2 Introduction

2.1 Swedish egg production

The number of holdings for laying hens has decreased by 77% from 1990 to 2017. On the other hand, the number of Swedish laying hens has increased by 8% from 8,567,619 in 1990 to 9,288,000 in 2017. Around 98% of the laying hens are found in flocks with at least 5,000 hens (SOS, 2018). This means that egg production is dominated by few but large flocks. Simultaneously, the number of organic laying hens has increased by 70% from 730,421 in 2010 to 1,241,010 in 2017. This corresponds to 5% more production of organic eggs in 2017 compared to 2010. The total egg production has increased by 13% from 122,000 ton in 1990 to 138,000 in 2017. In 2017, Swedish import of egg and egg albumen was 338 million SEK (≈19,000 tons) while the export was 238 million SEK (≈12,000 tons). The average egg consumption per capita has increased from 11.5 kg in 2003 to 15 kg in 2017. The mean supply of energy, protein, fat and carbohydrates by egg per capita and per day in 2016 was 162 KJ, 3 g, 3 g and 0 g respectively. The price for a kg of eggs has increased around 55% from 2000 to 2012 (SOS, 2018).

Following national legislation (1988:539) (Erlandsson & Österlund, 2007) and the EU ban on conventional battery cages (EU Council Directive 1999/74/EC), Swedish laying hens have been kept under following production systems: organic, free-range, single-tiered or multi-tiered indoor production, and enriched cages. Comparison of production systems between the years 2011 and 2016 shows that there has been a noticeable increase in barn and free-range chickens including organic from 64% in 2011 to 88% in 2018. What stands out in the figures is the continual decline of furnished cages from 35% in 2011 to 12 % in 2016 (Öberg, 2018).

2.2 *Ascaridia galli*

The parasitic roundworm *A. galli* belongs to the phylum Nematoda. It occurs worldwide in galliform birds of all ages. The body of the adult worms is semi-transparent, cylindrical and has a creamy-white colour (Figure 1). Like most nematodes, *A. galli* is diecious with distinct sexual dimorphism.

Figure 1. Female adult *A. galli.* Photo: Behdad Tarbiat

Females are longer than males with a length of 72–116 mm and have a straight posterior terminal, whereas males are around 51–76 mm in length 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. The entire body is covered with a cuticle, which is striated transversely throughout the length of the body (Hassanain *et al.*, 2009).

2.2.1 Life cycle

Ascaridia galli has a direct life cycle which includes two phases, the free-living nematode eggs in the environment and the larvae and adult worms in the gastrointestinal (GI) tract of the host (Figure 2). Infection takes place by ingestion of the embryonated parasite eggs from the environment. These eggs that contain third stage larvae, hatch in the proventriculus and the initial predilection site for newly hatched *A. galli* larvae is the anterior part of the jejunum (Ferdushy *et al.*, 2012). The next larval stage is called histotrophic. Luna-Olivares *et al.* (2012) reported that most larvae, three days post infection, were found in the lumen (63%) in close contact with the epithelium in the crypt zone. However, 37% of the larvae were found within the tunica mucosa. They also described that the highest number of larvae was located in the crypts (51%), followed by the transitional zone (31%) and the villus zone (18%). Herd and

McNaught (1975) reported that the duration of this phase was dose-dependent. Upon maturation, the adult female worms produce massive numbers of eggs which are passed to the environment through the faeces. The prepatent period is about 4–8 weeks (Taylor *et al.*, 2007; Anderson, 1992), this depending on the age of the host.

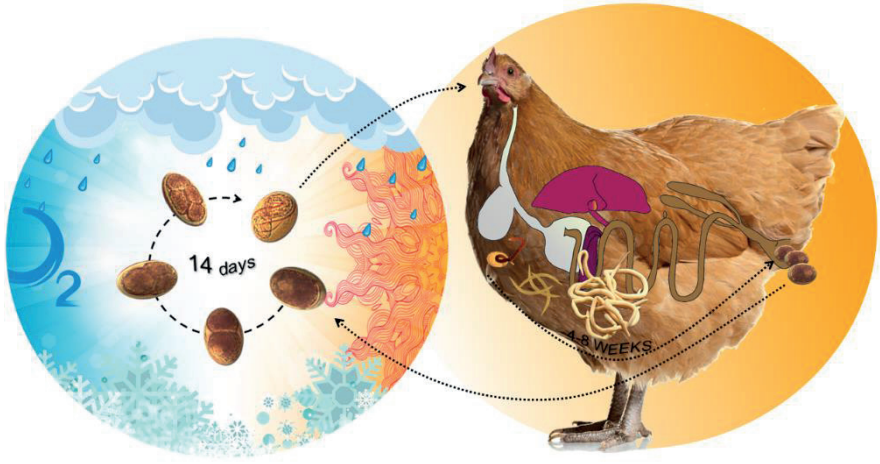

Figure 2. Life cycle of *A. galli.* Photo: Behdad Tarbiat

When laid, the eggs are covered with a resistant three-layered shell: the inner permeable layer called the vitelline membrane, a thick chitinous layer, and finally a thin, albuminous outer layer (Wharton, 1980). The egg shell is important to protect the developing larva against harsh environmental conditions (Tarbiat *et al.*, 2015) and to preserve infectivity (Hansen *et al.*, 1956).

2.2.2 Impact on the host

Despite the importance of *A. galli*, a search of the literature revealed surprisingly few studies which have systematically investigated the effects of this parasite on modern laying hybrid birds.

Clinical and histopathological manifestations

Penetration of the newly hatched larvae into the jejunal mucosa may cause haemorrhagic enteritis and anaemia. This is often associated with severe diarrhoea, loss of appetite and general weakness (Ikeme, 1971). In an analysis of chicken intestine, Luna-Olivares *et al.* (2015) reported that *A. galli* infection was associated with reduced length of villi.

Loss of muscle tone and the intestinal walls, haemorrhagic patches in the duodenum, scar tissue on the intestinal epithelium, extensive destruction and erosion of glandular epithelium, and proliferation of mucus-secreting cells which may result in the adhesion of the villi have been reported by Ikeme (1971). Thickening of the *tunica muscularis* of the infected hens has also been reported (Dänicke *et al.*, 2009). More recently, Hinrichsen *et al.* (2016) reported an association between combined helminth infection (*A. galli* and *Heterakis* species) and increased rate of mortality in Danish organic hens. However, other similar reports are limited.

Behaviour and welfare

There has been reports of behavioural changes in infected chickens. These include higher food intake and lower activities, as well as reduction in ground pecking and increase in nesting habits during both the prepatent and patent period (Gauly *et al.*, 2007).

Production losses

Reports on the effects of gastrointestinal helminths on egg production and reduced growth are generally scarce, both in commercial and backyard chickens. Having said that, infection with *A. galli* has been associated with reductions in egg production and overall growth in chickens (Soulsby, 2005; Ikeme, 1971; Reid & Carmon, 1958; Ackert & Herrick, 1928). A study by Phiri *et al.* (2007) on free-range chickens in central Zambia showed reduced weight gain in young birds harbouring multiple helminth species. Concurrent *A. galli* and *Escherichia coli* (Permin *et al.*, 2006) or *Pasteurella multocida* (Dahl *et al.*, 2002) were shown to have a significant impact on weight gain and egg production. By contrast, a study by Sharma *et al.* (2018) reached different conclusions, pointing out that egg production, feed intake and feed conversion ratio were not affected when hens were exposed to different levels of *A. galli*. Moreover, in their analysis of egg quality parameters e.g., shell thickness, albumin height, shell breaking strength, no differences were observed between groups with different exposure levels to *A. galli*. These results were similar to those reported by Gauly *et al.* (2007).

2.2.3 *Ascaridia galli* prevalence

Despite the description of *A. galli* in 1788, the extent of its prevalence was not the focus of many studies until recently. One possible reason could be that since mid-20th century many commercial laying hens have been housed in

conventional battery cages which lower the exposure of birds to faeces and parasite eggs. In a study conducted by Fossum *et al.* (2009) the occurrence of parasitic and bacterial disease was significantly lower in flocks housed in cages compared with those housed in barns or free-range. Infection with GI parasites in poultry, including *A. galli*, occurs through the faecal-oral route (Soulsby, 2005). That explains the low occurrence of helminth infection in conventional cages since they separate birds from their faeces.

However, animal welfare concerns resulted in an EU-wide ban on conventional battery cages for laying hens from 2012 (Appleby, 2003). In Sweden, the shift in housing systems for laying hens started already in 1988, when animal protection regulation (1988:539) forbade utilization of battery cages (Erlandsson & Österlund, 2007). These changes for the EU laying hen population has been associated with re-emerging infections with *A. galli* (Thapa *et al.*, 2015; Jansson *et al.*, 2010). Likewise, reports from other European countries indicated similar results in free-range including organic. These include 64% in Denmark (Permin *et al.*, 1999), 67–88% in Germany (Kaufmann *et al.*, 2011; Kaufmann & Gauly, 2009) and 84% in England (Sherwin *et al.*, 2013). Thapa *et al.* (2015) estimated the prevalence of *A. galli* in organic laying hens to be 97% in the Netherlands, 61% in Austria, 54% in Belgium and 50% in Italy.

2.3 Control strategies

2.3.1 On-farm management and prevention

Encountered diseases on a poultry farm is usually influenced by the type of production system. In commercial egg production, management practices essentially determine the magnitude of parasite infections. Given the ubiquitous presence of *A. galli* infection in most EU member countries and its potential negative impact both on production and animal welfare, effective control remains the utmost priority.

Stocking rate

Although Permin *et al.* (1998) did not observe significant difference between the influence of low, medium and high stocking rates on the availability of infective eggs in the environment, the trend was towards a higher worm burden and egg excretion in the medium stocking density flocks. Heckendorn *et al.* (2009) on the other hand, found a positive correlation between the risk of infection and stocking density in an outdoor run.

Flock structure

The pullets on arrival may look strong and healthy but they may be silent carriers of pathogens capable of infecting birds with which they come in contact. Likewise, the older birds in the existing flock may harbour diseases that can be passed on to the younger birds. Therefore, the all in-all out principle (Permin & Hansen, 1998), which means one age group on one site, has been widely implemented by modern egg producers. Having said that, it has been shown that age only partially (Idi *et al.*, 2004) or not at all determines susceptibility to *A. galli* infection (Gauly *et al.*, 2005).

Biosecurity, cleaning and disinfection of barns

Strict biosecurity routines such as disinfecting delivery vehicles before entering the production sites, using disinfection boxes and footwear exchange for personnel entering the barns, having a shower room and clean overalls, headgear and footwear supplies will reduce the risk of introduction of new infective agents to farms and between flocks. Hygienic measures between consecutive flocks also aim to interrupt transmission of pathogens, prevent re-infection and gradually minimize the infection level on the farm to an acceptable level. Thorough cleaning of the barn with high pressure hot water is recommended before placement of new pullets to reduce the level of parasite egg contamination. This together with a downtime period between consecutive flocks would theoretically reduce the infection level and delay the spread of the infection within the flock. The free-living stages of most parasite species including *A. galli* need high relative humidity to develop to the infective stage and must survive until they are ingested by the host. Therefore, keeping the floor and the litter dry is of utmost importance. If frequent removal of the litter is not possible, it has been suggested to remove the soiled and wet parts of the litter bed (Permin & Hansen, 1998). Having said that, findings of Maurer *et al.* (2009) indicated that there were no significant differences in helminth egg reduction in relation to different litter managements practices (adding, replacing or no management). However, they reported that in the group where fresh litter was added on top of the old litter, the FEC results were lower compared to the group with unmanaged litter. Reports on the effect of different disinfectants against helminths eggs are limited to field observations (Höglund & Jansson, 2011). However, they should be incorporated into the overall sanitation routine. Recently it has been shown that 1% dilution of chlorocresol (Interkokask®RTU) effectively eliminated all *A. galli* eggs *in vitro* (Tarbiat *et al.*, 2015). Whether this can be achieved in barns under commercial conditions remains to be determined. Other techniques such as lime-wash has been suggested after

cleaning and disinfection of the barn (Permin & Hansen, 1998). Overall, few systematic experimental studies are available from the field.

2.3.2 Host genetics and immunity

Little is known about genetic resistance to parasite infections in poultry. It has been demonstrated that worm burden and egg excretion can be significantly lower in Lohmann Brown hens compared to Danish Landrace (Permin & Ranvig, 2001). The authors suggested therefore the possibility of breeding for resistance to *A. galli* in chickens. Schou *et al.* (2003) correspondingly stated that genetic factors are involved in establishment and survival of *A. galli* in the GI tract of hens of different breeds.

The primary protection against infections in chicks are through maternal antibodies which are transferred via egg (Brambell, 1970). In chickens immunoglobulins are classified as IgY, IgA and IgM of which IgY is found primarily in egg yolk (Leslie & Clem, 1969). Even though maternally derived antibodies can provide partial protection against some bacterial and viral infections (Ahmed & Akhter, 2003), there is so far no evidence that maternally derived antibodies protect chickens against *A. galli* (Rahimian *et al.*, 2017). A strong immune response and intensive inflammatory reaction in the intestinal mucosa has been reported upon experimental infection of adult hens with *A. galli* (Marcos-Atxutegi *et al.*, 2009). Yet, the immune response does not protect the host against re-infection with *A. galli* (Andersen *et al.*, 2013; Norup *et al.*, 2013).

2.3.3 Anthelmintics

Anthelmintics are anti-parasitic drugs containing substances that are active against helminths including nematodes. These compounds either kill or remove the worms from any organs and tissues they may be present in. They are used to prevent clinical and subclinical symptoms, production losses and to minimize associated costs. Anthelmintic drugs are available in various forms such as add-on feed, oral suspensions, pre-mixtures for water or feed administration pour-on preparations, and injectable solution depending on target hosts and parasite species (Barragry, 1984). Anthelmintic drugs approved in the EU for commercial poultry are usually administered in feed or in drinking water to the flock rather than to individual birds.

The three major broad-spectrum drug classes of anthelmintics used in veterinary medicine are benzimidazoles (BZs), macrocyclic lactones such as ivermectin, and tetrahydropyrimidines such as levamisole (LEV) (Jacobs *et al.*, 2016). However, only BZ drugs i.e., flubendazole (FLBZ) (EMEA) and

fenbendazole (FBZ) (EMA) are approved for use in poultry against *A. galli* in the EU. It is worth noting that the amount of BZs sold for poultry in Sweden increased from 0.4 kg active substance in 2005 (Fag, 2006) to 46 kg in 2015 (Grima, 2016).

Benzimidazole mode of action

The α– and β–tubulin proteins bind to form heterodimers, the building blocks of microtubules. The functions of the microtubules at the cellular level include formation of the mitotic spindle, maintenance of cell shape, cellular secretion and nutrient absorption and intracellular transport (Dustin, 1984). Formation of microtubules is a dynamic process including polymerization of tubulin at one end and depolymerisation at the other (Martin, 1997). In nematodes, BZs selectively bind to the β-tubulin monomer causing disruption of microtubule equilibrium (Friedman & Platzer, 1980). The disruption of this equilibrium leads to a series of biochemical/physiological changes which in turn leads to loss of cellular homeostasis (Lacey, 1988) and interrupted transport of secretory vesicles. This results in cell lysis, and inhibition of motility and feeding (von Samson-Himmelstjerna *et al.*, 2007).

2.4 Anthelmintic resistance (AR)

One of the first reports of AR was from Kentucky, USA in the barber´s pole worm *Haemonchus contortus*, which is a strongyle nematode that mainly infects small ruminants (Drudge *et al.*, 1964). Since this report, AR has continued to increase globally and in severity in nematodes from a wide range of animals (Papadopoulos *et al.*, 2012; Sutherland & Leathwick, 2011; Kaplan, 2004; Sangster, 1999).

Anthelmintic resistance is defined as reduction of the efficacy of a chemical drug (anthelmintic) to a point where the majority of the worms survive the therapeutic dose (Sangster *et al.*, 2018). Once resistance is established the likelihood of reversion to susceptible strain is low (Wolstenholme *et al.*, 2004; Permin & Hansen, 1998).

Extensive use of anthelmintics has led to global spread of AR mainly in different strongyle nematodes of ruminant livestock and in horses (Gasbarre *et al.*, 2009; Lind & Christensson, 2009; Kaplan, 2004). Resistance to major classes of anthelmintics is common worldwide and the most serious problems have been encountered especially in nematodes of sheep and goats (Kaplan, 2004; Sangster & Gill, 1999). Currently the problem with cattle nematodes is less serious, despite resistance to multiple anthelmintic classes has been reported

both from farms in Europe and on the southern hemisphere (Sutherland & Leathwick, 2011; Loveridge *et al.*, 2003; Mejia *et al.*, 2003). In horses, there are problems with resistant nematodes to all available drug classes (Matthews, 2014).

To this day, AR in nematodes of poultry has not been reported yet. However, paper III in this thesis represents the first investigation of AR in *A. galli*. As stated by Knapp-Lawitzke *et al.* (2015), combination of high treatment frequency with subtherapeutic doses, which can be the case on poultry flocks, can lead to rapid selection for AR. With increased reliance on BZs against *A. galli* in laying hens during the past decades, and in the absence of new drug classes with novel modes of action, there is a risk of increased frequency of resistance alleles in the *A. galli* populations. Thus, it may only be a matter of time before resistance occurs in the field.

2.4.1 Mechanisms of AR

General mechanisms

The mechanisms of AR are complex and are likely to be different between drug classes with different modes of actions. Our understanding of AR, at least with strongyle nematodes, is more comprehensive with BZs compared with other major anthelmintic classes. However, information on how resistance alleles develop, undergo selection and spread within parasites populations is still scarce. There is also an ongoing debate about the means of measurement of drug efficacy and ongoing attempts to standardize these techniques (Levecke *et al.*, 2012; Miller *et al.*, 2006; Grimshaw *et al.*, 1996). The lack of reliable *in vitro* and *in vivo* tests means that we are most likely unable to detect the initial development and spread of resistance alleles in *A. galli*.

According to Wolstenholme *et al.* (2004), the resistance phenomenon is largely a random event. It is influenced by a variety of factors such as population size, diversity and mutation rate of the gene(s) in question, dosing and frequency of treatment (Knapp-Lawitzke *et al.*, 2015; Silvestre *et al.*, 2001), parasite genetic diversity (Prichard, 2001) and parasite population dynamics (van Wyk, 2001), to name but a few. These predisposing factors may cause: A) changes in drug targets i.e. reduction in drug binding affinity, B) changes in drug metabolism i.e. inactivation of the drug, and C) prevention of the drug to access the target site (Wolstenholme *et al.*, 2004).

Benzimidazole resistance

Structural changes in the β-tubulin molecules are associated with reduced BZ binding affinity (Lubega & Prichard, 1991). Strong evidence suggest that single nucleotide polymorphisms (SNPs) in the gene that codes for β-tubulin are associated with these structural changes and are correlated with BZ resistance, at least in *H. contortus* and other strongyles of veterinary interest (Wolstenholme *et al.*, 2004; Silvestre & Cabaret, 2002; Kwa *et al.*, 1993).

Several SNPs have been correlated with BZ resistance of which the Phe-Tyr polymorphism at codon 200 of the β-tubulin isotype 1 is the most well-known position (Kwa *et al.*, 1994). Prichard (2001) reported another Phe-Tyr polymorphism at codon 167 of the same isotype of the β-tubulin of *H. contortus*. Correlation between the SNP at codon 167 of isotype 1 β-tubulin and BZ resistance has also been confirmed by Silvestre and Cabaret (2002) in other strongylid nematodes. Moreover, a mutation at codon 198 of β-tubulin isotype 1 has also been suggested conferring BZ resistance. Accordingly, Ghisi *et al.* (2007) identified a Glu-Ala polymorphism at codon 198 in BZ-resistant *H. contortus* isolated from sheep in Australia and South Africa. The same SNP was later reported from Germany by von Samson-Himmelstjerna *et al.* (2007) in a BZ-resistant population of *H. contortus*.

2.4.2 Detection of AR

With the rapid development and spread of AR in nematodes of livestock, the methods to detect resistance have evolved, more or less, at the same pace. These methods are available as *in vivo* and *in vitro* tests. The *in vitro* tests are considered more reliable, accurate and practical compared to the *in vivo* tests. Besides, few *in vivo* tests are routinely used in veterinary diagnostic laboratories.

Faecal egg count reduction test (FECRT)

The FECRT is the gold standard and the most widely used test for detection of clinical resistance in nematodes of livestock. Results provides an estimate of anthelmintic efficacy. In principle, FECRT can be used with all types of anthelmintic drugs and nematode species in which parasite eggs are shed in faeces. It requires the comparison of faecal parasite egg counts (FEC) in at least ten animals. The FEC in pre-treatment faeces is compared with those obtained 7–14 days post treatment depending on the species and the drug in question. Due to the lack of correlation between egg output and actual worm numbers in some species (Martin *et al.*, 1985), the estimation may not always be fully accurate.

This technique was originally developed for the detection of AR in strongyle nematodes of sheep (Riffkin *et al.*, 1984), but has been further adapted for use in cattle, swine and horses according to guidelines provided by WAAVP (Coles *et al.*, 1992). The minimum recommended pre-treatment EPG levels is around 100–150 depending on the animal species (Coles *et al.*, 2006; Coles *et al.*, 1992). However, Levecke *et al.* (2018) has recently suggested a more dynamic range of required EPG based on the sample size. To the best of our knowledge, no official guidelines have yet been approved for use of FECRT in poultry.

Egg hatch test (EHT)

This test was designed to detect BZ resistance. The EHT was first developed to distinguish levels of resistance in strongyle nematodes such as *H. contortus*, *Teladorsagia circumcincta* and *Trichostrongylus columbriformis* (Hall *et al.*, 1978; Le Jambre, 1976), based on the calculation of the proportion of nematode eggs that fail to hatch in the presence of increasing concentrations of the BZ drug thiabendazole (TBZ). The percentage of eggs that hatch at each concentration is determined, and a dose-response curve is plotted against drug concentration. As with FECRT, guidelines for the use of EHT have been compiled (Coles *et al.*, 1992). From this, the LC_{50} (lethal dose 50: the concentration of the anthelmintic required to kill 50% of eggs) with the 95% confidence interval (CI) can be calculated.

Larval development test (LDT)

The LDT is an alternative tool to quantify the level of resistance against any anthelmintic. Parasite eggs are subjected to increasing concentration of anthelmintic dilutions in wells of microtiter plates. Embryonic development of the parasite to their infective larval stage is then measured during a specified period of time. Suitable controls are also run without the presence of anthelmintic. The LDT has been modified since it was first introduced by Coles *et al.* (1988) (Hubert & Kerboeuf, 1992; Lacey *et al.*, 1990; Taylor, 1990) and evaluated (Várady *et al.*, 2009; Amarante *et al.*, 1997). Like the EHT, the LC_{50} with their respective CI of 95% can be calculated using a logistic regression model. However, like with EHT a major limitation of the LDT is the lack of validated protocols for detection of AR resistance in *A. galli*.

Larval motility test

This test measures the ability of anthelmintics to paralyse the free-living stage larvae. Therefore, the principle relies on the microscopic assessment of larval

motility. The earliest test was developed to measure resistance to LEV and morantel, nicotinic receptor agonist drug class (Martin & Le Jambre, 1979). The procedure involves exposing parasite larvae to increasing dilutions of anthelmintics for 24 hours. Motility of these larvae is then assessed microscopically and the LC_{50} values are determined based on percentage of paralysed larvae. Like previous methods, the LMT has undergone evaluations and modifications. A micromotility meter was developed by Bennett and Pax (1986) where microprocessor technology was used to measure light reflection caused by larval movement in solutions. This technique was later used successfully by Folz *et al.* (1987) on *H. contortus*. Sutherland and Lee (1990) incubated TBZ resistant strongyle larvae in the presence of the acetylcholinesterase inhibitor and observed that they were paralyzed more slowly than the susceptible strain. More recently, Storey *et al.* (2014) developed a computer based high definition image processing technique to measure larvae motility in *Cooperia punctata*, *Brugia malayi* and *Dirofilaria immitis*.

Biochemical tests

A tubulin-binding test has been developed to detect BZ resistance. The principle is based on the reduction of the tubulin-binding affinity for BZs in the resistant isolates. The procedure includes the incubation of parasite tubulin extract with the tritium-labelled BZ until equilibrium is reached. The free drug is then removed and the drug binding is measured by a liquid scintillation counter (Lacey & Snowdon, 1988). Despite being a robust and reproducible technique, expensive facilities and the need for well-trained personnel makes it less useful for routine diagnostic work.

Molecular techniques

DNA alterations (e.g. mutations), changes in gene expression and genetic regulation (e.g. epigenetic modification) may explain phenotypic changes. Advancement in molecular techniques has made it easier to run fast and inexpensive tests, and therefore has deepened our knowledge about mechanisms of AR in recent decades, especially with regards to BZ resistance (von Samson-Himmelstjerna, 2006).

In one of the earliest studies, Roos *et al.* (1990) investigated DNA polymorphisms in the genome of BZ susceptible and resistant population of *H. contortus*. They found that BZ resistance was associated with an amino acid substitution at position 200 in the β-tubulin gene and showed that genetic assays can in fact be used successfully to detect AR to BZs. Development of the polymerase chain reaction (Saiki *et al.*, 1988) (PCR)-based tests has allowed the

detection of AR at lower levels than in classical methods (e.g. FECRT) (Elard *et al.*, 1999). Humbert *et al.* (2001) developed an allele-specific PCR to estimate the proportion of resistant and susceptible genotypes in strongylid worm population of ruminants. They reported a significant correlation between their molecular test and two classical methods (EHA and adult reduction test). This PCR-based technique was further modified by Silvestre and Humbert (2000) in order to identify strongylid species and to diagnose their BZ resistance or susceptibility in small ruminants. Major advancement in development of sequencing techniques from Sanger sequencing (Sanger *et al.*, 1973) to high-throughput sequencing (HTS) techniques (Heather & Chain, 2016) in recent years has led to their potential application in exploration of AR.

Genome sequences and transcriptomic data of both animal and plant parasites species are available on online data banks such as NCBI, Wormbase (www.wormbase.org) and helminth.net of which the later provides specific data-mining and comparative analysis tools to study helminths (Martin *et al.*, 2015). These have provided major insights into the biology of some parasitic nematodes. For a review of the recent advances in both candidate-gene and whole-genome approaches to discover AR refer to Kotze *et al.* (2014). Although each of these resources improves accessibility to existing data and can help users with their analysis of their own data, little is known about the genome of *A. galli* (Martis *et al.*, 2017).

3 Aims of the thesis

The overall aims of this thesis were two-fold: A) to develop a control strategy against *A. galli* based on the concept of strategic targeted treatment, and B) to incorporate available *in vitro* and *in vivo* AR detection methods into screening for BZ resistance in connection with regular use of anthelmintics.

The specific objectives of paper I–III were as follows:

I. To investigate whether BZs are capable of eliminating all larval stages, including histotrophic larvae, and adult stages of *A. galli* in the GI tract of hens. Therefore, the specific objective of this study was to answer this question. This paper also estimated the duration of the reinfection period after treatment with BZs in modern laying hen hybrids on a commercial farm.

II. This prospective study was designed to investigate the usefulness of a deworming programme based on a TT strategy in multiple flocks on a commercial laying hen farm where FECs were monitored on a weekly basis. The hypothesis was that by using this strategy we would reduce the barn contamination with *A. galli* eggs, which would in turn lead to lower worm burdens in the laying hens.

III. To evaluate anthelmintic resistance status in an *A. galli* population, which was exposed to various anthelmintic treatment frequency, by using a range of available *in vivo* and *in vitro* techniques. To generate novel information on the mechanisms that may underpin AR in *A. galli*.

4 Materials and methods

A brief description of materials and methods used in this thesis is presented below. More details can be found in papers I–III.

4.1 Study farms (paper I-III)

Primary inclusion criteria for the study farm were: A) mono-infection with *A. galli* B) shortest possible distance to the laboratory and C) aviary housing system and multiple flocks available for study. Two commercial laying hen farms were recruited. Both farms were located in the central part of Sweden. Investigated flocks (each held ≈7,000 hens) were housed indoors in NATURA-Nova aviary system with wood shavings covering the concrete floor. One flock from each farm was included in paper I whereas, six identical flocks from the second farm were included in paper II–III. In paper I, the experiment started when the hens were 67 (farm 1) and 70 (farm 2) weeks old. In contrast, the field experiment in paper II started post-placement and continued until slaughter.

4.2 Anthelmintic treatment

The authors provided the farmers with clear instructions on when and how to administer the anthelmintic drugs. In paper I, hens on farm 1 and 2 were treated with FLBZ (Verminator® Elanco Animal Health/Eli Lilly Danmark A/S, 1.43 mg/kg body weight) at 67 and 70 weeks age, respectively. The drug was administered daily for seven days via drinking water according to the manufacturer´s instructions.

In paper II, six flocks were randomly allocated between three treatment groups. The hens in the TT group were treated with FBZ (Panacur AquaSol®, Intervet AB, 1 mg/kg body weight) at 22, 27 and 36 weeks post placement (WPP). The hens in the conventional treatment (CT) group were treated with

FBZ at 27 WPP and the hens in the untreated (UT) group did not receive any anthelmintic drug (control group). The drug was administered daily for five days via drinking water according to the manufacturer´s instruction. In paper II, FBZ was chosen over FLBZ because of its shorter administration period.

4.3 Sample collection

4.3.1 Sampling of faeces and intestinal contents

Farmers received step-by-step instructions on when and how to collect, handle and post faecal samples. These samples were collected on specified occasions by placing clean plastic trays (20 × 27 cm) on the manure belts under the slats from the respective flocks. The manure belts had been operated before placement of trays to ensure that the collected faeces represented faeces produced during the last 24 hours. The faecal samples were then used to: A) assess FEC before, during and after treatment with FLBZ in paper I, B) assess the overall parasite egg expulsion in the barns in paper II and C) isolate *A. galli* eggs for LDT in paper III (the latter were collected by the authors).

Samples of cloacal contents were collected from ten randomly selected hens (the same hens under section 4.3.4) by the author on specified occasions to assess individual FECs in paper II. In addition, individual faecal samples were collected from 15 randomly selected hens before and ten days after the end of the treatment period to conduct FECRT in paper III.

Duodenal contents were collected from the same hens mentioned above to detect and quantify the concentrations of FLBZ in paper I.

4.3.2 Blood samples

Blood samples were collected from the wing vein (cutaneous ulnar vein) of ten randomly selected hens to determine the concentration of FLBZ in serum, as well as its reduced (R-FLBZ) and hydrolysed (H-FLBZ) metabolites in paper I.

4.3.3 Water samples

Water samples were collected from the middle and the end of the water lines, 2 and 4 h after start of FLBZ administration to quantify the water concentration of FLBZ in paper I.

4.3.4 Worm collection

All parasites and eggs used in this study were the results of natural infection of flocks on commercial farms. In the field trials (papers I–III), hens were most likely infected with *A. galli* eggs present in the barn environment, presumably from the preceding flock. Handling and euthanasia were approved by the Swedish Ethical Committee for Scientific Experiments (C108/14). Ten hens were selected randomly on specified occasions to obtain larvae and adult worms. Hens were euthanized by stunning and cervical dislocation according to national legislation and were necropsied following an in-house avian necropsy protocol of the National Veterinary Institute (SVA) in Sweden. The intestines were collected and transported to the laboratory at a temperature around 15–20 °C. Larvae and adult *A. galli* were then isolated to: A) assess the FLBZ efficacy in paper I, B) estimate worm burdens in paper II and C) extract DNA for molecular analysis of the β-tubulin gene in paper III.

4.4 Parasitological analyses

4.4.1 Faecal samples

Cloacal contents samples (≈0.5 g/hen) in paper I were analysed with MiniFlotac (miniFlotac®, University of Naples Federico) to assess the *A. galli* FEC. The MiniFlotac technique was used because only small amount of cloacal contents was recovered. In paper II, cloacal contents (≈3 g/hen) were analysed with McMaster technique due to the fact that in all cases desired amounts of sample material were collected. Pooled (3 g of approximately 800 g faeces collected on plastic trays) and individual faecal samples (≈3 g/hen) in papers I–III were analysed with McMaster technique. The sensitivity of both methods was adjusted to 50 EPG.

4.4.2 Worm isolation

The collected intestines in paper I and II were cut open in the laboratory and the intestinal contents from the small intestines were each washed with 1 L tap water in a plastic container. The contents of the containers were then sieved (mesh size 300 µm) and the adult worms were collected. The remaining liquid from the sieving process were collected in 1 L glass bottles and left to sediment for 30 min. The supernatant was then removed by a water aspirator and clean water was added to the bottles. The process was repeated several times in order to remove

most faecal matter. Larvae were then counted microscopically in approximately 100 ml intestinal suspensions.

The histotrophic larvae in paper I were detected with an agar gel-incubation method (Ferdushy *et al.*, 2012) with some modifications. For details refer to paper I.

4.4.3 *A. galli* eggs isolation

Parasite eggs were extracted from faecal samples using a modified flotation technique. Accordingly, parasite eggs were isolated through a series of sieves using cold tap water. The mesh sizes used in declining order were 300, 150 and 70 µm. Eggs were collected on a 35 µm sieve, transferred to several 50 ml Falcon tubes, and centrifuged (IES CL50 centrifuge, Techtum Lab AB®) for 1 min at 425 ×g. The supernatant was discarded and the tubes were filled with saturated salt. Tubes were vortexed vigorously and then centrifuged for 3 min at 425 ×g and the liquid phase containing parasite eggs were passed through a 25 µm sieve and washed several times with cold tap water. Eggs were collected in a 50 ml falcon tubes.

4.5 Pharmacological analyses

Blood samples were centrifuged in the laboratory to obtain serum. Experimental and fortified samples of serum, water, and intestinal contents were analysed for the parent FBZ, its reduced (R-FBZ) and hydrolysed (H-FBZ) metabolites by high performance liquid chromatography (HPLC) at the Laboratory of Farmacology, Facultad de Ciencias Veterinarias, UNCPBA, Tandil, Argentina. For further details refer to paper I.

4.6 Faecal egg count reduction (FECR) analyses

In order to assess the FECR, the mean number of parasite eggs per gram faeces (EPG) pre-treatment in 15 samples (T1) were compared with the mean EPGs in the same number of samples ten days post-treatment (T2) using the formula: FECR=100×(1-[T2/T1]) (McKenna, 2006).

4.7 Larval development analyses

4.7.1 Preparation of BZ dilutions

Thiabendazole (TBZ), due to its relatively high solubility in water, was used in the LDT. A stock solution (SS) of 5000 µg/ml was prepared by dissolving 50 mg thiabendazole in 10 ml dimethyl sulphoxide (DMSO). Working solution 1 (WS1) (50 µg/ml) was created from the stock solution by diluting 1 ml SS in 99 ml DMSO with a final concentration of. The WS2 (20 µg/ml) was prepared by diluting 4 ml WS1 in 6 ml DMSO. The WS1 and WS2 were then used to create serial dilutions of TBZ (0.02−0.2 µg/ml). For details refer to table 1 in paper III.

4.7.2 LC_{50} and LC_{99} calculation

The *A. galli* eggs were incubated at 25˚C for 2 weeks in a series of gradually increasing TBZ concentrations (0.02 µg/ml intervals) in microtiter plates with 12 replicates per interval. At the end of the incubation period plates were examined under an inverted microscope. A minimum of 200 eggs per well were counted and the number of L3 larvae at each TBZ concentration was determined. The percentage egg development of the replicates was corrected (baseline-corrected) based on the egg development rate of the control wells (in water). The data were analysed by a logistic regression model to determine LC_{50} (with their respective CI of 95% values). The LC_{99} was calculated from LC_{50}. For detailed statistical model refer to paper III.

4.8 Molecular analyses

4.8.1 *A. galli* DNA extraction

Twenty male and 20 female worms were retrieved from sacrificed hens from each flock (120 worms in total) at 45 WPP in paper II. The DNA was extracted from the anterior part of worms using a DNA purification kit (NucleoSpin® Tissue) according to the manufacturer's protocol and DNA concentrations were estimated using Picodrop Microliter UV Spectrophotometer (Picodrop Ltd).

4.8.2 Isolation of β-tubulin

A 630 base pair (bp) fragment of the β-tubulin gene was amplified using primers previously described by Tydén *et al.* (2013). For details of amplification conditions and cycling parameters refer to paper III. Prior to sequencing, 5 µl of

the PCR product was examined on a 1% agarose electrophoresis gel with TBE (Tris/Borate/EDTA) buffer to confirm the existence and the size of the PCR products.

4.8.3 SNPs analyses of the *A. galli* β-tubulin gene

In total 120 *A. galli* DNA-fragments were sequenced. Sequences were assembled and aligned, and gene organization was retrieved by blasting sequences against the reference *A. galli* β-tubulin isotype 1 gene (GenBank accession no. KC713796.1) using the NCBI Blast engine (version 2.6.0) and visualized using the web interface, NCBI Sequence Viewer (version 3.18.3). A partial gene map based on the sequenced DNA fragment was created, and regions for codons 167, 198 and 200 were identified using CLC Genomic Workbench 7 (version 7.2.5, CLCbio, Arhus, Denmark).

5 Results and discussion

The general outcomes of papers I–III of this thesis are briefly described below. More detailed information can be found in the respective paper.

5.1 Efficacy of BZs against *A. galli* (paper I-III)

In paper I, the efficacy of FLBZ against all stages of *A. galli* was systematically evaluated for the first time. We hypothesized that during the histotrophic larval stage, where larvae embed themselves in the epithelium of small intestine or are localized within the lumen of crypt, the parasite might possibly evade anthelmintic treatment. It was therefore important to know whether FLBZ was effective against this developmental stage of *A. galli* in addition to later larval and adult stages.

The results of paper I showed that FLBZ was highly effective against all life stages of *A. galli* within the host. Neither adult, nor luminal or histotrophic larvae were found on the seventh day of treatment. This was also supported by the results of paper III in which the percentage of FECR was 98–100% following treatment with FBZ.

Pharmacological findings in paper I indicated that there were variations in the water concentrations of FLBZ both within and between farms (1–4.72 µg/ml in flock 1 and 0–3.66 µg/ml in flock 2). Despite these variations, the desired duodenal FLBZ concentration to kill *A. galli* was achieved in all investigated hens (from 0.5 to 0.8 µg/g).

Perhaps the most important finding of paper I was that reinfection most likely occurred immediately after the end of the treatment period, as both luminal and histotrophic larvae were found seven days post treatment. This indicates that FLBZ, despite being highly efficacious against *A. galli*, has a short-lived effect. Thus, it is concluded that the residual activity of FLBZ against *A. galli* is poor.

5.2 Evaluation of the performance of the TT strategy against *A. galli* (paper II)

This investigation took the form of a prospective cohort study with in depth assessment of a FEC-based TT approach for control of ascaridiosis. Initially, some farmers dewormed their laying hens only once and quite late during the production cycle (Magnus Jeremiasson 2016, personal communication). Based on our results in paper I, it can be assumed that the effect of a single anthelmintic treatment late during the production cycle did not prevent build-up of large numbers of parasite eggs in the barn environment. These results highlighted the importance and the need for refinement of the current treatment strategy. The hypothesis of paper II was that by assigning a threshold level of parasite egg expulsion as an indicator for early and if necessary repeated deworming, the treatment would prevent the build-up of *A. galli* infection and lower the parasite eggs contamination of the barn which would in turn lower the worm burden.

The results of paper II showed significantly ($p< 0.001$) lower cumulative FECs in the TT flocks during week 22–45 compared with the other study flocks. The cumulative FECs were used as an indicator for environmental contamination with parasite eggs. The same pattern was observed when comparing the individual FECs. The FECs in the TT flocks were lower ($p< 0.05$) than both the CT and UT flocks at 35 and 45 WPP respectively. In paper II we also demonstrated that hens in the TT flocks in general harboured fewer worm than their counterparts in the CT flocks. This was in line with lower individual FECs observed in these flocks. Another important observation in paper II was that treating the flock once as we did in the CTs did not make any significant differences with regards to individual FECs and total adult worms at 35 and 45 WPP compared to UT flocks. In other words, treating the flocks once or not treating them at all yielded almost the same results in terms of parasite control.

5.3 No signs of development of AR in *A. galli* after repeated treatment with BZs (paper III)

The repeated extensive use of anthelmintic drugs in livestock is in general known to be a contributing factor for development of AR (Coles, 2005). It has also been shown that the combination of sub-therapeutic dosages with high treatment frequency lead to rapid selection for BZ resistance for example in the abomasal nematode *Ostertagia ostertagi* of cattle (Knapp-Lawitzke *et al.*, 2015).

We demonstrated variations in FLBZ concentration in the water samples in Paper I which suggests that laying hens are subjected to varying dose levels. This is a known risk factor for development of AR. In paper II, we showed that by

applying the TT strategy, build-up of *A. galli* eggs in the investigated poultry barns was prevented. This led to an overall lower parasite burden and egg expulsion however, at a cost of more frequent treatment occasions. In view of these results, it is of utmost importance to screen for early signs of AR in *A. galli*. This thesis thus, provides an initial step towards developing techniques that can be applied to chickens flocks for AR screening purposes, but further studies are necessary before they can be applied for field screening purposes. The hypothesis was that repeated treatment with anthelmintic would increase the frequency of a resistance allele, if it exists, in the investigated *A. galli* population.

The FECRT results in paper III indicated that the inspected *A. galli* population was susceptible to FBZ despite having been repeatedly exposed to BZs under commercial conditions. However, upon closer inspection of the lower confidence limit data it was evident that on three occasions in the TT flocks the lower confidence levels were near the border line (90% and below) for declaration of resistance in strongylid nematodes (refer to table 2 in paper III). As discussed in paper III, different cut-off values have been used for nematode parasites of horses and pigs. Even though the figures pointed to the susceptibility of *A. galli* to BZ, attention needs to be paid to the fact that lower CIs were observed in those flocks where anthelmintic were used repeatedly.

The susceptibility of *A. galli* eggs to TBZ were also investigated by an *in ovo* liquid-based LDT. In order to make an informed decision about existence of AR using LDTs, baseline values from resistant strains are needed. As discussed earlier, no specific guidelines for LDT have yet been developed for *A. galli*. As a result, it was not possible to solely rely on values provided in this thesis to declare resistance in *A. galli* even though the LC_{50} and LC_{99} values corresponded to values obtained from resistant nematode isolates of sheep and horses. However, no differences were observed in the LC_{50}s between groups due to repeated or no treatment. This means that an increase in treatment frequency within our specified production period did not decrease the susceptibility of *A. galli* eggs to BZ.

Much research is needed about the β-tubulin gene in *A. galli* and its involvement in AR to BZs. The work of Tydén *et al.* (2013) was the first attempt to sequence the *A. galli* complete β-tubulin isotype 1 gene. The results of paper III were therefore based on available data on the β-tubulin gene prior to the recent publication of Martis *et al.* (2017) who showed that there are at least five isoforms of the β-tubulin gene in *A. galli*. The results of the genetic study in paper III indicated lack of evidence of BZ resistance in *A. galli* from the study farm. All sequenced *A. galli* for the isoform 1 of the β-tubulin gene showed susceptible alleles in the studied codons. Genotype variations were observed at codons 167 and 200 of the β-tubulin gene between *A. galli* and *H. contortus*.

Based on our observations, all mutations were synonymous in the third position of these codons.

6 Summary and concluding remarks

The intestinal roundworm *A. galli* is an emerging problem in modern egg production. Infection with *A. galli* has been associated with reduced animal health, welfare and production losses. Worm control in modern livestock farming is in general based on biosecurity, hygienic measures and anthelmintic drugs. However, such measures have offered incomplete effect in controlling *A. galli* infection on European laying hen farms. There is currently little advice to offer to commercial egg producers in terms of improved worm control. Another concern is the annual sales of BZs in Sweden which have increased noticeably since FLBZ was first introduced in 2009.

This thesis therefore set out with the aim to gain more knowledge on optimal anthelmintic treatment strategies against *A. galli* by A) providing knowledge on the infection dynamics of *A. galli* in relation to deworming, B) validating a deworming strategy based on targeted treatment and C) applying tests for the detection and monitoring of AR. It provides the first comprehensive assessment of anthelmintic efficacy against *A. galli* and establishes a quantitative framework for detecting AR. The newly developed TT control strategy should help reduce parasite contamination of the barn environment and thus delay and reduce the infection of subsequent flocks. However, this strategy nees to be further refined. The following conclusions can be drawn from the present thesis.

* The BZ anthelmintic FLBZ is highly efficacious against all developmental stages of *A. galli*.
* One of the more significant findings to emerge from paper I was that reinfection with *A. galli* occurs immediately after the end of treatment. Therefore, deworming hens once around peak production is only a temporary solution to reduce worm burden as the effect lasts only while drug is being applied.
* The findings of paper I have important implications for the understanding of the infection dynamic in terms of timing of larval and adult stages and host-parasite interactions.

* The TT strategy should be considered as a better alternative to single treatments late during the production cycle.
* Lower worm burdens and significantly lower environmental parasite eggs contamination will justify the extra cost of frequent monitoring and administration of anthelmintics.
* It is of high importance to note that sporadic variations in drug distribution (paper I) combined with suggested frequent use of anthelmintic in paper II, may increase the risk for development of AR.
* In paper III, tools for the detection of AR in *A. galli* were applied for the first time. Such tools will need to be further refined and validated in order to be used for screening purposes for the presence of AR in the future studies.
* Whilst the FECRT results (paper III) did not indicate presence of AR in the *A. galli* population on the study farm, it did partially substantiate that AR might be slowly developing on the investigated study farm.
* In spite of the limitations in interpretation of the LDT results (paper III), it certainly adds to our understanding of parasite egg-drug interactions.
* The investigation of the β-tubulin gene (paper III) discovered no non-synonymous mutations in association with repeated treatment. These results add to the rapidly expanding field of genetic markers.
* In response to the question of whether AR against BZs is present in *A. galli*, this thesis could not provide a solid answer. Notwithstanding the boundaries, we found lack of evidence to declare AR in *A. galli*.

7 Future perspectives

Decades of research on *A. galli* has broadened our knowledge about this parasite in poultry. Despite the number of studies in literature, there is not a single study which provides guidelines on how to properly use anthelmintics to control this parasite on commercial laying hen farms. What is more, there have been few empirical investigations into AR and its underlying mechanisms. This thesis lays the groundwork for future research into AR in *A. galli*. It has also enhanced and further developed the existing control strategies against this parasite in laying hens. Although the work of this thesis has found and put together more pieces of the puzzle, there are still many gaps in our knowledge. Some potential areas for further research on this interesting parasite as well as improvement of the methods used in this thesis are listed below.

* Even though drug intake was clearly sufficient in paper I, drug distribution varied between the water samples. It seems like uneven drug distribution is inevitable when drugs are administered through the water pipes. Data on drug distribution from several farms will certainly give us a better picture on how big the extent of under-dosing is and how big the risk of developing AR is.
* Since the study described in paper II was conducted on a single farm with multiple flocks, the generalisability of these results is subject to certain limitations. Further studies on more farms with different management strategies should be performed to assess the long-term effect of the TT strategy in terms of reducing environmental parasite contamination, delaying the onset of the infection and reducing worm burdens.
* More research using controlled trials is required to compare the results of FECRT. What is now required is national and cross-national studies involving more populations of *A. galli* to provide meaningful comparison and perhaps more concrete evidence for AR.

* Considerably more work is required to determine the base line values in order to compare the LDT results. More information on sensitivity of *A. galli* eggs to anthelmintic drugs would help us establish a greater degree of accuracy on this matter.
* A question raised by paper III is whether the correct isotype of the β-tubulin gene was investigated. Further research and identification of SNP in the other isotypes is therefore an essential next step to shed more light on the role of tubulin molecule in AR.
* Studies regarding the role of other candidate genes would be worthwhile. Likely candidates are the ATP-binding cassette transporter proteins, such as P-glycoprotein (p-gp), which has been shown to confer AR in other nematode parasites of veterinary interest (Kerboeuf *et al.*, 2003).
* Further investigation in AR using non-candidate driven approach is another highly interesting area that can provide information about genes that are involved in drug binding and metabolism (Martis *et al.*, 2017).
* Another question that still remains to be answered is whether BZ resistance can be achieved by more than one mechanism simultaneously, and if so to what extent these combinations would affect the level of resistance.

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Populärvetenskaplig sammanfattning

Ascaridia galli (Nematoda: *Ascaridiidae*) är en rundmask som infekterar hönsfåglar. Parasiten har en direkt livscykel som inkluderar parasitägg i miljön (fjäderfästallar och inhägnader utomhus), samt larver och vuxna maskar i tunntarmen hos dess värddjur. Hos värphöns är kraftig maskinfektion förknippad med nedsatt djurhälsa och produktionsbortfall. Sedan de konventionella oinredda burarna för värphöns förbjöds inom EU 2012 har förekomsten av *A. galli* ökat i flera europeiska länder. Anthelmintikabehandling i kombination med rengöring och desinfektion av stallarna mellan olika produktionsomgångar är centralt för att bibehålla låg parasitbörda i infekterade flockar. Behandlingen (avmaskningen) påbörjas tidigast när parasitägg detekteras i träckprov eller när producenterna upptäcker maskar i avföring eller om hönsens hälsa är nedsatt. I de två senare fallen har hönsen oftast redan en hög parasitbörda vilket innebär att miljösmittan med parasitägg också är hög.

Avhandlingens mål var att öka kunskapen om behandlingsstrategier mot *A. galli* genom att; A) ta fram kunskap om infektionsdynamiken hos *A. galli* i relation till avmaskning i kommersiella värphönsbesättningar, B) validera en avmaskningsstrategi baserad på riktad behandling och C) utvärdera olika testmetoder för detektion och övervakning av anthelmintikaresistens.

Vår studie om effektiviteten hos avmaskningsmedlet flubendazol (FLBZ) som användes vid behandling av *A. galli* under pågående äggproduktion i två flockar på olika gårdar visade att FLBZ var effektivt mot samtliga parasitära utvecklingsstadier. Däremot återinfekterades hönorna inom en vecka efter avmaskningen vilket visar att effekten var kortvarig. Vår slutsats är att dagens användning av anthelmintika behöver förbättras.

Hos en svensk äggproducent jämfördes under 38 veckor effekterna av tidigarelagd riktad anthelmintikabehandling med de i en konventionell avmaskningsstrategi senare under produktionscykeln liksom i en obehandlad kontrollgrupp. Hönsen i stallet med riktad avmaskning hade ett signifikant lägre antal maskar och utskiljde färre ägg än de två övriga grupperna. Däremot

noterades inga skillnader avseende parasitförekomst eller äggutskiljning hos hönorna i den konventionella behandlingsgruppen och hos de i den obehandlade kontrollgruppen. Vår slutsats är att en enda behandling relativt sent under produktionscykeln är suboptimalt eftersom den inte förebygger uppbyggnaden av miljösmitta med parasitägg. Riktad tidigarelagd behandling är följaktligen att föredra framför en enstaka behandling senare under produktionscykeln. En nackdel är dock att det krävs flera behandlingar för att uppnå långsiktig parasitkontroll. Resultaten av äggräkning och parasitlarvsutveckling visade inga tecken på förekomst av anthelmintikaresistens på den undersökta gården. Även undersökning av sambandet mellan olika mutationer på β-tubulin isotyp 1 vid kodonen 167, 189 och 200 gav stöd för att anthelmintikaresistens inte förelåg.

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