Aus dem Institut für Parasitologie und Tropenveterinärmedizin
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Identification of β-tubulin Isotypes and Development of Pyrosequencing Assays for Benzimidazole Resistance in *Heterakis gallinarum* and *Ascaridia galli*

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Vahel J. Ameen
Tierarzt aus Amêdi, Kurdistan Region, Iraq

Berlin 2020
Journal-Nr.: 4188

mbvberlin | mensch und buch verlag

Aus dem Institut für Parasitologie und Tropenveterinärmedizin

des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Identification of β-tubulin Isotypes and Development of Pyrosequencing Assays for Benzimidazole Resistance in *Heterakis gallinarum* and *Ascaridia galli*

Inaugural-Dissertation

zur Erlangung des Grades eines

Doktors der Veterinärmedizin

an der

Freien Universität Berlin

vorgelegt von

Vahel J. Ameen

Tierarzt aus Amêdi, Kurdistan Region, Iraq

Berlin 2020

Journal-Nr.:4188

Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Dekan:	Univ.-Prof. Dr. Jürgen Zentek
Erster Gutachter:	Univ.-Prof. Dr. Georg von Samson-Himmelstjerna
Zweiter Gutachter:	Univ.-Prof. Dr. Marcus Fulde
Dritter Gutachter:	Univ.-Prof. Dr. Dr. Hafez Mohamed Hafez

Deskriptoren (nach CAB-Thesaurus):
Heterakis gallinarum; Ascaridia galli; benzimidazole; drug resistance; tubulin; DNA; RNA; DANN sequencing; nucleotid sequencing

Tag der Promotion: 27.02.2020

Bibliografische Information der *Deutschen Nationalbibliothek*
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <https://dnb.de> abrufbar.

ISBN: 978-3-96729-042-4
Zugl.: Berlin, Freie Univ., Diss., 2020
Dissertation, Freie Universität Berlin
D188

Choriner Str. 85 - 10119 Berlin
verlag@menschundbuch.de – www.menschundbuch.de

This dissertation is lovingly dedicated to

My mother and father and my beloved Aleen, Reveen,

Hazha and Malak

Table of Contents

Table of contents

List of Tables

List of Figures

Abbreviations

AR	Anthelmintic Resistance
ATP	Adenosine Triphosphate
aLRT	approximate Likelihood Ratio Test
BZs	Benzimidazoles
CET	Controlled Efficacy Test
CI	Confidence Interval
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleosidtriphosphate
ddPCR	Droplet digital PCR
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
EHA	Egg Hatch Assay
EPG	Eggs per Gram
E198A	Glutamic acid to Alanine substitution P198
E198L	Glutamic acid to Leucine
FAO	Food and Agriculture Organisation
FECs	Faecal Egg Counts
FECRT	Faecal Egg Count Reduction Test
F167Y	Phenylalanine to Tyrosine substitution P167
F200Y	Phenylalanine to Tyrosine substitution P200
GSTs	Glutathione S-transferases
IL	Interleukin
PPi	inorganic pyrophosphate
L	Larva
LDA	Larval Development Assays
MALDI	Matrix-Assisted Laser Desorption/Ionization
min	Minute
µl	Microliter
mM	Millimolar
mRNA	messenger Ribonucleic acid
NCBI	National Center for Biotechnology Information
NDV	Newcastle disease virus
PCR	polymerase chain reactions
RFLP-PCR	Restriction fragment length polymorphism-PCR
RNA	Ribonucleic acid
qPCR	quantitative PCR
RACE	Rapid Amplification of cDNA Ends
SNPs	single nucleotide polymorphisms
SH	Shimodaira-Hasegawa
L3	third stage larvae

TBZ	thiabendazole
TOF	Time of Flight
WAAVP	World Association for the Advancement of Veterinary Parasitology

1 Introduction

For thousands of years, poultry has been considered to be one of the main sources of animal protein providing high quality of protein for human consumption. The interest in poultry and poultry products has grown tremendously in the last years and poultry production is considered the fastest growing and most flexible compared to the other livestock sectors such as cattle, pig or sheep production. The increasing demand for poultry products has transformed poultry production activity into a fully-developed industry. To cover the global needs and keep poultry in high numbers, all the factors which constrain and limit poultry productivity should be controlled – including parasites.

Due to recent changes in consumer demands particularly in European Union (EU) states toward less intensive animal production systems including egg production and a focus more on animal welfare (Gauly et al., 2002, Blokhuis, 2004), the European directive 1999/74/EC for laying hens has forbidden conventional cage breeding systems for laying hens in the EU since 2012. Consequently, layer hens are kept using alternative production systems such as free-range outside and floor indoor husbandry, which provide environmental conditions in which layers can display all their natural behaviours.

This change in husbandry systems has resulted in an increase of the prevalence of gastrointestinal helminth infections in Germany and in the other European countries (Kaufmann et al., 2011, Sherwin et al., 2013, Wuthijaree et al., 2017). In chickens, these are mainly the two important ascarid nematodes, *Ascaridia galli* and *Heterakis gallinarum* (Kaufmann et al., 2011, Wongrak et al., 2014, Wongrak et al., 2015, Sherwin et al., 2013, Thapa et al., 2015, Wuthijaree et al., 2017). Helminth infections have an impact on poultry health, welfare and productivity (Skallerup et al.,

2005, Gauly et al., 2007). Basically, effective control of helminth infections in poultry farms relies on the use of broad-spectrum anthelmintic therapy in combination with other preventive measures, e.g. biosecurity and disinfection. Because of the wide-range action and good tolerability of benzimidazoles (BZs), they, together with the macrocylic lactones, became the drugs of choice for the treatment and control of many helminthoses in human and veterinary medicine (Horton, 2000).

Two BZs, flubendazole and fenbendazole, which have the same mode of action, have been certified for treatment of chicken nematodes within the European Union (EMA/73085/2018, 2018). Tarbiat et al. (2016) showed that flubendazole is highly effective against all developmental stages of *A. galli* in laying hens. The primary mode of action of BZs is based on the inhibitory effect on tubulin polymerization to form microtubules (Friedman, 1980). Microtubules play an essential role in a number of important cell functions such as cell division, shape and motility or intracellular transport.

In gastro-intestinal helminths of ruminants, resistance to BZs was observed within a short time after commercialized drugs came on the market (Conway, 1964). Many factors may promote the development of anthelmintic resistance (AR) such as treatment frequency, under-dosing or relying on a single-drug only (Prichard, 1990, Wolstenholme et al., 2004). Benzimidazole resistance has spread worldwide and creates an important problem for the livestock sector. It has been reported in various nematodes of ruminants and horses in different parts of the world (Ishii et al., 2017, Schwab et al., 2005, von Samson-Himmelstjerna, 2006, Kaplan et al., 2004, Ademola et al., 2015, Demeler et al., 2013, Ramünke et al., 2016). Recently, BZ resistance has also been suspected for *Ascaris lumbricoides* in school children in Rwanda (Krücken et al., 2017).

The mechanisms of BZ resistance have been comprehensively studied in the past decades (Whittaker et al., 2017). Based on broad research activities in various nematode species, it was

shown that BZ-resistance in strongyle nematodes is often correlated with single nucleotide polymorphisms (SNPs) at codons F167Y (TTC to TAC), E198A (GAA to GCA) or E198L (GAA to TTA) (Redman et al., 2015, Keegan et al., 2017, Avramenko et al., 2019) and F200Y (TTC to TAC) of the isotype 1 β-tubulin gene (Kwa et al., 1994, Kwa et al., 1995, Prichard, 2001, Ghisi et al., 2007, Von Samson-Himmelstjerna et al., 2007, Chaudhry et al., 2014, Chaudhry et al., 2015, Demeler et al., 2013). Consequently, SNPs in the β-tubulin gene particularly at the three specific codons have been used as markers for the molecular detection of BZ resistance in strongyle nematodes of veterinary importance. Regular surveys of drug efficacies on livestock farms are required to implement an effective anthelminthic management agenda (Kaplan and Vidyashankar, 2012, Sutherland and Leathwick, 2011). The established tests for detection of BZ resistance are grouped mainly into two phenotypic tests and molecular approaches. Phenotypic tests include *in vivo* methods, principally represented by the faecal egg count reduction test (FECRT) or the controlled efficacy test (CET) and *in vitro* methods such as the egg hatch assay (EHA) and the larval development assays (LDA) (Coles et al., 1992, Yazwinski et al., 2003, Coles et al., 2006). The *in vivo* FECRT is currently the most practical and comprehensive method for field diagnosis of AR. This approach is based on the quantitative assessment of nematode eggs per gram of faeces of the infected host before and after treatment. However, this approach is relatively cost-intensive due to repeated sampling and coproscopic analysis of at least 5-10 animals required to obtain a meaningful result. The reduction in faecal egg counts (FECs) is calculated either between pre and post treatment samples or between a treatment and a control group. A combination of advanced statistical analysis methods (Torgerson et al., 2014, Wang et al., 2018, Wang et al., 2017a) together with the use of sensitive coproscopical methods such as FLOTAC (Cringoli et al., 2010) and Mini-FLOTAC (Barda et al., 2013b) improved the egg count analysis and strongly improved the

calculation of 95% confidence intervals. Like the FECRT also the CET is suitable for all types of anthelmintic drugs. As it directly measure the respective worm burden it is considered the best test for evaluating their efficacy, particularly in birds (Yazwinski et al., 2003). This test is conducted by counting the parasites recovered during necropsy comparing a post anthelmintic treatment group with an untreated control group. However, the CET is only very rarely being performed due to the necessity to necropsy the tested animals. In comparison, the EHT is an inexpensive and relatively simple test. The EHT has been standardised for the detection of BZ-resistance development (von Samson-Himmelstjerna et al., 2009a). The LDT, as compared to the EHT, is very sensitive but more labour intensive and it also takes at least a week to obtain results. Generally, various molecular tests have been developed and used for diagnosis of AR against BZs including conventional PCR (Silvestre and Humbert, 2000, Njue and Prichard, 2003, Tydén et al., 2013, Tarbiat et al., 2016), real-time PCR (Alvarez-Sanchez et al., 2005, Walsh et al., 2007), droplet digital PCR (Baltrušis et al., 2018), deep-amplicon next-generation sequencing as well as pyrosequencing (von Samson-Himmelstjerna et al., 2009b, Skuce et al., 2010, Demeler et al., 2013, Morrison et al., 2014, Ramünke et al., 2016). All these techniques are based on the determination of polymorphisms in β-tubulin gene in the three above-mentioned codons. Droplet digital PCR (ddPCR), deep amplicon sequencing and pyrosequencing are considered an excellent molecular technique for BZ resistance detection even when the allele frequency is still quite low (Baltrušis et al., 2018, Avramenko et al., 2019, von Samson-Himmelstjerna et al., 2009b).

Taking into account the importance of *A. galli* and *H. gallinarum* for poultry health and production and the impact of AR on poultry industry as well as the requirement to develop sensitive molecular tests to detect the presence of resistant parasites in an early phase of resistance selection. the aim of this study, therefore, was (i) to identify β-tubulin isotypes in *A. galli* and *H. gallinarum*, (ii) to

develop pyrosequencing assays for quantitative analysis of BZ resistance associated SNPs in *A. galli* and *H. gallinarum*, (iii) to determine the frequency of BZ resistance associated SNPs in ascarids of naturally infected chickens. In the present study, pyrosequencing assays for quantitative analysis of BZ resistance associated SNPs at the codons F167Y, E198A and F200Y in β-tubulin of *A. galli* and *H. gallinarum* were developed. The assays were standardized and evaluated by analysing defined mixtures containing susceptible and resistant alleles.

2 Review of literature

2.1 Importance of poultry production

Poultry is an important source of high quality protein for human consumption (Farrell, 2005, Kaufmann et al., 2011, Ogbaje et al., 2012). The protein deficiency, especially lack of protein from animal sources, is one of the most important aspects of malnutrition and undernourishment afflicting many millions of people in different parts of the world (Ogbaje et al., 2012, Scanes, 2007). Globally, production of the primary poultry products (meat and eggs) has increased rapidly. This reflects increased consumption based, in turn, on consumer preference for these high-quality products and the relatively low price because of the efficiency of poultry production (Scanes, 2007). Chicken meat and eggs provide not only high-quality protein but also supply high-biological-value of important vitamins and minerals. Poultry plays a major role in developing countries. Production is relatively inexpensive and widely available even on a subsistence production level. The Food and Agriculture Organisation (FAO) of the United Nations stated that 7,144,416 ton s of eggs were produced in European Union countries in 2017 (FAO, 2017).

2.2 Constraints to poultry production

According to several studies, the major challenges affecting poultry production are shortage and/or cost of nutrition and diseases (Mohamed et al., 2016, Hinrichsen et al., 2016, Thapa et al., 2015). Based on the results of many researchers, a lot of losses in poultry have been linked to disease causing agents such as viruses, bacteria and parasites (Sayyed et al., 2000, Murrell, 1991). Although parasitic diseases are among the major causes that decrease productivity of chickens, they are often ignored since they are rarely lethal (Shiferaw et al., 2016). However, infections such as ascaridiosis are important since they can be associated with significant production losses (Reid and Carmon, 1958, Toledo and Castell, 1981, Skallerup et al., 2005). Moreover, it has been

suggested that helminth infections may compromise the immune response in the host leading to increased susceptibility to other pathogens, which would indirectly further decrease productivity (Degen et al., 2005, Schwarz et al., 2011, Pleidrup et al., 2014).

2.3 Important parasitic nematodes in domestic chickens

Parasitism is defined as '*a relationship between species, where one organism, the parasite, lives on or in another organism, the host, causing it some harm, and is adapted structurally to this way of life*' (Poulin, 2011) . According to this definition, all parasites are important and interfere with the performance of the host in many ways. Overall economic losses caused by individual parasite species depend on the severity of the clinical disease depending on the species-specific pathogenicity of the parasite and the prevalence of the parasite in poultry flocks, which again depends on the production system. Therefore, knowledge regarding the prevalence of important parasites in different production systems is a prerequisite to estimate their impact on productivity (Kaufmann, 2011). Parasites are a problem wherever poultry is raised and can have adverse economic effects on production parameters (Ruff, 1999). According to several studies, the most prevalent chicken nematodes in European farms are the nematodes *Ascaridia galli* and *Heterakis gallinarum* (Kaufmann et al., 2011, Permin and Hansen, 1998, Pleidrup et al., 2014, Permin et al., 1999, Irungu et al., 2004, Sherwin et al., 2013, Tarbiat et al., 2015).

2.4 Ascaridia galli

2.4.1 Taxonomy

The name *A. galli* was given by (Schrank 1788). The systematic division according to the National Center for Biotechnology Information (NCBI) is as follows (*Taxonomy ID:* 46685)

Cellular organisms

Eukaryota (Superkingdom)

Opisthokonta

Metazoa (Kingdom)

Eumetazoa

Bilateria

Protostomia

Ecdysozoa

Nematoda (Phylum)

Chromadorea (Class)

Ascaridida (Order)

Heterakoidea (Superfamily)

Ascaridiidae (Family)

Ascaridia (Genus)

2.4.2 Morphology

Adult worms are yellowish white in colour, semi-transparent, tapering towards both ends, the cuticle is distinctly striated, and cuticular alae are weakly developed, three prominent trilobed lips surrounded the oral opening, two conspicuous papillae occur on the dorsal lip and one on each of the subventral lips (Ramadan and Abouznada, 1992, Hassanen et al., 2009, Lalchhandama, 2010). The adult males reach a length of 42-76 mm (mean 63 mm) while the females appear longer and stouter than males and measure 72-108 mm (mean 85 mm) in length (Ramadan and Abouznada, 1992, Kates and Colglazier, 2011). The number of caudal papillae in males are ten pairs occurring on the ventral surface of the caudal end and they are arranged in distinct groups, *i.e* precloacal (three pairs), cloacal (one pair), postcloacal (three pairs) and subterminal (three pairs)(Ramadan and Abouznada, 1992, Lalchhandama, 2010). The number of caudal papillae in females is often difficult to determine and is probably not of much diagnostic value (Kates and Colglazier, 2011).

The male has well developed spicules measuring 1.2 to 2.9 mm (mean 2.4 mm) in length, in females vulva opens at a distance of 28.1 to 57.4 mm (mean 48.6 mm) from the anterior end (Ramadan and Abouznada, 1992). Sexual dimorphism in ascarids is morphologically characterized by a ventrally coiled tail with pre cloacal sucker in males, and a blunt and rounded posterior end in females (Lalchhandama, 2010). The eggs are elliptical and 77-94 by 43-55 µm in size. Like all eggs of ascarid nematodes, they possess a thick shell known to be highly resistant to environmental influences allowing the egg to withstand highly unfavourable conditions including the effects of ethanol and aldehyde-based disinfectants (Wharton, 1983, Katakam et al., 2014).

2.4.3 Pathogenicity of *Ascaridia galli*

Heliminthoses are considered important health problems for backyard chickens incriminated as major causes of illness and loss of productivity (Shiferaw et al., 2016). *Ascaridia galli* infects chickens of all ages, but the highest degree of damage is often found in young birds. The parasite can produce a devastating effect on growth and performance, egg production and over-all health (Junaidu et al., 2014, Höglund and Jansson, 2011, Permin et al., 2006) but even subclinical infections can increase serum testosterone concentrations and have a multitude of effects on hen behavior. Infested chickens showed a higher food intake and lower activity as well as changes in ground pecking and nesting activity during both, prepatent and patent periods (Gauly et al., 2007). *Ascaridia galli* may act as a predisposing factor for secondary bacterial infections (e.g. salmonellosis and fowl cholera) (Eigaard et al., 2006, Dahl et al., 2002, Chadfield et al., 2001). Ascarids may migrate up the oviduct (via the cloaca) to become enclosed within the laid egg (Hall, 1945). The presence of large numbers of adult parasites was associated with partial or total obstruction of the duodenum or the jejunum evantually leading to death of the host (Ikeme, 2009). In addition, *A. galli* infections, may have an immunosuppressive effect (Permin et al., 2006).

Pleidrup et al. (2014) showed that *A. galli* infection interfered with efficient vaccination and influenced both humoral and cell-mediated immune responses after vaccination against Newcastle disease virus (NDV). Thus, significantly lower NDV serum titres were found in the *A. galli*-infected group as compared to the non-parasitized group early after vaccination. Moreover, the *A. galli*-infected chickens showed significantly lower frequencies of NDV-specific T cells in peripheral blood three weeks after the first NDV vaccination as compared to non-parasitized chickens. The few immunological studies on *A. galli* infection in chickens demonstrated the systemic and local increase in interleukin (IL)-4 and IL-13 mRNA expression in splenic and ileal tissues (Degen et al., 2005, Kaiser, 2007) and Marcos-Atxutegi et al. (2009) indicated circulating IgG antibodies against the parasite starting 2 to 3 weeks post infection.

2.5 Heterakis gallinarum

2.5.1 Taxonomy

Cellular organisms

Eukaryota (Superkingdom)

Opisthokonta

Metazoa (Kingdom)

Eumetazoa

Bilateria

Protostomia

Ecdysozoa

Nematoda (Phylum)

Chromadorea (Class)

Ascaridida (Order)

Heterakoidea (Superfamily)

Heterakidae (Family)

Heterakis (Genus)

2.5.2 Morphology

Heterakis gallinarum has a typical roundworm morphology. The worm is small and white in colour, the mouth surrounded by three well-defined lips. The esophagus has a short narrow anterior portion (pharynx) and a long posterior part ending in a well-developed bulb containing a valvular apparatus. The cuticle usually has lateral flanges (Rahman and Manap, 2014, Echevarria et al., 1993). Adult males measure 7 to 13 mm in length, the tail of male has large caudal alae extending some distance down the sides of the posterior body, which bears a number of caudal papillae and a prominent pre-cloacal sucker. The two spicules are well developed, have unequal lengths and protrude out at anal opening. The female worms reach a length of 10 to 15 mm, the tail is also elongated, narrow and pointed and the vulva situated at the middle of the body (Rahman and Manap, 2014). The egg of *H. gallinarum* are approximately 55-77 by 35-48 µm, ellipsoidal, thick shelled, unsegmented when deposited and undistinguishable from those of *A. galli* (Park and Shin, 2010). Like Eggs of ascarid and due to the thick shell, *H. gallinarum* eggs known to be highly resistant to environmental influences (Barrett, 1976, Wharton, 1983).

2.5.3 Pathogenicity of *H. gallinarum*

Infection with *H. gallinarum* is generally subclinical and mildly pathogenic (Schwarz et al., 2011). Its chief pathogenic importance is as a vector of the protozoan parasite *Histomonas meleagridis*, the causal agent of blackhead disease (enterohepatitis) in turkeys. *Histomonas meleagridis* is highly pathogenic to turkeys and responsible for severe liver and caecal lesions (Brener et al., 2006, Lund and Chute, 1974, Lund and Chute, 1972, McDougald, 2005). Heavy infestation of *H.*

gallinarum is characterized by congestion, thickening and petechial haemorrhages of the mucosa as well as nodules in the caecal wall (Kaushik and Deorani, 1969, Riddell and Gajadhar, 1988).

In addition under microscopy, chronic diffuse typhlitis, haemosiderosis, granulomas with a necrotic center in the submucosa and leiomyomas in the submucosa, muscular and serosa associated with immature *H. gallinarum* worms were observed (Menezes et al., 2003).

2.6 Life cycle of ascarid poultry parasites

Ascaridia galli and *H. gallinarum* have a simple direct life cycle involving a single host (monoxen) (Figure 2.1). No intermediate host is needed to complete their lifecycle (McDougald, 2005, Herd and McNaught, 1975), which to a certain degree explains the high prevalence of these two nematodes in poultry. The susceptible host becomes infected by ingesting or drinking food or water contaminated with embryonated eggs containing infective third stage larvae (L3). The development of ascarid eggs into infective eggs containing L3 depends on several factors such as environmental temperature and relative humidity (Ackert, 1931). Tarbiat et al. (2015) observed that *A. galli* eggs developed rapidly within 7 days at 30 ˚C whereas the development time increased as the temperature was lower such as 14 days at 25 ˚C, 21 days at 20 ˚C and 90 days at 15 ˚C. In contrast, a temperature of 35 ˚C inhibited development of *A. galli* eggs (Reid, 1960, Tarbiat et al., 2015). Larvae of *A. galli* hatch in either the proventriculus or the duodenum of the infected host around 24 hours after ingestion. Larvae live in the lumen of the duodenum for several days and then penetrate the mucosa starting a tissue (histotropic) phase. The exact duration of the histotropic phase was defined by Herd and McNaught (1975) who found that is varies from 13 to 50 days depending on the infection dose. This wide variation is related to arrested development of some larvae in the tissue. Third-stage larvae return to the lumen where they moult to the fourth and later the preadult stage by 28–30 days post infection. Then, worms mature and female worms start

producing eggs (end of prepatent period) approximately 5 to 8 weeks post infection depending on host's immune status, age and length of the histotropic phase (Ramadan and Abouznada, 1992, Idi et al., 2004, Ikeme, 2009, Martis et al., 2017).

In case of *H. gallinarum*, eggs incubated at 25˚C reach the infective stage in approximately 2 weeks. When swallowed by a susceptible host, the larvae hatch in the upper intestine and reach the ceca within 24 h (Kaufmann, 2011, Permin and Hansen, 1998). The extend to which the *H. gallinarum* life cycle includes a tissue phase is not fully known. Some authors have described a histotropic phase (Van Grembergen, 1954) whereas others have recorded that the larvae are closely associated with the mucosa but a true tissue phase is uncommon, which may lead to misinterpretation and confusion surrounding this phenomenon (Kaufmann, 2011). The prepatent period of *H. gallinarum* varies between 21 and 34 days (Bauer, 2006, Fine, 1975).

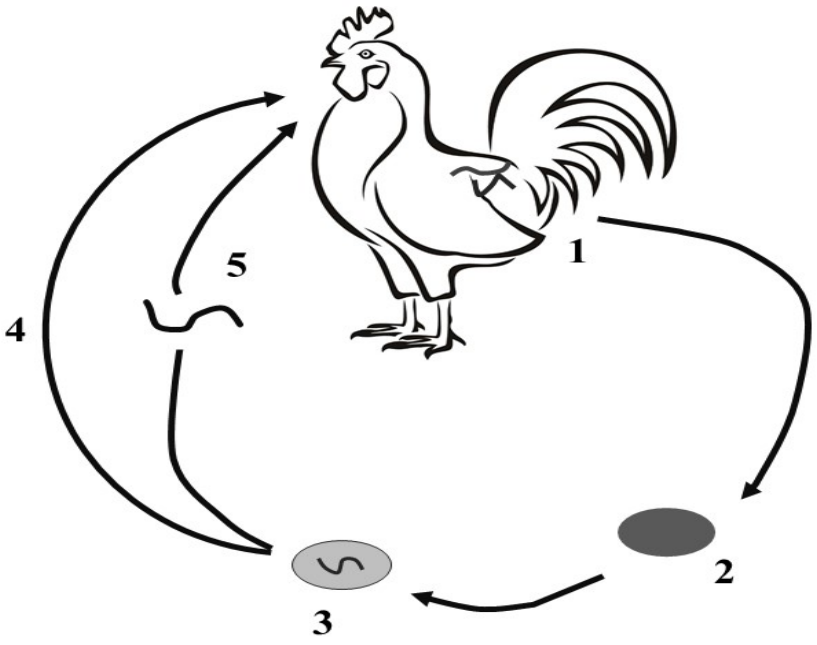

Figure 2.1 Life cycle of *H. gallinarum*. Mature female worms produce eggs that are excreted with faeces (1) and contaminate the environment (2). Eggs embryonate in the soil or litter (3) and the embryonated eggs, containing an infective L3, are ingested by hens, either directly (4) or indirectly with the intake of an earthworm as paratenic host (5).

Although not an obligate part of the life cycle, eggs of both ascarids may be ingested by paratenic hosts, i.e. earthworms, where they hatch and L3 live for months (Papini and Cacciuttolo, 2008). Earthworms may later be ingested by birds, resulting in infections with *A. galli* and/or *H. gallinarum*. Remarkably, infection of poultry with *H. meleagridis* after uptake of L3 with earthworms is also possible (Permin and Hansen, 1998, Lund et al., 1966, McDougald, 1998).

2.7 Role of management systems on the epidemiology of helminths

Ascaridia galli and *H. gallinarum* are considered the most common parasitic nematodes in birds worldwide. They infect a wide spectrum of hosts including chicken, goose, guinea fowl, turkey and various wild birds (Ackert, 1931, Ramadan and Abouznada, 1992, Skallerup et al., 2005, Lund and Chute, 1974, Daş et al., 2014). However, the economically and epidemiologically most important host is presumably the chicken (Daş and Gauly, 2014, Kates and Colglazier, 2011). Many factors contribute to the transmission and spread of these nematodes in poultry farms. Among others, the flock management system has a powerful effect on the particular extend of the problem that is seen (Ruff, 1999). Poultry eggs production systems, like the wide majority of other modern animal husbandry systems, are highly industrialized to increase the quantity and capacity of production. This infers restricted housing, power ventilation, mechanical feeding and automatic egg collection aiming at the reduction of production costs to increase economic gain at the market (Kaufmann, 2011). Therefore the majority of hens in important egg producing countries were kept in laying cages which fitted the mentioned requirements best and were (and still are) the most economical way to produce eggs (Van Horne and Achterbosch, 2008). Furthermore, these cage systems had a positive effect in providing good conditions for infectious disease prevention mainly parasitic infection (Hulzebosch, 2006). In the last two decades, animal welfare in a commercial poultry production is an important topic in Europe and in other parts of the world, too. Koknaroglu and Akunal (2013) defined animal welfare and stated that providing environmental conditions in

which animals can display all their natural behaviours is important. Changed consumer attitude demanded for changes towards less-intensive animal production systems with the intention that egg productions system should focus more on animal welfare (Van Horne and Achterbosch, 2008, Kaufmann et al., 2011). Thus, poultry welfare received more legislative attention in the EU than in many other countries of the world (Van Horne and Achterbosch, 2008). The behaviour of laying hens has been studied intensively and it has been shown that normal behavioural traits such as perching, nesting and dust bathing cannot be performed in conventional cage systems (Abrahamsson and Tauson, 1995). For that reasons, in 2012 the EU directive 1999/74/EC (Anonymous, 1999) entered into force prohibiting conventional cage systems for laying hens EU-wide. Consequently, the cage systems (excluding enriched cages) have been gradually replaced by alternative egg production systems (Hinrichsen et al., 2016, Kaufmann et al., 2011). In an enriched cage, each hen has at least 750 cm², a perch, a nest box and litter (Van Horne and Achterbosch, 2008). Alternative production systems provide highest animal welfare standards (Tuyttens et al., 2008). Germany is one country that has 'gold plated' the directive, banning conventional cages. Thus, in Germany, the percentage of alternative production system farms increased from 15% in 2001 to 63% in 2009 (Marktinfo Eier und Geflügel (MEG), 2010, Zentrale Mark und Preisberichtstelle (ZMP) GmbH, 2008). Since January 2011, all laying hens are kept in alternative husbandry systems as the German government and House of Representatives decided to overrule the directive with a relevant national directive (TierSchNutzV, 2009). The freedom of movement of poultry, as an important factor of alternative animal husbandry (Berg, 2002), increases the chance and risk of infection with several parasites including nematodes, as hens are in contact with faeces allowing helminths to complete their lifecycle, the chickens pick up the parasite eggs directly by ingesting contaminated feed, water and litter. Several authors (Permin et al., 1999,

Sherwin et al., 2013, Martín-Pacho et al., 2005, Kaufmann et al., 2011, Hinrichsen et al., 2016, Jansson et al., 2010) emphasized high prevalences and worm counts in alternative production systems and consequently biosecurity in these production systems seems to be fairly poor. However, as such husbandry systems established, researchers and experts are requested to study impacts, effects and constrains of such production systems, and provide the opportunity that these alternative production systems are able to meet their high expectations.

2.8 Diagnosis

Traditionally, infections with acarids in chicken can easily be diagnosed, either by identifying the eggs in the faeces using a simple flotation method, a modified McMaster method, FLOTAC or mini-FLOTAC. Furthermore, diagnosis can be made post mortem by identifying the worms directly in the intestine.

2.9 Prevention and control

Biosecurity, flock management systems and practices broadly determine the extent of helminthosis in poultry flocks. Total enclosure, improvement of cleaning, disinfection procedures and production according to the “all in - all out” principle have apparently decreased the significance of helminth infections in the modern industrial poultry production. With the ban on battery cages, new free-range systems have developed in which the prevention of helminth infections has proven to be considerably more difficult (Permin et al., 1999, Sommer and Vasicek, 2000, Pennycott and Steel, 2001, Shimmura et al., 2010, Kaufmann et al., 2011). The use of out-door areas, where parasite eggs may persist in the environment for years has increased the risk of helminth infections. Management practices including alternate use of the pen and strict disinfection of the house might subsequently reduce problems with helminth infections. In addition to management practices, prevention and control can also be obtained through a range of available drugs (Ruff, 1999).

Strategic use of broad-spectrum anthelmintics to either prevent parasite infections or minimize production losses is still the main cornerstone of parasite control on most poultry farms. It is important to mention that no vaccine has yet been developed against *A. galli* and *H. gallinarum* (Andersen et al., 2013).

2.10 Anthelmintics

Anthelmintic treatment has proven to be applicable to many kinds of livestock and has served to reduce parasitism in them to the point where it apparently does little or no harm. Practices to treat helminth infections in poultry are extremely variable and depend on many factors such as production type, species and type (e.g. broiler vs. layers) of bird, stage of production, producer and costumers preferences as well as legal obligations (Yazwinski et al., 2013). Anthelmintics for nematode infections are recommended only after diagnosis and when prevalence and intensity of infections may likely have economic effects. Alternatively, treatments can be a part of an existing program intended to prevent the emergence of significant helminthoses (Tucker et al., 2007). Generally, a group of a few broad-spectrum anthelmintic drugs is used for treatment and control of nematodes in poultry. Piperazine adipate, levamisole hydrochloride, and pyrantel compounds have been widely used for the treatment of turkey and chicken ascaridiosis for several years (Yazwinski et al., 2009, Cruthers, 1975, Verma et al., 1991). Domestic and game birds have also been dewormed successfully with benzimidazoles (BZs). Because of their broad spectrum and good tolerance, BZs, specially fenbendazole, became the drug class of choice for treatment of nematodes in commercial poultry and game birds (Kirsch, 1984, Yazwinsri et al., 1993, Yazwinski et al., 2002, Tucker et al., 2007). Currently, only BZ drugs, e.g., fenbendazole 200 mg/ml drinking

water suspension for chicken have been approved for treatment of *A. galli*, *H. gallinarum* and within the EU member states (EMA/73085/2018).

2.10.1 Benzimidazoles

Benzimidazoles is the general name for a class of drugs that have a similar chemical structure. Thiabendazole, mebendazole, fenbendazole, albendazole, oxibendazole and oxfenbendazole among others belong to the BZ group of drugs. These molecules all contain a heterocyclic aromatic organic structure. This bi-cyclic compound consists of the fusion of benzene and imidazole rings at the 4th and 5th positions (Figure 2.2) (Barker et al., 1960).

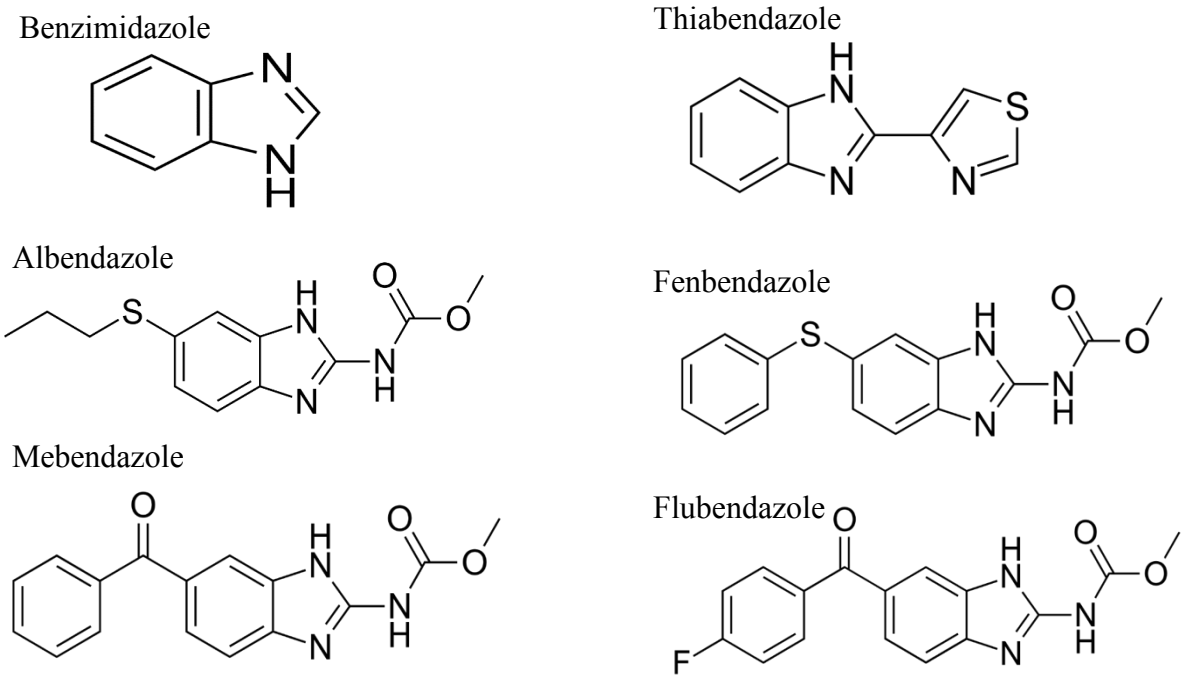

Figure 2.2 The structural formulas of BZs and some BZ group drugs (https://en.wikipedia.org/wiki/Benzimidazole) the copyright is under the Creative Commons CC0 1.0 Universal Public Domain Dedication license

Due to high potency and mild side effects of BZs, they have become the drugs of choice against most human and veterinary helminths (Horton, 2000). The first veterinary benzimidazoles introduced in the 1960's (e.g. thiabendazole, parbendazole, oxibendazole) were highly effective against adults and larvae of most gastrointestinal nematodes of livestock. In the 1970's, another group of BZs, sulphide and sulphoxide derivatives was introduced. These compounds such as albendazole, fenbendazole, flubendazole, mebendazole and oxfendazole (Figure 2.2) were not only successful in the treatment of all developmental stages of gastrointestinal nematodes but were also effective against non-gastrointestinal nematodes (e.g. in the lungs, kidneys, skin, etc., depending on compound and dose) as well as against tapeworms (Cestoda).

2.10.1.1 Spectrum of activity and mode of action of benzimidazoles

Benzimidazoles have a wide spectrum of activity against a considerable number of pathogenic internal parasites, mainly but not exclusively parasitic nematodes including *A. galli* and *H. gallinarum*. The principal mode of action of these compounds is similar, but the differences in their efficacy have been related to variability in their bioavailability (Gokbulut et al., 2007). The mechanism of action of BZs is based on the inhibitory effect on tubulin polymerization that results in the loss of cytoplasmic microtubules. Moreover, disturbance of helminth metabolic pathways was accounted as a second mechanism of action, leading to inhibition of metabolic enzymes, including malate dehydrogenase and fumarate reductase (Abongwa et al., 2017, McKellar and Scott, 1990, Prichard, 2001). Microtubules are intracellular filaments made from dimeric proteins known as α-tubulin and β-tubulin of approximately 50 kDa each. Microtubules serve a variety of functions such as movement of chromosomes during cell division, providing the structural skeleton to the cell, flagellar motility, intra-cellular transport of vesicles and organelles including axonal transport in neurons, transportation of intracellular particles including energy metabolites and

exocytosis. These microtubules are also used to develop thrusters that allow the movement of the cell (Lopus et al., 2008). The formation of microtubules is a dynamic equilibrium process, involving polymerization of tubulin dimers at one end (the positive pole) and depolymerization at the other end (the negative pole) being controlled by a range of endogenous regulatory proteins and co-factors (Lacey, 1990). Exogenous substances known as microtubuli inhibitors can disrupt this equilibrium. Most of such inhibitors apply their action by binding to tubulin dimers to prevent the self-association of dimers into growing microtubules. Thus, one of the mode of actions of the BZ-drugs is the selective binding to parasite β-tubulin following an inhibition of microtubule formation during mitosis causing a mitotic inhibition (Lacey, 1990, Martin, 1997, Lacey, 1988, Von Samson-Himmelstjerna et al., 2007). Because of the restraint of microtubulin polymerization while depolymerisation continues at the minus pole, BZs also disrupt the more stable cytoplasmic microtubules. Missing of cytoplasmic microtubules causes disruption in the uptake of glucose by the larval and adult stages of the susceptible parasites, followed by exhaustion of their glycogen stores. Degenerative changes in the endoplasmic reticulum, mitochondria and the subsequent release of lysosomes result in diminished generation of adenosine triphosphate (ATP), which is the energy required for the survival of cells. Due to diminished energy production, the parasite is immobilized and eventually dies (Lacey, 1988).

2.10.1.2 Efficacy

Because of the wide spectrum of activity of BZs for the treatment and control of helminth diseases, they are broadly used in several regimes and with high efficacy against larval and adult worms of livestock (Besier et al., 2016). The efficacy of BZs in poultry naturally infected with *A. galli* and *H. gallinarum* was determined in several studies. According to the World Association for the Advancement of Veterinary Parasitology (WAAVP) standards, anthelmintic drugs must have a

>90% efficacy to be considered "effective" (Yazwinski et al., 2003). In previous studies, fenbendazole was demonstrated to be 97% to 99% effective against all stages of *Asacriadia dissimilis* in turkey (Yazwinsri et al., 1993, Yazwinski et al., 2009). Tucker et al. (2007) gave albendazole at dose rates of 5.0, 10.0, and 20.0 mg/kg body weight to naturally infected hens and calculated anthelmintic efficacies based on geometric means of 87.7, 91.2, and 98.2% against *A. galli* larvae; 100.0, 100.0, and 100.0% against *A. galli* adults; 96.9, 95.7, and 98.9% against *H. gallinarum* larvae; and 92.7, 95.4, and 94.9% against *H. gallinarum* adults. Tarbiat et al. (2016) found that flubendazole was effective against all internal developmental stages of *A. galli* in laying hens in connection with routine deworming of two laying hen flocks on different commercial farms. In contrast, Perkins et al. (2012) found considerably lower efficacies of 80.9% and 92.3% against *A. dissimilis* in turkey treated with albendazole and fenbendazole, respectively. Yazwinski et al. (2013) demonstrated in naturally infected laying hens from an organic, free-range facility treated with fenbendazole as an oral drench at a dose rate of 5.0 mg/kg body weight efficacies of 85.5 and 89.5% against *A. galli* and *H. gallinarum* populations, respectively. Both previous studies suspected that the less than 90% anthelmintic efficacies of fenbendazole and albendazole was due to a rising resistance to benzimidazoles in *A. galli* and *H. gallinarum* and *A. dissimilis* populations. However, to date no reports of confirmed BZ resistance in *A. galli* and *H. gallinarum* have been published.

2.11 Anthelmintic resistance

2.11.1 Definition

The definition of resistance varies in different publications. The following definition is given in the Guideline on anthelmintic combination products targeting nematode infections of ruminants and horses published by the WAAVP (Geary et al., 2012) "*the ability of parasites to survive doses*

of drugs that would normally kill parasites of the same species and stage". Anthelmintic resistance (AR) has been defined as a reduction in the efficacy of a drug against populations of parasites, which are usually susceptible to this drug by a specific dose or concentration, or when a greater concentration of drug is required to reach a certain level of effectiveness (Paraud and Chartier, 2017). Prichard et al. (1980) defined resistance as: *"Resistance is present when there is a greater frequency of individuals within a population able to tolerate doses of compound than in a normal population and it is heritable".* The development of anthelmintic resistance in principle appears unavoidable, unless treatment is not applied or if the treatment kills all the targeted parasites (i.e. it is 100% efficient). Consequently, the high level of treatment of livestock with anthelmintic drugs has inevitably led to the development of resistance (Kaplan, 2004). Individual parasites that possess alleles that can disrupt the effects of the anthelmintic will have a higher chance of surviving the treatment. The survivors will be able to mate and pass these resistance alleles to their progeny. The high prevalence and severity (low efficacy) of AR is a threat to livestock production and the income of farmers.

2.11.2 Development of anthelmintic resistance

The evolution of AR is a highly complex process influenced by the host, the parasite, the environment, the treatment parameters and the anthelmintic product. At present, the resistance mechanisms for any of the number of anthelmintic substances/classes are not yet fully understood. However, the general consensus is that AR appears to be a pre-adaptive heritable phenomenon with the gene or genes conferring resistance being present within the parasite population even prior to the drug being used for the first time or readily occurring in a parasite population due to random mutation events at any time (Gilleard and Redman, 2016, Wolstenholme et al., 2004). Resistance-conferring alleles are then selected by anthelmintic pressure (Silvestre and Humbert, 2002). Under

these circumstances, resistance arises as a result of exposure of the worm population to an anthelmintic. After deworming, only worms that carry the resistance-conferring alleles should survive. For a short period, and until re-infected with anthelmintic-susceptible worms occurs, the resistant survivors are the only worms laying eggs and in this way the gene pool is enriched for alleles causing resistance (Shalaby, 2013).

2.11.3 Promoters of anthelmintic resistance in poultry

2.11.3.1 Treatment frequency

The frequent usage of the same group of anthelmintic may result in the development of AR. Resistance develops more rapidly in regions where animals are treated regularly (Leathwick and Besier, 2014, Martin et al., 1982, Coles, 1986, Gaba et al., 2006) but can also be selected for at low treatment frequencies when the same drug is used over many years (Shalaby, 2013). It has been reported that development of anthelmintic resistance can occur even when only two or three treatments were given annually (Coles et al., 1995).

2.11.3.2 Anthelmintics under-dosing

Under-dosing is the application of a lower dose (or dose rate) than that recommended by the manufacturer. Sub-therapeutic doses of anthelmintics might allow the survival of heterozygous-resistant worms, and accordingly, drug under-dosing is generally considered an important factor in the development of AR (Silvestre et al., 2001). Several laboratory experiments have shown that under-dosing contributes to the selection of resistant or tolerant strains (Woodgate et al., 2017, Knapp-Lawitzke et al., 2015).

2.11.3.3 Single-drug regimens

Frequent and continuous use of a single drug leads to the development of resistance. For example, a single drug, which is usually very effective in the first years and is continuously used will

eventually no longer work (Pal and Qayyum, 1996). Basically, long-term exposure to anthelmintic drugs will increase the frequency of resistance genes by selection of resistant mutants that will become the dominant genotype.

2.11.3.4 New animals

The introduction of new birds coming from another poultry farm, without appropriate quarantine before integration into the recipient flock, can contaminate the litter with any resistant parasites that they might carry.

2.11.3.5 Other factors

Other parameters concern the parasite population and genetics such as the initial frequency of the resistance allele within the population, the size of the population, the extent of genetic diversity, the number of genes involved in the mechanism of resistance and their relative contribution to the resistant phenotype.

2.12 Mechanism of benzimidazole resistance

Benzimidazoles as a class of broad-spectrum anthelmintics are widely used to treat parasitic nematode infections of humans and animals but resistance is more prevalent in veterinary parasites (von Samson-Himmelstjerna et al., 2009b, Krücken et al., 2017). Understanding the mechanisms and genetics of anthelmintic resistance is important to overcome resistance. Finally, understanding the processes leading to selection of resistant parasites is important to delay the development of resistance to new anthelmintic drugs, improve recommendations for use of new anthelmintics and to use better strategies for parasite control (Prichard, 2008). Benzimidazoles act by binding to the growth end of microtubules, preventing microtubules from adding new α-/ß-tubulin dimers (Sangster et al., 1985). The disruption of the tubulin polymerisation leads to the dysfunction of almost all vital processes associated with the microtubules such as cytoskeleton formation,

intracellular nutrient transport and energy consumption (Lubega and Prichard, 1991, Prichard, 1994). Nematodes have several different β-tubulin paralogs. In the model organism *Caenorhabditis elegans*, six isotypes are annotated in the genome (Zheng et al., 2017) while the ruminant parasite *Haemonchus contortus* encodes four paralogs (Saunders et al., 2013). In *Ascaris suum*, up to nine isotypes might be present in the genome (Kotze et al., 2014). In trichostrongyloid nematodes, the β-tubulin isotypes 1 and 2 have been implicated in BZ resistance (Kwa et al., 1993, Kotze et al., 2012, Keegan et al., 2017).

Based on broad research in various strongyle nematode species, BZ-resistance is associated to single nucleotide polymorphisms (SNPs) in the isotype 1 β-tubulin gene. BZ resistance is often associated with the exchange in codon 200 (F200Y, TTC to TAC) of isotype-1 β -tubulin of *H. contortus*, leading to the expression of tyrosine instead of phenylalanine (Kwa et al., 1994, Kwa et al., 1995). The same polymorphism F200Y (TTC to TAC) has been recorded in the β -tubulin of several cyathostomin species (Pape et al., 1999, von Samson-Himmelstjerna et al., 2002a). Further work identified the presence of a SNP in codon F200Y (TTC to TAC) also in association with BZ-resistance in some other trichostrongyloid species, including *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (Grant and Mascord, 1996, Silvestre and Humbert, 2002, Blackhall et al., 2011). Furthermore, (Prichard, 2001, Silvestre and Cabaret, 2002, Drogemuller et al., 2004, Melville et al., 2006, Schwab et al., 2005, Hodgkinson et al., 2008) found a SNP in codon 167 (F167Y, TTC to TAC) to be associated with BZ resistance. In *Trichuris trichiura*, Diawara et al. (2013) reported the presence of the SNP at codon 200 in worms that did not respond to albendazole treatment. Finally, a polymorphism in codon 198 (E198A, GAA to GCA) or (E198L GAA to TTA) was proposed to be involved in BZ-resistance (Ghisi et al., 2007, de Lourdes Mottier and Prichard, 2008, Keegan et al., 2017).

2.13 Detection of anthelmintic resistance

It is of high importance to diagnose AR as early as possible, whilst the frequency of resistance alleles in the parasite population is still low. In this way, the onset of AR could be delayed and the efficacy of the currently used anthelmintic drugs could be maintained for longer (Woodgate et al., 2017). The WAAVP provided guidelines on the detection of AR (Coles et al., 1992, Coles et al., 2006, Geary et al., 2012, Yazwinski et al., 2003). The established tests for detection of AR are grouped mainly into two types; phenotypic tests and molecular approaches.

2.13.1 Phenotypic tests

2.13.1.1 Controlled efficacy test/or slaughter trial

The controlled efficacy test (CET) is an in vivo test that is suitable for all types of anthelmintic drugs and considered the gold standard for evaluating their efficacy in poultry (Yazwinski et al., 2003). Because of the highly variable (over dispersed) number of parasite that may be present in individual hosts, it is recommended to have control and treatment groups containing at least 10 birds infected with each targeted parasite/stage at treatment time. Naturally or artificially infected birds are randomly assign into treated and untreated (control) groups. After a 7 days acclimatization period, the test anthelmintic is administered as recommended by the manufacturer. After treatment and an appropriate period, depending on the pharmacokinetics of the test compound, the birds are necropsied and the parasites recovered, identified and counted. The effectiveness of the compound is then calculated by the following formula (Yazwinski et al., 2003);

$$\text{percentage effectiveness} = \frac{\text{mean number of worms in controls} - \text{mean number of worms in treated animals}}{\text{mean number of worms in controls}} \times 100$$

2.13.1.2 Faecal egg count reduction test (FECRT)

This in vivo test, the FECRT, is currently the most universally applicable and practical method for field diagnosis of resistance against any anthelmintic drugs. This test is based on the microscopic detection and quantification of nematode eggs in faecal samples of the infected host before and after treatment, from which the reduction in faecal egg counts (FECs) is calculated. The WAAVP has compiled guidelines that provide details for estimating the drug efficacy with some variations between different host species. Within sheep a population of a worm species, which was fully susceptible to the drug when it was launched, is declared to be resistant if the percentage reduction is <95% and the lower 95% confidence limit is <90%. If the FECR and its lower 95% CI are higher than 95%, the population is considered to be susceptible. However, it needs to be mentioned that these guidelines were established for ruminant, horse and pig GI nematodes and until to date respective recommendations for poultry are not available. In general, detection of BZ resistance using FECRTs usually has limitations due to the difficulties (or impossibility) to differentiate parasite species in mixed infections, e.g. it is necessary to calculate one FECR over all trichostrongyloid species (Roeber et al., 2013). This results in a lack of sensitivity since a low intensity infection with a resistant worm population can be masked by co-infection with a highly fertile susceptible species.

An important factor associated with limitations in the reliability and reproducibility of FECRT data is the method used for egg count analysis. The modified McMaster technique, with a detection limit of 50 eggs per gram (EPG), often fails to detect low numbers of eggs. Therefore, an early diagnosis of resistance is impeded by low sensitivity (Levecke et al., 2009). To enhance the sensitivity of egg counting, new techniques were introduced, such as FLOTAC (Cringoli et al., 2010) and mini-FLOTAC (Barda et al., 2013a), which nowadays offer the possibility to determine

egg counts with theoretical detection limits of 1 and 5 EPG, respectively.

2.13.1.3 Egg hatch test

The egg hatch test (EHT) is a relatively simple and inexpensive in vitro test. The essential principle of the EHT is to incubate undeveloped helminth eggs in serial concentrations of the anthelmintic, for example thiabendazole (TBZ), and to examine the inhibitory effect on the hatching of the larvae from the eggs. The percentage of hatched larvae at each concentration is determined and a concentration-response curve plotting percentage of hatched larvae (corrected for the natural mortality of untreated eggs) is plotted against the $\log_{10}$ transformed drug concentrations (Coles et al., 1992). The EC_{50} (effective concentrations for 50% inhibition of egg hatching) can be calculated using either logit or probit regression analysis. The EHT has been standardized for the detection of BZ-resistance in *H. contortus* (von Samson-Himmelstjerna et al., 2009a). Regarding the nematocidal drugs on the market, the EHT is only suitable for detection of BZ resistance.

2.13.1.4 Larval development test (LDT)

The larval development test (LDT) is a very sensitive in vitro test for the detection of AR but more labour intensive than the EHT and it takes at least a week to obtain results. This test is based on the examination of the inhibition of the embryonic and postembryonic development of parasite eggs to third larval stage (L3) in the presence of increasing concentrations of an anthelmintic. Two versions of LDT have been established; a liquid- based test (Hubert and Kerboeuf, 1992) and an agar-based test (Gill et al., 1995, Coles et al., 2006). Statistical evaluation of data is performed as described for the EHT. In comparison to the EHT, the LDT has the advantage that it is applicable for all commercially available classes of nematocidal drugs.

2.13.2 Molecular detection of AR

The molecular approaches are capable to detect resistance-associated alleles even when the frequency of these alleles is still very low (Wolstenholme et al., 2004). However, a genetic test for resistance requires the knowledge of the molecular basis of resistance and a limited number of pathways leading to resistance. The identification of mutations in target genes or the detection of alterations in the expression of non-target genes such as genes involved in metabolism of xenobiotics could lead to the development of assays for resistance detection. Possible techniques for such assays include pyrosequencing or real-time polymerase chain reactions (PCR). Currently, molecular tests for detection of resistance are only available for BZ-resistance while the molecular mechanisms for resistance against levamisole and ivermectin are insufficiently understood (Choi et al., 2017). As the mechanism of BZ-resistance has been intensively studied in the past decades (Whittaker et al., 2017), research has focused on detecting resistance with molecular tools. In veterinary medicine, β-tubulin polymorphisms are widely used as molecular markers for detection and/or quantification of BZ-resistance alleles in different trichostrongyloid nematodes.

Molecular methods such as conventional PCR (Silvestre and Humbert, 2000, Njue and Prichard, 2003, Winterrowd et al., 2003), real-time PCR (Alvarez-Sanchez et al., 2005, Walsh et al., 2007) as well as pyrosequencing (von Samson-Himmelstjerna et al., 2009b, Demeler et al., 2013, Ramünke et al., 2016, Skuce et al., 2010, Morrison et al., 2014) are all based on the detection of SNPs in one or more of the three above-mentioned codons in the β-tubulin isotype 1 gene. Intially, conventional PCR such as allele-specific PCR-based tests were employed to determine the genotype and the known SNPs related to BZ resistance in *H. contortus* (Kwa et al., 1994, Mohanraj et al., 2017) and *Telodorsagia circumcincta* (Elard et al., 1999) through using a set of specific primers that were designed to allow amplification DNA only if the primer sequence is perfectly complementary with the respective sequence at the SNP site of the β-tubulin sequences in the

susceptible or resistant genotypes. Restriction fragment length polymorphism-PCR (RFLP-PCR) procedures combined with the allele-specific amplification of a β-tubulin were used for detecting BZ resistance allele in *H. contortus*, *Trichostrongylus colubriformis and Telodorsagia circumcincta* (Silvestre and Humbert, 2000) and several cyathostomin species (von Samson-Himmelstjerna et al., 2002a).

Real-time PCRs have been described for frequency quantification of BZ resistance alleles in *H. contortus* for SNP F200Y (Walsh et al., 2007) and SNP F167Y (Lambert et al., 2017) in small ruminants (Rashwan et al., 2017). Recently, developed a rapid genotyping assays to determining the BZ-resistance associated β-tubulin SNPs in *T. trichiura* and *Ascaris lumbricoides* based on the SmartAmp2 method. Digital droplet PCR (ddPCR) was used for the quantification of sequence variations, including SNPs, due to its high accuracy and precision in comparison to techniques such as quantitative PCR (qPCR) (Baltrušis et al., 2018). As it was initially described, ddPCR has been shown to have many advantages regarding the ability to detect and accurately quantify frequency of minor sequence variants in a predominating background of wild-type sequences (Hayden et al., 2013). Recently, Baltrušis et al. (2018) developed a ddPCR protocol for identification of BZ resistance in *H. contortus* detection and quantification of the polymorphism occurring at the β –tubulin isotype-1codon 200 (F200Y, TTC to TAC).

2.13.2.1 Pyrosequencing

The principle of pyrosequencing was first described by Nyren et al. (1993) and then further developed (Ronaghi et al., 1996, Ronaghi et al., 1998). Pyrosequencing is a sequencing technique for short DNA fragments on the basis of the detection of pyrophosphate, a normal by-product of DNA synthesis which is like Sanger sequencing based on the "*sequencing-by-synthesis*" principle. It is based on a cascade of enzymatic reactions during DNA synthesis when the inorganic

pyrophosphate (PPi) is released from a nucleotide incorporated by a DNA polymerase into the DNA polymer. Sulfurylase catalyzes the reaction of PPi with adenosine 5' phosphosulfate yielding adenosine triphosphate (ATP). The visible light is generated as the result of luciferin oxidation by luciferase with the concomitant emission of light proportional to the amount of ATP. A charge-coupled device (CCD) camera records the emitted light in the form of a peak in a pyrogram. The intensity of light produced and subsequently the height of the peak is dependent on the number of nucleotides added (Figure 2.2). After each peak, the reaction is stopped by apyrase leading to degradation of PPi and all nucleotide triphosphates.

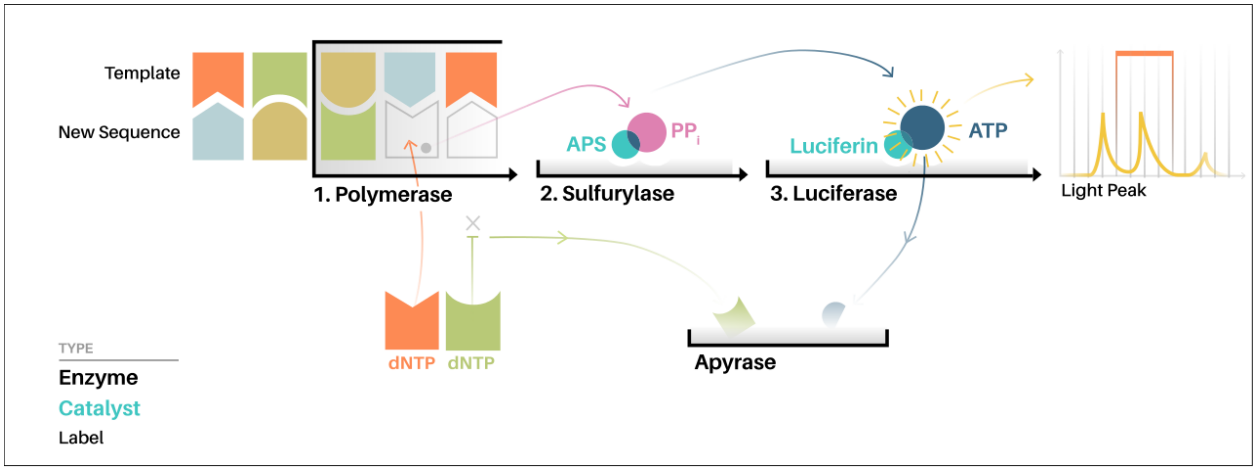

Figure 2.3 Inorganic pyrophosphate (PPi) in the presence of the substrate adenosine 5' phosphosulfate and the enzyme sulfurylase leads to ATP production, visible light is generated in proportion to the amount of ATP in the presence of luciferase and is detected as a peak on a pyrogram. Apyrase, a nucleotide-degrading enzyme, continuously degrades unincorporated nucleotides, ATP and PPi. https://en.wikipedia.org/wiki/Pyrosequencing. Image is released under CC-BY-SA licence.

The molecular techniques based on the analysis of β-tubulin isotype 1 BZ-resistance-associated SNPs through the use of PCR followed by pyrosequencing are highly sensitive for the detection of BZ-resistance development (von Samson-Himmelstjerna et al., 2009a, Demeler et al., 2013,

Ramünke et al., 2016, Morrison et al., 2014). The pyrosequencing approach principally has the capacity for quantitative analysis of SNP frequencies associated with resistance thus making it an excellent and molecular technique for BZ-resistance detection.

The pyrosequencing technique offers several advantages. It is a rapid and accurate technique to analyse allele frequencies in nematodes (Alderborn et al., 2000, Ronaghi et al., 1996), it can determine more than one SNP in one target (Lötsch et al., 2003, Pourmand et al., 2002), which makes it beneficial in the analysis of BZ resistance, as often more than one SNP is present in a parasite population. Another advantage of this technology compared with other techniques such as real-time PCR, matrix-assisted laser desorption/ionization (MALDI) followed by time of flight (TOF) analysis, mass spectrometry and mini-sequencing is that pyrosequencing allows to estimate the template quantity through the light signal intensity measurements (von Samson-Himmelstjerna, 2006). Pyrosequencing assays were developed and conducted for determining and monitor BZ-resistance in several nematodes of livestock. von Samson-Himmelstjerna et al. (2009b) concluded that drug sensitivity of pooled L3s isolates with different levels of resistance to BZ decreased with increasing F200Y (TAC) frequency. Demeler et al. (2013) established a pyrosequencing assay to determine BZ-resistance associated SNPs in isotype 1 β-tubulin codons 167, 198 and 200 of *Cooperia oncophora* and *Ostertagia ostertagi* in cattle. Species-specific BZ-resistance pyrosequencing assays have been established for a number of sheep nematodes species (e.g. *H. contortus*, *Teladorsagia circumcincta*, *Trichostrongylus* spp., *Nematodirus battus*) (Von Samson-Himmelstjerna et al., 2007, von Samson-Himmelstjerna et al., 2009b, Skuce et al., 2010, Morrison et al., 2014, Ramünke et al., 2016). However, to date no reports of developing a pyrosequencing assay to detect BZ-resistance associated SNPs in β-tubulin isotypes in any ascarid nematode infecting livestock including *A. galli* and *H. gallinarum* has been described.

3 Materials and methods

3.1 Materials

3.1.1 Chemicals

Name	Manufacturer
Agar-Agar Kobe I powder	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Bacillol® AF disinfection	BODE Chemie GmbH, Hamburg, Germany
Bromophenol	Sigma-Aldrich Corporation, St. Louis, USA
EDTA Disodium salt dihydrate ≥ 99 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Acetic Acid 100 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ethanol absolute ≥ 99,5 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Glycerin ROTIPURAN® ≥ 99,5 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
LB-Medium	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
LE Agarose powder	Biozym Scientific GmbH, Hess. Oldendorf, Germany
Methanol 100 %	Sigma-Aldrich Corporation, St. Louis, USA
Sodium hydroxide	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Tris (hydroxymethyl) aminomethane acetatePUFFERAN®, ≥ 99%	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sulfuric acid	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside)	Sigma-Aldrich, Taufkirchen, Germany

3.1.2 Consumables

Name	Manufacturer
Biosphere® pipette tips with filter 10 µl, 100 µl, 1000 µl	Sarstedt AG + Co., Nümbrecht, Germany
BZO Seal Film, Adhesive Optical Film	Biozym Scientific GmbH, Hess. Oldendorf, Germany
Falcons 50 ml	Sarstedt AG + Co., Nümbrecht, Germany
NitriCare® gloves nitrile size M	Showa Best Glove S.A.S., France
rotiprotect® gloves latex size M	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Slides	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
PARAFILM® M	Bemis Company, Inc., Neenah, USA
PCR SingleCap 8er-Softstrips 0,2 ml, farblos, dorned cap	Biozym Scientific GmbH, Hess. Oldendorf, Germany
Petri dishes Ø 9 cm	Sarstedt AG + Co., Nümbrecht, Germany
Pipette tips 5 ml, Typ Eppend.	Sarstedt AG + Co., Nümbrecht, Germany
Rotilabo® Sterile Indicator Tape for Steam Sterilization	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Microcentrifugation tubes 0.5 µl, 1 µl, 1,5 µl and 2µl	Sarstedt AG + Co., Nümbrecht, Germany
Pipette tips 0.5-10 µl, 10-100 µl, 100-1000 µl	Sarstedt AG + Co., Nümbrecht, Germany
Scalpel blades	Henry Schein Inc., Melville, USA
Disposal plastic bags	Sarstedt AG + Co., Nümbrecht, Germany
Ventilated cell culture flask	Sarstedt AG + Co., Nümbrecht, Germany

Wooden sticks	Roth, Karlsruhe GbmH, Germany
Beads innuSPEED Lysis Tubes F	Jena Bioscience, Jena, Germany
Adhesive PCR plate foil	Bio-Rad Laboratories GmbH, München, Germany

3.1.3 Enzymes

Name	Manufacturer
AccuPrime™ Taq polymerase	Invitrogen, by Thermo Fisher Scientific cat No. 1-12339-016, Van AllenWay Carlsbad, USA
*Eco*RI 10 U/µl Restriktionsenzym	Thermo Fisher Scientific, Inc., Waltham, USA
Phusion Hot Start II High-Fidelity DNA Polymerase 2 U/µl	Thermo Fisher Scientific, Inc., Waltham, USA
Proteinase K	Macherey und Nagel, Düren, Germany
Transcriptor Reverse Transcriptase	Roche Diagnostics GmbH, Mannheim,Germany
Terminal Transferase recombinant 80U/µl	Roche Diagnostics GmbH, Mannheim,Germany
Transcriptor Reverse Transcriptase	Roche Diagnostics GmbH, Mannheim,Germany

3.1.4 Devices and equipment

Name	Manufacturer
ALC-1100.2 balance	Acculab, Sartorius AG, Göttingen, Germany
Anxiostar plus light microscope	Carl Zeiss AG, Oberkochen, Germany
Centrifuge 54 30 R	EPPENDORF, Hamburg Germany
C1000™ Thermal Cycler	Bio-Rad Laboratories, Inc., Hercules, USA
Digital Graphic UP-D807 Printer	Sony Corporation, Tokio, Japan
Electrolux refrigerator	Elektrolux AB, Stockholm, Sweden
Epoch BioTek® Instrument	BioTek®, Bad Friedrichshall, Germany
Eppendorf Research pipettes 0,5 - 10 µl, 10 - 100 µl, 100 - 1000 µl und 500 - 5000 µl	Eppendorf AG, Hamburg, Germany
Forma 900 Series freezer	Thermo Fisher Scientific, Inc., Waltham, USA
G box	SynGene Europe, Cambridge, UK
HI208 Educational pH meter	Hanna® Instruments, Woonsocket, USA
IKA® COMBIMAG RCT Magnetic Stirrer	IKA® Works, Inc.,Wilmington, USA
IKA® Vortex Genius 3 Vortexer	IKA® Works, Inc.,Wilmington, USA
LABOKLAV autoclave	SHP Steriltechnik AG, Detzel Schloss/Satuelle
LIEBHERR freezer	Liebherr-International AG, Bulle, Switzerland
Max Q 6000 incubator	Thermo Fisher Scientific, Inc., Waltham, USA
Maxwell® 16 Research Instrument	Promega Corporation, Madison, USA
Microwave	Sharp Electronics GmbH, Hamburg, Germany
Microscope	Carl Zeiss, Jena, west Germany
Mini FLOTAC counting chamber	University of Naples Federico, Naples, Italy
PCR Chamber laboratory workbench with UV light	PLAS Labs, Inc., Lansing, USA
Power-Pac™ Universal Power-Supply	Bio-Rad Laboratories, Inc., Hercules, USA
PyroMarkQ24 work station, cartridge, instrument	QIAGEN, Hilden, Germany
ScanLaf Mars Safety Class 2 sterile bench	LaboGene Aps, Lynge, Denmark

Scotsman® AF 100 flake ice maker	Scotsman Ice Systems, Vernon Hills, USA
SpeedMill Plus Homogenizer	Analytik Jena AG, Jena, Germany
Stainless steel sieves 250µm, 160µm, 100µm, 25µm	Retsch® Hann, Germany
Take 3 ™ Multi-Volume Plate	BioTek® Instruments, Inc., Winooski, USA
TMix shaking incubator	Analytik Jena AG, Jena, Germany
Wide Mini-Sub® Cell GT gel electrophoresis chamber	Bio-Rad Laboratories, Inc., Hercules, USA
WNE 45 water bath	Memmert GmbH + Co. KG, Schwabach

3.1.5 Buffers and Solutions

Name	Manufacturer
Aqua bidest - double distilled H_2O from the TKA-Genpure ultrapure water system	Jens Löwe Water Treatment and Cleaning Systems, Berlin
DEPC - treated water, bioscience grade, nuclease free and autoclaved	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
dNTP Mix, 10 mM	Thermo Fisher Scientific, Inc., Waltham, USA
5 × Phusion HF Puffer	Thermo Fisher Scientific, Inc., Waltham, USA
50 × TAE Puffer	Own preparation
Roti® Nucleic Acid Free Spray	Carl Roth GmbH + Co. KG, Karlsruhe
6 × Loading Dye	Own preparation
10 × Puffer *Eco*RI	Thermo Fisher Scientific, Inc., Waltham, USA
5X Reaction Buffer	Thermo Fisher Scientific, Inc., Waltham, USA
RiboLock RNase Inhibitor (20 U/µL)	Thermo Fisher Scientific, Inc., Waltham, USA
RevertAid M-MuLV RT (200 U/µL)	Thermo Fisher Scientific, Inc., Waltham, USA
Oligo $(dt)_{18}$ primer	Thermo Fisher Scientific, Inc., Waltham, USA
cDNA Synthesis Buffer	Roche Diagnostics GmbH, Mannheim,Germany
Deoxynucleotide Mixture	Roche Diagnostics GmbH, Mannheim,Germany
10X Reaction Buffer	Roche Diagnostics GmbH, Mannheim,Germany
dATP 2mM	Roche Diagnostics GmbH, Mannheim,Germany
Oligo dT-Anchor Primer	Roche Diagnostics GmbH, Mannheim,Germany
PCR anchor Primer	Roche Diagnostics GmbH, Mannheim,Germany
PyroMark Binding Buffer, Wash Buffer, Annealing Buffer	QIAGEN, Hilden, Germany
Sodium Hydroxide solution 0.2M	Carl Roth GmbH + Co. KG, Karlsruhe

3.1.6 DNA-Marker

Name	Manufacturer
GeneRuler™ 100 bp Marker	Thermo Scientific™ Karlsruhe GmbH, Germany
GeneRuler™ 1 kb Marker	Thermo Scientific™ Karlsruhe GmbH, Germany
λ *Eco*RI/HindIII Marker	Thermo Fisher Scientific, Inc., Waltham, USA
GR Green Nucleic Acid Stain 10.000 × stock	LABGENE Scientific, Châtel-St-Denis,Switzerland

3.1.7 Reaction Kits

Name	Manufacturer
AccuPrime™ Taq DNA Polymerase System	Invitrogen, by Thermo Fisher Scientific cat No. 12339-016, Van AllenWay Carlsbad, USA
EasyPrep Pro Plasmid Miniprep Kit	Biozym Scientific GmbH, Hess. Oldendorf
Maxwell® 16 LEV simplyRNA Tissue Kit	Promega Corporation, Madison, USA
NucleoSpin® Tissue Kit	Macherey und Nagel, Düren, Germany
NucleoSpin® Tissue XS Kit	Macherey und Nagel, Düren, Germany
Phusion® Hot Start II	Fermentas/ Thermo Scientific, Waltham, USA
PyroMark Gold Q24 reagents	QIAGEN, Hilden, Germany
StrataClone Blunt PCR Cloning Kit	Agilent Technologies, Santa Clara, USA
StrataClone Solopack® competent cells	Agilent Technologies, Santa Clara, USA
Thermo Scientific RevertAid First strand cDNA synthesis Kit	Thermo Fisher Scientific, Inc., Waltham, USA
ZymoPURE™ Plasmid Midiprep Kit	Zymo Reasearch, Irvine, USA
Zymoclean™ Gel DNA Recovery Kit	Zymo Reasearch, Irvine, USA
5'/3' RACE kit 2nd generation version 13	Roche Diagnostics GmbH, Mannheim,Germany

3.1.8 Software

Name	Manufacturer
BLAST Software	(Altschul et al., 1990) National Center for Biotechnology Information, Bethesda, USA
Clone Manager 9 Professional Edition	Scientific & Educational Software, USA
Endnote X8	Thomson Reuters, New York, USA
Gen5™ Datenanalyse	BioTek® Instruments, Inc.,Winooski, USA
GeneSnap Version 7.09.06	SynGene Europe, Cambridge, UK
GraphPad Prism5 Version 5.03	GraphPad Software, Inc. USA
MEGA7 Version 7.0.26	(Kumar et al., 2016)
Microsoft Excel 2010	Microsoft Corporation, Redmond, USA
Microsoft Word 2010	Microsoft Corporation, Redmond, USA
PyroMarkQ24 software	QIAGEN, Hilden, Germany

3.1.9 Preparation of solutions, buffers and reagents

If not explizitly stated different, all solutions were prepared using water from a TKA Genpure water purification system and had an electrical conductivity below 0.055 µS/cm.

3.1.9.1 TAE Puffer (50×)

For this purpose, 242 g of Tris base were transferred via a funnel into a 1000 ml Erlenmeyer flask. To prepare the 500 mM EDTA, 186.1 g of EDTA disodium dihydrate were stirred in 800

ml of water in an Erlenmeyer flask and sodium hydroxide pellets were added until a pH of 8.0 was reached and the EDTA was dissolved. Subsequently, 100 ml of 500 mM EDTA were added to the Erlenmeyer flask containing the Tris base. This was followed by addition of 57.1 ml of acetic acid, and the mixture was made up to 1000 ml with water. Before use, the buffer was diluted 1:50 with water.

3.1.9.2 100 bp, 1 kb und λ*Eco*RI/HindIII Marker

Water (94 µl) was pipetted together with 20 µl Loading Dye (6x) and 6 µl of the respective marker into a 1.5 ml eppendorf tube and mixed with a vortexer.

3.1.9.3 LB-Agar

Agar-agar powder (10 g) were mixed with 12.5 g of LB medium powder and 500 ml of water in a laboratory glass bottle and then autoclaved.

3.1.9.4 Loading Dye (6×)

Glycerol (100%, 60 ml) were added to 1 ml of 3% bromophenol blue and 10 ml of TrisCl buffer (pH 7.6) in an Erlenmeyer flask made up to 100 ml with water and mixed with the magnetic stirrer until the mixture was homogeneous. Subsequently, the Loading Dye was aliquoted into 1.5 ml tubes.

3.1.9.5 Primers

Table 3.1 List of the primers

	Primer name	Oligonucleotide sequence (5'–3')	Ta[a] °C
Degenerate primers	Primer Forward	GARGARTAYCCICGIATHATG	57.1
	Primer Reverse	IGGRTCRCAIGCIGCCATCAT	57.1
5' RACE	Hetbtub5RACE1	CTGCTTCCTTGCGAATCACATC	-
	Hetbtub5RACE2	TCTAGCACGTTGTCGACAAGCTCTGCGCCTT	60.1
	Hetbtub5RACE nested1	GCGCCTTCTGTGTAGTGACCTTTCGCCCAGTT	60.1
	Hetbtub5RACE nested2	CGATGGGACAACTGAGAACGAGCT	60
3' RACE	Hetbtub3RACE1	GTACCATTCCCACGTCTTCACTTCTTCAT	60
	Hetbtub3RACE nested1	TTCTTCATGCCTGGCTTTGCACCA	60
	Hetbtub3RACE nested2	TTCTTCATGCCCGGCTTTGCTCCG	60
***H.gallinarum* full β-tubulin**	HetbtubFull F	ATTACCCAAGTTTGAGAATCGCCTGGTTGGTT	65
	HetbtubFull R	GAAGGGAGCTGCAAGCGCAATGATGTGTGCAT	

***H.gallinarum* and *A. galli* genomic DNA**	HgbtDNAF1	GATTGTCTCCAGGGATTCCAACTGACCCATTC	65
	HgbtDNAR1	CGGACATCGTTACAGACACAAGATGGTTAAGG	
	HgbtDNAF2	GGTACTGGATCTGGAATGGGTACGCTTTTGAT	65
	HgbtDNAR2	GGGTTGGTAAGTTTCAACGTCCTGAAGCAGAT	
	AgbtDNAF1	GTCACTATACAGAAGGCGCAGAGCTTGTTGAC	47.6
	AgbtDNAR1	AAGATGATTAAGATCGCCATAGGTCGGGTTG	
	AgbtDNAF2	GCTGGTAACAACTGGGCGAAAGGTCACTATAC	47.6
	AgbtDNAR2	GCCGGACATCGTCACAGATACAAGATGATTAAG	

PCR primer sequences used for generation of the biotinylated target strand employed in the pyrosequencing assay

	Primer name	codon	Oligonucleotide sequence 5' – 3'	Amplicon length	
H. gallinarum	H.gPYR167for	167	TTGATATCTAAGATCCGCGAAGAG	122 bp	63.6
	H.gPYR167rev+B	167	Biotin- ATCCAATAGCAAACCCGATAAC		
	H.gPYR198_200for	198/200	TTTGCGCTCTCCATCCCA	214 bp	63.6
	H.gPYR198_200rev+B	198/200	Biotin-TCGGGTTGGTAAGTTTCAACG		
	H.gPYR200for	200	TTTGCGCTCTCCATCCCA	214 bp	63.6
	H.gPYR200rev	200	Biotin-TCGGGTTGGTAAGTTTCAACG		
A. galli	A.gPYR167for+B	167	Biotin-GCTTCCAATTGACGCACTCG	132 bp	63.9
	A.gPYR167rev	167	AAACCTTCGGCGATGGAACA		
	A.gPYR198_200for	198/200	GGTATTCTGGCTCCTGTTAATCAA	159 bp	56
	A.gPYR198_200rev+B	198/200	Biotin-GCGTCCTGAAGCAGATATCGTATA		
	A.gPYR200for	200	GGTATTCTGGCTCCTGTTAATCAA	159 bp	56
	A.gPYR200rev+B	200	Biotin-GCGTCCTGAAGCAGATATCGTATA		

List of the sequences of the sequencing primers applied in pyrosequencing assays

	Primer name	Oligonucleotide sequence 5' – 3'
H. gallinarum	HgPYRSeq167	AGATCGCATTATGAGCT
	HgPYRSeq198_200	CGTGGAGAACACTGATG
	HgPYRSeq200	TGGAGAACACTGATGAGA
A. galli	AgPYRSeq167	GATGGAACAACCGAA
	AgPYRSeq198_200	TCGTCGAGAACACTGAT
	AgPYRSeq200	CGAGAACACTGATGAGACAT

Species abbreviations: *H.g. Heterakis gallinarum, A.g Ascaridia galli*. Ta[a] annealing temperature, (+B) primers carried a biotin tag.

Table 3.2 Analysed sequences and programmed dispersions of dNTPs

Analysed sequences and programmed dispensations of dNTPs applied in pyrosequencing assays			
Assay	**codon**	**Sequence to analyse**	**Dispensation order**
H.gPYR167	167	CGT**W**CTCGGTTGTCCCATCGCCGAAGGTT	GCAGTACTC
H.gPYR198_200	198/200	**M**GACTT**W**CTGCATCGACAATGAAGCTTTATAT	TACGAGCTACTG
H.gPYR200	200	CTT**W**CTGCAT CGACAATGAA GCTTTATAT	GCTACTGC
A.gPYR167	167	**W**ACGAGCTCA TGATGCGATC GGGATA	GTACGAGC

A.gPYR198_200	198/200	G**M**GACAT**W**CT GCATCGATAA TGAAGCT	TGACGACGATACTG
A.gPYR200	200	**W**CTGCATCGA TAATGAAGCT TTATAC	GATCTGCA

Species abbreviations: *H.g. Heterakis gallinarum, A.g Ascaridia galli*. SNPs analysed during genotyping of β-tubulin alleles are shown in bold.

3.2 Methods

3.2.1 Samples

3.2.1.1 Collection of poultry faecal samples

In coordination with the Institute of Poultry Diseases (Freie Universität Berlin), 50 samples of at least 100 g faeces were collected from different 10 poultry farms around Berlin. Random selection was not achievable because of lack of information on number of flocks on the farms so all the faecal samples have been collected based on convenience. The farmers were asked to collect the faecal samples from multiple sites of litter belts and several manure belts in the poultry house. Each farmer was encouraged to collect five-pooled samples per flock, each consisting of at least 100 g faeces in sterile, properly labelled polythene bags. The samples were sent to the Institute of Poultry Diseases. The positive samples were identified using the Mini- FLOTAC technique with a sensitivity of 10 EPG (Rinaldi et al., 2014, Barda et al., 2013a) and as described in section 3.2.1. It was not attempted to differentiate ova of *Ascaridia* spp. and *Heterakis* spp. Thus, in most infected flocks it was not known whether *A. galli* and/or *H. gallinarum* were present, they were only recorded as positive for ascarids.

3.2.1.2 Adult worms

For the present study, 30 adult worms (male and female) from both *A. galli* and *H. gallinarum* were obtained from the Department of Animal Sciences, University of Göttingen (Germany). Worms were collected at necropsy from experimentally infected chicken. Collected worms were washed immediately with tap water and finally washed thoroughly by deionized water, all isolated worms were preserved separately in sterile cryotubes and stored at -80 °C. Samples were sent to

the Institute for Parasitology and Tropical Veterinary Medicine on dry ice. From the field, 150 *A.galli* adult worms were collected at necropsy from naturally infected hens from three different commercial laying hen farms in Sweden. Collected worms were washed immediately with tap water and finally washed thoroughly with deionized water, all isolated worms were preserved separately in sterile plastic containers with screw cap and stored at -20 °C. All samples were maintained at the Department of Biomedical Sciences and Veterinary Public Health, Uppsala, Sweden.

3.2.2 Parasitological techniques

3.2.2.1 Mini-FLOTAC technique

All faecal samples were analysed using the standard Mini-FLOTAC protocol. Each faecal sample was mixed thoroughly before 10 g were weighted and homogenized in 40 ml saturated NaCl solution (specific gravity 1.18 – 1.20 g per cm^3) and filtered through a stainless steel sieve (mesh size 250 µm). The counting chambers of the Mini-FLOTAC device were loaded with the suspension using a Pasteur pipette and left for 10 min to allow floatation of the eggs. Then, the upper part of the device was turned by 90° and samples were examined under 400× magnification (Rinaldi et al., 2014). Analytic sensitivity calculation: 40 ml of suspension contained 10 g of faeces, one filling chamber contained 1 ml of suspension, containing 0.25 g of faeces. Two filling chambers contained 0.5 g of faeces together. Assuming all eggs in this 0,5 g of faeces floated to the surface, than one egg found is equivalent to 5 eggs per gram of faeces. If no eggs were found, the faeces contained less than 5 EPG. The $EPG_{Mini\text{-}FLOTAC}$= amount of eggs found were multiplied by 10. The positive faecal samples were collected for embryonation and molecular studies.

3.2.2.2 Isolation and embryonation of nematode eggs and larval culturing

For isolation of ascarid eggs, approximately ~98 g of positive faecal samples were homogenized properly in 900 ml tap water and filtered through a 250 µm stainless steel sieve. The flow through was further washed by serially passing it through stainless steel sieves with mesh sizes of 160 µm and 100 µm before eggs were collected on a 25 µm mesh size sieve. The retained material was washed with tap water and transferred to a 50 ml screw-cap (Falcon) centrifuge tube, diluted with tap water to 50 ml and centrifuged at 190 × g for 10 min (Centrifuge 54 30 R, EPPENDORF, Hamburg Germany). The pellet was resuspended in 50 ml saturated NaCl flotation fluid (specific gravity 1.81 -1.20 g/cm^3), gently mixed and again centrifuged at 190 × g for 10 min. The supernatant containing the eggs was washed twice with tap water and centrifugation at 190 × g for 10 min to obtain clean eggs. After washing, the material was retained on a 25 µm mesh size sieve, thoroughly washed with deionised water and flushed in to a 15 ml falcon tube for centrifugation. The supernatant was discard and the sediment was collected with 1 ml water in a 1.5 ml microcentrifuge tube. For each sample, 0.5 ml aliquots were used for embryonation of the eggs by dilution into 100 ml 0.05 M H_2SO_4 in a cell culture flask with filter cap. The eggs were incubated for three weeks in the dark at room temperature and aerated once a week for 15 min. Then, the embryonated eggs were collected on a 25 µm mesh size sieve washed properly with water, transferred with 1 ml water to 1.5 ml microcentrifuge tubes, counted and stored at -80 ˚C for molecular studies. For larval culturing, the rest (0.5 ml) of the retained material was suspended into a culture flask with 100 ml tap water and incubated at 25 ˚C for 3 weeks in the dark. During this time, they were checked periodically and aerated once a week for 15 min. Then, the larvae were retained on the 25 µm mesh size sieve, washed thoroughly with water and counted before storage in 1.5 microcentrifuge tubes at -80 ˚C. The whole procedures are summarised in the process flow diagram shown in Figure 3.1

3.2.3 Molecular Methods

3.2.3.1 RNA extraction and cDNA synthesis

The Maxwell 16 LEV simplyRNA Tissue kit (Promega co. cat No. AS1280, Madison, USA) was used for extraction of RNA from the embryonated eggs, larvae and adult worms following the instructions of the manufacturer. Briefly, samples were incubated in 200 µl of chilled 1-thioglycerol/homogenization solution and further homogenized mechanically. Adult worms were homogenized using sterilized pestles in 1.5 ml Safe-Lock Eppendorf® tubes (Sigma-Aldrich Chemie Gmbh, Munich, Germany) whereas the embryonated eggs and larvae were disrupted mechanically by rapid shaking in the presence of beads. For the latter approach, samples were placed in innuSPEED Lysis Tubes F (Jena Bioscience, Jena, Germany) and shaken five times for 1 min with 1 min pausing between shaking intervals using a SpeedMill (Jena Bioscience, Jena, Germany). Afterwards, 200 µl of lysis buffer were added to the homogenate, mixed vigorously, followed by transfer of the total 400 µl into well 1 of the Maxwell 16 LEV cartridge. Genomic DNA in the extracted matter was treated using 5 µl recombinant DNase solution as described in the user bulletin. Nuclease-free water (50 µl) was added to elution tubes and the RNA extraction program was started at the Maxwell 16 instrument. The concentration of total RNA was determined photospectrometrically using a Take3® plate in a microplate reader (Epoch®, Bio Tek®) and the quality of the total RNA was assessed based on the ratio of absorbance at 260/280 nm. Approximately 100-800 ng RNA were used for cDNA synthesis using Thermo Scientific RevertAid First strand cDNA synthesis Kit and the Oligo $(dt)_{18}$ primer according to the manufacturer's recommendation. For a 20 µl reaction mixture, 4 µl of 5× Reaction Buffer, 2 µl of 10 mM dNTP Mix, 1 µl of RiboLock RNase Inhibitor (20 U/µL), 1 µl of RevertAid M-MuLV RT (200 U/µL), 1 µl of Oligo $(dt)_{18}$ primer and 11 µl of RNA were combined. The entire reagents were from Thermo Fisher Scientific, Inc. (Waltham, USA). cDNA was synthesised by incubating the

mixture at 50 °C for 45 min, followed by 60 °C for 15 min and 85 °C for 5 min. All RNAs and cDNAs were stored at -80 °C.

3.2.3.2 DNA extraction

Genomic DNA was isolated from nematode eggs, larvae and adult worms using NucleoSpin® XS Tissue Kits. Homogenization of the samples was performed as described in the previous section. DNA extraction was performed according to the manufacturer's recommendations for the purification of genomic DNA from small tissue samples. For the adult A. galli samples collected in Sweden, from each farm, five chicken were selected. Handling of the birds and euthanasia were approved by the Swedish Ethical Committee for Scientific Experi- ments (C24/10). DNA was isolated separately from the anterior portion of 10 adult worm from each chicken using the NucleoSpin® Tissue Kit following to the manufacturer's protocol for the elution of high quality and quantity of DNA. To improve DNA yield, samples were initially homogenised in 1.5 ml Safe-Lock Eppendorf® tubes in the presence of T1 Lysis buffer (180 µl) plus Proteinase K (25 µl) using a sterilised pestle. The tubes were then placed at 56 ˚C for overnight incubation in a TMix shaking incubator. The quality and quantity of the extracted DNA samples were measured in terms of 260/280 ratio using an Epoch Microplate Spectrophotometer and then the DNA was stored at -20 °C until further use.

3.2.3.3 Isolation of β-tubulin cDNAs

For the initial isolation of *A. galli* and *H. gallinarum* β-tubulin amplicons, cDNA fragments of ~444 bp were amplified after designing degenerated primers (Table 3.1) based on highly conserved regions of β-tubulin proteins of clade III nematodes. These degenerated primers spanned the entire region covering the codons 167, 198 and 200 SNPs. In the first PCR, the cDNA was amplified with AccuPrime™ Taq polymerase enzyme (Invitrogen, Thermo Fisher Scientific). PCR reactions were performed in a 25 µl volume with 2.5 µl of 10 × Accu Prime PCR buffer I, 5 µl of 100 pM

of sense primer, 2.5 µl of 100 pM antisense primer, 0.2 µl of 5 U/µl AccuPrime™ Taq polymerase, 1 µl of template cDNA and 13.8 µl nuclease-free water.

The cycling parameters for the amplification consisted of an initial denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 15 s, primer annealing at 57.1 °C for 30 s, and extension at 68 °C for 30 s, with a prolongation of the final extension of 5 min. All amplifications were carried out in a C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories GmbH, München, Germany). The amplified PCR product was separated on 1.5% agarose electrophoresis gels stained with GRGreen Nucleic Acid Stain (LABGENE Scientific, Châtel-St-Denis, Switzerland) and buffered with 1× TAE buffer. A GeneRuler 100 bp DNA Ladder (Thermo Scientific™ Karlsruhe GmbH, Germany) was used to estimate the size of the amplicons. Because of the high concentration of primers and consequently accumulation of unspecific products such as primer dimers, the DNA fragments of expected size were cut out of agarose gels under exposure to an excitation wavelength of 488 nm (blue light converter) and then purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research) according to the manufacturer's recommendations. The quantity and quality of the purified PCR products (~444 bp) were confirmed by loading onto 1.5% agarose gels with GRGreen Nucleic Acid Stain and estimated in comparison with a 100 bp marker separated in parallel. The purified DNA fragments were subjected to cloning followed by sequencing.

3.2.3.4 Gene cloning and sequencing

In the present study, all amplified DNA fragments whether derived from cDNA or genomic DNA were confirmed by gene cloning and sequencing. Amplified DNA fragments were analysed by electrophoresis on 1.5% agarose gels and bands of the expected size were excised and purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research). Purified PCR products were cloned into the StrataClone blunt-end PCR cloning vector 'pSC-B-amp/kan' supplied in the StrataClone Blunt PCR cloning kit (Agilent Technologies, CA, USA) and recombinant plasmid

vectors were transformed into StrataClone Solopack® competent cells according to the manufacturer's instructions. The cells were spread on LB agar plates mixed with chromogenic substrate X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and incubated overnight at 37 ˚C. White colonies were picked and cultured overnight in LB medium. Plasmid DNA was isolated using the Plasmid Mini Prep or Medi Prep Kit EasyPrep® Pro. Then, an aliquot of the plasmid DNA was digested using EcoRI restriction enzyme at 37 ˚C for 2 h and analysed by electrophoresis in 1.5% agarose gels. Clones with inserts were sequenced by LGC Genomics (Berlin, Germany). The resulting sequences were then identified by BLASTn and BLASTx searches in the National Center for Biotechnology Information (NCBI) database GenBank™ (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2.3.5 Development of RACE protocols for generating a full-length of *H. gallinarum* β-tubulin cDNA sequence

Since no *H. gallinarum* β-tubulin cDNA sequence was available in GenBank™ at the start of the project, the Rapid Amplification of cDNA Ends (RACE) protocol was applied for completion of coding sequences of *H. gallinarum* β-tubulin gene staring from the partial fragment generated using degenerated primers. This process was performed using the 5'/3' RACE kit 2nd generation version 13 according to the manufacturer's recommendations. cDNAs derived from the extracted RNA from embryonated eggs, larvae and *H. gallinarum* adult worms were used as template. Based on cloning and sequencing results mentioned above, gene specific primers were designed individually for the 5' and 3' ends of the of β-tubulin gene of *H. gallinarum* (Table 3.1). For the nested PCR and based on the sequence diversity of three isolated clones, two individual specific primers designed in order to determine the possible presence of different β-tubulin isotypes. AccuPrime polymerase (1 µl) was used for amplification of the 5' and 3' RACE fragments, the other reagents were from 5'/3' RACE kit 2nd generation version 13 (Roche Diagnostics GmbH).

PCR products of 3' and 5' RACE were verified by cloning and sequencing. Ten clones from 3' RACE PCR products and ten clones from 5' RACE PCR products were sequenced. After RACE PCR, partial sequences of the target cDNA were obtained that shared an overlapping region with the initial product obtained with degenerated primers. The full-length cDNA was generated by direct PCR using specific primers (Table 3.1) designed based on 5' and 3' RACE results. PCR reactions were performed in a 20 µl volume with 4 µl of 5 × Phusion HF buffer, 0.4 µl of 10 mM dNTPs, 1 µl of 10 pM of each forward and reverse primer, 0.2 µl of 2 U/µl Phusion Hot Start II High-Fidelity DNA polymerase, 2 µl of cDNA template and 11.4 µl of deionized water. The entire reagents used for PCR were obtained from Thermo Fisher Scientific. Cycling conditions were initial denaturation at 98 ˚C for 1 min, followed by 39 cycles of 98 ˚C for 30 s, annealing temperature 65˚C for 30 s and elongation at 72 ˚C for 1 min followed by a final elongation at 72 ˚C for 5 min. The resulting PCR products were purified from agarose gels and then cloned using StrataClone Blunt PCR cloning kit as described above. Plasmid DNA was isolated using the Plasmid Mini Prep Kit EasyPrep® Pro, clones with inserts were sequenced by LGC Genomics (Berlin, Germany).

3.2.3.6 Development of pyrosequencing assays for *H. gallinarum* and *A. galli*

Pyrosequencing assays targeting codons F167Y, E198A and F200Y of the isotype 1 β-tubulin genes of *H. gallinarum* and *A. galli* were developed. To develop suitable pyrosequencing assays for the analysis of *H. gallinarum,* four individual specific primers (Table 3.1) based on an alignment of the previous cDNA and RACE results were designed to amplify *H. gallinarum* genomic DNA spanning exons and intervening intron of the β-tubulin gene covering codons 167, 198 and 200. For *A. galli*, also four separated specific primers were designed (Table 3.1) to amplify *A. galli* β- tubulin genomic DNA based on an alignment of the sequences of isotype 1 from A. galli (GenBank Accession number KC713796). PCR was performed in a total reaction volume of

20 µl, which consisted of 4 µl of 5 × Phusion HF buffer, 0.4 µl of 10 mM dNTPs, 1 µl of 10 pM of each primer, 0.2 µl of 2 U/µl Phusion Hot Start II High-Fidelity DNA polymerase, and 12.4 µl of nuclease-free water and 1 µl of DNA template extracted from adult *H. gallinarum* and *A. galli* worms as previously described in section 3.3.2. The entire reagents used for these PCRs were from Thermo Fisher Scientific, Inc., Waltham, USA. Cycling conditions were initial denaturation at 98 ˚C for 1 min, followed by 39 cycles of 98 ˚C for 20 s, a primer pair-specific annealing temperature (Table 3.1) for 30 s and elongation at 72 ˚C for 1 min followed by a final elongation at 72 ˚C for 5 min. Amplification of template DNA was verified by gel electrophoresis by loading 5 µl PCR product on a 1.5% agarose gel. The DNA fragments amplified with the primers **HgbtDNAF1 and HgbtDNAR1** for *H. gallinarum* and **AgbtDNAF2** and **AgbtDNAR1** for *A. galli* (Table 3.1) were isolated from the gel, purified and cloned into StrataClone blunt-end PCR cloning vector (Agilent Technologies, CA, USA). Sequences were determined by Sanger sequencing (LGC Genomics Berlin). Based on the sequencing result, pyrosequencing assays were designed for each, *H. gallinarum* and *A. galli* β-tubulin using the PyroMark Assay Design software 2.0 (Qiagen): one assay for codon 167, a combined assay for codons 198 and 200 and one assay for codon 200 (Table 3.1). For each nematode separately, biotinylated PCR products were generated using a Biotin-labelled primer (Table 3.1). PCRs were carried out in 50 µl containing 10 µl of 5 × Phusion HF buffer, 1 µl of 10 mM dNTPs, 1.5 µl of 10 pM of each primer, 2 µl 2 U/µl Phusion Hot Start II High-Fidelity DNA polymerase, 33.5 µl of nuclease-free water and 2 µl of plasmid DNA template from the previous cloning reaction. Cycling conditions were initial denaturation at 98 ˚C for 2 min, followed by 39 cycles of 98 ˚C for 10 s, a primer pair-specific annealing temperature (Table 3.1) for 10 s and elongation at 72 ˚C for 15 s followed by a final elongation at 72 ˚C for 5 min. Amplification of template DNA was verified by gel electrophoresis before proceeding with

positive reactions to pyrosequencing. The pyrosequencing assays were conducted on the PyroMark Q24 instrument using the PyroMark Gold Q24 reagents following the manufacturer's protocols with slight modifications. In brief, the PCR plates were prepared by mixing 3 µl Streptavidin-coated Sepharose beads (Streptavidin Sepharose High Performance, GE Healthcare) with 40 µl binding buffer and 45 µl of a biotinylated PCR product (approximately 50 - 250 ng) in a 24 well polystyrene PCR plate.

The 24 well plate was sealed properly with adhesive PCR plate foil to ensure that no leakage is possible between the wells followed by agitation for 5 min at room temperature to allow binding of the biotin-labelled DNA to the beads. Using the PyroMark Q24 Vacuum Workstation, the beads together with the DNA were aspirated to a 24-format filter tool. Attached to the filter by vacuum suction, beads are washed and denaturated by successive transfer to plates containing ethanol (70%), 0.2 M sodium hydroxide solution and finally 1× wash buffers to remove the non-biotinylated DNA strand. Subsequently, the beads were released by switching off the vacuum and shaking the tool gently in a 24-well sequencing plate containing 25 µl of 0.4 mM sequencing primer (Table 3.1) in annealing buffer. The single-stranded DNA in the 24-well sequencing plate was denatured at 80 ˚C for 2 min by placing the plate on the block of a thermocycler (C1000 Touch^{TM} Thermal Cycler Bio-Rad Laboratories GmbH, München, Germany) using the PyroMark plate holder adapter. Then samples were cooled to room temperature for at least 5 min to allow annealing of the sequencing primer before the pyrosequencing run was initiated. The enzyme, substrate and nucleotides were pipetted on a PyroMark Q24 cartridge according to the volume information given in the pre run information report of PyroMark Q24 software version 2.0.8. Following the completion of the pyrosequencing reaction, the software of the instrument produces a 'pyrogram' and, based on the relative peak heights for the different alleles, a percent

quantification for the frequency of each polymorphism can be established. To determine the accuracy of the measurements and the technical background of the pyrosequencing assays, DNA fragments with artificially inserted combinations of SNPs in the codons 167, 198 and 200 were obtained by custom DNA synthesis (General Biosystems,Inc. Morrisville, USA), two fragments of β-tubulin for each parasite were amplified containing either the susceptible or the resistance associated allele at the respective codon.

Plasmid DNAs containing either the susceptibility or resistance associated allele in the target codons were mixed in 11 different ratios (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100), PCRs and pyrosequencing assays were performed using the same protocol, primers and enzymes used for generating biotinylated PCR products for pyrosequencing (see above).

3.2.3.7 Pyrosequencing assays for *A. galli* isolated from Sweden farms

For detection of BZ resistance-associated alleles in the *A. galli* β-tubulin 1 from worms collected from naturally infected chickens, adult worms were collected from three different commercial poultry farms in Sweden and the *A. galli* pyrosequencing assays developed above were used. From each farm, five birds were selected and from each bird DNA from 10 adult worms was isolated separately. As initial step prior to pyrosequening PCRs specific for *A. galli* isotype 1 β-tubulin were performed on DNA samples pooled on the chicken level and run essentially as described above. Pyrosequencing reactions for each SNP were run in three replicates and contained 40 µl (approximately 50-200 ng DNA) of the initial PCR reaction.

3.2.4 Phylogenetic analysis

The cDNA sequences for β-tubulin genes from various nematodes belonging to clade I, III and V were obtained from GeneBank. The accession number of all cDNA sequences used are shown in

Figure 4.2. Untranslated regions were removed from sequences, sequences were aligned in MEGA7 (Kumar et al., 2016) using MUSCLE (Edgar, 2004) on the protein level but displayed and exported to interleaved Phylip format as aligned codons. A maximum likelihood phylogenetic tree was calculated using RAxML 8.2.6 (Stamatakis, 2014) on the CIPRES Science Gateway server (Miller et al., 2010) fitting separate GTRGAMMA models for each partition. RAxML was used in the rapid bootstrapping mode with 1,000 replicates. The resulting tree was used as additional input to serve as a fixed tree topology for a second RAxML run to estimate node support via the Shimodaira-Hasegawa (SH) approximate likelihood ratio test (aLRT). The resulting tree with the highest likelihood was visualized and processed using MEGA7 (Kumar et al., 2016).

3.3 Statistical analysis

The prevalence of ascarid eggs in pooled faecal samples collected from poultry farms around Berlin was calculated as the number of infected faecal pools divided by total number of faecal pools (n = 50). The binomial confidence intervals for proportions were estimated with „binom.wilson“ function from epitools 0-5.10. package with R for Windows software 3.5.2. To evaluate the performance of the newly developed pyrosequencing assays for the assessment of putatively BZ-resistance associated β-tubulin SNPs of *A. galli* and *H. gallinarum*, measured allele frequencies were plotted against given frequencies of plasmid mixtures in GraphPad Prism5 Software version 5.03. Linear regressions with 95% confidence bands and Pearson correlation coefficients were calculated. In addition, the mean frequencies (in %) ± standard deviation of BZ-resistance associated SNPs in isotype 1 β-tubulin genes of the *A. galli* collected from naturally infected chicken from Swedish farms were analysed in GraphPad Prism5 Software version 5.03.

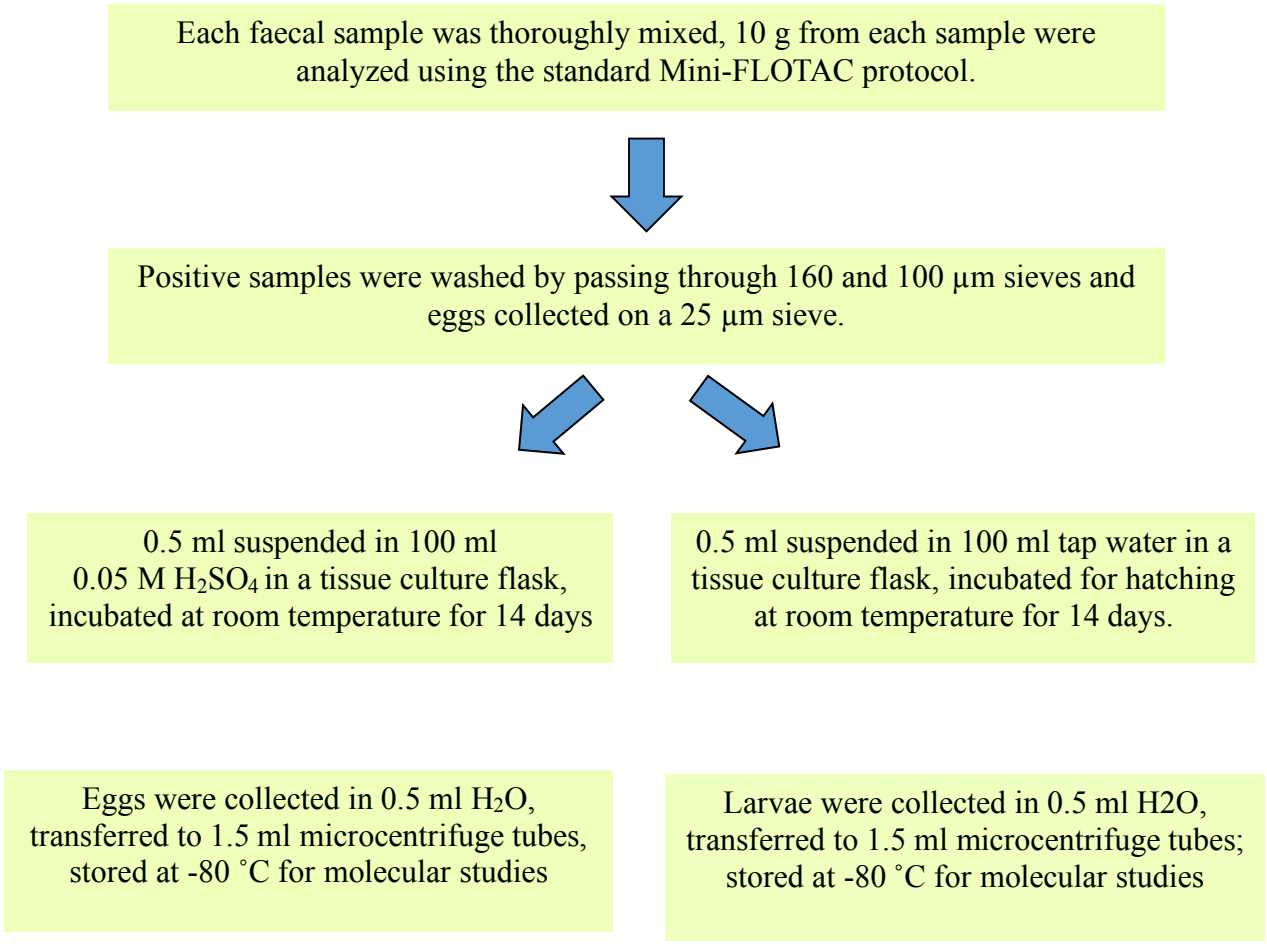

Figure 3.1 Flow chart summarizing the protocols used for isolation and embryonation of nematode egg and larval culturing.

4 Results

4.1 Faecal examination result

Out of 50 faecal samples collected from domestic layer farms that were examined using the Mini-FLOTAC method, 9 (18%) were found positive for ascarid eggs (18%, 95% CI 9.8-30.8%). The EPG of the positive faecal samples was calculated based on the analytic sensitivity of the Mini-FLOTAC method as mentioned previously in section 3.2.1 and according to the following formula:

$EPG_{Mini\text{-}FLOTAC}$= amount of eggs found × 10.

The number of ascarid EPGs of faecal samples are summarizes in Table 4.1.

Table 4.1 Number of ascarid eggs (EPG) detected using the Mini-FLOTAC procedure.

Farm code	EPG
1	200
2	400
3	250
4	150
5	350
6	250
7	250
8	650
9	400
Overall mean	**322.22**

4.2 Molecular study results

4.2.1 Characterization of β-tubulin cDNAs

For both nematode species, cDNAs representing a single β-tubulin isotype were obtained using degenerated primers designed based on highly conserved regions of β-tubulin of related nematodes. Initially, RT-PCRs were performed using cDNA derived from RNA extracted from

pooled eggs and in vitro hatched larvae using AccuPrime™ Taq polymerase and degenerated primers covering SNPs 167, 198 and 200 as mentioned in section 3.3.3. PCR reactions resulted in amplification of a partial cDNA fragment of ~444 bp which was analysed by gel electrophoresis (Figure 4.1) and confirmed by gene cloning and sequencing. Pooled eggs and larvae sequences were confirmed by comparing against the adult worm sequences and the *A. galli* β-tubulin sequence submitted previously to GenBank. A BLAST search of nine clones of *A. galli* β-tubulin fragments showed 100% identity at the nucleotide level to a β-tubulin isotype 1 sequence from *A. galli* (accession number KC713796). In contrast, at the beginning of this study, no *H. gallinerum* β-tubulin sequence was available in GenBank. To determine the possible presence of different isotypes of β-tubulin in *H. gallinarum*, 40 clones derived from eight independent RT-PCRs were sequenced. The obtained partial *H. gallinarum* sequences were 93-99% identical to each other and shared ~88% identity to the β-tubulin isotype 1 cDNA of *A. galli* (accession number KC713796), ~81% to the β-tubulin isotype 1 of *Ascaris lumbricoides* (accession number EU814697) and ~79% to the *Parascaris equorum* β-tubulin isotype 1.

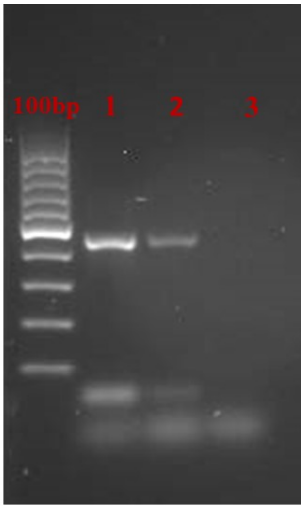

Figure 4.1 Agarose gel electrophoresis (2% agarose) of PCR amplified products using degenerated primers. Lanes 1 and 2 DNA fragment of β- tubulin using cDNA derived from *Ascaris* eggs and larvae respectively. Lane 3 nuclease free water used as negative control. A 100 bp DNA ladder was used as marker.

4.2.2 RACE results for Heterakis gallinarum

The sequencing of 3'/5' RACE PCR-products resulted in the assembly of one full-length cDNA of the β-tubulin gene of *H. gallinarum*. The complete coding sequence was 1497 bp in length.

A BLAST search using the *H. gallinarum* full-length β-tubulin open reading frame as query revealed 87.9% (1187/1349 nt) identity to *A. galli* β-tubulin isotype 1 (accession number KC713796), 83.3% (914/1137 nt) identity to *A. lumbricoides* β-tubulin isotype 1 (accession number EU814697) and 79.0% (1070/1353 nt) identity to the *P. equorum* β-tubulin isotype 1 (accession number KC713797). Based on the protein level, the identity was 98.8% (445/450), 99.7% (366/367) and 99.3% (436/439) to the *A. galli* β-tubulin isotype 1 (accession number AGM37948), *A. lumbricoides* β-tubulin isotype 1 (accession number ACJ01792) and *P. equorum* β-tubulin isotype 1 (accession number AGM37949), respectively. Although two different nested primers were used in the second PCR for amplification of 3'/5' RACE products and ten clones were selected from each 3'/5' RACE products aiming to amplify specifically minor sequence variants present in some of the 40 partial cDNA clones no other variants were found. Nucleotide sequence data is available in the GenBank™ database under the accession number MF578746.

4.2.3 Phylogenetic analysis

The full-length cDNA sequence (1353 bp) was compared to other nematode tubulins and a phylogenetic tree was calculated using the maximum likelihood method. The resulting phylogram (Figure 4.2) shows a classification of all β-tubulin isotypes. The maximum-likelihood phylogenetic analysis of *H. gallinarum* data revealed that the newly identified sequence clustered together with the previously described *A. galli* β-tubulin isotype 1 (Accession Number KC713796) with high statistical support values 100% (Figure 4.2).

Overall, there is a clear separation between β-tubulins from clade V (strongylids and *Caenorhabditis*) and clade III nematodes. The latter form a highly supported group (100% and 99% in the bootstrap analysis and the Shimodaira-Hasegawa likelihood test SH aLRT). This together with the fact that in both clusters several β-tubulin paralogs are found per species prevents to identify clear one-to-one orthologs between the ascarid β-tubulins and those of *C. elegans*. Furthermore, such one-to-one orthologs cannot even be identified in comparison to *A. suum* since the sister group in the phylogenetic tree to the *A. galli* and *H. gallinarum* group containing *A. suum* tbb-1 and tbb-2 cDNAs.

The phylogenetic maximum-likelihood analysis moreover showed that β-tubulin isotype 1 and 2 of *H. contortus*, *O. ostertagi*, *C. oncophora* and *C. catinatum* clustered together with high statistical support values of 97% by bootstrapping and 98% SH LRT tests. This branch is opposed by a branch carrying the *C. elegans* ben-1, tbb-1 and tbb-2 cDNAs. Together, these branches form the group of clade V specific paralogs. Resistance to BZs has been associated with polymorphism in the tbb-1 paralog in strongyle nematodes such as *H. contortus* and with loss-of-function in ben-1 in *C. elegans* (Kwa et al., 1994, Driscoll et al., 1989). In contrast, the only *Trichuris trichiura* cDNA encoded in the genome of this clade I parasitic nematodes, which has been associated with resistance in this parasite (Kotze et al., 2014), turned out to be closely related to a sister group containing cDNA of *T. canis* tbb-4, *A. suum* tbb4 and one *A. suum* β –tubulin. This cDNA sequence is also closely related to a sister group containing cDNAs of *Caenorhabditis* spp. tbb-4 and mec-7. This whole branch formed the mec7/tbb4-like group and did not include any of the rhabditid tbb-1, tbb-2 or ben-1 cDNAs. Overall, the phylogram reveals a considerable diversity within the nematode β–tubulin gene family.

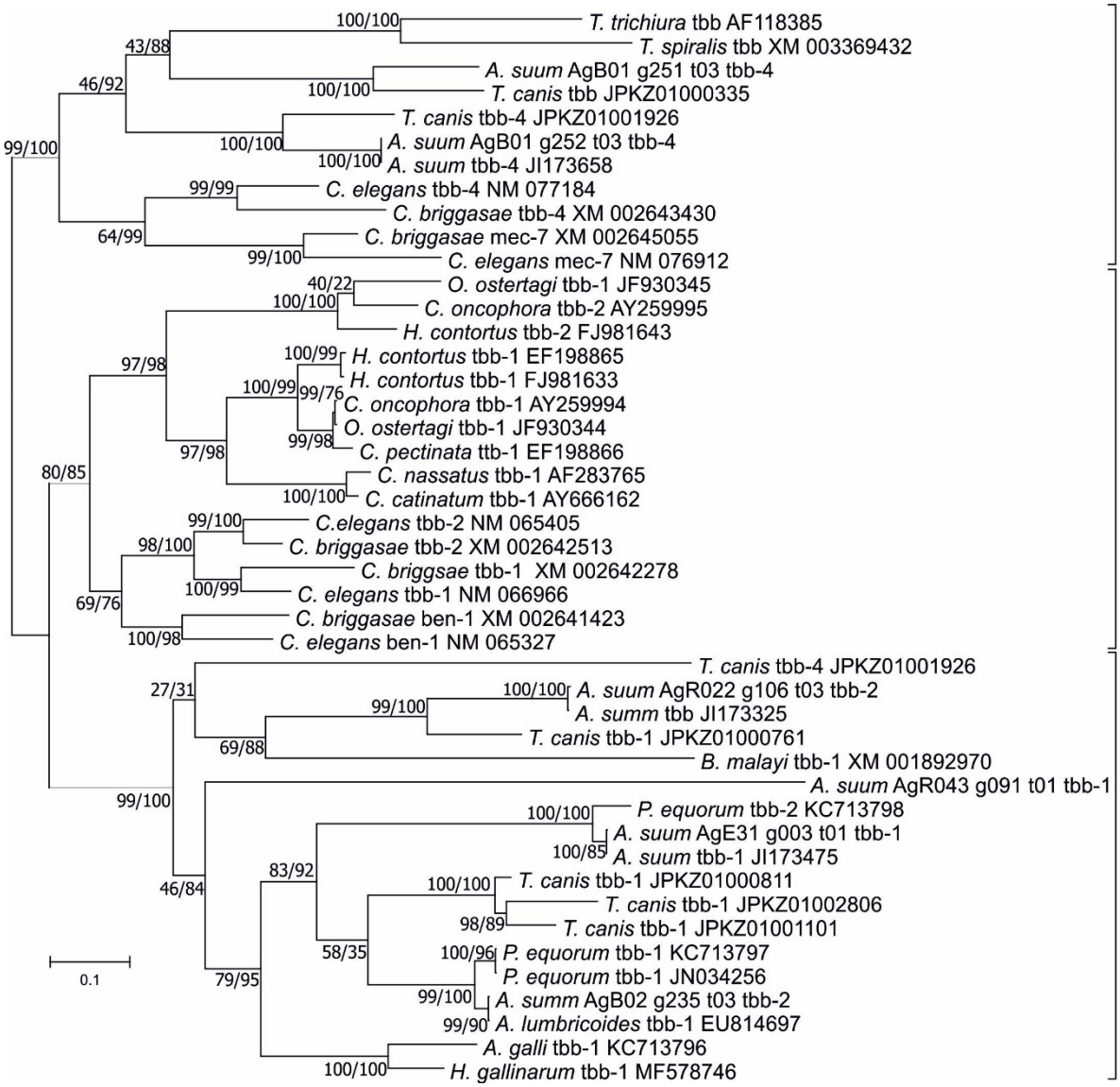

Figure 4.2 Phylogram showing the relationship between nematode β-tubulin cDNAs. Deduced proteins were aligned and the corresponding alignment of cDNAs with aligned codons was analysed by maximum likelihood analysis as described in material and methods. Statistical support according to bootstrapping and Shimodaira-Hasegawa modification of the approximate likelihood test are reported before and after the slash. The scale bar represents 0.1 substitutions per site. The cDNA sequences of *A. suum* with abbreviations like *A. suum* AgB01 g251 t03 were obtained from an *A. suum* genome (Wang et al., 2017b) deposited in GenBank under BioProject number PRJNA63155 and accession number AEUI00000000.

4.2.4 Pyrosequencing assay evaluation

To determine potential BZ-resistance associated polymorphisms in the β-tubulin genes of *H. gallimarum* and *A. galli*, pyrosequencing assays were developed for the quantitative analysis of BZ-resistance associated SNPs in the codons F167Y, E198A and F200Y.

Initially, the sensitivity of pyrosequencing assays was assed using PCRs amplifying a 122 bp and a 214 bp fragment encompassing *H. gallinarum* codons 167 and 198 & 200 respectively, and a 132 bp and a 159 bp fragment encompassing *A. galli* codons 167 and 198 & 200, respectively. Serial dilutions of 40000, 4000, 400, 40 and 4 copies of plasmid DNA containing the target sequence as insert were used as template. The reactions were analysed by gel electrophoresis and products were further used as templates for pyrosequencing. The pyrosequencing result showed that at least 40 DNA copies of *H. gallinarum* and *A. galli* β-tubulin gene were required to obtain sufficient PCR products to produce a clear, accurate pyrosequencing pattern clearly quantifiable from background noise in all three BZ resistance associated SNPs (Figure 4.4).

Standardization and evaluation of the assays were performed using plasmid DNA as template. These plasmids contained inserts with artificially synthesized genomic DNA fragments carrying combinations of SNPs in the codons 167, 198 and 200. Defined mixtures of two β-tubulin plasmids for both *H. gallinarum* and *A. galli* were amplified containing either the susceptibility- or the resistance-associated allele at the respective codon.

PCRs products were analysed by gel electrophoresis (Figure 4.3) before pyrosequencing assays were performed. Representative pyrograms are shown in Figure 4.6.

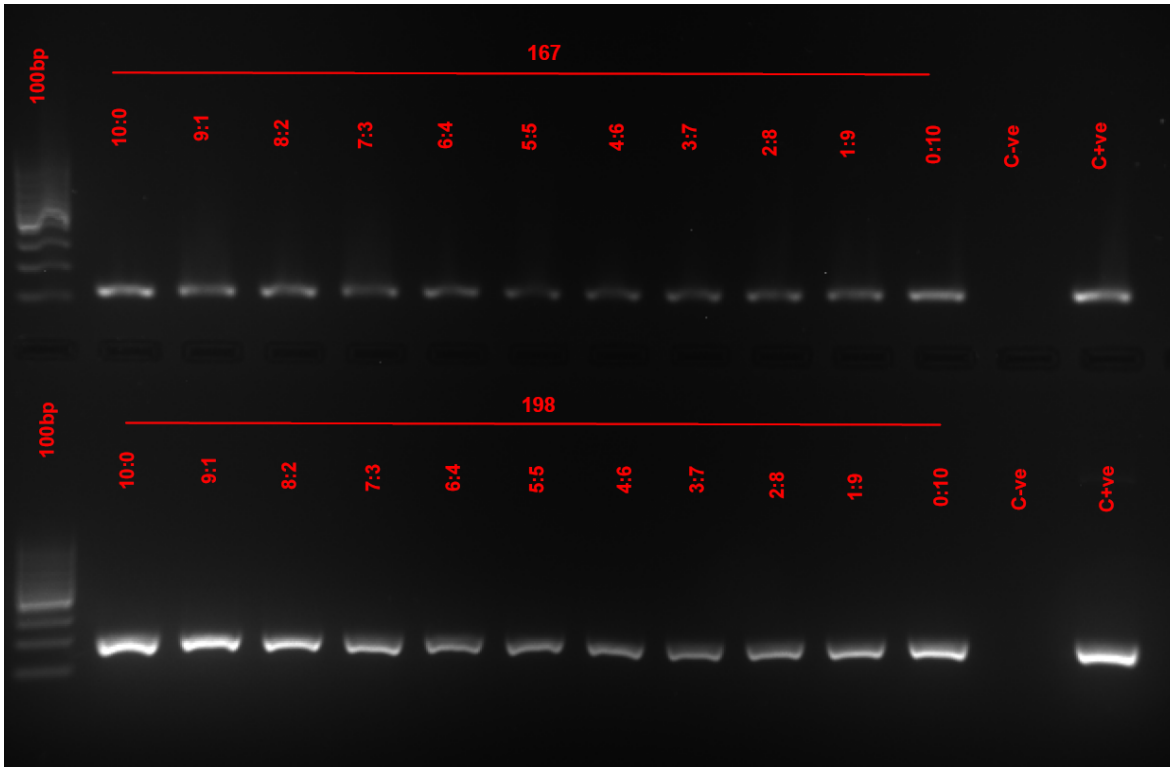

Figure 4.3 Gel electrophoresis of PCR amplified products using 2 ng of the artificially combined resistant and the susceptible alleles with the following ratios: 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10. Nuclease free water was used as negative control (C-ve) and diluted plasmid DNA (1:1000) was used as positive control (C+ve).

For analysis, measured frequencies were plotted against calculated input frequencies and linear regression analyses were performed. Excellent correlations ($R^2 > 0.98$) of calculated and observed frequencies for the SNP determination at codons 167, 198 and codon 200 in the β-tubulin 1 genes were obtained for *H. gallinarum* (Figure 4.5A) and *A. galli* (Figure 4.5B).

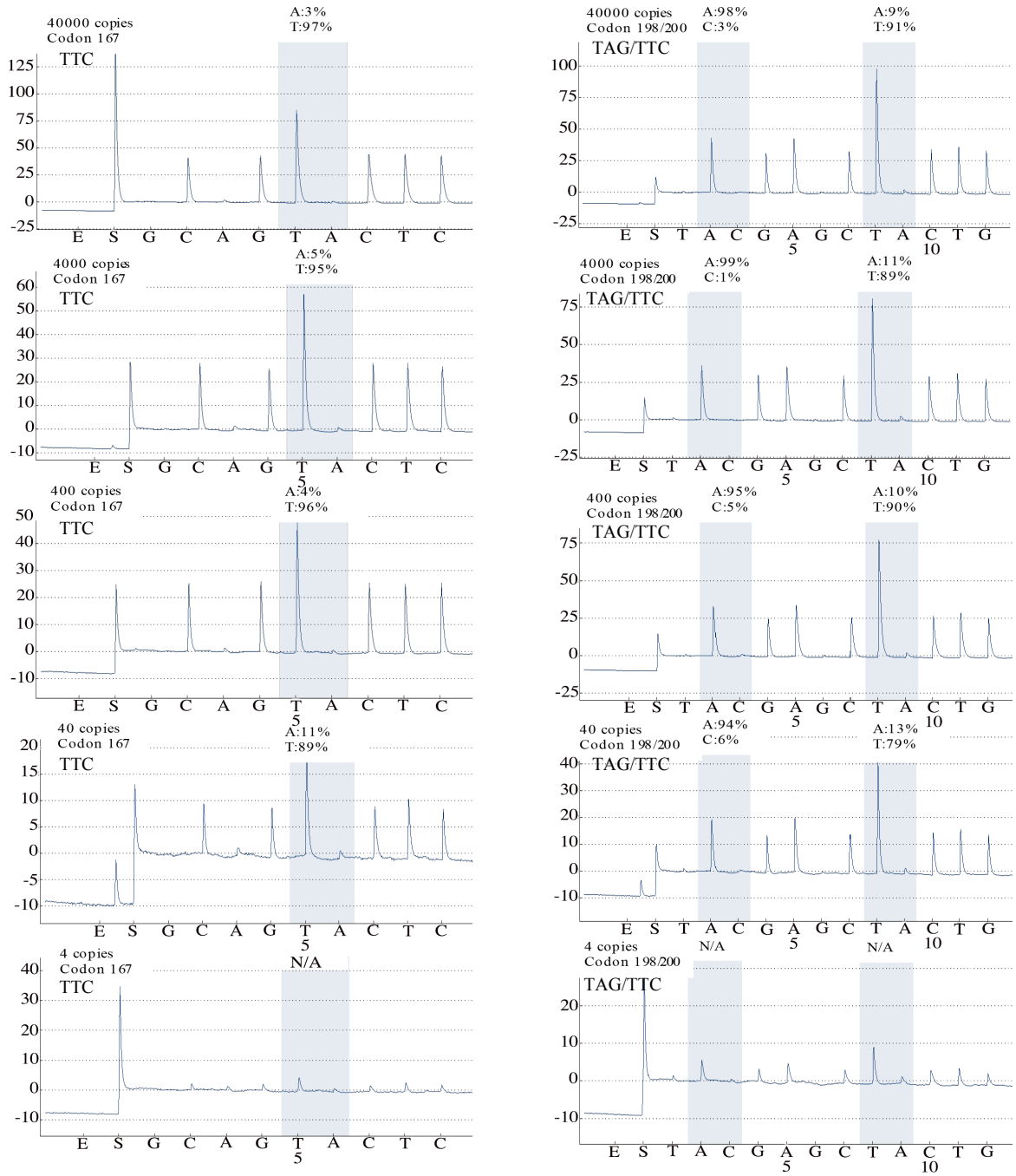

Figure 4.4 Pyrograms representing SNP data obtained by pyrosequencing using serial dilution of (40000, 4000, 400, 40 and 4) DNA copies of the plasmid DNA derived from *H. gallinarum* worm. Dispensions are shown below the pyrograms and are abbreviated as follows: E, enzyme; S, substrate, A, dATP, C, dCTP, G, dGTP, T, dTTP and N/A, Not analyzable.

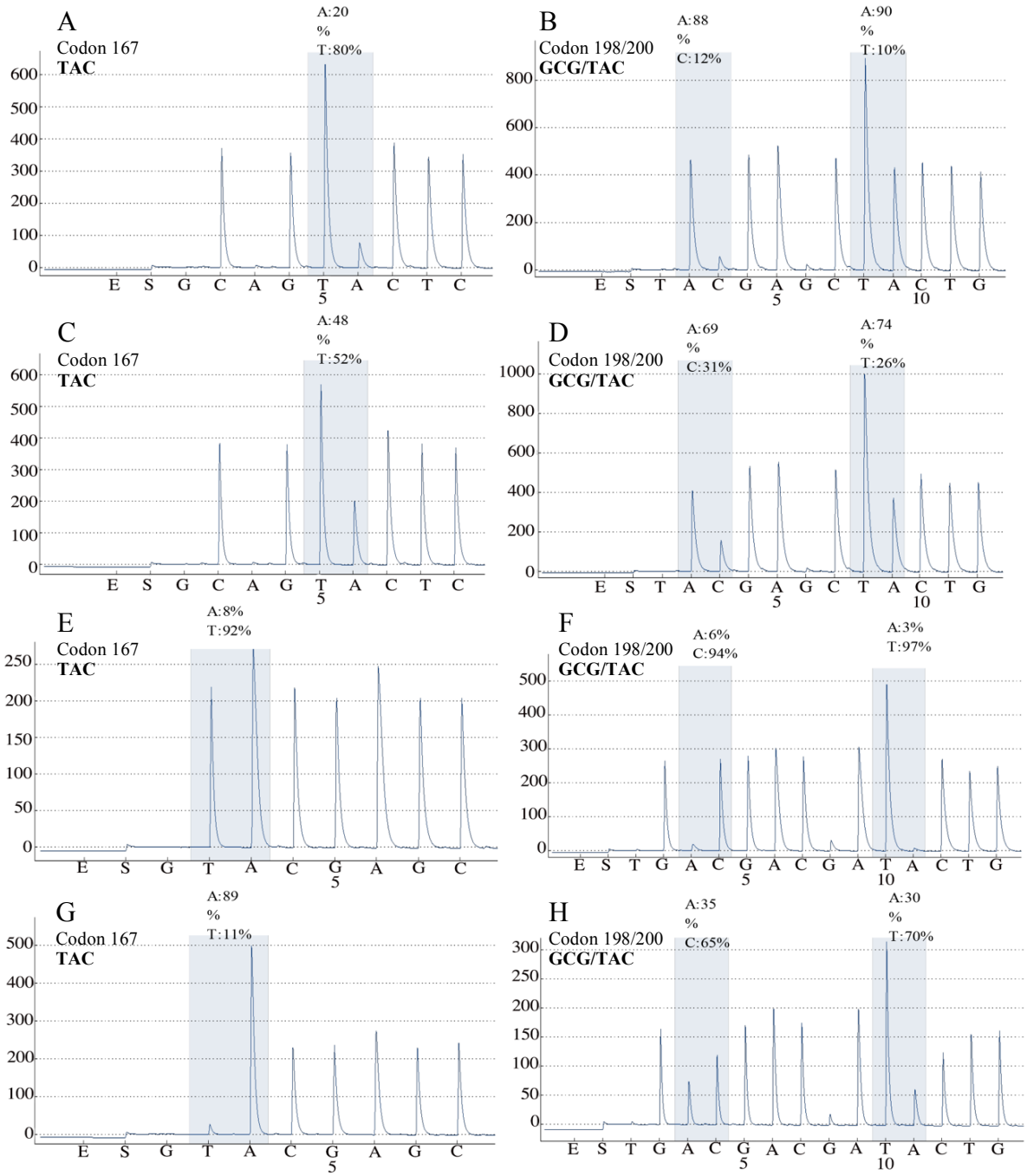

Figure 4.5 Representative pyrograms representing SNP data obtained by pyrosequencing using synthetic fragments containing the relevant SNPs (P1: 167Y,198E and 200Y, P2: 167F,198A and 200F) in various mixtures. (A and C) *H. gallinarum* codon 167 using artificial mixture with ratio of 2:8 and 5:5 (P1:P2) respectively. (B and D) *H. gallinarum* codons 198-200 using ratio of 9:1 and 7:3 (P1:P2) respectivily. (E) *A. galli* codon 167 using artificial plasmid P1. (F) *A. galli* codons 198-200 using artificial plasmid P2. (G and H) *A. galli* codon 167, 198 and 200 using artificial mixture with ratio of 1:9 and 3:7 (P1:P2) respectively. Dispersions are shown below pyrograms and are abbreviated as follows: E, enzyme; S, substrate, A, dATP, C, dCTP, G, dGTP, T, dTTP.

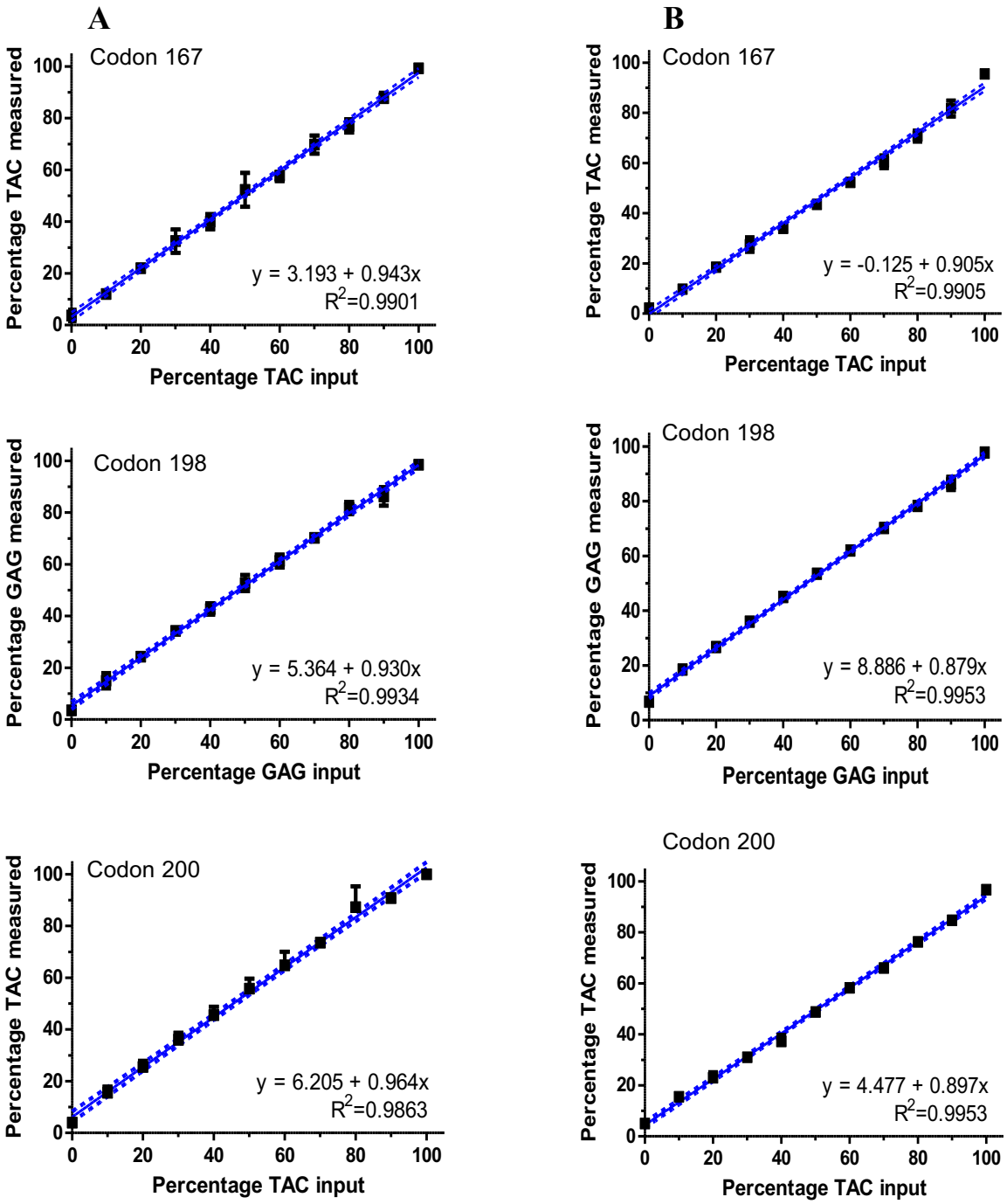

Figure 4.6 Regression analysis for pyrosequencing assays targeting *H. gallinarum* (A) and *A. galli* (B) β -tubulin assays for codons F167Y (TTC to TAC), E198A (GAG to GCA) and F200Y (TTC to TAC). Mixtures of plasmids containing the target sequences as templates were prepared and analysed by pyrosequencing. Observed frequencies (mean of 4 replicates ± SEM) were plotted against calculated input frequencies. Regression plots with 95% confidence bands and Pearson correlation coefficients are shown.

4.2.5 Pyrosequencing results of *A. galli* from Swedish field population

Pyrosequencing results of *A. galli* from naturally infected chicken in Swedish farms showed no evidence for increased frequencies in BZ resistance associated SNPs. Only very low allele frequencies for BZ resistance associated SNPs between 1.2 and 6.4% were obtained (Table 4.1). All these values are well within the technical background of the method. Moreover, sequencing primer designed for detection of the allele frequency for both codons 198 and 200 showed lower technical background for codon 200 compared to the sequencing primer designed for detection of the allele frequency at the codon 200 separately.

Table 4.2 Mean frequencies (in %) ± standard deviation of BZ resistance-associated SNPs in the *A. galli* β-tubulin 1 gene of the field samples collected from Sweden.

Poultry Farm	Codon 167 (TAC)	Codon 198 (GCA)	Codon 200[a] (TAC)	Codon 200[b] (TAC)
Sörmland Ägg	1.26 ± 0.30	2.73 ± 0.23	4.13 ± 0.23	5.20 ± 0.00
Linnebjörke	1.20 ± 0.35	2.67 ± 0.42	5.27 ± 0.90	5.80 ± 0.72
Gräsljunga	1.33 ± 0.30	3.20 ± 0.72	6.27 ± 1.53	6.40 ± 0.87
Total	1.26 ± 0,32	2.87 ± 0.38	5.22 ± 0.87	5.80 ± 0.53

[a]Allele frequency detected using sequencing primer of codons 198 and 200.
[b]Allele frequency detected using sequencing primer of codons 200.

5 Discussion

5.1 Importance and implications of anthelmintic resistance for parasite control in poultry

Chicken egg production in litter-based and free-range housing systems profoundly differs from the conventional battery cages in the environmental conditions provided to the poultry. Infection with nematodes is one of the most important economic constraints on the poultry industry in Europe and possibly worldwide (Thapa et al., 2015, Sherwin et al., 2013, Katoch et al., 2012). Changing poultry breeding and egg production from conventional cage production systems to alternative production systems, particularly organic farming, increased prevalence of *A. galli* and *H. gallinarum* in commercial poultry farms in some European countries (Thapa et al., 2015, Kaufmann et al., 2011). The freedom of movement as one main characteristic of free-range housing systems increased re-emergence and risk of nematode infections in domestic chickens, since the birds are in contact with faeces which increases the chance for the helminths to complete their life cycle, which depend mainly on the faecal-oral transmission route (Kaufmann, 2011, Sherwin et al., 2013, Hinrichsen et al., 2016). Incidence of infection and increases in worm counts from cage over to free range systems cannot only be assigned to poor biosecurity in free-range systems but must also be attributed to the characteristics of organic farming that seem to provide favourable conditions for helminth infections. Improved hygienic measures and high level of biosecurity may reduce the risk of pathogen infections in poultry industry including nematode infections in non-cage housing systems for laying hens. However, implementing good biosecurity measures in litter-based or free range systems to reduce environmental faecal contamination and thereby minimize the exposure to infectious parasite eggs or larvae is difficult (Wongrak et al., 2014). The two ascarid poultry nematode species, *A. galli* and *H. gallinarum*, have a direct life cycle, therefore, any factor which enhances the probability of a hen to ingest contaminated faeces

increases the risk of infection. Concerning the reduction of environmental contamination with faeces containing eggs and larvae of nematodes in ruminants, Thamsborg et al. (2010) concluded that pasture rotation should be considered as an important non-chemical method which can be used for nematode control and minimize the environmental contamination. Indeed, Maurer et al. (2013) recorded that a rotationally used paddock with wood chips appeared to reduce the ascarid faecal egg counts significantly compared with unmanaged paddocks. Nevertheless, it is a fact that the control of endoparasites to reduce their negative effects on poultry production in various species is heavily dependent on the use of anthelmintics. Currently, BZs, mainly flubendazole and fenbendazole in drinking water, are being used for treatment of chicken nematodes within the EU member states due to their high potency and mild side effects. Based on the provided residue depletion studies, a withdrawal period of 6 days for slaughter and zero days for eggs is enforced (EMA/73085/2018, 2018). A recent study concluded that flubendazole apparently still shows excellent efficacy against different developmental stages of *A. galli* in poultry (Tarbiat et al., 2016). Benzimidazole resistance now is a sincere problem in various livestock in the veterinary field corresponding to the decades of use of this drug class for mass treatment against different gastrointestinal parasites. There is a large number of reports on nematode resistance in different livestock that have been published (Waghorn et al., 2006, Gasbarre et al., 2009, Rendell, 2010, Anziani et al., 2001, Condi et al., 2009, Ramünke et al., 2016, von Samson-Himmelstjerna et al., 2009b). Based on broad research activities in various nematode species, it was shown that BZ-resistance in strongyle nematodes is correlated with SNPs at codons F167Y (TTC to TAC), E198A (GAA to GCA) or E198L (GAA to TTA) (Redman et al., 2015, Keegan et al., 2017, Avramenko et al., 2019) and F200Y (TTC to TAC) of the isotype 1 β-tubulin gene (Kwa et al., 1994, Kwa et al., 1995, Prichard, 2001, Ghisi et al., 2007, Von Samson-Himmelstjerna et al., 2007, Chaudhry et

al., 2014, Chaudhry et al., 2015, Demeler et al., 2013). It is important to mention here that up to date it is unclear what the relevance of β-tubulin SNPs in ascarids in BZ resistance mechanism is and which isotypes are involved. In European and US cattle, many factors have been considered as explanation for the so far limited prevalence of anthelmintic resistance as for example use of different breeding systems and less intensive anthelmintic use (Coles, 2002, McArthur and Reinemeyer, 2014). In South America the situation is clearly different and anthelmintic resistance is widespread in cattle parasitic nematodes. The situation differs from Europe by inefficient management systems and inadequate control of the use of anthelmintic drugs (Jaeger and Carvalho-Costa, 2017).

Thes widespread occurrence of anthelmintic resistance in nematodes demonstrates the need to develop sensitive and suitable methods for early detection of anthelmintic resistance in the veterinary field which can be used to regularly monitor anthelmintic efficacy aiming at the prevention or at least postponement of further resistance development (von Samson-Himmelstjerna et al., 2009b). Accordingly, a cost efficient and reliable BZ-resistance test for poultry nematodes would be of real advantage for the poultry industry in order to help implementing effective nematode control programs to prevent development of clinically relevant BZ resistance and spread of resistance.

5.2 Incidence of ascarid eggs in faecal samples collected from poultry farms using the mini-FLOTAC technique

Several parasitological techniques are used for the qualitative and quantitative diagnosis of helminth infections in veterinary practice. The mini-FLOTAC method is an innovative technique based on floatation of helminth eggs and combines good sensitivity with quantitative performance and low costs. The technique is a more sensitive method for quantification of FECs than the widely

used McMaster method and has a detection limit of 5 or 10 EPG, depending on the number of chambers counted per sample (Barda et al., 2013a). It is important to mention that the faecal samples collected from the poultry flocks around Berlin for this study are not representative of the poultry population due to lack of information, e.g. regarding the number of flocks, type of husbandry system, anthelmintic therapy and other management and environmental factors. Therefore, the FEC results of this study can only be compared with those of others to see if the data are within the range of previous observations. Furthermore, risk factors associated with the presence or absence of ascarid eggs were not included and analysed. The present study revealed presence of ascarid eggs in 18% of the flocks sampled using the mini-FLOTAC technique. The mean ascarid FEC of the present study was 322.2 EPG and the maximum was 650 (Table 4.1). Pennycott and Steel (2001) reported *Ascaridia* and *Heterakis* FECs in England and Wales with an overall mean of 179 EPG and a maximum of 1100 EPG. The prevalence of *A. galli* and *H. gallinarum* was demonstrated in previous reports from Germany and other European countries. For example, in Germany prevalences of *A. galli* and *H. gallinarum* were found in organic/free-range flocks was 98% and 88%, respectively (Kaufmann et al., 2011), Moreover, 89% of the studied free-range flocks were infected with *H. gallinarum* in England and Wales (Sherwin et al., 2013). In Denmark, the prevalence on flock level was found to be 63.8% for *A. galli* and 72.5% for *H. gallinarum* in free-range/organic flocks and 41.9% for *A. galli* and 19.4% for *H. gallinarum* in deep-litter systems (Permin et al., 1999). Thapa et al. (2015) reported results of a study across eight European countries in which *A. galli* and *H. gallinarum* were highly prevalent with an overall mean prevalence of 69.5% and 29%, respectively. This difference may reflect that the management factors play a role in the spread of nematode infections within poultry flocks. Skallerup et al. (2005) concluded that the environmental conditions must be considered to be among the most

important determining factors for transmitting infective eggs in natural helminth infections. Several studies demonstrated that the increasing prevalence of ascarids in poultry flocks can be correlated with the use of alternative husbandry systems since the ban of conventional cages in 2011 (Hafiz et al., 2015, Wuthijaree et al., 2017, Sherwin et al., 2013). The freedom of movement for the birds is an important constituent of alternative poultry husbandry systems but increases the chance of infection with parasites, as hens are in direct contact with and exposed to faeces. Soil contaminated with nematode eggs and/or intermediate host such as earthworms allows ascarids to complete their life cycle (Kaufmann et al., 2011, Duffy et al., 2005, Hauck and Hafez, 2013, Thapa et al., 2017).

5.3 Characterization of β-tubulin gene in *A. galli* and *H. gallinarum* and generation the full–length *H. gallinarum* β-tubulin cDNA sequence

The present study describes for the first time the sequence of a β-tubulin cDNA from *H. gallinarum*. Partial cDNA fragments of closely related *A. galli* and *H. gallinarum* β-tubulins were obtained using degenerate primers covering a region containing all three SNPs that have been associated with BZ resistance in nematodes. Cloning and sequencing of nine clones of *A. galli* β-tubulin fragments showed 100% identity at both the nucleotide and protein level to a β-tubulin isotype 1 sequence from *A. galli* deposited in GenBank. However, sequences of 40 clones representing a *H. gallinarum* β-tubulin cDNA fragment derived from different PCRs were 93-99% identical on the nucleotide level to each other and 88% identical to the *A. galli* β-tubulin isotype 1 sequence of the previous study(Tydén et al., 2013).

In general, there is a considerable lack of knowledge and only ambiguous information about the number and phylogenetic relationship of β-tubulin paralogs in nematodes, their expression pattern and function in different cell types, tissues and life cycle stages in particular (Tyden et al., 2016). In the present study, RNA was isolated from ascarid eggs, larvae and adults worms. Tyden et al.

(2016) described that there is a differential expression of two β-tubulin gene isotypes in *Parascaris* sp. after exposure to various concentrations of thiabendazole during worm development. They showed that isotype1 and isotype 2 were expressed at the same level during the early phase of embryogenesis up to 24 h after egg shedding (morula stage). While isotype 1 continued to be expressed at high levels during all life cycle stages, isotype 2 expression decreased between 40 and 120 h by 150 fold to very low levels during embryogenesis and low expression continued until the adult worm stage. Fuchs et al. (2013) described that the β-tubulin repertoire in the common liver fluke *Fasciola hepatica* consists of six isotypes, which are all expressed in the adult stage. They showed that the isotype 1 is dominant in adults. In contrast, isotype 1 was not found to be expressed in the immature stages, whereas isotypes 2, 3 and 4 was expressed in all life cycle stages. In the present study, β-tubulin isotype 1 of *H. gallinarum* and *A. galli* were identified while no other β-tubulin isotypes were detected although variation of the PCR protocol was performed and RNA from different life cycle stages, i.e. eggs, larvae and adults, was used as template.

Full-length *H. gallinarum* β-tubulin cDNA was successfully generated using RACE-PCR. Only a single full-length cDNA of the β-tubulin gene of *H. gallinarum* was generated even though ten clones were selected from each 3'/5' RACE PCR-products and two different primer pairs were designed for the nested RACE PCR based on the variation in the sequences of the clones derived from amplification of the partial cDNA fragment using degenerated primers with the aim to obtain also full-length variants. The complete coding sequence of the newly described *H. gallinarum* β-tubulin cDNA is 1497 bp in length.

5.4 Phylogenetic analysis

The maximum likelihood phylogenetic analysis on cDNA level presented here showed that there is a large diversity and complex phylogenetic relationship between the tubulin paralogs found in different nematode species and within a single nematode species. A sufficient resolution of the

phylogenetic relationship could be obtained by combination of alignment on protein level followed by maximum likelihood analysis on the cDNA level as described for nematode tubulins previously (Demeler et al., 2013). Interestingly, the *H. gallinarum* sequence clustered together with *A. galli* β-tubulin isotype 1 with high statistical support values. Consequently, we have inspected the phylogenetic relationship of this branch with the other ascarid nematode β-tubulins such as *A. suum* tbb-1, *P. equorum* tbb-1 and -2, *A. lumbrioides* tbb-1 and *T. canis* tbb-1. Together with the tbb-1 of the two poultry parasites they form an clade III-specific group (also including a *B. malayi* β-tubulin) which was a sister cluster to β-tubulins (tbb-1, tbb-2 and ben-1) of clade V nematodes. Moreover, this clade V specific group contained the tbb-1 isotypes of important parasitic nematode species infecting livestock, such as *H. contortus, C. oncophora* and *O. ostertagi.* Single nucleotide polymorphisms in these isotypes are well known to confer BZ-resistance to the parasitic worms (von Samson-Himmelstjerna et al., 2009b, Demeler et al., 2013). Indeed, the phylogenetic diversity of β-tubulin genes among the nematode species could lead to differences in BZ-resistance mechanisms or pathways concerning isotypes and/or SNPs location within genes.

5.5 Application of pyrosequencing assays for determination of BZ-resistance in *Ascaridia galli* from field population in Sweden

This study investigated for the first time the susceptibility of *A. galli* to BZs using highly sensitive molecular pyrosequencing assays in flocks of commercial laying hens in the field. The field study investigated the presence of putatively BZ-resistance associated β-tubulin alleles in *A. galli* in chickens from naturally infested Swedish farms using pyrosequencing but found no evidence for increased frequencies in such putatively BZ-resistance associated SNPs in the β-tubulin isotype under investigastion. The respective allele frequencies observed in *A. galli* (between 1.2 and 6.4%) were within the range of the technical background observed during assay evaluation and there was as well no history of reduced BZ-efficacy reported by these farmers and therefore these result were

in line with the corresponding phenotypic observations. In the current study, only *A. galli* isolates from three different commercial poultry farms with unknown history of exposure to anthelmintics were investigate. Recently, Tarbiat et al. (2017) investigated FECR and LDT data of *A. galli* in a commercial laying hen farm in Sweden treated with flubendazole and fenbendazole. They showed that the the FECRs were above 95% and no significant difference was observed among obtained EC_{50} values in the LDT. This indicated that *A. galli* is still phenotypically susceptible to BZ chemotherapy in Sweden. All Swedish chicken flocks from which samples were collected used an all-in all-out management system. The all-in all-out replacement system (particularly in indoor breeding systems) as one of the predominant biosequrity programms, provides a much higher power than other flock management systems in the control and prevention of diseases. Furthermore, it does not give a sufficient time and opportunity to nematodes to establish BZ-resistance and transfer the resistant alleles among generations. Frequent changes of poultry litter in combination with effective disinfection after each breeding helps to keep the infection pressure by many pathogens including parasitic nematodes low. Here, its important to mention that all-in all-out may not be as helful in free-range systems since the ascarid eggs have the ability to survive long peroid in the soil and the eggs cannot easily removed or destroyed by disinfection. Recently, Thapa et al. (2017) found that a small proportion of poultry ascarid eggs may survive and remain infective for at least 2 years.

5.6 Potential BZ-resistance mechanisms not involving isotype 1 β-tubulin of *A. galli* and *H. gallinarum*

There is a high possibility that other *A. galli* and *H. gallinarum* β-tubulin isotypes are encoded in the parasites' genomes as known for other ascarids such as *P. equorum* and *A. suum*. Recently, Martis et al. (2017) identified six novel *A. galli* β-tubulin isotyps of which one candidate is most similar in its sequence to the *A. galli* β-tubulin isotype 1 which was previously identified by Tydén

et al. (2013). Currently, it is unclear if β-tubulin isotype 1 in *A. galli* is involved in BZ-resistance mechanism or not. In non-strongyle nematodes in general, there is insufficient knowledge about β-tubulin isotypes and their participation in BZ-resistance mechanism. Tyden et al. (2016) observed the differential β-tubulin gene expression of isotypes 1 and 2 in different life cycle stages of *Parascaris* sp. and they concluded that exposure to thiabendazole only changed expression of β-tubulin isotype 1. In adult female *A. galli*, Martis et al. (2017) showed no differences in the gene expression levels of different β-tubulin isotyps before and after exposure to flubendazole. Recent advances in the application of genomic tools provide additional insight and increased our knowledge about the phylogenetic diversity of the β-tubulin gene family and their possible association with drug resistance of parasitic nematodes of veterinary importance. Noteworthy, other anthelmintic resistance mechanisms which play a role in decreasing the concentration of the drug in the target tissue were established. These include drug efflux pumps (e.g. P-glycoproteins, Pgsp) and detoxification enzymes (e.g. cytochrome P450 monooxygenases). Membrane P-glycoproteins are able to transport a variety of substrates across cell membranes including many different drugs (Lespine et al., 2012). P-glycoproteins have been found to bind the drug and/or are stimulated to hydrolyse ATP in the presence of anthelmintics. This might reduce the drug concentration at the target site leading to therapy failure (Lespine et al., 2012, Jones et al., 2015). Detoxification of the substance by enzymes e.g. cytochrome P450 or glutathione S-transferases (GSTs) is another pathway reducing the drug efficacy. (James et al., 2009). Cvilink et al. (2009) have reported that anthelmintics are metabolized by cytochrome P450 monooxygenases and GSTs enzymes. After initial oxidation of the anthelmintic by the an oxidase such as a cytochrome P450, GST enzymes would be able to conjugate the activated drug to glutathione followed by eliminatation of the metabolized anthelmintics from the cell by the efflux pumps. Recenlty, Martis

et al. (2017) compared the transcriptome between flubendazole treated and untreated *A. galli*. They showed a significant down-regulation of transcripts annotated as mitochondrial glutamate dehydrogenase and cytochrome P450 in treated adult worms. Mitochondrial glutamate dehydrogenase has a role in nitrogen and glutamate (Glu) metabolism and energy homeostasis in other nematodes such as *T. circumcincta* (Muhamad et al., 2011). Hanser et al. (2002) concluded that exposure of *T. canis* and *A. suum* to flubendazole causes cellular destruction including disruption of mitochondria in the hypodermis, muscle cells and intestine. However, Martis et al. (2017) found significant up-regulation in transcripts homologous to catalase, phosphofructokinase, heat shock proteins (HSPs), , and a multidrug resistance P-glycoprotein (PGP-1) in treated worms. The heat shock proteins such as HSP70 are produced in most organisms in response to exposure to a range of stressful conditions (Sørensen et al., 2003).

The abcence of field poultry population which were characterized as phenotypically resistant to BZ has to be considered as a major current constraint for the analysis of BZ-resistance machanisms in poultry nematodes. Based on many studies in other nematodes e.g *H. conturtus,* cyathostomin and trichostrongylus, the mechanism of BZ-resistance was mostly identified genotypically from the samples which were eiher derived from the field or from experimentally selected populations with BZ-resistance phenotype (Kwa et al., 1994, Kwa et al., 1995, Pape et al., 2002, Von Samson-Himmelstjerna et al., 2007, Silvestre and Humbert, 2000, von Samson-Himmelstjerna et al., 2002b). It is therefore one of the key outstanding research objectives in this context to improve our understanding of the mechanisms of BZ-resistance in poultry nematodes.

All of the above mentioned mechanisms might somehow be involved in BZ-metabolism in ascarids and thus in BZ-resistance mechanisms. Therefore, the recognition of the most relavant

mechanisms and the development and evaluation of molecular markers that could indicate BZ-resistance especially in ascarids may form the basis for development of sensitive diagnostic tests. Since there is little information available about the β-tubulin isotypes and BZ-resistance machanisms in *A. galli* and *H. gallinarum*, future studies should focus to characterise the entire family of β-tubulin isotypes in these two important parasitic nematodes in poultry and use this information to elucidate the pathway leading to BZ-resistance.

5.7 Conclusions and recommendations

- Mini-FLOTAC is a good, sensitivity and low-cost alternative technique that can be used for diagnosis and monitoring helminth infections in control programs in poultry. Using this technique for the phenotypic analysis of BZ-susceptibility/resistance in a FECRT has the limitation that in mixed infections with *A. galli* and *H. gallinarum* discrimination of both parasites by egg morphology is not reliably possible in practice.
- For the first time, a *H. gallinarum* full-length β-tubulin cDNA was sequenced and deposited in GenBank. The genomic sequence data presented here provide the basis for the determination of possible SNPs within the β-tubulin gene.
- More investigations are needed for the identification of other possible present *H. gallinarum* β-tubulin isotypes that could be involved in the BZ-resistance pathway.
- Phylogenetic maximum-likelihood analysis revealed that both *A. galli* and *H. gallinarum* β-tubulin cDNAs cluster together with high statistical support values and share a phylogenetic relationship with the other ascarid nematode β-tubulins. Moreover, it must be stated that the clade III specific group does not belong to the same phylogenetic cluster as the clade V specific group that includes nematode species in which presence of SNPs in this particular β-tubulin isotypes was shown to confer resistance to BZs. This diversity could reflect the differences in BZ-resistance mechanisms or pathways concerning isotypes and/or SNP location.

- Pyrosequencing assays for the quantitative analysis of potentially BZ resistance associated SNPs in the β-tubulin isotype 1 genes of *A. galli* and *H. gallinarum* were successfully developed. The developed assays offer the tools to screen for the presence and to quantify the relative frequency of BZ-resistance associated SNPs in populations of important poultry parasites. The assays will potentially allow detecting the development of resistance in an early phase before it becomes clinically apparent. This offers the chance to implement measures to counteract further selection before resistance becomes widespread.
- Pyrosequencing results of pools from *A. galli* collected from naturally infected chicken on Swedish farms showed no evidence for increased frequencies of BZ resistance associated SNPs. More investigations are needed from many other poultry flocks in different geographical regions particularly in Europe to link the phenotypic data with the molecular examination using highly sensitive techniques such as pyrosequencing to evaluate BZ-resistance in poultry field samples.
- In poultry nematodes, a major constrain for the analysis of BZ-resistance mechanisims is that there are thus far no populations with BZ-resistance phenotype available. In fact, the key to understanding the mechanisms of BZ-resistance in poultry is the comparative analyzis of nematodes derived from phenotypically susceptible and resistant populations.

6 Summary

Poultry is considered to be one of the best sources of high quality protein for humans. Following recent changes in husbandry systems for poultry in Europe from cage to other, alternative husbandry systems such as free-range systems, increased prevalence of helminth infections, predominantly *Ascaridia galli* and *Heterakis gallinarum*, was observed. The basic principles for the effective control of helminth infections in poultry farms are a combination of preventive measures such as biosecurity and disinfection with broad-spectrum anthelmintic therapy. The benzimidazoles (BZs) flubendazole and fenbendazole have been certified for treatment of chicken nematodes within the European Union and are widely used as save broad-spectrum anthelmintics in poultry. Benzimidazole (BZ)-resistance is widespread and a significant problem in several parasitic nematodes of ruminants and horses and was correlated with the presence of three single nucleotide polymorphisms (SNPs) in the β-tubulin isotype 1 gene of strongyle nematodes. However, BZ-resistance has so far not been reported in the chicken parasitic nematodes but the risk for resistance development has to be considered as existant and will increase with parasite infection intensity and treatment frequencies. Therefore, it is necessary to establish sensitive and reliable diagnostic tests for detection of BZ-resistance at least for the most important nematodes of chicken. Single nucleotide polymorphisms in the β-tubulin gene, particularly at the three specific codons F200Y, E198A and F200Y, are used as markers for the molecular detection of BZ-resistance in-several strongyle nematodes in veterinary medicine. Among the molecular tests, pyrosequencing is considered an excellent technique for quantification of BZ-resistance associated β-tubulin alleles even when the allele frequency is still quite low. Here, partial cDNAs representing a single β-tubulin isotype were obtained using degenerated primers. The obtained nine *A. galli* sequences were 100% identical to a previously published sequence while the 40 new *H. gallinarum*

sequences were 93-99% identical to each other and 88% identical to the *A. galli* sequence. The 1497 bp full-length β-tubulin isotype 1 cDNA sequence with an 1353 bp open reading frame of poultry the nematode *H. gallinarum* was identified using 3'/5' RACE protocols. Phylogenetic maximum-likelihood analysis revealed that both *A. galli* and *H. gallinarum* β-tubulin cDNAs cluster together with high statistical support values and share a close phylogenetic relationship with the other ascarid nematode β-tubulins. Overall, there is a clear separation between β-tubulins from clade V and clade III nematodes. The latter form a highly supported group. This together with the fact that in both clusters several β-tubulin paralogs are found per species prevents to identify clear one-to-one orthologs between the ascarid β-tubulins and those of *Caenorhabditis elegans* and strongyles. Even worse, such one-to-one orthologs cannot even be identified in comparison to the pig roundworm *A. suum* since the sister group in the phylogenetic tree to the *A. galli* and *H. gallinarum* group contains *A. suum* tbb-1 and tbb-2 cDNAs. Pyrosequencing assays for the quantitative analysis of potentially BZ-resistance associated SNPs in the β-tubulin isotype 1 genes of *A. galli* and *H. gallinarum* were successfully developed. Plasmids with artificially synthesised inserts carrying combinations of SNPs were combined in defined ratios to evaluate sensitivity and linearity of the assays. The correlation of calculated and observed frequencies for the SNP determination at codons F167Y, E198A and F200Y was very high. Pyrosequencing assays carried out for *A. galli* collected from naturally infected chicken on Swedish farms showed no evidence for increased frequencies of potentially BZ-resistance associated SNPs. The developed assays offer the tools to screen for the presence and to quantify the relative frequency of BZ-resistance associated SNPs in populations of important poultry parasites. The assays have the potential of detecting the development of resistance in an early phase before it becomes clinically

apparent. This offers the chance to implement measures to counteract further selection before resistance becomes widespread.

7 Zusammenfassung

Identifizierung von β-Tubulin Isotypen und Entwicklung von Pyrosequenzierungs-Assays für die Analyse von Benzimidazole-Resistenz bei *Heterakis gallinarum* und *Ascaridia galli*

Geflügel wird als eine der besten tierischen Proteinquellen für Menschen angesehen. Nach kürzlichen Änderungen in den Haltungssystemen für Geflügel in Europa von Käfig- zu alternativen Haltungsformen wie Freilandhaltung, wurden erhöhte Prävalenzen von Infektionen mit Helminthen, insbesondere *Ascaridia galli* und *Heterakis gallinarum*, beobachtet. Die grundlegenden Prinzipien für eine effektive Kontrolle von Helmintheninfektionen beim Geflügel vereinen eine Kombination von präventiven Maßnahmen wie Biosicherheit und Desinfektion mit der Anwendung von Breitspektrum-Anthelminthika. Die Benzimidazole Flubendazol und Fenbendazol wurden für die Behandlung von Hühnernematoden in der Europäischen Union zertifiziert und werden weithin als sichere Breitspektrum-Anthelminthika beim Geflügel eingesetzt. Benzimidazol(BZ)-Resistenz ist ein weit verbreitetes und bedeutendes Problem bei zahlreichen parasitischen Nematoden von Wiederkäuern und Pferden und wurde insbesondere mit dem Vorhandensein von drei Einzelnukleotidpolymorphismen (engl. Single Nucleotide Polymorphisms, SNPs) im β-Tubulin Isotyp 1 Gen von Strongyliden korreliert. Benzimidazol-Resistenz wurde jedoch bisher nicht für parasitische Nematoden der Hühner beschrieben. Grundsätzlich ist allerdings das Risiko für die Entwicklung von BZ-Resistenz bei Geflügelnematoden als gegeben einzuschätzen und wird mit erhöhter Infektionsintensität und Behandlungsfrequenz steigen. Daher ist es notwendig, sensitive und zuverlässige diagnostische Tests für die Detektion von BZ-Resistenz zumindest für die wichtigsten Nematoden der Hühner zu etablieren. Einzelnukleotidpolymorphismen im β-Tubulin Isotyp 1 Gen, insbesondere in den drei spezifischen Codons F200Y, E198A und F200Y, werden in der Veterinärmedizin als Marker zur Detektion von BZ-Resistenz in einigen Strongylidenspezies verwendet. Von den verfügbaren molekularen Verfahren wird Pyrosequenzierung als eine exzellente Technik für die Quantifizierung von mit BZ-Resistenz assoziierten β-Tubulinallelen angesehen, selbst wenn die Häufigkeit dieser Allele noch gering ist. Hier wurden partielle cDNAs eines bestimmten β-Tubulinisotyps mit Hilfe degenerierter Primer erhalten. Die neun gewonnenen *A. galli* cDNA-Sequenzen waren 100 % identisch untereinander und zu einer zuvor publizierten Sequenz während die 40 neuen *H. gallinarum* 93-99% identisch untereinander und 88% identisch zu der *A. galli*

Sequenz waren. Es wurde eine 1497 bp umfassende, vollständige β-Tubulin Isotyp 1 cDNA-Sequenz mit einem offenen Leseraster von 1353 bp des Geflügel Nematoden *H. gallinarum* identifiziert. Eine phylogenetische Maximum-Likelihood Analyse zeigte, dass die beiden *A. galli* und *H. gallinarum* β-Tubulin cDNAs zusammen eine Gruppe bildeten und die gleiche phylogenetische Beziehung zu den anderen β-Tubulinen aus Ascariden aufweisen. Insgesamt ergab sich eine klare Aufteilung zwischen β-Tubulinen von Klade V- und Klade III-Nematoden. Die letzteren formten eine Gruppe mit hoher statistischer Unterstützung. Dies, zusammen mit der Tatsache, dass in beiden Gruppen mehrere Paraloge je Spezies gefunden wurden, verhindert die Identifizierung von eins-zu-eins Orthologen zwischen den β-Tubulinen von Ascariden und denen von *Caenorhabditis elegans* sowie denen der weiteren Strongylidenspezies. Zudem können solche eins-zu-eins Orthologe auch nicht im Vergleich zum Schweinespulwurm *Ascaris suum* identifiziert werden, denn die Schwestergruppe im phylogenetischen Baum zu der Gruppe aus *A. galli* und *H. gallinarum* enthält die *A. suum* tbb-1 und tbb-2 cDNAs. Pyrosequenzierungsassays für die quantitative Analyse der potentiell mit BZ-Resistenz assoziierten SNPs in den β-Tubulin Isotyp 1-Genen von *A. galli* und *H. gallinarum* wurden erfolgreich entwickelt. Plasmide mit artifiziell synthetisierten Insertionen wurden in definierten Verhältnissen gemischt, um die Sensitivität und Linearität der Assays zu evaluieren. Die Korrelation zwischen berechneter und gemessener Frequenz für die Bestimmung der SNPs in den Codons F167Y, E198A und F200Y war sehr hoch. Pyrosequenzierungsassays, die mit *A. galli* aus natürlich infizierten Hühnern von schwedischen Farmen durchgeführt wurden, ergaben keinen Nachweis für erhöhte Häufigkeiten von mit BZ-Resistenz assozierten β-Tubulin SNPs. Die entwickelten Assays erlauben es, die Entwicklung von Resistenz schon in einer frühen Phase nachzuweisen, bevor die Resistenz klinisch sichtbar wird. Dies eröffnet die Chance Maßnahmen zu implementieren, die einer weiteren Resistenzentwicklung entgegenwirken.

8 Literature cited

ABONGWA, M., MARTIN, R. J. & ROBERTSON, A. P. 2017. A brief review on the mode of action of antinematodal drugs. *Acta Veterinaria,* 67**,** 137-152.

ABRAHAMSSON, P. & TAUSON, R. 1995. Aviary systems and conventional cages for laying hens: Effects on production, egg quality, health and bird location in three hybrids. *Acta Agriculturae Scandinavica A-Animal Sciences,* 45**,** 191-203.

ACKERT, J. E. 1931. The morphology and life history of the fowl nematode Ascaridia lineata (Schneider). *Parasitology,* 23**,** 360-379.

ADEMOLA, I. O., KRÜCKEN, J., RAMÜNKE, S., DEMELER, J. & VON SAMSON-HIMMELSTJERNA, G. 2015. Absence of detectable benzimidazole-resistance associated alleles in Haemonchus placei in cattle in Nigeria revealed by pyrosequencing of β-tubulin isotype 1. *Parasitology Research,* 114**,** 1997-2001.

ALDERBORN, A., KRISTOFFERSON, A. & HAMMERLING, U. 2000. Determination of single-nucleotide polymorphisms by real-time pyrophosphate DNA sequencing. *Genome Research,* 10**,** 1249-1258.

ALVAREZ-SANCHEZ, M., PEREZ-GARCIA, J., CRUZ-ROJO, M. & ROJO-VAZQUEZ, F. 2005. Real time PCR for the diagnosis of benzimidazole resistance in trichostrongylids of sheep. *Veterinary Parasitology,* 129**,** 291-298.

ANDERSEN, J. P., NORUP, L. R., DALGAARD, T. S., ROTHWELL, L., KAISER, P., PERMIN, A., SCHOU, T. W., FINK, D. R., JUNGERSEN, G., SØRENSEN, P. & JUUL-MADSEN, H. R. 2013. No protection in chickens immunized by the oral or intra-muscular immunization route with Ascaridia galli soluble antigen. *Avian Pathology,* 42**,** 276-282.

ANZIANI, O. S., GUGLIELMONE, A., ZIMMERMANN, G., VAZQUEZ, R. & SUAREZ, V. 2001. Avermectin resistance in Cooperia pectinata in cattle in Argentina. *Veterinary Record,* 149**,** 58-59.

AVRAMENKO, R. W., REDMAN, E. M., MELVILLE, L., BARTLEY, Y., WIT, J., QUEIROZ, C., BARTLEY, D. J. & GILLEARD, J. S. 2019. Deep amplicon sequencing as a powerful new tool to screen for sequence polymorphisms associated with anthelmintic resistance in parasitic nematode populations. *International Journal for Parasitology,* 49**,** 13-26.

BALTRUŠIS, P., HALVARSSON, P. & HÖGLUND, J. 2018. Exploring benzimidazole resistance in Haemonchus contortus by next generation sequencing and droplet digital PCR. *International Journal for Parasitology: Drugs and Drug Resistance,* 8**,** 411-419.

BARDA, B., ZEPHERINE, H., RINALDI, L., CRINGOLI, G., BURIONI, R., CLEMENTI, M. & ALBONICO, M. 2013a. Mini-FLOTAC and Kato-Katz: helminth eggs watching on the shore of lake Victoria. *Parasites & Vectors,* 6**,** 220-225.

BARDA, B. D., RINALDI, L., IANNIELLO, D., ZEPHERINE, H., SALVO, F., SADUTSHANG, T., CRINGOLI, G., CLEMENTI, M. & ALBONICO, M. 2013b. Mini-FLOTAC, an innovative direct diagnostic technique for intestinal parasitic infections: experience from the field. *PLoS Neglected Tropical Diseases,* 7**,** e2344.

BARKER, H., SMYTH, R., WEISSBACH, H., TOOHEY, J., LADD, J. & VOLCANI, B. 1960. Isolation and properties of crystalline cobamide coenzymes containing benzimidazole or 5, 6-dimethylbenzimidazole. *Journal of Biological Chemistry,* 235**,** 480-488.

BARRETT, J. 1976. Studies on the induction of permeability in Ascaris lumbricoides eggs. *Parasitology,* 73**,** 109-121.

Literature cited

BAUER, C. 2006. Helminthosen des Nutztgeflügels. *Veterinärmedizinische Parasitologie. Parey, Stuttgart,* 6**,** 600-630.

BERG, C. 2002. Health and welfare in organic poultry production. *Acta Veterinaria Scandinavica,* 95**,** 37-45.

BESIER, R., KAHN, L., SARGISON, N. & VAN WYK, J. 2016. Diagnosis, treatment and management of Haemonchus contortus in small ruminants. *Advances in Parasitology,* 93**,** 181-238.

BLACKHALL, W. J., KUZMINA, T. & VON SAMSON-HIMMELSTJERNA, G. 2011. β-Tubulin genotypes in six species of cyathostomins from anthelmintic-naive Przewalski and benzimidazole-resistant brood horses in Ukraine. *Parasitology Research,* 109**,** 1199-1203.

BLOKHUIS, H. 2004. Recent developments in European and international welfare regulations. *World's Poultry Science Journal,* 60**,** 469-477.

BRENER, B., TORTELLY, R., MENEZES, R. C., MUNIZ-PEREIRA, L. C. & PINTO, R. M. 2006. Prevalence and pathology of the nematode Heterakis gallinarum, the trematode Paratanaisia bragai, and the protozoan Histomonas meleagridis in the turkey, Meleagris gallopavo. *Memorias do Instituto Oswaldo Cruz,* 101**,** 677-681.

CHADFIELD, M., PERMIN, A., NANSEN, P. & BISGAARD, M. 2001. Investigation of the parasitic nematode Ascaridia galli (Shrank 1788) as a potential vector for Salmonella enterica dissemination in poultry. *Parasitology Research,* 87**,** 317-325.

CHAUDHRY, U., MILLER, M., YAZWINSKI, T., KAPLAN, R. & GILLEARD, J. 2014. The presence of benzimidazole resistance mutations in Haemonchus placei from US cattle. *Veterinary Parasitology,* 204**,** 411-415.

CHAUDHRY, U., REDMAN, E. M., ABBAS, M., MUTHUSAMY, R., ASHRAF, K. & GILLEARD, J. S. 2015. Genetic evidence for hybridisation between Haemonchus contortus and Haemonchus placei in natural field populations and its implications for interspecies transmission of anthelmintic resistance. *International Journal for Parasitology,* 45**,** 149-159.

CHOI, Y. J., BISSET, S. A., DOYLE, S. R., HALLSWORTH-PEPIN, K., MARTIN, J., GRANT, W. N. & MITREVA, M. 2017. Genomic introgression mapping of field-derived multiple-anthelmintic resistance in Teladorsagia circumcincta. *PLoS Genetic,* 13**,** e1006857.

COLES, G. 1986. Anthelmintic resistance in sheep. *Veterinary Clinics of North America: Food Animal Practice,* 2**,** 423-432.

COLES, G. C. 2002. Cattle nematodes resistant to anthelmintics: why so few cases? *Veterinary Research,* 33**,** 481-489.

COLES, G. C., BAUER, C., BORGSTEEDE, F. H. M., GEERTS, S., KLEI, T. R., TAYLOR, M. A. & WALLER, P. J. 1992. World association for the advancement of veterinary parasitology (WAAVP) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology,* 44**,** 35-44.

COLES, G. C., JACKSON, F., POMROY, W. E., PRICHARD, R. K., VON SAMSON-HIMMELSTJERNA, G. & SILVESTRE, A. 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology,* 136**,** 167-185.

COLES, G. C., PAPADOPOULOS, E. & HIMONAS, C. A. 1995. Tubulin, resistance and worms. *Parasitology today,* 11**,** 183-184.

CONDI, G., SOUTELLO, R. & AMARANTE, A. F. T. D. 2009. Moxidectin-resistant nematodes in cattle in Brazil. *Veterinary Parasitology,* 161**,** 213-217.

Literature cited

CONWAY, D. 1964. Variance in the effectiveness of thiabendazole against Haemonchus contortus in sheep. *American Journal of Veterinary Research,* 25**,** 844-846.

CRINGOLI, G., RINALDI, L., MAURELLI, M. P. & UTZINGER, J. 2010. FLOTAC: new multivalent techniques for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans. *Nature Protocols,* 5**,** 503-515.

CRUTHERS, L. 1975. Efficacy of Levamisole (Tramisol®) 1 in Drinking Water Against Some Nematodes of Chickens. *proceedings of the Oklahoma Academy of Science,* 55**,** 119-121.

CVILINK, V., LAMKA, J. & SKÁLOVÁ, L. 2009. Xenobiotic metabolizing enzymes and metabolism of anthelminthics in helminths. *Drug Metabolism Reviews,* 41**,** 8-26.

DAHL, C., PERMIN, A., CHRISTENSEN, J. P., BISGAARD, M., MUHAIRWA, A. P., PETERSEN, K. M. D., POULSEN, J. S. D. & JENSEN, A. L. 2002. The effect of concurrent infections with Pasteurella multocida and Ascaridia galli on free range chickens. *Veterinary Microbiology,* 86**,** 313-324.

DAŞ, G., ABEL, H., SAVAŞ, T., SOHNREY, B. & GAULY, M. 2014. Egg production dynamics and fecundity of Heterakis gallinarum residing in different caecal environments of chickens induced by fibre-rich diets. *Veterinary Parasitology,* 205**,** 606-618.

DAŞ, G. & GAULY, M. 2014. Response to Ascaridia galli infection in growing chickens in relation to their body weight. *Parasitology Research,* 113**,** 1985-1988.

DE LOURDES MOTTIER, M. & PRICHARD, R. K. 2008. Genetic analysis of a relationship between macrocyclic lactone and benzimidazole anthelmintic selection on Haemonchus contortus. *Pharmacogenetics and Genomics,* 18**,** 129-140.

DEGEN, W. G. J., DAAL, N. V., ROTHWELL, L., KAISER, P. & SCHIJNS, V. E. J. C. 2005. Th1/Th2 polarization by viral and helminth infection in birds. *Veterinary Microbiology,* 105**,** 163-167.

DEMELER, J., KRÜGER, N., KRÜCKEN, J., VON DER HEYDEN, V. C., RAMÜNKE, S., KÜTTLER, U., MILTSCH, S., LOPEZ CEPEDA, M., KNOX, M., VERCRUYSSE, J., GELDHOF, P., HARDER, A. & VON SAMSON-HIMMELSTJERNA, G. 2013. Phylogenetic characterization of beta-tubulins and development of pyrosequencing assays for benzimidazole resistance in cattle nematodes. *PLoS One,* 8**,** e70212.

DIAWARA, A., HALPENNY, C. M., CHURCHER, T. S., MWANDAWIRO, C., KIHARA, J., KAPLAN, R. M., STREIT, T. G., IDAGHDOUR, Y., SCOTT, M. E. & BASÁÑEZ, M.-G. 2013. Association between response to albendazole treatment and β-tubulin genotype frequencies in soil-transmitted helminths. *PLoS Neglected Tropical Diseases,* 7**,** e2247.

DRISCOLL, M., DEAN, E., REILLY, E., BERGHOLZ, E. & CHALFIE, M. 1989. Genetic and molecular analysis of a Caenorhabditis elegans beta-tubulin that conveys benzimidazole sensitivity. *The Journal of Cell Biology,* 109**,** 2993-3003.

DROGEMULLER, M., FAILING, K., SCHNIEDER, T. & VON SAMSON-HIMMELSTJERNA, G. 2004. Effect of repeated benzimidazole treatments with increasing dosages on the phenotype of resistance and the beta-tubulin codon 200 genotype distribution in a benzimidazole resistant cyathostomin population. *Veterinary Parasitology,* 123**,** 201-213.

DUFFY, C., SIMS, M. & POWER, R. 2005. Evaluation of dietary Natustat™ for control of Histomonas meleagridis in male turkeys on infected litter. *Avian Diseases,* 49**,** 423-425.

Literature cited

ECHEVARRIA, F. A., ARMOUR, J., BAIRDEN, K. & DUNCAN, J. L. 1993. Laboratory selection for ivermectin resistance in Haemonchus contortus. *Veterinary Parasitology,* 49**,** 265-270.

EDGAR, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research,* 32**,** 1792-1797.

EIGAARD, N. M., SCHOU, T. W., PERMIN, A., CHRISTENSEN, J. P., EKSTRØM, C. T., AMBROSINI, F., CIANCI, D. & BISGAARD, M. 2006. Infection and excretion of Salmonella Enteritidis in two different chicken lines with concurrent *Ascaridia galli* infection. *Avian Pathology,* 35**,** 487-493.

ELARD, L., CABARET, J. & HUMBERT, J. 1999. PCR diagnosis of benzimidazole-susceptibity or-resistance in natural populations of the small ruminant parasite, *Teladorsagia circumcincta*. *Veterinary Parasitology,* 80**,** 231-237.

EMA/73085/2018 2018. Committee for Medicinal Products for Veterinary Use CVMP assessment report for type II variation for Panacur AquaSol. *Retrievedfrom:* https://www.ema.europa.eu/documents/variation-report/panacur-aquasol-v-c-2008-ii-0015-epar-assessment-report-variation_en.pdf.

FAO. 2017. *FAOSTAT* [Online]. Retrieved from http://www.fao.org/faostat/en/ - data/QL. [Accessed].

FARRELL, D. 2005. Matching poultry production with available feed resources: issues and constraints. *World's Poultry Science Journal,* 61**,** 298-307.

FINE, P. E. 1975. Quantitative studies on the transmission of Parahistomonas wenrichi by ova of Heterakis gallinarum. *Parasitology,* 70**,** 407-417.

FRIEDMAN, P. A. 1980. *The molecular mechanism of action of benzimidazole drugs in embryos of Ascaris suum.* Review of literature. PhD Disseration. University of Califoria, USA. 8-11.

FUCHS, M.-A., RYAN, L. A., CHAMBERS, E. L., MOORE, C. M., FAIRWEATHER, I., TRUDGETT, A., TIMSON, D. J., BRENNAN, G. P. & HOEY, E. M. 2013. Differential expression of liver fluke β-tubulin isotypes at selected life cycle stages. *International Journal for Parasitology,* 43**,** 1133-1139.

GABA, S., CABARET, J., GINOT, V. & SILVESTRE, A. 2006. The early drug selection of nematodes to anthelmintics: stochastic transmission and population in refuge. *Parasitology,* 133**,** 345-356.

GASBARRE, L. C., SMITH, L. L., LICHTENFELS, J. R. & PILITT, P. A. 2009. The identification of cattle nematode parasites resistant to multiple classes of anthelmintics in a commercial cattle population in the US. *Veterinary Parasitology,* 166**,** 281-285.

GAULY, M., BAUER, C., PREISINGER, R. & ERHARDT, G. 2002. Genetic differences of Ascaridia galli egg output in laying hens following a single dose infection. *Veterinary Parasitology,* 103**,** 99-107.

GAULY, M., DUSS, C. & ERHARDT, G. 2007. Influence of Ascaridia galli infections and anthelmintic treatments on the behaviour and social ranks of laying hens (Gallus gallus domesticus). *Veteterinary Parasitology,* 146**,** 271-80.

GEARY, T. G., HOSKING, B. C., SKUCE, P. J., VON SAMSON-HIMMELSTJERNA, G., MAEDER, S., HOLDSWORTH, P., POMROY, W. & VERCRUYSSE, J. 2012. World Association for the Advancement of Veterinary Parasitology (WAAVP) Guideline:

Anthelmintic combination products targeting nematode infections of ruminants and horses. *Veterinary Parasitology,* 190**,** 306-316.

GHISI, M., KAMINSKY, R. & MASER, P. 2007. Phenotyping and genotyping of Haemonchus contortus isolates reveals a new putative candidate mutation for benzimidazole resistance in nematodes. *Veterinary Parasitology,* 144**,** 313-320.

GILL, J. H., REDWIN, J. M., VAN WYK, J. A. & LACEY, E. 1995. Avermectin inhibition of larval development in Haemonchus contortus—effects of ivermectin resistance. *International Journal for Parasitology,* 25**,** 463-470.

GILLEARD, J. & REDMAN, E. 2016. Genetic diversity and population structure of Haemonchus contortus. *Advances in Parasitology,* 93**,** 31-68.

GOKBULUT, C., BILGILI, A., HANEDAN, B. & MCKELLAR, Q. 2007. Comparative plasma disposition of fenbendazole, oxfendazole and albendazole in dogs. *Veterinary Parasitology,* 148**,** 279-287.

GRANT, W. N. & MASCORD, L. J. 1996. Beta-tubulin gene polymorphism and benzimidazole resistance in Trichostrongylus colubriformis. *International Journal for Parasitology,* 26**,** 71-77.

HAFIZ, A. B., MUHAMMAD, A. R., MUHAMMAD, A. A., IMRAN, A. K., ABDUL, A., ZAHID, M. & SHAUKAT, H. M. 2015. Prevalence of Ascaridia galli in white leghorn layers and Fayoumi-Rhode Island red crossbred flock at government poultry farm Dina, Punjab, Pakistan. *Tropical Biomedicine* 32**,** 11-16.

HALL, G. O. 1945. A Large Roundworm Ascaridia Lineata Found in Egg of Fowl. *Poultry Science,* 24**,** 496-498.

HANSER, E., MEHLHORN, H., HOEBEN, D. & VLAMINCK, K. 2002. In vitro studies on the effects of flubendazole against Toxocara canis and Ascaris suum. *Parasitology Research,* 89**,** 63-74.

HASSANEN, M., RAHMAN, E. A. & KHALIL, A. 2009. New Scanning Electron Microscopy Look of Ascaridia galli (Schrank, 17SS) Adult Worm and its Biological Control. *Research Journal of Parasitology,* 2**,** 21-32.

HAUCK, R. & HAFEZ, H. M. 2013. Experimental infections with the protozoan parasite Histomonas meleagridis: a review. *Parasitol Res,* 112**,** 19-34.

HAYDEN, R., GU, Z., INGERSOLL, J., ABDUL-ALI, D., SHI, L., POUNDS, S. & CALIENDO, A. 2013. Comparison of droplet digital PCR to real-time PCR for quantitative detection of cytomegalovirus. *Journal of clinical microbiology,* 51**,** 540-546.

HERD, R. P. & MCNAUGHT, D. J. 1975. Arrested development and the histotropic phase of Ascaridia galli in the chicken. *International Journal for Parasitology,* 5**,** 401-406.

HINRICHSEN, L. K., LABOURIAU, R., ENGBERG, R. M., KNIERIM, U. & SORENSEN, J. T. 2016. Helminth infection is associated with hen mortality in Danish organic egg production. *Veterinary Record,* 179**,** 196-199.

HODGKINSON, J. E., CLARK, H., KAPLAN, R., LAKE, S. & MATTHEWS, J. 2008. The role of polymorphisms at β tubulin isotype 1 codons 167 and 200 in benzimidazole resistance in cyathostomins. *International Journal for Parasitology,* 38**,** 1149-1160.

HÖGLUND, J. & JANSSON, D. S. 2011. Infection dynamics of Ascaridia galli in non-caged laying hens. *Veterinary Parasitology,* 180**,** 267-273.

HORTON, J. 2000. Albendazole: a review of anthelmintic efficacy and safety in humans. *Parasitology,* 121**,** S113-S132.

HUBERT, J. & KERBOEUF, D. 1992. A microlarval development assay for the detection of anthelmintic resistance in sheep nematodes. *Veterinary Record,* 130**,** 442-446.

HULZEBOSCH, J. 2006. Wide range of housing options for layers. *World Poultry,* 22**,** 20-22.

IDI, A., PERMIN, A. & MURRELL, K. D. 2004. Host age only partially affects resistance to primary and secondary infections with Ascaridia galli (Schrank, 1788) in chickens. *Veterinary Parasitology,* 122**,** 221-231.

IKEME, M. M. 2009. Observations on the pathogenicity and pathology of Ascaridia galli. *Parasitology,* 63**,** 169-179.

IRUNGU, L. W., KIMANI, R. & KISIA, S. 2004. Helminth parasites in the intestinal tract of indigenous poultry in parts of Kenya. *Journal of the South African Veterinary Association,* 75**,** 58-59.

ISHII, J. B., ARENAL, A., FELIX, A., YOSHITANI, U., BEECH, R. & MOLENTO, M. B. 2017. Diagnosis of resistance alleles in codon 167 of the beta-tubulin (Cya-tbb-1) gene from third-stage larvae of horse cyathostomins. *Research in Veterinary Science,* 115**,** 92-95.

JAEGER, L. H. & CARVALHO-COSTA, F. A. 2017. Status of benzimidazole resistance in intestinal nematode populations of livestock in Brazil: a systematic review. *BMC Veterinary Research,* 13**,** 358-367.

JAMES, C. E., HUDSON, A. L. & DAVEY, M. W. 2009. Drug resistance mechanisms in helminths: is it survival of the fittest? *Trends in Parasitology,* 25**,** 328-335.

JANSSON, D. S., NYMAN, A., VAGSHOLM, I., CHRISTENSSON, D., GORANSSON, M., FOSSUM, O. & HOGLUND, J. 2010. Ascarid infections in laying hens kept in different housing systems. *Avian Pathology,* 39**,** 525-32.

JONES, L. M., FLEMMING, A. J. & URWIN, P. E. 2015. NHR-176 regulates cyp-35d1 to control hydroxylation-dependent metabolism of thiabendazole in Caenorhabditis elegans. *Biochemical Journal,* 466**,** 37-44.

JUNAIDU, H., LUKA, S. & MIJINYAWA, A. 2014. Prevalence of gastrointestinal helminth parasites of the domestic fowl (Gallus-gallus domesticus) slaughtered in Giwa market, Giwa local government, area, Kaduna state, Nigeria. *Journal of Natural Sciences Research,* 4**,** 121-126.

KAISER, P. 2007. The avian immune genome-a glass half-full or half-empty? *Cytogenet Genome Research,* 117**,** 221-30.

KAPLAN, R. M. 2004. Drug resistance in nematodes of veterinary importance: a status report. *Trends in Parasitology,* 20**,** 477-481.

KAPLAN, R. M., KLEI, T. R., LYONS, E. T., LESTER, G., COURTNEY, C. H., FRENCH, D. D., TOLLIVER, S. C., VIDYASHANKAR, A. N. & ZHAO, Y. 2004. Prevalence of anthelmintic resistant cyathostomes on horse farms. *Journal of the American Veterinary Medical Association,* 225**,** 903-910.

KAPLAN, R. M. & VIDYASHANKAR, A. N. 2012. An inconvenient truth: global worming and anthelmintic resistance. *Veterinary Parasitology,* 186**,** 70-78.

KATAKAM, K. K., MEJER, H., DALSGAARD, A., KYVSGAARD, N. C. & THAMSBORG, S. M. 2014. Survival of Ascaris suum and Ascaridia galli eggs in liquid manure at different ammonia concentrations and temperatures. *Veterinary Parasitology,* 204**,** 249-257.

KATES, K. & COLGLAZIER, M. 2011. Differential morphology of adult Ascaridia galli (Schrank, 1788) and Ascaridia dissimilis Perez Vigueras, 1931. *Proceedings of the helminthological society of Washington, USA,* 37**,** 80-84.

KATOCH, R., YADAV, A., GODARA, R., KHAJURIA, J. K., BORKATAKI, S. & SODHI, S. S. 2012. Prevalence and impact of gastrointestinal helminths on body weight gain in backyard chickens in subtropical and humid zone of Jammu, India. *Journal of Parasitic Diseases,* 36**,** 49-52.

KAUFMANN, F. 2011. *Helminth infections in laying hens kept in alternative production systems in Germany-Prevalence, worm burden and genetic resistance.* in General introduction. PhD Disseration, University of Goettingen, Germany. 6-18.

KAUFMANN, F., DAŞ, G., SOHNREY, B. & GAULY, M. 2011. Helminth infections in laying hens kept in organic free range systems in Germany. *Livestock Science,* 141**,** 182-187.

KAUSHIK, R. & DEORANI, V. S. 1969. Studies on tissue responses in primary and subsequent infections with Heterakis gallinae in chickens and on the process of formation of caecal nodules. *Journal of Helminthology,* 43**,** 69-78.

KEEGAN, J. D., GOOD, B., DE WAAL, T., FANNING, J. & KEANE, O. M. 2017. Genetic basis of benzimidazole resistance in Teladorsagia circumcincta in Ireland. *Irish Veterinary Journal,* 70**,** 8-11.

KIRSCH, R. 1984. Treatment of nematodiasis in poultry and game birds with fenbendazole. *Avian Diseases,* 28**,** 311-318.

KNAPP-LAWITZKE, F., KRÜCKEN, J., RAMÜNKE, S., VON SAMSON-HIMMELSTJERNA, G. & DEMELER, J. 2015. Rapid selection for β-tubulin alleles in codon 200 conferring benzimidazole resistance in an Ostertagia ostertagi isolate on pasture. *Veterinary Parasitology,* 209**,** 84-92.

KOKNAROGLU, H. & AKUNAL, T. 2013. Animal welfare: An animal science approach. *Meat Science,* 95**,** 821-827.

KOTZE, A. C., COWLING, K., BAGNALL, N. H., HINES, B. M., RUFFELL, A. P. & HUNT, P. W. 2012. Relative level of thiabendazole resistance associated with the E198A and F200Y SNPs in larvae of a multi-drug resistant isolate of Haemonchus contortus. *International Journal for Parasitology: Drugs and Drug Resistance,* 2**,** 92-97.

KOTZE, A. C., HUNT, P. W., SKUCE, P., VON SAMSON-HIMMELSTJERNA, G., MARTIN, R. J., SAGER, H., KRÜCKEN, J., HODGKINSON, J., LESPINE, A. & JEX, A. R. 2014. Recent advances in candidate-gene and whole-genome approaches to the discovery of anthelmintic resistance markers and the description of drug/receptor interactions. *International Journal for Parasitology: Drugs and Drug Resistance,* 4**,** 164-184.

KRÜCKEN, J., FRAUNDORFER, K., MUGISHA, J. C., RAMÜNKE, S., SIFFT, K. C., GEUS, D., HABARUGIRA, F., NDOLI, J., SENDEGEYA, A. & MUKAMPUNGA, C. 2017. Reduced efficacy of albendazole against Ascaris lumbricoides in Rwandan schoolchildren. *International Journal for Parasitology: Drugs and Drug Resistance,* 7**,** 262-271.

KUMAR, S., STECHER, G. & TAMURA, K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution,* 33**,** 1870-1874.

KWA, M. S., VEENSTRA, J. G. & ROOS, M. H. 1994. Benzimidazole resistance in Haemonchus contortus is correlated with a conserved mutation at amino acid 200 in β-tubulin isotype 1. *Molecular and Biochemical Parasitology,* 63**,** 299-303.

KWA, M. S., VEENSTRA, J. G., VAN DIJK, M. & ROOS, M. H. 1995. β-Tubulin Genes from the Parasitic NematodeHaemonchus contortusModulate Drug Resistance inCaenorhabditis elegans. *Journal of Molecular Biology,* 246**,** 500-510.

KWA, M. S. G., VEENSTRA, J. G. & ROOS, M. H. 1993. Molecular characterization of beta-tubulin genes present in benzimidazole resistant populations of Haemonchus contortus. *Molecular and Biochemical Parasitology,* 60**,** 133-144.

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

LACEY, E. 1990. Mode of action of benzimidazoles. *Parasitology Today,* 6**,** 112-115.

LALCHHANDAMA, K. 2010. On the structure of Ascaridia galli, the roundworm of domestic fowl. *Science Vision* 10**,** 20-30.

LAMBERT, S. M., NISHI, S. M., MENDONÇA, L. R., DA SILVA SOUZA, B. M. P., DA SILVA JULIÃO, F., DA SILVA GUSMÃO, P. & DE ALMEIDA, M. A. O. 2017. Genotypic profile of benzimidazole resistance associated with SNP F167Y and F200Y beta-tubulin gene in Brazilian populations of Haemonchus contortus of goats. *Veterinary Parasitology: Regional Studies and Reports,* 8**,** 28-34.

LEATHWICK, D. & BESIER, R. 2014. The management of anthelmintic resistance in grazing ruminants in Australasia—strategies and experiences. *Veterinary Parasitology,* 204**,** 44-54.

LESPINE, A., MÉNEZ, C., BOURGUINAT, C. & PRICHARD, R. K. 2012. P-glycoproteins and other multidrug resistance transporters in the pharmacology of anthelmintics: prospects for reversing transport-dependent anthelmintic resistance. *International Journal for Parasitology: Drugs and Drug Resistance,* 2**,** 58-75.

LEVECKE, B., DE WILDE, N., VANDENHOUTE, E. & VERCRUYSSE, J. 2009. Field validity and feasibility of four techniques for the detection of Trichuris in simians: a model for monitoring drug efficacy in public health? *PLoS Neglected Tropical Diseases,* 3**,** e366.

LOPUS, M., YENJERLA, M. & WILSON, L. 2008. Microtubule dynamics. *Wiley Encyclopedia of Chemical Biology,* 3**,** 153-160.

LÖTSCH, J., SKARKE, C. & GEISSLINGER, G. 2003. Simultaneous screening for three mutations in the ABCB1 gene. *Genomics,* 82**,** 503-510.

LUBEGA, G. W. & PRICHARD, R. K. 1991. Beta-tubulin and benzimidazole resistance in the sheep nematode Haemonchus contortus. *Molecular and Biochemical Parasitology,* 47**,** 129-137.

LUND, E. E. & CHUTE, A. M. 1972. Heterakis and Histomonas infections in young peafowl, compared to such infections in pheasants, chickens and turkeys.. *Journal of Wildlife Diseases,* 8**,** 352-358.

LUND, E. E. & CHUTE, A. M. 1974. The reproductive potential of Heterakis gallinarum in various species of galliform birds: Implications for survival of H. gallinarum and Histomonas meleagridis to recent times. *International Journal for Parasitology,* 4**,** 455-461.

LUND, E. E., WEHR, E. E. & ELLIS, D. J. 1966. Earthworm transmission of Heterakis and Histomonas to turkeys and chickens. *The Journal of parasitology,* 52**,** 899-902.

MARCOS-ATXUTEGI, C., GANDOLFI, B., ARANGÜENA, T., SEPÚLVEDA, R., ARÉVALO, M. & SIMÓN, F. 2009. Antibody and inflammatory responses in laying hens with experimental primary infections of Ascaridia galli. *Veterinary Parasitology,* 161**,** 69-75.

MARKTINFO EIER UND GEFLÜGEL (MEG) 2010. Marktbilanz Eier und Geflügel 2010. Ulmer, Stuttgart, Deutschland.

MARTIN, P., ANDERSON, N., JARRETT, R., BROWN, T. & FORD, G. 1982. Effects of a preventive and suppressive control scheme on the development of thiabendazole-resistance in Ostertagia spp. *Australian Veterinary Journal,* 58**,** 185-190.

MARTIN, R. 1997. Modes of action of anthelmintic drugs. *The Veterinary Journal,* 154**,** 11-34.

MARTÍN-PACHO, J., MONTOYA, M., ARANGÜENA, T., TORO, C., MORCHÓN, R., MARCOS-ATXUTEGI, C. & SIMON, F. 2005. A coprological and serological survey for the prevalence of Ascaridia spp. in laying hens. *Zoonoses and Public Health,* 52**,** 238-242.

MARTIS, M. M., TARBIAT, B., TYDEN, E., JANSSON, D. S. & HOGLUND, J. 2017. RNA-Seq de novo assembly and differential transcriptome analysis of the nematode Ascaridia galli in relation to in vivo exposure to flubendazole. *PLoS One,* 12**,** e0185182.

MAURER, V., HERTZBERG, H., HECKENDORN, F., HÖRDEGEN, P. & KOLLER, M. 2013. Effects of paddock management on vegetation, nutrient accumulation, and internal parasites in laying hens. *Journal of Applied Poultry Research,* 22**,** 334-343.

MCARTHUR, M. & REINEMEYER, C. 2014. Herding the US cattle industry toward a paradigm shift in parasite control. *Veterinary Parasitology,* 204**,** 34-43.

MCDOUGALD, L. R. 1998. Intestinal protozoa important to poultry. *Poultry Science,* 77**,** 1156-1158.

MCDOUGALD, L. R. 2005. Blackhead disease (histomoniasis) in poultry: a critical review. *Avian Diseases,* 49**,** 462-476.

MCKELLAR, Q. & SCOTT, E. 1990. The benzimidazole anthelmintic agents-a review. *Journal of Veterinary Pharmacology and Therapeutics,* 13**,** 223-247.

MELVILLE, L. A., SYKES, A. M. & MCCARTHY, J. S. 2006. The β-tubulin genes of two Strongyloides species. *Experimental Parasitology,* 112**,** 144-151.

MENEZES, R. C., TORTELLY, R., GOMES, D. C. & PINTO, R. M. 2003. Nodular typhlitis associated with the nematodes Heterakis gallinarum and Heterakis isolonche in pheasants: frequency and pathology with evidence of neoplasia. *Mem Inst Oswaldo Cruz,* 98**,** 1011-1016.

MILLER, M. A., PFEIFFER, W. & SCHWARTZ, T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Proceedings of thegateway computing environments workshop (GCE). *New Orleans, LA***,** 1-8.

MOHAMED, A., HAILEMARIAM, S., GEBREMEDHIN, G. & GEBEYEW, K. 2016. Challenges and Opportunities of Small Scale Poultry Production System in Jigjiga Zone, Somali Regional State, Ethiopia. *Poultry, Fisheries and Wildlife Science,* 4**,** 144.

MOHANRAJ, K., SUBHADRA, S., KALYANASUNDARAM, A., ILANGOPATHY, M. & RAMAN, M. 2017. Genotyping of benzimidazole resistant and susceptible isolates of Haemonchus contortus from sheep by allele specific PCR. *Journal of Parasitic Diseases,* 41**,** 282-288.

MORRISON, A. A., MITCHELL, S., MEARNS, R., RICHARDS, I., MATTHEWS, J. B. & BARTLEY, D. J. 2014. Phenotypic and genotypic analysis of benzimidazole resistance in the ovine parasite Nematodirus battus. *Veterinary Research,* 45**,** 116-125.

MUHAMAD, N., SIMCOCK, D. C., PEDLEY, K. C., SIMPSON, H. V. & BROWN, S. 2011. The kinetic properties of the glutamate dehydrogenase of Teladorsagia circumcincta and their significance for the lifestyle of the parasite. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 159**,** 71-77.

Literature cited

MURRELL, K. D. 1991. Economic losses resulting from food-borne parasitic zoonoses. *Southeast Asian Journal and Tropical Medicine Public Health,* 22**,** 377-381.

NJUE, A. & PRICHARD, R. 2003. Cloning two full-length beta-tubulin isotype cDNAs from Cooperia oncophora, and screening for benzimidazole resistance-associated mutations in two isolates. *Parasitology,* 127**,** 579-588.

NYREN, P., PETTERSSON, B. & UHLEN, M. 1993. Solid Phase DNA Minisequencing by an Enzymatic Luminometric Inorganic Pyrophosphate Detection Assay. *Analytical Biochemistry,* 208**,** 171-175.

OGBAJE, C., AGBO, E. & AJANUSI, O. 2012. Prevalence of Ascaridia gafli, Heterakis gaflinarum and Tapeworm Infections in Birds Slaughtered in Makurdi Township. *International Journal of Poultry Science,* 11**,** 103-107.

PAL, R. A. & QAYYUM, M. 1996. A review of anthelmintic resistance in small ruminants: prevalence, recent understanding and future prospects for the nematodes control. *Pakistan Veterinary Journal,* 16**,** 107-114.

PAPE, M., SCHNIEDER, T. & VON SAMSON-HIMMELSTJERNA, G. 2002. Investigation of diversity and isotypes of the beta-tubulin cDNA in several small strongyle (Cyathostominae) species. *Journal of Parasitology,* 88**,** 673-677.

PAPE, M., VON SAMSON-HIMMELSTJERNA, G. & SCHNIEDE, T. 1999. Characterisation of the beta-tubulin gene of Cylicocyclus nassatus1Note: Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers AF181092 and AF181093.1. *International Journal for Parasitology,* 29**,** 1941-1947.

PAPINI, R. & CACCIUTTOLO, E. 2008. Observations on the occurrence of Heterakis gallinarum in laying hens kept on soil. *Italian Journal of Animal Science,* 7**,** 487-493.

PARAUD, C. & CHARTIER, C. 2017. Facing Anthelmintic Resistance in Goats. In: Simões J, Gutiérrez C, editors. Sustainable goat production in adverse environments: welfare, health and breeding. Springer International Publishing,Switzerland. 1, 267-292.

PARK, S.-I. & SHIN, S.-S. 2010. Concurrent Capillaria and Heterakis infections in zoo rock partridges, Alectoris graeca. *The Korean Journal of Parasitology,* 48**,** 253-257.

PENNYCOTT, T. & STEEL, F. 2001. Parasitic worms in commercial free-range poultry flocks in England and Wales. *The Veterinary Record,* 149**,** 428.

PERKINS, J., YAZWINSKI, T. & TUCKER, C. 2012. Efficacies of fenbendazole and albendazole in the treatment of commercial turkeys artificially infected with Ascaridia dissimilis. *The Student Journal of Dale Bumpers College of Agricultural, Food and Life Sciences,* 13**,** 61-66.

PERMIN, A., BISGAARD, M., FRANDSEN, F., PEARMAN, M., KOLD, J. & NANSEN, P. 1999. Prevalence of gastrointestinal helminths in different poultry production systems. *British Poultry Science,* 40**,** 439-443.

PERMIN, A., CHRISTENSEN, J. P. & BISGAARD, M. 2006. Consequences of concurrent Ascaridia galli and Escherichia coli infections in chickens. *Acta Veterinaria Scandinavica,* 47**,** 43-54.

PERMIN, A. & HANSEN, J. W. 1998. Epidemiology, diagnosis and control of poultry parasites. *In: FAO Animal Health Manual,* 4**,** 15-37.

PLEIDRUP, J., DALGAARD, T. S., NORUP, L. R., PERMIN, A., SCHOU, T. W., SKOVGAARD, K., VADEKÆR, D. F., JUNGERSEN, G., SØRENSEN, P. & JUUL-MADSEN, H. R. 2014. Ascaridia galli infection influences the development of both

humoral and cell-mediated immunity after Newcastle Disease vaccination in chickens. *Vaccine,* 32**,** 383-392.

POULIN, R. 2011. *Evolutionary ecology of parasites*, Princeton University Press. 2, 8-40.

POURMAND, N., ELAHI, E., DAVIS, R. W. & RONAGHI, M. 2002. Multiplex pyrosequencing. *Nucleic acids Research,* 30**,** e31-e33.

PRICHARD, R. 1990. Anthelmintic resistance in nematodes: extent, recent understanding and future directions for control and research. *International journal for parasitology,* 20**,** 515-523.

PRICHARD, R. 1994. Anthelmintic resistance. *Veterinary Parasitology,* 54**,** 259-268.

PRICHARD, R., HALL, C., KELLY, J., MARTIN, I. & DONALD, A. 1980. The problem of anthelmintic resistance in nematodes. *Australian Veterinary Journal,* 56**,** 239-250.

PRICHARD, R. K. 2001. Genetic variability following selection of Haemonchus contortus with anthelmintics. *Trends Parasitology,* 17**,** 446-453.

PRICHARD, R. K. 2008. Mechanisms of anthelmintic resistance: implications for the future of parasite control. *Congresso Brasileiro De Parasitologia Veterinária,* 21**,** 62-68.

RAHMAN, W. A. & MANAP, N. H. 2014. Descriptions on the morphology of some nematodes of the Malaysian domestic chicken (Gallus domesticus) using scanning electron microscopy. *Malaysian Journal of Veterinary Research,* 5**,** 35-42.

RAMADAN, H. H. & ABOUZNADA, N. Y. 1992. Morphology and life history of Ascaridia galli in the domestic fowl that are raised in Jeddah. *Science,* 4**,** 78-99.

RAMÜNKE, S., MELVILLE, L., RINALDI, L., HERTZBERG, H., DE WAAL, T., VON SAMSON-HIMMELSTJERNA, G., CRINGOLI, G., MAVROT, F., SKUCE, P., KRUCKEN, J. & DEMELER, J. 2016. Benzimidazole resistance survey for Haemonchus,

Teladorsagia and Trichostrongylus in three European countries using pyrosequencing including the development of new assays for Trichostrongylus. *International Journal for Parasitology: Drugs and Drug Resistance,* 6**,** 230-240.

RASHWAN, N., SCOTT, M. & PRICHARD, R. 2017. Rapid Genotyping of β-tubulin Polymorphisms in Trichuris trichiura and Ascaris lumbricoides. *PLoS Neglected Tropical Diseases,* 11**,** e0005205.

REDMAN, E., WHITELAW, F., TAIT, A., BURGESS, C., BARTLEY, Y., SKUCE, P. J., JACKSON, F. & GILLEARD, J. S. 2015. The emergence of resistance to the benzimidazole anthlemintics in parasitic nematodes of livestock is characterised by multiple independent hard and soft selective sweeps. *PLoS neglected tropical diseases,* 9**,** e0003494.

REID, W. M. 1960. Effects of temperature on the development of the eggs of Ascaridia galli. *The Journal of Parasitology,* 46**,** 63-67.

REID, W. M. & CARMON, J. L. 1958. Effects of numbers of Ascaridia galli in depressing weight gains in chicks. *The Journal of Parasitology,* 44**,** 183-186.

RENDELL, D. 2010. Anthelmintic resistance in cattle nematodes on 13 south-west Victorian properties. *Australian Veterinary Journal,* 88**,** 504-509.

RIDDELL, C. & GAJADHAR, A. 1988. Cecal and hepatic granulomas in chickens associated with Heterakis gallinarum infection. *Avian Diseases,* 32**,** 836-838.

RINALDI, L., LEVECKE, B., BOSCO, A., IANNIELLO, D., PEPE, P., CHARLIER, J., CRINGOLI, G. & VERCRUYSSE, J. 2014. Comparison of individual and pooled faecal samples in sheep for the assessment of gastrointestinal strongyle infection intensity and

anthelmintic drug efficacy using McMaster and Mini-FLOTAC. *Veterinary Parasitology,* 205**,** 216-223.

ROEBER, F., JEX, A. R. & GASSER, R. B. 2013. Impact of gastrointestinal parasitic nematodes of sheep, and the role of advanced molecular tools for exploring epidemiology and drug resistance-an Australian perspective. *Parasites & Vectors,* 6**,** 153-166.

RONAGHI, M., KARAMOHAMED, S., PETTERSSON, B., UHLÉN, M. & NYRÉN, P. 1996. Real-time DNA sequencing using detection of pyrophosphate release. *Analytical Biochemistry,* 242**,** 84-89.

RONAGHI, M., UHLÉN, M. & NYRÉN, P. 1998. A sequencing method based on real-time pyrophosphate. *Science,* 281**,** 363-365.

RUFF, M. D. 1999. Important parasites in poultry production systems. *Veterinary Parasitology,* 84**,** 337-347.

SANGSTER, N. C., PRICHARD, R. K. & LACEY, E. 1985. Tubulin and benzimidazole-resistance in Trichostrongylus colubriformis (Nematoda). *The Journal of Parasitology,* 71**,** 645-651.

SAUNDERS, G. I., WASMUTH, J. D., BEECH, R., LAING, R., HUNT, M., NAGHRA, H., COTTON, J. A., BERRIMAN, M., BRITTON, C. & GILLEARD, J. S. 2013. Characterization and comparative analysis of the complete Haemonchus contortus β-tubulin gene family and implications for benzimidazole resistance in strongylid nematodes. *International Journal for Parasitology,* 43**,** 465-475.

SAYYED, R., PHULAN, M., BHATTI, W., PARDEHI, M. & SHAMSHER, A. 2000. Incidence of nematode parasites in commercial layers in Swat. *Pakistan Veterinary Journal,* 20**,** 107-108.

SCANES, C. G. 2007. The Global Importance of Poultry. *Poultry Science,* 86**,** 1057-1058.

SCHWAB, A. E., BOAKYE, D. A., KYELEM, D. & PRICHARD, R. K. 2005. Detection of benzimidazole resistance–associated mutations in the filarial nematode Wuchereria bancrofti and evidence for selection by albendazole and ivermectin combination treatment. *The American Journal of Tropical Medicine and Hygiene,* 73**,** 234-238.

SCHWARZ, A., GAULY, M., ABEL, H., DAS, G., HUMBURG, J., WEISS, A. T., BREVES, G. & RAUTENSCHLEIN, S. 2011. Pathobiology of Heterakis gallinarum mono-infection and co-infection with Histomonas meleagridis in layer chickens. *Avian Pathology,* 40**,** 277-287.

SHALABY, H. A. 2013. Anthelmintics Resistance; How to Overcome it? *Iranian Journal of Parasitology,* 8**,** 18-32.

SHERWIN, C., NASR, M., GALE, E., PETEK, M., STAFFORD, K., TURP, M. & COLES, G. 2013. Prevalence of nematode infection and faecal egg counts in free-range laying hens: relations to housing and husbandry. *British Poultry Science,* 54**,** 12-23.

SHIFERAW, S., TAMIRU, F., GIZAW, A., ATALEL, D., TERFA, W., DANDECHA, M. & MEKIBIB, A. 2016. Study on prevalence of helminthes of local backyard and exotic chickens in and around ambowest shoa zone, Oromia regional state, Ethiopia. *Journal of Veterinary Science and Medecine,* 4**,** 11-14.

SHIMMURA, T., HIRAHARA, S., AZUMA, T., SUZUKI, T., EGUCHI, Y., UETAKE, K. & TANAKA, T. 2010. Multi-factorial investigation of various housing systems for laying hens. *British Poultry Science,* 51**,** 31-42.

SILVESTRE, A. & CABARET, J. 2002. Mutation in position 167 of isotype 1 beta-tubulin gene of trichostrongylid nematodes: role in benzimidazole resistance? *Molecular and Biochemical Parasitology,* 120**,** 297-300.

SILVESTRE, A., CABARET, J. & HUMBERT, J.-F. 2001. Effect of benzimidazole under-dosing on the resistant allele frequency in Teladorsagia circumcincta (Nematoda). *Parasitology,* 123**,** 103-111.

SILVESTRE, A. & HUMBERT, J. 2002. Diversity of benzimidazole-resistance alleles in populations of small ruminant parasites. *International Journal for Parasitology,* 32**,** 921-928.

SILVESTRE, A. & HUMBERT, J.-F. 2000. A Molecular Tool for Species Identification and Benzimidazole Resistance Diagnosis in Larval Communities of Small Ruminant Parasites. *Experimental Parasitology,* 95**,** 271-276.

SKALLERUP, P., LUNA, L. A., JOHANSEN, M. V. & KYVSGAARD, N. C. 2005. The impact of natural helminth infections and supplementary protein on growth performance of free-range chickens on smallholder farms in El Sauce, Nicaragua. *Preventive Veterinary Medicine,* 69**,** 229-244.

SKUCE, P., STENHOUSE, L., JACKSON, F., HYPŠA, V. & GILLEARD, J. 2010. Benzimidazole resistance allele haplotype diversity in United Kingdom isolates of Teladorsagia circumcincta supports a hypothesis of multiple origins of resistance by recurrent mutation. *International journal for parasitology,* 40**,** 1247-1255.

SOMMER, F. & VASICEK, L. 2000. Management and state of health in free-range poultry flocks. *Wiener Tierärztliche Monatsschrift,* 87**,** 202-212.

SØRENSEN, J. G., KRISTENSEN, T. N. & LOESCHCKE, V. 2003. The evolutionary and ecological role of heat shock proteins. *Ecology Letters,* 6**,** 1025-1037.

STAMATAKIS, A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics,* 30**,** 1312-1313.

SUTHERLAND, I. A. & LEATHWICK, D. M. 2011. Anthelmintic resistance in nematode parasites of cattle: a global issue? *Trends in Parasitology,* 27**,** 176-181.

TARBIAT, B., JANSSON, D., TYDÉN, E. & HÖGLUND, J. 2017. Evaluation of benzimidazole resistance status in Ascaridia galli. *Parasitology,* 144**,** 1338-1345.

TARBIAT, B., JANSSON, D. S. & HÖGLUND, J. 2015. Environmental tolerance of free-living stages of the poultry roundworm Ascaridia galli. *Veterinary Parasitology,* 209**,** 101-107.

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.

THAMSBORG, S. M., ROEPSTORFF, A., NEJSUM, P. & MEJER, H. 2010. Alternative approaches to control of parasites in livestock: Nordic and Baltic perspectives. *Acta Veterinaria Scandinavica,* 52**,** 12-16.

THAPA, S., HINRICHSEN, L. K., BRENNINKMEYER, C., GUNNARSSON, S., HEERKENS, J. L., VERWER, C., NIEBUHR, K., WILLETT, A., GRILLI, G., THAMSBORG, S. M., SORENSEN, J. T. & MEJER, H. 2015. Prevalence and magnitude of helminth infections in organic laying hens (Gallus gallus domesticus) across Europe. *Veterinary Parasitology* 214**,** 118-124.

THAPA, S., THAMSBORG, S. M., MEYLING, N. V., DHAKAL, S. & MEJER, H. 2017. Survival and development of chicken ascarid eggs in temperate pastures. *Parasitology,* 144**,** 1243-1252.

TIERSCHNUTZV 2009. Verordnung zum Schutz landwirtschaftlicher Nutztiere und anderer zur Erzeugung tierischer Produkte gehaltener Tiere bei ihrer Haltung (Tierschutz-Nutztierhaltungsverordnung – TierSchNutztV), 5, 9-11.

TOLEDO, R. & CASTELL, S. 1981. Effect of Ascaridia galli on the weight gain in broilers. *Revista Cubana de Ciencias Veterinarias (Cuba),* 12**,** 274-278.

TORGERSON, P. R., PAUL, M. & FURRER, R. 2014. Evaluating faecal egg count reduction using a specifically designed package "eggCounts" in R and a user friendly web interface. *International Journal for Parasitology,* 44**,** 299-303.

TUCKER, C. A., YAZWINSKI, T. A., REYNOLDS, L., JOHNSON, Z. & KEATING, M. 2007. Determination of the Anthelmintic Efficacy of Albendazole in the Treatment of Chickens Naturally Infected with Gastrointestinal Helminths. *The Journal of Applied Poultry Research,* 16**,** 392-396.

TUYTTENS, F., HEYNDRICKX, M., DE BOECK, M., MOREELS, A., VAN NUFFEL, A., VAN POUCKE, E., VAN COILLIE, E., VAN DONGEN, S. & LENS, L. 2008. Broiler chicken health, welfare and fluctuating asymmetry in organic versus conventional production systems. *Livestock Science,* 113**,** 123-132.

TYDÉN, E., ENGSTRÖM, A., MORRISON, D. A. & HÖGLUND, J. 2013. Sequencing of the β-tubulin genes in the ascarid nematodes Parascaris equorum and Ascaridia galli. *Molecular and Biochemical Parasitology,* 190**,** 38-43.

TYDEN, E., SKARIN, M., ANDERSSON-FRANKO, M., SJOBLOM, M. & HOGLUND, J. 2016. Differential expression of beta-tubulin isotypes in different life stages of Parascaris spp after exposure to thiabendazole. *Molecular and Biochemical Parasitology,* 205**,** 22-28.

VAN GREMBERGEN, G. 1954. Hæmoglobin in Heterakis gallinae. *Nature,* 174**,** 35-35.

VAN HORNE, P. & ACHTERBOSCH, T. 2008. Animal welfare in poultry production systems: impact of EU standards on world trade. *World's Poultry Science Journal,* 64**,** 40-52.

VERMA, N., BHATNAGAR, P. & BANERJEE, D. 1991. COMPARATIVE EFFICACY OF 3 BROAD-SPECTRUM ANTHELMINTICS AGAINST ASCARIDIA-GALLI IN POULTRY. *Indian Veterinary Journal of Animal Sciences,* 61**,** 834-835.

VON SAMSON-HIMMELSTJERNA, G. 2006. Molecular diagnosis of anthelmintic resistance. *Veterinary Parasitology,* 136**,** 99-107.

VON SAMSON-HIMMELSTJERNA, G., BLACKHALL, W. J., MCCARTHY, J. S. & SKUCE, P. J. 2007. Single nucleotide polymorphism (SNP) markers for benzimidazole resistance in veterinary nematodes. *Parasitology,* 134**,** 1077-86.

VON SAMSON-HIMMELSTJERNA, G., COLES, G. C., JACKSON, F., BAUER, C., BORGSTEEDE, F., CIRAK, V. Y., DEMELER, J., DONNAN, A., DORNY, P., EPE, C., HARDER, A., HÖGLUND, J., KAMINSKY, R., KERBOEUF, D., KÜTTLER, U., PAPADOPOULOS, E., POSEDI, J., SMALL, J., VÁRADY, M., VERCRUYSSE, J. & WIRTHERLE, N. 2009a. Standardization of the egg hatch test for the detection of benzimidazole resistance in parasitic nematodes. *Parasitology Research,* 105**,** 825-834.

VON SAMSON-HIMMELSTJERNA, G., PAPE, M., VON WITZENDORFF, C. & SCHNIEDER, T. 2002a. Allele-specific PCR for the beta-tubulin codon 200 TTC/TAC

polymorphism using single adult and larval small strongyle (Cyathostominae) stages. *Journal of Parasitology,* 88**,** 254-257.

VON SAMSON-HIMMELSTJERNA, G., VON WITZENDORFF, C., SIEVERS, G. & SCHNIEDER, T. 2002b. Comparative use of faecal egg count reduction test, egg hatch assay and beta-tubulin codon 200 genotyping in small strongyles (cyathostominae) before and after benzimidazole treatment. *Veterinary Parasitology* 108**,** 227-235.

VON SAMSON-HIMMELSTJERNA, G., WALSH, T., DONNAN, A., CARRIERE, S., JACKSON, F., SKUCE, P., ROHN, K. & WOLSTENHOLME, A. J. 2009b. Molecular detection of benzimidazole resistance in Haemonchus contortus using real-time PCR and pyrosequencing. *Parasitology,* 136**,** 349-358.

WAGHORN, T., LEATHWICK, D., RHODES, A., LAWRENCE, K., JACKSON, R., POMROY, W., WEST, D. & MOFFAT, J. 2006. Prevalence of anthelmintic resistance on 62 beef cattle farms in the North Island of New Zealand. *New Zealand Veterinary Journal,* 54**,** 278-282.

WALSH, T. K., DONNAN, A. A., JACKSON, F., SKUCE, P. & WOLSTENHOLME, A. J. 2007. Detection and measurement of benzimidazole resistance alleles in Haemonchus contortus using real-time PCR with locked nucleic acid Taqman probes. *Veterinary Parasitology,* 144**,** 304-312.

WANG, C., TORGERSON, P. R., HOGLUND, J. & FURRER, R. 2017a. Zero-inflated hierarchical models for faecal egg counts to assess anthelmintic efficacy. *Veterinary Parasitology,* 235**,** 20-28.

WANG, C., TORGERSON, P. R., KAPLAN, R. M., GEORGE, M. M. & FURRER, R. 2018. Modelling anthelmintic resistance by extending eggCounts package to allow individual efficacy. *International Journal for Parasitology: Drugs and Drug Resistance,* 8**,** 386-393.

WANG, J., GAO, S., MOSTOVOY, Y., KANG, Y., ZAGOSKIN, M., SUN, Y., ZHANG, B., WHITE, L. K., EASTON, A. & NUTMAN, T. B. 2017b. Comparative genome analysis of programmed DNA elimination in nematodes. *Genome Research,* 27**,** 2001-2014.

WHARTON, D. 1983. The production and functional morphology of helminth egg-shells. *Parasitology,* 86**,** 85-97.

WHITTAKER, J., CARLSON, S., JONES, D. & BREWER, M. 2017. Molecular mechanisms for anthelmintic resistance in strongyle nematode parasites of veterinary importance. *Journal of Veterinary Pharmacology and Therapeutics,* 40**,** 105-115.

WINTERROWD, C., POMROY, W., SANGSTER, N., JOHNSON, S. & GEARY, T. 2003. Benzimidazole-resistant β-tubulin alleles in a population of parasitic nematodes (Cooperia oncophora) of cattle. *Veterinary Parasitology,* 117**,** 161-172.

WOLSTENHOLME, A. J., FAIRWEATHER, I., PRICHARD, R., VON SAMSON-HIMMELSTJERNA, G. & SANGSTER, N. C. 2004. Drug resistance in veterinary helminths. *Trends in Parasitology,* 20**,** 469-476.

WONGRAK, K., DAS, G., MOORS, E., SOHNREY, B. & GAULY, M. 2014. Establishment of gastro-intestinal helminth infections in free-range chickens: a longitudinal on farm study. *Berliner und Munchener Tierarztleche Wochenschrift,* 127**,** 314-321.

WONGRAK, K., DAŞ, G., VON BORSTEL, U. K. & GAULY, M. 2015. Genetic variation for worm burdens in laying hens naturally infected with gastro-intestinal nematodes. *British Poultry Science,* 56**,** 15-21.

WOODGATE, R., CORNELL, A. & SANGSTER, N. 2017. Occurrence, measurement and clinical perspectives of drug resistance in important parasitic helminths of livestock.In: Mayers DL, Sobel JD, Ouellette M, Kaye KS, Marchaim D (eds). Antimicrobial Drug Resistance. Springer International Publishing, Switzerland, 2, 1305-1326.

WUTHIJAREE, K., LAMBERTZ, C. & GAULY, M. 2017. Prevalence of gastrointestinal helminth infections in free-range laying hens under mountain farming production conditions. *British Poultry Science,* 58**,** 649-655.

YAZWINSKI, T., TUCKER, C., REYNOLDS, J., JOHNSON, Z. & PYLE, D. 2009. Efficacies of fenbendazole and levamisole in the treatment of commercial turkeys for Ascaridia dissimilis infections. *Journal of Applied Poultry Research,* 18**,** 318-324.

YAZWINSKI, T., TUCKER, C., STELZLENI, A., JOHNSON, Z., ROBINS, J., DOWNUM, K., FINCHER, M., MATLOCK, J. & CHAPMAN, H. 2002. Subclinical effects and fenbendazole treatment of turkey ascaridiasis under simulated field conditions. *Avian Diseases,* 46**,** 886-892.

YAZWINSKI, T. A., CHAPMAN, H. D., DAVIS, R. B., LETONJA, T., POTE, L., MAES, L., VERCRUYSSE, J. & JACOBS, D. E. 2003. World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for evaluating the effectiveness of anthelmintics in chickens and turkeys. *Veterinary Parasitology,* 116**,** 159-173.

YAZWINSKI, T. A., TUCKER, C. A., WRAY, E., JONES, L. & CLARK, F. D. 2013. Observations of benzimidazole efficacies against Ascaridia dissimilis, Ascaridia galli, and Heterakis gallinarum in naturally infected poultry. *The Journal of Applied Poultry Research,* 22**,** 75-79.

YAZWINSRI, T., ROSENSTEIN, M., SCHWARTZ, R., WILSON, K. & JOHNSON, Z. 1993. The use of fenbendazole in the treatment of commercial turkeys infected with Ascaridia dissimilis. *Avian Pathology,* 22**,** 177-181.

ZENTRALE MARK UND PREISBERICHTSTELLE (ZMP) GMBH 2008. Marktbilanz, Eier und Geflügel 2008. Bonn, 213pp.

ZHENG, C., DIAZ-CUADROS, M., NGUYEN, K. C., HALL, D. H. & CHALFIE, M. 2017. Distinct effects of tubulin isotype mutations on neurite growth in Caenorhabditis elegans. *Molecular Biology of the Cell,* 28**,** 2786-2801.

Publications

Published conference proceedings

1. Vahel J. Ameen, Jürgen Krücken, Gürbüz Daş, Hafez M. Hafez, Jabbar Ahmed, Georg von Samson-Himmelstjerna (2018) Development of Pyrosequencing Based Assays for Benzimidazole Resistance Detection in *Heterakis gallinarum* and *Ascaridia galli*. In: The 28th Annual Meeting of the German Society for Parasitology. March 21-24, 2018 Berlin, Germany. Abstract band ISBN: 978-3-9816508-7-7, page No. 299.
2. Vahel J. Ameen, Jürgen Krücken, Gürbüz Daş, Hafez M. Hafez, Jabbar Ahmed, Georg von Samson-Himmelstjerna (2018) Identification of β-tubulin Isotypes and Development of Pyrosequencing Assays for Benzimidazole Resistance in *Heterakis gallinarum* and *Ascaridia galli*. In: Tagung der Deutschen Veterinärmedizinischen Gesellschaft (DVG), Fachgruppe Parasitologie und parasitäre Krankheiten. June 2-4, 2018. Gießen, Germany. Abstract band ISBN: 976-3-86345-420-3, page No. 50-51.

Acknowledgments

I thank the Almighty ALLAH for giving me the strength and patience to work through all these years so that today I can stand proudly with my head held high.

I take benefit of this auspicious opportunity to express with great pleasure, my deepest and intense sense of indebtedness to my respected supervisor Professor Dr. Georg von Samson-Himmelstjerna. His expert advising, untiring support with anything, anytime, anywhere, excellent patience, persistent guidance during the whole span of this program, valuable and prompt feedback with intellectual criticism made it all possible for me to successfully accomplish this work. Their immense knowledge, motivation and patience have given me more power and spirit to excel in the research.

I would like to express my sincere gratitude to Dr. Jürgen Krücken. Your advice on both research as well as on my career have been priceless. Thanks for giving the encouragement and sharing insightful suggestions. Your close watch, continuous patience, excellent guidance and valuable suggestions without the help of which this effort would never become so comprehensive and apt. Your endless guidance is hard to forget throughout my life.

I would like to express my deepest sense of gratitude and honor to Professor Dr. Jabbar S. Ahmed untiring support and constant encouragement throughout my study. I am also very thankful to Professor Dr. Hafez M. Hafez and Dr. Gürbüz Daş for providing me specimens for analysis, insightful comments and suggestions for conferences manuscripts.

I am very grateful to Professor Dr. Johan Höglund, Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences and all his laboratory staff for providing me field samples and for his warm hospitality.

Acknowledgments

I am also very thankful to Mrs. Sabrina Ramünke and all the former and present PhD colleagues and staff members at the Institute for Parasitology and Tropical Veterinary medicine, Freie Universität Berlin for their cooperation, good advices and suggestions.

I feel a momentous serenity in transcribing my sincere thankfulness to Dr. Abdul REHMAN for his continuous support in the Lab. and for all the fun, we have had at the time of living in Berlin.

I am also pleased to say thank you to Dr. Jassim Abdo, director of Duhok Research Center for his continuous support. I have a great sense of obligation to reverend Serwan Taib Amedi for his warm hospitality. You are the one who is ready to help anyone in any situation. I cannot tell you how much I appreciated you and your family kindness.

Special thanks are due to the Ministry of Higher Education and Scientific Research, Kurdistan Region Government and Human Capacity Development Program in Higher Education (HCDP) for providing me with a PhD fellowship.

I also feel my indebtedness to my family. Words cannot express how grateful I am to my parents, my beloved wife, son and daughters for all the sacrifices that you have made on my behalf and for showing faith in me and giving me liberty to choose what I desired. Your prayer for me was what sustained me thus far. I consider myself the luckiest in the world to have such a lovely and caring family, standing beside me with their love and unconditional support.

Thanks for Peshmarga, we proud of you.

Vahel J. Ameen,

Berlin

Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Dissertation mit dem Titel „Identification of β-tubulin Isotypes and Development of Pyrosequencing Assays for Benzimidazole Resistance in *Heterakis gallinarum* and *Ascaridia galli*“ selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe. Keine entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder anderer Personen) wurde in Anspruch genommen. Niemand hat von mir mittelbar oder unmittelbar entgeltliche Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Ich habe die Dissertation am Institute for Parasitology and Tropical Veterinary Medicine des Freie Universität Berlin. Die vorliegende Arbeit wurde bisher nicht für eine Prüfung oder Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht.

Berlin, den 27.02.2020

Vahel J. Ameen

mbvberlin mensch und buch verlag

49,90 Euro | ISBN: 978-3-96729-042-4