# Metabolism of macrocyclic lactones and benzimidazoles in parasitic nematodes

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**ESRA YILMAZ**

**from Berlin**

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This work was prepared from April 2015 to April 2019 under the supervision of PD Dr. Jürgen Krücken at the Institute for Parasitology and Tropical Veterinary Medicine of Freie Universität Berlin, Germany.

1st Reviewer:

**PD Dr. Jürgen Krücken**

Institute for Parasitology and Tropical Veterinary Medicine

Department of Veterinary Medicine

Freie Universität Berlin

Germany

2nd Reviewer:

**Prof. Dr. Rupert Mutzel**

Institute of Biology

Department of Biology, Chemistry and Pharmacy

Freie Universität Berlin

Germany

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# List of abbreviations

| | |
|---|---|
| ABZ | albendazole |
| ABZ-SO | albendazole sulfoxide |
| AUC | area under the concentration *vs* time curve |
| BZ | benzimidazole |
| CGC | Caenorhabditis Genetic Center |
| CNV | copy number variation |
| COPD | cyclooctadepsipeptide |
| COWPs | copper oxide wire particles |
| CYP | cytochrome P450 monooxygenase |
| DALYs | disability- adjusted life years |
| DMSO | dimethyl sulfoxide |
| $EC_{50}$ | half maximal effective concentration |
| EHA | egg hatch assay |
| EPG | eggs per gram |
| E/S | excretory/secretory |
| ET | extracellular trap |
| FEC | fecal egg count |
| FECRT | fecal egg count reduction test |
| FLU | flubendazole |
| GABA channels | ɣ-aminobutyric-acid gated chloride channels |
| GluCl channels | glutamate-gated chloride channels |
| GI | gastro-intestinal |
| IVM | ivermectin |
| L1 | first stage larvae |
| L2 | second stage larvae |
| L3 | third stage larvae |
| L4 | fourth stage larvae |

| | |
|---|---|
| LDA | larval development assay |
| LEV | levamisole |
| LMIA | larval migration inhibition assay |
| ML | macrocyclic lactone |
| MMT | micromotility meter test |
| MON | monepantel |
| MOX | moxidectin |
| nAChR | nicotinic acetylcholine receptors |
| NGM | nematode growth medium |
| PBO | piperonyl butoxide |
| PCR | polymerase chain reaction |
| Pgp | P-glycoprotein |
| PCV | packed cell volume |
| rDNA | ribosomal DNA |
| Rh123 | rhodamine 123 |
| SNP | single-nucleotide polymorphisms |
| STE | sheath-tail extension |
| STH | soil-transmitted helminth |
| TBZ | thiabendazole |
| TT | targeted treatment |
| TST | targeted selective treatment |
| VPL | verapamil |
| WAAVP | World Association for the Advancement of Veterinary Parasitology |

# 1 Introduction

With over 25,000 species described and estimates of global diversity reaching up to 1 million species, nematodes are the second diverse group of diploid animals, surpassed only by arthropods. While most nematodes are free-living and beneficial, around 15% of the described species are known to parasitize on terrestrial animals and/or humans and 10% are plant parasitic nematodes. Infections with gastro-intestinal nematodes (GI nematodes) are among the most common worldwide. It is estimated that over 50% of the world's human population are affected by GI nematodes (Horton, 2003). Although losing over 3 million disability- adjusted life years (DALYs) to four of the most important human nematodes *Necator americanus*, *Ancylostoma duodenale*, *Ascaris lumbricoides* and *Trichuris trichiura* according to the Global Burden of Disease Study 2015 (DALYs GBD, 2016) and *A. lumbricoides* causing 60.000 deaths per year (http://www.who.int/water_sanitation_health/diseases-risks/diseases/ascariasis/en/, last accessed 31.03.2019), public awareness remains surprisingly low, possibly because they are more frequently associated with chronic diseases rather than mortality.

GI nematodes that affect livestock, however, receive a great deal of attention as they can pose a serious threat to animal welfare and lead to significant production losses. Most pathogenic GI nematodes able to infest small and large ruminants are members of the suborder Trichostrongyloideae, order Strongylida, such as *Haemonchus contortus* and *Ostertagia ostertagi*. Unfortunately, the widespread emergence of drug resistance has made the treatment of GI nematode infections an enormous challenge in livestock. This problem particularly involves the sheep industry in Australia, New Zealand and South Africa (van Wyk et al., 1999; Besier and Love, 2003; Waghorn et al., 2006) and is becoming an increasing issue in the UK (Papadopoulos et al., 2012).

Resistance has emerged to all three main drug classes: the macrocyclic lactones (MLs), benzimidazoles (BZs) and the nicotinergic drugs with the tetrahydropyrimidines and levamisole (LEV). Due to the overwhelming success of the MLs in combating nematode infections for over twenty years, combined with challenges of drug discovery and costs of development and approval well over $100 million, only two new anthelmintic drugs have been introduced into the livestock market. Both Zolvix® (agent: monepantel; Elanco, formerly Novartis) and Startect® (agent: derquantel; Zoetis) are approved for veterinary use on sheep and goats against GI nematodes. The first report on field resistance against the former was published just four years after its market introduction (Scott et al., 2013). Consequently, concerns about long-lasting losses in profitability and animal health are widespread in the livestock industry.

The call for more efforts in drug discovery is justified but this will only provide temporary relief because parasites, although initially amenable to new chemotherapy, are typically prone to develop resistance. Further research and concurrent strategies, such as pasture management to avoid the peak in larval contamination on pasture, need to be undertaken to prolong or maintain the effectiveness of already

existing and future anthelmintics. One vital research focus is the investigation of mechanisms of drug resistance as this will not only help to develop more tests capable of diagnosing resistance in the field and help with proper pasture management but also simplify the search for potential new drug targets. Even though some resistance mechanisms have been validated and more proposed, the overall picture remains fragmentary and co-occurrence of different mechanisms has barely been elucidated. While most research has been conducted on target-site related mechanisms, non-target-site related mechanisms are increasingly recognized as putative contributors of resistance. In this context, drug-metabolizing enzymes may reduce anthelmintic efficacy and thereby contribute to development of resistance. Cytochrome P450 monooxygenases (CYPs) represent the largest group of metabolizing enzymes and have been confirmed as contributors or mediators of resistance in mammals and insects (Rochat, 2005; David et al., 2013). Ever since CYP activity was shown in parasitic nematodes (Kotze, 1997), attention gradually increased, and they have now become an integral part of anthelmintic resistance research.

The objective of the PhD project was to understand the role of CYPs in the metabolization of the most important anthelmintic drugs: BZs and MLs. The free-living model nematode *Caenorhabditis elegans* has been shown to metabolize the BZ representative thiabendazole via CYP35D1 (Jones et al., 2015). Genomic, metabolomic and *in vitro* studies suggest that CYPs might be involved in anthelmintic resistance in parasitic nematodes just as well (AlGusbi et al., 2014; Laing et al., 2015; Stuchlíková et al., 2018).

Through approaches involving determination of gene expression profiles of the parasitic *Haemonchus contortus* as well as changes in susceptibility of *C. elegans*, it was intended to further understand the relevance of detoxification for the emergence of anthelmintic resistance.

# 2 Literature review

## 2.1 The phylum Nematoda

The phylum Nematoda describes an exceptionally diverse group of animals. Nematodes have a deceptively similar morphological pattern, which masks how diverse these animals are in terms of morphological details and size. While being commonly round in cross-section, nematodes can display diversity in every other aspect of their morphology, particularly in their pharynx and posterior end and they can range in size from microscopic to several meters. With up to 8.4 m in length, the sperm whale nematode *Placentonema gigantissima* is by far the largest representative (Gubanov, 1951).

The body wall of a nematode is composed of three layers: the cuticle, epidermis and longitudinal muscle cells. In combination with the fluid-filled pseudocoel the body wall makes up the hydrostatic skeleton which generates a pressure, known to be higher than in other invertebrates. This hydrostatic pressure is essential for movement and for maintaining their round body structure.

The quite noticeable digestive system starts with a buccal cavity, which may contain blades or teeth and opens into a muscular pharynx. As already mentioned, the pharynx is of variable form. The intestine is essentially a tube consisting of a single layer of epithelial cells. In female worms, it stretches from the pharynx to the anus, whereas in male worms it terminates in a cloaca.

The reproductive system is the second prominent organ system. While protandric hermaphroditism is present within the Rhabditoidea in a minority of the species, most nematodes are dioecious. The basic female reproductive organs include ovary (paired or single), oviduct and uterus (paired or single), which ends in an unpaired vagina. The latter opens at the vulva and the location of the vulva can be used as a diagnostic feature. Male reproductive organs are comprised of a single testis and a vas deferens ending in a cloaca. Some species have accessory male reproductive organs, which are helpful in diagnosis. Examples to be named in this context are the chitinous spicules, which are usually present in pairs and help open the vulva and the gubernaculum, which guides the spicules. The gubernaculum is not necessarily a concomitant of spicules. The cuticle at the posterior end of males can have various shapes and is often modified into a bursa copulatrix, which embraces the female. This modification is very distinct in strongylid nematodes and helps with species identification.

Nematodes live in a variety of habitats with most species residing in a marine environment where they outnumber all other animals. All nematodes follow a life cycle involving four larval stages from egg until adulthood. Parasitic nematodes constitute a small fraction of nematodes, but they are extremely complex and have developed considerable variations in their life cycles. Common to all parasitic nematodes, some development needs to take place for the parasite to become infectious. Life cycle patterns involving development in the environment are direct life cycles. In the most common form of these, eggs will hatch in the environment and molt to infectious third-stage larvae (L3) which are then

ingested by the host. In some cases, infection of the host can occur by ingesting eggs containing a larva. In indirect life cycles, moulting takes place inside an intermediate host and the definitive host is infected by ingesting the intermediate host or by the intermediate host feeding on the definitive host. Until recently, the phylogenetic framework of the phylum Nematoda heavily relied on morphological traits. The introduction of DNA sequences, in particular small subunit (SSU) ribosomal DNA sequences, has changed perspectives on nematodes systematics. Currently, molecular phylogenetics suggest three major branches named Enoplia, Dorylaimia and Chromadoria, whereas traditional classification divides the phylum into the Adenophorea and Secernentea (Blaxter et al., 1998). Blaxter´s pioneering work on nematode phylogeny introduced five major clades and provided evidence for the paraphyly of the Adenophorea (Blaxter et al., 1998). The five-clade system has emerged as the more popular scheme when explaining a nematodes phylogenetic context than the later on published 12-clade system by Helder and colleagues (Holterman et al., 2006; van den Elsen et al., 2009).

## 2.2 *Haemonchus contortus*

### 2.2.1 Taxonomy and phylogenetic context

The taxonomic description of the parasitic nematode *H. contortus,* first described as *Strongylus contortus* by Rudolphi in 1803, is as follows:

Eukaryota

Opisthokonta

Metazoa

Eumetazoa

Bilateria

Protostomia

Ecdysozoa

Nematoda

Chromadorea

Rhabditida

Strongylida

Trichostrongyloidea

Haemonchidae

Haemonchinae

Haemonchus

Currently, twelve *Haemonchus* species are regarded as valid. There are no comprehensive molecular data available to provide a phylogenetic framework for all species. However, a phylogenetic study

based on 25 morphological characters places *H. contortus* basal to a group consisting of seven species including *Haemonchus placei* which *H. contortus* is most closely related to (Hoberg et al., 2004). Other medically noteworthy members of the family Haemonchidae are species of *Teladorsagia* and *Ostertagia*. The superfamily Trichostrongyloidea comprises two further families of very high veterinary importance: the Trichostrongylidae and the Cooperiidae, which is the sister taxon of the Haemonchidae (Durette-Desset et al., 1999).

### 2.2.2 General

*Haemonchus contortus* is mainly found in tropical and sub-tropical regions in the world, albeit with increasing numbers in Europe (Rinaldi et al., 2015). While female worms may reach up to 18-30 mm, male worms are smaller and have a length of 10-16 mm. Sexes are shaped differently with males possessing a bursa copulatrix with barbed spicules and females having a prominent vulva flap and the characteristic red and white striped appearance from which it takes its common name, Barber´s pole worm. The characteristic stripes stem from the ovaries spirally encircling the blood-filled intestine.

As most clade V nematodes, *H. contortus* has a direct life cycle involving several molts from first stage larvae (L1) to adulthood (Fig.1). Given the right climatic conditions, fecally-shed eggs hatch into L1 in the environment and molt to second (L2) and then infectious third stage larvae (L3). The latter are still covered by the cuticle of the L2 and this so-called sheath protects them from environmental influences. Once ingested by grazing ruminants, L3s exsheath in the rumen and move to the abomasal glands where they molt to L4 and further develop to adults. Once mature, worms mate and lay eggs which are passed out in the feces. To ensure survival under adverse conditions, early L4s may arrest their development. Most often this arrest, called hypobiosis, occurs in cold and arid zones. Development is resumed when conditions turn favorably.

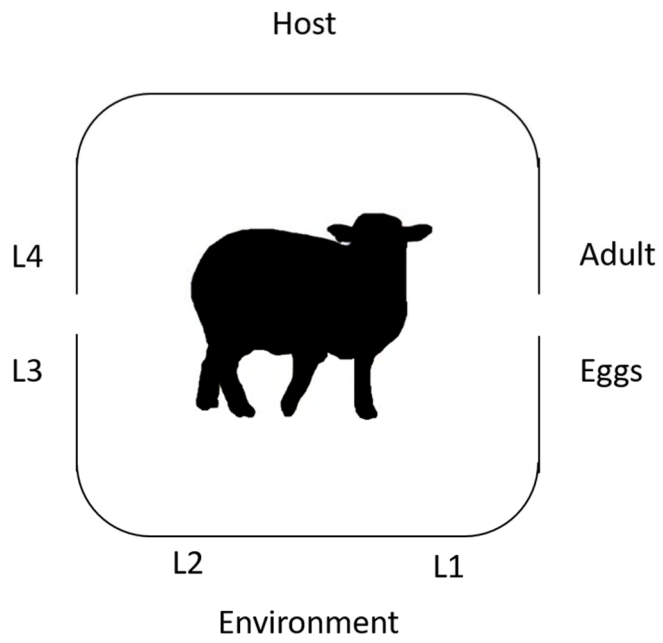


**Fig. 1.** A simplified depiction of the life-cycle of *Haemonchus contortus*

## 2.2.3 Haemonchosis

### 2.2.3.1 Pathology and clinical signs

By far, *H. contortus* is the most feared parasitic nematode of sheep and goats due to its blood-sucking behavior and the female´s prolific breeding habits with up to 10,000 eggs laid per day. While parasitizing in the abomasum one adult worm may suck up to 50 µl blood per day. However, blood loss commences earlier with the development of fourth-stage larvae. The pathological effects of *Haemonchus* is hence dependent on the amount of blood loss, which strongly correlates with worm burden. Sheep infected with at least 2000 worms are under serious risk of developing acute haemonchosis characterized by severe anaemia, which usually becomes apparent 2 weeks after infection. The anemia is characterized by an initial fall in hematocrit (packed cell volume, PCV) and a stabilization thereof in the subsequent weeks as compensatory erythropoiesis takes place. However, the host´s ability to reabsorb hemoglobin iron is severely compromised and the iron reserves in the bone marrow become depleted causing the hematocrit to fall again. The most common clinical signs of anemia are pale mucous membranes. The concomitantly occurring hypoproteinemia very often leads to the formation of submandibular edema. Without treatment, this form of haemonchosis is usually lethal. In the rarely occurring hyperacute form with up to 30,000 worms or more, animals might succumb suddenly to infection due to sever hemorrhagic gastritis without showing clinical symptoms. In tropical areas, a third form of infection can develop during dry seasons when the pasture becomes deficient in nutrients so that several hundred worms are enough to cause symptoms including weight loss, weakness and inappetence, but no anemia. This form is known as chronic haemonchosis.

### 2.2.3.2 Identification of *Haemonchus contortus* and diagnosis of haemonchosis

Morphology is the gold standard for ante-mortem identification of *H. contortus*. Since *Haemonchus* eggs are virtually indistinguishable from many other nematode eggs, commonly described as strongyle-type eggs, morphological criteria are usually applied to L3. Hereby, two of the most important features that are used for differentiation of L3 to the genus level is the sheath-tail extension (STE, distance from caudal tip of larvae to the tip of the sheath) and the proportion of the STE comprising the filament (thin end of sheath). The STE of *H. contortus* is 74 µm in length (medium sized) and 10-15% of the STE comprises the filament (van Wyk and Mayhew, 2013). One major drawback to this method is the time required for cultivating larvae from eggs recovered in the feces, which takes about 7-10 days at 25-27 °C.

Molecular methods for identifying *Haemonchus* have made considerable progress and protocols for traditional-, multiplex- and real-time polymerase chain reactions (PCRs) targeting nuclear ribosomal DNA (rDNA) isolated from larvae have been published (Bisset et al., 2014; von Samson-Himmelstjerna et al., 2002; Schnieder et al., 1999). PCRs for crude fecal egg preparations are also available (Demeler et al., 2013d), but particularly these may suffer from inhibitory substances, which are present in complex biological materials such as feces.

Alternatively, recovered eggs can be stained with peanut lectin, which specifically binds carbohydrates present on the surface of *Haemonchus* eggs (Colditz et al., 2002). As both PCR and lectin staining are laboratory methods and costly, they are not suitable for on-farm diagnosis of *Haemonchus* infections. As a consequence, the clinical diagnosis of haemonchosis mostly relies on the detection of anemia in combination with fecal egg counts (FEC) to exclude non-nematode related causes of anemia. The FAMACHA© eye color system, developed in South Africa (van Wyk and Bath, 2002), provides a fast, practical and low-cost approach to identify individual sheep which are affected by blood loss caused by *H. contortus*. Identification is achieved by comparing the color of eyelid mucous membranes with a chart depicting pictures of sheep eyes with progressive stages of anemia. Chemotherapeutic intervention is recommended with scores of four and five and a FEC positive for strongyle-type eggs (Kaplan and Miller, FAMACHA© Information Guide) In case of lambs, depending on the overall health status, deworming may be advisable with scores of three (Kaplan and Miller, FAMACHA© Information Guide).

Immunodiagnosis of haemonchosis has sporadically been described as a promising tool but antigen-based assays are still associated with several challenges such as cross-reactivity and a lack of uniform expression of antigens in different life cycle stages (Raleigh et al., 1996; Raleigh and Meeusen, 1996; Roeber et al., 2013). Similarly, antibody-based assays are not favored because antibodies can be maintained at detectable levels following clearance of infection or the host might show clinical signs

before antibody levels reach their lowest detection limit (Roeber et al., 2013). As such, these assays are currently more suitable for research purposes rather than for clinical diagnosis.

#### 2.2.3.3 Host immunity and other host-related determinants of disease outbreak

The establishment of worm burden is primarily dependent on host-parasite dynamics such as immunity. Both innate and adaptive immune responses help protect the host from *H. contortus* infections whereby progressive stages of immune responses aim at preventing L3 establishment, arresting L4 development, reducing fecundity and expelling adult worms. In immune and naïve animals, the initial defense is suggested to be a pro-inflammatory innate immune response involving an increase in mucosal mast cells and globule leucocytes, which specifically contribute to rapid rejection of L3s by degranulation and concomitantly releasing inflammatory mediators. This mechanism targets L3s, which have not yet reached their destined tissue niche (Bowdridge et al., 2015; Douch et al., 1996; Huntley et al., 1987; Huntley et al., 1992; Kemp et al., 2009). By contrast, a delayed rejection of L3s, which occurs only in immunized sheep, is characterized by an increase in eosinophils and is known to occur when L3s successfully reach their destination (Balic et al., 2002). Eosinophils are capable of immobilizing larvae and killing them *in vitro* (Rainbird et al., 1998). Since studies showed significant recruitment but varying degrees of contact of tissue eosinophils with damaged/dead larvae – depending on the immunization history of the host – it is assumed that eosinophilia-mediated *in vivo* killing of larvae is conditional on further factors which for the most part remain unknown (Balic et al., 2006). In naïve animals, eosinophil response is cytokine-independent and weaker in terms of rapidity and magnitude of recruitment and lacks close association with larvae (Balic et al., 2002). Extracellular traps (ETs), which are a network of extracellular fibers, mostly composed of DNA, have been shown to entrap L3s of *Schistosoma japonicum* and *Strongyloides stercoralis* and very recently, neutrophil- and eosinophil-derived ovine ETs have been demonstrated to bind to *H. contortus* L3s (Bonne-Année et al., 2014; Chuah et al., 2013; Muñoz-Caro et al., 2015). *In vitro*, neutrophil-derived ET formation occurs as early as one hour after exposure to larval antigen or to *H. contortus* L3 but does not result in killing (Garza et al., 2018; Muñoz-Caro et al., 2015). Therefore, it is hypothesized that neutrophil-derived ETs occur primarily during early infection and aim at preventing migration and larval establishment.

Larvae surviving both phases of expulsion will be continuously targeted by different phases of the adaptive immune system, which is triggered following recognition of *H. contortus* antigens by antigen-presenting cells including activated dendritic cells and macrophages. These cells migrate to lymph nodes and are recognized by naïve T-cells whose differentiation into Th2 cells is induced by IL-4 – a cytokine, which is released by mast cells during the initial immune response. Activated Th2 cells release a range of cytokines including IL-4, IL-5, IL-9, IL-10 and IL-13. The production of IL-4 results in the differentiation of B cells into plasma cells and the subsequent production of IgE and IgG antibodies.

Antigen bound IgE activates further mast cells which in turn degranulate and release even more cytokines. IgE also mediates the above-mentioned degranulation of eosinophils whose development are promoted by IL-5. The latter also triggers plasma cells to produce IgA antibodies (McRae et al., 2015).

Depending on the age, reproductive status and breed of host, the immune system may respond differently and hence influence the host's susceptibility to infection. Younger animals are more prone to infection because their innate immune system responds slower and less efficiently in terms of eosinophil and mast cell numbers and production of Th2 cytokines (Schallig, 2000). Lambs become less susceptible after puberty (Schallig, 2000).

The periparturient relaxation of immunity in pregnant and lactating ewes is associated with higher fecal egg counts reflecting their lower resistance against infections (Fleming et al., 1988). While changes in immunological parameters have not been investigated in pregnant and lactating sheep infected with *H. contortus*, experimental infections with *Trichostrongylus colubriformis* revealed lower numbers of circulating eosinophils and lower IgG1 titers for pregnant ewes and lower numbers of mast cells, globules leucocytes and lower titers of IgG1, IgM, IgA and IgE in lactating ewes (Beasley et al., 2010). The periparturient rise in fecal egg count is a major source of larval pasture contamination and can be reduced by dietary supplementation of ewes with metabolizable protein (Kahn et al., 2003).

It is well recognized that some sheep breeds are resistant or more resilient to *Haemonchus* infections than others. This includes but is not limited to indigenous breeds coming from areas where nematodes can prosper due to favorable climatic conditions leading to positive selection for resistance and resilience in sheep. On a parasitological level, resistance usually manifests itself as lower fecal egg counts and lower numbers of adults and larvae within the host and resilience is characterized by higher PCV and/or a faster recovery of PCV values following infection (Saddiqi et al., 2011). While a number of studies reported higher levels for eosinophils, mucosal mast cells, global leukocytes, antibodies and Th2 cytokines in resistant breeds (Guo et al., 2016; MacKinnon et al., 2010; Shakya et al., 2009; Terefe et al., 2009), other studies have failed to report differences in immune parameters between resistant and susceptible breeds (Amarante et al., 2005; Gauly et al., 2002).

#### 2.2.3.4 Climatic determinants of disease outbreak

Whilst best adapted to warm moist climates, *H. contortus* can be found in most climatic zones including cool and arid zones. Correspondingly, the epidemiology of *Haemonchus* and probability of disease outbreaks varies. Moisture is a critical requirement for the development of eggs. Even at adequate temperatures of 20-30 °C, most eggs will fail to develop if relative humidity is below 85% (Hsu and Levine, 1977). As for infective larvae, survival can vary tremendously depending on climatic conditions. Controlled studies have revealed that, in water, larvae may survive for 3-4 months at 30 °C and for

more than 12 months at 20 °C, provided that moisture conditions are suitable (Boag and Thomas, 1985).

In tropical regions with wet and dry seasons, a seasonal plasticity of larval development has been observed with peak larval development and survival coinciding with rainy seasons. No substantial development or survival has been detected in dry and warm conditions (Onyali et al., 1990) or dry and cool conditions (Tembely et al., 1997). On the other hand, haemonchosis is a continuous threat in the wet tropics, where year-round rainfall occurs (Banks et al., 1990). In summer rainfall zones, risk of haemonchosis is highest in warmer months (Southcott et al., 1976); however, larvae may survive mild winters. A very sharp seasonal pattern is present in Mediterranean regions, which are characterized by hot and dry summers and relatively mild winters. Here, typically, larval development peaks in spring and autumn (Besier et al., 2016). *Haemonchus contortus* is less feared in cool zones, such as northern Europe, for larval development is severely compromised for the most part of the year and only occurs during warmer summer months with sufficient rainfall (Gibson and Everett, 1976; Rose JH, 1963). However, prediction models suggest the emergence of a bimodal seasonal occurrence of infective L3 for northern Europe, as is the case for Mediterranean regions (Rose et al., 2016). In arid zones, outbreaks of haemonchosis are mostly confined to short periods of rainfall and are generally considered a minor threat (Biggs and Anthonissen, 1982; El-Azazy, 1995).

## 2.2.4 Control strategies

### 2.2.4.1 Anthelmintic control

Despite increasing reports of drug resistance, chemotherapy remains the frontline of intervention. Anthelmintic drugs can be administered in different formulations including drenches, plunge dips, pour-ons and injections. Market authorization and hence legal availability of anthelmintic drugs may vary strongly between countries. Nonetheless, a wide variety of drugs from different substance classes is available for the treatment of haemonchosis in most countries. With a few exceptions, these drugs are broad-spectrum in nature. The spiroindole derquantel is the most recently introduced anthelmintic compound on the market and while being mid-spectrum itself, it is available only in combination with abamectin, a well-known broad-spectrum ML. Monepantel, the only commercialized representative of the amino-acetonitrile derivatives, is also used against *Haemonchus* infections. However, it is approved only for use in sheep, which are reared for meat production.

Another compound, which is only licensed to be used in sheep reared for their meat, is the imidazothiazole LEV, which has been available in different formulations since the 1980s.

Considering their decades-long presence on the market, far more approved drugs are available from the ML- and BZ group of substances. Among the MLs, several compounds are used for the treatment of haemonchosis and two of these–ivermectin (IVM) and moxidectin (MOX)–are approved in most

countries. BZs were the first commercialized anthelmintics and they remain big sellers in the veterinary pharmaceutical industry. In Germany, for instance, no other group of substances has as many licensed formulations for use in sheep as the BZs.

### 2.2.4.2 Vaccines

First immunization experiments to protect sheep against *H. contortus* infections were performed with irradiated infective L3. While protective effects were shown in older sheep, the effects in lambs were too weak (Smith and Angus, 1980; Urquhart et al., 1966) eventually leading to a discontinuation of such vaccine projects. Nowadays, attention has turned to subunit vaccines which are characterized by longer shelf lives and bear no risk of spreading the parasite as they have no live components. Principally, two types of parasite antigens qualify as targets - natural antigens and hidden antigens. The former are readily accessible to the immune system of the host and include excretory/secretory (E/S) products and somatic antigens. Hidden antigens, on the other hand, do not trigger an immune response under natural conditions since they are located inside the parasite. Inherently, hidden antigens do not enable a natural boosting of the host´s immunity upon reinfection and repeated vaccinations are required. One advantage of using hidden antigens over natural antigens is that they cannot have evolved immune evasion mechanisms.

Natural antigens were of early interest for vaccine research, since they are readily recognized by the immune system. Research on E/S products has identified two low molecular weight antigens (Schallig et al., 1994). Both the 15 and 24 kDa E/S antigens are expressed in L4 and adult worms only and trigger strong immune responses in mature sheep and lambs leading to reduction in fecal egg counts and worm burden of more than 70% (Schallig et al., 1997). Among the somatic antigens, three are considered very promising: a high molecular weight L3 surface antigen (Hc-sL3) and two low molecular weight peptides from adult worms (Hc23 and Hc26). In the presence of aluminium hydroxide as an adjuvant, Hc-sL3 was able to reduce fecal egg counts and worm burden up to 69% and 55%, respectively (Jacobs et al., 1999). As for Hc23 and Hc26, more encouraging studies were performed. Initial vaccination trials with the respective somatic fraction showed a reduction in worm burden and fecal egg count of more than 60% (Domínguez-Toraño et al., 2000). When using purified Hc23 in combination with aluminium hydroxide or commercially available bacterial adjuvants, a reduction of 67-86% in worm burden and 70-85% in fecal egg counts was achieved (Fawzi et al., 2014). Similar reductions in worm burden and fecal egg counts were observed with recombinant Hc26/23 (Fawzi et al., 2015; Kandil et al., 2017). Despite intensive research, however, none of these antigens have led to commercial products.

Among the hidden antigens, two gut derived proteins have been incredibly successful in protecting sheep from *H. contortus*. H11 is a glycoprotein complex found on the intestinal microvilli of blood-

sucking stages of *H. contortus* and has consistently been shown to reduce fecal egg counts and worm burdens by ca. 90% and 70-90%, respectively (Bassetto and Amarante, 2015). Unlike natural antigens, vaccination with H11 especially affects female worms (Smith and Smith, 1993). An early study by Andrews et al. revealed significant protection for five to six months (Andrews et al., 1997) and vaccination was associated with a rise in antibody response to glycan or glycan-linked epitopes on H11 (Roberts et al., 2013). The second gut protein, termed H-gal-GP, has proven to be equally protective (Bassetto and Amarante, 2015). Consisting largely of proteases it is believed that H-gal-GP digests ovine haemoglobin and albumin (Ekoja and Smith, 2010). Worms feeding on vaccinated sheep ingest host antibodies targeted against these antigens and this uptake results in antibodies blocking the activity of the enzymes, essentially starving the parasite.

While recombinant H11 or H-gal-GP were not capable of reducing fecal egg counts or worm burdens could be successfully developed, the combination of native H11 and H-gal-GP has proven to be a very effective vaccination approach and has been commercialized for sheep under the name Barbervax- the first and only available vaccine against *H. contortus* and only the third non-live vaccine against any metazoan parasite after *Taenia ovis* and *Echinococcus granulosus*. Following a series of efficacy and safety trials between 2011 and 2012, which revealed a mean reduction in fecal egg counts of 80% and commercially acceptable adverse local reactions (Smith, 2014), the Australian Pesticide and Veterinary Medicines Authority granted the registration of Barbervax for use in lambs in October 2014 and for use in sheep of all ages in December 2015. In order to reach sufficient levels of circulating antibodies, three priming injections at a dose of 5 µg of purified antigen are needed for immunization of previously unvaccinated animals. A detailed vaccination schedule for lambs, hoggets and ewes previously vaccinated and not vaccinated is available under www.barbervax.com.au. Because Barbervax harbors complex hidden antigens, worms are not expected to develop resistance to it. While the vaccine certainly has become a valid control option, it cannot completely replace anthelmintic treatment.

### 2.2.4.3 Alternative control strategies

A good pasture management is of primary importance for reducing the number of infective larvae on pasture. A key concept is rotational grazing, whereby a pasture is divided into several paddocks and sheep are allowed to graze on a paddock for a certain period of time before being moved on to the next paddock. This ensures that by the time sheep return to the initially used paddock most infective larvae have died. In tropical areas, a rest of 9 weeks should be enough to lower larval burden on pasture between September to March, whereas a rest of 14 weeks is necessary from April onwards (Banks et al., 1990). An equally long rest period of 14 weeks should be implemented in summer rainfall zones (Colvin et al., 2008). Alternatively, worm-resistant sheep or adult cattle or horses, which are

naturally less or not susceptible to *H. contortus*, may be used on pasture to clear it from the bulk of infective larvae before other sheep are introduced (Rocha et al., 2008).

Control of *Haemonchus* and other gastro-intestinal (GI) nematodes via biological agents, especially nematophagous fungi, is viewed as a conceivable scenario. Most well studied nematophagous fungi are those of predatory nature, which, depending on species, produce different kinds of traps (Braga and Araújo, 2014). The genera *Duddingtonia* and *Arthrobotrys* are particularly well investigated and have been shown to pass the GI tract of ruminants unaffected and to subsequently trap hatched larvae in faeces with trapping efficacies of more than 90% in some cases (Acevedo-Ramírez et al., 2015; Flores-Crespo et al., 2003; Kahn et al., 2007; Silva et al., 2017). They harbor a potential for significant pasture decontamination, but further research needs to be performed in order to overcome uncertainties over practical implementation and environmental risks.

The anthelmintic activity of copper (Cu) has been historically known but owing to sheep's Cu sensitivity Cu-based treatments against nematodes had never substantialized. Their use in husbandry management were practically limited to treatment of Cu deficiency. In recent years, however, researchers have come to the conclusion that attempts to control abomasal nematodes, specifically *H. contortus*, may benefit from copper oxide wire particles (COWPs). Under experimental conditions and following a single infection with *H. contortus*, activity of a single dose COWP of approximately 2 g has been demonstrated to persist at least 4-5 weeks (Watkins, 2003). COWPs are most efficacious during the first two weeks after administration and depending on the dose reductions in worm burden and fecal egg count of up to more than 90% can be achieved (Bang et al., 1990; Watkins, 2003). However, considerable knowledge gaps concerning their efficacy under natural conditions call for continued research efforts.

## 2.3 *Caenorhabditis elegans*

### 2.3.1 Taxonomy and phylogenetic context

The full lineage of the free-living *Caenorhabditis elegans*, as depicted in relevant databases, is as follows:

Eukaryota

Opisthokonta

Metazoa

Eumetazoa

Ecdysozoa

Nematoda

Chromadorea

Rhabditida

Rhabditoidea

Rhabditidae

Peloderinae

Caenorhabditis

*C. elegans* was first described by Maupas in 1900. Naming an exact figure of existing *Caenorhabditis* species proves to be difficult, because for a very long time, the scientific curiosity on the ecology of *Caenorhabditis* had been very low. As of 2010, 38 *Caenorhabditis* species are mentioned, 16 of which were discovered between 2005 and 2010 (Kiontke et al., 2011). However, the validity of some of these species is questionable as most are insufficiently described or not at all.

The phylogenetic relationship within *Caenorhabditis* is well resolved for the *Elegans* group, which includes the species *C. elegans*, *Caenorhabditis briggsae*, *Caenorhabditis remanei* and *Caenorhabditis brenneri*, all of which are maintained at and purchasable from the Caenorhabditis Genetics Center (CGC). In the monophyletic framework of the *Elegans* group, *C. elegans* respresents an outgroup to the other three species and *C. briggsae* and *C. remanei* are most closely related. The positioning of *Caenorhabditis japonica* as an outgroup and sister species to the *Elegans* group is strongly favored (Kiontke et al., 2004; Sudhaus and Kiontke K, 2007). Phylogenetic reconstructions of *Caenorhabditis* species less closely related to *C. elegans* have been done. However, their phylogeny is anticipated to change as more species and DNA sequences are incorporated. Current knowledge positions *C. elegans* in clade V, which also includes the Strongylida. Among them are parasites of human and veterinary importance.

### 2.3.2 General

With 1-2 mm in length, *C. elegans* is a tiny nematode. It is found worldwide and traditionally described as a soil-nematode, but its natural habitats are decomposing plant materials rather than soil. Decomposing material is a rich source for microorganisms which *C. elegans* feeds upon (Félix and Duveau, 2012).

Under favorable conditions, *C. elegans* will complete its life cycle in about three to four days while undergoing four larval stages (L1, L2, L3 and L4) before maturing to adult hermaphrodites or males. Overcrowding, limited food and high temperatures, however, may trigger L1s to enter a predauer stage (L2d) and finally the so-called dauer stage, which is commonly described as an alternative L3. These dauers may be found in soil (Barrière and Félix, 2007) and are known to be stress-resistant and survive several months without food. They will resume development and start feeding again once conditions improve sufficiently (Cassada and Russell, 1975). The critical stimulus for dauer arrest is overcrowding

which is conveyed by a pheromone, recently discovered to be a complex mixture of ascarosides (Golden and Riddle, 1982; Pungaliya et al., 2009). *Caenorhabditis elegans* can also arrest its development during the L1 stage in response to starvation (Baugh, 2013) without compromising its adult life span when resuscitated (Johnson et al., 1984). L1 arrest is distinct from dauer arrest in that no morphological modifications occur, and survival is possible for several weeks compared to months. *Caenorhabditis elegans* has an unusual breeding system called androdioecy whereby hermaphrodites (XX) can reproduce by self-fertilizing or by mating with males (X0). In the late L4 stage, XX animals will initially produce and store sperms before irreversibly switching to oocyte production. While being self-fertile, these hermaphrodites cannot inseminate other animals. A spontaneous non-disjunction of X-chromosomes at meiosis generates males. The proportion of males in a laboratory population seldomly exceeds 0.1% with estimations concerning natural populations being similar (Félix and Duveau, 2012). The reproductive success of hermaphrodites is limited by the number of sperms produced, which is around 200-350. However, insemination by males may increase offspring production to 1000 and beyond.

Unquestionably, *C. elegans* is one of the most profoundly studied organisms in history and has been adapted for use as a model organism in different fields. Yet, knowledge on its life outside the laboratory remains very sketchy. It was not until the 2000s that researchers realized exploring the ecology of *C. elegans* might enable its adaptation as a model organism for evolutionary biology and ecology as well (Frézal and Félix, 2015).

### 2.3.3 *Caenorhabditis elegans* as a model organism in parasitology

Ever since its introduction as a model organism by Sydney Brenner in 1963 (Goldstein, 2016), *C. elegans* has enjoyed great popularity in research. It has been used as a tool in developmental biology, neurology, genetics, toxicology, parasitology and many other research fields. This worm owes its initial appeal as a model organism to its unsophisticated nature. Cheaply maintained on petri-dishes with laboratory *E. coli* strains, they are easy to cultivate in large numbers and complete their life cycle in just three days at 20 °C (Byerly et al., 1976). Moreover, due to the diligent work of John Sulston, the complete cell lineage has been elucidated in the 1980s. Male individuals contain a constant 1031 cells. Out of the 1090 somatic cells of a hermaphrodite 131 undergo programmed cell death (Sulston et al., 1983). Because the worm is transparent, cell death and cell lineage can be tracked easily. This has tremendously advanced research on developmental biology and apoptosis (Arvanitis et al., 2013; Sulston et al., 1983; Sulston and Horvitz, 1977). But perhaps more important in consolidating the status of *C. elegans* as a model organism is its self-fertilization. It not only simplifies maintaining stocks but also facilitates finding and maintaining mutants because it follows the Mendelian rules of segregation. *Caenorhabditis elegans* was the first multi-cellular organism whose genome was sequenced

completely (Consortium CeS, 1998). Understanding the potential the latter might offer for genetic analyses, a consortium of laboratories was set up to mutate every gene. Initiated in 1998, the *C. elegans* Deletion Mutant Consortium is continuing its efforts (Consortium CeDM, 2012). Together with the "Million Mutation Project", led by Donald Moerman and Robert Waterston, loss-of-function alleles are available for 13,760 of 20,514 protein-coding genes (Thompson et al., 2013). Responsible for strain distribution are the Caenorhabditis Genetics Center (CGC) and the National Bioresource Project (NBRP). It is possible to freeze starved L1 at - 80 °C for several years.

Studies on parasitic helminths are complicated and hampered because of the difficulty in cultivating them *in vitro*. Despite being able to maintain some developmental stages of certain helminths in media or even, trigger parasite growth to some extent, *in vitro* systems that maintain complete life cycles have not been established. Life cycles of parasitic helminths are complex and hence a complete parasite development under artificial conditions will most likely not be achieved in the immediate future making surrogate systems essential.

According to Blaxter (Blaxter et al., 1998) the phylum Nematoda consists of five clades (designated I-V), whereby most clades include parasitic and free-living nematodes. *Caenorhabditis elegans* is in clade V as are important parasites of humans and animals including *Necator*, *Ancylostoma* and the trichostrongyloid nematodes *Haemonchus*, *Ostertagia*, *Cooperia* and *Trichostrongylus*. Therefore, it is generally assumed that *C. elegans* is more suitable as a model for nematodes of clade V than for parasites residing in any of the other remaining clades (Gilleard, 2004). This, however, does not mean that all findings in *C. elegans* can be extrapolated to parasitic nematodes of clade V. At the same time, it does not preclude the value of *C. elegans* biology for those more distantly related nematodes as some pathways and organs systems may be fairly conserved. In fact, it has been shown that the nervous system and the major neurotransmitters of *C. elegans* share great similarities with those of *Ascaris suum* (clade III) (Holden-Dye and Walker, 2014). Such being the case, research on *C. elegans* to uncover modes of action of anthelmintic drugs is generally considered justified because most exert their effects on neuromuscular targets (except BZs, which target ß-tubulin). The large body of methods available for *C. elegans*, such as forward and reverse genetic approaches and its usability as an expression system, has enabled or contributed to elucidate the mechanisms of action of the anthelmintic drugs MLs (Ardelli et al., 2009; Cully et al., 1994; Janssen et al., 2015; Lynagh and Lynch, 2012; Pemberton et al., 2001), BZs (Driscoll et al., 1989; Kwa et al., 1995; Saunders et al., 2013), LEV (Holden-Dye et al., 2013; Martin et al., 2012) and emodepside (Guest et al., 2007; Kulke et al., 2014b; Welz et al., 2011). The model worm may also help in elucidating the mechanisms of anthelmintic resistance. For example, the BZ resistance-conferring nature of a *H. contortus* β-tubulin allele was confirmed by expressing it in BZ-resistant *C. elegans* mutants and showing that it did not change the

drug resistant phenotype as opposed to alleles present in susceptible *H. contortus* populations (Kwa et al., 1995).

In view of the deterioration of anthelmintic effectiveness due to drug resistance, new anthelmintically effective compounds are urgently needed. Compound screening using parasitic nematodes is difficult in high throughput formats as the only available assays, such as larval-development assays and egg hatch assays are laborious and have only a low to medium throughput. In addition, target-based approaches have not delivered an anthelmintic product. In recent years, whole-worm screening approaches using *C. elegans* have been thoroughly discussed. *Caenorhabditis elegans* has a profound resistance to pharmacological perturbation suggesting one might miss anthelmintically active compounds during screening (Burns et al., 2010). Fortunately, the efficacy of most anthelmintics is conserved throughout different clades. Therefore, compounds that do kill *C. elegans* might likely kill parasitic nematodes as well. The extent to which positive hits might have broad-spectrum activity remains unknown, though. Nonetheless, as nicely demonstrated by Burn et al. (Burns et al., 2015), pre-screening with the model worm will increase the chance of finding compounds which are lethal to parasitic nematodes as opposed to randomly selected molecules. Despite having certain limitations, *C. elegans* is consensually considered a suitable alternative for anthelmintic drug discovery which allows for high-throughput screening with rates of up to 1,900 compounds per hour (Mathew et al., 2016).

Even though comparative studies on the conservation of metabolic pathways in *C. elegans* and parasitic nematodes are largely missing, the few studies that exist on this subject suggest that *C. elegans* is a valid model organism for drug metabolism studies (Laing, 2010).

Ultimately, the applicability of *C. elegans* as a surrogate system depends on the given question and the decision needs to be made on a case-to-case basis while considering aspects such as phylogenetic distance and organ system.

## 2.4 Anthelmintics used against nematodes

Three main classes of anthelmintic drugs are used against various helminth infections in companion animals and livestock. The period between the 1960s and the 1980s is generally considered the golden era of anthelmintic drug discovery since each decade a new class of anthelmintic drug was introduced into the market for veterinary use. BZs were the first available anthelmintics followed by the tetrahydropyrimidines and LEV in the 1970s and MLs in the 1980s. In practice, BZs and MLs have emerged as the most popular drug classes, particularly against *Haemonchus* spp. and other gastro-intestinal nematodes of small and large ruminants.

## 2.4.1 Benzimidazoles

### 2.4.1.1 Discovery and anthelmintic activity

Initial interest in BZs was fueled when they were found to be a naturally occurring part of vitamin B12 and resembling purine nucleotides. Speculating they might serve as antimetabolites, their antifungal and antimicrobial efficacies have been demonstrated in the mid-1940s (Woolley, 1944). It was in the late 1950s, however, when researchers at Merck Animal Health began their routine screening that a BZ (2-phenylbenzimidazole) was shown to exert anthelmintic activity. Discovered in 1961, thiabendazole (TBZ) was the first BZ derivative that was marketed for veterinary use and remains one of the most potent chemotherapeutic agents ever discovered. Thousands of BZ derivatives were screened throughout the years and around a dozen have been commercialized. Thiabendazole and cambendazole, which belong to the thiazolyl BZs, are not as frequently used as methylcarbamate BZs. The latter are usually more potent and include fenbendazole (and its metabolite oxfendazole), albendazole (and its metabolite ricobendazole), mebendazole, oxibendazole and flubendazole. Fenbendazole and albendazole are also available as pro-dugs (febantel and netobimin, respectively).

Other BZs have a broad-spectrum activity and they are used to treat a wide range of nematode infections from different clades including economically important helminths such as GI nematodes of ruminants. Several BZs have some additional activity against trematodes and cestodes and certain others, for example mebendazole, are considered future alternatives for the treatment of giardiosis and trichomoniosis in humans (Katiyar et al., 1994; Solaymani-Mohammadi et al., 2010).

### 2.4.1.2 Pharmacology

The therapeutic effect of BZs is based on their binding to helminth β-tubulin. This binding unfolds the carboxy terminal region of β-tubulin, which renders further addition of α/β-tubulin dimers impossible and results in progressive loss of microtubules in worms (Harder, 2016). Consequently, all tubulin-based processes, such as motility, cell division and transport of nutrients, come to a halt leading to an expulsion of paralyzed and dead worms from within the host. Although mammalian and helminth β-tubulins are well conserved, BZs display a low host toxicity. BZs have a higher binding affinity to helminth tubulin than to mammalian tubulin, which is attributable to a tyrosine at codon 200 (Lacey and Gill, 1994). When present in parasitic nematodes, the same codon causes BZ resistance (the relevance of tyrosine at codon position 200 for BZ resistance is discussed in chapter 2.5.3.1).

The accumulation of BZs or any other drugs in parasites depends on their physiochemical properties and pharmacokinetic disposition in the host. BZs have a limited solubility in water and are administered as oral or intraruminal suspensions. They need enough time in order to dissolve and become available for absorption. It is therefore essential that BZs pass the gastrointestinal tract slowly, particularly the abomasum, since the low pH of the abomasum helps with dissolution of BZs (Lifschitz et al., 2017) and

in the case of *H. contortus* is the site of residence of parasitic stages. The importance of a long residence time is accentuated by the occasionally occurring esophageal groove reflex following oral administration which leads to a ruminal bypass and can result in treatment failure (Prichard and Hennessy, 1981).

Once dissolved, parent drugs can be taken up by *H. contortus* transcuticularly from the abomasal fluid or from plasma while feeding on blood. However, most parent BZs are readily metabolized to less anthelmintically active sulfoxide and sulfide metabolites and to a lesser extent to inactive sulfone metabolites by host liver enzymes.

The relationship between host pharmacokinetics and bioavailability has been best described for albendazole (ABZ). When administered orally, ABZ is subjected to extensive first-pass metabolism in the liver of sheep and goats and is not detectable in the bloodstream. Instead, albendazole sulfoxide (ABZ-SO), also known as ricobendazole, serves as the anthelmintically active compound. At the recommended dose of 7.5 mg/kg ABZ for sheep, ABZ-SO reaches its peak plasma concentration 12 h post administration and remains detectable for 60 h (Moreno et al., 2004). Plasma concentrations of BZ metabolites have been shown to correlate well with tissue/fluid availability and to be a suitable indicator of anthelmintic efficacy (Moreno et al., 2004). Numerous studies have demonstrated that the plasma availability of ABZ-SO and the area under the concentration *vs* time curve (AUC), which is a measure of systemic availability, can be enhanced by increasing the dosage. Administration of ABZ at 15 mg/kg increases the peak plasma concentration of ABZ-SO from 1.23 µg/mL at a dosage of 7.5 mg/kg to 3.3 µg/mL and the AUC from 31.5 µgh/ mL to 159 µgh/ mL (Alvarez et al., 2012; Moreno et al., 2004). Additionally, a 12-24 h fasting period prior to treatment has been shown to increase the peak plasma concentration and the AUC of ABZ-SO as well. Because upon starvation, the flow rate of digesta decreases enabling a longer dissolution time (Lifschitz et al., 1997).

## 2.4.2 Macrocyclic lactones

### 2.4.2.1 Discovery and anthelmintic activity

The collaboration between Merck and the Kitasato Institute of Japan, which ultimately lead to the awarding of the Nobel Prize in Medicine for the discovery of the avermectin MLs to Merck's William C. Campbell and Kitasato Institute's Satoshi Omura in 2015, initially had the objective to identify antimicrobial agents to be put into feed-additives for use in livestock. Eventually, the screening of fermentation metabolites from microorganisms was expanded to test for antiparisitic activity. One of the tested batches, which had shown activity against the mouse nematode *Heligmosomoides polygyrus* in 1974, originated from a new species of *Streptomyces* (now called *Streptomyces. avermitilis*) and later was determined to harbor avermectins as the anthelmintically active metabolites (Campbell, 2012). After the discovery of their anthelmintic activity, the semi-synthetic IVM had been

synthesized and within a year of extensive screening both the semi-synthetic IVM and a naturally occurring avermectin (abamectin) had been shown to be active against various nematodes in different host species. Their range of activity was complemented in 1976, when they were found to be highly active against ectoparasites (Campbell, 2012). IVM was first introduced in 1981 as a subcutaneous injection for use in cattle in France and as an intramuscular injection for use in horses in New Zealand. Interestingly, avermectins were not the first MLs shown to have antiparasitic activity. Milbemycins had already been demonstrated to be active against plant mites in the mid-1960s. However, it was during the course of avermectin discovery that milbemycins were found to be active against nematodes as well. In contrast to some BZs, MLs are neither active against trematodes nor cestodes.

#### 2.4.2.2 Pharmacology

MLs exert their neurotoxic effects by activating/potentiating primarily glutamate-gated (GluCl) chloride channels but can also potentiate ɣ-aminobutyric-acid (GABA)-gated chloride channels. The influx of chloride ions leads to a flaccid paralysis of the worm. Because GluCl channels are unique to invertebrates and MLs do not cross the blood-brain barrier and hence cannot target mammalian GABA-gated channels, MLs are usually well-tolerated drugs. Prominent exceptions to this are certain herding dog breeds which harbor a deletion mutation in the *mdr-1* gene, which is heavily expressed at the blood-brain barrier and prevents entry of therapeutic drugs into the brain. This mutation, leading to a truncated, non-functional efflux protein, was first described in Collie dogs and is responsible for the observed toxicity against avermectin-type MLs. IVM at a single dose as little as 100 µg/kg causes sensitivity in Collies homozygous for the mutation (Paul et al., 1987). A less toxic response has been observed for MOX in *mdr-1* deficient and normal dogs when orally administered at a dose 20 times higher than the lowest IVM dose causing adverse effects and when topically administered at a dose 270 times higher (Paul et al., 2000; Paul et al., 2004). Similarly, in mice, MOX has been shown to have a 5 times lower acute toxicity than IVM in *mdr-1* deficient animals (Ménez et al., 2012). The same study has also shown that when administered at an equimolar dose IVM is more rapidly accumulated in the brain and has a higher brain-to-plasma concentration than MOX in *mdr-1* deficient mice. Because increasing IVM and MOX to respective sublethal doses has shown that a 3 times higher accumulation of MOX is required to provoke the same degree of neurotoxicity as IVM, it was assumed that drug accumulation in the brain cannot be the primary explanation for the observed differences in toxicity. While both IVM and MOX have been shown to act as allosteric modulators of GABA-receptors via electrophysiological recordings of rat GABA-receptors expressed in *Xenopus* oocytes, IVM has been shown to have stronger potentiating effects (Ménez et al., 2012). This difference in interaction with GABA receptors is hypothesized to be the main reason for differential neurotoxicity in mammals.

Changes in pharmacokinetic behavior of MLs due to different routes of administration have been shown by numerous studies. Subcutaneous administration of MLs results in considerably higher plasma concentrations compared to oral treatment (Gayrard et al., 1999; Gokbulut et al., 2006; Gokbulut et al., 2010). This increase equates to a higher systemic availability and consequently to higher tissue concentrations and a longer persistence in hosts which is ideal for the treatment of tissue nematodes such as lungworms and migrating larvae and whenever a prolonged protection against reinfection is needed. In small ruminants, horses and companion animals, it is, however, the oral route of administration, which is preferred. Besides being more practical in application, oral treatment has the advantage of resulting in enhanced drug concentrations in the gastro-intestinal tract which is of great importance for combating infections with susceptible and in particular resistant GI nematodes (Lloberas et al., 2012). Systemic availability following oral administration may be improved by fasting (Alvinerie et al., 2000) and, as with BZs, caution is needed to avoid triggering the groove closure reflex. In monogastric species, bioavailability after oral administration is much higher than in polygastric species (ca. 70% in dogs vs. 25% in sheep) and has been shown to be improvable by co-administration of lipids (Lespine et al., 2006; Prichard et al., 1985). Pour-on formulations are used in cattle only and have low AUC values (Laffont et al., 2001). Parent MLs show a longer residence time in the adipose tissue than do BZs since they are more lipophilic and very limitedly metabolized in the host. Excretion of MLs primarily occurs unchanged through feces via P-glycoprotein (Pgp) efflux. There seems to be a negative correlation between lipophilicity and affinity to Pgps. Moxidectin has the highest lipophilicity among MLs and a lower affinity to Pgps (Lespine et al., 2007), which contributes to its longer residence time and higher AUC values compared to avermectin-type MLs. It has become apparent, that the pharmacokinetics of MLs are not only dependent on the lipophilicity of the compound but also on the body fat of the host. It has consistently been shown that lean animals absorb and excrete drugs faster than fat animals and hence have a lower systemic availability of MLs (Lespine, 2013).

## 2.4.3 Other anthelmintics with activity against nematodes

### 2.4.3.1 Piperazine

Piperazine was introduced as an anthelmintic in 1953 and used in humans to treat infections with *A. lumbricoides* and *Enterobius vermicularis* (BUMBALO and PLUMMER, 1957). In veterinary medicine it can be used against *Ascaris suum* in pigs, *Ascaridia galli* in poultry, some intestinal worms in horses, such as *Parascaris equorum* and *Oxyuris equi* and against ascarids of dogs and cats (Riviere and Papich, 2018). In addition, it can also be administered to ruminants to control *Oesophagostomum* spp. infections (Campbell, 1960). Piperazine is not active against the major intestinal worms of ruminants (i.e. *Haemonchus* spp., *Ostertagia* spp. and *Trichostrongylus* spp.) and therefore has been used very scarcely in ruminants.

Using electrophysiological recordings in *A. suum*, piperazine was shown to reversibly bind to $GABA_A$ channels and cause a flaccid paralysis of body wall muscle (Martin, 1985) leading to an expulsion of worms. Following the introduction of broad-spectrum anthelmintics its use has drastically declined.

### 2.4.3.2 Emodepside (Cyclooctadepsipeptide)

PF1022A is the first anthelmintically active cyclooctadepsipeptide (COPD) that was discovered in 1992 in Japan (Sasaki et al., 1992). It is an active metabolite from *Mycelia sterilia* (*Rosellinia* sp.), a fungus colonizing the leaves of *Camellia japonica.* PF1022A has been proven to exert a broad-spectrum activity against various nematodes in cats, dogs, rodents, horses and ruminants (Conder et al., 1995; Fukashe et al., 1990; Kachi et al., 1998; Samson-Himmelstjerna et al., 2000) while having low toxicity to mammalian hosts (von Samson-Himmelstjerna et al., 2000; Sasaki et al., 1992). A semi-synthetic derivative of PF1022A has been introduced and named emodepside, the activity of which has been shown to be more wide-ranging in terms of targeted nematodes and applicable hosts (Krücken et al., 2012; Kulke et al., 2013a; Kulke et al., 2013b; Kulke et al., 2014a). Emodepside is the first and only commercially available COPD, currently purchasable as Profender® (agents: emodepside and praziquantel, Bayer), approved for cats and dogs and Procox® (agent: emodepside and toltrazuril, Bayer), approved for dogs.

It has been considered a potential drug for the treatment of onchocerciosis in humans (Olliaro et al., 2011) and has recently entered Phase I studies in 2016 (https://www.dndi.org/diseases-projects/portfolio/emodepside/, last retrieved 31.03.2019).

Numerous studies suggest SLO-1, a large-conductance calcium- and voltage-gated potassium channel, to be the target of emodepside (Crisford et al., 2015; Kulke et al., 2014b). This novel mode of action may explain the observed resistance-breaking properties of emodepside and PF1022A (von Samson-Himmelstjerna et al., 2005).

### 2.4.3.3 Levamisole

Discovered in 1966 and introduced for veterinary use in the early 1970s, levamisole (LEV) is a synthetic imidazothiazole derivative. Its spectrum of activity does not cover as many nematode species as BZ or MLs, but it is effective against mature stages of major GI nematodes (not *Trichuris* spp.) and mature and immature lungworms (Riviere and Papich, 2018). In dogs, LEV has also been shown to be effective against microfilaria of *Dirofilaria immitis* (Carlisle et al., 1984; Mills and Amis, 1975). LEV has been shown to target nicotinic acetylcholine receptors (nAChR), which are transmembrane proteins found at neuromuscular junctions and are comprised of five different subunits. Activation of the receptors by LEV leads to sustained membrane depolarization resulting in the spastic paralysis of body wall

muscle (Martin and Robertson, 2007), initially observed in *A. suum* (Aceves et al., 1970). These receptors are commonly described as L-type nAChRs (L for LEV).

Because LEV has immunomodulatory effects, it was used to treat cancer in humans (Miwa and Orita, 1978; Mutch and Hutson, 1991). For safety reasons it has been withdrawn in many countries but is still being researched for use against human diseases other than cancer (Gruppen et al., 2018).

#### 2.4.3.4 Monepantel (Aminoacetonitrile derivative)

The amino-acetonitrile derivative monepantel (MON) is a recent addition to the existing chemotherapeutic options for the treatment of nematode infections in sheep. Its discovery was reported in 2008 (Kaminsky et al., 2008) and it has been introduced to the market as Zolvix® (Novartis) the following year. Its range of activity includes both adults and L4s of major GI nematodes (Kaminsky et al., 2008) and also inhibited L4s of *H. contortus*, *Teladorsagia* spp. and *Trichostrongylus axei* (Ramage et al., 2012; Stein et al., 2010)(https://www.ema.europa.eu/en/documents/product-information/zolvix-epar-product-information_en.pdf/, last retrieved on 04.04.2019). Like LEV, MON is a nAChR agonist, but is thought to target specific nAChR subunits that are not found in levamisole receptors (Bagnall et al., 2017; Rufener et al., 2009; Rufener et al., 2013). That is why MON is not only effective against nematodes resistant against anthelmintic drugs with entirely different modes of action but also against LEV-resistant nematodes (Kaminsky et al., 2011; Sager et al., 2012). To avoid the excessive use of MON and thereby conserve its efficacy, Zolvix® is sold as a prescription only drug. Nevertheless, MON resistance has already been reported within a few years after market release. Initial reports came from goats for which the drug is not licensed and that were probably underdosed (Scott et al., 2013). However, this was followed by several reports about MON resistance in sheep flocks from all over the world (Cintra et al., 2016; Mederos et al., 2014; van den Brom et al., 2015) and Raza et al. (Raza et al., 2016b) could show that resistance of *H. contortus* can be selected very rapidly.

#### 2.4.3.5 Tribendimidine (Symmetric diamidine)

While most anthelmintics that are authorized for use in humans were first introduced to the veterinary market, tribendimidine was directly approved for human use. So far, it is only approved by Chinese authorities and in use to treat soil-transmitted helminths (STH) since 2004. The drug´s activity against most common STHs, except *Trichuris* spp., had already been discovered in the 1980s (Xiao et al., 2005). Its mode of action has not been completely elucidated but is known to also involve nAChRs. Observations in *C. elegans* suggest that tribendimidine is an L-type nAChR agonist (Hu et al., 2009) while in *A. suum* it preferentially agonizes B-type nAChRs (receptors that are activated by bephenium, an anthelmintic formerly used against ascariosis and hookworm infections) (Robertson et al., 2015).

#### 2.4.3.6 Derquantel (Spiroindole)

Derquantel is a semi-synthetic member of the new class of spiroindole anthelmintics. It has been shown to selectively antagonize B-type nAChRs, which produces flaccid paralysis of worms (Puttachary et al., 2013). Because derquantel was found to be a mid-spectrum anthelmintic with activity against adult stages and L4s of *Trichostrongylus* spp., *Nematosirus* spp., *H. contortus* and *T. circumcincta* in sheep (Little et al., 2011) it has been developed in combination with the broad-spectrum abamectin. In 2010, it was launched under the tradename Startect® (Zoetis).

## 2.5 Anthelmintic resistance

The introduction of broad-spectrum anthelmintics had revolutionized parasite control in livestock. For a long time, anthelmintics were regarded as an easy and inexpensive tool to eliminate a range of parasites from a host. With only a few vaccines available for a limited number of parasites and the ever-increasing demand for animals and animal products, farmers today are still reliant on anthelmintic drugs to maintain productivity and competitiveness. Unfortunately, the intense chemotherapeutic control of parasites has quickly led to the emergence of resistance, which, in simple terms, can be described as a worm's heritable ability to survive treatment at the recommended therapeutic dose (Geary et al., 2012). First reports of treatment failure were reported often only 3-10 years after market introduction (De Graef et al., 2013a) and field resistance against all three main drug classes is now widely documented in different parasites and expected to develop against more recently introduced drugs as well – unless deworming strategies are meaningfully changed.

Studies from Australia and New Zealand have repeatedly evaluated productivity parameters in sheep exposed to anthelmintic resistant GI nematodes and have consistently shown a significant impact on productivity. In sheep infected with BZ-resistant GI nematodes and treated with albendazole, Sutherland et al (Sutherland et al., 2010), have observed a 1.3-fold lower live-weight gain and a 1.2-fold lower carcass weight compared to animals treated with the effective monepantel. The monetary value of animals treated with albendazole was estimated to be 14% lower than that of the other animals. With a 1.55-fold difference in live-weight gain and a 1.25-fold difference in carcass weight between animals treated with effective and ineffective anthelmintics, losses in productivity were even more apparent in the study of (Miller et al., 2012). These studies clearly show that anthelmintic resistance, if not resolved or counteracted, bears the risk of persistent constraints to animal health and production.

### 2.5.1 Prevalence of macrocyclic lactone and benzimidazole resistance in *Haemonchus contortus*

The problem of anthelmintic resistance is undoubtedly the most severe in GI nematodes of small ruminants. While resistance has been detected in essentially all GI nematodes of small ruminants, the prevalence is highest in *H. contortus*. BZ and ML resistance was first reported in *H. contortus* in 1964 and 1988, respectively (DRUDGE et al., 1964; van Wyk and Malan, 1988). It would later become clear that the parasite's propensity to develop anthelmintic resistance can be partially attributed to large population sizes and very high levels of genetic diversity within populations which enable this parasite a high adaptive capacity (Ali et al., 2018; Gilleard and Beech, 2007; Gilleard and Redman, 2016).

In the Americas, anthelmintic resistant *H. contortus* seem to be more prevalent in southern countries where sheep farming is an important industry (Torres-Acosta et al., 2012). Among those countries, Brazil stands out particularly: not only has ML and BZ resistance been reported from the clear majority of investigated farms, but also multi-drug resistance (Torres-Acosta et al., 2012). There are not as many reports available for the rest of South America, but studies suggest a similarly severe situation for Uruguay, particularly in terms of BZ resistance (Nari et al., 1996; Torres-Acosta et al., 2012). As for North America, most reports on anthelmintic resistance have been provided from Mexico but with the conclusion that the severity of anthelmintic resistance is not as high as in the USA (Howell et al., 2008; Torres-Acosta et al., 2003; Torres-Acosta et al., 2012). Resistance to MLs and BZs in *H. contortus* is not widespread in Canada but appears to be an emerging problem in Ontario sheep flocks (Falzon et al., 2013).

In Australia, sheep usually carry mixed roundworm infections. In summer rainfall areas of Australia (northern New South Wales, southern Queensland and south-west Western Australia), these infections are dominated by *H. contortus*. BZ resistance is already established in ca. 90% of all farms throughout Australia and ML resistance is becoming more and more common in northern New South Wales and southern Queensland (on an estimated more than 70+% of farms) (Besier and Love, 2003; Love, 2011). Multi-drug resistant *H. contortus* strains have also been reported from these areas (Green et al., 1981; Lamb et al., 2017). In New Zealand, BZ- and ML resistant *H. contortus* has been reported from the North Island (Waghorn et al., 2006).

Prevalence of BZ and ML resistance in *H. contortus* has not been extensively studied in Africa and the full extent of resistance is unknown. BZ resistance has been reported from 6 countries and ML resistance from 2 countries with most reports originating from Kenya and South Africa (Vattaa and Lindberg, 2006). South African farmers are suspected to be among the most heavily affected worldwide by resistant *Haemonchus* populations (Vattaa and Lindberg, 2006).

Hardly any information is available from Asia. So far, resistance reports have only been published from Malaysia (Chandrawathani et al., 1999; Chandrawathani et al., 2003), China (Zhang et al., 2016) and India (Mohanraj et al., 2017).

Within Europe, prevalence of anthelmintic resistance in *H. contortus* is best understood in western, central and northern regions. While reports have emerged from almost all countries of these regions, the overall extent of resistance has not been categorized as alarming (Rose et al., 2015). As for East Europe, BZ-resistant *H. contortus* were detected on multiple sheep farms in Hungary (Nagy et al., 2017) the Czech Republic (Vernerova et al., 2009) and Bulgaria (Iliev et al., 2014), whereas only one anecdotal report is available from Poland (Kowal et al., 2016). At present, resistance to MLs is only reported from the Slovak Republic (Cernanská et al., 2006).

### 2.5.2 Resistance-enabling factors and management of anthelmintic resistance in sheep

Drug resistance is a genetic trait and attributable to the emergence of mutations such as single-nucleotide polymorphisms (SNPs), deletions and copy number variations (CNVs). Depending on the genes the mutations occur in, they can lead to functional alterations of the drug target or to changes in drug metabolism and efflux (i.e. changes in gene expression and/or substrate affinity). The excessive use of anthelmintics in the past decades has enabled artificial selection to act on resistance-associated alleles and has given resistant parasites a survival and reproductive advantage which ultimately lead to the spread of resistance over entire nematode populations (Falzon et al., 2014). Other administration practices that have been identified as risk factors for the selection of resistant alleles include under dosing due to the misestimation of an animal's weight or the incorrect use of a drench-gun (Calvete et al., 2012; Niciura et al., 2012; Smith et al., 1999), as well as long-lasting formulations (Barnes et al., 2001; Falzon et al., 2014; Smith et al., 1999). Under dosing exposes worms to sub-lethal concentrations of an anthelmintic drug immediately, while long-lasting formulations do so belatedly ("tailing off" effect). This allows for both homozygous and heterozygous resistant nematodes to survive treatment. Often in livestock industries, all animals will be treated, irrespective of worm burden and clinical manifestation. Again, this will result in all susceptible worms dying and all resistant worms surviving and passing resistance to the next generation.

A key counter measure to slow down the development of anthelmintic resistance is therefore diluting the proportion of resistance alleles in the population by allowing susceptible worms to survive– a concept known as refugia (Besier, 2012; van Wyk, 2001). In practical terms, this means a less frequent administration of anthelmintics, correct weighing of lambs, restriction of long-lasting formulations on peri-parturient ewes which are known to have higher nematode egg outputs for 6-8 weeks after lambing (Sargison et al., 2012) and discontinuation of drench-and-shift regimes whereby animals are

transferred to clean pastures with very few susceptible worms immediately after anthelmintic treatment. In theory, refugia can also be created by actively infecting animals with susceptible parasites which would contaminate the pasture with their susceptible eggs (Moussavou-Boussougou et al., 2007). Because susceptible larvae are not readily available and often contaminated with additional pathogens, this approach has not been established in practice and would only be successful if used concomitantly with other refugia-based strategies, particularly if anthelmintics against which resistance is already established are planned to be used for treatment.

Targeted treatment (TT) and targeted selective treatment (TST) are two further refugia concepts which are particularly promoted by experts. TTs are whole-flock treatments that are given only at times of high infection risks as opposed to prophylactic treatments which are sometimes given as frequently as every 4 weeks. Since TTs are given to whole groups of animals, they aim at maintaining refugia by reducing the number of treatments rather than creating it. By basing the time of treatment on clinical symptoms, Kenyon et al. (Kenyon et al., 2013) were able to reduce treatment frequency in sheep by a factor of 2.8 per season and maintain high levels of IVM efficacy even after five years when compared to monthly whole-flock treatments (4.0 and 1.4 treatments per season and mean IVM efficacy of 62% and 83% after five years for whole-flock and targeted whole-flock treatment, respectively). Mean FECs have also been used to identify risk periods and shown to reduce treatment frequency by 35% and create savings of around €790 per year (Kenyon and Jackson, 2012). Using FEC as an indicator for TTs is sometimes criticized since it requires gathering of animals twice (once for determination of FEC and then for treatment) and might not always correlate well with GI worm burden (Kenyon et al., 2009). For example, in infections with *H. contortus* (Coadwell and Ward, 1982; Oliveira-Sequeira et al., 2000), *T. colubriformis* (Amarante et al., 2007) and *T. circumcincta* (McRae et al., 2014; Stear et al., 1995) FECs are strongly correlated with worm burden, whereas in infections with *Nematodirus battus* they are not (McKenna, 1981). Possibly due to an individual variation in the host immune response, nematodes have a skewed distribution whereby most worms are found within a small proportion of the flock (Sréter et al., 1994; Stear et al., 1998). It is this overdispersion of worms which makes TST strategies, where only individual animals are treated, an even better option than TTs. Targeted selected treatments can be given based on individual FECs (Cringoli et al., 2009; Gallidis et al., 2009), but for reasons of economic efficiency and time saving, performing FECs on all animals may not be considered feasible in large production systems. Certain restrictions may also apply when using pathophysiological indicators. For instance, the FAMACHA eye color chart can only be used as an indicator for anemia in animals on farms where *H. contortus* is the dominating species (see chapter 2.2.3.2). However, if applicable, it is an excellent tool especially for resource-poor communities as the chart needs to be purchased only once and has been shown to reduce treatment in sheep by 40-60% (Cringoli et al., 2009). Dag scores and body condition scores have also been investigated but judged to be less reliable

(Besier, 2008; Gallidis et al., 2009). Performance based indicators, such as milk yield and weight gain, may help identify animals in need of treatment before production losses become too apparent and hence be more readily acknowledged by farmers. Gallidis et al (Gallidis et al., 2009) have treated sheep which produce more than 2 L of milk per day and have shown a reduction of anthelmintic treatment by ca. 80% when compared to the traditional regime of two treatments per year. In studies where the fastest-growing animals were left untreated, treatments could be reduced by up to 50% when compared to the control group treated every 6 weeks (Busin et al., 2014; Stafford et al., 2009). Alternatively, the heaviest 10% of animals may be left untreated to provide refugia (Leathwick et al., 2006).

So far, TTs and TSTs based on FECs, FAMACHA, milk yield and weight gain have not been shown to have any detrimental effects on milk production or live weight gain. Somewhat surprisingly, the consequence of these applications on the rate of anthelmintic resistance development has been mostly neglected. Nonetheless, it has been shown that leaving 10% of all animals untreated and treating the remaining animals with a combination product will maintain high efficacy levels when compared to all animals treated with only one anthelmintic at the same treatment frequency (Leathwick et al., 2012). Using combinations of anthelmintics as part of a worm control program may not always be regarded as a good practice as there is a potential to select for resistance to multiple classes of anthelmintics. This may happen if the different classes share similar resistance mechanisms and if the frequency of resistance alleles in a population is already very high (Bartram et al., 2012).

Although less responsible for the spread of resistance, according to Redman et al. (Redman et al., 2015), animal movement between farms can facilitate the spread of resistant parasites.

Rotational grazing and mixed-species grazing, similar in concept to what has been described for *H. contortus* in chapter 2.2.4.3, are also considered useful management tools (Shalaby, 2013).

The simultaneous implementation of several practices is the most advisable approach and has been shown to reverse anthelmintic resistance over a period of several years (Leathwick et al., 2015).

## 2.5.3 Proposed mechanisms for macrocyclic lactone and benzimidazole resistance

### 2.5.3.1 Target-site related mechanisms

The initial course of action for the elucidation of resistance mechanisms usually is to determine whether changes at the drug target site are present and, if so, whether they are proportionally enriched in resistant populations. Despite ongoing research, the involvement of such target-site related mechanisms in the emergence of ML resistance remains unclear. MLs activate GluCl channels that contain α-subunits. For *C. elegans,* four genes encoding ML sensitive α-subunits have been shown to be present in its genome, namely *avr-14*, *avr-15*, *glc-1* and *glc-3* with the first two being alternatively

spliced to produce two different subunits. Products of two further genes have been deemed ML insensitive (*glc-2* and *glc-4*) (Glendinning et al., 2011). It was reported that a simultaneous occurrence of a triple mutation in the genes *avr-14*, *avr-15* and *glc-1* conferred >4000-fold resistance compared to wild-type *C. elegans* (Dent et al., 2000). The GluCl family has not been elucidated for each and every veterinary important parasitic nematode. However, it has become increasingly evident that GluCl subunits may substantially vary between nematode species. In *H. contortus*, for instance, there are six genes encoding at least seven subunits, but two genes found in *H. contortus* (*glc-5* and *glc-6*) are missing in *C. elegans* (Laing et al., 2013). On the other hand, *C. elegans glc-1* is missing in *H. contortus* (Laing et al., 2013). However, orthologs of *avr-14* have been identified in several strongylid parasitic nematodes and an L256F substitution in this gene has been associated with IVM resistance in *Cooperia oncophora* and *H. contortus* (McCavera et al., 2009; Njue et al., 2004; Njue and Prichard, 2004). But the relevance of this allele is controversial as it could not be found in field isolates of *C. oncophora* or the closely related *Ostertagia ostertagi* (El-Abdellati et al., 2011). Once suspected to play a role in ML resistance (Blackhall et al., 1998b), no loci in the *Haemonchus* specific and ML-sensitive *glc-5* (Forrester et al., 2002) have been found to be consistently selected in ML-resistant field isolates (Laing et al., 2016; Williamson et al., 2011) and although *glc-6* has been shown to rescue a triple mutant *C. elegans* (*avr-14, avr-15, glc-1*) from its IVM resistance, it has not been further investigated (Glendinning et al., 2011). The secondary target of MLs are GABA-gated channels. In *H. contortus*, *lgc-37* encodes a GABA-gated channel subunit and has been found to harbor four substitutions in IVM and MOX selected isolates (Feng et al., 2002). In a recent publication, however, no apparent selection at any locus in *lgc-37* could be determined (Laing et al., 2016).

Research on target-site changes possibly associated with BZ resistance has progressed faster and more fruitfully. Mutations in genes encoding β-tubulin, the target of BZ, had been anticipated to play a role in BZ resistance in *C. elegans* and *H. contortus* early on (Driscoll et al., 1989; Kwa et al., 1993). In 1994, a SNP in the β-tubulin isotype 1 gene leading to a phenylalanine to tyrosine substitution at codon 200 (F200Y) had been shown to correlate with BZ resistance in *H. contortus* (Kwa et al., 1994) and later, along with two other SNPs at codons 198 and 167 resulting in the replacement of glutamate by alanine and phenylalanine by tyrosine (E198A and F167Y), also shown to be present and correlate with BZ resistance in other trichostrongylid nematodes (Ghisi et al., 2007; Ramünke et al., 2016; Redman et al., 2015; Samson-Himmelstjerna et al., 2007), but not in ascarid nematodes (Krücken et al., 2017; Tydén et al., 2013). In none of the studies in which all three SNPs were examined has the simultaneous occurrence of all three SNPs and homozygosity at more than one locus in nematode populations been shown. It has transpired that in all resistant trichostrongylid nematode populations investigated so far, the F200Y substitution is the most common and, when present in combination with one of the other SNPs, usually the highest in frequency (Esteban-Ballesteros et al., 2017; Ramünke et al., 2016; Redman

et al., 2015). Exceptions hereto were reported from Brazil and China where F167Y and E198A, respectively, prevailed over F200Y in resistant *H. contortus* (Santos et al., 2017; Zhang et al., 2016). A possible contribution of β-tubulin isotype 2 has not been thoroughly investigated but currently does not seem to be of major importance for BZ resistance in *H. contortus* and *T. colubriformis* (Ramünke et al., 2016; von Samson-Himmelstjerna et al., 2009b).

### 2.5.3.2 Non-target-site related mechanisms

In *C. elegans*, sensory amphids are involved in ML uptake and defects in genes related to these amphid structures have several times been shown to increase or to autonomously confer IVM resistance (Dent et al., 2000; Page, 2018; Urdaneta-Marquez et al., 2014). Among these, the dye filling gene *dyf-7* has also been investigated in *H. contortus*. Urdaneta-Marquez et al (Urdaneta-Marquez et al., 2014) have observed a certain *dyf-7* haplotype in IVM and MOX selected *H. contortus* and have shown that most worms bearing this haplotype have amphid defects. These defects are thought to be based on the observed lower expression of *dyf-7* in resistant worms. No amino acid changes were predicted in the haplotype associated with ML resistance. Contrary to the study of Urdaneta-Marquez et al., other studies did not find evidence for selection at any locus in *dyf-7* in ML resistant populations (Elmahalawy et al., 2018; Laing et al., 2016).

Drug efflux via pumping by transmembrane P-glycoproteins (Pgp), members of the ATP-binding cassette (ABC) transporter superfamily, has also been proposed to play a role in resistance. More precisely, expression of *pgp-1* has been shown to be increased in IVM selected *C. elegans* and together with *pgp-6* has also been shown to be inducible by MOX in the wild-type N2 strain (Bygarski et al., 2014; James and Davey, 2009). Expression of several others has been successfully induced by IVM in an IVM-resistant triple-mutant (Bygarski et al., 2014). The apparent involvement of Pgps in ML resistance in *C. elegans* has further been corroborated by verapamil (VPL)-mediated inhibition of Pgp activity and a subsequent increase in susceptibility towards MLs in the wild-type N2 strain and several individual loss-of-function strains (Bygarski et al., 2014; Janssen et al., 2013b). Although Pgps had already been suspected as mediators of resistance in *H. contortus* in the late 1990s (Blackhall et al., 1998a; Xu et al., 1998), it is only in the past ten years that research has intensified. P-glycoprotein inhibition by VPL has been shown to enhance susceptibility towards IVM in susceptible and resistant worms and other studies have identified Pgps that are inducible by IVM and have a higher constitutive expression in resistant isolates; hereby, *Hco-pgp-1*, *Hco-pgp-2* and *Hco-pgp-9* seem to be the most promising candidates for functional studies (Bartley et al., 2009; Raza et al., 2016a; Williamson et al., 2011). Susceptible and resistant *T. circumcincta* exposed to VPL have been shown to become more susceptible towards IVM and an increased constitutive expression of *Tci-pgp-9* has consistently been observed in resistant isolates (Bartley et al., 2009; Choi et al., 2017; Dicker et al., 2011; Turnbull et al.,

2018). P-glycoproteins may potentially be involved in ML resistance in *C. oncophora*, too. Again, inhibition of Pgps has been used as the approach of choice and shown to enhance susceptibility in both susceptible and resistant isolates (AlGusbi et al., 2014; Demeler et al., 2013b). Furthermore, *Con-pgp-11* was observed to be more highly expressed in resistant- and inducible by MLs in susceptible worms (Graef et al., 2013b).

An association of Pgps with ML resistance has also been reported in cyathostomins (Peachey et al., 2017), the filarial nematodes *Onchocerca volvulus* and *Dirofilaria immitis (Ardelli et al., 2006; Bourguinat et al., 2008; Bourguinat et al., 2016)* and in the ascarid nematode *Parascaris equorum* (Janssen et al., 2013a; Janssen et al., 2015).

Studies on Pgps and BZ resistance are considerably fewer in number and not as conclusive. While selection at a *pgp-2* locus has been shown to occur in *H. contortus* selected with BZs (Blackhall et al., 2008) and larval development of BZ-susceptible and resistant *O. ostertagi* and *C. oncophora* was shown to cease by co-incubation with TBZ and VPL (AlGusbi et al., 2014), effects of Pgp inhibition in *H. contortus* on TBZ susceptibility by several inhibitors, including VPL, were revealed to be not as pronounced as for IVM or LEV (Raza et al., 2015). Furthermore, BZs had no stimulatory effects on Pgp activity in *H. contortus* eggs which was shown by a lack of effect on the efflux of rhodamine 123, a Pgp substrate (Kerboeuf and Guégnard, 2011). Possibly, BZs are "weak" ligands for Pgps of *H. contortus*, as suggested to be the case in *C. elegans* by *in silico* analysis (David et al., 2016).

While drug metabolizing enzymes and their role in drug resistance in humans has been thoroughly addressed in research, they have only been superficially investigated in parasitic nematodes. Resistance mediated by drug metabolism particularly by cytochrome P450 monooxygenases (CYPs), which comprise the biggest group of drug metabolizing enzymes, are discussed in depth in chapter 2.6.

## 2.5.4 Diagnosis of benzimidazole and macrocyclic lactone resistance

On a farm level, resistance is considered to be present when more than 5% of the worms survive treatment. However, at this level, resistance is imperceptible to the "naked eye" as animals still perform well. Production losses become noticeable only when at least half of the nematode population is drug resistant (http://www.scops.org.uk/about/what-is-anthelmintic-resistance/, last retrieved on 31.03.2019). For this reason, regular testing for the presence of anthelmintic resistance is essential and can be done using different methods.

### 2.5.4.1 Coprological methods

The most commonly used test is the fecal egg count reduction test (FECRT). This test assesses anthelmintic efficacy by comparing egg counts in feces before and after treatment. In small ruminants, according to the World Association for the Advancement of Veterinary Parasitology (WAAVP)

guidelines, a reduction in egg counts of less than 95% and a lower 95% confidence limit of less than 90% is indicative of resistance (Coles et al., 1992). Eggs can be counted using the McMaster counting technique, which was developed in 1939 (Gordon and Whitlock, 1939) and of which several modifications have been presented over the years (Pereckiene et al., 2007) or the FLOTAC method and its derivative the Mini-FLOTAC. The latter are more accurate and sensitive counting techniques, allowing a detection limit of one egg per gram (EPG) and 5 EPG, respectively, whereas the McMaster technique, depending on its version, has a sensitivity of between 10-50 EPG (Cringoli et al., 2010; Cringoli et al., 2017). One major disadvantage of the FECRT is that it can only be used reliably when the proportion of resistant nematodes exceeds 25% (Martin et al., 1989). On the other hand, the FECRT can be used for all anthelmintic groups and, once trained, can also be performed by farmers who, except for the one-time purchase of the necessary equipment, would not have to continuously invest large amounts of money for resistance testing.

#### *2.5.4.2. In vitro* methods

Several tests employing the ovicidal or larvicidal properties of anthelmintics have been developed to discriminate between susceptible and resistant trichostrongyloid nematode populations. The egg hatch assay (EHA), in which nematode eggs are exposed to serial concentrations of a BZ in order to determine the proportion of hatched larvae and calculate the half maximal effective concentration ($EC_{50}$ value) (Le Jambre, 1976), has been standardized a few years ago (von Samson-Himmelstjerna et al., 2009a) adhering to the recommended discriminatory dose of 0.1 µg/ml TBZ (Coles et al., 1992). This test cannot be used for the detection of ML resistance since this drug class is not sufficiently ovicidal.

The Larval Development Assay (LDA) is an *in vitro* test system that can be used to detect resistance against any drug class (Demeler et al., 2013a; Lacey et al., 1990; Raza et al., 2016b; Schürmann et al., 2007; Taylor, 1990). It measures the inhibitory effects of anthelmintics on the development of nematodes from eggs or L1 to L3 by exposing them to a concentration range for 6-8 days and subsequently determining the proportion of undeveloped stages (eggs, L1 and L2). The Larval Migration Inhibition Assay (LMIA) and the Micromotility Meter Test (MMT) can also be used for the detection of ML resistance in L3. It is not suitable for BZ resistance testing, since BZ are slow-acting drugs and cannot paralyze L3 within 24 h of exposure, which is the standard exposure time in LMIAs and MMTs to measure motility by migration through a sieve and electronic detectors, respectively (Demeler et al., 2010). When using LMIAs for the detection of ML resistance, it must be kept in mind that LMIAs require much higher concentrations for the discrimination of susceptible and resistant isolates than LDAs or MMTs, and this may not reflect the true resistance status of field populations. Neither LDAs nor LMIAs or MMTs have been standardized by a ring test and studies with field

populations are largely missing. Nevertheless, they are thought to have a lower detection limit for resistant nematodes than FECRTs. LMIAs, for instance, are thought to detect a proportion of resistant nematodes as low as 10% (Kotze et al., 2006).

### 2.5.4.3. Molecular methods

Three SNPs leading to amino acid changes in the β-tubulin isotype-1 gene (F167Y, E198A and F200Y) have been identified as the molecular basis of BZ resistance (see chapter 2.5.3.1). Derivatives of traditional sequencing and PCR-based approaches not only allow the detection of these SNPs but also enable quantitative measurements when using pooled samples.

Similar to the chain-terminating Sanger sequencing, pyrosequencing is based on the sequencing by synthesis principle but relies on the detection of light, which is generated by a chain reaction involving a cocktail of 4 enzymes and 2 substrates. The addition of a complementary nucleotide to a sequencing primer by a DNA polymerase results in the release of inorganic pyrophosphate that is converted to ATP by an ATP-sulfurylase. The ATP drives the luciferase-based conversion of luciferin to oxyluciferin which produces light that is proportional to the amount of ATP and is shown as a peak in the data output. The reaction is terminated by degradation of all nucleotides and pyrophosphate by apyrase. SNP allele frequencies can then be derived from the height of these peaks. Pyrosequencing assays have been successfully established for the detection of all three BZ resistance-associated SNPs in nematodes of ruminants including *Teladorsagia circumcincta*, *H. contortus*, *T. colubriformis*, *C. oncophora* and *O. ostertagi* and has also been extensively validated with field samples of the first three mentioned nematodes (Demeler et al., 2013c; Höglund et al., 2009; Ramünke et al., 2016; Samson-Himmelstjerna et al., 2009b). The sensitivity of pyrosequencing has been conservatively set at 10% (Samson-Himmelstjerna et al., 2009b).

Several PCR methods have been adapted to detect the F200Y SNP in *H. contortus*. These include restriction-fragment length polymorphism analyses of PCR products, allele-specific PCRs as well as real-time PCRs with allele-specific probes (Samson-Himmelstjerna et al., 2009b) (Alvarez-Sánchez et al., 2005; Mohanraj et al., 2017; Tiwari et al., 2006; Walsh et al., 2007). The newest addition to the range of available PCRs is droplet digital PCR which has been shown to reliably detect the F200Y SNP in *H. contortus* at frequencies as low as 2.6% (Baltrušis et al., 2018).

Very recently, Avramenko et al. (Avramenko et al., 2019) have introduced a deep amplicon sequencing protocol that allows the detection of all three SNPs in seven different trichostrongyloid nematode species of sheep at a sensitivity of 0.1% via a single MiSeq sequencing run. This method has been shown to yield results that are in strong agreement with pyrosequencing results (Avramenko et al., 2019). It remains to be seen to what extent SNP detection by Next-Generation Sequencing will be adapted in practice.

## 2.6 Cytochrome P450 monooxygenases

In metazoans, most drugs and other xenobiotics undergo several phases of processing. Hereby, cytochrome P450 monooxygenases (CYPs) catalyze most reactions in phase 1 – the initial phase of xenobiotic metabolism rendering compounds more reactive for further modifications in phase 2 via typical reactions such as oxidation, reduction, hydrolysis and hydration. Reactions in phase 2 are conjugations catalyzed by diverse enzymes including UDP-glucuronosyltransferases (UGTs), glutathione S-transferases (GSTs) and many other enzymes, mostly transferases. While many drugs are subject to this sequential metabolism, some drugs may undergo either phase 1 metabolism or phase 2 metabolism. The last phase of a drug´s metabolism is its expulsion from cells by Pgps and other efflux transporters.

### 2.6.1 Structure, function and enzyme mechanism

CYPs are cross-kingdom enzymes with conserved ancestral functions in endogenous substrate metabolism, which in humans include, steroid, fatty acid and cholesterol metabolism (Tralau and Luch, 2013). In higher eukaryotes, they are also part of the biotransformation cascade of xenobiotics. Having been shown to be responsible for up to 75% of all drug metabolism in humans, CYPs are among the best-studied proteins (Tralau and Luch, 2013). This functional variability is conferred by a considerable degree of sequence and structural diversity (Mestres, 2005).

While members of a family share at least 40% and members of a subfamily at least 55% amino acid identity, CYPs from different families may show a sequence homology as low as 10-30% (Mestres, 2005). The absolute numbers of α-helices and β- sheets may differ in different CYPs, but all CYPs have a heme-binding core consisting of a four α-helices bundle (α-D, α-E, α-I, α-L), two further α-helices (α-J and α-K), β- sheets 1-5, a cysteine pocket and a coil, termed the meander loop (Werck-Reichhart and Feyereisen, 2000). Conserved motifs that can be found within these regions are the ExxR motif at the C-terminal end of the α-K helix, proposed to stabilize the core structure, and the CXG motif located in the cysteine pocket just before the α-L helix, the cysteine of which serves as fifth ligand to the heme iron. These three amino acids (glutamic acid, arginine and cysteine) are the only absolutely conserved amino acids in CYPs (Werck-Reichhart and Feyereisen, 2000). Most variable regions are found at the distal part of the protein where, so far in humans, six substrate recognition sites have been identified (Werck-Reichhart and Feyereisen, 2000).

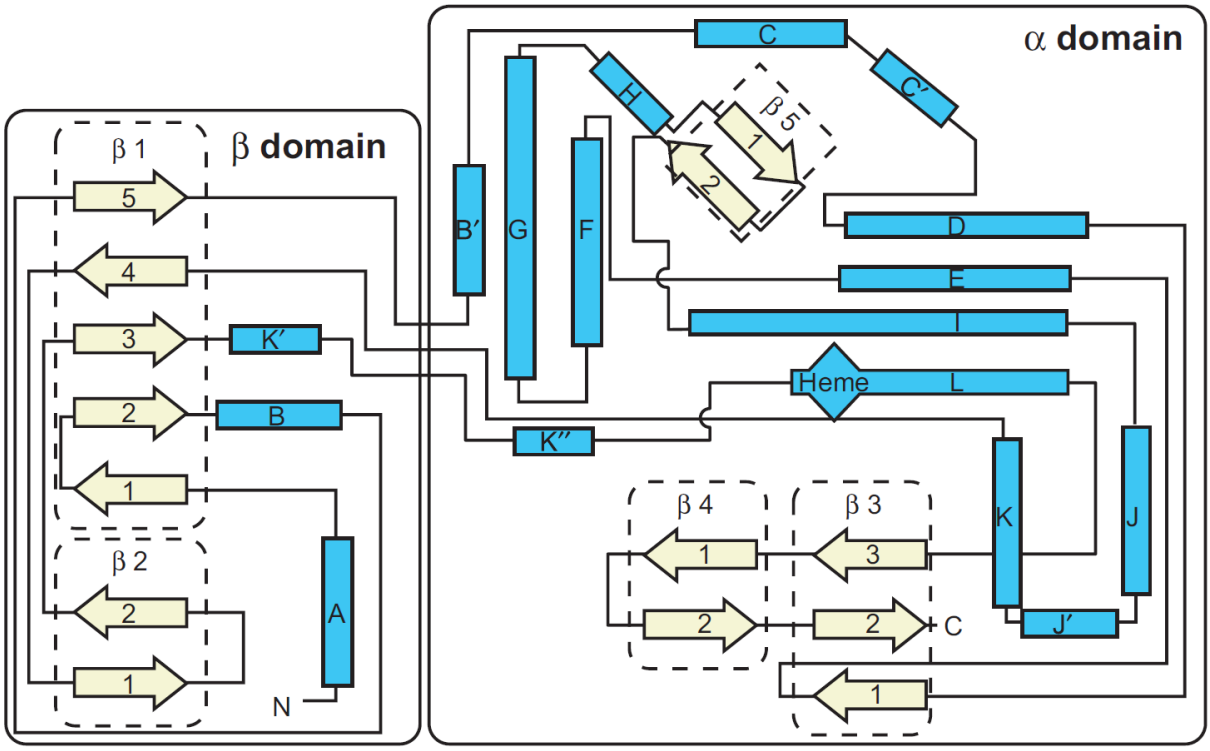


**Fig.2.** Typical secondary structure of a cytochrome P450 protein. Adapted from Werck-Reichhart and Feyereisen, 2000

The most frequently catalyzed reaction is hydroxylation, which is the best understood type of reaction CYPs can catalyze. Oxidation of substrates involves the displacement of the sixth water ligand by the substrate and is followed by a one-electron reduction of the ferric iron in the active center (heme b). The next step is the binding of molecular oxygen and subsequently a further reduction of the complex. Following this second reduction, the oxygen double bond is cleaved upon which one oxygen atom is reduced to water and the other is inserted into the substrate (Werck-Reichhart and Feyereisen, 2000). Cytochrome P450s have also been found to catalyze dehydrations, dehalogenations, decarboxylations, reductions, epoxidations, isomerizations and other specialized reactions (Tralau and Luch, 2013). As these reactions as well as substrate specificity are controlled by the less conserved distal regions of the protein, they are not well understood.

Cytochrome P450s are often categorized in four classes, depending on how they recruit electrons from NAD(P)H for oxidative reactions (Werck-Reichhart and Feyereisen, 2000). Class I enzymes, which are found in soluble state in prokaryotes and mitochondrial membranes of eukaryotes, require a FAD-containing reductase as well as an iron-sulfur redoxin. Class II proteins are membrane-bound microsomal enzymes of eukaryotes and only need an FAD/FMN-containing reductase. Together with class I enzymes they constitute 90% of all CYPs. Class III CYPs are present in mammal and plants. Because they bind oxygen-containing substrates, they do not require an electron donor and are

described “self-sufficient”. A fungal CYP that receives electrons directly from NAD(P)H has also been described and assigned to class IV.

## 2.6.2 CYP-mediated drug resistance

### 2.6.2.1 CYP-mediated drug resistance in humans

There are 57 active CYP genes in the human genome, organized in 18 families and 43 subfamilies (Wang and Chou, 2010). Among these, members of the CYP1, CYP2, CYP3 and CYP4 families have been recognized as key enzymes in the bioactivation and -transformation of drugs. Many polymorphisms have been identified that, depending on allele combinations, result in four metabolic phenotypes: poor metabolizers with two nonfunctional genes; intermediate metabolizers with one nonfunctional gene; extensive metabolizers with two normal genes and ultra-rapid metabolizers with multiple copies of genes. These phenotypes are associated with interindividual differences in drug response. Hence, they have been of great interest in clinical therapy, particularly in anticancer therapy, since CYPs have been shown to be involved in the development of resistance to anticancer drugs. Cancer cells can develop resistance through increased metabolic inactivation of drugs or decreased metabolic activation of prodrugs. Members of the CYP3A family metabolize at least 50% of all human drugs (Rodriguez-Antona and Ingelman-Sundberg, 2006). Increased activity of CYP3A4 has been shown to influence the outcome of anticancer therapy. For instance, breast cancer patients with a lower cyp3a4 mRNA expression have been found to respond better to docetaxel treatment (Miyoshi et al., 2002). Of the CYP2 family, several members have been implied in the metabolic activation and inactivation of anticancer drugs (Rodriguez-Antona and Ingelman-Sundberg, 2006). CYP2A6 catalyzes the activation of tegafur, which is used for the treatment of colorectal cancer. Although the functional consequences of the many polymorphisms of *cyp-2a6* on the metabolism of tegafur or other anticancer agents have not been completely elucidated, certain polymorphisms are suspected to worsen therapeutic outcome through insufficient levels of bioactivation (Daigo et al., 2002). Equally, the impact of polymorphisms found in *cyp-2b6* on the metabolic activation of prodrugs remains largely unknown but allelic variants may potentially hinder treatment success (Rodriguez-Antona and Ingelman-Sundberg, 2006). CYP2C19 and CYP2C9 are further highly polymorphic CYPs and in contrast to other CYPs allelic variants have clearly been associated with differences in the activation of anticancer prodrugs (Sim et al., 2006; Timm et al., 2005). CYP2D6 is a key metabolizer of tamoxifen, a drug used to treat breast cancer, and patients with defective *cyp-2d6* alleles have been shown to have lower levels of the main metabolites of tamoxifen (Jin et al., 2005). CYP1B1 of the CYP1 family is mainly expressed extra-hepatically and overexpression in tumor tissues may be responsible for reduced efficacy of anticancer treatment (McFadyen et al., 2004). Members of the CYP4 family do not seem to play a major role in the biotransformation of anticancer drugs (Rodriguez-Antona and Ingelman-Sundberg, 2006).

### 2.6.2.2 CYP-mediated drug resistance in arthropods

Plant chemicals that have insecticidal and acaricidal activities are thought to be evolutionary driving forces responsible for the enormous diversification of arachnid and insect CYPs (Nkya et al., 2013). Mosquito genomes, for instance, have been shown to have a large expansion of the CYP family with more than 100 CYPs in *Anopheles gambiae* and even more than 200 CYPs in *Culex quinquefasciatus* (Alphey et al., 2002; Yang and Liu, 2011). Because many plant toxins served as templates for the development of synthetic drugs, including the commonly used pyrethroid, reports of drug resistance in arthropods are not surprising. Target site alterations have been identified that lead to insecticide and acaricide resistance (Nkya et al., 2013), but enhanced drug detoxification has been implied in resistance, too. In Anopheline mosquitos, CYP-mediated resistance is even considered to be the dominating mechanism by which resistance develops to all major classes of insecticides (organophosphates, organochlorines, carbamates and pyrethroids) (Hemingway, 2014). In most studies, an overexpression of several members of CYP6 family have been shown to exist in resistant field mosquito populations and to be inducible by insecticides (Chandor-Proust et al., 2013). Some of these constitutively overexpressed and inducible CYPs have been identified as pyrethroid metabolizers (Chandor-Proust et al., 2013). While most research has clearly been done on mosquitos, CYPs have also been implied in drug resistance in other insects such as the planthoppers *Nilaparvata lugens* and *Laodelphax striatellus* (Miah et al., 2017; Zimmer et al., 2018), the fruit fly *Drosophila melanogaster* (Seong et al., 2018) and the beetle *Leptinotarsa decemlineata* (Kaplanoglu et al., 2017), to name a few. Enhanced metabolism is considered a contributing factor to drug resistance in ticks and mites as well. Elevated levels of CYP expression have been found in pyrethroid-resistant field populations of *Rhipicephalus microplus* (Cossío-Bayúgar et al., 2018) and several acaricides including the ML abamectin have been shown to be metabolized by individual members of the CYP39 family in the mite *Tetranychus urticae* (Demaeght et al., 2013; Riga et al., 2014; Riga et al., 2015).

### 2.6.2.3 Drug resistance by CYPs in nematodes

Compared to other putative resistance mechanisms, CYP-mediated biotransformation of anthelmintic drugs has received little attention in nematodes and consequently the understanding of its relevance for anthelmintic drug resistance remains unsatisfactory.

Investigations into CYPs and their role in BZ resistance in *C. elegans* have disclosed the inducibility of gene expression of several CYPs from the CYP35 family by ABZ and TBZ (Jones et al., 2013; Laing et al., 2010) and were completed with evidence that TBZ is metabolized by CYP35D1 (Jones et al., 2015). As for MLs, an inducible expression of *cyp-33C7*, *cyp-34A9*, *cyp-34A4*, *cyp-37B1* and *cyp-36B2* by IVM has been shown in an IVM-resistant *C. elegans* strain (Laing, 2010) and wild-type worms selected with IVM

and MOX over many generations have shown an overexpression of *cyp-14A2* and *cyp-14A5* (Ménez et al., 2016). However, so far, there are no studies to suggest a metabolism of IVM or MOX in *C. elegans*. The production of BZ metabolites in *H. contortus*, some of which are consistent with CYP activity, has been determined several times (Cvilink et al., 2008; Munguía et al., 2015; Stuchlíková et al., 2018). Metabolic studies with a focus on ML in *H. contortus* were less indicative of CYP activity as no IVM metabolites were found (Vokřál et al., 2013) and the features of one detected MOX metabolite remain unknown (Alvinerie et al., 2001). *In vitro* data, that is limited to the study of AlGusbi et al., (AlGusbi et al., 2014), argue that CYPs might be involved in BZ and ML resistance in *C. oncophora* and *O. ostertagi* since co-incubation of BZ or MLs with the CYP-inhibitor PBO has been shown to completely inhibit larval development. In addition, the study has observed a significant reduction in larval migration when larvae were co-exposed to MLs and PBO and an increase in susceptibility of eggs when co-exposed to BZs and PBO. In conclusion, contrary to *C. elegans,* no breakthrough has been achieved in case of parasitic nematodes. However, many studies that have been published suggest a role of CYPs in the metabolism of anthelmintic drugs.

# 3. Publications

## 3.1 Comparison of constitutive and thiabendazole-induced expression of five cytochrome P450 genes in fourth-stage larvae of *Haemonchus contortus* isolates with different drug susceptibility identifies one gene with high constitutive expression in a multi-resistant isolate

**Esra Yilmaz, Sabrina Ramünke, Janina Demeler, Jürgen Krücken**

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## 3.1.1 Abstract

Benzimidazoles (BZs) remain amongst the most widely used anthelmintic drug classes against gastro-intestinal nematode infections, although their efficacy is increasingly compromised by resistance. The primary underlying mechanisms for BZ resistance are single-nucleotide polymorphisms (SNPs) in the isotype 1 β-tubulin gene causing the substitutions F167Y, E198A or F200Y. However, resistance is believed to be multi-genic and previous studies have shown that isolates carrying 90-100% F200Y can vary considerably in their resistance level in the egg hatch assay (EHA). Cytochrome P450 monooxygenases (CYPs) are associated with drug resistance in mammals and arthropods and have been considered as mediators of anthelmintic resistance. In Caenorhabditis elegans, several members of the CYP34/35 and CYP31 families are BZ and/or xenobiotic inducible and thiabendazole (TBZ) is metabolised by CYP35D1. Here, expression of all 5 CYPs closely related to the *C. elegans* CYP34/35 and CYP31 families was investigated in fourth-stage larvae of two susceptible and three BZ-resistant *Haemonchus contortus* isolates following *in vitro* exposure to TBZ for 3 and 6 h using real-time RT-PCR. The resistance status of all isolates was determined using EHAs and quantification of resistance-associated β-tubulin SNPs using pyrosequencing. While none of the CYPs was TBZ inducible, constitutive expression of CYP34/35 family member HCOI100383400 was significantly 2.4-3.7-fold higher in the multi-drug resistant WR isolate with the strongest BZ resistance phenotype compared to susceptible and intermediate-level BZ-resistant isolates. Although this increase is only moderate, HCOI100383400 might still be involved in high-level BZ resistance by further decreasing susceptibility in isolates already carrying 100% of a β-tubulin SNP causing BZ resistance. Lower transcript levels were observed for all CYPs in the intermediately resistant IRE isolate in comparison to the susceptible HcH isolate, which, except for CYP HCOI01579500, were statistically non-significant. This suggests that none of the investigated CYPs may contribute to protection against TBZ in this particular isolate.

## 3.1.2 Introduction

In small ruminants, *Haemonchus contortus* is one of the most pathogenic gastrointestinal nematodes and infections with this parasite cause considerable economic losses (Perry et al., 2002; Peter and Chandrawathani, 2005). Treatment of *H. contortus* and other trichostrongyloids has become increasingly difficult due to the emergence of anthelmintic resistance, particularly in countries that heavily relied on use of anthelmintics in livestock production. Despite intensive research, anthelmintic resistance mechanisms are not fully understood yet. Recently, a study reported insufficient activity of the benzimidazole (BZ) albendazole against human *Ascaris lumbricoides* infections (Krücken et al., 2017).

Thiabendazole (TBZ) belongs to the class of benzimidazole (BZ) anthelmintics which have been in veterinary use against nematode infection since the 1960s. Resistance to BZs has been reported to

emerge through single nucleotide-polymorphisms (SNPs) in the isotype 1 β-tubulin gene in codons 167, 198 and 200 in a number of trichostrongyloid nematodes (Grant and Mascord, 1996; Elard and Humbert, 1999; von Samson-Himmelstjerna et al., 2007; Skuce et al., 2010; Demeler et al., 2013; Ramünke et al., 2016) including *H. contortus* (Kwa et al., 1994; Prichard, 2001; Ghisi et al., 2007). However, no such SNPs in potential BZ target sites could be identified in the recent report on insufficient albendazole efficacy in *A. lumbricoides* (Krücken et al., 2017).

In addition to changes in the BZ target sites, non-target related alterations have been considered to be involved in anthelmintic resistance. In particular, drug efflux pumps, such as P-glycoproteins (Pgps), have also been addressed as possible mediators of anthelmintic resistance (Prichard, 2001; Janssen et al., 2013; Kotze et al., 2014; Janssen et al., 2015; Kaschny et al., 2015) – particularly against the macrocyclic lactones where no target-site changes have been associated with resistance. Regarding BZs, selection at the pgp-2 locus in BZ-selected *H. contortus* has been described (Blackhall et al., 2008) and AlGusbi et al. (2014) have shown that the Pgp inhibitor verapamil potentiates the effects of TBZ on larval stages of BZ-susceptible and -resistant *Cooperia oncophora* and *Ostertagia ostertagi* leading to complete impairment of larval development. However, no activation of the rhodamine 123 (Rh123) binding site of Pgps and hence no increased efflux of Rh123 has been observed in eggs of a resistant *H. contortus* isolate following incubation with TBZ or albendazole while most macrocyclic lactones stimulated Pgp activity as revealed by significantly elevated efflux of rhodamine 123 (Kerboeuf and Guegnard, 2011). It is possible that BZs first need to undergo biochemical modifications to render them better substrates for efflux pumps. Moreover, in silico analysis of the binding properties of Pgp-1 of the free-living nematode *C. elegans* has predicted binding sites for TBZ and triclabendazole, though with less binding energy than drugs belonging to other classes of anthelmintics (David et al., 2016).

In recent years, the possibility of resistance via phase I (modifying) and phase II (conjugating) drug-metabolising enzymes and phase III transporters has received increasing attention. Regarding phase I, the largest group of drug-metabolising enzymes, the cytochrome P450 monooxygenases (CYPs), are known to be responsible for the biotransformation of a vast number of xenobiotics in mammals and insects and their association with insecticide and cancer drug resistance is well established (Rochat, 2005; David et al., 2013).

For a long time, it was assumed that CYPs exhibit no or very low activities in nematodes (Barrett, 1998). However, this general assumption has been weakened by a number of reports and attention devoted to CYPs is gradually increasing. The genome of *C. elegans* encodes more than 80 CYP proteins and several of these, in particular members of the family CYP35, have been shown to be inducible by xenobiotics (Menzel et al., 2001; Menzel et al., 2005). Increased expression of cyp35C1, cyp35A5 and cyp35A2 mRNAs has been reported in *C. elegans* upon albendazole exposure (Laing et al., 2010) and recently TBZ has been shown to induce *cyp-35A3, cyp-35A5, cyp-35C1* and *cyp-35D1* (Jones et al., 2013)

and to be metabolised by CYP35D1 in *C. elegans* (Jones et al., 2015). Other CYP families that are inducible by xenobiotics are CYP34, CYP31 and CYP33 (Menzel et al., 2001).

In parasitic nematodes, studies on the xenobiotic response to BZs have progressed less far and focused mainly on *H. contortus*. One of the first reports on CYP activity was provided by Kotze (Kotze, 1997) using microsomal preparations of *H. contortus*. Consistent with phase I followed by phase II metabolism, *ex vivo* and *in vivo* studies have furthermore shown that in this parasite albendazole and fenbendazole are metabolised to albendazole- and fenbendazole-sulfoxide (Cvilink et al., 2008; Munguia et al., 2015). Also corroborating the hypothesis of CYP-mediated BZ resistance in gastro-intestinal nematodes, eggs and larvae of *C. oncophora* and *O. ostertagi* isolates with differing BZ susceptibility have been shown to become more susceptible to TBZ in the presence of the CYP inhibitor piperonyl butoxide (AlGusbi et al., 2014).

The CYP superfamily of *H. contortus* has recently been described in detail by Laing et al. (Laing et al., 2015). They found that the CYP superfamily encoded in the *H. contortus* genome is considerably smaller than that of C. elegans and apparently lacks the extensive duplication of genes with function in metabolism of exogenous substrates. However, they identified four *H. contortus* CYPs that form a sister family to the xenobiotic-inducible *C. elegans* CYP families CYP34 and CYP35 (each with 10 members in *C. elegans*). This family will be designated as CYP34/35 family in the following. In C. elegans, one member of the CYP35 family, CYP35D1, has been shown to be inducible by TBZ and to contribute to detoxification of this anthelmintic (Jones et al., 2013; Jones et al., 2015). The *H. contortus* CYP34/35 family has apparently evolved through relatively recent gene duplications since HCOI01928800a and HCOI01928800b are located directly adjacent to each other on scaffold_930 in the *H. contortus* genome while HCOI100383700 and HCOI100383400 are separated by only two unrelated genes on scaffold_1500 (Laing et al., 2015). Other xenobiotic-inducible *C. elegans* CYP families have either no (CYP33) or only one gene (CYP31) in *H. contortus* (HCOI01579500) that has a corresponding position in the phylogenetic tree (see Fig. 3 in (Laing et al., 2015).

Since CYP31A and CYP35D1 have been shown to be xenobiotic- and TBZ-inducible, respectively, and the latter was able to use the drug as substrate, this study aimed to investigate the expression patterns of the above mentioned, most closely related *H. contortus* CYPs in the fourth-stage larvae (L4) following *in vitro* exposure to TBZ using qRT-PCR. In order to determine any potential effects of unspecific (CYP-mediated) and specific (β-tubulin-mediated) resistance mechanisms, the current phenotypic and target-site related genetic level of resistance of five *H. contortus* isolates was determined by egg hatch assays (EHA) and pyrosequencing, respectively. For all three approaches, material from the same parasite passage was used.

## 3.1.3 Materials and Methods

Parasites

Five isolates of *H. contortus* with differing susceptibility to BZs and other anthelmintics were incorporated in this study.

i) *H.c* Hannover (HcH, MHco9): susceptible to all anthelmintics.

ii) CAVR (MHco10) (Chiswick-Avermectin-Resistant): highly ivermectin (IVM) resistant, albeit BZ susceptible.

iii) IRE (inbred-resistant-Edinburgh; MHco5): highly IVM resistant; partially BZ resistant.

iv) TBZ (Thiabendazole): TBZ resistant; was originally obtained by Bayer Animal Health

v) WR (White River; MHco4): highly IVM and BZ resistant; moderately levamisole resistant

All isolates have been maintained at the Institute for Parasitology and Tropical Veterinary Medicine of the Freie Universität in Berlin for several years. Resistant isolates were regularly challenged by treatment of infected animals with the respective anthelmintic. All animal experiments were in agreement with the European directive 2010/63/EU and the German law ("Tierschutzgesetz") and were approved by the responsible local authorities (LAGeSo Berlin) under the reference number L0088/10. Individual sheep were infected with approximately 6000 third stage larvae (L3) of one of the isolates and faeces were collected for egg recovery and larval cultures. For EHAs, only animals with at least 500 eggs/g faeces (epg) were used. Animals were treated orally with Cydectin 0.1% (0.2 mg/kg KGW) three weeks after begin of patency to clear the infections.

Eggs were purified from fresh faeces using a sucrose step gradient. Briefly, faeces were homogenised and passed through a 100 µm sieve. Eggs in the flow through were collected on a 25 µm sieve followed by centrifugation and flotation with saturated sodium chloride solution. Then, the egg suspension was laid on the top of a sucrose step gradient containing 10%, 25% and 40% of a saturated sucrose solution and centrifuged at 2000×g and 4 °C for 5 min. Eggs floated between the 10% and 25% layer. They were collected and washed with de-ionised water.

Egg hatch assay

Egg hatch assays (EHA) were principally carried out as described by Demeler et al. (2012). Recovered eggs were suspended in sodium phosphate buffer (10 mM NaPOi buffer, pH 7) and adjusted to approximately 100 eggs/ml. TBZ (Sigma-Aldrich, T8904) was dissolved at a concentration of 10063 µg/ml in DMSO. From this stock solution, working solutions were prepared by dilution with DMSO. Stock and working solutions were prepared at least 24 h prior to use. Assays were set-up in 24-well plates with each well consisting of 1990 µl of egg suspension and 10 µl of drug solution or DMSO (vehicle control). A dilution range with final concentrations of 0.01, 0.024, 0.05, 0.077, 0.101, 0.157,

0.177, 0.201, 0.252 and 0.503 µg/ml of TBZ and positive control of 5.0315 µg/ml TBZ were used in assays with the isolates HcH, IRE and CAVR. For the highly resistant strains TBZ and WR, additional final concentrations of 0.75, 0.9, 1.0, 1.25 and 5.0315 µg/ml of TBZ and a positive control of 50.315 µg/ml TBZ were used.

Plates were incubated for 48 h at 25-27 °C and stopped with a drop of Lugol´s iodine. All assays were performed at least in duplicate and repeated five times independently. Eggs and larvae were counted and the number of hatched larvae was calculated as percentage. EC50 values were determined by four parameter logistic regression using GraphPad Prism 5.0.3. Top constraints were restricted to values between 0 and 100% and bottom constraints were set to equal 0%.

Sums of square F tests were conducted to determine significant differences between isolates. All p values were corrected for multiple testing using the Holm correction as implemented in the p.adjust command in R statistics version 3.3.1.

DNA extraction and pyrosequencing of isotype 1 β-tubulin gene

Approximately 10,000 L3 of each isolate were concentrated using a Baermann Apparatus and kept at -80 °C until usage. DNA was isolated using the NucleoSpin Tissue XS kit (Macherey Nagel) according to manufacturer´s instructions. To improve DNA yield, samples were initially thawed and homogenised in the presence of T1 Lysis Buffer (Macherey Nagel) using the SpeedMill P12 and innuSPEED Lysis Tubes F (both Analytik Jena) for three cycles of 1 min.

PCRs specific for *H. contortus* isotype 1 β-tubulin were run essentially as described by Ademola et al. (Ademola et al., 2015) by setting up 50 µl reactions containing 1 × HF Buffer, 1 U Phusion II High Fidelity DNA polymerase (Thermo Scientific), 0.2 mM dNTPs, 0.25 µM of each primer (HcPY2PCRFor: 5'-GACGCATTCACTTGGAGGAG-3' and HcPY2PCRRev: 5'-Biotin-CATAGGTTGGATTTGTGAGTT-3') and 2 µl of DNA (ca. 10 ng). Cycling conditions were as follows: initial denaturation at 98 °C for 30 s was followed by 40 cycles of 98 °C for 10 s, 56 °C for 30 s and 72 °C for 30 s. PCRs were terminated by a final elongation at 72 °C for 10 min. PCR products were used as templates for the subsequent pyrosequencing assays using the PyroMark Q24 system and the PyroMark Gold Q24 kit following the manufacturer´s instructions. Sequencing primers used for measurement of allele frequencies were Hc167PySeq1: (5'-ATAGAATTATGGCTTCGT-3'), Haemcon198Seq-Pr: (5'-GGTAGAGAACACCGATG-3'), and Hc200PySeq1: (5'-TAGAGAACACCGATGAAACAT-3').

*In vitro* cultivation of third to fourth stage larvae

The *in vitro* protocol for cultivation of *H. contortus* was modified from Rothwell and Sangster (1993) and Song (2003). Exsheathed L3s were obtained by incubating 30 ml of L3 suspension with 1 ml of 12.5% sodium hypochlorite for 10-15 min at 37 °C and 200 rpm. After microscopical control of

exsheathment success, exsheathed L3 were washed twice with 0.9% NaCl solution by rapid mixing of larvae with solution and removal of solution by filtration through a bottle top sterile filter collecting the larvae on the top of the filter. Then, larvae were transferred to a Baermann Apparatus equipped with a 22 µm precision-woven nylon mesh and containing 0.9% NaCl solution. After incubation for at least 60 min, L3s were collected in a falcon tube and filled up with pre-warmed (37.5 °C) sterile axenisation fluid (136 mM NaCl, 5 mM KCL, 0.05 mM NaH2PO4, 0.1 mM Na2HPO4, 2 mM glucose, 0.024 mg/ml benzylpenicillin, 0.064 mg/ml streptomycin and 0.0008 mg/ml amphotericin B). Exsheated L3s were kept in this fluid at 37.5 °C and 90 rpm for 3 h. During this period, the axenisation fluid was replaced three times by removing the old solution by filtration on a bottle top filter.

Larvae were finally concentrated by filtration and maintained in pre-warmed RPMI 1460 culture medium (Lonza), supplemented with 20% foetal bovine serum (FBS) (Biochrom) and 0.06 mg/ml benzylpenicillin, 0.1 mg/ml streptomycin and 0.002 mg/ml amphotericin B, at a density of approximately 2080 larvae per ml culture medium at 41 °C and 20% CO2 for 2 days. On day 3, culture medium was replaced with fresh culture medium and larvae were incubated for another two days until exposure to TBZ.

Thiabendazole exposure of fourth stage larvae

Larvae were microscopically controlled to ensure that at least 80% had moulted to the L4 stage and displayed active pharynx pumping. Then, larvae were split into three groups of approximately 100,000 L4s each. Two groups were exposed to TBZ (0.5 µg/ml) for 3 h and 6 h, respectively. One group served as vehicle control and was incubated with 0.05% DMSO for 3 h. After incubation, L4s were washed with 4 °C cold 0.9% NaCl solution via filtration and collected in a falcon tube. L4s were pelleted through centrifugation at 4 °C and maximum speed for 5 min, re-suspended in cold 0.9% NaCl solution and again pelleted. The pellet was transferred to a lysis tube and frozen at -80 °C until used.

RNA extraction and cDNA synthesis

For optimal RNA isolation, larvae were rapidly thawed in the presence of T1 Lysis Buffer (Macherey Nagel) and homogenised using the SpeedMill P12 and innuSPEED Lysis Tube F (Analytik Jena) for three cycles of 1 min. The remaining steps were performed with the NucleoSpin RNA kit from Macherey Nagel according to manufacturer´s instructions. Approximately 125 ng of RNA were treated with DNase I (Thermo Fisher) and reverse transcribed to cDNA using Thermo Fisher's Dynamo cDNA Synthesis Kit. For a 20 µl reaction mixture, 2 µl of M-MuLV RNase H+ reverse transcriptase and 1 µl of random hexamers were added to 10 µl RT buffer and RNA. cDNA was synthesised by incubating the mixture at 25 °C for 10 min, followed by 39 °C for 30 min and 85 °C for 5 min. Then, cDNA was diluted 1:3 and 1:6 for subsequent expression analysis of CYP- and reference genes, respectively.

Quantitative reverse-transcription PCR

Reference genes superoxide dismutase (*sod*), fatty acid retinol binding protein (*far*) and glyceraldehyde-3P-dehydrogenase (*gpd*) were adopted from Lecova et al. (Lecova et al., 2015) who had shown stable expression of these transcripts in adult *H. contortus* following varying treatments with albendazole and ivermectin. Primer sequences of both reference and candidate CYP genes can be found in Table 3.1S1. along with PCR efficiencies for each primer set. PCR reactions were run using the GoTaq qPCR Master Mix (Promega) on the CFX96 real-time PCR cycler (Biorad). A 50 µl reaction mixture was set up containing 25 µl of the GoTaq qPCR Master Mix for Dye-Based Detection, 21 µl of nuclease-free water, 0.2 µM of each primer and 2 µl of cDNA. Cycling conditions included initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A melt curve was generated by heating from 60 °C to 95 °C with increments of 0.5 °C each 5 s.

Samples were run in 2-5 biological and two technical replicates. All real-time PCRs were performed twice independently and primer efficacies were evaluated in every run via ten-fold serial dilutions with plasmid DNA carrying the respective genes (4×108 - 4×101 copies). Melt curves indicated absence of unspecific products. To account for run-to-run variation, a pool of plasmid DNA was used as a calibrator on each plate and data was analysed with the CFXTM Manager Software 2.0 which considers PCR efficiencies when calculating relative expression levels (Pfaffl, 2001). Statistical analysis to compare the gene expression level of target genes between treatment groups within one isolate and between different isolates without drug exposure was carried out using the one-way ANOVA with Tukey´s post hoc test in GraphPad Prism 5.0.3

**Table 3.1S1.**

**Primer sequences for quantitative reverse transcription PCR**

| Primer | Sequence | Amplicon length (bp) | Source | Mean PCR efficiency (in %) ± standard deviation |
|---|---|---|---|---|
| Sod for | GCTGGCACTGATGATTTGGG | 73 | Lecova et al., 2015 | 95.7% ± 7.3 |
| Sod rev | CGCCAGCATTTCCTGTCTTC | | | |
| Gpd for | ACGAGACCTACAATGCAGCC | 67 | Lecova et al., 2015 | 90.4% ± 10.3 |
| Gpd rev | GCGAGACAGTTGGTGGTACA | | | |
| Far for | TGCCAAGGACTATGCCAAGT | 128 | Lecova et al., 2015 | 88.6% ± 6 |
| Far rev | TGAGTGCGTCGATCTTTCCC | | | |
| | | | | |
| HCOI100383400 for | CGTTGCTGGACCTATGGTTT | 115 | Own design | 87.7% ± 5.5 |
| HCOI100383400 rev | AGCAACTCCTCTTCCACTCG | | | |
| HCOI100383700 for | CAGCGATCTTCCCAATGAAT | 160 | Own design | 89.6% ± 5.1 |
| HCOI100383700 rev | TATCGGTCCGGATTGAACTC | | | |
| HCOI01928800a for | CGACCAGGACCAAGCCC | 201 | Laing et al., 2015 | 88.5% ± 6.6 |
| HCOI01928800a rev | TCGCCTTGTTAGCTTCTTGAAA | | | |
| HCOI01928800b for | TCGAACCAAGGAAGGCAAAC | 201 | Laing et al., 2015 | 89% ± 4.9 |
| HCOI01928800b rev | CACCGAGACATGACCGTTTC | | | |
| HCOI01579500 for | GCTGTGCATACTGTCAACGAT | 170 | Laing et al., 2015 | 82% ± 2.3 |
| HCOI01579500 rev | CCTGCTCCTGGATCTCGC | | | |

### 3.1.4 Results

Phenotypic resistance status of isolates against thiabendazole determined by egg hatch assay

Phenotypic resistance towards TBZ was determined by calculating EC50 values of all isolates. A wide range of EC50 values was found. For the BZ-susceptible HcH and CAVR isolates, EC50 values of 0.018 and 0.024 µg/ml, respectively, were obtained. With an EC50 value of 0.96 µg/ml, the WR isolate displayed the highest resistance towards TBZ. For the other two isolates of *H. contortus*, intermediate resistances were determined (EC50 values of 0.118 and 0.189 µg/ml for IRE and TBZ) (Fig. 3.1.1). The difference in EC50 values among all isolates were statistically highly significant (Table 3.1.1).

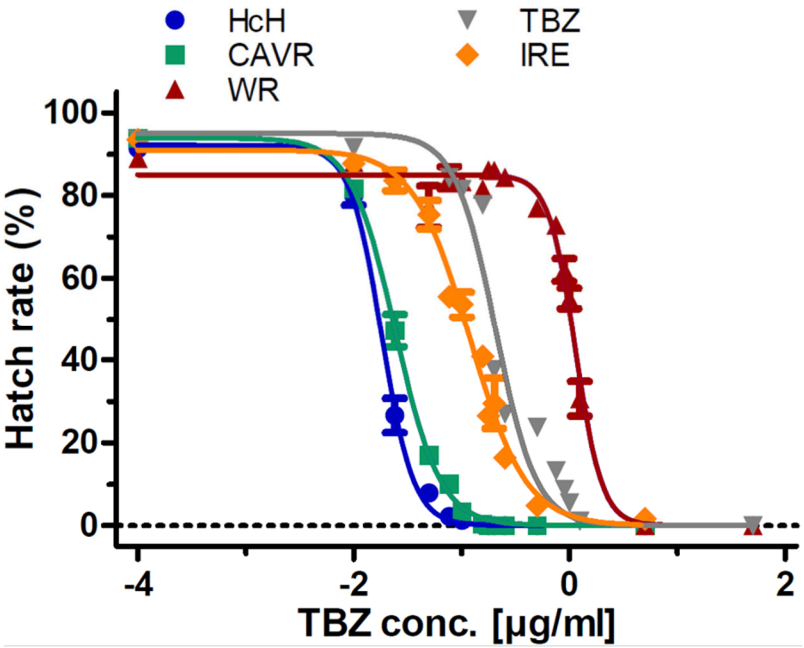


**Fig. 3.1.1.** Concentration-response-curves for thiabendazole using different *Haemonchus contortus* isolates in an egg hatch assay. All $EC_{50}$ values are significantly different from each other with a $p$ = 0.0009.

**Table 3.1.1**
Efficacy of thiabendazole against *Haemonchus contortus* in an egg hatch assay

| Isolate | $EC_{50}$ [µg/ml][a] | 95% CI [µg/ml][b] | $R^{2c}$ |
|---|---|---|---|
| *H.c* Hannover | 0.01809 | 0.01708 - 0.01916 | 0.9787 |
| *H.c* CAVR | 0.02434 | 0.02293 - 0.02584 | 0.986 |
| *H.c* IRE | 0.1186 | 0.1054 - 0.1335 | 0.9448 |
| *H.c* TBZ | 0.1891 | 0.1817 - 0.1967 | 0.9399 |
| *H.c* WR | 0.9637 | 0.5814 - 1.597 | 0.9889 |

[a] Effective concentration 50%.
[b] 95% confidence interval.

[c] Coefficient of determination.

Frequency of benzimidazole-resistance associated single nucleotide polymorphisms determined by pyrosequencing

The frequency of the resistance-associated TAC SNP at codon 200 was strongly elevated in the BZ-resistant isolates WR (93.5 ± 2%, mean ± SD), TBZ (74.8 ± 2.7%) and IRE (64.5 ± 3.1%) compared to the BZ-susceptible HcH and CAVR isolates with frequencies of 6 ± 0.9% and 10 ± 1.4%, respectively, which corresponded to the technical background (Table 3.1.2). No evidence for increased frequencies of resistance-associated alleles at codons 167 and 198 was found, which is in agreement with previous results using the IRE and WR isolates (von Samson-Himmelstjerna et al., 2009).

**Table 3.1.2**
Mean frequencies (in %) ± standard deviation of benzimidazole resistance-associated alleles in the β-tubulin 1 gene

| Isolate | Codon 200 (TAC) | Codon 198 (GCA) | Codon 167 (TAC) |
|---|---|---|---|
| *H.c* Hannover | 6 ± 0.9 | 13.5 ± 1 | 5.5 ± 0.6 |
| *H.c* CAVR | 10 ± 1.4 | 15.5 ± 1.7 | 10 ± 1.8 |
| *H.c* IRE | 64.5 ± 3.1 | 14.3 ± 1.5 | 6.3 ± 2.1 |
| *H.c* TBZ | 74.8 ± 2.7 | 13.2 ± 1.5 | 11.7 ± 1.3 |
| *H.c* WR | 93.5 ± 2 | 14.7 ± 1.3 | 2.6 ± 0.6 |

CYP expression in susceptible and resistant isolates of *Haemonchus contortus* following thiabendazole exposure

Due to insufficient *in vitro* development rates of L3 during the establishment phase of the cultivation method causing a limitation in available number of L3 and, more importantly, very low yield of RNA for some replicates, it was not possible to achieve five biological replicates for all isolates and time points. Since the experiments required large numbers of larvae (100,000 per replicate) and the number of available L3 was limited, no additional experiments were possible. For most isolates and experimental conditions, at least four replicates were available while only three were analysed for the IRE isolate and the TBZ isolate after 3 h exposure to TBZ and only two for the TBZ isolate after 6 h TBZ exposure.

Transcript levels of investigated CYPs in all isolates showed no significant changes following 3 or 6 h exposure to 0.5 µg/ml TBZ when compared to worms of the corresponding isolate treated for 3 h with DMSO alone (Fig. 3.1.2 and 3.1.3). However, only two biological replicates were available for the resistant TBZ isolate at the 6 h time point, which in case of CYP HCOI100383400, HCOI100383700 and HCOI01928800b showed a high degree of variability.

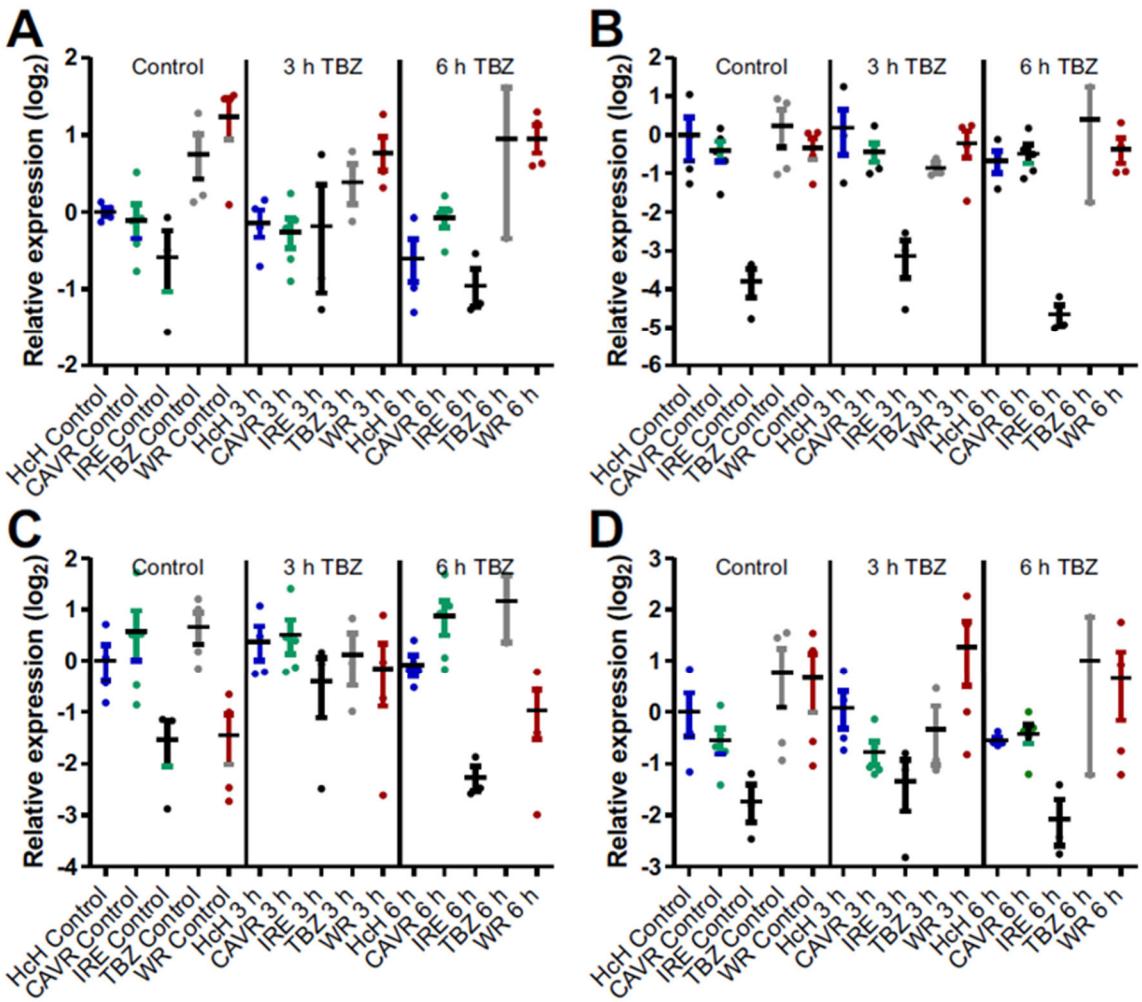


**Fig. 3.1.2.** Expression levels of cytochrome P450 family 34/35 (Cyp34/35) mRNA orthologues in response to thiabendazole. Fourth stage larvae obtained by *in vitro* culture were exposed to 0.05% DMSO (vehicle control) for 3 h or 0.5 µg/ml thiabendazole (TBZ) in 0.05% DMSO for 3 or 6 h. Expression of the CYPs HCOI100383400 (A) HCOI100383700 (B), HCOI01928800a (C), HCOI01928800b (D) was analysed using the housekeeping genes superoxide dismutase (*sod*), fatty acid retinol binding protein (*far*) and glyceraldehyde-3P-dehydrogenase (*gpd*) as reference genes and the HcH isolate as control for normalisation.

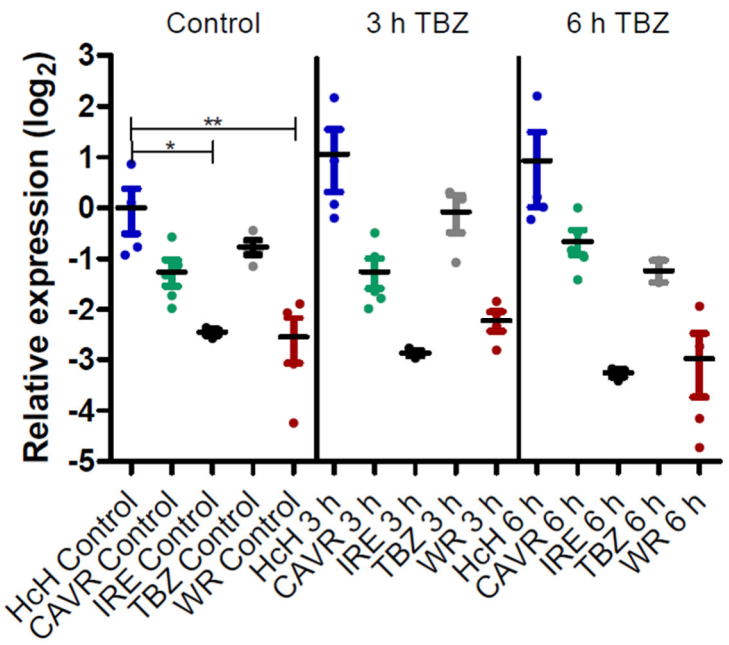


**Fig. 3.1.3.** Expression levels of cytochrome P450 family 31 (CYP31) mRNA orthologue in response to thiabendazole. Fourth stage larvae obtained by *in vitro* culture were exposed to 0.05% DMSO (for 3 h, vehicle control) or 0.5 µg/ml thiabendazole (TBZ) for 3 or 6 h. Expression of the CYP HCOI01579500 was analysed using the housekeeping genes superoxide dismutase (*sod*), fatty acid retinol binding protein (*far*) and glyceraldehyde-3P-dehydrogenase (*gpd)*as reference genes and the HcH isolate as control for normalisation.

Comparison of basal transcript levels between isolates in the absence of drugs

Comparison of the basal transcript levels revealed no significant differences for HCOI100383700, HCOI01928800a, HCOI01928800b in any of the isolates (Fig. 3.1.2 B, C, D). In contrast, significantly elevated transcript levels were observed for HCOI100383400 in the highly TBZ resistant WR isolate compared to both susceptible isolates, HcH (2.4-fold) and CAVR (2.7-fold), and the intermediately resistant IRE (3.7-fold) isolate (Fig. 3.1.2 A). Transcript levels were slightly but not significantly elevated for the TBZ isolate. For HCOI01579500, transcript levels were significantly lower (approximately 5.7-fold) in the IRE and WR isolates compared to the HcH isolate (Fig. 3.1.3). The IRE isolate displayed lower transcript levels for the other CYPs as well when compared to the HcH isolate. However, these differences were not statistically significant.

## 3.1.5 Discussion

Cytochrome P450 monooxygenases have several times been considered as players in the evolution of anthelmintic resistance. However, the most convincing studies were conducted with *C. elegans* while

knowledge of CYPs present in parasitic nematodes is still very scarce. For *H. contortus*, this has changed only recently, when Laing et al. (Laing et al., 2015) characterised its CYP superfamily and provided a basis to assay specific CYPs. In the present study, five *H. contortus* isolates were evaluated regarding their resistance status by EHAs and pyrosequencing assays targeting codons 200, 198 and 167 of the isotype 1 β-tubulin gene. Then, the constitutive and TBZ-inducible expression of HCOI100383700, HCOI01928800a, HCOI01928800b, HCOI100383400 and HCOI01579500 were measured in L4 obtained by *in vitro* culture. The first four of the chosen CYPs are orthologues of the BZ- and/or xenobiotic-inducible CYP34 and CYP35 families in *C. elegans* while HCOI01579500 is an orthologue of the xenobiotic-inducible CYP31 family. Therefore, all closest phylogenetic relatives to the xenobiotic-inducible CYP families in the *H. contortus* genome have been included in the present study.

In vertebrates, the CYP gene superfamily is known to include so-called evolutionary stable and unstable genes. While stable CYP genes are typically the only members in their CYP family, are evolutionary conserved and function in biosynthesis and metabolism of endogenous substrates, unstable CYP genes generally show a high rate of gene duplication and deletion and encode enzymes acting on xenobiotic substrates (Thomas, 2007). The latter birth-death evolutionary scenario has been proposed to apply also to the evolutionary history of CYP genes in *C. elegans* (Thomas, 2007). Among the investigated *H. contortus* CYPs, HCOI100383700, HCOI01928800a, HCOI01928800b and HCOI100383400 all cluster in the same family, whereas HCOI0579500 appears to be a member of a CYP family containing no other members. Consistent with the above interpretation, all CYPs from the CYP34/35 family are more highly expressed in the intestine of adult *H. contortus* than in the soma (Laing et al., 2015). Since the intestine is hypothesised to be the major site of detoxification in nematodes (Lindblom and Dodd, 2006; McGhee, 2007; Rosa et al., 2015), this expression pattern would be in agreement with a role in detoxification.

Although exposure to TBZ did not induce a significant change in transcript level of any of the CYPs in the CYP34/35 family, basal expression of HCOI100383400 was significantly elevated in the highly resistant WR compared to the susceptible HcH, CAVR and the moderately resistant IRE isolates. However, the rather small difference in basal transcript level of this CYP in the resistant WR isolate suggests merely a minor role in resistance. For comparison, induction of *cyp-35D1* by TBZ in *C. elegans* was 257-fold (1h; 0.125 mM TBZ) and thus considerably higher albeit at a 50-fold higher TBZ concentration. Nevertheless, such a constitutive overexpression has the potential to protect against the effects of BZs at a very early time-point after exposure whereas inducible expression should be assumed to take several hours until enough functional enzyme has been synthesised to achieve considerable protection.

In this context, it should be noted that the WR isolate is resistant to IVM and also partially resistant to levamisole and increased expression levels of HCOI100383400 might also be a result of selection by

these anthelmintics. However, in the absence of an inducibility by TBZ, investigations on potential effects of other anthelmintics on transcript levels and any functional data on drug binding or metabolism by HCOI100383400, it is impossible to assess which explanations are more likely. Another important aspect is that the isolates included in the study have completely independent genetic backgrounds. Thus, finding higher or lower expression level as for HCOI100383400 in WR and for HCOI01579500 in IRE and WR isolates, respectively is in no way conclusive. Differences between isolates might simply be by chance and completely independent of the resistance status.

The expression of the CYP31 orthologue HCOI01579500 is evidently higher in the soma of *H. contortus* than in the intestine and highest in adult females (Laing et al., 2015). Further, its *C. elegans* orthologues *cyp-31A2* and *cyp-31A3* are expressed in gonads and oocytes and have been shown to be indispensable for proper development of the embryo (Benenati et al., 2009). Even though there is no functional information available for HCOI01579500, the possibility of a similar critical function in development in *H. contortus* should not be completely excluded. Similarly, a role in xenobiotic metabolism should not be ruled out entirely, since CYPs may have activity against both endogenous and xenobiotic substrates as implied by the inducibility of *C. elegans cyp-31A* by the xenobiotics PCB52, β-naphtoflavone, phenobarbital, atrazine, pyrazol and toluene (Menzel et al., 2001). Interestingly, the IRE isolate showed lower transcript levels for the other CYPs as well – all of which are expected to have exogenous substrates. However, these differences were non-significant which can most likely be attributed to the small number of biological replicates that were available for the IRE isolate since no other technical issues were encountered. In the absence of any known function of these CYP enzymes and considering that most differences were not significant it is not possible to conclude if lower expression levels in the IRE isolate might be physiologically relevant.

While the picture concerning the basal transcript levels of the five CYPs in the L4 stage of these isolates is clear, the picture concerning induced expression by TBZ is by far incomplete. The negative results obtained in the present study do not exclude that induction of CYP expression is possible under different experimental conditions. For instance, it cannot be excluded that an induction was not detected because up-regulation and return to basal levels might have occurred outside of the 3 - 6 h time window used here. Alternatively, CYPs might be responsive to TBZ in a concentration-dependent manner. Due to limitations in the number of larvae that were available, it was not possible to use a gradient of TBZ concentrations as previously done in the case of C. elegans for different albendazole concentrations (Laing et al., 2010). Here, a concentration of 0.5 µg/ml TBZ was chosen as a compromise aiming to use a concentration as high as possible while ensuring that susceptible isolates survive for the incubation time. Apart for a small number of individuals, larvae of both susceptible and resistant isolates were alive until the moment of incubation termination and the very few exceptions in all isolates most likely had no influence on the outcome of the analysis. However, due to the wide range

of phenotypical BZ resistance within the five isolates, it might well be that the concentration window needed for induced expression of CYPs differs between isolates and that this window was missed. Another potential explanation for missing inducibility is the very artificial experimental setup. Since obtaining L4 in sufficient numbers from *in vivo* experiments is extremely difficult, *in vitro* culturing of L3 to L4 is a reasonable alternative to study responses of parasitic life cycle stages. Nevertheless, *in vitro* culture is a highly artificial system that might have unpredictable effects on the physiology of the L4s. Additionally, it remains to be elucidated whether constitutive expression of these CYPs is different in adult worms or other larval stages and whether expression can be induced by TBZ in adults or after *in vivo* treatment of the host.

Of course, the involvement of other CYPs that were not investigated here is also possible. This would be particularly plausible for genes belonging to rapidly evolving gene families with multiple members that have relatively recently evolved by gene duplication (Laing et al., 2015). This property again points to the four orthologues of the CYP34/35 family, as revealed by the analysis presented by Laing et al. (Laing et al., 2015). Together, these data suggest that all members of rapidly evolving CYPs in the *H. contortus* genome have been included here. Nevertheless, the draft genome used to predict the *H. contortus* CYP gene family represents 93% of the conserved eukaryotic genes (Laing et al., 2013). Although this is a very good value for a nematode draft genome, the missing 7% suggest that also some of CYP gene superfamily members might have been missed.

In principle, mechanisms other than regulation of gene expression might be involved. Genome sequence analysis suggests that unstable vertebrate CYPs are subject to significant positive selection for amino acid changes possibly driven by xenobiotic substrates (Thomas, 2007). Although highly speculative, such a mechanism might be present in *H. contortus* or might eventually become relevant with the ongoing wide use of BZs. However, it cannot be excluded that all the CYPs that were investigated in the present study have no major function in xenobiotic mechanism at all. All currently available information provides only indirect arguments such as phylogenetic position, subfamily size and tissue distribution while direct evidence such as induction by and in particular metabolism of individual xenobiotics by particular CYPs is still completely missing for *H. contortus*.

The frequency of the resistance-associated SNP in codon F200Y was elevated in the phenotypically resistant IRE, TBZ and WR isolates. The rank order of the EC50 values in the EHA and the frequency of the resistance-associated SNP were identical over all five isolates investigated. This confirms that the genotype of the isotype 1 β-tubulin gene is the major parameter determining the BZ resistance status of *H. contortus*. However, comparison of the three phenotypically resistant lines identifies that a 10% increase in F200Y between the IRE (63.5% F200Y) and TBZ (73.5% F200Y) isolates is associated with an increase in the EC50 value by 1.6-fold. A further increase of the SNP frequency by 19% (92.5% F200Y in WR) is accompanied by a further increase in the EC50 value by 8.1-fold. Two phenomena might

contribute to this dramatic additional increase. First, the frequency of homozygous worms carrying the allele associated with resistance is estimated to be 40.3% (IRE), 54.0% (TBZ) and 85.5% (WR) if random mating is assumed. Considering the technical background of pyrosequencing, data for the WR isolates do not even exclude that the isolate carries nearly exclusively the resistant allele. Nevertheless, it is hard to imagine that the dramatic increase in the EC50 value can be entirely attributed to increased frequency in F200Y. von Samson-Himmelstjerna et al. (2009) reported a F200Y frequency of 44% and an EC50 value in the EHA of 0.117 for the WR isolate. Since then, the isolate has gone through several *in vivo* selections using albendazole. While comparing EHA EC50 values for TBZ with F200Y frequencies of several isolates, von Samson-Himmelstjerna et al. (2009) identified eight isolates or field samples with F200Y frequencies between 90 and 100%. The EC50 values for these *H. contortus* populations ranged between 0.13 and 0.66 µg TBZ/ml – all lower than the WR isolate reported here. The high variability in phenotypic resistance between isolates with 90-100% F200Y suggests that selection at additional loci is able to further increase resistance levels. These might involve the isotype 2 β-tubulin gene (Kwa et al., 1993) but also other genes. If higher resistance levels are due to multi-genic effects, even moderate changes in constitutive expression of several genes could have considerable additive or synergistic effects on the resistance phenotype. In such a multi-genic context, constitutive or inducible overexpression of genes involved in detoxification of anthelmintics might occur only in isolates with the highest resistance level and different isolates with high resistance might differ in the set of overexpressed genes.

In conclusion, the results of the present study do not provide clear evidence that any of the *H. contortus* CYPs closely related to those involved in metabolism of xenobiotics or specifically TBZ in *C. elegans* has a role in resistance of the parasite. However, in combination with previous EHA and pyrosequencing data, the results also clearly show that (i) the isotype 1 β-tubulin gene locus cannot be the only one with influence on the phenotypic resistance level and (ii) that the *H. contortus* CYP gene HCOI100383400 should be considered as a candidate that might be involved in very high-level BZ resistance in a multi-genic context as well as in resistance to other anthelmintics.

## 3.1.6 Conflict of interest

The authors declare no conflicts of interest.

## 3.1.7 Financial Support

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## 3.1.8 Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2017.10.001.

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## 3.2 Orthologs of anthelmintic resistance-associated cytochrome P450 13A genes of *Teladorsagia circumcincta* are not thiabendazole-inducible in cultured fourth-stage larvae of *Haemonchus contortus*

**Esra Yilmaz, Sabrina Ramünke, Janina McKay-Demeler, Jürgen Krücken**

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## 3.2.1 Abstract

Background: Cytochrome P450 monooxygenase (CYP) activity has long been recognized as an important player in the development of drug resistance in mammals and arthropods. In addition, there is reason to assume that CYPs might also be involved in anthelmintic resistance in nematodes. In *Caenorhabditis elegans*, xenobiotics, including anthelmintic drugs, can induce the expression of selected CYPs and CYP35D1 has been shown to mediate resistance against the benzimidazole thiabendazole (TBZ). Recently, introgression of multi-drug resistant *Teladorsagia circumcincta* into a susceptible background has identified two CYPs that were highly overexpressed in resistant worms and represent orthologs of the xenobiotic-inducible CYP13A family in *C. elegans*.

Results: The expression of Cyp13A genes was investigated in susceptible and resistant *H. contortus* isolates using *in vitro* generated fourth stage larvae following 3 and 6 h exposure to TBZ. Essentially, TBZ did not result in induction of CYP expression in any of the isolates at the chosen concentration and inter-isolate differences in expression were not observed.

Conclusions: While the study does not suggest a CYP13A-mediated TBZ resistance in *H. contortus*, only functional studies can conclusively demonstrate whether these CYPs are part of drug resistance pathways.

## 3.2.2 Background

Anthelmintic therapy is the most important pillar in parasitic nematode management in livestock. Being the first of its kind, benzimidazoles (BZ) were quickly embraced by practicing veterinarians and farmers alike when they became available more than 40 years ago and are still in use against a variety of nematode infections. However, decades of indiscriminate use have selected BZ-resistant nematode populations, which is becoming a significant impediment to successful therapy in intensive animal farming and wherever multi-drug resistance occurs. In sheep and goat production systems, BZ-resistant *Haemonchus contortus* populations are considered particularly problematic (1).

There is clear evidence that single nucleotide polymorphisms (SNPs) in the isotype 1 β-tubulin gene in codons 167, 198 and 200 confer resistance to BZs in a number of gastrointestinal nematodes, including *H. contortus* (2). While changes in β-tubulin are thought to be the major determinant for BZ resistance, in particular the substitution of phenylalanine to tyrosine at codon position 200 (2), non-target site related mechanisms are suspected to be involved as well.

We have recently shown that isolates of *H. contortus* with the F200Y substitution can substantially vary in their phenotypic resistance levels (up to 6.9–fold) while having similar F200Y frequencies between 90-100% (3, 4). This suggests that an additional locus or loci other than the isotype 1 β-tubulin locus might be involved in determining high-resistance phenotypes in *H. contortus*.

In mammals and arthropods, phase-1 detoxification systems involving cytochrome P450 monooxygenase enzymes (CYPs) have been associated with drug resistance (5, 6) and recent findings suggest that CYPs are also implicated in BZ resistance in nematodes. In the free-living nematode *Caenorhabditis elegans*, several members of the CYP35 family are inducible by BZs, and thiabendazole (TBZ) has been shown to serve as a substrate for CYP35D1 (7). Studies on the metabolism of different BZs in *H. contortus* advocate a role of both phase-1 and phase-2 metabolizing enzymes, including CYPs, in this parasitic nematode (8, 9). Therefore, CYP encoding genes must be considered important candidate genes that might contribute to anthelmintic drug and in particular BZ resistance in parasitic nematodes.

In a previous study, the expression of five CYPs closely related to the xenobiotic- and BZ-inducible CYP34/35 and CYP31 families of *C. elegans* was investigated in fourth stage larvae of two BZ-susceptible and three BZ- resistant isolates of H. contortus following *in vitro* exposure to TBZ (4). While no TBZ-inducible expression was observed for any of the investigated CYPs, one member of the CYP34/35 family was shown to have a moderate constitutive overexpression in a multi-drug resistant isolate with high-level resistance against TBZ.

Recently, after introgression of *Teladorsagia circumcincta* with triple resistance against BZs, ivermectin and levamisole into a susceptible background, two CYPs, representing orthologs of the CYP13A subfamily in *C. elegans*, have been shown to be approximately 20-fold overexpressed in resistant worms (10). The CYP13A subfamily has been shown to be also inducible by some xenobiotics (11) but were not considered as xenobiotic-inducible CYPs in a publication describing the complete CYP repertoire of *H. contortus*. The present study aimed to complete the picture regarding the inducibility of two additional candidate CYP- encoding genes in the context of BZ resistance using the material collected previously for analysis of initially proposed five Cyp genes (4). Therefore, the present work investigated the basal expression and possibly TBZ-inducible expression of two Cyp13A orthologs, termed HCOI00284400 and HCOI00827700, in *in vitro* cultivated fourth stage larvae of five *H. contortus* isolates displaying varying resistance phenotypes against TBZ.

## 3.2.3 Material and Methods

Isolates

As previously determined in Yilmaz et al. (4) in an egg hatch assay, isolates used in this study showed three different levels of resistance to TBZ. While the HcH and HcCAVR isolates were categorized as susceptible, moderate TBZ resistance was determined for the HcIRE and HcTBZ isolates and high level TBZ resistance was found for the WR isolate (Table 3.2.1).

**Table 3.2.1.** Resistance status against thiabendazole of included *Haemonchus contortus* isolates revealed in an egg hatch assay

| Isolate | $EC_{50}$ [µg/ml][a] | 95% CI [µg/ml][b] |
|---|---|---|
| *H.c* Hannover | 0.01809 | 0.01708 - 0.01916 |
| *H.c* CAVR | 0.02434 | 0.02293 - 0.02584 |
| *H.c* IRE | 0.1186 | 0.1054 - 0.1335 |
| *H.c* TBZ | 0.1891 | 0.1817 - 0.1967 |
| *H.c* WR | 0.9637 | 0.5814 - 1.597 |

[a] Effective concentration 50%.
b 95% confidence interval.
Data reproduced from Yilmaz et al. (4)

*In vitro* cultivation and exposure of fourth stage larvae to thiabendazole

RNA material from *in vitro* cultivated fourth stage larvae exposed and non-exposed to TBZ were the same samples described in Yilmaz et al. (4). In brief, third stage larvae were exsheathed by sodium hypochlorite treatment and washed with 0.9% NaCl solution. Larvae were concentrated using a Baermann Apparatus and maintained in sterile axenization fluid at 37.5 °C and 90 rpm for 3 h with medium exchange every hour. Subsequently, larvae were concentrated by filtration and *in vitro* cultivated to fourth stage larvae (L4) in RPMI 1460 culture medium, supplemented with fetal bovine serum and antibiotics. During the five-day cultivation process at 41 °C and 20% CO2, culture medium was changed once. Finally, fourth stage larvae were split into two treatment and one vehicle control group of 100,000 L4s each and exposed to 0.5 µg/ml TBZ for 3 and 6h and 0.05% DMSO for 3 h, respectively.

Analysis of cytochrome P450 expression levels by real-time qPCR

After RNA isolation using the SpeedMill P12 homogenizer and Macherey Nagel's NucleoSpin RNA kit, cDNA was synthesized with approximately 125 ng of RNA according to manufacturer's instructions (Dynamo cDNA Synthesis Kit, Thermo Fisher). Reference genes superoxide dismutase *(sod)*, fatty acid retinol binding protein *(far)* and glyceraldehyde-3P-dehydrogenase *(gpd)* were chosen according to Lecova et al. (12). For the amplification of CYP and reference genes, each qRT-PCR contained 25 µl of GoTaq qPCR Master Mix for Dye-Based Detection (Promega), 21 µl of nuclease-free water, 0.2 µM of each primer and 2 µl of previously 1:6 diluted and 1:3 diluted cDNA, respectively. Cycling conditions were comprised of an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min and were complemented by a melt curve analysis. PCRs were run twice independently with 2-5 biological and two technical replicates and further included pooled plasmid DNA as an inter-run calibrator. Amplification efficiencies were calculated from the slope of standard curves generated via ten-fold serial dilutions of plasmid DNA. The CFX™ Manager Software 2.0 was used to analyze data

and the one-way ANOVA with Tukey's post hoc test to investigate statistical differences in gene expression. Primer sequences and efficiencies for CYP and reference genes are listed in Table 3.2.2.

**Table 3.2.2. Primer sequences for quantitative reverse transcription PCR**

| Primer | Sequence | Amplicon length (bp) | Source | Mean PCR efficiency (in %) ± standard deviation |
|---|---|---|---|---|
| Sod for | GCTGGCACTGATGATTTGGG | 73 | Lecova et al., 2015 | 95.7% ± 7.3 |
| Sod rev | CGCCAGCATTTCCTGTCTTC | | | |
| Gpd for | ACGAGACCTACAATGCAGCC | 67 | Lecova et al., 2015 | 90.4% ± 10.3 |
| Gpd rev | GCGAGACAGTTGGTGGTACA | | | |
| Far for | TGCCAAGGACTATGCCAAGT | 128 | Lecova et al., 2015 | 88.6% ± 6 |
| Far rev | TGAGTGCGTCGATCTTTCCC | | | |
| HCOI100284400 for | CGGAATTTGCGGCAACCAA | 100 | Own design | 107.5% ± 4.6 |
| HCOI100284400 rev | TCTGACGGAAGGGAGCAGTA | | | |
| HCOI100827700 for | TGGGCCAACAAGGTTCTCAG | 149 | Own design | 93.8% ± 4.5 |
| HCOI100827700 rev | GGCCATGAAGAACTGGCTCA | | | |

### 3.2.4 Results

No evidence of induced expression of HCOI00284400 following 3 or 6 h exposure to 0.5 µg/ml TBZ was observed in any of the isolates (Fig. 3.2.1A). Interestingly, the basal transcript levels were slightly but not significantly elevated in the BZ susceptible HcH and HcCAVR isolates when compared to all three BZ resistant isolates. Likewise, no TBZ-inducible expression nor differences in basal transcript levels were found for HCOI00827700 (Fig. 3.2.1B). Here, it should be noted that due to limited amounts of RNA, it was no longer possible to incorporate all isolates in the expression analysis of HCOI00827700 and data for the CAVR- and TBZ isolate are missing.

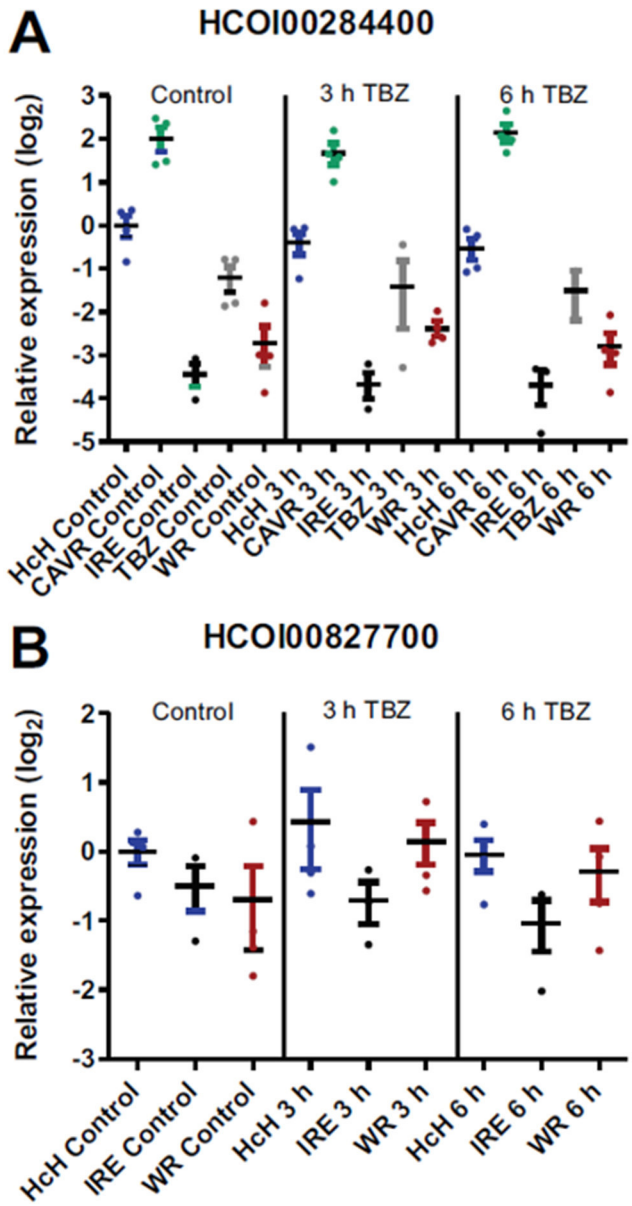

**Fig. 3.2.1. *Haemonchus contortus* cytochrome P450 family 13 (CYP13) mRNA expression levels in response to thiabendazole (TBZ).** Fourth stage larvae of the isolates HcH, CAVR, IRE, TBZ and WR were obtained by *in vitro* culture from L3 and then exposed to 0.05% DMSO (for 3 h, vehicle control) or 0.5 µg/ml TBZ for 3 or 6 h. Using the housekeeping genes superoxide dismutase (*sod*), fatty acid retinol binding protein (*far*) and glyceraldehyde-3P-dehydrogenase (*gpd*) as reference genes, expression of the CYPs HCOI002824400 (A) and HCOI00827700 (B) was analyzed. The drug susceptible HcH isolate was used as a control for normalization of data.

## 3.2.5 Discussion

CYPs are NADPH-dependent enzymes that are ubiquitously distributed throughout kingdoms of life and their ancestral function has been postulated to be the metabolization of endogenous substances such as steroids or fatty acids (13). Consensually considered to be among the most rapidly evolving genes, CYPs in higher eukaryotes are thought to have expanded through repetitive gene duplications resulting in new CYP families and subfamilies (so-called “blooms”) with functions in xenobiotic metabolism (13).

While *C. elegans* displays such an expansion with several large blooms and a number of CYPs from different subfamilies have been shown to be strongly inducible by xenobiotics, the parasitic *H. contortus* does not seem to have undergone multiple rounds of gene duplication (14). Except for one small bloom, which is comprised of the above-mentioned four orthologues to the xenobiotic-inducible CYP34/35 families of *C. elegans*, all other CYPs in the *H. contortus* genome are either singletons or form subfamilies with only two members. This lineage-specific inventory of CYPs is compatible with different lifestyles of these nematodes. As a free-living organism, *C. elegans* experiences selection pressure on xenobiotic defense mechanisms, whereas the free-living stages of *H. contortus* are only exposed to environmental stress after hatching and during development in feces (L1-L2) while parasitic stages are protected by host homeostasis – until the discovery of anthelmintics – and while eggs and L3 are protected against many xenobiotics by the egg shell and the sheath. The efficacy of these mechanical diffusion barriers is exemplified by the much higher EC50 values observed for trichostrongyloid nematodes in egg hatch and larval migration inhibition assays compared to larval development assays (15).

Even though the CYP repertoire of *H. contortus* is much smaller than that of *C. elegans*, CYPs of *H. contortus* (and other parasitic nematodes) might nonetheless have adopted a role in detoxification of xenobiotics. A range of metabolites of flubendazole (FLU) and albendazole (ABZ) has recently been identified in *H. contortus* (8). During phase I, the primary metabolite of FLU has been shown to be FLU with a reduced carbonyl group (FLU-R) which most likely was catalyzed by a carbonyl reductase rather than a CYP. However, CYP-consistent hydroxylation has also been shown to take place. In contrast, ABZ

was mainly metabolized to ABZ-sulfoxide, which represents a typical reaction catalyzed by CYPs. In the same study, a significantly higher amount of the main metabolite of FLU and ABZ has been reported in the BZ-resistant IRE isolate of *H. contortus* when compared to the fully susceptible ISE isolate of the same genetic background. The metabolism of TBZ has not been investigated in *H. contortus*; however, TBZ possesses a sulfur atom and might be targeted for oxidation similar to ABZ.

Tissue specific and life-stage specific expression of CYPs in *H. contortus* has been investigated and several CYPs, including HCOI00284400, have been shown to be highly enriched in the intestine of adult *H. contortus* (14) which has been suggested to be the main organ responsible for xenobiotic detoxification in nematodes (16). In addition, the above- mentioned introgression studies using multi-drug resistant and susceptible *T. circumcincta* have revealed significantly higher transcript levels of two CYPs representing orthologs of the *C. elegans* CYP13A subfamily in resistant worms (10). Due to this finding, CYP13A orthologs must be considered candidate genes that might be involved in anthelmintic resistance.

In *C. elegans*, CYP13A12 metabolizes polyunsaturated fatty acids to signaling molecules involved in the modulation of locomotor behavior (17) but has also been implicated in xenobiotic metabolism due to its inducibility by triclosan (11). The miscellaneous role of certain *C. elegans* CYPs has also clearly been shown for CYP35A2. This CYP is involved in fat storage and in increasing the toxicity of the pesticide fenitrothion (18, 19). Although no functional information is available for HCOI002824400 or HCOI00827700 and the present observations do not corroborate the hypothesis that these two CYPs might be involved in BZ resistance in *H. contortus*, the possibility of them partaking in both, endogenous and xenobiotic metabolism, as shown for *C. elegans*, cannot be entirely ruled out.

In general, strong inducibility by xenobiotics is a hallmark of genes involved in phase I and phase II xenobiotic metabolism (20-22). The fact that a gene is induced by a xenobiotic does not necessarily mean that the gene product is involved in metabolism of the inducer. The opposite relationship that a gene is induced by the xenobiotic against which it protects, is frequently but not always true. In the present study, the lack of inducibility does therefore not contradict a role in detoxification of xenobiotics. In *C. elegans*, for instance, CYP34A6 has been identified as a metabolizer of PCB52 (23), whilst previously no inducible expression by the xenobiotic was shown (24). Identification of BZ-inducible genes is thus a suitable method to identify good candidates to be further evaluated but not sufficient to formally confirm or exclude candidate genes that might be involved in drug metabolism

## 3.2.6 Conclusion

The chosen experimental design is not comprehensive enough to exclude any role of the CYP13A orthologs in BZ resistance in the analyzed *H. contortus* isolates, since there is a considerable possibility that the chosen TBZ concentration, the exposure times, the live cycle stage and the artificial culture

system are not compatible with induced expression (4). However, the data presented here completes a previous data set involving the CYP31 and CYP34/35 orthologs. Due to the fact that none of the potentially xenobiotic-inducible CYPs show any response to thiabendazole a role of CYPs in BZ resistance of these *H. contortus* isolates is quite unlikely. Nevertheless, it might well be that for different drug classes and/or in different isolates (and species) different resistance mechanisms contribute to the overall phenotype. Particularly, involvement of the CYP13A genes in resistance against macrocyclic lactones or levamisole remains to be investigated since high expression of the *T. circumcinta* orthologs was observed in a multi-drug-resistant isolate (10). Functional studies such as overexpression of the CYP genes in C. elegans followed by determination of the effect of the transgene on drug susceptibility, in addition to studies on expression level in response to multiple drugs will be required to complete the overall picture.

## 3.2.7 Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

EY and SR performed the experiments. EY, JK, SR and JD were involved in study design. EY and JK analysed the data and drafted the first version of manuscript. All authors edited the first draft and read and approved the final manuscript.

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# 3.3 Minimal modulation of macrocyclic lactone susceptibility in *Caenorhabditis elegans* following inhibition of cytochrome P450 monooxygenase activity

**Esra Yilmaz[1], Bastian Gerst[1], Janina Demeler, Jürgen Krücken**

**[1] Contributed equally**

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# 4 General Discussion

The administration of anthelmintic therapy without any consideration of parasitic nematode ecology has inherently created one of the most difficult challenges in modern sheep industry: treatment failure due to drug resistance. In large production systems, where resistance emerges and spreads quickly, lack of treatment success can be business-threatening. Understanding resistance mechanisms is considered vital for the identification of putative drug targets and for the development of tools able to diagnose resistance early on and to maintain animal health and profitability.

Research into mechanisms underlying BZ and ML resistance are at different stages of progression. Target-site related mechanisms have been identified for BZs involving SNPs in the β-tubulin isotype 1 gene while for MLs it still is a subject of ongoing effort (Doyle et al., 2019). On the other hand, there are substantial indications for an involvement of drug efflux by Pgps in ML resistance; however, no conclusive indications are available regarding BZ resistance (see chapter 2.5.3 for an in-depth coverage of topic).

In recent years, biotransformation of MLs and BZs by CYPs have come to receive more attention and the current study aimed to contribute to a better understanding on how CYPs might mediate anthelmintic resistance in parasitic nematodes. One reason why CYPs were subject of investigation is that they are involved in drug resistance in humans and arthropods. A phylogenetic comparison between CYPs of nematodes and humans or arthropods does not provide sufficient information to assign typical biochemical and enzymatic properties such as substrate specificity. Indeed, it has been shown that CYP3A4 which metabolizes MLs and BZs in humans is not an ortholog of CYP35D1, which has been shown to metabolize CYPs that are inducible by MLs and BZs in the nematode *C. elegans* (Jones et al., 2015). This lack of correlation between phylogenetic signal and functional properties is due to the fact that CYPs are amongst the most rapidly evolving genes and show extreme sequence diversity mediated by gene duplications and gene conversions followed by sequence diversification due to rapid accumulation of mutations in one of the gene copies (Sezutsu et al., 2013). In particular, there seem to be differences in metabolic capabilities between *C. elegans* (and potentially other nematodes) and mammals and some substrates have been shown to be metabolized by CYPs that do not represent the closest orthologs in each species (Harlow et al., 2018). However, the presence of xenobiotic-metabolizing CYPs in distantly related organisms, such as humans and arthropods, demonstrates the often-cited functional promiscuity of CYPs in higher eukaryotes and gives reason to believe that they might also be able to participate in anthelmintic resistance in parasitic nematodes and that there might be selection pressure on certain CYPs under constant drug pressure in the field. Indeed, the first conclusive evidence regarding the biotransformation of an anthelmintic drug by CYPs

in nematodes was published in 2015, in the *C. elegans* models. These worms were shown to metabolize the BZ representative TBZ by CYP35D1 (Jones et al., 2015). The knock-down of CYP35D1 resulted in a decrease of the TBZ $EC_{50}$ in an egg laying assay about 2-fold, i.e. the worms were twice as susceptible to the effects of TBZ when *cyp-35d1* was knocked down (Jones et al., 2015).
In *C. elegans*, the nuclear hormone receptor NHR-8 has been shown to regulate CYP gene expression and modulate the metabolism of BZs and IVM (Jones et al., 2013; Jones et al., 2015; Ménez et al., 2019). By rescuing an *nhr-8 C. elegans* mutant with the *H. contortus* homolog and by showing that silencing of *Hco-nhr-8* by RNAi on L2 of *H. contortus* results in an increase in IVM susceptibility, Menez et al (Ménez et al., 2019) have demonstrated its relevance for *H. contortus* as well.

Since research into metabolism-based resistance of nematodes is still in its infancy, only little functional information is available for CYPs of *C. elegans* and none for CYPs of parasitic nematodes. The latter is foremost attributable to the lack of sequence information for CYPs due to insufficient quality of available genome data leading to a lack of genes in the assemblies or wrong annotation of genes in the genomes. *Haemonchus contortus* is an exception for which two draft genomes have been published and the CYP gene superfamily has been resolved recently (Laing et al., 2013; Laing et al., 2015; Schwarz et al., 2013). It was therefore promising to further analyze CYPs of *H. contortus* in order to assess whether a CYP-mediated BZ resistance pathway might also play a role in resistant isolates of parasitic nematodes. Very often, but not always, xenobiotic substrates of CYPs behave as inducers, typically by binding to nuclear receptors following binding to and activation of the target gene. Therefore, this research question was approached via gene expression studies (chapters 3.1 and 3.2) in which both the constitutive and TBZ-inducible expression of CYPs was investigated in five isolates of *H. contortus* which differed in their susceptibility to TBZ.
From the total of 21 CYPs that have been identified in *H. contortus* so far (Laing et al., 2015), seven CYPs have been included in the gene expression analyses that was a part of this study. CYPs HCOI100383700, HCOI01928800a, HCOI01928800b and HCOI100383400 are orthologs of the *C. elegans* CYP34 and CYP35 families (Laing et al., 2015), of which several members have been shown to be inducible by xenobiotics, including several BZs and IVM (Jones et al., 2013; Laing, 2010). As for the other chosen CYPs, HCOI00284400 and HCOI00827700 are orthologs of the xenobiotic-inducible CYP13A family of *C. elegans* and HCOI01579500 is an ortholog of the xenobiotic-inducible CYP31 family of *C. elegans*. At first glance, one may question whether the selection of *H. contortus* CYP genes basing primarily on orthologs of a free-living nematode is adequate. In fact, there are fundamental differences when looking at the CYP repertoire of the two nematode species. With around 80 genes, the number of CYPs in *C. elegans* is very high and the CYP gene family is characterized by an extensive expansion of certain CYP subfamilies into so-called blooms, i.e. diversification and distribution of CYPs in families

and subfamilies. In contrast, the CYP gene family of *H. contortus* includes only one bloom consisting of the four CYP genes that are ortholog to the *C. elegans* CYP34 and CYP35 families and expression of these genes was included in the present study. A higher number of rapidly evolving CYP genes in blooms is expected since *C. elegans* is a free-living organism and continuously exposed to numerous environmental xenobiotics including presumably highly variable secondary metabolites from microbes fermenting the rotting fruits that are the typical *C. elegans* habitat (Félix and Duveau, 2012). Despite these differences, however, principal pathways of biotransformation can be expected to be similar considering that both *C. elegans* and *H. contortus* are clade V nematodes and have been shown to produce the same and/or similar ABZ metabolites (ABZ-SO and two albendazole-glucoside conjugates) (Cvilink et al., 2008; Laing et al., 2010; Stuchlíková et al., 2018). However, the intricacies of the regulation of gene expression and biotransformation may be different between nematodes with fundamentally different life styles and the studies underlying this thesis have verified that a direct extrapolation of findings from *C. elegans* to *H. contortus* may sometimes be an oversimplification that needs to be treated with caution.

In the course of the investigation of a CYP-mediated BZ resistance in *C. elegans* two characteristics have been uncovered. First, the expression of CYP35 genes has been shown to be inducible by BZs (Jones et al., 2013; Laing et al., 2010), and second, the potency of induction has been determined to be concentration-dependent (Laing et al., 2010). Further, an introgression of multi-drug resistant *Teladorsagia circumcincta* into a susceptible background has found two CYPs that were highly overexpressed in resistant worms and represent orthologs of the xenobiotic-inducible CYP13A family in *C. elegans* (Choi et al., 2017).

In L4s of *H. contortus*, an induction of specific CYP transcripts after a three- and six-hour *in vitro* exposure to 0.5 µg/ml TBZ could not be observed in any of the isolates. In the free-living *C. elegans,* CYP alleles showing an induction of CYP transcription even at very low concentrations of a xenobiotic substrate might have been selected and fixed in the genome, whereas in *H. contortus,* the threshold concentration for the induction of CYP expression might simply be higher and not represented by the chosen concentration of TBZ. This might particularly be the case for parasitic life cycle stages as the host provides a much more stable environment and the parasite, except during drug treatments, is not continuously exposed to diverse harmful conditions. In addition, similar to what has been described for *C. elegans*, CYP induction in *H. contortus* may be dynamic and respond in a concentration-dependent manner, which due to restrictions of time and particularly biological material could not be elucidated in the current studies. In this context it is important to emphasize that at least a million of fresh *H. contortus* L3 were required to obtain successful *in vitro* L4 cultures. This number was too high to use larvae that were produced during routine passage of *H. contortus* isolates in the Institute for Parasitology and Tropical Veterinary Medicine. Therefore, a lamb was required for each of the isolates

and animal experiments had to be conducted specifically for this study. Due to the limited and only seasonal availability of lambs and the aim to keep the number of animals in experiments as low as possible, it was impossible to evaluate more than one concentration of TBZ, more time points or additional drugs.

Another reason why it is not yet possible to completely negate the involvement of CYPs in BZ resistance in *H. contortus*, and of course even less in other parasitic nematode species, is the fact that CYPs might still be involved in the biotransformation of a xenobiotic compound without being first induced (Menzel et al., 2007). The transcriptional induction of CYP expression usually serves the purpose of an increased production of the enzyme and its catalytic activity and hence an acceleration of the metabolism of the xenobiotic. However, this concept is not in strict contradiction to some alternative, although rarer scenarios. It may well be that the basal activity of the enzyme is not increased by higher (inducible or constitutive) RNA expression levels which would have been detectable in the studies described in chapter 3.1 and 3.2. In addition, there might be scenarios such as (i) alleles with in general increased enzymatic activity towards all potential substrates including the anthelmintic (i.e. higher turnover number) (Zanger and Schwab, 2013) or (ii) increased metabolism of the particular xenobiotic by drug selected alleles presumably involving increased substrate affinity or (iii) even simply increased translation efficacy that can even lead to higher catalytic activity although mRNA and primary structure are identical.

As discussed in chapter 3.1, further considerations that need to be taken into account are the artificial experimental approach as well as the limited time frame. As already discussed above, it was not possible to do a full kinetic time curve experiment and therefore the chosen time points were instructed by extrapolating previous results particularly from *C. elegans* assuming that parasitic nematodes such as *H. contortus* show similar kinetics which might be oversimplified as discussed below. The fact that in *C. elegans* BZ exposure induces CYP expression very quickly and upholds elevated levels of expression even after six hours (Jones et al., 2015) might again represent a particular evolutionary adaptation of a free-living nematode to its highly variable evironment. It might well be that induction is a slower process in parasitic (clade V) nematodes such as *H. contortus* than in *C. elegans*. In any case, whether a particular CYP gene shows a fast/quick or late-onset induction, it is important to realize that it may take some time until enough protective enzyme has been produced to substantially increase detoxification. Due to this considerable time lag, a constitutive overexpression of CYPs can be expected to be more relevant than inducibility, particularly for fast-acting anthelmintic drugs such as MLs and other anthelmintics targeting neuronal ion channels such as levamisole, pyrantel, emodepside or derquantel. The constitutively overexpressed HCOI100383400 in the highly multi-drug resistant WR isolate of *H. contortus* and the absence of such a constitutive overexpression

in less resistant and susceptible isolates might indicate that CYP-mediated resistance to BZs and possibly to other anthelmintic drugs might occur isolate-specific and particularly play a role in isolates with a highly resistant phenotype.

A central question that remains to be elucidated is whether other CYPs, not analyzed in the context of this study, might potentially be involved in BZ resistance. The selection of CYPs of *H. contortus* was based on their similarity to xenobiotic-metabolizing *C. elegans* CYPs and on their distribution patterns into families and subfamilies. CYPs that show variation in their family or subfamily size such as rapid expansion by duplication followed by diversification are often associated with functions in xenobiotic metabolism (Thomas, 2007). While several subfamilies with more than one member have been included in the study, there are four subfamilies with two members in the *H. contortus* genome that were not part of the study, primarily because no xenobiotic-metabolizing ortholog had been identified in *C. elegans*. However, a possible contribution of these CYPs to anthelmintic drug resistance cannot be ruled out entirely. It is equally important to consider the quality of the *H. contortus* genome and how this might have affected the overall outcome of the study. As mentioned previously, two draft genomes from two distinct isolates (MHco3 and McMaster), both published in 2013, are available for *H. contortus* (Laing et al., 2013; Schwarz et al., 2013). Interestingly, a recent study that analyzed and compared the two genomes has found several quality issues in and substantial differences between the two genomes (Wintersinger et al., 2018). For instance, an underrepresentation of core eukaryotic genes (CEG) has been observed in both genomes. Of the 248 CEGs that have been identified in most eukaryotic organisms (Parra et al., 2009), including *C. elegans*, only 216 (87%) complete CEGs were present in the genome derived from the MHco3 isolate, whereas the percentage was even lower for the genome of the McMaster isolate (ca. 70%). Both figures contrast with an expected minimum of 95% representation of CEGs in *H. contortus*. The phylogenetic calculations for the identification of ortholog CYP genes between *C. elegans* and *H. contortus* which was published by Laing et al. (Laing et al., 2015) and of which basis the selection of candidate genes was chosen for the current studies are based on genome sequences of MHco3. This means that around 13% of CEGs are missing in the MHco3 genome. Even though multi-gene families (which CYPs typically are in animals and plants (Parra et al., 2009)have been significantly reduced for the set-up of the 248 CEGs (Parra et al., 2009), the lack of CEGs still is an indication of incompleteness of genomic information, possibly leading to a non-representation of CYPs in the annotated genome. Furthermore, an unexpected lack of orthology has been found. Only 66% of MHco3 genes were ortholog to McMaster genes and 45% of McMaster genes were ortholog to MHco3 genes (Wintersinger et al., 2018). These numbers are thought to be attributable to technical errors rather than biological differences between isolates. Despite these shortcomings, however, the MHco3 isolate still represents a better option for the depiction of small-

scale genomic features such as genes than the McMaster isolate. This might be explained by the inbred nature of the MHco3 isolate resulting in less heterozygosity and, therefore, facilitated assembly of sequences, in comparison to McMaster, which is a field isolate. A very recent article describes resequencing of the MHco3 isolate and the new assembly has an N50 value of 47.4 Mbp (Doyle et al., 2019), which is orders of magnitude higher than the N50 value of the assembly used by Laing et al (Laing et al., 2013) to predict the CYP repertoire of *H. contortus* (N50 = 0.083 Mbp). The complete genome is not available so far since its annotation is ongoing and the publication does not focus on the genome quality but on the use of the genome in backcrossing experiments to identify markers associated with IVM resistance. Therefore, no data on completeness of the genome and representation of the 248 CEGs is provided (Doyle et al., 2019). Nevertheless, it can be easily imagined that such a huge increase in contig length will correspond to a much better representation of all gene families and genes. However, the authors identified the same approximately 5 Mbp quantitative trait locus region in the genome of two independent IVM resistant isolates (HcCAVR and HcWR both also used in the present study regarding BZ resistance) (Doyle et al., 2019). The authors state that none of the leading candidate genes proposed in previous studies was shown to be under selection in these two *H. contortus* isolates but CYPs were not on their list of such candidate genes. Since this region is not annotated so far, the results do not exclude that selection occurs at a CYP or on a master regulator that drives expression of many genes such as shown for NHR-8 and transcriptional responses to IVM in *C. elegans* (Ménez et al., 2019).

Although the results gained so far do not indicate a substantial involvement of CYPs in TBZ resistance in *H. contortus*, a significance of CYPs for the metabolism of and consequently resistance to other BZ representatives cannot be ruled out. Equally, it remains to be elucidated whether CYPs of *H. contortus* might contribute to resistance against MLs or other anthelmintic drugs. The importance of an individual evaluation of a possible CYP involvement in the resistance to different drugs of the same drug class and of distinct drug classes, is stressed by an apparent lack of IVM metabolism in *H. contortus* whilst MOX metabolism could be determined (Alvinerie et al., 2001; Vokřál et al., 2013). Similarly, the study on the involvement of CYPs in the metabolism of MLs in *C. elegans* performed in the course of this doctorate (chapter 3.3) suggests that CYPs might have different affinities to MLs, since genetic ablation of CYP activity did not result in an increase of MOX susceptibility, however in a minimal but significant increase of IVM susceptibility. Differences in the interaction of IVM and MOX have been described in terms of their interaction with GluCl channels (Prichard et al., 2012) as well as Pgps (Prichard et al., 2012) and this might be possible in regard to CYPs as well, particularly when considering their much higher diversity in comparison to the Pgps. While BZ metabolism has clearly been shown to be mediated by CYPs in *C. elegans* (Jones et al., 2015), the experiments here do not suggest a

metabolism of IVM to the same or a similar extent. Taken together, it becomes clear that an intra-species and inter-species extrapolation of drug metabolism should not be applied blindly.

There is little dispute that gene duplication events are major evolutionary mechanisms that allow for the adaptation to a changing environment (Magadum et al., 2013; Sezutsu et al., 2013). For *C. elegans*, a very high spontaneous rate of gene duplications ($10^{-7}$ duplications/gene/generation) has been determined (Lipinski et al., 2011). This high rate of gene duplication along with high rates of point mutations and chromosomal rearrangements (Cutter et al., 2009) and finally its hermaphrodite mode of reproduction relying predominantly on selfing might have been important factors in the fixation and divergence of duplicated CYP genes, particularly with positive selection acting on them. The occurrence and preservation of gene duplications on a scale that has been observed for *C. elegans*, do not seem to hold true for *H. contortus*. Even though the parasite has apparently not adapted the proliferation of CYP gene families as a major xenobiotic defense mechanism, the future relevance of CYPs in anthelmintic drug resistance should not be entirely disregarded. Its obligate sexual reproduction and the female´s high egg production rate have led to very high effective population sizes even for local populations of *H. contortus* such as those on a single farm (Gilleard and Redman, 2016). The large effective population size is considered a major determinant of the observed high levels of genetic diversity within *H. contortus* populations (Gilleard and Redman, 2016) and high mutation rates might further contribute to that. An assumed mutation rate of 2.1 mutations/genome/generation (assumed from the mutation rate in *C. elegans*) and a progeny of billions of eggs every couple of days are expected to create an extremely large pool of mutations, which selection can act on. Conceivably, selection pressure on parasitic and free-living stages might be caused by the ongoing use of anthelmintics and by residual anthelmintics, respectively, possibly even by residual herbicides, insecticides and environmental pollutants. Studies on anopheline mosquitos have shown, for example, that pre-exposure to pollutants increases the resistance to insecticides and has been linked to elevated levels of CYP activity (David et al., 2013). Although there are no studies which have investigated the cross response between pollutants and anthelmintics in nematodes, it needs to be considered a possible scenario – for both the free-living *C. elegans*, for which the induction of the same CYP gene by different xenobiotics have been shown (Menzel et al., 2001; Menzel et al., 2005), as well as free-living stages of parasitic nematodes.

A mutation that has been selected in a population might spread widely since a high gene flow among *H. contortus* populations, due to host movement, is known to exist (Gilleard and Redman, 2016). Host movements in combination with insufficient quarantine for newly introduced animals in a flock, do allow that even rare mutations have a sufficient likelihood to spread. The unusually high frequency of the E198A SNP in the β-tubulin isotype 1 gene in *H. contortus* populations in China is an example of

that (Zhang et al., 2016). Recently it has even been shown that this resistant genotype has spilled over into *H. contortus* infecting wild blue sheep (*Pseudois nayaur*) (Shen et al., 2019)

# 5 Conclusions and outlook

The co-governance of anthelmintic resistance by different, unrelated mechanisms, although not yet systematically investigated, is considered to be very likely in nematodes. The fact that mutations in the ben-1 β-tubulin gene (Kwa et al., 1995) and expression level of *cyp-35d1* (Jones et al., 2015) can both modulate the susceptibility of *C. elegans* to BZs can be considered to be a paradigm that is of relevance for other drug classes as well. If multiple pathways can lead to phenotypic resistance against a particular drug or drug class, the significance of different mechanisms may vary not only from parasitic nematode species to species but also between different isolates of the same species. Moreover, different mechanisms can have different quantitative and, in the end, additive effects contributing to different levels of phenotypic resistance between isolates. Despite the fact that multi-drug resistance is a widespread phenomenon, resistance to different drugs or drug classes is expected to also involve different xenobiotic metabolic pathways and enzymes. The studies described in this thesis provided no clear evidence that CYPs are directly involved in anthelmintic resistance. The most relevant effects that were observed are a minimal increased efficacy of IVM but not MOX in *C. elegans* deleted for activity of the whole CYP family and increased expression of a CYP of the CYP34/35 in a multi-drug resistant *H. contortus* isolate. Absence of larger effects does not exclude that CYPs are contributing at least in some resistant parasitic nematode isolates to anthelmintic resistance.

Future research could involve overexpression of the CYP34/35 member HCOI100383400 in *C. elegans* to determine if the allele from the multi-drug resistant WR isolate is able to protect nematodes against various anthelmintics. Although a role of CYPs cannot completely ruled out at the moment, other pathways of xenobiotic metabolism should also be taken into consideration. These include among others phase I Flavin-containing monooxygenases and phase II UDP-glucuronosyltransferases and GST transferases. Since these enzymes also constitute multi gene families in *C. elegans* as well as in parasitic nematodes, transcriptomic analyses should be applied in order to identify candidates that exhibit inducible expression upon exposure of resistant parasitic nematodes to anthelmintics. This approach should be able to identify candidates to choose from for functional analyses.

# 6 Summary in English and German

Anthelmintic resistance is an accompaniment to modern livestock industry, which heavily relies on chemotherapy and chemoprophylaxis for maintaining animal health and productivity. While anthelmintic resistance is already established in horses and is a growing issue in cattle, it is, by far, the most severe and most prevalent in the sheep industry. To combat the spread of resistance, researchers are investigating alternative ways of nematode control and formulate recommendations to move towards more sustainable patterns of chemotherapeutic control. A further pillar in the management of nematodes is the understanding of the mechanisms underlying anthelmintic resistance. This knowledge is necessary to implement resistance diagnosis tests and might offer potential new drug targets. Macrocyclic lactones (MLs) and benzimidazoles (BZs) are the most frequently used anthelmintics in the sheep industry and resistance against them has been reported worldwide. Target-site related mechanisms usually are investigated first. For BZs, mutations in the ß-tubulin isotype 1 gene leading to amino acid changes at the drug target site have been found to confer resistance in trichostrongyloid nematodes, which are among the most pathogenic parasites of small ruminants. These mutations have been exploited to set up several tests capable of diagnosing BZ resistance even at very low frequencies of resistance alleles in nematode populations. So far, no target-site related mechanisms have been identified in case of MLs and diagnosis of ML resistance is limited to *in vivo* and *in vitro* tests. Drug efflux by P-glycoproteins is a non-target-site related mechanism that has been proposed to play a role in ML resistance particularly. A third possibility that has come to receive more attention in recent years is bioinactivation of MLs and BZs by cytochrome P450 monooxygenases (CYPs). In cancer drug resistance research as well as in the field of insecticide and acaricide resistance CYPs and their contribution to drug resistance are well established concepts.

Reasons to assume that CYPs might also be involved in anthelmintic resistance include, for instance, the metabolism of thiabendazole, a BZ representative, by CYP35D1 in the free-living nematode *Caenorhabditis elegans* and the detection of BZ metabolites, consistent with CYP activity in the parasitic nematode *Haemonchus contortus*. Despite these and many other indications, the role of CYPs in anthelmintic resistance is not fully clear.

The present cumulative doctoral thesis elaborates on the likelihood of an involvement of CYPs in ML metabolism in *C. elegans* and BZ metabolism in *H. contortus*. The first part was primarily processed by an *in vitro* approach whereby the larval development of mutant strains was compared to that of the wild-type N2 strain under different ML exposures. In particular, a strain with a temperature-sensitive mutation in the cytochrome reductase gene *emb-8* was compared at a non-permissive temperature to the wild-type regarding ivermectin (IVM) and moxidectin (MOX) susceptibility. The second part focused on expression patterns of CYPs in *in vitro* cultured fourth larvae of *H. contortus*. Here,

constitutive and TBZ-inducible expression was investigated in five different *H. contortus* isolates with varying levels of phenotypic BZ resistance.

Essentially, the ablation of CYP enzymatic activity by use of an inhibitor and a genetic approach was found to minimally alter the susceptibility to IVM in *C. elegans*, but not to MOX, both of which are MLs but belong to different groups. CYP14-A5, initially suspected to be involved in ML resistance in *C. elegans*, is most likely not involved, as its loss of activity was not shown to modulate IVM susceptibility and inducibility of gene expression by IVM or MOX was not observed. Therefore, CYPs can be expected to play no major role in ML resistance in *C. elegans*. As for BZ resistance in *H. contortus*, exposure to TBZ did not induce expression of selected CYPs in any of the isolates. However, the highly resistant WR isolate showed a 2.4- 3.7- fold higher constitutive expression of CYP HCOI100383400 in comparison to BZ susceptible isolates. In a previously published study, this CYP has been shown to have elevated transcript levels in the gut – the hypothesized major site of detoxification in nematodes. This is a first hint towards a possible involvement of CYPs in BZ resistance in a parasitic nematode. The WR isolate was also shown to be considerably more resistant than known resistant field isolates while having similar frequencies of the F200Y single nucleotide polymorphism (SNP) in the β-tubulin isotype 1 gene which along with E198A and F167Y is the primary cause for BZ resistance. The comparison of the frequency of F200Y and phenotypic resistance levels corroborates the long-held assumption of a multi-genic context for BZ resistance. Whether a co-governance of BZ resistance by several mechanisms in general and by the involvement of CYPs in particular is true, cannot be guaranteed with absolute certainty at the present state of research. However, the possibility of such should not be thoughtlessly abandoned, particularly in case of high resistance phenotypes as suggested by the present thesis. There is no question that further experimental investigations are necessary to improve our understanding of metabolism-based detoxification in nematodes.

Resistenzen gegenüber Anthelmintika sind eine Begleiterscheinung der modernen Viehindustrie, die für die Sicherstellung des Tierwohls und der Ertragsfähigkeit auf therapeutische und prophylaktische Entwurmung zurückgreift. Während Resistenzen gegenüber Anthelmintika in Pferden mittlerweile weit verbreitet sind und ein zunehmendes Problem in Rindern darstellen, sind sie in der Schaftindustrie am gravierendsten und weitverbreitetsten. Um einer Verbreitung der Resistenzen entgegenzuwirken, forschen Wissenschaftler an alternativen Methoden der Nematodenkontrolle und sprechen Empfehlungen für nachhaltige Entwurmungsstrategien aus. Weiterhin ist die Aufschlüsselung von Resistenzmechanismen eine wichtige Voraussetzung für die Kontrolle von Nematoden. Dieses Wissen ist notwendig, um Resistenztests zu entwickeln und potenzielle Angriffspunkte für Medikamente zu identifizieren. Makrozyklische Laktone (MLs) und Benzimidazole (BZs) gehören zu den am meisten

eingesetzten Anthelmintika in der Schafindustrie und Resistenzen gegenüber diesen sind weltweit verbreitet. In der Regel werden zuallererst Zielort-gebundene Resistenzmechanismen erforscht. Mutationen im β-Tubulin Isotyp 1 Gen, die zu Aminosäureaustauschen am Zielprotein von BZs führen, wurden als Resistenzverursacher in trichostrongyloiden Nematoden, die zu den pathogensten Parasiten von kleinen Wiederkäuern gehören, identifiziert. Diese Mutationen dienten als Grundlage für die Etablierung von mehreren Resistenztests, die in der Lage sind Resistenzen gegenüber BZs auch bei geringerer Häufigkeit der entsprechenden Resistenzallele in der Population nachzuweisen. Im Falle von MLs wurden bislang keine Zielort-gebundenen Resistenzmechanismen entdeckt und die Diagnosemöglichkeiten beschränken sich auf *in vitro* und *in vivo* Tests. Das Ausschleusen von Anthelmintika durch Efflux-Pumpen wie z.B. P-Glykoproteinen stellt einen nicht-Zielprotein assozierten Mechanismus dar und wird als ein möglicher Resistenzmediator, insbesondere der Resistenz gegenüber MLs, angesehen. Eine dritte Möglichkeit, die in jüngster Zeit stärker Beachtung fand, ist die Bioinaktivierung von MLs und BZs durch Cytochrom-P450-Monoxygenasen (CYPs). In der Forschung zu Resistenz bei Krebszellen sowie bei Insektizid- und Akarizidresistenzen sind CYPs als Resistenzmediatoren bereits bekannt.

Hinweise, die für eine Beteiligung von CYPs auch bei der Resistenzentwicklung gegenüber Anthelmintika sprechen, sind beispielsweise die Metabolisierung von TBZ, einem Vertreter der BZs, durch CYP35D1 im frei-lebenden Nematoden *Caenorhabditis elegans* und der Nachweis von BZ-Metaboliten im parasitischen *Haemonchus contortus*. Trotz dieser und weiterer Hinweise ist die Rolle von CYPs in der Resistenzentwicklung gegenüber Anthelmintika jedoch nicht völlig klar.

Die vorliegende kumulative Doktorarbeit betrachtet die Wahrscheinlichkeit einer Beteiligung von CYP in der Resistenz gegenüber ML in *C. elegans* und in der Resistenz gegenüber BZ in *H. contortus* näher. Die erste Fragestellung wurde mithilfe eines *in vitro*- Ansatzes bearbeitet, bei dem die larvale Entwicklung von *C. elegans* Mutantenstämmen mit der des Wildtyps (N2) unter verschiedenen ML Exposition verglichen wurde. Insbesondere wurde ein Stamm mit einer Temperatur-empfindlichen Mutation im Cytochrom Reduktase Gen emb-8 bei einer nicht-permissiven Temperatur mit dem Wildtyp hinsichtlich Suszeptibilität gegen Ivermectin (IVM) und Moxidectin (MOX) verglichen.

Im zweiten Teil der Doktorarbeit wurden anhand *in vitro* kultivierter vierter Larven von *H. contortus* Expressionsmuster von CYPs untersucht. Hierbei wurden die konstitutive Expression sowie die Induzierbarkeit der Expression durch TBZ in fünf verschieden *H. contortus* Isolaten untersucht, die eine unterschiedliche Ausprägung ihrer Resistenz gegenüber BZs haben.

Zusammenfassend lässt sich sagen, dass die Inhibierung enzymatischer Aktivität durch einen Inhibitor und einen genetischen Ansatz zu einer minimalen Modulation der Suzeptibilität gegenüber IVM in *C. elegans* führte, jedoch nicht gegenüber MOX. Sowohl IVM als auch MOX sind MLs, gehören jedoch zu unterschiedlichen Wirkstoffgruppen. CYP14A5, welches zunächst verdächtigt wurde an der

Resistenzentwicklung gegenüber ML in *C. elegans* beteiligt zu sein, ist höchstwahrscheinlich nicht involviert, da dessen Aktivitätsverlust zu keiner Modulation der Suszeptibilität gegenüber IVM geführt hat und keine Induzierbarkeit durch IVM oder MOX beobachtet werden konnte. Es kann davon ausgegangen werden, dass CYPs bei der Resistenz gegenüber MLs in *C. elegans* keine große Rolle spielen. Hinsichtlich der BZ- Resistenz in *H. contortus*, lässt sich festhalten, dass die Exposition mit TBZ zwar keine Induzierbarkeit der ausgewählten CYPs zur Folge hatte, jedoch CYP HCOI100383400 im hochresistenten WR Isolat eine im Vergleich zu BZ -suszeptiblen Isolaten 2.4-bis 3.7-fach höhere konstitutive Expression aufwies. In einer bereits veröffentlichten Studie konnte für dieses CYP ein höherer Transkriptlevel im Darm, dem hypothetischen Detoxifizierungsorgan in Nematoden, gezeigt werden. Dies ist ein erster Hinweis auf eine mögliche Beteiligung von CYPs in der Resistenzentwicklung gegenüber BZs in parasitischen Nematoden. Das WR Isolat zeigte zudem eine weitaus höhere phänotypische Resistenz als bislang untersuchte Feldisolate, obgleich es eine ähnliche Häufigkeit des F200Y Einzelnukleotidpolymorphismen (SNP im β-tubulin Isotyp 1 Gen aufweist. Dieser SNPs sowie die SNPs E198A und F167Y werden in erster Linie für die Resistenz gegenüber BZs verantwortlich gemacht. Der Vergleich der F200Y Häufigkeit mit phänotypischen Resistenzleveln bestärkt die allgemein akzeptierte Annahme, dass die Resistenz gegenüber BZs multi-genischer Natur ist. Ob sie durch eine Beteiligung mehrerer Mechanismen im Allgemeinen und einer Beteiligung von CYPs im Speziellen gekennzeichnet ist, kann beim gegenwärtigen Forschungsstand nicht mit absoluter Sicherheit gesagt werden. Basierend auf den Ergebnissen der Doktorarbeit, sollte die Möglichkeit eines solchen multigenischen Mechanismus jedoch nicht verworfen werden, insbesondere im Hinblick auf stark resistente Phänotypen. Die Notwendigkeit weiterer Experimente für ein besseres Verständnis Metabolismus-basierter Detoxifizierung in Nematoden steht außer Frage.

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# 8 List of Publications

**Yilmaz, E**., Gerst, B., McKay-Demeler, J., Krücken, J., 2019. Minimal modulation of macrocyclic lactone susceptibility in *Caenorhabditis elegans* following inhibition of cytochrome P450 monooxygenase activity. Experimental Parasitology, 200, 61-66.

**Yilmaz, E**., Wongkamchai, S., Ramünke, S., Koutsovoulos, GD., Blaxter, ML., Poppert, S., Schaper, R., von Samson-Himmelstjerna, G., Krücken, J., 2019. High genetic diversity in the *Dirofilaria repens* species complex revealed by mitochondrial genomes of feline microfilaria samples from Narathiwat, Thailand. Transboundary and Emerging Diseases, 66(1), 389-399.

**Yilmaz, E**., Ramünke, S., Demeler, J., Krücken., J., 2017. Comparison of constitutive and thiabendazole-induced expression of five cytochrome P450 genes in fourth-stage larvae of *Haemonchus contortus* isolates with different drug susceptibility identifies one gene with high constitutive expression in a multi-resistant isolate. International Journal for Parasitology: Drugs and Drug Resistance, 7(3), 362-369.

**Yilmaz, E**., Fritzenwanker, M., Pantchev, N., Lendner, M., Wongkamchai, S., Otranto, D., Kroidl, I., Dennebaum, M., Hoa Le, T., Anh Le, T., Ramünke, S., Schaper, R., von Samson-Himmelstjerna, G., Poppert, S., Krücken, J., 2016. The mitochondrial genomes of the zoonotic canine filarial parasites *Dirofilaria (Nochtiella) repens* and *Candidatus* Dirofilaria (Nochtiella) hongkongensis provide evidence for presence of cryptic species. PLoS Neglected Tropical Diseases 10(10):e0005028

**Yilmaz, E**., Kulke D., von Samson-Himmelstjerna, G., Krücken, J., 2015. Identification of novel splice variants of the voltage- and Ca(2+)-dependent K(+)- channel SLO-1 of *Trichuris muris*. Molecular und Biochemical Parasitology, 199, 5-8.

# 9 Declaration

I hereby declare that the submitted thesis is my own original work and that I have used no sources and aids other than indicated and have appropriately declared all citations.

This work has not been presented to any other examination board.

Berlin, April 2019

Esra Yilmaz