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der Freien Universität Berlin

Analyse putativer Inhibitoren von Anthelminthika-Resistenz-Mechanismen in gastrointestinalen Nematoden des Rindes

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Institute for Parasitology and Tropical Veterinary Medicine
Faculty of Veterinary Medicine
Freie Universität Berlin

Analysis of putative inhibitors of anthelmintic resistance mechanisms in cattle gastrointestinal nematodes

Thesis submitted
for the fulfilment of a doctoral degree in Veterinary Medicine at
the Freie Universität Berlin

Submitted by
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DEDICATED

To my parents, my husband Hussein and my beloved children (Maab and Moaed)

TABLE OF CONTENTS

LIST OF ABBREVIATIONS VI

LIST OF FIGURES VIII

LIST OF TABLES X

1 INTRODUCTION 1

2 LITERATURE REVIEW 3

2.1 Biology of nematodes 3

2.1.1 Structure and function of nematodes 3
2.1.2 Cuticle and epidermis 3
2.1.3 Digestive system 4
2.1.4 Nervous system 6
2.1.5 Reproductive system 7

2.2 Parasitic nematodes in cattle 8

2.2.1 Trichostrongylidae 8
2.2.1.1 *Cooperia oncophora* 9
2.2.1.2 *Ostertagia ostertagi* 9
2.2.2 Disease 10
2.2.2.1 Cooperiosis 10
2.2.2.2 Ostertagiosis 10
2.2.3 Epidemiology 11
2.2.4 Treatment and control 12

Table of Contents

2.3 Anthelmintics **13**

2.3.1 Benzimidazole 15

2.3.2 Imidazothiazole 17

2.3.3 Macrocyclic lactones 17

2.3.4 Cyclooctadepsipeptides 19

2.3.5 New compounds 20

2.4 Anthelmintic resistance **20**

2.4.1 Types of resistance 21

2.4.2 Selection for resistance 21

2.4.3 Specific resistance mechanisms 22

2.4.3.1 Benzimidazole 22

2.4.3.2 Levamisole 23

2.4.3.3 Macrocyclic lactones 24

2.4.4 Non-specific resistance mechanisms 25

2.4.4.1 P-glycoproteins 26

2.4.4.2 Cytochrome P450 28

2.5 Scientific research methods for the detection of resistance **28**

2.5.1 *In vivo* tests 29

2.5.1.1 The controlled test 29

2.5.1.2 The faecal egg count reduction test (FECRT) 30

2.5.2 *In vitro* tests 30

2.5.2.1 Egg Hatch Assay (EHA) 30

2.5.2.2 Larval Development Assay (LDA) 31

2.5.2.3 Larval Migration Inhibition Assay (LMIA) 32

2.5.2.4 The Micromotility Meter (MM) 33

3 MATERIALS AND METHODS **34**

3.1 Materials **34**

3.1.1 Chemicals 34

3.1.2 Buffer and Solutions 34

3.1.3 Equipment 35

3.1.4 Software 36

3.2 Methods **37**

3.2.1 Animals 37

3.2.2 Parasites populations used for experiments 37

3.2.2.1 *Cooperia oncophora* populations 37

3.2.2.2 *Ostertagia ostertagi* populations 38

3.2.3 Parasitological techniques 38

3.2.3.1 Animal infection 38

3.2.3.2 Faecal egg count 38

3.2.3.3 Egg recovery 39

3.2.3.4 Faecal and larval cultures 39

3.2.4 Egg Hatch Assay (EHA) 40

3.2.4.1 Parasites 40

3.2.4.2 Test substances 40

3.2.4.3 Assay performance 42

3.2.4.4 Toxicity testing of inhibitors 42

3.2.4.5 Data analysis 42

3.2.5 Larval Development Assay (LDA) 43

3.2.5.1 Parasites 43

3.2.5.2 Test substances 43

3.2.5.3 Assay performance 45

3.2.5.4 Toxicity testing of inhibitors 46

3.2.5.5 Data analysis 46

3.2.6 Larval Migration Inhibition Assay (LMIA) 46

3.2.6.1 Parasites 46

3.2.6.2 Test substances 46

3.2.6.3 Assay performance 48

3.2.6.4 Toxicity testing of inhibitors 49
3.2.6.5 Data analysis 50

4 RESULTS 51

4.1 Effect of Verapamil on egg hatching 51

4.2 Effect of Piperonyl butoxide on egg hatching 52

4.3 Effect of Verapamil on larval development 53
4.3.1 Thiabendazole and Verapamil (VPL) 54
4.3.2 Ivermectin and Verapamil (VPL) 55

4.4 Effect of Piperonyl butoxide on larval development 57
4.4.1 Thiabendazole and Piperonyl butoxide (PBO) 57
4.4.2 Ivermectin and Piperonyl butoxide (PBO) 58

4.5 Effect of Verapamil on larval migration 59
4.5.1 Ivermectin and Verapamil (VPL) 60
4.5.2 Levamisole and Verapamil (VPL) 61

4.6 Effect of Piperonyl butoxide on larval migration 63

5 DISCUSSION 64

5.1 Effect of Verapamil 67
5.1.1 Verapamil in combination with TBZ 67
5.1.2 Verapamil in combination with IVM 69
5.1.3 Verapamil in combination with LEV 71

5.2 Effect of Piperonyl butoxide 72
5.2.1 Piperonyl butoxide in combination with TBZ 72
5.2.2 Piperonyl butoxide in combination with IVM 73

6 CONCLUSIONS 75

7 SUMMARY 76

8 ZUSAMMENFASSUNG **78**

9 REFERENCES **80**

10 APPENDIX **128**

ACKNOWLEDGMENTS **135**

SELBSTÄNDIGKEITSERKLÄRUNG **136**

List of Abbreviations

AADs	Amino-acetonitrile derivatives
Amp B	Amphotericin B
AM	Anthelmintics
AR	Anthelmintic resistance
A. suum	*Ascaris suum*
BZ	Benzimidazole
C°	Degree Celsius
C. oncophora	*Cooperia oncophora*
C. elegans	*Caenorhabditis elegans*
CODPS	Cyclooctadepsipeptides
CI	Confidence interval 95%
DMSO	Dimethyl sulphoxide
EC_{50}	Effective concentration 50%
EHA	Egg Hatch Assay
E. coli	*Escherichia coli*
et al.	Et alia
e.g.	Example given
FECRT	Faecal egg count reduction test
Fig.	Figure
GABA	γ-aminobutyric acid
GIT	Gastro-intestinal tract
GluCl	Glutamate-gated chloride channel
h	hour
H. contortus	*Haemonchus contortus*
IVM	Ivermectin
L1	First larval stage
L2	Second larval stage
L3	Third larval stage
L4	Fourth larval stage
LEV	Levamisole

LDA	Larval Development Assay
LMIA	Larval Migration Inhibition Assay
M	Molar
ML	Macrocyclic lactone
O. circumcincta	*Ostertagia circumcincta*
O. ostertagi	*Ostertagia ostertagi*
PBO	Piperonyl butoxide
Pgp	P-glycoproteins
p.i.	Post infection
PC	Positive control
PCR	polymerase chain reaction
p-value	P-value
res.	resistant
RR	Resistance ratio
s.c.	subcutaneous
sus.	susceptible
spp.	several species
T. colubriformis	*Trichostrongylus colubriformis*
VPL	Verapamil
WAAVP	World Association for the Advancement of Veterinary Parasitology
xg	gravitational force (g-force)

List of Figures

Figure 1:	Schematic representation of the body of nematodes modified from Lee (1965)	4
Figure 2:	Types of nematodes oesophagus: a. rhabditiform, b. filariform, c. oxyurid, d. trichuroid; modified from Hiepe et al. (1985)	5
Figure 3:	Nervous system of *A. suum* modified from Bulock and Horridge (1965)	7
Figure 4:	Bursa copulatrix modified from Hiepe et al. (1985)	7
Figure 5:	Trichostrongylidae life cycle. Modified from the University of Pennsylvania, School of Veterinary Medicine, Parasitology Website (www.stanford.edu/.../Incubation.html) Depending on the species Trichostrongylidae settle in the stomach or small intestine (A/B). Eggs are passed out with the faeces (C). The parasite then hatches and develops via two larval stages (L1, L2) through to the infective third larval stage (L3)	8
Figure 6:	Calves suffering from ostertagiosis (A) with bottle neck (www.kstate.edu/.../625tutorials/Ostertagia.html); (B) with profuse diarrhoea (Institute of Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, Germany)	11
Figure 7:	The chemical structure of some Benzimidazoles	16
Figure 8:	Chemical structures of Levamisole and Tetramisole	17
Figure 9:	Chemical structure of Ivermectin	18
Figure 10:	Chemical structures of PF1022A and Emodepside	19
Figure 11:	Chemical structure of Amino-Acetonitrile- Derivatives (AADs)	20
Figure 12:	(A) Schematic presentation of P glycoprotein (University of Veterinary Medicine Hannover, Germany); (B) ATP-binding cassette, sub-family B (MDR/TAP), member 1. Crystallographic structure of the mouse MDR3 protein. The approximate positioning of the protein in the cell membrane is indicated by the blue (extracellular face) and red (cytoplasmic face) lines. The protein is depicted as a rainbow coloured cartoon (N-terminus = blue, C-terminus = red). A cyclic peptide inhibitor QZ59 is represented by a space-filling model (http://en.wikipedia.org/wiki/P-glycoprotein)	27

Figure 13:	Structure of a Cytochrome P450eryF (PDB: 1EGY) (http://de.wikipedia.org/wiki/Cytochrom_P450)	28
Figure 14:	Migration plate with sieves for the LMIA (Demeler, 2005)	49
Figure 15:	The dose-response-curves for TBZ for *C. oncophora* (A), *O. ostertagi* (B) and for TBZ+125 µM VPL for *C. oncophora* (C) and *O. ostertagi* (D)	52
Figure 16:	The dose-response-curves for TBZ, TBZ+PBO for *C. oncophora* (A) and *O. ostertagi* (B) isolates in the EHA	53
Figure 17:	The dose-response-curves for TBZ, TBZ+125 µM VPL for *C. oncophora* (A+C) and *O. ostertagi* (B+D) isolates in the LDA	55
Figure 18:	The dose-response-curves for IVM for *C. oncophora* (A) and *O. ostertagi* (B), IVM+125 µM VPL for *C. oncophora* (C) and *O. ostertagi* (D) isolates in the LDA	56
Figure 19:	The dose-response-curves for TBZ, TBZ+PBO for *C. oncophora* (A) and *O. ostertagi* (B) isolates in the LDA	58
Figure 20:	The dose-response-curves for IVM, IVM+PBO for *C. oncophora* (A) and for *O. ostertagi* (B) isolates in the LDA	59
Figure 21:	The dose-response-curves for IVM, IVM+25 µM VPL for *C. oncophora* (A & C) and *O. ostertagi* isolates (B & D) in the LMIA	61
Figure 22:	The dose response curves for LEV, LEV+25 µM VPL for *C. oncophora* (A) and *O. ostertagi* (B) isolates in the LMIA	62
Figure 23:	The dose-response-curves for IVM, IVM+85 µg/ml PBO for *C. oncophora* (A) and *O. ostertagi* isolates (B) in the LMIA	63

List of Tables

Table 1:	Main anthelmintics, spectrum of activity and their modes of action	15
Table 2:	Thiabendazole solutions (and preparations) for the EHA	40
Table 3:	Preparation of VPL for the toxicity test in the EHA	41
Table 4:	Preparation of PBO for the toxicity test in the EHA	41
Table 5:	Preparation of TBZ for the LDA	44
Table 6:	IVM dilution series for the LDA	44
Table 7:	Final concentrations of IVM in the wells in the LDA	44
Table 8:	Preparation of PBO for the toxicity test in the LDA	45
Table 9:	Concentrations of IVM for the LMIA	47
Table 10:	Concentrations of LEV for the LMIA	47
Table 11:	Final concentrations of LEV achieved in the wells	47
Table 12:	Preparation of VPL for the toxicity test in the LMIA	48
Table 13:	Preparation of PBO for the toxicity test in the LMIA	48
Table 14:	EC_{50} values, 95% CI and R^2 of TBZ (in µg/ml) alone and in combination with VPL (125 µM) for *C. oncophora* and *O. ostertagi* isolates in the EHA. For statistical comparison of the two curves obtained with and without VPL for each isolate, a p-value was calculated	51
Table 15:	EC_{50} values and 95% CI for TBZ (in µg/ml) alone and in combination with PBO (8.5 µg/ml and 0.266 µg/ml) for *C. oncophora* and *O. ostertagi* isolates in the EHA. P-values for each isolate were calculated for statistical differences between TBZ and TBZ+PBO	53
Table 16:	EC_{50} values, 95% CI and p-values for TBZ (in µg/ml) alone and in combination with VPL (125 µM) for *C. oncophora* and *O. ostertagi* in the LDA	54
Table 17:	EC_{50} values, 95% CI, R^2 and p-values for IVM (in nM) and IVM + 125 µM VPL for the *C. oncophora* and *O. ostertagi* isolates in the LDA	56
Table 18:	EC_{50} values, 95% CI and p-values for TBZ (in µg/ml), TBZ+ (85 & 5.33 µg/ml PBO) for each isolate of *C. oncophora* and *O. ostertagi* isolates in the LDA	58

Table 19:	EC_{50} values for IVM (in nM) alone and combined with 85 & 5.33 µg/ml PBO, 95% CI and p-values obtained in the LDA	59
Table 20:	EC_{50} values for IVM (in nM) and IVM with 25 µM VPL including 95% CI, R^2 and p values for both isolates of *C. oncophora* and *O. ostertagi* in the LMIA	60
Table 21:	EC_{50} values for LEV (in nM) and LEV+25 µM VPL, 95% CI, R^2 and calculated p values for the two curves of all isolates of *C. oncophora* and *O. ostertagi* in the LMIA	62
Table 22:	EC_{50} values for IVM (in nM) and IVM+85 µg/ml PBO including 95% CI and p-values for the two curves for all isolates of *C. oncophora* and *O. ostertagi* in the LMIA	63

1 Introduction

Gastrointestinal nematodes of livestock cause serious economic losses, mainly in countries which depend on intensive grazing management. In temperate regions the most common nematodes of cattle are *Ostertagia ostertagi* (brown stomach worm) and *Cooperia oncophora* (cattle bankrupt worm) which live in the abomasum and small intestine, respectively. Signs of infection include profuse watery diarrhoea, progressive emaciation, reduced feed consumption, weight loss, anaemia and hypoproteinaemia. Management of gastrointestinal nematodes in grazing livestock continues to rely heavily on the use of chemotherapy to control parasites. The continued use of anthelmintics led to an increased development of anthelmintic resistance (AR), which is meanwhile a world wide problem in a substantial number of economically important parasites of livestock. Reports of resistance to the most commonly used classes of broad-spectrum anthelmintics, the benzimidazoles (BZs), levamisole (LEV) and the macrocyclic lactones (MLs) become more common (Besier et al., 1993; Leathwick, 1995; Waller, 1997; Leathwick et al., 2000; Echevarria et al., 2001; Mason et al., 2001; Vickers et al., 2001; Kaplan, 2004; Stenhouse, 2007; Demeler et al., 2009).
To be able to detect AR in nematodes of farm animals several techniques which differ in their sensitivity are available. A number of *in vitro* techniques such as the Egg Hatch Assay (EHA), Larval Development Assay (LDA) and Larval Migration Inhibition Assay (LMIA) had been developed in free-living nematodes or non-parasitic stages and were reviewed by Johansen (1989). While the EHA can only be used for the detection of BZ resistance, the LDA is suitable for the detection of resistance against most anthelmintics, (BZ, LEV and MLs) and the LMIA is so far only suitable for MLs and, to a lesser extend, also for LEV.
In order to reduce the development of AR, we need a better understanding of the mechanism and the mode of action of anthelmintic drugs to improve the way in which a drug class is used. Accordingly, research was recently increased in the field of resistance mechanisms. In addition to specific mechanisms (e.g. specific receptor binding sites), non-specific mechanisms involving detoxification systems such as P-glycoporteins (Pgp; transmembrane transporter) and Cytochrome P450 (CYP) oxidative enzymes have been shown to be involved in anthelmintic resistance (Beugnet et al., 1997; Kerboeuf et al., 1999; Molento et al., 2003; Kotze et al., 2006; Dicker, 2010). The inhibition of these pathways by the use of chemicals *in vitro* was reported to reverse resistance in free-living stages (eggs and larvae) of parasitic nematodes (Kotze et al., 1997; 1999; 2000; Kerboeuf et al., 2002; Stenhouse, 2007).

Therefore, verapamil (VPL) was chosen as an inhibitor for Pgps and piperonyl butoxide (PBO) was chosen as an inhibitor for CYPs. The objective of this study was to investigate the effects of a combined usage of anthelmintics and the inhibitors VPL and PBO on nematode eggs or larvae *in vitro*. In total, 5 different isolates of *C. oncophora* and *O. ostertagi* were chosen to enable the comparison of measured effects between susceptible and drug resistant isolates of the same species as well as between species itself.

Three *in vitro* techniques, the EHA, LDA and LMIA were used for testing anthelmintic efficacy and providing a better understanding of mechanisms of drug resistance to improve drug efficacy. To include the three major drugs classes BZs, MLs and imidazothiazoles, representatives of each class were chosen: thiabendazole (TBZ) for BZs, ivermectin (IVM) for MLs and LEV for imidazothiazoles.

2 Literature review

2.1 Biology of nematodes

2.1.1 Structure and function of nematodes

Nematodes are elongate, bilaterally symmetrical in shape and the body surface is a transparent cuticle. They have a large body cavity (pseudocoelom) containing fluid which surrounds the digestive tract and parts of the reproductive system. Nematodes have no circular muscle layers; therefore all somatic musculature is oriented longitudinally and divided into dorsal and ventral fields. They have a tubular digestive tract from the mouth through the oesophagus and intestine to the anus. The nervous system consists basically of a ring of nerve tissue around the oesophagus and another nerve ring around the posterior region of the intestine. A schematic drawing of a nematodes body is shown in Fig. 1.

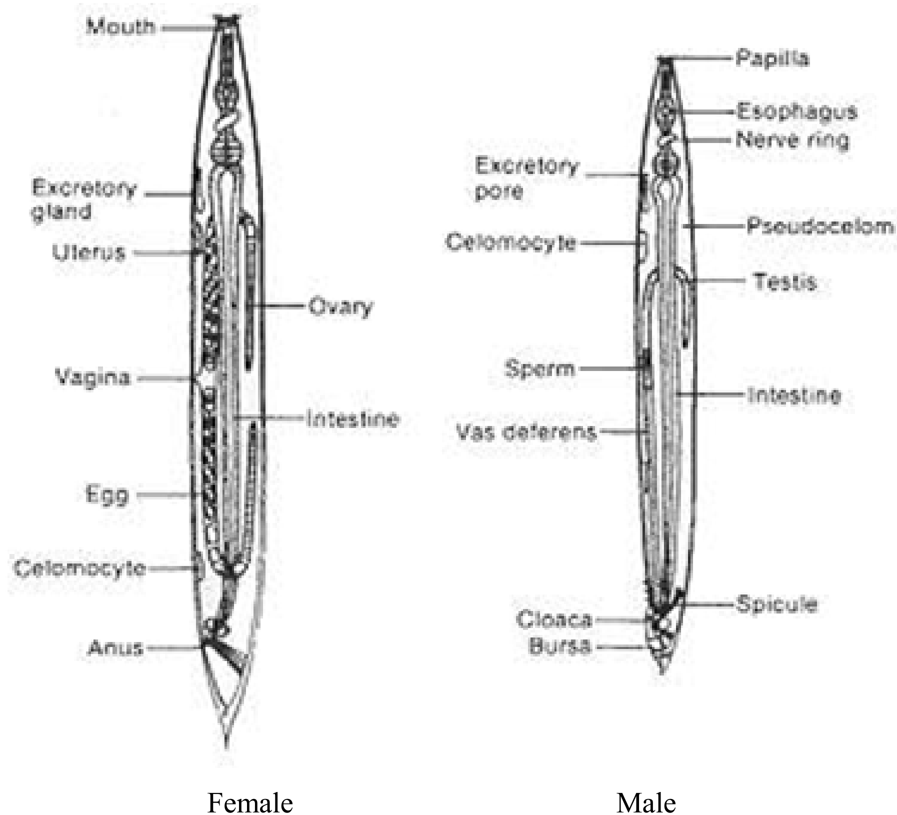
The parasitic nematodes feed on intestinal debris, mucus, bacteria and cells of the intestinal mucosa and blood. The sexes are separate, with male nematodes usually smaller than females. This chapter is mainly based on information provided by Chen et al. (1966) and Soulsby et al. (1982).

2.1.2 Cuticle and epidermis

The body surface of parasitic nematodes is composed of a smooth, thick, elastic cuticle which covers the external surface and lines the buccal cavity, oesophagus, vagina, excretory pore, cloaca and anus. It is smooth but usually covered with spines or has longitudinal and transvers ridges. It serves as a tough, flexible and protective covering, resistant to digestive enzymes and gives rigidity to the worm and its elasticity allows abundant movement.

The cuticle consists of three layers (cortical, middle and basal layer from outside to inside), which are subdivided. The subdivisions vary in numbers and structure among the species of nematodes. Beneath the basal layer of the cuticle lies the epidermis, which is thickened into

four longitudinal lines, two laterals, one dorsal and one ventral and between these the muscles of the body are disposed. The epidermis aids in regulating body wall permeability.
The cuticle and epidermis of the nematodes give structural support, protection from the environment and contribute to nutrition and excretion.



Female Male

Figure 1: Schematic representation of the body of nematodes; modified from Lee (1965)

2.1.3 Digestive system

The digestive tract of nematodes is a simple canal extending from the mouth to the anus, which is divided into the mouth cavity, oesophagus, intestine, rectum and cloaca. The mouth is surrounded by three lips. Lateral to the mouth opening pairs of small chemoreceptor organs (amphids) are located.
The oesophagus is a long cylindrical tube and it has a well developed structure surrounded by muscles. It varies characteristically in shape and structure in different species and developmental stages. It can be divided into three regions: the anterior corpus is located at the

top end of the body; the median isthmus is located along the median bulb and the posterior bulb which is situated at the posterior end of oesophagus. The oesophagus is variable in its form which is a useful tool for the identification of different species. It may be rhabditiform, with slight anterior and posterior swellings which is present in first stage larvae of many nematodes and adult stages of Trichostrongylidae. The oxyurid-shape, with a large posterior swelling is present in the ascarid nematodes. The trichuroid oesophagus has a capillary form, passing through a single column of cells (stichosome). Filariform is club-shaped without a posterior bulb and found in the second and third stage larvae of nematodes and in individuals of parasitic generations of the Rhabditida. The different types of the oesophagus are presented in Fig. 2. The oesophagus is responsible for sucking food into the oral cavity and pumping it into the intestine where further transport to the cloaca takes place. This process is regulated through pharyngeal pumping events.

The wall of the oesophagus contains three oesophageal glands, one is located dorsal and opens into the mouth and two subventral pharyngeal glands are involved in digestion through secretion of lytic substances. The oesophagus is joined to the intestine by the oesophago-intestinal valve. Following the oesophagus, the intestine is a simple cylindrical tube with a non-muscular wall composed of a single layer of columnar cells standing on a basal membrane. It leads into the rectum, which is a short, flattened tube lined with cuticle and connecting the intestine and the anus. In females the intestine terminates in an anus while in males the intestine joins with the genital duct to form a cloaca which opens through the anus, and into which opens the vas deferens and through which the copulatory spicules may be extruded.

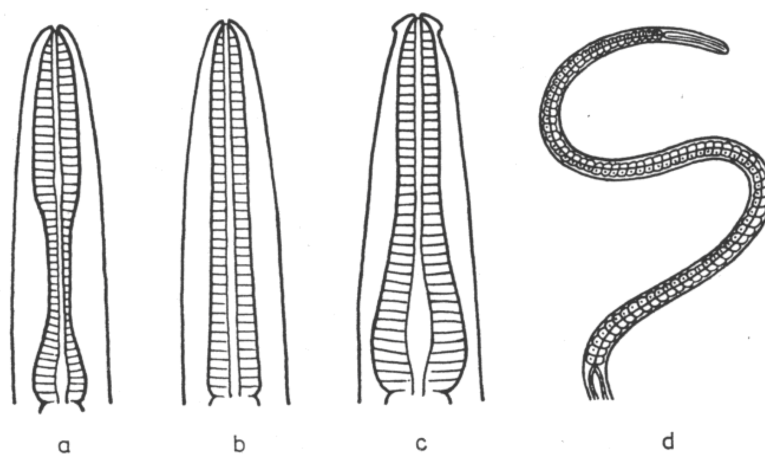


Figure 2: Types of nematodes oesophagus: a. rhabditiform, b. filariform, c. oxyurid, d. trichuroid; modified from Hiepe et al. (1985)

2.1.4 Nervous system

The nervous system of nematodes is composed of the central and the peripheral nervous system, and various sense organs. The central nervous system includes the nerve ring, encircling the anterior oesophagus. It is composed of nerve fibres and few ganglia that surround the oesophagus. Connected to this ring are a number of ganglia which are also connected to each other by commissures. Six nerves extend anteriorly from the ring; two ventrolateral, two lateral and two dorsolateral. These six fibres and their branches innervate the various structures and tissues in the anterior portion of the body. A mid-dorsal nerve, a mid-ventral nerve, and one of three pairs of lateral nerves are directed posteriorly. These nerves and their branches innervate the various structures and tissues posterior to the nerve ring. Posterior to this nerve ring is the ventral nerve bundle, which extends to the anus. The smaller dorsal nerve bundle is comprised of motor axons. As an example the diagrammatic structure of nervous system of *Ascaris suum* is shown in Fig. 3.

The ventral nerve cord is composed of two subventral branches which fusion into a single ventral branch. There are four submedial nerves and one or more pairs of lateral nerve bundles. The peripheral nerve system is located mainly in the anterior region of the worm. There are netlike structures consisting of various peripheral nerves which are associated by commissures.

The sense organs of nematodes are quite diverse and allow the perception of mechanical, thermal, photo-sensitive and chemical signals. They are present particularly in the head region and in the genital area. The former include the amphids which are located near the mouth. They represent the largest sensory cells of the nematodes enabling direct contact with the environment and receiving thermal and chemical stimuli (Ashton & Schad, 1996). The phasmids are paired and found in the ventral posterior end of the genital region.

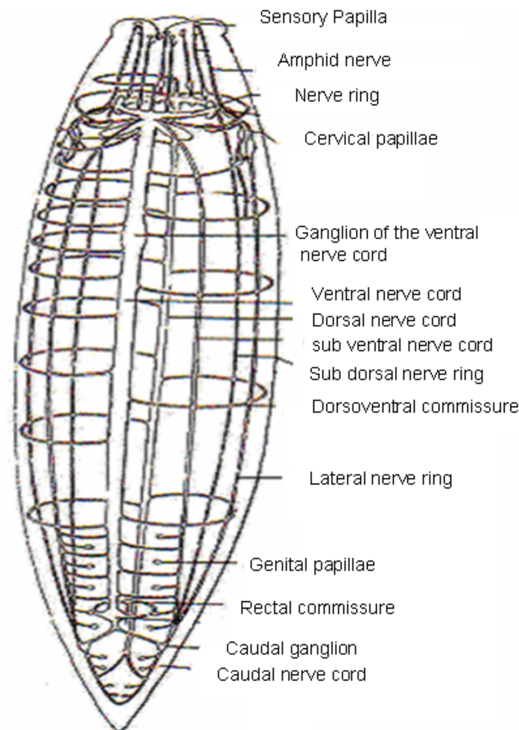


Figure 3: Nervous system of *A. suum*; modified from Bulock and Horridge (1965)

2.1.5 Reproductive system

The male reproductive system has single testis and a slender vas deferens which is enlarged terminally to form the seminal vesicle. This vesicle leads into the cloaca. In strongylids there is dorsal to the cloaca a large copulatory structure, the “bursa copulatrix” (Fig. 4), which contains one or two spicules. It varies among species and can be used for differentiation.
The female reproductive system possesses two ovaries leading into the uterus, which terminates in the vulva. The vagina is a highly muscular organ (“ovijector”) and ejects the eggs by contraction.

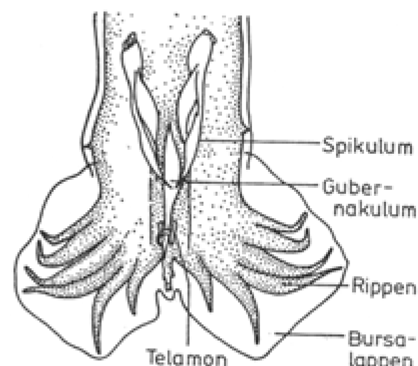


Figure 4: Bursa copulatrix; modified from Hiepe et al. (1985)

2.2 Parasitic nematodes in cattle

Gastrointestinal nematode parasites appear to be widespread in most cattle populations. In moderate climate regions (20-23 °C) the most common gastrointestinal nematodes are *C. oncophora* and *O. ostertagi* which cause economically important infectious diseases in large ruminants.

2.2.1 Trichostrongylidae

C. oncophora and *O. ostertagi* occur in the gastrointestinal tract of cattle. They both have a direct life cycle. Eggs are laid by the adult female worms and passed out with the host faeces into the environment. Under appropriate environmental conditions they develop via two moults through to an infective third larval stage (L3). The host becomes infected after ingesting the L3 while grazing on pasture. Further development takes place in the crypts of the gastric glands or in the mucosa of the intestine. After further moults, the fourth (L4) larval stage and finally the adult stage is reached. Completion of this life cycle in the host takes approximately 3 weeks. The eggs are again passed out with the faeces of the host and the cycle commences (Fig. 5).

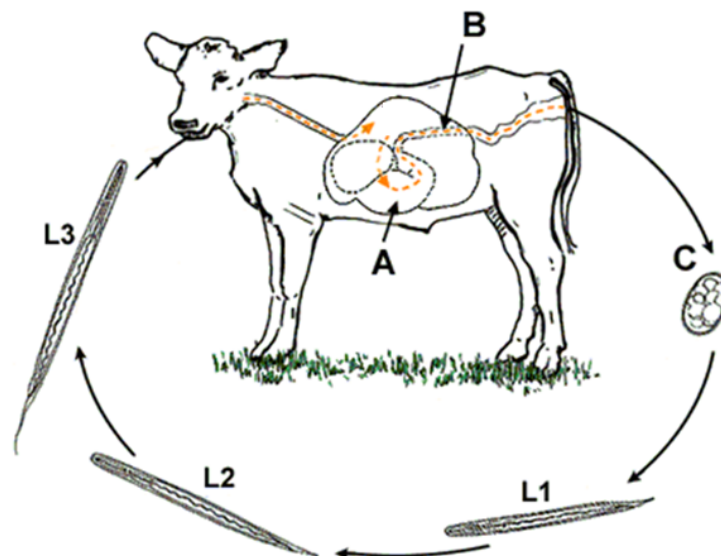


Figure 5: Trichostrongylidae life cycle. Modified from the University of Pennsylvania, School of Veterinary Medicine, Parasitology Website (www.stanford.edu/.../Incubation.html)
Depending on the species Trichostrongylidae settle in the stomach or small intestine (A/B). Eggs are passed out with the faeces (C). The parasite then hatches and develops via two larval stages (L1, L2) through to the infective third larval stage (L3)

2.2.1.1 *Cooperia oncophora*

Cooperia species are nematodes of the small intestine of ruminants and rarely the horse. The males are 5.5-9 mm, the females are 6-8 mm long.

They have a rounded relatively thick head with a small mouth cavity. *C. oncophora* is easily recognised in microscopic examination by its coiled appearance that has been described as like a "watch spring". The cephalic vesicle is found in all *Cooperia* species, giving the head end a slight bulbous look. In addition the anterior cuticle has transverse striations in the oesophageal region. The mouth cavity is small and cervical papillae are present.

The bursa of the male possesses two lateral lobes and one dorsal lobe, and the ray is characteristically divided into two branches which may be parallel or curved so as forming a lyre or horseshoe-shaped structure. The spicules and gubernaculums of the males are usually short.

The vulva of the females is covered by a flap and situated in the last third of the body.

The manifestation of clinical signs depends on the severity of infection and the susceptibility of the infected animal. Clinical signs include diarrhoea, dull hair coat and poor weight gain.

2.2.1.2 *Ostertagia ostertagi*

O. ostertagi occurs in the abomasums of cattle. They are medium in size measuring 6 to 10 mm in length and are usually reddish brown worms. Therefore, they are usually referred to as “medium brown stomach worms”. They are the major cause of parasitic gastritis (Ostertagiosis) of ruminants in temperate climate regions. The buccal cavity is short and broad, the cuticle at the anterior end may be slightly inflated and the rest of the cuticle bears longitudinal ridges but they have no transverse striations.

The males are 6.5 to 7.5 mm long. They have short, thin spicules divided into two or more processes. The bursa consists of two lateral lobes and one smaller dorsal lobe. The females are 8.3 to 9.2 mm long, the tail terminates in a rather blunt rounded point, slightly swollen and is located a short distance away from the tip, where the vulva is covered with a cuticular flap.

2.2.2 Disease

2.2.2.1 Cooperiosis

Cooperia is generally considered to be of mild pathogenicity in calves, but often secondary effects as such resulting from infections with *Ostertagia* and *Haemonchus* contribute to the primary parasitic pathogen in parasitic gastroenteritis. The worms penetrate into the mucosa of the small intestine and cause changes in the mucosal structure. A light infection is not problematic, but young cattle may be severely affected by heavy infections, which are usually acquired on moist pastures. Clinical signs are relatively unspecific and include loss of appetite, poor weight gains, diarrhoea, and weight loss.

2.2.2.2 Ostertagiosis

O. ostertagi is the most common cause of parasitic gastritis in cattle. The disease ostertagiosis is characterised by weight loss and diarrhoea. Typically young cattle are affected during their first grazing season, although herd outbreaks and sporadic individual cases have also been reported in adult cattle (Eckert & Bürger, 1979).
Bovine ostertagiosis is known to occur in two clinical forms. In temperate climate regions with cold winters the seasonal occurrence of these is as follows:

The Type I
Disease is seen in calves during the first grazing season as a result of large numbers of larvae ingested 3-4 weeks previously and is due to the development of these ingested larvae through to adult parasites. This type of ostertagiosis normally occurs from mid-July onwards.

The Type II
Disease occurs in yearlings, usually in late winter or spring following their first grazing season and results from the maturation of larvae which were ingested during the previous autumn and subsequently inhibited their development.
Examples of clinical signs are shown in photos in Fig. 6.

The main clinical sign in both, Type I and Type II disease, is profuse diarrhoea and in Type I, where calves are on pasture, this is usually persistent, watery and has a characteristic bright green colour. In the majority of animals with Type II the diarrhoea is often intermittent and anorexia and thirst are usually present. The coat of affected animals is often dull and the hind quarters heavily soiled with faeces (Schillinger & Barth, 1993).
In both forms of the disease, the loss of body weight is considerable during the clinical phase and may reach 20% (Coop & Kyriazakis, 1999). Carcass quality may also be affected since there is a reduction in total body solids relative to total body water. In Type I disease, the morbidity is usually high, often exceeding 75%, but mortality is rare provided treatment is administered within a reasonable time. In Type II disease only a proportion of animals in the group are affected but mortality in such animals can be high unless early treatment with an anthelmintic effective against both inhibited and developing larval stages is given.

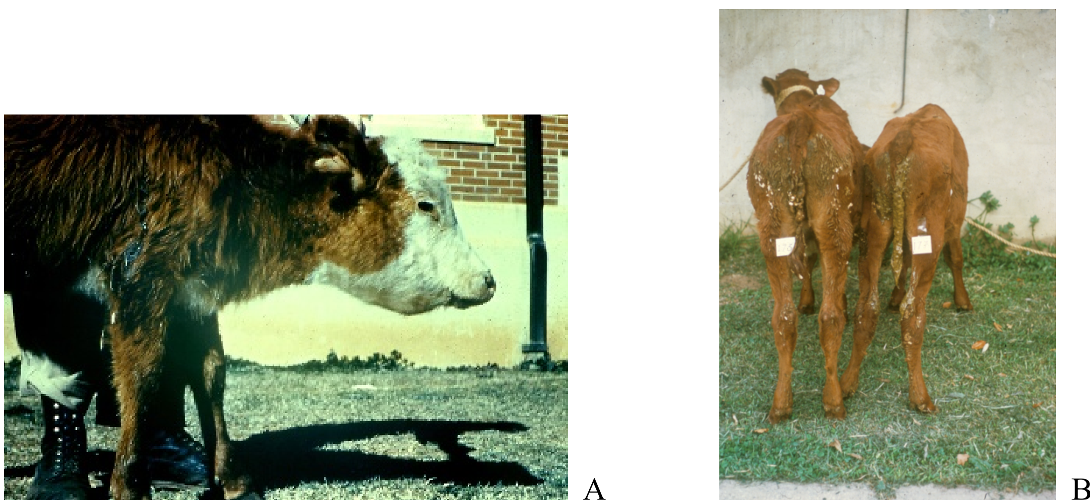


Figure 6: Calves suffering from ostertagiosis
(A) with bottle neck (www.kstate.edu/.../625tutorials/Ostertagia.html); (B) with profuse diarrhoea (Institute of Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, Germany)

2.2.3 Epidemiology

The transmission of gastrointestinal parasites depends mainly on humidity, temperature, host susceptibility, management and the overwintering proportion of the population.
In Germany, first season grazing calves are typically infected with infective larvae during the grazing season, especially in summer and early autumn. The larvae are ingested with grass by the host. It takes approximately three weeks for larvae to develop into adults and start laying

eggs. The rate of hatching of these eggs depends on climatic conditions, usually reaching a peak in midsummer. Other factors such as wet summers will produce another early peak. In dry summers, larvae are released from the faecal pats. A higher proportion of larvae die due to dessiccation and development is slowed down.
In infected calves the larval stages of *O. ostertagi* have the possibility of inhibition to overwinter in the animal and be re-activated in the proceeding year. Calves excreting larvae lead to early contamination of "clean" pasture, resulting in more heavily contaminated pastures. Additionally, L3s can overwinter in the soil. The proportion of surviving larvae is dependent on the average temperatures and freezing of the ground. This part of the larval population is also re-activated in spring and contributes to the early contamination of pastures.

2.2.4 Treatment and control

Anthelmintic treatment should be integrated in pasture management in order to provide the best efficacy of parasite control. Part of the nematodes life cycle is on pasture and pasture management is indicated to reduce the number of infective larvae.
The aim of most anthelmintic treatments is the use of a drug with broad spectrum activities against most species of nematodes and also to kill inhibited larval stages.
Strategic treatment of anthelmintics is designed to reduce worm burdens and thereby, the contamination of pastures. Timing of administration is based on knowledge of the seasonal changes in infection and the regional epidemiology of the various helminths. Prompt recognition of circumstances likely to favour development of parasitic disease, e.g. weather, grazing behaviour and loss of weight and condition, is essential.
In the northern USA, Canada, or Western Europe, usually pasture levels of *Ostertagia* and other parasites increase substantially after mid July. The general pattern of infectivity is minimal in spring but increases rapidly to peak levels in late summer and early autumn rainfall. Current practices in most areas include the administration of 2 or more anthelmintic treatments (usually at intervals of 3-5 weeks) given when cattle first go onto pasture in spring.
Routine deworming of dairy cattle at calving has been shown to have a significant response to treatment.
If prophylactic medication is applied, use of anthelmintics should be restricted to the young animals, requiring some protection because of their low ability to resist the acquisition and the effects of parasites. Single treatments with subsequent transfer of animals to safe pasture also

have been effective, but increase the risk of resistance development in the treated parasite population. Decreased numbers of treatment associated with delayed spring turn-out have been reported to effectively control parasitic burdens in first season grazing calves. Late or delayed turn-out should be combined with at least one cut of grass before animals are allowed on pasture. With this procedure, the proportion of larvae which survived over winter is removed from the paddock. The cut grass should not be given to stabled animals but can be used for silaging, because the larvae are killed in the silaging process. Generally, grazing management is directed to the limitation of the infective larvae on pasture. Heavily contaminated pasture can for example be cleared by a) keeping them free of animals for an extended period of time (at least one year), b) rotational grazing with different species, c) agricultural use.

In other countries of either cool or warm temperate climate, similar controls may be used if the seasonal patterns of the disease are known, but in most regions the prophylactic use of anthelmintics is common, e.g. in warm and moist conditions.

2.3 Anthelmintics

Anthelmintic drugs are chemicals commonly used to treat and control parasitic nematodes of grazing animals. They have been grouped according to their chemical structures and modes of action (Table 1).

Piperazine was first used as an anthelmintic in 1953 (White, 1953) and is effective against adult stages of most nematodes. It acts on GABA receptors, causing blocking of neuromuscular transmission in the parasite through hyperpolarisation of the nerve membrane, leading to flaccid paralysis. It also blocks succinate production by the worm. The parasites, paralysed and depleted of energy, are expelled with the ingesta by peristalsis of the host.

The anthelmintic thiabendazole (TBZ) belongs to the group of BZs and was discovered in 1961 (Brown et al., 1961). Further BZs were introduced as broad spectrum anthelmintics. They were much more efficient, stable and had high anthelmintic activity against a wide range of gastro-intestinal nematodes. They can be administered orally or mixed with food. In addition to their wide therapeutic use no toxic effects have been recorded.

Imidazothiazoles and tetrahydropyrimidines (LEV, pyrantel and morantel) were discovered between 1962 and 1966 and introduced into the market in 1966 and 1969 (Mehlhorn, 2008). They are active against gastrointestinal nematodes and lung worms and often used

anthelmintics. They act as nicotinic agonists at acetylcholine receptors of the nematode muscle membranes (Martin et al., 1996), causing muscle contraction and spastic paralysis.
A more recent class of broad spectrum anthelmintic drugs are the MLs which are comprised of the avermectins and the milbemycins. They were discovered in 1979 and ivermectin (IVM, an avermectin) was introduced as an anthelmintic in 1981 by Merck (Campbell, 1989). MLs are highly potent, broad-spectrum nematocides, insecticides and acaricides and therefore known as endectocides. They presumably act by binding to GABA or glutamate-gated chloride channel receptors (GluCl) in nematode and arthropod nerve cells (Campbell, 1989). This causes the channel to open, allowing an influx of chloride ions, which causes hyperpolarisation of the membrane, leading to the paralytic effects on different neuromuscular systems of the worms.
The two most recently discovered anthelmintic drug classes, the cyclooctadepsipeptides and the amino acetonitrile derivates (AADs) where discovered in 1993 (Harder & von Samson-Himmelstjerna, 2003) and 2008 (Kaminsky et al., 2008), respectively. The cyclooctadepsipeptide emodepside was first launched as an anthelmintic in combination with parziquantel in 2005, while the first AAD was marketed first in 2009.

Table 1: Main anthelmintics, spectrum of activity and their modes of action

Class	Spectrum of activity	Mode of action
Benzimidazoles: Albendazole Fenbendazole Flubendazole Mebendazole Oxfendazole **Probendazoles:** Febantel	GI nematodes, lungworms and cestodes	Inhibit tubulin polymerisation to microtubules, causing energy depletion
Imidazothiazole: Levamisole	GI nematodes and lungworms	Act as agonist on acetylcholinesterase receptor, cause spastic paralysis
MLs: Avermectins: Doramectin, Eprinomectin, Ivermectin, Selamectin Milbemycins: Milbemycin-Oxim Moxidectin	GI nematodes and lungworms	Selective binding to glutamate-gated chloride channels in nerve and muscle cells, cause spastic paralysis and death
Piperazine:	GI nematodes	GABA-ergic action at neuromuscular junction of nematodes
Pyrimidine: Pyrantel, Morantel	GI nematodes	Act as agonists on acetylcholinesterase receptors, cause spastic paralysis
Salicylanilides	GI nematodes	Interfering with ATP
AADs: Monepantel	GI nematodes	Interfere with acetylcholine receptor of nematode, hypercontraction, leads to paralysis
CODPs Emodepside	GI nematodes	Interfere with potassium gated calcium channel receptors, lead to paralysis

2.3.1 Benzimidazole

TBZ was the first broad-spectrum anthelmintic used to treat nematode and trematode infections in domestic animals. The BZs are characterised by a broad spectrum of activity against roundworms (nematodes), an ovicidal effect and a wide safety activity. Some BZs also

have activity against cestodes and trematodes. Those of interest are: mebendazole, flubendazole, fenbendazole, oxfendazole, oxibendazole, albendazole, albendazole sulfoxide, thiophanate, febantel, netobimin, and triclabendazole. The latter is active against liver flukes; however, unlike all the other BZs, triclabendazole has no activity against roundworms. Examples of structures of BZs are shown in Fig. 7.

The target site of BZs is the ß-tubulin which is essential to form microtubules, responsible for vital cell functions as motility, cellular shape, mitosis, coordination, absorption, transport and secretion in nematodes (Dustin, 1984). BZs cause inhibition of polymerisation of the microtubules while depolymerisation goes on naturally and thus interferes with cellular transport and energy metabolism (Lacey, 1990; Lacey & Gill, 1994). Inhibition of these processes appears to play an essential role in the lethal effect on worms. BZs progressively deplete energy reserves and inhibit excretion of waste products and protective factors from parasite cells. Therefore, prolongation of contact time between drug and parasite is an important factor regarding their efficacy.

Metabolism of the BZs is variable and may alter their activity, e.g. albendazole is rapidly and reversibly oxidised to its sulfoxide. The sulfoxide may be irreversibly oxidised to sulfone, which is significantly less active than the sulfoxide. Similarly, fenbendazole and oxfendazole (fenbendazole sulfoxide) are interchangeable, but the oxidation product fenbendazole sulfone is less active and is not reduced back to the sulfoxide or thio metabolites. In ruminants, the BZs are most effective if deposited directly into the rumen. Administration directly into the abomasum, via the oesophageal groove may shorten the duration for drug absorption and increase the rate of excretion in the faeces, which may reduce efficacy. Drug residues persist for many days.

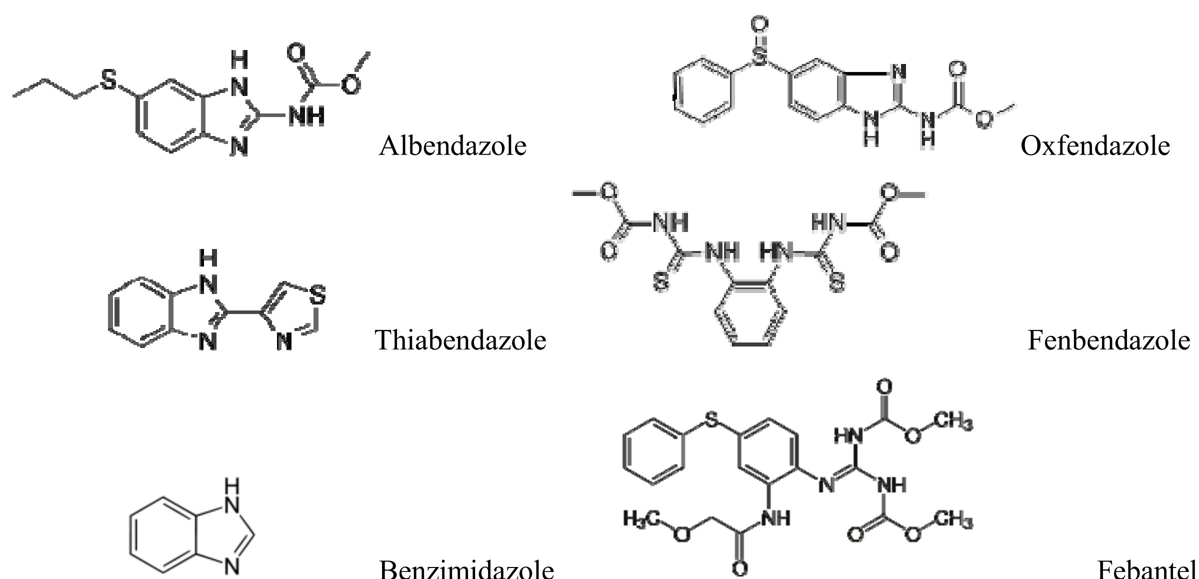


Figure 7: The chemical structure of some Benzimidazoles

Relatively shortly after the introduction of BZs in 1961, the first occurrence of resistance was already reported 1964 in *Haemonchus contortus* (Conway, 1964) and is meanwhile widespread (Wolstenholme et al., 2004).

2.3.2 Imidazothiazoles

The synthetic imidazothiazole tetramisol and LEV are nicotinic anthelmintics which act as agonists on acetylcholine receptors of the nematode muscle membranes (Martin et al., 1996) causing spastic paralysis. Their anthelmintic activity is mainly attributed to their ganglion-stimulant (cholinomimetic) activity, whereby they stimulate ganglion-like structures in somatic muscle cells of nematodes (Fig. 11). This stimulation first results in sustained muscle contractions, followed by a neuromuscular depolarising blockade resulting in paralysis. In higher concentrations LEV also inhibits the activity of the acetylcholine esterase. Hexamethonium, a ganglionic blocker, inhibits the action of LEV. The compounds are effective against adult and larval gastrointestinal worms and lungworms. The absorption and excretion of LEV is rapid whether given orally or by injection because it is highly soluble. In cattle, peak blood levels within 1 h after subcutaneous (s.c.) administration are reached. LEV resistance has been commonly reported in sheep and goat trichostrongylid nematodes, (Coles et al., 1994).

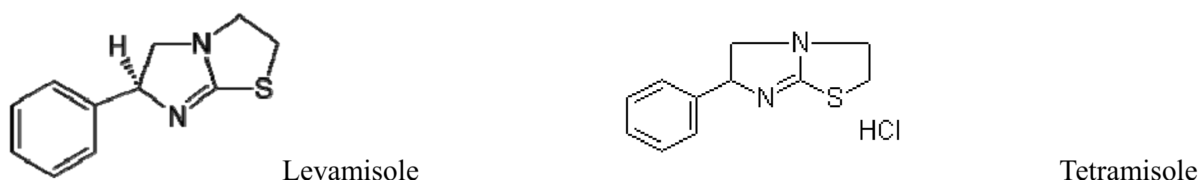


Figure 8: Chemical structures of levamisole and tetramisole

2.3.3 Macrocyclic lactones

One of the most recent classes of anthelmintics are the MLs. They consist of two main subclasses, the avermectins and the milbemycins. They are natural products derived from *Streptomyces avermitilis* (abamectin, doramectin) or semi-synthetic substances which include moxidectin, milbemycin, milbemycin oxime, eprinomectin, IVM and selamectin. The chemical structure of IVM is shown in Fig. 9.

MLs are highly potent, broad-spectrum endectocides. The milbemycins were discovered in 1973 as acaricidal and insecticidal compounds, while IVM was introduced into the market in 1981. MLs act by binding to ligand-gated ion channels (Cully et al., 1994) in nematode and arthropod nerve cells, including glutamate- (GluCl) and GABA-gated (GABACl) chloride channels (Blackhall et al., 1998; 2003). Receptor binding causes the channel to open, allowing an influx of chloride ions. By irreversibly opening these channels the permeability of the cell membrane for chloride ions is increased. This leads to a hyperpolarisation of the cell membrane, contributing to the inhibition of nerve signal transduction. Different chloride channel subunits may show variable susceptibility to MLs and different sites of expression, which could account for the paralytic effects of MLs on different neuromuscular systems at different concentrations. GluCls and GABACl are widely distributed in nematodes and regulate locomotion, feeding and reproduction (Yates et al., 2003). Therefore they can paralyse the pharynx, the body wall and the uterine muscles of nematodes. Flaccid paralysis of body wall muscle may be critical for rapid expulsion, even though paralysis of pharyngeal muscles is more sensitive. As the ML concentration decreases, motility may be regained, but paralysis of the pharynx, inhibition of feeding and egg laying in *C. elegans* and *H. contortus* (Avery et al., 1990; Geary et al., 1993) may last longer and are irreversible, resulting in non-spastic paralysis and death of the parasite. Two sites, the somatic muscles and the pharynx are thought to be the major site of action of MLs in the worms. The pharyngeal and somatic muscle receptors may differ in their binding properties in parallel with their sensitivity to IVM.

ML resistance is complex, with mechanisms varying both within and between species. Several *in vitro* assays for the detection of resistance in trichostrongyloids have been developed. *C. oncophora* in cattle have the ability to survive a recommended dose of IVM that would be effective against susceptible parasites (Vermunt et al., 1995) and are therefore referred to as the dose limiting species.

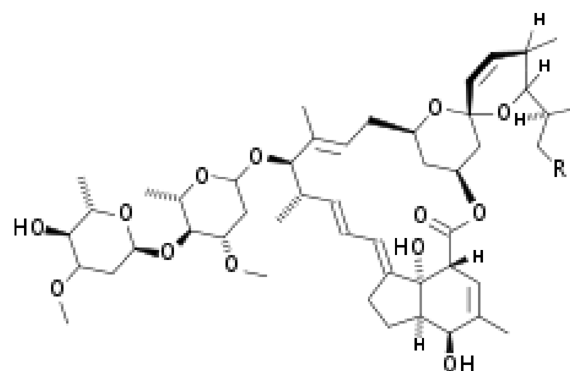


Figure 9: Chemical structure of Ivermectin

2.3.4 Cyclooctadepsipeptides

Emodepside is a member of a novel group of compounds called cyclooctadepsipeptides and was discovered in 1993 (Fig. 10) and introduced in 2005. It has a broad spectrum anthelmintic activity as a semi-synthetic derivative of PF1022A which is obtained from the natural secondary metabolite of the fungus *Mycelia sterilia*, growing for example on the leaves of *Camellia japonica*. The discovery and anthelmintic activity has been described by Harder & von Samson-Himmelstjerna (2002). Emodepside is effective against a variety of gastrointestinal nematodes and filarial parasites (Zahner et al., 2001; Harder et al., 2003), also against IVM and LEV-resistant *H. contortus*, IVM-resistant *T. colubriformis* in sheep and IVM-resistant *C. oncophora* in cattle (von Samson-Himmelstjerna et al., 2005).
Emodepside interferes with latrophilin receptors (Willson et al., 2003) which bind the main vertebrate-specific neurotoxic protein latroxin. This relaxes both pharyngeal and somatic muscles, resulting in paralysis of the nematode. Furthermore, it has been shown that emodepside acts through a potassium-gated calcium channel in parasitic nematodes (Welz et al., 2011).
The effects of Emodepside were shown in *C. elegans* (Bull et al., 2007), including the inhibition of pharyngeal pumping, decreasing forward movement and inhibition of egg-laying in adults. The studies in *A. suum* revealed clear muscle paralysis (Willson et al., 2003) demonstrating that emodepside is a potent anthelmintic drug.

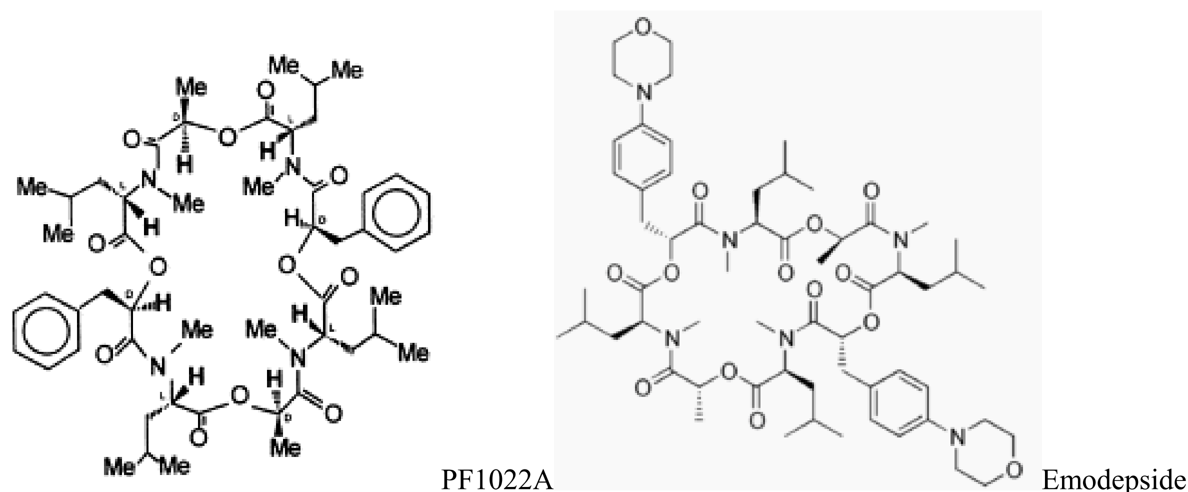


Figure 10: Chemical structures of PF1022A and Emodepside

2.3.5 New compounds

The most recently introduced anthelmintic drug Monepantel was launched under the name Zolvix® and is the first product of a new anthelmintic class, the AADs. Their anthelmintic activity was demonstrated first in sheep (Kaminsky et al., 2008). The AADs are a class of low molecular mass compounds bearing different aryloxy and aroyl moieties on an amino-acetonitrile core (Ducray et al., 2008). They have high activity against all gastro-intestinal nematodes in sheep, including resistant isolates. They paralyse worms by binding to a specific receptor which is only found in nematode parasites. The chemical structure of an AAD is shown in Fig. 11.

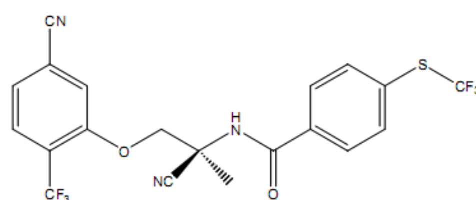


Figure 11: Chemical structure of Amino-Acetonitrile- Derivatives (AADs)

2.4 Anthelmintic resistance

The extensive regular use of anthelmintic treatments has resulted in the development of anthelmintic resistance to the commonly used drugs in most livestock animals such as sheep, goats, horse and cattle, particularly in the southern hemisphere but also in the US and Europe (Bjorn et al., 1990; Vermunt et al., 1995; Waller et al., 1995; 1996; Ihler et al., 1996; Coles et al., 1999; Van Wyk et al., 1999; Coles et al., 2001; Familton et al., 2001; Fiel et al., 2001; Coles et al., 2002; Mejia et al., 2003; Yue et al., 2003; Anziani et al., 2004; Smith & Gasbarre, 2004; Demeler et al., 2009; Gasbarre, 2009; Rendell, 2010). The first resistance was reported for phenothiazine, followed by BZs, imidazothiazoles and MLs (Prichard et al., 1980). Resistance to piperazine has been reported in small strongyles in horses (Drudge et al., 1988) and some populations of *H. contortus* resistant to salicylanilides and organophosphate compounds have also been reported (Sangster, 1999).

The progressive development of resistance to commonly used anthelmintic is globally a major problem for livestock, people and economy.

2.4.1 Types of resistance

Resistance in parasitic nematodes has been defined by Prichard et al. (1980): "resistance is present, when there is a greater frequency of individuals within a population able to tolerate doses of a compound than in a normal population of the same species and is heritable".

Side-resistance: where resistance to a compound is the result of selection by another compound with a similar mode of action.

Multiple-resistance: when parasites are resistant to two or more anthelmintics through either selection by each group independently or by side-resistance.

Reversion: refers to the return of resistant individuals/worm populations to susceptibility, with a decrease in the frequency of resistant individuals following the removal of the selecting agent.

2.4.2 Selection of resistance

Resistance is a hereditary phenomenon. Resistant genes are present in almost every population in only some worms, which principally can pass these genes on to the next generation.

The control of parasites with drugs leads to the survival of resistant worms and can therefore lead to the development of resistance. The proportion of genes for susceptibility in the worm population becomes smaller with increasingly higher frequencies of resistance genes. Experience has shown that anthelmintic resistance generally develops more slowly than, for example, antimicrobial resistance because of the longer generation times in nematodes. When using anthelmintics only the parasites within the host are exposed to the selecting agent. The free-living part of the population living on pasture is not selected and is referred to as the "refugium". It plays therefore a major role in the selection for resistance.

2.4.3 Specific resistance mechanisms

2.4.3.1 Benzimidazole

The BZs act by binding to the β-tubulin, which interferes with the polymerisation of the microtubules (Lacey, 1990). The microtubules are heterodimeric proteins of tubulin. Tubulin is composed of α and ß- protein which are about the same size (450 kDa) and consist of 450 amino acids. Together, these tubulin units are ring-shaped, caused by the tubular joint storage of microtubules, with an inside diameter of 15 nm and an outer diameter of 25 nm (Dustin, 1984). The polymerisation of tubulin to microtubulin is a continuous process of proliferation and dissociation. On one side of the dimers dissociation takes place while on the opposite side proliferation occurs, keeping a dynamic equilibrium. The process of polymerisation is influenced by some endogenous factors. These factors include microtubule-associated proteins which are responsible for the stability of microtubules, GTP, Mg^{2+}, Ca^{2+}, calmodulin, and temperature changes (Martin et al., 1997). The microtubules serve as structural components within cells and are involved in important cell functions, including the structural formation of the cytoskeleton, the formation of cell processes, the formation of the spindle apparatus during cell division, intracellular transport, exocytosis and the movement of flagella (Sullivan, 1988).

As explained above, the action of the benzimidazoles is based on the binding of ß-tubulin, so that the microtubule proliferation at the positive pole is inhibited, but dissociation continues. This attachment is described by Lacey (1988) as “capping” and causes the depolymerisation of the microtubulin structure. This leads to loss of their function, therefore the formation of the cytoskeleton is impaired, uptake decreased and the intracellular transport of nutrients and metabolic substrates reduced, so that the nematode dies following the consumption of energy reserves (Lacey, 1988).

Some authors suggest that a loss of high affinity for tubulin in resistant isolates is involved in the mechanism of resistance (Lubega & Prichard, 1991). BZ resistance associated with alteration in the β-tubulin gene sequence has been reported in *C. elegans* (Driscoll et al., 1989), in fungi (Fujimura et al., 1992; Jung et al., 1992) and in resistant isolates of *H. contortus* (Kwa et al., 1994). Roos et al. (1990) showed that there were specific differences in DNA of benzimidazole-resistant isolates of *H. contortus*, which indicated that alterations in the ß-tubulin gene might be accompanied with resistance to BZs. Cross-resistance can exist

among all members of this group because they act on the same receptor protein, β-tubulin, which is altered in resistant organisms so that BZs cannot bind to the receptor with high affinity.

2.4.3.2 Levamisole

LEV acts on nicotinic acetylcholine receptors (Lewis et al., 1987), which can be found in the somatic musculature of the parasite (Martin et al., 1997). A depolarisation block which occurs in the ganglia of the parasite leads to a spastic paralysis (Harrow & Gration, 1985). The effect of LEV depends on the parasite species, the life cycle status, the concentration of LEV and thus the ACh esterase inhibition.

The regular use of LEV as the most widely used cholinergic anthelmintic (Wolstenholme et al., 2004) has led to a significant development of resistance, so that the drug has no effect against a number of different isolates (Sangster et al., 1999). The LEV resistant nematodes no longer respond to other nicotinic agonists such as morantel and pyrantel (Sangster et al., 1998). There is genetic evidence that resistance in *T. colubriformis* and *Oesophagostomum dentatum* is the result of single sex-linked genes. In *H. contortus* it is likely that multiple genes are involved. Genetic screens for mutants that survive exposure to LEV in the free-living nematode *C. elegans* have led to the identification of five genes (unc-38, unc-63, unc-29, lev-1 and lev-8) that encode a LEV-sensitive acetylcholine receptor (L-AChR). Loss of these genes leads to LEV resistance. Orthologues of these genes were identified in three species of *H. contortus*, *T. circumcincta* and *T. colubriformis*. Polymorphism associated with LEV resistance by comparing transcripts of these subunits in LEV susceptible and LEV-resistant isolates of the three strongylid species was investigated by Neveu et al. (2010). The study revealed an unexpected high diversity of L-AChR subunits specific to the trichostrongylid parasites that are associated with LEV resistance which are a principal target for the LEV. Abbreviated variants, predicted to produce non-functional unc-63, were associated with LEV resistance.

In *C. elegans*, resistance in mutants is due to a lack of acetylcholine receptors causing altered or no response to cholinergic agonists which normally cause contractions, also the binding of LEV to the receptor differs between resistant and susceptible *C. elegans* (Lewis et al., 1987). The inheritance of LEV resistance in *H. contortus* was investigated using egg hatching (Sangster et al., 1991). These authors concluded that LEV resistance is attributed to the loss of

cholinergic receptors. In the past decade research in the field of the mode of action of levamisole and its resistance mechanisms was increased and several structures, including receptors, were reported.

2.4.3.3 Macrocyclic lactones

Generally, the action of MLs is characterised by the immobilisation of the parasite in the form of a rapid paralysis of somatic muscles (Campbell et al., 1995; Shoop et al., 1995; Feng et al., 2002). The pharyngeal muscles are the most sensitive site to MLs and respond with a decreased pump activity (Avery & Horvitz, 1990; Brownlee et al., 1997).

The first problems regarding IVM resistance were reported for gastrointestinal nematodes of sheep and goats in South Africa and Brazil (Carmichel et al., 1987; Van Wyk et al., 1988; Echevarria & Trindade, 1989). The mechanism of resistance to IVM has been studied in detail in *C. elegans* (Rohrer et al., 1992) and revealed, that IVM interacts with certain membrane receptors. GABACl receptors which are found in neuromuscular cells of invertebrates and in neurons of the central nervous system in vertebrates have been identified as a potential target of MLs. IVM has no effect on cestodes and trematodes because these species do not possess these receptors (Martin et al., 2002; Ungemach, 2002). The MLs have additionally been found to bind with high affinity to other chloride channels. Further studies on the free-living nematode *C. elegans* suggested that additionally to GABACls, the GluCls are the major site of action of IVM (Holden-Dye & Walker, 1990; Arena et al., 1995; Cully et al., 1996). Cully et al. (1994) reported GluCl channels in invertebrates, which have been found to be similar to GABA receptors with respect to their structure and function (Dent et al., 1997; Vassilatis et al., 1997; Njue et al., 2004). As a result of the interaction of MLs with these channel receptors the cell is hyperpolarised, which causes a blockage of conduction, leading to a flaccid paralysis and death of the parasite.

Resistance has also been found to be associated with pharyngeal pumping events in *C. elegans* (Laughton et al., 1995) and *A. suum* (Martin et al., 1996). A study on the inheritance of avermectin/milbemycin resistance in free-living stages of trichostrongyloid nematodes revealed important differences in the development process of resistance. *In vivo*, the efficacy of MLs against e.g. *O. circumcincta* may be different to that in *H. contortus* and *T. colubriformis* (Gill et al., 1998). Demeler (2005) used several *in vitro* techniques to measure the effects of six different ML's on the somatic and pharyngeal musculature in larvae

and adult worms of the sheep parasitic nematodes *H. contortus, T. colubriformis* and *T. circumcincta*. Differences between the various life cycle stages were evaluated and showed that the appearance of resistance to MLs anthelmintics differs between the two sites, pharynx and somatic muscles. It was also observed that the effects of MLs vary between life cycle stages of the parasites.

2.4.4 Non-specific resistance mechanisms

Non-specific mechanisms involved in resistance have been shown to contribute to the occurrence of multi-drug resistance (MDR). Similar mechanisms are also responsible for the development of chemotherapy-resistant tumour cells. Cell membrane pumps such as Pgps appear to contribute to anthelmintic resistance, as they were found to be activated in resistant populations.

Localisation of Pgp has been studied in the free-living nematode *C. elegans* and in *H. contortus* in sheep using specific monoclonal antibodies or lectins (Kerboeuf et al., 2002). Alterations in expression levels and allele frequencies of Pgp in anthelmintic-resistant populations have been observed in nematodes (Blackhall et al., 2003; 2008). Inhibitory substances used in human studies, such as the calcium-channel blocker VPL, appear to have similar effects in helminths as in human cancer cells (Beugnet et al., 1997; Kerboeuf et al., 1999) when reversing agents are co-administered with the anthelmintic.

The involvement of a second potential mechanism, the function of CYPs, has also been suggested to play a role in resistance mechanisms. They are involved in the oxidation of endogenous compounds and xenobiotic agents (Guengerich, 1991) and most drugs are suitable for oxidative metabolism by CYPs. By inhibition of these pathways through the use of specific inhibitors it is possible to show comparative effects of these transporters on anthelmintic resistance of parasitic nematodes *in vitro*.

VPL is a calcium channel blocker which inhibits mainly the function of Pgp (Shoji et al., 1991), probably by indirect hyperphosphorylation of the glycoprotein (Hamada et al., 1987). In MDR cancer cells, VPL and other Pgp inhibitors have been demonstrated to be able to reverse multi-drug resistance (Baumert & Hilgeroth, 2009). To investigate the role of the inhibitors in drug-transport mechanisms in parasites, Kerboeuf et al. (1999) studied the effects of two Pgp substrates, VPL and Rhodamine 123, in combination with BZs by flow cytometry in eggs of susceptible and resistant *H. contortus* isolates. The results revealed that VPL

increased susceptibility to BZs. Accordingly, a higher affinity of the resistant *H. contortus* eggs for Rhodamine 123 in the presence of VPL compared to susceptible ones was observed. Similar results were obtained by Beugnet et al. (1997) who found that VPL was able to partially reverse BZ resistance in free-living stages of *H. contortus* when the eggs were exposed to TBZ and TBZ with VPL, suggesting that a Pgp-mediated mechanism plays a role in BZ resistance. Stenhouse (2007) investigated the role of Pgps and CYPs in BZ resistance mechanisms in *T. circumcincta*. Molento and Prichard (1999) treated jirds infected with *H. contortus* with a combination of IVM or Moxidectin (MOX) and VPL or analogues of VPL. The results supported *in vivo* experiments, where the activity of IVM and MOX was enhanced by the addition of VPL.

PBO acts as synergist for insecticides by inhibiting the CYPs of insects (Jones, 1998). The anthelmintic activity of rotenone in *in vitro* assays with adults and larvae of *H. contortus* and *T. colubriformis*, respectively, was enhanced when combined with PBO but the toxicity of rotenone was increased in presence of PBO by 3-4 fold in both, adult and larval stages of *H. contortus* and in larval stages of *T. colubriformis* (Kotze et al., 2006). In the LDA and the LMIA the Pgp inhibitor VPL enhanced susceptibility of parasitic stages of the cattle nematode *C. oncophora* to IVM (Demeler et al., 2009).

Interestingly, rotenone/PBO combinations have been widely used for management of fish populations (Ling, 2002).

2.4.4.1 P-glycoproteins

Pgps are members of the ATP binding cassette (ABC) superfamily of cell membrane transporters (Fig. 12) which protect cells from exogenous and endogenous cytotoxic compounds (Bode et al., 2003). They are found in high quantities in the intestine, kidney, liver and in blood tissue barriers. In the free-living nematode *C. elegans*, Pgps were found in excretory cells, the pharynx and in intestinal cells. The localisation appeared to be different, depending on the Pgp isoform.

Pgp was found to play a major role in IVM transport (Pouliot et al., 1997) and therefore contributing to IVM resistance in parasitic nematodes. Pgps are also believed to be the major cause of multi-drug resistance in mammals. Noteworthy, Schinkel et al. (1994) found that mice showed symptoms of neurotoxicity when exposed to IVM due to disrupture in the mdr1a

Pgp gene. Didier & Loor (1996) and Pouliot et al. (1997) confirmed that IVM is a substrate for Pgps in mammalian cells.

In *C. elegans* this was analysed by Broeks et al. (1995) and in protozoan parasites by Ekong et al. (1993), investigating their potential contribution to anthelmintic resistance. 14 Pgp genes have so far been described in *C. elegans* (Geerts & Gryseels, 2000), one of which may be a pseudogene, 7 Pgp genes in *H. contortus* compared with only two in humans and three genes in rodents (Kerboeuf et al., 2003). Furthermore, Kerboeuf et al. (1999) investigated the functional role of Pgp in BZ resistance in *H. contortus*. Addition of VPL as inhibitor of Pgps increased the susceptibility of jirds to IVM in a moxidectin resistant strain (Xu et al., 1998). Beugnet et al. (1997) showed that addition of VPL to eggs partially reversed resistance to TBZ and this was attributed to the presence of Pgps. Other studies found that Pgps are involved in ML resistance (Kerboeuf et al., 2003).

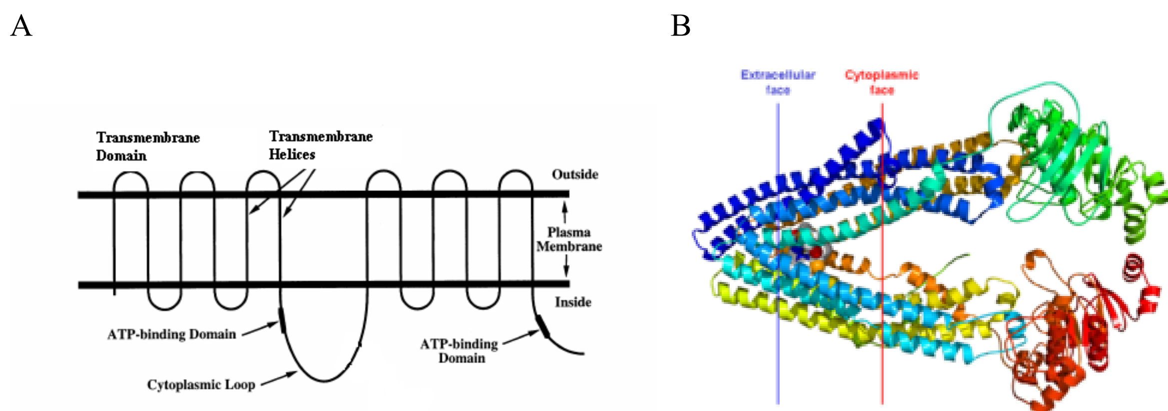


Figure 12: (A) Schematic presentation of P glycoprotein (University of Veterinary Medicine Hannover, Germany); (B) ATP-binding cassette, sub-family B (MDR/TAP), member 1. Crystallographic structure of the mouse MDR3 protein. The approximate positioning of the protein in the cell membrane is indicated by the blue (extracellular face) and red (cytoplasmic face) lines. The protein is depicted as a rainbow coloured cartoon (N-terminus = blue, C-terminus = red). A cyclic peptide inhibitor QZ59 is represented by a space-filling model (http://en.wikipedia.org/wiki/P-glycoprotein)

2.4.4.2 Cytochrome P450

Cytochrome P450 (CYPs) monooxygenases are enzymes which catalyse the oxidation of endogenous and exogenous compounds (Estabrook, 1984; Nelson et al., 1993). They are hemoproteins (Fig. 13) which act as the terminal oxidase in the monooxygenase system. The first indication of CYPs in parasitic nematodes came with metabolism of aminopyrine during *in vivo* studies of larvae of *Heligmosomoides polygyrus* (Kerboeuf et al., 1995) and CYPs have also been found in larvae of *H. contortus* (Kotze, 1999). However, no association was seen between the activity of them and ML resistance in a resistant isolate of *H. contortus* compared to a susceptible isolate (Kotze, 2000). However, the metabolism of moxidectin by homogenates of *H. contortus* adults suggests the activities of P450 in this parasite (Alvinerie et al., 2001), indicating a potential role of CYP activity in the development of anthelmintic resistance in nematodes.

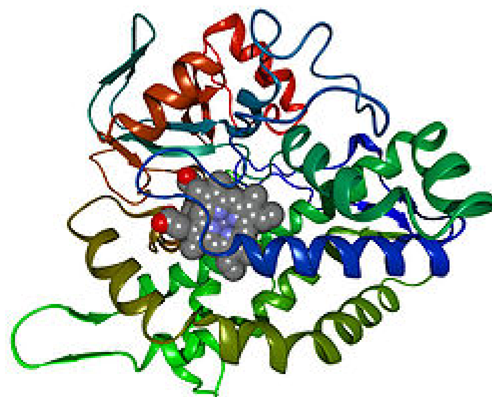


Figure 13: Structure of a Cytochrome P450eryF (PDB: 1EGY) (http://de.wikipedia.org/wiki/Cytochrom_P450)

2.5 Scientific research methods for the detection of resistance

To detect AR in parasitic nematodes several methods have been developed over the past 50 years. Generally they can be divided into two main groups, *in vivo*, involving the living host and *in vitro* tests, involving the living parasites. A variety of techniques have been reviewed by Johansen (1989). In the last two decades the development of molecular based techniques became more and more important, particularly as they have been shown to often be more accurate and sensitive than classical parasitological methods. Generally, two different *in vivo* tests, the controlled test which was introduced by Whitlock et al. (1980) and the faecal egg

count reduction test (FECRT) are available. The controlled test is the most reliable test for detection of resistance. However, it is expensive and requires a large number of animals. In the FECRT, AR is estimated by comparing faecal egg counts of animals before and after treatment (Boersema, 1983; Presidente, 1985). For both tests accounts that the degree of resistance can unfortunately not be easily measured with either of these methods (Hubert et al., 1992).

Compared to the *in vivo* methods, *in vitro* tests have several advantages. They are less expensive, relatively simple, reliable and often require only a single sampling from the farms (Lacey, 1990). They usually involve incubation of one of the free-living stages of the parasites under controlled conditions in different concentrations of the tested anthelmintic. From these data a dose-response-curve can be obtained and e.g. an EC_{50} (the concentration of drug required to prevent 50% of eggs from hatching) can be calculated.

The first *in vitro* test, the egg hatch assay (Le Jambre, 1976) has been developed for the detection of resistance to TBZ and LEV. These drugs are ovicidal (inhibit hatching) and hatch rates were measured by counting the proportion of eggs and hatched larvae after an overnight culture at 27 °C. Another common method for the detection of AR is the LDA (Lacey, 1990), where the inhibition of the development from L1 through to L3 is measured. The first larval motility assay was developed by Martin & Le Jambre (1979). Meanwhile, various different methods have been published. All include the incubation of larvae in the presence of an anthelmintic and the motility is then measured by observation (Gill et al., 1991), migration through a sieve (Sangster et al., 1988) or by electronic detectors (Folz et al., 1987).

In addition, molecular based methods provide often more sensitive possibilities for the detection of resistance. The genetic basis of anthelmintic resistance must be known in order to develop these tests systems. This is currently only known for resistance against BZs, where PCR-based tests (Silvestre et al., 2000; Humbert et al., 2001; von Samson-Himmelstjerna, 2006) were developed for the detection of BZ resistance.

2.5.1 *In vivo* tests

2.5.1.1 The controlled test

In this test the animals are infected with a designated dose of infective larvae and after the infection, the animals are treated with the recommended dose of an anthelmintic. Faecal egg

counts (FECs) are regularly monitored before and after treatment. In the post mortem examination worm burdens in parts of the body (according to the location of the parasite of interest) are examined and resistance is confirmed when the reduction in worm counts is less than 90% or more than 1000 worms surviving after treatment (Presidente, 1985).

2.5.1.2 The faecal egg count reduction test (FECRT)

The FECRT involves the comparison of the FECs before and after treatment with an anthelmintic (Boersema, 1983; Presidente, 1985). For the detection and monitoring of anthelmintic resistance, recommendations regarding the performance of a FECRT have been given by the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Coles et al., 1992). For example in sheep, resistance is declared if the reduction in faecal egg counts is less than 95% and the lower 95% confidence limit is below 90%.
FECRT have some disadvantages as they require at least two faecal samples, weighing and treating of the animals, counting techniques and laboratories. Additionally, nematode egg output does not necessarily correlate well with actual worm numbers and therefore the test may not estimate anthelmintic resistance correctly. Anthelmintic resistance below 25% cannot be detected with this test due to a lack of sensitivity (Martin et al., 1989). The test can be easily applied for sheep (Coles et al., 2006), but there are no current recommendations how to overcome the difficulties associated with cattle.

2.5.2 *In vitro* tests

2.5.2.1 Egg Hatch Assay (EHA)

The EHA was first described by Le Jambre for the detection of BZ resistance in sheep (Le Jambre, 1976), while an egg embryonation test was reported by Coles and Simpkin (1977). The use of an EHA for detecting LEV resistance in nematodes has been described by Dobson et al. (1986), involving a more or less complicated protocol. In the past 35 years this assay has been modified by a number of workers, reviewed by Taylor et al. (2002). It has been used for the detection of BZ resistance in nematodes of ruminants (Jackson et al., 1987; Hunt & Taylor, 1989; Johansen & Waller, 1989; Martin et al., 1989; Borgsteede et al., 1992; 1997;

Hoekstra et al., 1997; Coles et al., 2006; Várady et al., 2006; Ghisi et al., 2007), horses (Whitlock et al., 1980; Ullrich et al., 1988; Ihler & Bjorn, 1996; Várady et al., 2000; von Samson-Himmelstjerna et al., 2002; Wirtherle et al., 2004), pigs (Várady et al., 1996) and humans (Albonico et al., 2005). Recently, standardisation of the EHA has been achieved by a ring test of one protocol in different European laboratories using susceptible and resistant isolates of *H. contortus* (von Samson-Himmelstjerna et al., 2009). The EHA is only suitable for the evaluation of certain drug classes and can for example not be used for the detection of ML resistance (Prichard, 1994).

The assay is based on the ability of eggs to hatch under standardised conditions and the ovicidal effect of anthelmintics. It requires fresh eggs no more than 4 h old (Coles & Simpkin, 1977) or, alternatively, eggs obtained from faecal samples stored under anaerobic conditions for up to 7 days (Smith-Buijs & Borgsteede, 1986; Taylor & Hunt, 1989) can be used. The essential aim is to incubate undeveloped eggs in different concentrations of TBZ for 48 h at 27 °C in order to establish a dose-response-curve. To obtain a linear regression from which the EC_{50} (the concentration of anthelmintic required to kill 50% of eggs) can be calculated, transformation and fitting of data either by the use of a log-probit model (Healy, 1988), an arcsine model (Cawthorne & Whitehead, 1983) or a four parameter logistic model must be performed. An EC_{50} value > 0.1 µg/ml TBZ indicates resistance to BZs (Coles et al., 1992; Ihler & Bjorn, 1996; Graven et al., 1999). However, limitations also apply to this assay which has been reported not to be suitable for the detection of less than 25% resistance (Martin et al., 1989).

2.5.2.2 Larval Development Assay (LDA)

The first larval development test was described by Coles et al. (1988) for the detection of resistance to the commonly used anthelmintics. Several different types of larval development assays have been reported since then, based on either liquid or microagar (Taylor, 1990; Hubert & Kerboeuf, 1992; Gill et al., 1995; Coles et al., 2006).

The assay can be performed with BZs and LEV (Gill et al., 1995) and was improved by adding IVM and other MLs using *H. contortus*. That led to the development of a commercially available assay, the Drenchrite® LDA (Microbial Screening Technologies, Kemps Creek, NSW, Australia), which was developed and introduced by the Commonwealth Scientific Industrial Research Organization (CSIRO) in Australia. This assay can be used for

detecting resistance to four anthelmintic classes: BZ, LEV, BZ/LEV combination and avermectins (Lacey, 1990; Lacey et al., 1991; Gill et al., 1995). The LDA was also used for the detection of AR in nematodes in the field (Mitchell et al., 1991; Hong et al., 1992; Praslička et al., 1994; Bartley et al., 2003; Ancheta et al., 2004). In horses, Young et al. (1999) reported the use of the DrenchRite assay as promising for the detection of anthelmintic resistance in cyathostomine nematodes while Tandon & Kaplan (2004) revealed limitations, explicitly for the detection of resistance to IVM and LEV.

The LDA protocol published by Demeler et al. (2010a) was used for the detection of anthelmintic resistance in the cattle gastrointestinal nematodes *C. oncophora* and *O. ostertagi.* The LDA is based on the development of eggs through to infective third stage larvae (L3) under controlled conditions. In comparison to the EHA, the age of eggs has no to little effect on the assay performance (Coles et al., 2006). After incubation of eggs in different concentrations of an anthelmintic for 7 days at 27 °C, the assay is stopped by adding lugol´s iodine. The proportion of non-developed stages (unhatched eggs, L1 and L2) to the total of all stages in each well is counted and corrected by the number of stages not developing in the control wells. The EC_{50} (the concentration of drug required to prevent the development of 50% of the eggs to infective L3) can be determined as described in chapter 2.4.2.1 for the EHA. The discriminating doses for anthelmintic resistance for ovine nematodes have been established from sensitive cultures of *H. contortus* (Gill et al., 1995).

2.5.2.3 Larval Migration Inhibition Assay (LMIA)

The LMIA is an *in-vitro* assay, which measures the effect of anthelmintics on the motility of L3. Compared to the EHA or the LDA where fresh faeces are required, the advantage of this assay is that L3s can be easily collected and stored before use.

The first larval paralysis assay for the detection of resistance to LEV and morantel tartrate in *Teladorsagia* spp. was described by Martin and Le Jambre (1979). This assay has also been used by others (Barton, 1983; Geerts et al., 1987; 1989; Sutherland & Lee, 1990), but was considered to be subjective and not reproducible (Boersema, 1983). Douch et al. (1983) achieved an objective assessment of L3 motility by measuring the inhibition of the migration of larvae through agar blocks. This assay was modified to detect anti-parasitic components in the mucus of sheep (Douch et al., 1986) and the effect of different anthelmintics on the migration of nematode larvae of sheep (Douch et al., 1994). Gill et al. (1991) reported a

motility assay for the detection of IVM resistance in third larval stage of *H. contortus*. A number of similar assays have been used to evaluate anthelmintic activity in gastrointestinal mucus (Kimambo et al., 1988; Gamble et al., 1992). Sangster et al. (1988) reported an agar-free system for the use of LEV in *T. colubriformis* and *H. contortus*, where sieves were suspended in water to allow migration. The detection of resistance here is based on the ability of larvae to migrate through fine nylon sieves. The mesh size of sieves was chosen so that L3 can actively migrate (Rabel et al., 1994). A variety of methods containing the use of sieves were published in the past 20 years for the detection of resistance to closantel, IVM, TBZ, LEV (Wagland et al., 1992; Rothwell et al., 1993; Rabel et al., 1994; Gatongi et al., 2003) and the inhibitory activity of other extracts (Lorimer et al., 1996). Demeler et al. (2010a) established EC_{50} values for *C. oncophora* and *O. ostertagi* in cattle. Recently, the LMIA has been evaluated for detection IVM resistance in field populations (Kleinschmidt, 2007).
The performance of the LMIA is divided in two phases, incubation and migration. In the incubation phase, L3s are incubated under different concentrations of drugs at 27 °C for 24 h prior to migration. Another 24 h are given to allow migration through the sieves. The migration of larvae can be influenced by several parameters such as temperature, drugs, incubation time and numbers of tested larvae. The incubation with the possibility of migration is depending on the used anthelmintic substance and should take place for at least 14-17 up to 24 hours (Wagland et al., 1992; Rabel et al., 1994; Gatongi et al., 2003; Demeler et al., 2010a). Gatongi et al. (2003) showed that a higher temperature can increase the proportion of migrated L3.

2.5.2.4 The Micromotility Meter (MM)

A Micromotility Meter was developed for measuring the motility of larval and adult nematodes after incubation with anthelmintics (Bennett & Pax, 1986; 1987).
Anthelmintic effects on the parasite were detected using the instrument (Micromotility Meter TM , B and P Instruments, 324 West South Street, Mason, MI 48854) designed to monitor helminth motor activity (movement) in a test solution/suspension (Bennett & Pax, 1986; Folz et al., 1987). Differences in motility were observed between susceptible and resistant strains (Folz et al., 1988). The MM was evaluated for detection of IVM resistance in adult *C. oncophora* of cattle parasitic nematodes and in adult *T. colubriformis* and *T. circumcincta* of sheep nematodes (Demeler et al., 2005; 2010a).

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

Bacto Agar 454 g (T8904)	**Sigma Aldrich**
Piperonyl butoxide 100 mg PS-100	**Sigma Aldrich**
E. coli (K12) 10 g Ec-9637	**Sigma Aldrich**
Amphotericin B (A9528)	**Sigma, Steinheim**
Ivermectin (T8898)	**Sigma, Steinheim**
Levamisole (L9756)	**Sigma, Steinheim**
Thiabendazole (T8904)	**Sigma, Steinheim**
Verapamil hydrochloride (V4629)	**Sigma, Steinheim**
DMSO (Dimethyl sulfoxide>-99.5% (A994.2)	**Carl Roth, Karlsruhe**
di-Sodium Hydrogen Phosphate anhydrous 500 gm Na_2HPO_4 (P0301) M 141.96 gm/mol	**Carl Roth, Karlsruhe**
Sodium dihydrogen phosphate monohydrate GR 1KG $NaH_2Po_4H_2O$ (A912946) M 137.99g/mol	**Merck, Darmstadt**
NaCl	**VWR, Hannover**
Yeast	**Difco, Detroit Michigan USA**

3.1.2 Buffer and solutions

Lugol's iodine solution (N0523)	**Roth GmbH & Co. KG, Karlsruhe**
Earls balanced solution 10x (E7510)	**Sigma, Steinheim**
Sugar solution	**60 g sugar in 40 ml water**
Yeast extract	**1 g yeast in 90 ml NaCl (0.9%)**
water	**deionised water**

3.1.3 Equipment

Drying oven	**Memmert, Schwabach**
Incubator	**Memmert, Schwabach**
Inverted microscope (40/0.65; 6.3/0.020; 100/1.25; 2.5/0.45)	**Zeiss, Jena**
Stereo microscope	**Zeiss, Jena**
Centrifuge Omnifuge 2.ORS	**Heraeus, Osterode**
Vortexer	**Heidolph, Schwabach**
Shaker Vortex Genius 3	**Werke, Staufen**
Laboratory balance	**Sartorius-H. Jürgens & CO**
Eppendorf Multipipette 4780	**Eppendorf Netheler Hinz, Hamburg**
Parafilm®	**Pechiney Plastic Packaging, Chicago**
McMaster counting (2 chambers)	**MSD-AGVET, München**
24-cell culture plates	**Biochrom AG, Berlin**
Dispenser tips 5 ml	**Biochrom AG, Berlin**
Syringe 2 ml, 5 ml, 10 ml, 60 ml	**Biochrom AG, Berlin**
Pipette tips 0.5-10µl,10-100µl, 100-1000µl, 1000-5000µl	**Roth, Karlsruhe**
Counting chamber	**Roth, Karlsruhe**
48-cell culture plates	**Roth, Karlsruhe**
Magnetic stirrer	**Roth, Karlsruhe**
Weighing scale	**Roth, Karlsruhe**
Eppendorf tubes 50 ml	**Roth, Karlsruhe**
Hose clamping	**VWR, Hannover**
Funnels	**VWR, Hannover**
Glas Pasteur pipettes 200 mm, 150 mm	**VWR, Hannover**
Tube silicone 12x15	**VWR, Hannover**
Hand counter	**VWR, Hannover**
Kitchen sieve	**VWR, Hannover**
Vacuette	**WDT, Garbsen**

Test tubes 15 ml	**Sarstedt, Nümbrecht**
Petri dishes	**Sarstedt, Nümbrecht**
24-cell culture plates	**Sarstedt, Nümbrecht**
Disposable Pasteur pipettes	**Sarstedt, Nümbrecht**
Tissue culture flask 200 ml	**Sarstedt, Nümbrecht**
Rubber-O-Rings	**Hand arbeitshaus, Karlsruhe**
Measure band 250 cm	**AgrarGIGANT, Elsfleth**
Needle 0.90x40 mm	**B. Braun, Melsungen**
Migration sieve 28 µm	**Self made**
Pipettes 10µl, 100µl, 1000µl, 5000µl	**Eppendorf Research, Hamburg**
Gauze	**Wilh. Weisweiler, Münster**
Food colouring	**Schwartau, Bad Schwartau**
Mortar and pestle	**Neolab, Berlin**
Sawdust	**Ruckdeschel & Söhne, Berlin**
Honey jars 500 ml	**Deutscher Imkerbund e. V.**
Microscope slides	**Glaswarenfabrik Karl Hecht KG, Sondheim**

3.1.4 Software

GraphPad Prism 5.0 ®	**GraphPad Software, California, USA**
Microsoft Excel	**Microsoft Inc.**
Microsoft Word	**Microsoft Inc.**
Microsoft Power Point	**Microsoft Inc.**

3.2 Methods

3.2.1 Animals

The calves used in these experiments were 3-6 months old and mainly Holstein Friesian calves. They were obtained from the University of Hannover Research Farm in Ruthe at the University of Hannover or from Hammink GbR, Agragenossenschaft eG and Agrar GmbH Bliesdorf, Berlin. The calves were kept in the animal house in concrete pens in pairs of 2 up to 4 animals. After arrival they were health-checked, and treated with anthelmintics (IVM or albendazole) to ensure that the animals had no gastrointestinal parasites before infection. One week after treatment FECs were performed to ensure the absence of worm eggs. Infections with parasites were usually performed after a minimum of 7 up to 14 days post treatment. The calves were fed once daily with pellets, water and autoclaved hay was given *ad libitum.* They were monitored and the stables were cleaned daily, FECs were performed weekly and the calves were weighed fortnightly.

3.2.2 Parasite populations used for experiments

3.2.2.1 *Cooperia oncophora* populations

The susceptible *C. oncophora* isolate (*C. o*. sus.) used in this study is a Weybridge-isolate obtained from Bayer Animal Health in 2002. This isolate has no history of exposure to anthelmintics. An IVM-resistant *C. oncophora* isolate (*C. o.* IVM-res.) was obtained from Bristol (Coles et al., 1998) and is resistant against IVM *in vivo* where therapeutic doses failed to remove the parasites. Resistance to other anthelmintics is not known. A third *C. oncophora* isolate was obtained from New Zealand (provided by Prof. Pomroy, Massey University, Palmerston North, New Zealand). This isolate showed resistance against BZs and MLs *in vivo*.

All isolates were individually and regularly passaged in 3-6 month old calves. The resistant isolates were additionally challenged with a) the recommended therapeutic dose of IVM (Ivomec®, 0.2 mg IVM/kg bodyweight) b) with 50% of the recommended dose of either IVM or albendazole in regular intervals.

3.2.2.2 *Ostertagia ostertagi* populations

The susceptible *O. ostertagi* (*O. o.* sus.) is a Weybridge-isolate, also obtained from Bayer Animal Health in 2002. The other *O. ostertagi* (*O. o.* sel.) isolate originates from this susceptible Weybridge isolate and was selected for BZ-resistance in the Institute for Parasitology, University of Veterinary Medicine in Hannover since 2003 (Pachnicke, 2009).
The *O. o*. sel. isolate tolerates a sub-therapeutic dose of 30% albendazole (Valbazen®) at current.

3.2.3 Parasitological techniques

3.2.3.1 Animal infection

The calves were infected orally by delivering the third stage larvae (L3) with a syringe directly into the oesophagus.
For infection with *C. oncophora and O. ostertagi*, approximately 35.000 infective larvae were given to each calf. After 3-4 weeks, faecal egg counts were performed regularly to monitor the egg numbers.

3.2.3.2 Faecal egg count

FECs were estimated using a modified McMaster technique (Whitlock et al., 1980) with a sensitivity of 50 eggs per gram (epg). 4 gram of fresh faeces were weighed out and thoroughly mixed in a few ml saturated salt solution. The resulting solution was poured through a kitchen sieve and the volume filled up to the total of 60 ml. A pipette sample was taken and placed into a counting chamber.
The number of eggs in both chambers was counted under a microscope. The epg was calculated by multiplying the number of eggs in both chambers by 50 (Maff, 1986).

3.2.3.3 Egg recovery

The faeces were collected directly from the rectum of infected calves and homogenised with tap water until all faecal pellets had been broken. The solution was than subsequently poured through a 100 µm sieve and collected in a bucket. The collected fluid was than poured through a 25 µm sieve, the sediment was collected from the sieve and centrifuged for 10 minutes at 200 xg. The supernatant was removed by sucking it off and the centrifuge tubes were refilled with saturated chloride solution. This was again centrifuged for 10 minutes at 200 xg. The supernatant was poured through a 25 µm sieve and the sediment on the sieves collected with deionised water into a 50 ml falcon tube. From a saturated sugar solution, 3 sugar gradients were prepared with concentrations of 10%, 25% and 40%. Food colouring (yellow, red and blue) were added to the sugar gradients to simplify the differentiation between the different layers. First 5 ml of the 10% gradient were filled into a 50 ml falcon tube followed by underlaying of the 25% and the 40% gradient. 10 ml of egg suspension was added on top of the 10% solution and the falcon then centrifuged for 7 min at 300 xg. The eggs settled in the layer between the 10% and 25% gradient. The egg suspension was carefully sucked off using a glass Pasteur pipette. The eggs were washed with tap water through a 25 µm sieve, recollected from the sieve and placed in a 15 ml falcon tube. A subsample of 10 µl was counted to obtain the mean number of eggs per ml and the egg suspension was adjusted to the required number of eggs divided by 3 per ml.

3.2.3.4 Faecal and larval cultures

The infected larvae were obtained from faecal cultures. Faeces were collected rectally and mixed with sawdust to allow flow of oxygen. The mixture was filled into jars and closed loosely. The culture was incubated for 7 days (25 °C and 80% humidity). On the 8th day the jars were filled with tap water till it covered the suspended faeces, a petri dish was placed on top and the jars were inverted. The rim of the petri dish was filled with water and left overnight. The larvae migrate from the wrapped faeces and settle on the rim of the petri dish. From there the larvae were collected with a plastic pipette and transferred into 50 ml falcon tubes. After sedimentation of larvae the supernatant was removed. To clean up the solution containing the larvae, the remaining fluid was passed over a Baerman funnel system using either 28 µm sieves or several layers of gauze. The purification was carried out by active

migration of larvae through the sieve and sinking down to the hose clamp. After a maximum of 24 hours the larvae were collected, transferred into ventilated cell culture flasks, labelled and stored at 8-10 °C in the refrigerator until further use.

3.2.4 Egg Hatch Assay (EHA)

3.2.4.1 Parasites

The nematode eggs were recovered from fresh faeces as described in section 2.3.3. *C. oncophora* (sus., IVM-res. and NZ-res.) and *O. ostertagi* isolates (sus. and sel.) were used in this assay.

3.2.4.2 Test substances

TBZ, VPL (an inhibitor of Pgp) and PBO (an inhibitor of CYPs) were used in this assay in the following concentrations (Table 2, 3 & 4):

<u>Thiabendazole</u>:

Sol A: 50 mg Thiabendazole + 5 ml DMSO = 10 mg TBZ/ml DMSO

Sol B: 1 ml sol A +9 ml DMSO = 1 mg TBZ/ml DMSO

Table 2: Thiabendazole solutions (and preparations) for the EHA

Sol. B	DMSO	Prepared concentrations	Conc. TBZ µg/ml in well (after a final dilution 1:200)
20 µl	9980 µl	2 µg/ml	0.01 µg/ml
60 µl	9940 µl	6 µg/ml	0.03 µg/ml
100 µl	9900 µl	10 µg/ml	0.05 µg/ml
150 µl	9850 µl	15 µg/ml	0.075 µg/ml
200 µl	9800 µl	20 µg/ml	0.1 µg/ml
300 µl	9700 µl	30 µg/ml	0.15 µg/ml
370 µl	9630 µl	37 µg/ml	0.185 µg/ml
600 µl	9400 µl	60 µg/ml	0.3 µg/ml
1000 µl	9000 µl	100 µg/ml	0.5 µg/ml

Verapamil:

VPL stock solution: 24.55 mg/1000 µl DMSO = $5x10^{-2}$ M

Table 3: Preparation of VPL for the toxicity test in the EHA

	µl water		**Conc. VPL µM in well (after a final dilution 1:20)**
100 µl Stock sol.	900 µl	5000 µM	250 µM
500 µl 50µM	500 µl	2500 µM	125 µM
500 µl 25µM	500 µl	1250 µM	62.5 µM
500 µl 12.5µM	500 µl	625 µM	31.25 µM
500 µl 6.25µM	500 µl	312.5 µM	15.625 µM
500 µl 3.125µM	500 µl	156.3 µM	7.815 µM
500 µl 1.563µM	500 µl	78.1 µM	3.91µM
500 µl 0.78µM	500 µl	39.1 µM	1.953 µM

Piperonyl butoxide:

Solution A: 16.05 µl PBO + 983.95 µl DMSO = 17000 µg/ml

Solution B: 100 µl Sol. A + 900 µl DMSO = 1700 µg/ml

DMSO-water: 50 ml deionised water + 50 ml DMSO

Table 4: Preparation of PBO for the toxicity test in the EHA

	µl 50% DMSO-water		**Conc. PBO µg/ml in well (after a final dilution 1:100)**
250 µl Stock sol. B	250 µl pure water	850 µg/ml	8.5 µg/ml
250 µl 850 µg/ml	250 µl	425 µg/ml	4.25 µg/ml
250 µl 425 µg/ml	250 µl	212.5 µg/ml	2.125 µg/ml
250 µl 212.5 µg/ml	250 µl	106.3 µg/ml	1.063 µg/ml
250 µl 106.3 µg/ml	250 µl	53.3 µg/ml	0.533 µg/ml
250 µl 53.3 µg/ml	250 µl	26.55 µg/ml	0.266 µg/ml
250 µl 26.55 µg/ml	250 µl	13.275 µg/ml	0.133 µg/ml
250 µl 13.275 µg/ml	250 µl	6.64 µg/ml	0.066 µg/ml
250 µl 6.64 µg/ml	250 µl	3.325 µg/ml	0.033 µg/ml

3.2.4.3 Assay performance

The aim of this assay is to determine, if nematode eggs are resistant to BZs. Freshly isolated eggs should be used within 3 h of collection. The egg suspension was adjusted to 80-100 egg/10 µl. Stock solutions of TBZ were dissolved in DMSO (100%). Sol. B was diluted in DMSO to give final concentrations. These concentrations were prepared one day before the assay was performed. 10 µl of TBZ (of the different prepared concentrations with increasing concentrations from left to right) in 1990 µl of the egg suspension (1980 µl deionised water + 10 µl egg suspension) were dispensed in each well. Negative controls (DMSO-water only) were included in the assay. If VPL was included, 100 µl of VPL solution (concentration 2), 10 µl drug solution and 1890 µl egg suspension were used; if PBO was included, 20 µl of the PBO solution, 10 µl drug solution and 1970 µl egg suspension were used. The plates were incubated for 48 h at 25-27 °C and the assay stopped by adding two drops of lugol´s iodine. The eggs and larvae in each well were counted under an inverted microscope. For final analysis a minimum of 5 data sets (each in duplicates) was used.

3.2.4.4 Toxicity testing of inhibitors

The toxicity tests of inhibitors were performed following the same method as described in section 3.2.4.3 except that TBZ was replaced with an inhibitor. For VPL, concentrations ranged from 250-1.935 µM and for PBO concentrations between 8.5-0.266 µg/ml were used. The results of these tests showed VPL had no toxic effect on the development and hatching of eggs up to a concentration of 250 µM. Therefore, a concentration 125 µM was selected for the combination with TBZ. PBO appeared to be not toxic at all concentrations tested. Therefore, the assay was performed with TBZ and the first 6 concentrations of PBO.
For both substances, positive (stock solution) and negative controls (no substance) were included.

3.2.4.5 Data analysis

The numbers of larvae relative to the total (larvae + eggs) present at each drug concentration were counted in each well. The percentage of larvae was calculated using Microsoft Excel®

software. Data were corrected for P0, the mean number of eggs not hatching in the control wells. The GraphPad Prism software 5.0 ® was used to fit a sigmoidal (logistic) curve to the dose-response data and to compare the curves statistically.
A sigmoidal dose-response model with a variable slope was chosen to allow fitting of the Hill slope. TOP and BOTTOM values were defined as 0 and 100 to allow the exact calculation of EC_{50}. This model is also known as a “four-parameter-logistic model” and calculates the mean of all repeats, the exact EC_{50} and the standard error. Additionally we looked at R^2 which quantifies the goodness of fit and the 95% confidence intervals. For the test of statistical difference between the mean values for the EC_{50} of the populations, the p-value for EC_{50} was calculated to compare the means of both populations. The p-value is a probability with a value ranging from 0 to 1. Is the p-value small enough, it can be stated that the difference between the mean of the samples is unlikely to be due to chance. The threshold value in this analysis-program is set to 0.05 (an arbitrary–value that has been widely adopted).
GraphPad Prism defines the p-values as following:

P-value	**Definition of wording**
< 0.001	Extremely significant
0.001-0.01	Very significant
0.01-0.05	Significant
> 0.05	Not significant

3.2.5 Larval Development Assay (LDA)

3.2.5.1 Parasites

Faeces were freshly collected from calves and eggs recovered as described in 3.2.3.3. All available isolates (*C. o.* sus. IVM-res. and NZ-res., *O. o.* sus. and sel.) were used in this assay.

3.2.5.2 Test substances

Two drugs and two inhibitors were tested in this assay: TBZ & IVM and VPL & PBO (see tables 3, 5, 6, & 8).

Thiabendazole:

Solution A: 0.05 g TBZ + 5 ml DMSO = 10 mg TBZ/ml

Solution B: 0.1 ml sol. A + 9.9 ml DMSO = 0.1 mg TBZ/ml DMSO

Solution C: 1 ml sol. B + 9 ml H_2O = 0.01 mg TBZ/ml H_2O

Table 5: Preparation of TBZ for the LDA

µl Sol. C	µl deionised water	conc. µg/ml TBZ in well
18	9982 µl	0.001
45	9955 µl	0.0025
90	9910 µl	0.005
180	9820 µl	0.01
360	9640 µl	0.02
720	9280 µl	0.04

Ivermectin:

Stock-solution: 10^{-2} M (8.71 mg/ml)

Table 6: IVM dilution series for the LDA

	µl DMSO	Concentrations
10 µl stock. sol	990 µl DMSO	10^{-4} M
100 µl 10^{-4} M	400 µl DMSO	$2x10^{-5}$ M
100 µl 10^{-4} M	900 µl DMSO	10^{-5} M
200 µl 10^{-4} M	800 µl DMSO	$2x10^{-6}$ M
10 µl 10^{-4} M	990 µl DMSO	10^{-6} M
20 µl 10^{-5} M	980 µl DMSO	$2x10^{-7}$ M
10 µl 10^{-5} M	990 µl DMSO	10^{-7} M
20 µl 10^{-6} M	980 µl DMSO	$2x10^{-8}$ M
10 µl 10^{-6} M	990 µl DMSO	10^{-8} M
20 µl 10^{-7} M	980 µl DMSO	$2x10^{-9}$ M

To achieve the final concentration in the wells, 100 µl of each concentration was diluted in 900µl deionised water (Table 7).

Table 7: Final concentrations of IVM in the wells in the LDA

Conc. 1	$5x10^{-7}$ M	Conc. 4	10^{-8} M	Con. 7	$5x10^{-10}$ M
Conc. 2	10^{-7} M	Conc. 5	$5x10^{-9}$ M	Con. 8	10^{-10} M
Conc. 3	$5x10^{-8}$ M	Conc. 6	10^{-9} M		

Verapamil:

The VPL concentrations for the LDA were used as given in Table 3.

Piperonyl butoxide:

Stock-solution: A: 16.05 µl PBO + 983.95 µl DMSO = 17000 µg/ml

Table 8: Preparation of PBO for the toxicity test in the LDA

	µl DMSO	Conc. PBO in well
10 µl Stock sol. A	990 µl	170 µg/ml = con. 1
100 µl con. 1	100 µl	85 µg/ml = con. 2
100 µl con. 1	100 µl	42.5 µg/ml = con. 3
50 µl con. 1	350 µl	21.25 µg/ml = con. 4
50 µl con. 1	750 µl	10.63 µg/ml = con. 5
20 µl con. 1	620 µl	5.33 µg/ml = con. 6

3.2.5.3 Assay performance

This assay was performed to determine whether the developing larvae of nematodes are resistant to the anthelmintics. Stock solutions of TBZ have to be prepared 24 h before performance of the assay.

For TBZ, 16.6 µl (+ 213.4 µl deionised water) and for IVM 30 µl (+ 200 µl deionised water) were used in each well to achieve the concentrations given in tables 5 and 6. 50 µl of nutritive medium (containing 20 µl Yeast/Earles solution, 20 µl Amphotericin and 10 µl *E. coli*) was added to inhibit fungal growth. Finally, 20 µl of egg suspension, adjusted to contain 80-100 eggs was added. If VPL was included, 60 µl of VPL solution (concentration 2) and for PBO 30 µl of the PBO solution were used and, according to the drug tested (TBZ or IVM), the total amount of deionised water was adjusted. The plates were sealed with parafilm to prevent drying as well as fungal proliferation during incubation and placed in an incubator for 6-7 days at 25 °C. After incubation the assay was terminated by adding two drops of lugol´s iodine into each well. The eggs, L1, L2 and L3 of each well were counted.

3.2.5.4 Toxicity testing of inhibitors

In this assay eggs of the *C. o.* sus. isolate were used in the same manner as described in section 3.2.5.3 except that TBZ was replaced with an inhibitor. The concentrations of VPL and PBO were the same as in the toxicity tests described for the EHA.
As negative control 30 µl of 10% DMSO and in the positive control 30 µl of VPL stock-solution were included.
The concentration at which no significant effect of VPL on development of larvae was observed was 250 µM. A concentration of 125 µM was therefore selected for further use in the LDA with TBZ and IVM. All tested PBO concentrations had a similar effect as in the EHA; therefore the assay was performed accordingly.

3.2.5.5 Data analysis

The percentage of undeveloped stages (eggs, L1 and L2) and L3 of the total (all stages) at each concentration were calculated using Excel® software. The data were analysed as described in capital 3.2.4.5.

3.2.6 Larval Migration Inhibition Assay (LMIA)

3.2.6.1 Parasites

The LMIA was performed using larvae of susceptible and resistant/selected isolates of *C. oncophora* and *O. ostertagi* which were passed over a Baerman funnel filter system for a minimum of 3 h prior to their use in the test to ensure that only viable larvae were used.

3.2.6.2 Test substances

Two drugs and two inhibitors were tested in this assay: IVM & LEV and VPL & PBO (see tables 9, 10, 12 & 13). All solutions were prepared at the day of performance.

Ivermectin:

Stock solution: 10^{-2} M (8.71 mg/ml)

Table 9: Concentrations of IVM for the LMIA

	µl DMSO	**Concentration I**
50 µl stock sol	450 µl DMSO	10^{-3} M
200 µl10^{-3} M	800 µl DMSO	$2x10^{-4}$ M
100 µl 10^{-3} M	900 µl DMSO	10^{-4} M
20 µl 10^{-7} M	980 µl DMSO	$2x10^{-5}$ M
10 µl 10^{-7} M	990 µl DMSO	10^{-5} M
20 µl 10^{-4} M	980µl DMSO	$2x10^{-6}$ M
10 µl 10^{-4} M	990 µl DMSO	10^{-6}M
20 µl 10^{-5} M	980 µl DMSO	$2x10^{-7}$ M
10 µl 10^{-5} M	990 µl DMSO	10^{-7} M

To achieve the final concentrations which were added into each well, 100 µl of each concentration I (given in the last column of the table) were diluted in 900 µl deionised water.

Levamisole:

Stock solution: 10^{-1} M (24.1 mg/ml)

Table 10: Concentrations of LEV for the LMIA

	Water µl	
100 µl stock. sol	900 µl dist. water	10^{-2} M
200 µl 10^{-2} M	800 µl dist. water	$2x10^{-3}$ M
100 µl 10^{-2} M	900 µl dist. water	10^{-3} M
20 µl 10^{-2} M	980 µl dist. water	$2x10^{-4}$ M
10 µl 10^{-2} M	990 µl dist. water	10^{-4} M
400 µl 10^{-4} M	600 µl dist. water	$4x10^{-5}$ M
200 µl 10^{-4} M	800 µl dist. water	$2x10^{-5}$ M
100 µl10^{-4} M	900 µl dist. water	10^{-5} M
20 µl 10^{-4} M	980 µl dist. water	$2x10^{-6}$ M
10 µl 10^{-4} M	990 µl dist. water	$10^{-6}0$ M

Table 11: Final concentrations of LEV achieved in the wells

Con. 1	$5x10^{-4}$ M	Con. 4	10^{-5} M	Con. 7	10^{-6} M	Con. 10	$2x10^{-8}$ M
Con. 2	10^{-4} M	Con. 5	$5x10^{-6}$ M	Con. 8	$2x10^{-7}$ M		
Con. 3	$5x10^{-5}$ M	Con. 6	$2x10^{-6}$ M	Con. 9	10^{-7} M		

The concentrations 1-8 were used in resistant isolates and in susceptible isolates the concentrations 3-10 were used.

Verapamil:

Stock solution: 4.9105 mg/ml = 10^{-2} M in 100% DMSO

Table 12: Preparation of VPL for the toxicity test in the LMIA

	µl water (=10% DMSO)		**Conc. VPL in well (after final dilution 1:20)**
100 µl Stock sol.	900 µl deionised water	1000 µM	50 µM
500 µl 1000 µM	500 µl 10% DMSO	500 µM	25 µM
500 µl 500 µM	500 µl 10% DMSO	250 µM	12.5 µM
500 µl 250 µM	500 µl 10% DMSO	125 µM	6.25 µM
500 µl 125 µM	500 µl 10% DMSO	62.5 µM	3.125 µM
500 µl 62.5 µM	500 µl 10% DMSO	31.25 µM	1.563 µM
500 µl 31.25 µM	500 µl 10% DMSO	15.625 µM	0.78 µM
500 µl 15.63 µM	500 µl 10% DMSO	7.813 µM	0.39 µM

Piperonyl butoxide:

Stock-solution: A: 16.05 µl PBO + 983.95 µl DMSO = 17000 µg/ml

Table 13: Preparation of PBO for the toxicity test in the LMIA

	µl 50% DMSO-water		**Conc. PBO in well (after final dilution 1:50)**
250 µl Stock sol. A	250 µl pure water	8500 µg/ml	170 µg/ml
250 µl 8500 µg/ml	250 µl	4250 µg/ml	85 µg/ml
250 µl 4250 µg/ml	250 µl	2125 µg/ml	42.5 µg/ml
250 µl 2125 µg/ml	250 µl	1063 µg/ml	21.25 µg/ml
250 µl 1063 µg/ml	250 µl	532 µg/ml	10.63 µg/ml
250 µl 532 µg/ml	250 µl	266 µg/ml	5.32 µg/ml
250 µl 266 µg/ml	250 µl	133 µg/ml	2.66 µg/ml
250 µl 133 µg/ml	250 µl	66.5 µg/ml	1.33 µg/ml

3.2.6.3 Assay performance

This assay was performed to determine whether L3 of nematodes are resistant to the anthelmintic. The incubation plates were prepared with 1700 µl distilled water, 90 µl of the prepared drug concentrations (IVM or LEV) and 10 µl of larval suspension (contain 80-100 L3) in each well (1800 µl final volume). For negative controls, 90 µl of distilled water and for positive controls, 90 µl of IVM or LEV stock solution were added. In case of LEV, the

concentrations from 1-8 for resistant isolates and the concentrations from 3-10 for susceptible isolates were used. If IVM or LEV were tested together with VPL, 1682 µl deionised water, 90 µl drug solution, 18 µl VPL solution (concentration 2) and 10 µl larval suspension were used. For the combination with PBO, 1664 µl deionised water, 90 µl drug solution, 36 µl PBO solution (concentration 2) and 10 µl larval suspension were used.

After 24 hours the migration plates were prepared by filling with 400 µl Bacto agar 1.5% and allowed to cool. After 24 h incubation time the whole contents were transferred onto the sieves in Row A and C. Larvae were allowed to migrate for 24 hours at 28 °C. After incubation, the assay was stopped by lifting the sieves and gently washing them from the outside into the wells A and C, then flipping the sieves over into the row B and D (see Fig. 14); the contents of the sieves were washed with water into the wells of these rows. Then the migrated and non-migrated larvae for each concentration were counted under the stereo microscope. Each assay was performed in duplicates for susceptible and resistant isolates.

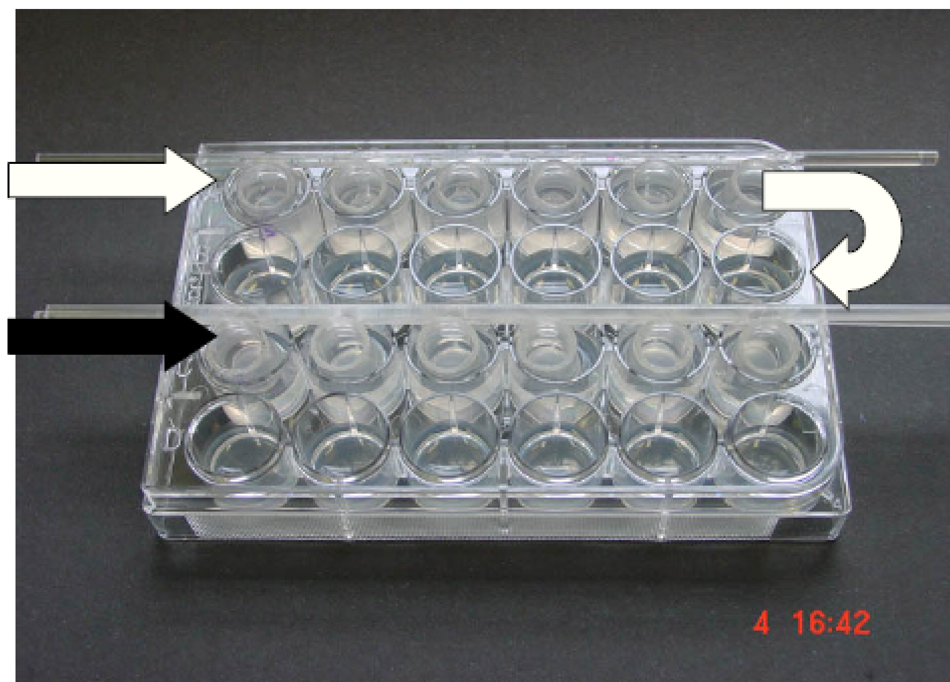


Figure 14: Migration plate with sieves for the LMIA (Demeler, 2005)

3.2.6.4 Toxicity testing of inhibitors

The aim of this assay was to establish a concentration of inhibitors which does not effect the tested larval population. The toxicity test was carried out using the same method as described

above, except that IVM was replaced by inhibitors. The maximum concentration of the inhibitor without effect on larval migration was determined by using dilutions ranging from 50-0.39 µM (VPL) and 85-1.33 µg/ml (PBO). The concentrations of 25 µM VPL had no effect on larval migration; therefore this concentration was used in combination with IVM. PBO had no effect on larval migration at all tested concentrations; therefore the LMIA was carried out with all possible combinations, using the prepared concentrations of PBO (table 13) in combination with all prepared concentrations of IVM (table 9).

3.2.6.5 Data analysis

The migrated and non-migrated larvae were counted and the percentages of non-migrated larvae were calculated by dividing the number of non-migrated larvae by the total number of migrated and non-migrated larvae × 100. These data were then analysed using GraphPad Prism® software as described for the EHA (details see section 3.2.4.5).

4 Results

4.1 Effect of Verapamil on egg hatching

The EHA was carried out using TBZ to investigate the effects of TBZ alone (without inhibitors) and in combination with inhibitors of Pgps and CYP P450s on the hatching of nematode eggs using susceptible and resistant isolates of *C. oncophora* and *O. ostertagi*.

The two isolates of *C. oncophora* susceptible to BZs (*C. o.* sus. and *C. o.* IVM-res.) showed similar sensitivities with EC_{50} values for TBZ of 0.043 µg/ml and 0.045 µg/ml, respectively (Fig. 15). In contrast, resistance to BZs was detected in the *C. o*. NZ-res. isolate with an EC_{50} of 0.11 µg/ml. For the susceptible *O. ostertagi* isolate an EC_{50} value of 0.037 µg/ml was obtained. Despite selection for BZ-resistance, the selected isolate had an EC_{50} of 0.039 µg/ml before treatment and directly post treatment of 0.063 µg/ml for TBZ. In the presence of VPL, the dose-response-curves of all isolates for TBZ (Fig. 15) showed a significant ($p<0.0001$) shift to the left (the EC_{50} decreased), except for the susceptible isolate of *C. oncophora* where the difference was not highly significant ($p = 0.03$). The EC_{50} values, 95% CI and R^2 for TBZ and TBZ+VPL are shown in Table 14. Dose-response-curves for all isolates using TBZ alone and in combination with VPL are presented in Fig. 15.

Table 14: EC_{50}values, 95% CI and R^2 of TBZ (in µg/ml) alone and in combination with VPL (125 µM) for *C. oncophora* and *O. ostertagi* isolates in the EHA. For statistical comparison of the two curves obtained with and without VPL for each isolate, a p-value was calculated

Isolates	**EC_{50} TBZ**	**95% CI**	**R^2**	**EC_{50} TBZ+VPL**	**95% CI**	**R^2**	**p-value**
C. o. sus.	0.043	0.042 - 0.045	0.874	0.041	0.037 - 0.046	0.809	0.03
C. o. IVM-res.	0.045	0.041 - 0.049	0.884	0.034	0.031 - 0.036	0.926	0.002
C. o. NZ-res.	0.11	0.106 - 0.120	0.929	0.022	0.019 - 0.025	0.923	< 0.0001
O. o. sus.	0.037	0.036 - 0.038	0.977	0.034	0.033 - 0.035	0.844	0.001
O. o .sel.	0.039	0.037 - 0.041	0.912	0.030	0.018 - 0.032	0.975	0.0001
O. o .sel. p.t	0.063	0.057 - 0.070	0.771	0.022	0.019 - 0.026	0.854	< 0.0001

p.t.= post treatment

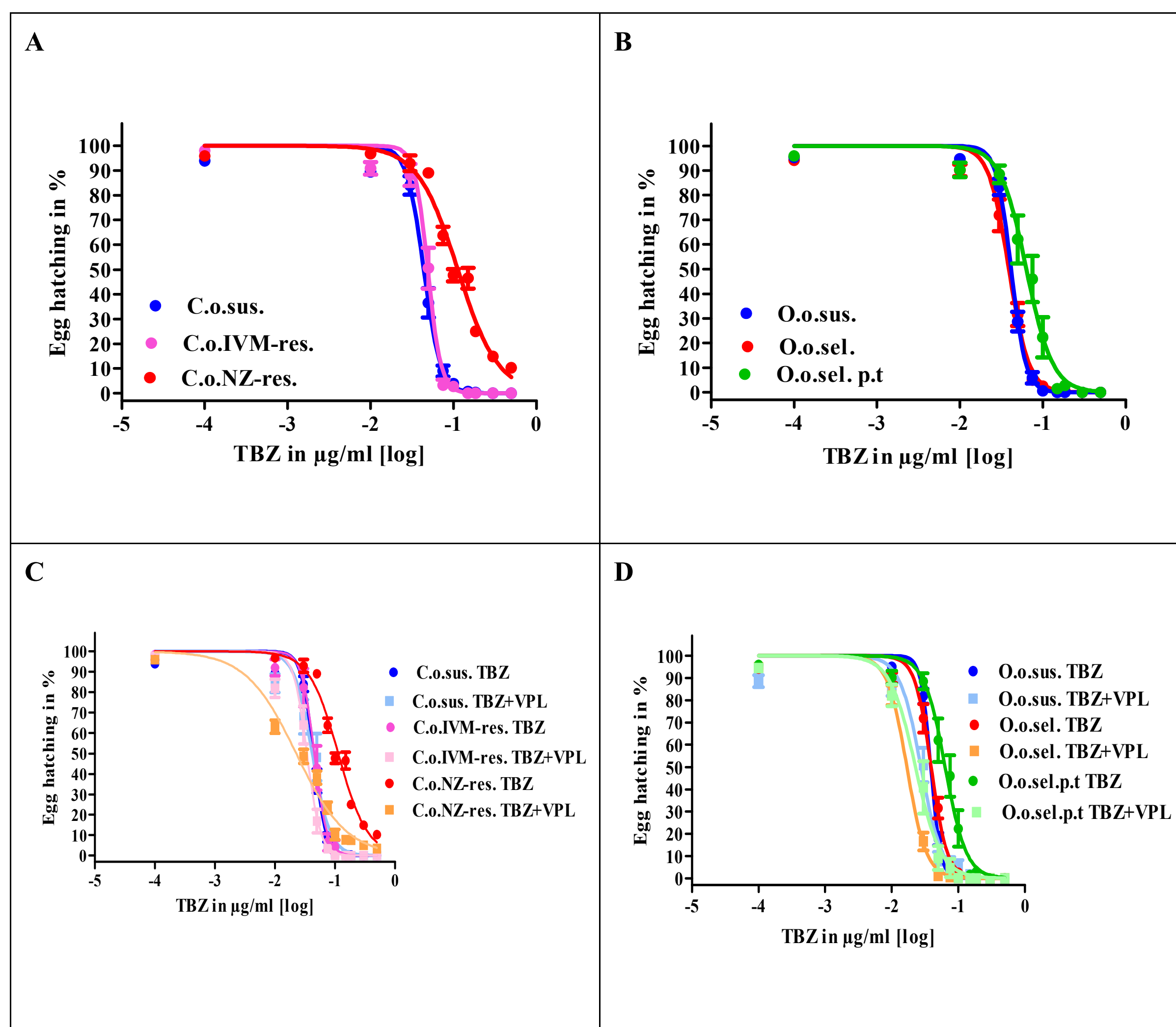


Figure 15: The dose-response-curves for TBZ for *C. oncophora* (A), *O. ostertagi* (B) and for TBZ + 125 µM VPL for *C. oncophora* (C) and *O. ostertagi* (D)

4.2 Effect of Piperonyl butoxide on egg hatching

In this assay only *C. oncophora* isolates (sus. and NZ-res.) were tested, as the *C. o*. IVM-res. isolate is only resistant to IVM. PBO had a clear effect on egg hatching when it was used in combination with TBZ in both isolates, with EC_{50} values of 0.013 µg/ml and 0.046 µg/ml TBZ, respectively. In combination with all tested concentrations of PBO, the EC_{50} values (~0.01 µg/ml TBZ) obtained for TBZ in both *O. ostertagi* isolates were similar to those obtained in the susceptible *C. oncophora*. The R^2 values for all tested combinations were between 0.912 - 0.991 for all isolates, indicating good reproducibility. In Table 15 the EC_{50} values, 95% CI and p-values for the lowest and highest tested concentration of PBO are

shown. Results for all other concentrations tested are given in Appendix 10. The dose-response-curves for all tested isolates are shown in Fig. 16, again exemplary for the highest and lowest concentration of PBO only (for complete results see Appendix 10).

Table 15: EC_{50} values and 95% CI for TBZ (in µg/ml) alone and in combination with PBO (8.5 µg/ml and 0.266 µg/ml) for *C. oncophora* and *O. ostertagi* isolates in the EHA. P-values for each isolate were calculated for statistical differences between TBZ and TBZ+PBO

Isolates	EC_{50} TBZ	EC_{50} TBZ+8.5 µg/ml PBO	95% CI	EC_{50} TBZ+0.266 µg/ml PBO	95% CI	p-value
C. o. sus.	0.043	0.013	0.012 - 0.013	0.015	0.014 - 0.016	< 0.0001
C. o. NZ-res.	0.11	0.020	0.017 - 0.022	0.046	0.042 - 0.050	< 0.0001
O. o. sus	0.038	0.015	0.014 - 0.016	0.014	0.013 - 0.016	< 0.0001
O. o. sel.	0.039	0.010	0.009 - 0.011	0.013	0.012 - 0.014	< 0.0001

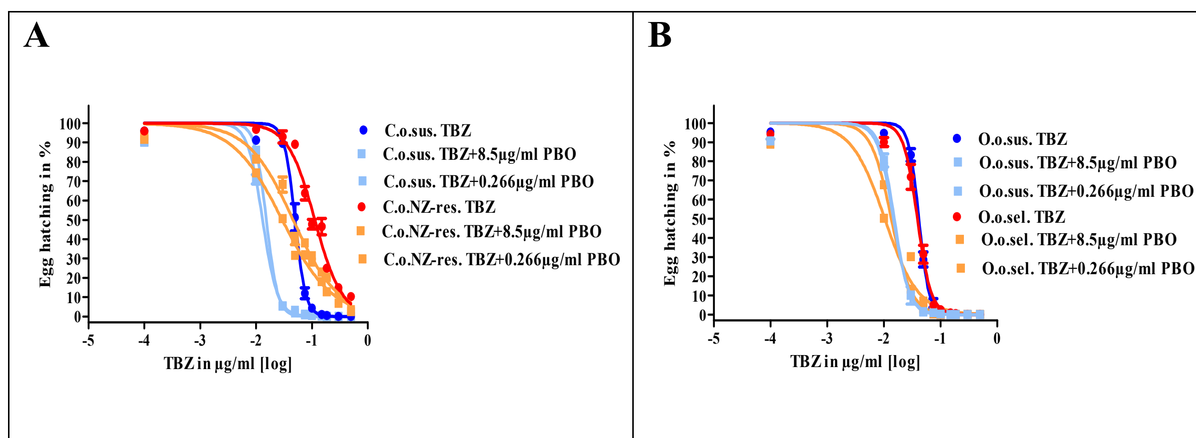


Figure 16: The dose-response-curves for TBZ, TBZ + PBO for *C. oncophora* (A) and *O. ostertagi* (B) isolates in the EHA

4.3 Effect of Verapamil on larval development

In the LDA, TBZ and IVM were tested using all isolates of *C. oncophora* and *O. ostertagi*. Both anthelmintics were also tested in combination with VPL and PBO.

4.3.1 Thiabendazole and Verapamil

For the susceptible and IVM-resistant *C. oncophora* isolates EC_{50} values of 0.0037 µg/ml and 0.0062 µg/ml were obtained for TBZ, respectively. For the NZ-resistant isolate, the dose-response-curve obtained for TBZ (Fig. 17A) showed a higher EC_{50} (0.0547 µg/ml) resulting in a significant ($p<0.0001$) shift to the right. The *O. ostertagi* isolates showed a similar pattern to the *C. oncophora* isolates. EC_{50} values with TBZ alone were 0.0062 µg/ml TBZ for *O. o*. sus. and 0.0078 µg/ml for *O. o*. sel. The EC_{50} value of 0.0095 µg/ml TBZ for *O. o.* sel. was obtained after treatment. R^2 values were between 0.0873 and 0.987. EC_{50} values, 95% CI and p-values for TBZ alone and in combination with VPL, obtained for all isolates, are presented in Table 16.

Table 16: EC_{50} values, 95% CI and p-values for TBZ (in µg/ml) alone and in combination with VPL (125 µM) for *C. oncophora* and *O. ostertagi* in the LDA

Isolates	EC_{50} TBZ	95% CI	EC_{50} TBZ+VPL	95% CI	p-value
C. o. sus.	0.0037	0.0035 - 0.0039	0.00026	0.00024 - 0.00027	< 0.0001
C. o. IVM-res.	0.0062	0.0057 -0.0067	0.00023	0.00021 - 0.00025	< 0.0001
C. o. NZ-res.	0.0547	0.0509 - 0.0588	0.00025	0.00024 - 0.00026	< 0.0001
O. o. sus.	0.0062	0.0056 - 0.0068	0.00025	0.00022 - 0.00028	< 0.0001
O. o. sel.	0.0078	0.0072- 0.0083	0.00023	0.00022 - 0.00025	< 0.0001
O. o. sel. p.t	0.0095	0.0087 - 0.0103	0.00025	0.00023 - 0.00026	< 0.0001

p.t.= post treatment

In the presence of VPL, the dose-response-curves obtained for TBZ of all tested isolates of *C. oncophora* and *O. ostertagi* (Fig. 17C & D) shifted far to the left, resulting in an EC_{50} of 0.0002 µg/ml. The differences in EC_{50} values were approximately 15-30 fold in the susceptible isolates and 30-270 fold in the resistant isolates, for the selected isolate the difference was 35-45 fold.

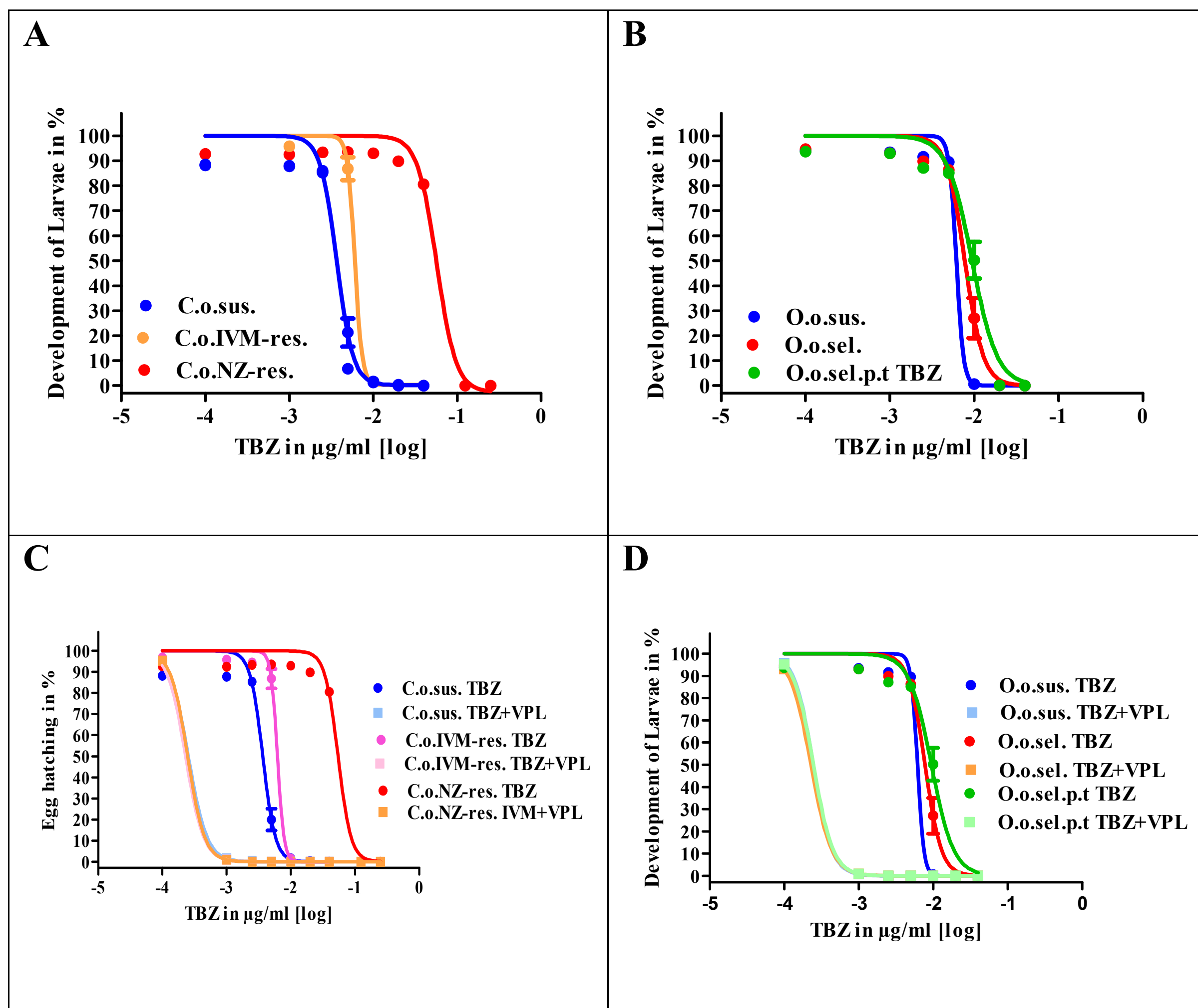


Figure 17: The dose-response-curves for TBZ, TBZ + 125 µM VPL for *C. oncophora* (A+C) and *O. ostertagi* (B+D) isolates in the LDA

4.3.2 Ivermectin and Verapamil

In comparison to the susceptible isolate of *C. oncophora* (EC_{50} 0.12 nM), significant ($p<0.0001$) shifts to the right (Fig. 18) were obtained for the two resistant isolates *C. o*. IVM-resistant and *C. o*. NZ-resistant, with EC_{50} values of 0.52 nM and 0.93 nM, respectively (Table 17).

Table 17: EC_{50} values, 95 % CI, R^2 and p-values for IVM (in nM) and IVM + 125 µM VPL for the *C. oncophora* and *O. ostertagi* isolates in the LDA

Isolates	EC_{50} IVM	95% CI	R^2	EC_{50} IVM+VPL	95% CI	R^2	p-value
C. o. sus.	0.12	0.11 - 0.14	0.938	0.00049	0.00043 - 0.00057	0.998	< 0.0001
C. o. IVM-res.	0.52	0.42 - 0.64	0.905	0.00054	0.00030 - 0.00069	0.998	< 0.0001
C. o. NZ-res.	0.93	0.82 - 1.05	0.967	0.00042	0.00041 - 0.00042	0.1	< 0.0001
O. o. sus.	0.18	0.17 - 0.2	0.977	0.00057	0.00051 - 0.00063	0.999	< 0.0001
O. o .sel.	0.20	0.17 - 0.23	0.915	0.00061	0.00057 - 0.00066	0.999	< 0.0001

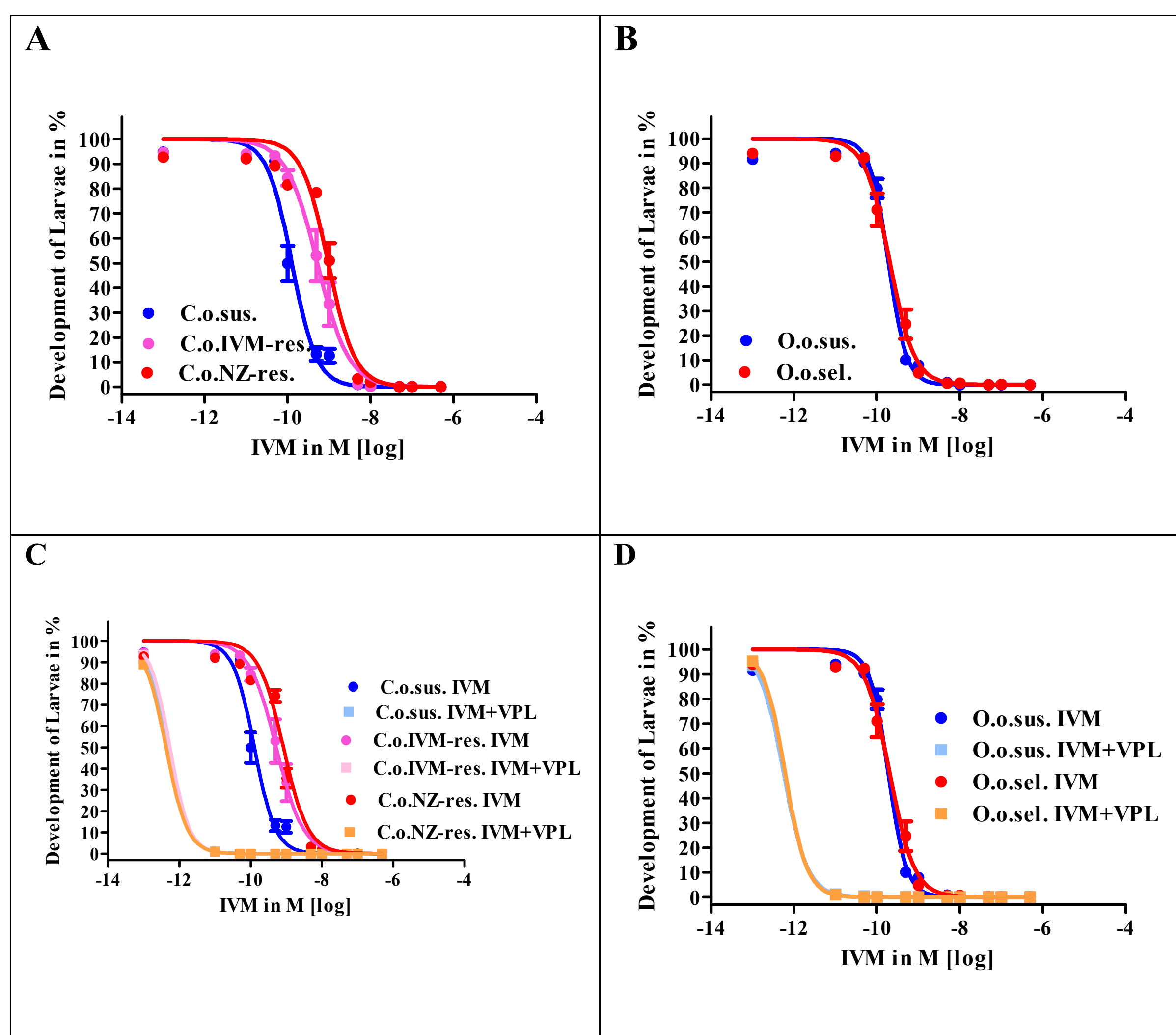


Figure 18: The dose-response-curves for IVM for *C. oncophora* (A) and *O. ostertagi* (B), IVM + VPL (125 µM) for *C. oncophora* (C) and *O. ostertagi* (D) isolates in the LDA

The addition of VPL to the assay significantly enhanced the susceptibility to IVM in all isolates. The effect of VPL was clear for the two resistant *C. oncophora* isolates with a shift of the dose response curve to the left, resulting in a 963 - 1976 fold decrease of the EC_{50}. For the susceptible isolate, the shift was also significant ($p<0.0001$) but only 245 fold. For the two isolates of *O. ostertagi* (sus. and selected), EC_{50} of 0.18 nM and 0.20 nM, respectively, were obtained for IVM. The shift to the left in the presence of VPL was similar to that obtained for the susceptible *C. oncophora* isolate with differences between 316 and 328 fold, respectively.

4.4 Effect of Piperonyl butoxide on larval development

4.4.1 Thiabendazole and Piperonyl butoxide (PBO)

PBO significantly ($p<0.0001$) increased the susceptibility to TBZ in all isolates of *C. oncophora*, resulting in EC_{50} values of 0.0002 µg/ml TBZ. All tested concentrations of PBO resulted in the same EC_{50}. Therefore, only results for the highest (85 µg/ml PBO) and lowest (5.33 µg/ml PBO) concentration tested are presented in Table 18 (for detail results see Appendix 10). The dose-response-curves were shifted to the left in the presence of PBO, similar to those obtained in the LDA with VPL, with differences from 15 to 270 fold (Fig. 19). Both *Ostertagia* (sus. and sel.) isolates appeared susceptible to TBZ in the LDA (EC_{50} 0.0062 µg/ml and 0.0074 µg/ml, respectively). In the presence of PBO they followed the same pattern as the *Cooperia* isolates (30-35 fold). The assays gave reproducible results; R^2 values for all tested combinations were between 0.999-1.000 for all isolates, indicating a good reproducibility.
EC_{50} for TBZ (in µg/ml), TBZ + PBO (85 & 5.33 µg/ml) including 95% CI and p-values obtained for all isolates are given in Table 18.

Table 18: EC_{50} values, 95% CI and p-values for TBZ (in µg/ml), TBZ+ (85 & 5.33 µg/ml PBO) for each isolate of *C. oncophora* and *O. ostertagi* isolates in the LDA

Isolates	EC_{50} TBZ	EC_{50} TBZ+85 µg/ml	95% CI	EC_{50} TBZ+5.33 µg/ml	95% CI	p-value
C. o. sus.	0.0036	0.00021	0.00020 - 0.00023	0.00020	0.00019 - 0.00021	<0.0001
C. o. IVM-res.	0.0061	0.00023	0.00022 - 0.00024	0.00022	0.00021 - 0.00022	< 0.0001
C. o. NZ-res.	0.0547	0.00022	0.00021 - 0.00023	0.00023	0.00022 - 0.00023	< 0.0001
O. o. sus.	0.0062	0.00023	0.00022 - 0.00024	0.00022	0.00020 - 0.00023	< 0.0001
O. o. sel.	0.0074	0.00021	0.00020 - 0.00022	0.00021	0.00019 - 0.00022	< 0.0001

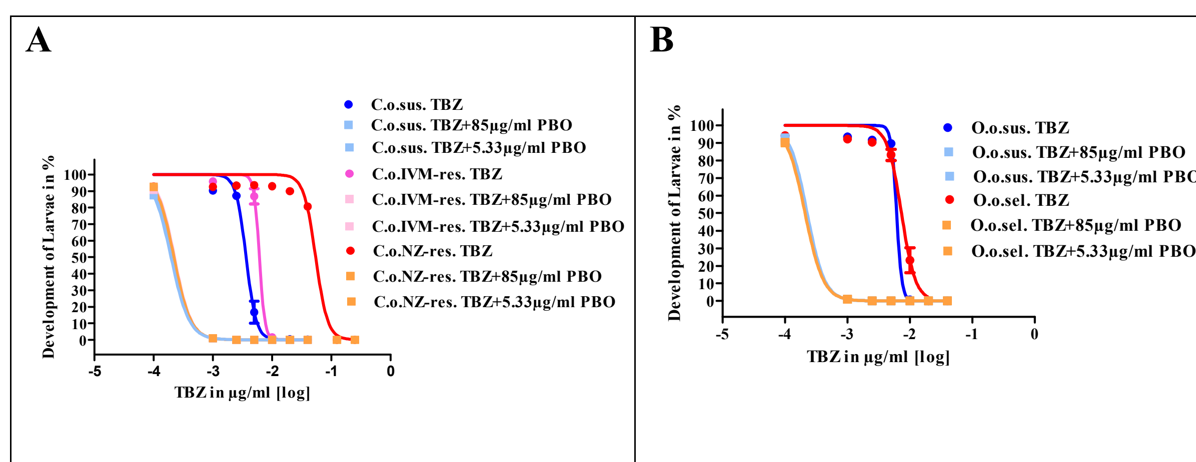


Figure 19: The dose-response-curves for TBZ, TBZ + PBO for *C. oncophora* (A) and *O. ostertagi* (B) isolates in the LDA

4.4.2 Ivermectin and Piperonyl butoxide (PBO)

The use of PBO in combination with IVM rendered the isolates of *C. oncophora* (sus. and res.) susceptible ($p<0.0001$) at all tested concentrations of PBO (Fig. 20). The differences in the EC_{50} values were 231-1694 fold, similar to those obtained with VPL. All EC_{50} values for IVM (in nM) and in combination with the highest and lowest concentration of PBO (85 µg/ml and 5.33 µg/ml), 95% CI, and p values are presented in Table 19. Similar to VPL, PBO shifted the dose-response-curves of both isolates of *O. ostertagi* far to the left in the presence of PBO ($p<0.0001$), resulting in increased susceptibility to IVM at tested concentrations of PBO. The goodness of fit for all tested isolates was between 0.905 and 0.999.

Table 19: The EC_{50} values and 95% CI for IVM (in nM) alone and combined with 85 & 5.33 µg/ml PBO, 95% CI and p-values obtained in the LDA

Isolates	EC_{50} IVM	EC_{50} IVM + 85 µg/ml PBO	95% CI	EC_{50} IVM + 5.33 µg/ml PBO	95% CI	p-value
C. o. sus.	0.12	0.00047	0.00033 - 0.00069	0.00052	0.0004 - 0.00068	< 0.0001
C. o. IVM-res.	0.52	0.00048	0.00044 - 0.00052	0.00048	0.00045 - 0.00051	< 0.0001
C. o. NZ-res.	0.93	0.00049	0.00044 - 0.00054	0.00049	0.00046 - 0.00052	< 0.0001
O. o. sus.	0.18	0.00049	0.00041 - 0.00058	0.00049	0.00046 - 0.00052	< 0.0001
O. o. sel.	0.20	0.00043	0.0004 - 0.00047	0.00044	0.00041 - 0.00047	< 0.0001

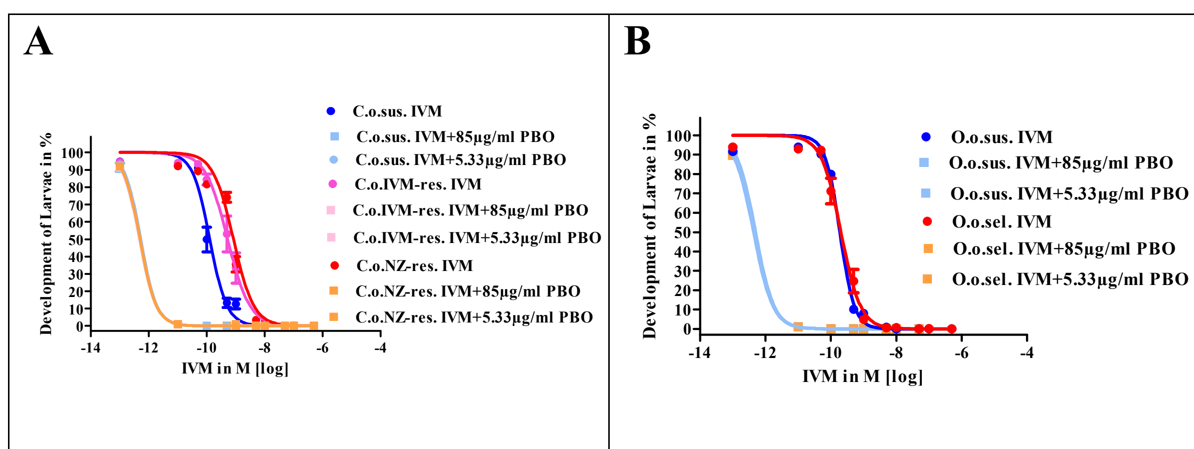


Figure 20: The dose-response-curves for IVM, IVM+PBO for *C. oncophora* (A) and for *O. ostertagi* (B) isolates in the LDA

4.5 Effect of Verapamil on larval migration

The LMIA was carried out using IVM and LEV to determine whether third stage larvae of *C. oncophora* (sus. IVM-res. and NZ-res.) and *O. ostertagi* (sus. and selected) are resistant to IVM and LEV. A dose-response-curve was obtained based on the inhibitory effect of different concentrations of the anthelmintics on the motility of larvae. IVM and LEV were used alone and in combination with 25 µM VPL.

4.5.1 Ivermectin and Verapamil

For all *Cooperia* isolates dose response curves for IVM (Fig. 21) were obtained with EC_{50} values of 118 nM and 348 nM for *C. o.* sus. and *C. o.* IVM-res., respectively, while the resistant isolate *C. o.* NZ-res. showed a significantly (p<0.0001) increased EC_{50} of 2490 nM (Table 20). The addition of VPL to the assay resulted in increased susceptibility to IVM (p<0.0001) with a shift to the left of all isolates. The dose response curves were shown in (Fig. 21). The differences were 27 and 45 fold for the IVM-res. and NZ-res. isolate and only 2 fold for the susceptible isolate, which was not significant.

The EC_{50} values obtained for *O. ostertagi* isolates (*O. o.* sus. and *O. o.* sel.) were 398 nM and 200 nM, respectively. In the presence of VPL the dose response curves were shifted significantly to the left (p-value <0.0001) in both isolates, *O. o.* sus. and *O. o*. sel., where EC_{50} values of 33.4 nM and 56.5 nM, were obtained, resulting in a difference of 12 fold for the susceptible and 3.5 fold for the selected isolate. The EC_{50} values for IVM, IVM with 25 µM VPL for both isolates including 95% CI, R^2 and p values are presented in Table 20.

Table 20: The EC_{50} values for IVM (in nM) and IVM with 25 µM VPL including 95% CI, R^2 and p-values for the two curves of each isolate of *C. oncophora* and *O. ostertagi* in the LMIA

Isolates	EC_{50} IVM	95% CI	R^2	EC_{50} IVM+VPL	95% CI	R^2	p-value
C. o. sus.	118	106 - 133	0.923	52.5	43.5 - 63.5	0.917	<0.0001
C. o. IVM-res.	348	308 - 393	0.933	13	10.7 - 15.9	0.861	<0.0001
C. o. NZ-res.	2490	1338 - 4633	0.788	61.5	46.9 - 80.7	0.875	<0.0001
O. o. sus.	398	341 - 463	0.873	33.4	25.1 - 44.3	0.851	<0.0001
O. o .sel.	200	145 - 226	0.914	56.5	41.8 - 76.3	0.791	<0.0001

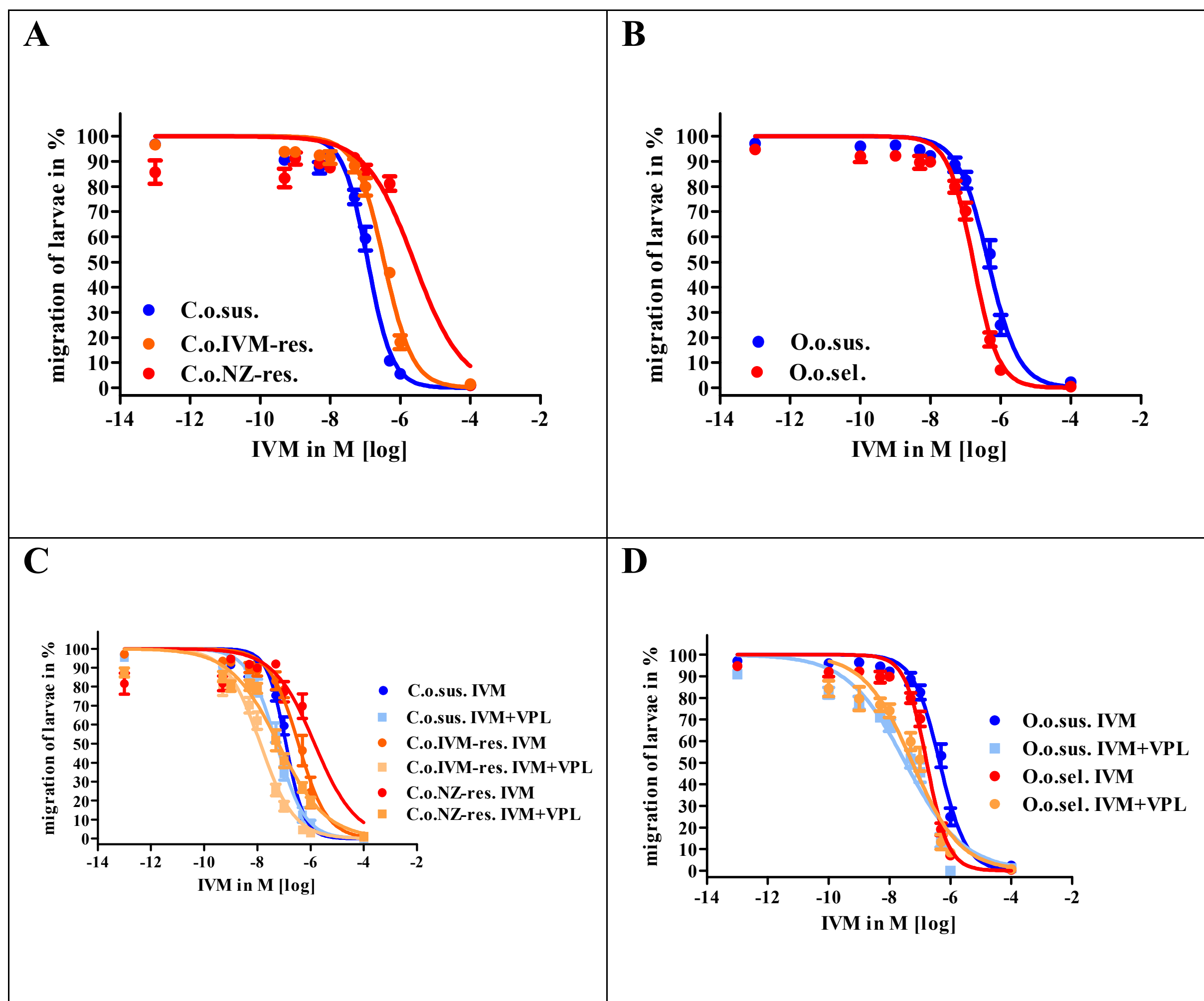


Figure 21: The dose-response-curves for IVM, IVM + 25 µM VPL for *C. oncophora* (A & C) and *O. ostertagi* isolates (B & D) in the LMIA

4.5.2 Levamisole and Verapamil

The LMIA was performed with larval stages of *C. oncophora* (sus. and IVM-res.) and *O. ostertagi* (sus. and sel.). For LEV the concentrations used ranged from 5×10^{-4} M to 5×10^{-7} M for resistant isolates and from 5×10^{-5} M to 5×10^{-8} M for the susceptible isolates.

For the susceptible *C. oncophora* isolate, the dose-response-curve showed an EC_{50} of 989 nM, for the *C. o.* IVM res. isolate 1015 nM. The EC_{50} values for LEV between the two *C. oncophora* isolates were not different ($p = 0.777$) (Fig. 22).

Table 21: EC_{50} values for LEV (in nM) and LEV+25 µM VPL, 95 % CI, R^2 and calculated p-values for the two curves of all isolates of *C. oncophora* and *O. ostertagi* in the LMIA

Isolates	EC50 LEV	95% CI	R^2	EC_{50} LEV+VPL	95% CI	R^2	p-value
C. o. sus.	989	854 - 1144	0.853	1152	1005 - 1322	0.922	0.171
C. o. IVM-res.	1015	924 - 1116	0.859	884	791 - 987	0.889	0.071
O. o. sus.	1117	964 - 1295	0.903	962	800 - 1157	0.909	0.218
O. o .sel.	293	272 - 339	0.920	226	119 - 267	0.911	0.01

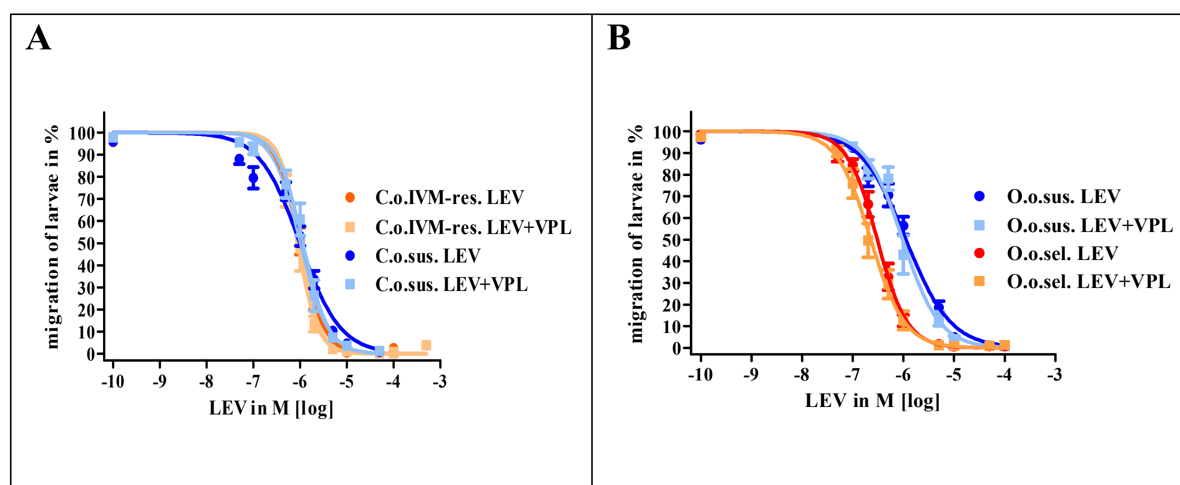


Figure 22: The dose-response-curves for LEV, LEV+25 µM VPL for *C. oncophora* (A) and for *O. ostertagi* (B) isolates in the LMIA

The addition of VPL to the assay caused no changes in the inhibitory effect of LEV on larval migration of *C. o.* sus. with an EC_{50} of 1152 nM (p = 0.171). In the *C. o.* IVM-res., the EC_{50} value for LEV+VPL was slightly reduced, resulting in an EC_{50} of 884 nM (Table 21), but this difference was still not significant (p = 0.071).

The *O. o.* sel. isolate appeared to be more susceptible than *O. o.* sus. for which an EC_{50} of 1117 nM was obtained. In comparison, *O. o.* sel. had an EC_{50} of 293 nM (p-value<0.0001).

For *O. o.* sus. no increased effect of LEV in the presence of VPL on the inhibition of larval migration (EC_{50} = 962 nM) was observed (p = 0.218). An apparently slight pronounced effect on the larval migration of *O. o* sel., with an EC_{50} of 226 nM, was seen (p = 0.01). The EC_{50} for LEV alone and in combination with 25 µM VPL, the R^2, 95% CI and p values for both isolates are found in Table 21.

4.6 Effect of Piperonyl butoxide on larval migration

IVM in combination with VPL had no different effect on the larval migration of *C. oncophora* and *O. ostertagi* than IVM alone; therefore the assay was performed only using IVM + PBO. In combination with IVM most of the lower concentrations of PBO revealed almost no increased effect on the inhibition of migration of larvae in all isolates of *C. oncophora* and *O. ostertagi* (data not shown). Therefore, the assay was performed using all concentrations of IVM in combination with PBO only in the concentration of 85 µg/ml. The dose-response-curves (Fig. 23) obtained for IVM for all isolates were shifted clearly to left ($p < 0.0001$). EC_{50} values for IVM (in nM) and IVM+PBO obtained for all isolates including 95% CI are shown in Table 22. The R^2 values of all isolates for IVM+PBO were between 0.729 - 0.923. The differences in EC_{50} were 45-226 fold in resistant isolates, 7-104 fold in susceptible isolates and 87 fold for the *O. o.* sel. isolate.

Table 22: EC_{50} values for IVM (in nM) and IVM+85 µg/ml PBO including 95% CI and p-values for the two curves for all isolates of *C. oncophora* and *O. ostertagi* in the LMIA

	***C. o.* sus.**	***C. o.* IVM-res.**	***C. o.* NZ-res.**	***O. o.* sus.**	***O. o.* sel.**
EC_{50} IVM	118	348	2490	398	200
95% CI	106 - 133	308 - 393	1338 - 4633	341 - 463	177 - 226
IVM+85µg/ml PBO	17.7	7.77	11.1	3.84	2.30
95% CI	11.5 - 27	5.10 - 11.8	6.48 - 19	2.07 - 7.11	1.45 - 3.66
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

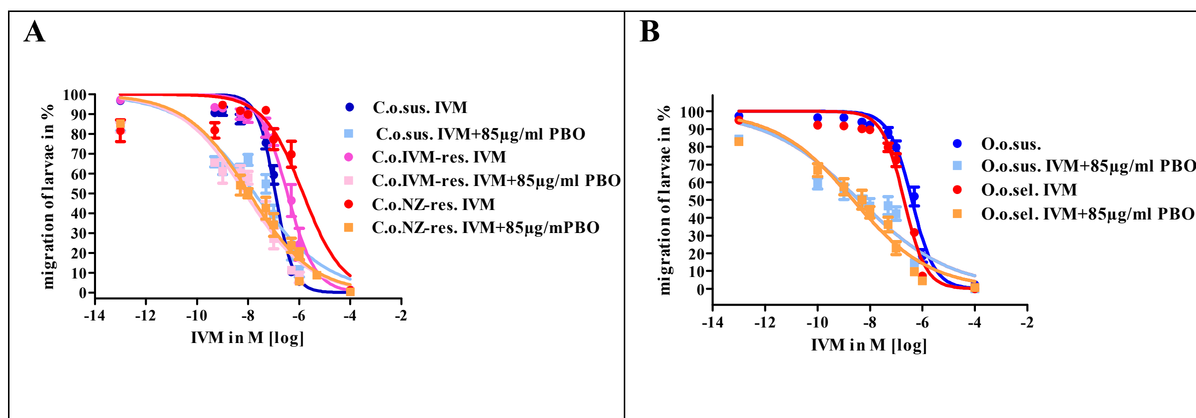


Figure 23: The dose-response-curves for IVM, IVM + 85 µg/ml PBO for *C. oncophora* (A) and *O. ostertagi* (B) isolates in the LMIA

5 Discussion

The family *Trichostrongylidae* contains the majority of species of gastrointestinal parasites in ruminants and other grazing animals. *Cooperia oncophora* and *Ostertagia ostertagi* are the most important pathogenic parasites of cattle, leading to inappetence, watery diarrhoea and, in some cases, death. Resistance to anthelmintic drugs has become a serious problem, particularly in small ruminants (Jackson & Coop, 2000). Resistance has been reported for all three broad spectrum anthelmintic drug classes available for the control of gastrointestinal parasites in small ruminants (Sangster & Gill, 1999), the BZ, LEV and MLs. Recently, an increasing amount of cattle nematodes becoming resistant to multiple anthelmintic classes in New Zealand, South America and Europe (Loveridge et al., 2003; Mejia et al., 2003; Demeler et al., 2009) and in horses (Kaplan, 2004; Molento et al., 2008; Traversa et al., 2009) have been reported. Resistance to anthelmintics can develop through different mechanisms such as changes in the target sites of drugs (Roos et al., 1990), detoxifying processes which increase the activity of specific enzymes (Sutherland et al., 1989) and increased drug efflux by membrane transporters, causing changes in metabolism and distribution of drugs in the target organism (Kerboeuf et al., 2003).

Specific and non-specific mechanisms are thought to be involved in the development of anthelmintic resistance. The specific mechanisms of anthelmintic resistance have been attributed to several changes in the intracellular target sites, e.g. the β-tubulin for BZs, nicotinic acetylcholine receptors for LEV and pyrantel and ligand gated chloride channel receptors for MLs. These receptors can be found in neuromuscular cells and in the central nervous system. In nematodes particularly GluCl channels have been identified as targets for MLs. By the binding of anthelmintic drugs to these target sites they are irreversibly opened and cause hyperpolarisation of cells. Certain GluCl channel variants have been implicated in ML resistance in *C. oncophora* (Njue & Prichard, 2004).

Anthelmintics have to be absorbed or ingested by the parasite to be transported into the cells. The P-glycoproteins (Pgp) are members of the ATP-binding cassette (ABC) transporter superfamily and are known to play an important role in the transport of endogenous and exogenous xenobiotic substances in nematodes (Lespine et al., 2009). They have been studied in several free-living and parasitic stages of nematodes (Lincke et al., 1992; 1993; Sangster, 1994; Broeks et al., 1995) which are quite different in their morphology and biology. Nematodes possess some structures of protection such as sheaths and cuticle, which

complicate the detection of the presence of Pgp in their tissue. Nevertheless, Pgps have been identified in organisms such as *C. elegans* and *H. contortus* using specific monoclonal antibodies or lectins and in a number of studies their presence and involvement in anthelmintic resistance have been suggested. Additional evidence that Pgp may play a role in the mechanism of resistance has been reported also in *Leishmania donovani* (Henderson et al., 1992; Chow et al., 1993) and *Plasmodium falciparum* (Foote et al., 1989; Wilson et al., 1989). In studies in mice where the Pgp-encoding mdr-1 gene had been disrupted, neurological symptoms in animals carrying the mutation were observed. This suggests the interaction of Pgps in the blood-brain barrier with IVM (Schinkel et al., 1994).

The first report of presence of Pgps in the free-living nematode *C. elegans* was given by Lincke et al. (1992). Compared to only one Pgp in mammalian cells, fourteen genes that encode Pgps have so far been identified in the *C. elegans*. Up to now only Pgp-1 and Pgp-3 have been studied in more detail. Followed by their localisation in nematodes, the involvement of Pgps in resistance to xenobiotics in *C. elegans* was analysed by Broeks et al. (1995). While Pgp-1 has been shown to contribute to resistance to heavy metal ions (Broeks et al., 1996), loss of Pgp-3 was found to lead to hypersensitivity to chloroquine and colchicine (Broeks et al., 1995). In further studies it was revealed the IVM acts as a substrate for mammalian Pgp (Didier et al., 1995).

Flow cytometry analysis indicated the increased presence of Pgp in eggs of a *H. contortus* isolate which is resistant to IVM and BZ when compared with eggs from a susceptible isolate (Kerboeuf et al., 1999). Pgps are most likely located in the egg shell which has previously been recognised as a protective structure for the parasite against environmental conditions. The egg shell may additionally contain molecules which are responsible for the detoxification of drugs (Kerboeuf et al., 1999).

VPL has been shown to inhibit the function of Pgps and simultaneously cause hyperphosphorylation of serin residues (Hamada et al., 1987). This effect has been suggested as an explanation for the action of VPL on voltage-gated Ca^{2+} channels (Nygren & Larsson, 1990) as well as in human cancer cells (Beugnet et al., 1997; Kerboeuf et al., 1999; 2002) and also in protozoa. The co-administration of VPL as an inhibitor for Pgps with IVM or MOX has been shown to increase the efficacy of both drugs against moxidectin-selected *H. contortus* (Xu et al., 1998), indicating that Pgp may be involved in ML resistance.

Beugnet et al. (1997) reported that VPL was able to partially reverse BZ resistance in free-living stages of *H. contortus*. The resistance can be associated with decreased affinity of

lectin for the Pgp site with 50% reversion in the resistance to BZ, indicating the functional role of the Pgp glycosylation (Kerboeuf et al., 2003). Reports of Pgp-mediated resistance to BZ, imidazothiazole and tetrahydropyrimidines underline the involvement of Pgps in resistance to these anthelmintics in parasitic nematodes (Kerboeuf et al., 2003). Facing the increasing problem of anthelmintic resistance worldwide, these phenomenons need to be further studied.

Detoxification of xenobiotics is not only mediated by extrusion but frequently also by hydroxylation, catalysed by monooxygenases such as CYPs. MLs are also known to be metabolised by mammalian cytochrome P450 enzymes (Chiu & Lu, 1989). Similarly, CYP has been shown to play an important role in the mechanism of resistance in insects (Daborn et al., 2002). Earlier studies of Kotze (1998; 2000) suggested no role for the involvement of CYPs in ML, BZ or imidazothiazole resistance. In contrast, Alvinerie et al. (2001) found evidence for the ML metabolism mediated by nematode CYPs. Moreover it was shown that the efficacy of rotenone can be improved by the use of the CYP inhibitor PBO in *H. contortus* and *T. colubriformis in vitro*, suggesting an important role of CYP enzyme systems in drug-detoxification in both species (Kotze et al., 2006).

Experiments to investigate the potential role of CYPs in anthelmintic resistance were therefore included in the current study.

To increase knowledge regarding the understanding of the mechanism of resistance is a key requirement in order to minimise the spread of anthelmintic resistance. In the present study, the putative involvement of these mechanisms was investigated using specific inhibitors of Pgps (VPL) and CYPs (PBO) in combination with anthelmintics (TBZ, IVM and LEV) in the egg hatch assay (TBZ), larval development assay (TBZ and IVM) and larval migration inhibition assay (IVM and LEV). Free-living stages of *C. oncophora* and *O. ostertagi* isolates were employed to determine whether the response to the drugs was changed when either Pgps or CYPs were inhibited.

The use of the inhibitors studied in this work in combination with anthelmintics have been suggested in the past due to their potential action in reducing the function of Pgp-mediated (Stenhouse, 2007) and CYP-mediated detoxification (Benchaoui & McKellar, 1996; Kotze et al., 2006) of anthelmintics. In *in vitro* tests, i.e. the larval development assay and a larval migration inhibition assay using parasitic stages of *C. oncophora*, the Pgp inhibitor VPL enhanced the susceptibility of *C. oncophora* larvae to IVM (Demeler, 2009). In the present study increased susceptibility of all isolates to IVM and TBZ in combination with 125 µM

VPL and all tested concentrations of PBO was observed in all *in vitro* assays performed. The comparison of EC_{50} values of both, susceptible and resistant isolates, indicates a significant role of drug transport and/or enzymatic metabolism in the susceptibility of helminths to different anthelmintic drugs.

5.1 Effect of Verapamil

5.1.1 Verapamil in combination with TBZ

Egg hatch assays (EHA) were carried out using TBZ to investigate the effect of TBZ alone and in combination with VPL on the egg hatching of nematodes using susceptible and resistant isolates of *C. oncophora* and *O. ostertagi.* For all isolates sigmoidal dose-response curves were obtained. The aim was to study the inhibitory effect of VPL on egg hatching. The EHA for benzimidazole resistance was first described by (Le Jambre, 1976) and has since been used with various modifications by a number of workers (Taylor et al., 2002).
The inhibitory concentration value for TBZ (0.1 µg/ml) was defined by Taylor (1990) as the percentage of eggs hatching in the discriminating dose (EC_{50}) indicating resistance to BZs. This discriminating dose for TBZ in the EHA has been established in sheep parasitic nematodes but is to our knowledge not confirmed for cattle parasitic nematodes yet. The EC_{50} values for TBZ in the susceptible and IVM-resistant isolates of *C. oncophora* used in this study were 0.043 µg/ml and 0.045 µg/ml, respectively. These values are both below 0.1 µg/ml, indicating susceptibility to TBZ. This was confirmed in an *in vivo* study, where treatment with albendazole eliminated the worms (FECR = 100%) in the calves (data not shown). The obtained EC_{50} values for TBZ for *C. oncophora* and *O. ostertagi* are similar to the ones obtained in previous ring tests which were carried out in different laboratories (von Samson-Himmelstjerna et al., 2009). These authors reported EC_{50} values for *C. oncophora* of 0.046 µg/ml and for *O. ostertagi* of 0.028 µg/ml. For the susceptible *O. ostertagi* isolate, an EC_{50} value of 0.037 µg/ml TBZ and for the BZ-selected *O. ostertagi* isolates EC_{50} values of 0.039 µg/ml (pre treatment) and 0.063 µg/ml (post treatment) were obtained. The FECR in animals infected with the BZ-selected *O. ostertagi* isolate showed that despite the increased EC_{50} values (following treatment with 35% of the recommended dose), the parasites were eliminated when the full recommended dose of albendazole (data not shown) was used. The fact, that the EC_{50} value for the IVM-res. *C. oncophora* isolate is similar to those obtained for

the susceptible isolates is in accordance with the *in vivo* observations (FECR = 100%) following treatment with albendazole (data not shown). In comparison to the susceptible isolates, the obtained EC_{50} value for the *C. oncophora* NZ-res. isolate is significantly higher (0.11 µg/ml), indicating resistance to BZs *in vivo*. This was confirmed in a FECRT, where treatment with the full dose (100%) and a double dose (200%) of albendazole failed to remove the parasites (data not shown).

From a field study in New Zealand, EC_{50} values for oxfendazole-resistant (0.52 mg TBZ/l) and susceptible (0.023 mg TBZ/l) *Cooperia* spp. isolates were published by Jackson et al. (1987). Borgsteede et al. (1992) investigated a mixed population derived from the field which included *Ostertagia leptospicularis* and *O. ostertagi*, which were suspected to be fenbendazole resistant. The EHA performed with *O. ostertagi* revealed an EC_{50} value of 0.032 µg/ml suggesting that this isolate was susceptible.

The congruence of the present *in vitro* and *in vivo* data for *C. oncophora* and *O. ostertagi* suggests that the discriminating dose of 0.1 µg/ml TBZ can also be used for cattle parasites. It obviously demonstrates that the onset of resistance in a population, which is not detectable *in vivo* (FECRT), can be diagnosed and quantified using the EHA.

The addition of VPL to the assay had no highly significant effect ($p = 0.03$) on egg hatching of *C. oncophora* sus., with an only minimal decreased EC_{50} of 0.041 µg/ml. A slightly bigger effect was obtained for *C. oncophora* IVM-res. (0.034 µg/ml TBZ), but still not highly significant ($p = 0.002$). Only for the *C. oncophora* NZ-res. isolate the EC_{50} of 0.022 µg/ml for TBZ in the presence of VPL was statistically significantly different ($p < 0.0001$). Out of all isolates used in this study, this is the only one with confirmed BZ-resistance *in vivo* and *in vitro*, suggesting that a mechanism potentially involved in resistance against BZs is not generally present in the eggs of the susceptible isolates studied herein.

For *O. ostertagi* (sus. and sel.) isolates similar data to those in *C. oncophora* were obtained. In the presence of VPL the EC_{50} values were reduced to 0.034 µg/ml and 0.030 µg/ml TBZ concerning the susceptible and selected *O. ostertagi* isolates, respectively. For the EHA, which was performed initially after treatment, the EC_{50} was significantly reduced ($p<0.0001$) from 0.063 to 0.022 µg/ml when VPL was added, indicating that VPL increased susceptibility to TBZ.

In contrast, in a recent study, 25 µM VPL had no effect on the egg hatching of resistant and susceptible field populations of *T. circumcincta* in sheep (Sargison et al., 2011).

The LDA, a liquid based assay, was carried out as previously described by Demeler et al. (2010a) to investigate the effect of TBZ on the development of larvae of *C. oncophora* and

O. ostertagi isolates alone and in the presence of VPL. The aim was to investigate whether a combined use of TBZ with VPL has an additional inhibitory effect on larval development of these isolates. For all isolates tested, dose response curves were obtained.
When TBZ was used alone, the EC_{50} for *C. o.* sus. and *C. o*. IVM-res. for TBZ (0.0037 µg/ml and 0.0062 µg/ml) indicated a similar susceptibility. Resistance to TBZ was detected in the *C. o.* NZ-res. isolate by in an EC_{50} of 0.0547 µg/ml. Both isolates of *O. ostertagi* (sus. and sel.) showed a similar susceptibility to TBZ as the BZ-susceptible *C. oncophora* isolates. The EC_{50} values were 0.0062 µg/ml and 0.0078 µg/ml (0.0095 µg/ml TBZ post treatment), respectively. These data are in agreement with the data obtained from the EHA, where the IVM-res. *C. oncophora* isolate was fully susceptible to TBZ and the BZ-selected isolate of *O. ostertagia* showed an increased EC_{50} value. In another recent study, EC_{50} values for TBZ of susceptible and IVM-resistant isolates of *C. oncophora* were 0.0051 µg/ml and 0.0056 µg/ml and for a susceptible and BZ-selected of *O. ostertagi* 0.0018 µg/ml and 0.0036 µg/ml, respectively (Demeler et al., 2010a).
A combined use of VPL with TBZ rendered all isolates, susceptible, selected and resistant, completely susceptible to TBZ, demonstrated by the dose-response-curve-shifting far to the left ($p < 0.0001$). In the presence of VPL, no significant difference between the EC_{50} values of all, susceptible, selected and resistant isolates (EC_{50} 0.0002 µg/ml TBZ) was detected. The differences between TBZ alone and TBZ + VPL were between 15-270 fold for all tested isolates, indicating an inhibitory effect of VPL on larval development.

5.1.2 Verapamil in combination with IVM

In contrast to the LMIA, the LDA measures the effect of IVM on pharyngeal muscles (Gill et al., 1995). In the present study the effect of IVM in combination with VPL on the larval development of *C. oncophora* (sus. IVM-res. and NZ-res.) and *O. ostertagi* (sus. and sel.) was demonstrated. The assay gave reproducible results similar to those obtained in the LDA with TBZ.
For the susceptible *C. oncophora* and *O. ostertagi* isolates EC_{50} values of 0.12 nM and 0.18 nM were obtained for IVM. For the BZ-selected *O. ostertagi* isolate an EC_{50} of 0.20 nM was obtained. The EC_{50} values for the *C. o.* IVM-res. and *C. o.* NZ-res. isolates were significantly higher (0.52 nM and 0.93 nM), respectively. In all tested isolates, the dose-response-curves for IVM were shifted to the left ($p<0.0001$) when IVM was combined with

VPL. No significant differences between the EC_{50} values for IVM in the presence of VPL between all isolates (0.00042-0.00061 nM) were detected.
The present results showed that the inhibition of Pgp increases the susceptibility to IVM. Using the LDA, Demeler (2009) reported that in *C. oncophora* the EC_{50} value for IVM was shifted to the left when IVM was combined with VPL. In the presence of VPL similar EC_{50} values for a susceptible and a resistant isolate of *C. oncophora* were detected. The results suggest that the inhibition of Pgps does increase the susceptibility to IVM, which contributes to the increased response of IVM-resistant isolates.

In the LMIA, the effects of IVM on the motility of third larval stages of all nematode species alone and in presence of VPL were examined. The LMIA is a suitable *in vitro* test for the detection of IVM and LEV resistance, since the paralysing effect of these drugs on the somatic muscles of L3s can be investigated. The LMIA was performed according to a protocol recently standardised for the LMIA in cattle nematodes (Demeler at el., 2010a). Sigmoid dose-response-curves were obtained for all isolates and drugs investigated in the LMIA.
In the present study the use of the LMIA to investigate a potential effect of VPL was tested. In the LMIA the ability of L3s to migrate through fine nylon mesh filters was analysed for a range of IVM or LEV concentrations, using larvae obtained from coprocultures. The LMIA was standardised by Demeler et al. (2010a) for cattle nematodes, where a mesh size was chosen to allow active migration but prevent passive falling of larvae through the sieve. The EC_{50} values obtained for an IVM-susceptible and an IVM-resistant *C. oncophora* isolate were 123 nM and 621 nM. For an IVM-susceptible *O. ostertagi* isolate, an EC_{50} value of 404 nM was obtained. These results showed that susceptible *O. ostertagi* exhibit a higher EC_{50} value than susceptible *C. oncophora*. In the present study, EC_{50} values of 118 nM for *C. o*. sus. and 398 nM for *O. o*. sus. were determined. The EC_{50} of the IVM resistant *C. oncophora* isolate (348 nM) was lower than the concentration which was previously reported for resistant *Cooperia* spp. (621 nM) (Demeler et al., 2010a). Other authors (Fritzen, 2005) determined an IVM EC_{50} value of 1230 nM for a susceptible *C. oncophora* isolate. This discrepancy can be explained by the fact, that in the latter study within the LMIA procedure the exposure to ivermectin before migration was limited to 2 hours. Since initial (<3 h) paralysing effects of IVM are reversable up to 12 h (Geary et al., 1993; Demeler, 2005), this protocol is unable to detect irreversible medium-term (>18 h) effects, resulting in apparently decreased susceptibility to IVM in this study.

Compared to the *C. o.* IVM-res. isolate (RR of 3), the *C. o.* NZ-res. isolate was highly resistant (EC_{50} of 2490 nM) to IVM in the LMIA, with a shift in the dose-response-curve far to the right (RR of 20.9). For the two isolates of *O. ostertagi* (sus. and sel.) EC_{50} values of 398 nM and 200 nM were obtained. In the presence of VPL the dose-response-curves for all isolates were shifted to the left.
When VPL was combined with IVM, an effect on larval migration within the *C. o*. sus., *C. o*. IVM-res. and *C. o.* NZ-res. isolates was observed. The EC_{50} values for IVM with VPL were significantly reduced ($p<0.0001$). This was least pronounced in the *C. o.* sus. isolate (2.2 fold), followed by the *O. o.* sel. isolate (3.5 fold) and the *O. o.* sus. isolate (12 fold). For the *C. o.* IVM-res. isolate a fold of 26.8 and for the *C. o.* NZ-res. isolate a fold of 40.5 was observed. The dose-response-curves were shifted significantly to the left (p-value <0.0001) in all isolates. These results indicate that in isolates resistant to IVM the addition of VPL leads to more drastic effects (increased susceptibility). In a recent study Demeler (2009) investigated the role of VPL in the LMIA, where it enhanced the susceptibility of parasitic stages of *C. oncophora* (sus. and IVM-res.) to IVM similarly to the results of the present study.

The response of nematodes to anthelmintics in the presence of VPL suggests an important role for detoxifying mechanisms such as of Pgps in drug resistance. The observed effects could be due to the location of Pgp in either the mid-gut epithelium or the H-shaped excretory cells, where at least some of the Pgps have been localised in *C. elegans* (Broeks et al., 1995).
Overexpression of Pgps might enhance extrusion of anthelmintics in resistant isolates as proposed by Prichard & Roulet (2007). Additional evidence for the role of Pgps in ML resistance comes from several studies indicating selection on Pgp loci in different parasitic nematode species frequently exposed to MLs (Blackhall et al., 1998; Ardelli et al., 2005).

5.1.3 Verapamil in combination with LEV

The effect of VPL on the larval migration of *C. oncophora* (sus. and IVM-res.) and *O. ostertagi* (sus. and sel.) isolates in the presence of LEV was tested. A minor inhibitory effect on LEV in the presence of VPL was observed for larval migration of *C. o*. IVM-res. and *O. o.* sel., with EC_{50} values of 1015 nM and 293 nM for LEV being slightly reduced to 884 nM and 226 nM in the presence of VPL ($p = 0.071$ & 0.01). The obtained EC_{50} value for LEV and VPL for the susceptible *C. oncophora* was slightly higher than with LEV alone, EC_{50}

values were 989 nM and 1152 nM, respectively, but this difference was not significant (P = 0.171).

In a previous study no association between Pgp and resistance to closantel (tetrahydropyrimidine) and LEV (imidazothiazole) in three resistant isolates of *H. contortus* was observed (Kwa et al., 1998).

5.2 Effect of Piperonyl butoxide

5.2.1 Piperonyl butoxide in combination with TBZ

The EHA with PBO was performed using stages of *C. oncophora* (sus. and NZ-res.) and *O. ostertagi* (sus. and sel.) as well as *C. oncophora* only resistant to IVM. The inhibitory effect of TBZ on egg hatching was clearly observed for all susceptible isolates with EC_{50} values between 0.038 and 0.043 µg/ml. For the *C. o.* NZ-res. isolate, an EC_{50} of 0.11 µg/ml was observed. All tested concentrations of PBO increased the susceptibility to TBZ ($p< 0.0001$) but no dose-dependent response was observed for the BZ-susceptible isolates. The effects were similar at the highest (8.5 µg/ml) and lowest concentration (0.266 µg/ml). The obtained EC_{50} values for TBZ + PBO for the *C. o.* NZ-res. isolate tested were similar to the EC_{50} values of the susceptible isolates when the highest concentration of PBO was used (0.02 µg/ml). However, at the lowest PBO concentration a higher EC_{50} value of 0.046 µg/ml was obtained, indicating a dependency on the concentration of PBO.

Stenhouse (2007) investigated the effects of the same two inhibitors (VPL and PBO) on non-specific xenobiotic removal mechanisms in BZ-resistant and susceptible isolates of *T. circumcincta* and showed that the inhibition of these pathway reversed the phenotype of the resistant isolate in an *in vitro* assay (EHA). In contrast, Sargison et al. (2011) could not find PBO induced changes in the EC_{50} value or dose-response-curve of a field population of the sheep parasitic nematode *T. circumcincta*.

Using TBZ in the LDA, sigmoid dose-response-curves were obtained for all isolates with only the *C. o* NZ-res. isolate showing resistance to TBZ (EC_{50} of 0.054 µg/ml). For TBZ, a potent additional effect of PBO on the drug-mediated inhibition of development of *C. oncophora* (sus., IVM-res. and NZ-res.) and *O. ostertagi* (sus. and sel.) isolates, similar to those in the LDA with TBZ + VPL, was observed. All tested concentrations of PBO had the significant

effect of reducing the EC_{50} values to 0.0002 µg/ml TBZ for all isolates. A combined use of TBZ with PBO rendered all isolates completely susceptible in the presence of PBO.

5.2.2 Piperonyl butoxide in combination with IVM

In the LDA with IVM and PBO, similar results than for TBZ + PBO where obtained. EC_{50} values were reduced 244 and 455 fold for the susceptible and between 1083 and 1898 fold for the resistant isolates resulting in EC_{50} values of 0.0004 nM for all isolates. In the presence of PBO, the dose-response-curves of all isolates were therefore shifted far to the left ($p<0.0001$), indicating an increased susceptibility to IVM.

In the LMIA, a change of response to IVM of the larval migration of *C. oncophora* (sus. IVM-res. and NZ-res.) and *O. ostertagi* (sus. and sel.) isolates was only observed at the highest concentrations of 85 µg/ml PBO (other data not shown). The decrease in EC_{50} values were 45-226 fold in the resistant isolates and 7-104 fold in susceptible isolates. For the TBZ selected isolate of *O. ostertagi* it was 87 fold. The addition of inhibitors in the LDA increased the susceptibility to the tested anthelmintics much more pronounced than in the LMIA which is independent of the inhibitor used. This effect might be due to the different life cycle stages exposed to the drugs and/or to the inhibitors in these two assays. While the LDA measures effects related to pharyngeal pumping, the L3s used in the LMIA do not feed. Generally two different factors can contribute to these findings: increased expression of detoxifying proteins (Pgps, CYPs) or decreased permeability of the L3s compared to L1s and L2s. The latter might be particularly important, as drugs and inhibitors are supposed to have free access to the mid-gut epithelium in feeding larvae but have to diffuse through the cuticle in non-feeding L3s.

The objective of this study was to use the two inhibitors VPL and PBO to evaluate, if Pgps and CYPs are involved in anthelmintic resistance processes. The results show that the *C. oncophora* NZ-res. isolate, which is resistant against IVM and TBZ, is comparatively more efficient in the detoxification of drugs through non-specific resistance mechanisms than the other isolates. Despite a higher resistance against IVM, in the presence of the tested inhibitors the dose-response-curves for the *C. o.* NZ-res. isolate were shifted back to a similar level as all other isolates.

MLs are known to be metabolised by mammalian CYPs (Chiu & Lu, 1989), however, the present study suggests that parasitic nematode CYPs have the same capability. Kotze (1997;

1998; 2000) found evidence for enzymatic activity of CYPs parasitic nematodes using various classical CYP substrates (e.g. aldrin, 7-ethoxycoumarin, VPL, Nicardipine, PBO, Tridiphane) but detected no differences between anthelmintic resistant and susceptible isolates. PBO was shown to increase susceptibility to the CYP substrate rotenone but could again not be correlated with the resistance status of the tested isolates in terms of their EC_{50} values for the response to BZ and IVM *in vitro* (Kotze et al., 2006). On the other hand, Alvinerie et al. (2001) demonstrated direct metabolism of MOX by CYPs in *H. contortus.* Since different substrates are presumably metabolised by different CYPs, results obtained using one particular chemical compound can not automatically be transferred to even structurally closely related compounds.

6 Conclusions

In the present thesis, three *in vitro* tests (EHA, LDA and LMIA) for the detection of anthelmintic resistance in cattle were successfully established for the investigation of alterations in the susceptibility to anthelmintics. There was strong evidence for the role of two inhibitors (VPL and PBO) in the reversion of anthelmintic resistance. The difference between susceptible and resistant isolates may be the result of a higher expression of Pgps in resistant isolates than in susceptible ones. In addition, drug metabolism by oxidative pathways apparently differs between susceptible and resistant isolates and might therefore also contribute to the overall resistance level.

The combination of the inhibitors with an anthelmintic drug on egg hatching, development of larvae and on larval migration increased the susceptibility towards the drugs. The effect of both inhibitors on TBZ and IVM responses were particularly marked for larval development. In this experimental set-up both VPL and PBO were able to completely eliminate all differences between susceptible and resistant isolates. In contrast, the addition of VPL in the EHA only altered the susceptibility to TBZ in the highly resistant *C. o.* NZ-res. isolate but had no effect on responses to TBZ in all other isolates. PBO caused parallel translation of the dose-response-curves towards a slightly lower EC_{50} without changing relative differences between isolates.

Further investigations will be necessary to study more resistant populations in order to document the potent role of these inhibitors in increasing the susceptibility to anthelmintics and their potential to contribute to a better control of parasitic infection in the cattle livestock.

In the future, the *in vitro* assays described in this study might be valuable to screen for and identify new inhibitors with equivalent activities. Such drugs could be used in combination with commonly used drug classes to potentiate their anthelmintic activity in nematodes in order to prolong sustainability.

7 Summary

Analysis of putative inhibitors of anthelmintic resistance mechanisms in cattle gastrointestinal nematodes

Infections of the gastrointestinal tract with parasitic nematodes in livestock animals lead to significant impact in animal health and welfare and to enormous economic damage. The therapeutic agents commonly used for the control of gastrointestinal (GI) parasites can be divided into three main chemical groups: the benzimidazoles (BZs), the macrocyclic lactones (MLs) and the imidazothiazoles. Resistance to all three drug classes jeopardises chemical control of GI nematodes in livestock, particularly in countries with intensive grazing. While facing increasing resistance worldwide, knowledge of the mechanism of action of the currently used anthelmintics is crucial in order to develop strategies for their sustainable use. While mutations in the β-tubulin coding sequences have been detected to confer resistance to BZs, the resistance mechanism of the MLs still remains mainly unknown. Apart from specific resistance mechanisms (e.g. glutamate gated chloride channels), P-glycoproteins (Pgps), transmembrane proteins belonging to the family of multidrug transporters (ABC-transporters) and enzymes of the cytochrome P450 family (CYP P450s) have been reported to be involved in resistance to MLs.

The effects of the Pgp inhibitor verapamil (VPL) and the CYP P450 inhibitor piperonyl butoxide (PBO) on the efficacy of ivermectin (IVM), thiabendazole (TBZ) and levamisole (LEV) were studied *in vitro* in susceptible and resistant isolates of the cattle parasitic nematodes *Cooperia oncophora* and *Ostertagia ostertagi*. The effects of the combined use of drugs and inhibitors on egg hatching, larval development and larval motility were investigated in the egg hatch assay (EHA), the larval development assay (LDA) and the larval migration inhibition assay (LMIA).

In the present study it was shown, that all three *in vitro* tests are suitable for the detection of resistance against MLs and BZs. Resistance to LEV was not further investigated due to the lack of a resistant isolate.

In the EHA, the addition of VPL caused almost no changes regarding the inhibitory effect of TBZ on egg hatching in the susceptible isolates of both parasite species. A minor but not significant effect appeared in a *C. oncophora* isolate resistant to MLs and in the BZ-selected

O. ostertagi isolate. A significant inhibitory effect was obtained in the multi-resistant *C. oncophora* isolate where resistance to BZs was completely reversed. In contrast, the dose-response-curve of TBZ was shifted to the left in the presence of PBO in all tested isolates, demonstrating an increase in susceptibility towards TBZ.
In the LDA, both TBZ and IVM had a potent and significantly increased inhibitory effect on larval development in the presence of VPL and PBO. While the shift in the dose-response-curves of TBZ using VPL and PBO in the EHA changed with the degree of resistance of the isolate tested, similar EC_{50} values were obtained across all isolates tested in the LDA, indicating an increase in susceptibility to the tested anthelmintics by 15-270 fold for TBZ and 231-1694 fold for IVM.
The LMIA revealed that the use of VPL in combination with IVM led to changed responses of larval migration which was generally similar to that observed in the LDA. However, the susceptibility to the tested anthelmintics was here only increased 2-27 fold, indicating that uptake or intake of the inhibitory substances is lower than in earlier developmental stages of the nematodes life cycle. A change of the inhibitory effect of IVM in the presence of PBO on larval migration was observed only at the highest concentration (85 µg/ml PBO) used for all isolates tested in the assay.
The effect of the addition of VPL to the tested anthelmintics appeared more pronounced in the LDA than in the LMIA. For LEV with VPL, only a minor inhibitory effect was detected. The effect of the addition of PBO was most apparent in the EHA and LDA and only present to a lesser extend in the LMIA.
In the present thesis the three *in vitro* tests EHA, LDA and LMIA were successfully employed to study the effect of inhibitors of putative unspecific anthelmintic resistance mechanisms in gastrointestinal nematodes of cattle. Further studies for the detection of anthelmintic resistance in more resistant nematode populations and the identification and characterisation of Pgps and CYPs involved in AR are required in order to improve options to reduce the development of anthelmintic resistance.

8 Zusammenfassung

Analyse putativer Inhibitoren von Anthelminthika-Resistenz-Mechanismen in gastrointestinalen Nematoden des Rindes

Infektionen des Magen-Darm-Trakts mit Nematoden bei Nutztieren führen zu erheblichen Auswirkungen im Bereich der Tiergesundheit und zu enormen wirtschaftlichen Schäden. Die therapeutischen Mittel, welche häufig für die Kontrolle der Magen-Darm-Parasiten verwendet werden, können in drei chemische Hauptgruppen unterteilt werden: die Benzimidazole (BZ), die Makrozyklischen Laktone (MLs) und die Imidazothiazole. Sich ausbildende Resistenzen gegen alle drei Substanzklassen gefährden die chemische Kontrolle von Magen-Darm-Nematoden in Tierbeständen, insbesondere in Ländern mit intensiver Weidewirtschaft. Vor dem Hintergrund sich rasant ausbreitender Anthelminthika-Resistenzen weltweit ist die Kenntnis der Wirkungsweise der derzeit verwendeten Anthelminthika entscheidend, um Strategien für ihre nachhaltige Nutzung zu entwickeln. Während Mutationen im für das β-Tubulin kodierenden Gen nachgewiesen wurden, welche mit BZ-Resistenz korrelieren, ist der Resistenzmechanismus der MLs noch immer weitgehend unbekannt. Abgesehen von den spezifischen Resistenzmechanismen (z. B. Glutamat-gesteuerten Chloridkanälen) wurden auch P-Glykoproteine (Pgps), Transmembranproteine aus der Familie der Multi-Drug-Transporter (ABC-Transporter), und Enzyme der Cytochrom-P450-Familie (CYP P450) mit der Resistenz gegen MLs in Verbindung gebracht.
Die Auswirkungen des Pgp-Inhibitors Verapamil (VPL) und des CYP P450-Hemmers Piperonyl butoxid (PBO) auf die Effektivität von Ivermectin (IVM), Thiabendazol (TBZ) und Levamisol (LEV) wurden *in vitro* in empfindlichen und resistenten Isolaten der Rinder-nematoden *Cooperia oncophora* und *Ostertagia ostertagi* untersucht. Die Effekte der kombinierten Verwendung von Anthelminthikum und Inhibitor auf den Larvenschlupf, die Larvenentwicklung und Migrationsfähigkeit von Larven wurden im Larvenschlupfhemmtest (LSHT), Larvenentwicklungshemmtest (LEHT) und Larvenmigrationsinhibitionstest (LMIT) untersucht.
In der vorliegenden Studie konnte gezeigt werden, dass alle drei *in vitro*-Verfahren für den Nachweis der Resistenz gegen MLs und BZs geeignet sind. Resistenzen gegen LEV wurden aufgrund der Nichtverfügbarkeit von resistenten Isolaten nicht weiter untersucht.

Im LSHT wurde durch die Zugaben von VPL fast kein vermehrt hemmender Effekt der verwendeten Anthelminthika auf die Schlupfrate der empfindlichen Isolate beider Parasitenspezies beobachtet. Ein geringer, aber nicht signifikanter Effekt konnte in einem IVM-resistenten *C. oncophora* Isolat und in einem auf BZ-Resistenz selektierten *O. ostertagi* Isolat nachgewiesen werden. Eine signifikante Wirkung wurde bei der Verwendung des multi-resistenten *C. oncophora* Isolates nachgewiesen, wo die Resistenz gegenüber MLs und BZs fast vollständig aufgehoben werden konnte. Im Gegensatz dazu wurden in Gegenwart von PBO die Dosis-Wirkungs-Kurven von TBZ in allen getesteten Isolaten nach links verschoben, was für eine erhöhte Empfindlichkeit gegenüber diesen Stoffen spricht.
Im LEHT wurde durch Einsatz beider Inhibitoren, VPL und PBO, eine potente und signifikante Steigerung der Anthelminthikawirkung auf die Entwicklung der Larven beobachtet. Während sich die Verschiebung der Dosis-Wirkungs-Kurven in Gegenwart von VPL und PBO im LSHT mit dem Grad der Resistenz des getesteten Isolates veränderte, wurden im LEHT extrem ähnliche EC_{50}-Werte für alle Isolate erzielt, wobei die Empfindlichkeit gegenüber den getesteten Anthelminthika 15-270 fach für TBZ und 231-1694 fach für IVM erhöht wurde.
Die Ergebnisse im LMIT ergaben, daß die Wirkung von VPL auf die Hemmung der Migrationsfähigkeit der Larven durch die Anthelminthika im Allgemeinen ähnlich zu der im LEHT war. Allerdings war die Empfindlichkeit gegenüber den getesteten Anthelminthika hier nur etwa 2-27-fach erhöht was dafür spricht, dass die Aufnahme der hemmenden Substanzen in L3 geringer ist als die in früheren Entwicklungsstadien. Der Effekt von PBO auf die Wirkung von IVM konnte nur bei der höchsten eingesetzten Konzentration (85 µg/ml PBO) bei allen in diesem Test verwendeten Isolaten beobachtet werden.
Der Effekt von VPL war im LEHT deutlicher ausgeprägt als im LMIT. Für die Kombination mit LEV konnte nur eine geringradige Steigerung der hemmenden Wirkung nachgewiesen werden. Der Effekt von PBO war am deutlichsten im LSHT und LEHT und war im LMIT nur geringgradig.
In der vorliegenden Arbeit konnten die drei *in vitro*-Verfahren LSHT, LEHT und LMIT erfolgreich für Untersuchung der Effekte von Inhibitoren möglicher unspezifischer Anthelminthikaresistenz-Mechanismen bei gastrointestinalen Nematoden von Rindern angewendet werden. Weitere Studien unter Verwendung zusätzlicher resistenter Populationen sowie die Identifizierung und Charakterisierung von Pgps und CYPs, welche in AR beteiligt sind, sind erforderlich, um die Möglichkeiten der Verhinderung einer Entwicklung von Anthelminthika-Resistenzen zu verbessern.

9 References

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10 Appendix

- **Tables:**

Table 1: EC_{50} values, 95% confidence interval (CI) and R^2 of thiabendazole (TBZ) and piperonyl butoxide (PBO) for isolate of *C. oncophora* sus. in the egg hatch assays (EHA)

***C. o.* sus.**	**EC_{50}**	**95% CI**	**R^2**
TBZ+8.5 µg/ml	0.013 µg/ml	0.013 - 0.014	0.973
TBZ+4.25 µg/ml	0.012 µg/ml	0.012 - 0.013	0.987
TBZ+2.125 µg/ml	0.013 µg/ml	0.012 - 0.013	0.984
TBZ+1.063 µg/ml	0.014 µg/ml	0.013 - 0.014	0.984
TBZ+0.533 µg/ml	0.015 µg/ml	0.014 - 0.016	0.987
TBZ+0.266 µg/ml	0.016 µg/ml	0.015 - 0.016	0.988

Table 2: EC_{50} values, 95% confidence interval (CI) and R^2 of thiabendazole (TBZ) and piperonyl butoxide (PBO) for isolate of *C. oncophora* NZ-res. in the egg hatch assay (EHA)

***C. o.* NZ-res.**	**EC_{50}**	**95 % CI**	**R^2**
TBZ+8.5 µg/ml	0.022 µg/ml	0.018 - 0.027	0.867
TBZ+4.25 µg/ml	0.024 µg/ml	0.019 - 0.030	0.774
TBZ+2.125 µg/ml	0.027 µg/ml	0.021 - 0.033	0.778
TBZ+1.063 µg/ml	0.034 µg/ml	0.029 - 0.041	0.839
TBZ+0.533 µg/ml	0.036 µg/ml	0.031 - 0.043	0.842
TBZ+0.266 µg/ml	0.046 µg/ml	0.042 - 0.050	0.951

Table 3: EC_{50} values, 95% confidence interval (CI) and R^2 of thiabendazole (TBZ) and piperonyl butoxide (PBO) for isolate of *O. ostertagi* sus. in the egg hatch assay (EHA)

***O. o.* sus.**	**EC_{50}**	**95% CI**	**R^2**
TBZ+8.5 µg/ml	0.016 µg/ml	0.015 - 0.018	0.945
TBZ+4.25 µg/ml	0.016 µg/ml	0.014 - 0.017	0.951
TBZ+2.125 µg/ml	0.015 µg/ml	0.013 - 0.016	0.943
TBZ+1.063 µg/ml	0.017 µg/ml	0.015 - 0.019	0.934
TBZ+0.533 µg/ml	0.018 µg/ml	0.016 - 0.020	0.944
TBZ+0.266 µg/ml	0.014 µg/ml	0.013 - 0.016	0.974

Table 4: EC_{50} values, 95% confidence interval (CI) and R^2 of thiabendazole (TBZ) and piperonyl butoxide (PBO) for isolate of *O. ostertagi* sel. in the egg hatch assay (EHA)

***O. o.* sel.**	**EC_{50}**	**95% CI**	**R^2**
TBZ+8.5 µg/ml	0.010 µg/ml	0.009 - 0.011	0.962
TBZ+4.25 µg/ml	0.016 µg/ml	0.008 - 0.011	0.941
TBZ+2.125 µg/ml	0.011 µg/ml	0.010 - 0.013	0.918
TBZ+1.063 µg/ml	0.013 µg/ml	0.011 - 0.015	0.914
TBZ+0.533 µg/ml	0.013 µg/ml	0.012 - 0.015	0.915
TBZ+0.266 µg/ml	0.013 µg/ml	0.012 - 0.014	0.925

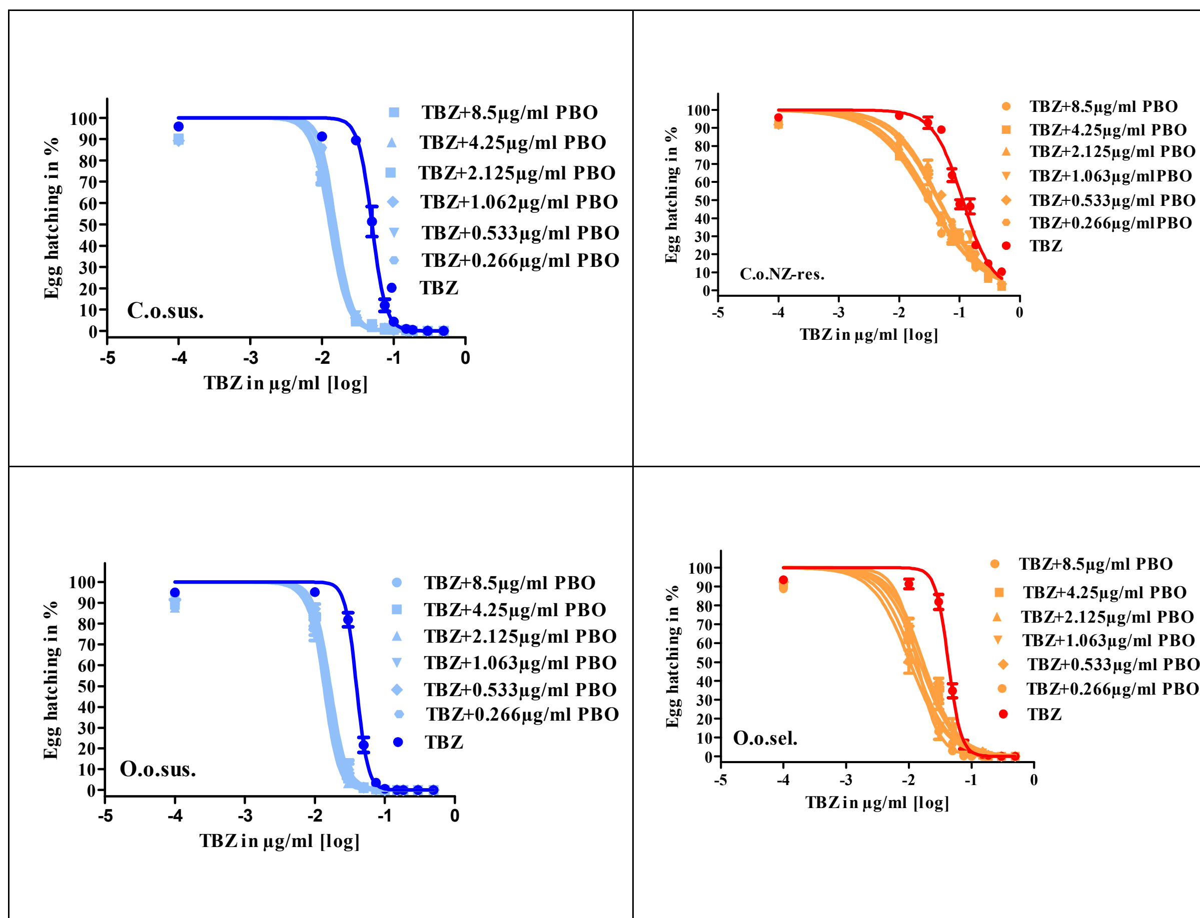

Figure 1: The dose-response-curves for TBZ (in µg/ml), TBZ+PBO for *C. oncophora* and *O. ostertagi* isolates in the EHA

Table 5: EC_{50} values, 95% confidence interval (CI) and R^2 of thiabendazole (TBZ) and piperonyl butoxide (PBO) for isolate of *C. oncophora* sus. in the larval development assay (LDA)

***C. o.* sus.**	**EC_{50}**	**95% CI**	**R^2**
TBZ+85 µg/ml	0.0002 µg/ml	0.0001 - 0.0002	0.999
TBZ+42.5 µg/ml	0.0002 µg/ml	0.0001 - 0.0002	0.999
TBZ+21.25 µg/ml	0.0002 µg/ml	0.0001 - 0.0002	0.999
TBZ+10.63 µg/ml	0.0002 µg/ml	0.0001 - 0.0002	0.999
TBZ+5.33 µg/ml	0.0002 µg/ml	0.0001 - 0.0002	0.999

Table 6: EC_{50} values, 95% confidence interval (CI) and R^2 of thiabendazole (TBZ) and piperonyl butoxide (PBO) for isolate of *C.oncophora* IVM-res. in the larval development assay (LDA)

***C. o.* IVM-res.**	**EC_{50}**	**95% CI**	**R^2**
TBZ+85 µg/ml	0.0002 µg/ml	0.0001 - 0.0002	0.999
TBZ+42.5 µg/ml	0.0002 µg/ml	0.0001 - 0.0002	0.999
TBZ+21.25 µg/ml	0.0002 µg/ml	0.0001 - 0.0002	0.999
TBZ+10.63 µg/ml	0.0002 µg/ml	0.0001 - 0.0002	0.999
TBZ+5.33 µg/ml	0.0002 µg/ml	0.0001 - 0.0002	0.999

Table 7: EC_{50} values, 95% confidence interval (CI) and R^2 of thiabendazole (TBZ) and piperonyl butoxide (PBO) for isolate of *C. oncophora* NZ-res. in the larval development assay (LDA)

***C. o.* NZ-res.**	**EC_{50}**	**95% CI**	**R^2**
TBZ+85 µg/ml	0.0002 µg/ml	0.0002 - 0.0002	0.999
TBZ+42.5 µg/ml	0.0002 µg/ml	0.0002 - 0.0002	0.999
TBZ+21.25 µg/ml	0.0002 µg/ml	0.0002 - 0.0002	0.999
TBZ+10.63 µg/ml	0.0002 µg/ml	0.0002 - 0.0002	0.999
TBZ+5.33 µg/ml	0.0002 µg/ml	0.0002 - 0.0002	0.999

Table 8: EC_{50} values, 95% confidence interval (CI) and R^2 of thiabendazole (TBZ) and piperonyl butoxide (PBO) for isolate of *O. ostertagi* sus. isolate in the larval development assay (LDA)

***O. o.* sus.**	**EC_{50}**	**95% CI**	**R^2**
TBZ+85 µg/ml	0.0001 µg/ml	0.0005 - 0.0004	0.999
TBZ+42.5 µg/ml	0.0002 µg/ml	0.0002 - 0.0002	0.999
TBZ+21.25 µg/ml	0.0002 µg/ml	0.0002 - 0.0002	0.999
TBZ+10.63 µg/ml	0.0001 µg/ml	0.0004 - 0.0004	0.999
TBZ+5.33 µg/ml	0.0002 µg/ml	0.0002 - 0.0002	0.999

Table 9: EC_{50} values, 95% confidence interval (CI) and R^2 of thiabendazole (TBZ) and piperonyl butoxide (PBO) for isolate of *O. ostertagi* sel. isolate in the larval development assay (LDA)

***O. o.* sel.**	**EC_{50}**	**95% CI**	**R^2**
TBZ+85 µg/ml	0.0002 µg/ml	0.0001 - 0.0002	0.999
TBZ+42.5 µg/ml	0.0002 µg/ml	0.0002 - 0.0002	1.000
TBZ+21.25 µg/ml	0.0002 µg/ml	0.0001 - 0.0002	0.999
TBZ+10.62 µg/ml	0.0002 µg/ml	0.0002 - 0.0002	0.999
TBZ+5.33 µg/ml	0.0002 µg/ml	0.0002 - 0.0002	0.999

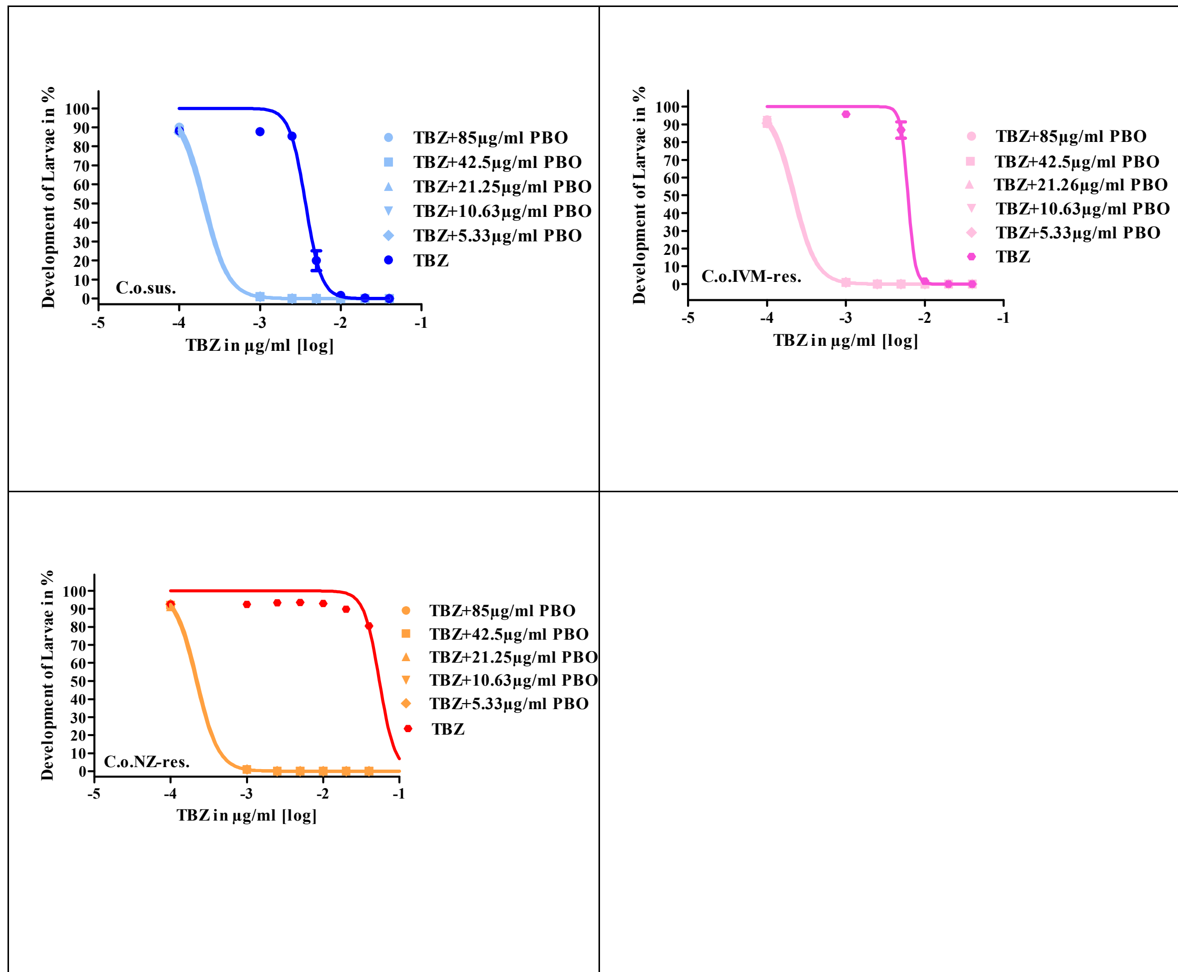

Figure 2: The dose-response-curves for TBZ (in µg/ml), TBZ+PBO for *C. oncophora* and isolates in the LDA

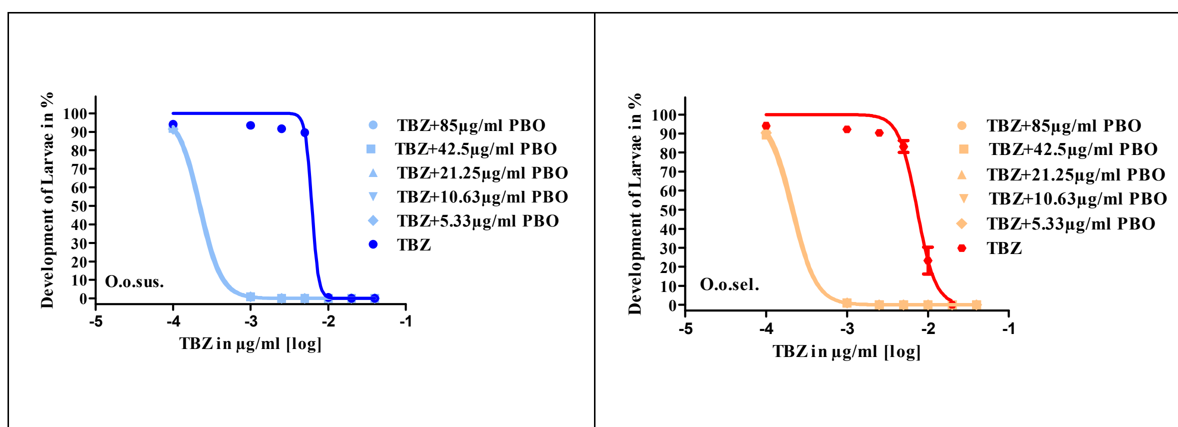

Figure 3: The dose-response-curves for TBZ (in µg/ml), TBZ+PBO for *O. ostertagi* isolates in the LDA

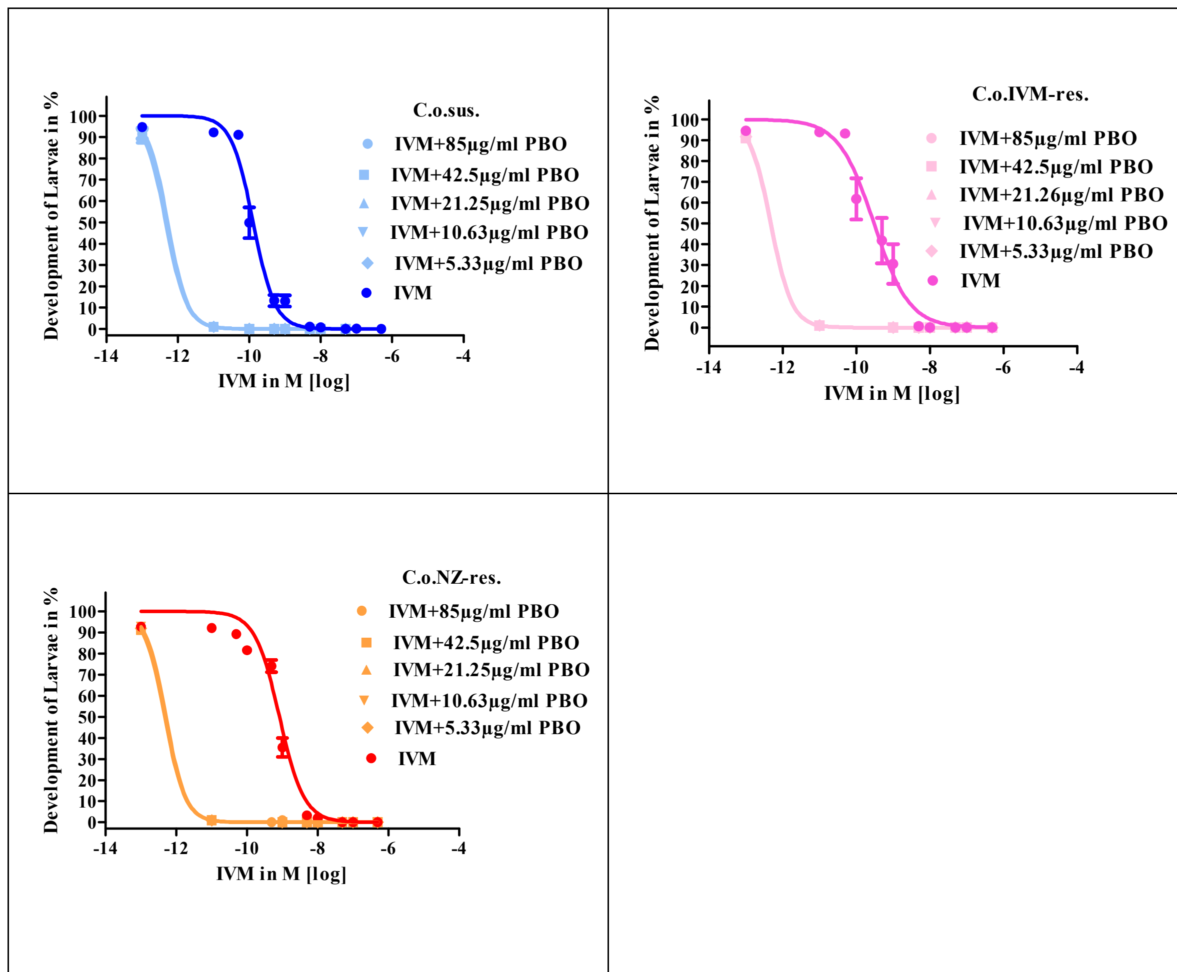

Figure 4: The dose-response-curves for IVM (in nM), IVM+PBO for *C. oncophora* and isolates in the LDA

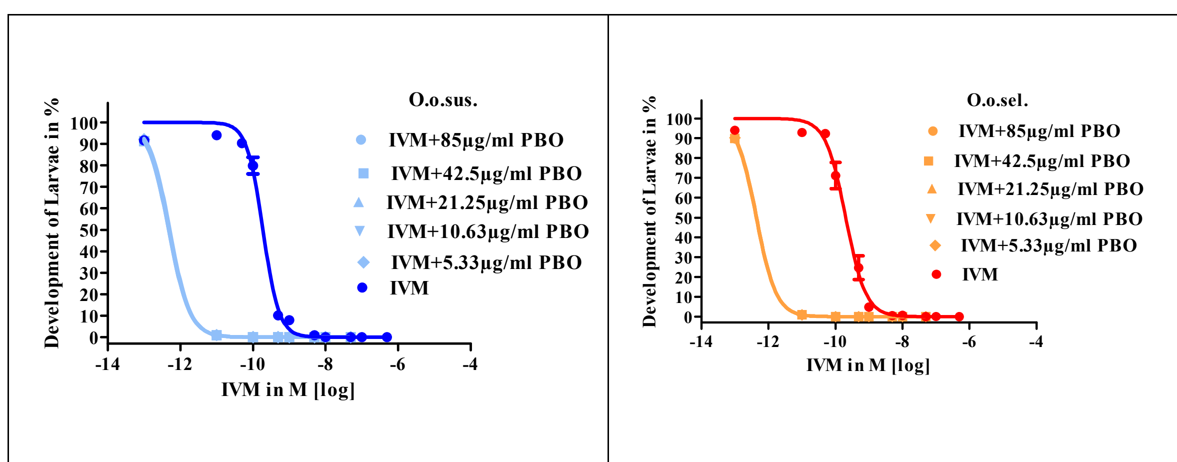

Figure 5: The dose-response-curves for IVM (in nM), IVM+PBO for *O. ostertagi* isolates in the LDA

Table 10: EC_{50} values, 95% confidence interval (CI) and R^2 of ivermectin (IVM) and piperonyl butoxide (PBO) for isolate for isolate of *C. oncophora* sus. in the larval development assay (LDA)

***C. o.* sus.**	**EC_{50}**	**95% CI**	**R^2**
IVM+85 µg/ml	0.00047 nM	0.00033 - 0.00069	0.996
IVM+42.5 µg/ml	0.00045 nM	0.00027 - 0.00075	0.993
IVM+21.25 µg/ml	0.00048 nM	0.00033 - 0.00069	0.997
IVM+10.62 µg/ml	0.00049 nM	0.00033 - 0.00073	0.995
IVM+5.33 µg/ml	0.00058 nM	0.00040 - 0.00068	0.998

Table 11: EC_{50} values, 95% confidence interval (CI) and R^2 of ivermectin (IVM) and piperonyl butoxide (PBO) for isolate of *C. oncophora* IVM-res. in the larval development assay (LDA)

***C. o.* IVM-res.**	**EC_{50}**	**95% CI**	**R^2**
IVM+85 µg/ml	0.00048 nM	0.00044 - 0.00052	0.996
IVM+42.5 µg/ml	0.00047 nM	0.00044 - 0.00051	0.993
IVM+21.25 µg/ml	0.00046 nM	0.00044 - 0.00048	0.997
IVM+10.62 µg/ml	0.00047 nM	0.00043 - 0.00052	0.995
IVM+5.33 µg/ml	0.00048 nM	0.00045 - 0.00052	0.998

Table 12: EC_{50} values, 95% confidence interval (CI) and R^2 of ivermectin (IVM) and piperonyl butoxide (PBO) for isolate of *C. oncophora* NZ-res. in the larval development assay (LDA)

***C. o.* NZ-res.**	**EC_{50}**	**95% CI**	**R^2**
IVM+85 µg/ml	0.00049 nM	0.00044 - 0.00054	0.999
IVM+42.5 µg/ml	0.00047 nM	0.00018 - 0.00024	0.999
IVM+21.25 µg/ml	0.00048 nM	0.00043 - 0.00053	0.999
IVM+10.62 µg/ml	0.00051 nM	0.00046 - 0.00056	0.999
IVM+5.33 µg/ml	0.00049 nM	0.00046 - 0.00052	0.999

Table 13: EC_{50} values, 95% confidence interval (CI) and R^2 of ivermectin (IVM) and piperonyl butoxide (PBO) for isolate of *O. stertagi* sus. in the larval development assay (LDA)

***O. o.* sus.**	**EC_{50}**	**95% CI**	**R^2**
IVM+85 µg/ml	0.00049 nM	0.00041 - 0.00058	0.999
IVM+42.5 µg/ml	0.00047 nM	0.00043 - 0.00051	0.999
IVM+21.25 µg/ml	0.00051 nM	0.00045 - 0.00056	0.999
IVM+10.62 µg/ml	0.00047 nM	0.00041 - 0.00054	0.999
IVM+5.33 µg/ml	0.00049 nM	0.00046 - 0.00052	0.999

Table 14: EC_{50} values, 95% confidence interval (CI) and R^2 of ivermectin (IVM) and piperonyl butoxide (PBO) for isolate of *O. ostertagi* sel. isolate in the larval development assay (LDA)

***O. o.* sel.**	**EC_{50}**	**95% CI**	**R^2**
IVM+85 µg/ml	0.00043 nM	0.0004 - 0.00047	0.999
IVM+42.5 µg/ml	0.00044 nM	0.00038 - 0.0005	0.999
IVM+21.25 µg/ml	0.00044 nM	0.00042 - 0.00046	0.999
IVM+10.62 µg/ml	0.00043 nM	0.00039 - 0.00047	0.999
IVM+5.33 µg/ml	0.00044 nM	0.00042 - 0.00047	0.999

- **Buffered water:**

141.96 g/mol/Na_2HPO_4+ 200 ml H_2O autoclaved for 20 min.

137.99 g/mol/$NaH_2Po_4H_2O$ + 200 ml H_2O autoclaved for 20 min.

5.77 ml NaH_2Po_4 + 4.23 ml NaH_2PO_4 in 100 ml distilled water. Autoclaved for 20 min

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, den 04 Mai 2011

Saleha Alarabi M. Algusbi