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From field to genetics

Anthelmintic resistance in the equine roundworm *Parascaris univalens*

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Parascaris univalens

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Abstract

The equine roundworm *Parascaris univalens* is a common parasite of foals. Most foals show mild clinical symptoms, but large worm burdens can lead to severe colic and even death. Regular treatment with anthelmintic drugs has resulted in resistance development, mainly to ivermectin but also to pyrantel and fenbendazole in sporadic cases. In Sweden, resistance to ivermectin is considered to be widespread.

The aim of the thesis was to examine the efficacy of anthelmintic drugs on stud farms in Sweden and Iceland, develop novel models for research and study genetic mechanisms potentially involved in drug metabolism and anthelmintic resistance.

Faecal egg count reduction tests showed that resistance to both pyrantel and fenbendazole has emerged on Swedish stud farms, and that ivermectin resistance is common on Icelandic farms. Due to the potentially lethal consequences of infection, this is a serious situation. We developed a novel method to hatch *P. univalens* eggs in order to use larvae in *in vitro* experiments to study resistance mechanisms. Quantitative PCR, RNA sequencing and amplicon sequencing were used to study genetic and transcriptomic mechanisms behind anthelmintic resistance in *P. univalens*. Several genes coding for drug metabolising enzymes, transport proteins and a possible drug target for ivermectin were found to be differentially expressed in *P. univalens* after exposure to anthelmintic drugs. However, mutations in β-tubulin genes responsible for benzimidazole resistance in many other parasitic nematodes were not present in a fenbendazole-resistant *P. univalens* population.

In conclusion, the current level of resistance in *P. univalens* has been updated in this thesis, a novel research method has been developed and novel candidate genes for future research have been identified.

Keywords: macrocyclic lactone, ivermectin, benzimidazole, fenbendazole, thiabendazole, pyrantel, efficacy, foal, hatching, amplicon sequencing

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Läkemedelsresistens hos hästens spolmask

Sammanfattning

Hästens spolmask, *Parascaris univalens*, är en inälvsparasit som infekterar föl och unghästar. Eftersom infektionen kan få allvarliga konsekvenser och till och med vara dödlig, så behandlas de flesta föl regelbundet under det första levnadsåret. Detta har bidragit till utveckling av resistens, framförallt mot ivermektin, men enstaka fall av resistens mot pyrantel och fenbendazol har även rapporterats världen över. I Sverige har utbredd resistens mot ivermektin påvisats i tidigare studier.

Målet med avhandlingen var att undersöka effekten av avmaskningsmedel vid svenska och isländska stuterier, att utveckla nya modeller för forskning, samt att studera mekanismer involverade i läkemedelsmetabolism och resistens.

Resultaten visade att resistens mot både pyrantel och fenbendazol har utvecklats på svenska gårdar, medan ivermektinresistens är vanligt på isländska gårdar. På grund av de potentiellt dödliga konsekvenserna av spolmaskinfektion är denna utveckling mycket allvarlig. Vi har utvecklat en metod för att kläcka *P. univalens* ägg och använda larver i *in vitro* experiment för att studera resistensmekanismer. Genetiska mekanismer och förändringar i genuttryck som kan ligga till grund för läkemedelsresistens hos *P. univalens* har undersökts med olika PCR- och sekvenseringstekniker. Uttryck av flera gener kodande för enzymer inblandade i läkemedelsmetabolism, transportproteiner och en möjlig målmolekyl för ivermektin var förändrat i *P. univalens* efter exponering för olika läkemedel. Däremot upptäcktes inga mutationer i gener kodande för β-tubuliner, vilket är en känd orsak till benzimidazolresistens hos många andra parasitmaskar.

Sammanfattningsvis har resistensläget för hästens spolmask uppdaterats, en ny modell för spolmaskforskning har utvecklats och ett antal kandidatgener för anthelmintikaresistens har identifierats.

Nyckelord: makrocykliska laktoner, ivermectin, benzimidazol, fenbendazol, thiabendazol, pyrantel, föl, kläckning, anthelmintikaresistens

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Dedication

To all cute foals out there - stay healthy!

Contents

List of publications

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

I. Martin, F., Höglund, J., Bergström, T. F., Karlsson Lindsjö, O. & Tydén, E. (2018). Resistance to pyrantel embonate and efficacy of fenbendazole in *Parascaris univalens* on Swedish stud farms. Veterinary Parasitology 264, 69-73.

II. Martin, F., Svansson, V., Eydal, M., Oddsdottir, C., Ernback, M., Persson, I. & Tydén E. (2021) First Report of Resistance to Ivermectin in *Parascaris univalens* in Iceland. Journal of Parasitology 107, 16-22.

III. Martin, F., Dube, F., Karlsson Lindsjö, O., Eydal, M., Höglund, J., Bergström, T. F. & Tydén, E. (2020) Transcriptional responses in *Parascaris univalens* after *in vitro* exposure to ivermectin, pyrantel citrate and thiabendazole. Parasite & Vectors 13, 342.

IV. Martin, F., Eydal, M., Höglund, J. & Tydén, E. (2021). Constitutive and differential expression of transport protein genes in *Parascaris univalens* larvae and adult tissues after *in vitro* exposure to anthelmintic drugs. Veterinary Parasitology 298, 109535

V. Martin, F., Halvarsson, P., Delhomme, N., Höglund, J. & Tydén, E. (2021) Exploring the β-tubulin gene family in a benzimidazole-resistant *Parascaris univalens* population. International Journal for Parasitology: Drugs and Drug Resistance 17, 84-91.

The contribution of Frida Martin to the papers included in this thesis was as follows:

I. Took major part in planning and designing the study, collection and analysis of samples. Analysed the data and had the main responsibility in writing the manuscript.

II. Took part in planning and designing the study. Analysed the data and had the main responsibility in writing the manuscript.

III. Took major part in planning and designing the study, collection and preparation of samples. Had the main responsibility in writing the manuscript.

IV. Took major part in planning and designing the study, development of novel techniques and analysis of samples. Analysed the data and had the main responsibility in writing the manuscript.

V. Took major part in planning and designing the study, collection and analysis of samples. Analysed the data together with co-authors and had the main responsibility in writing the manuscript.

Abbreviations

AAEP	American Association for Equine Practitioners
ABC	Adenosine triphosphate-binding cassette
ALMA	Automate larval migration assay
ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
CCS	Circular consensus
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EPG	Eggs per gram
FBZ	Fenbendazole
FEC	Faecal egg count
FECRT	Faecal egg count reduction test
FMO	Flavin containing monooxygenase
GABA	Gamma-aminobutyric acid
GST	Glutathione S-transferase
INRAE	French Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement
IVM	Ivermectin
PYR	Pyrantel
MFS	Major facilitator superfamily
nAChR	Nicotinic acetylcholine receptors
PCR	Polymerase chain reaction
Pgp	Permeability glycoprotein
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid

RNA-Seq	Ribonucleic acid-sequencing
SDR	Short-chain dehydrogenase/reductase
SLC	Solute carrier
SNP	Single nucleotide polymorphism
Spp.	Species
SVA	National veterinary institute
TBZ	Thiabendazol
UGT	Uridine 5'-diphospho-glucuronosyltransferase
WAAVP	World Association for the Advancement of Veterinary Parasitology
WHO	World Health Organization

1. Introduction

Gastrointestinal nematodes are common in all grazing animals and lead to reduced animal welfare and production losses. The major method for control of gastrointestinal nematodes is treatment with anthelmintic drugs several times during the grazing season. Broad spectrum anthelmintic drugs were released on the market in the 1960s and only a few years later the first case of anthelmintic resistance was reported in the sheep parasite *Haemonchus contorts* (Conway 1964).

As with other grazing animals, horses are infected with gastrointestinal nematodes and are usually treated with anthelmintic drugs throughout their life. Up until 2007, anthelmintic drugs were bought over the counter at Swedish pharmacies and owners were recommended to treat their horses routinely several times per year. An overuse of anthelmintic drugs selects for worms carrying favourable genes that survive treatment and pass the resistance genotype to their offspring (Sangster 1999). In 2007 novel regulations were introduced in Sweden, where anthelmintic drugs would only be prescribed by veterinarians after faecal examination (SJV 2010). By then however, anthelmintic resistance had already been introduced in several equine parasites (Nilsson *et al.* 1989; Lind *et al.* 2007).

Parascaris spp. are the most pathogenic parasites of juvenile equids, since large worm burdens can cause impaction in the small intestine and even death (Reinemeyer 2009). Foals are therefore usually dewormed at regular intervals during their first year. The first case of resistance in *Parascaris* spp. was reported 2002 in the Netherlands (Boersema *et al.* 2002). Since then reports of resistance to several drug classes have been made across the world and anthelmintic resistance is now a major threat to foal health and welfare (Nielsen 2016a). So far anthelmintic resistance in other species of the roundworm parasite family *Ascarididae* is rare, with single reports of

resistance in the human parasite *Ascaris lumbricoides* (Krucken *et al.* 2017) and *Ascaridia dissimilis*, a parasitic roundworm of turkeys (Collins *et al.* 2019).

The development of anthelmintic resistance is a dynamic process and the resistance status of parasites therefore needs to be monitored regularly (Sangster 1999). The mechanisms causing resistance to the different anthelmintic substances have not yet been elucidated in ascarid parasites. Such knowledge would however be important to slow down the spread of resistance and to develop molecular markers for early resistance detection. Advances in molecular techniques and genetics, such as the recent publication of a draft genome of *P. univalens* (Wang *et al.* 2017), have provided new possibilities to investigate responses in potential drug targets and pathways that may lead to resistance. However, *Parascaris* spp. research is complicated by the difficulty in parasite collection as the adult roundworm resides in the small intestine of the host. Further research would therefore be greatly simplified by the development of *in vitro* methods based on parasite eggs that can be collected from foal faeces.

Considering the risk of lethal complications of *Parascaris* spp. infection and the lack of new anthelmintic drugs for the equine market, the development of resistance to all available drug classes is a major threat to equine health and the equine industry. More research in this area is therefore crucial to combat the resistant parasites.

2. Background

2.1 *Parascaris* spp.

Parascaris spp. are large roundworms belonging to the family *Ascarididae* and are considered to be the most pathogenic parasites of juvenile horses (Nielsen 2016a). The adult worms have a whitish colour and are 15-50 cm long, whereas the eggs are round with a thick brownish shell, measuring around 100 µm in diameter (Reinemeyer & Nielsen 2018).

The equine parasitic roundworm has traditionally been referred to as *Parascaris equorum*, but it has long been known that the closely related species *Parascaris univalens* may also infect horses. The two species are undistinguishable by eye but can be separated by karyotyping as *P. equorum* has two pairs of chromosomes while *P. univalens* only has one pair (Goday & Pimpinelli 1984). However, recent studies have suggested that *P. univalens* is the dominating species infecting horses (Jabbar *et al.* 2014; Nielsen *et al.* 2014; von Samson-Himmelstjerna *et al.* 2021).

2.1.1 Life cycle

Most foals are exposed to *Parascaris* spp. infection from their first days of life and prevalence up to 83 % has been reported (Laugier *et al.* 2012; Relf *et al.* 2013; Armstrong *et al.* 2014).

Parascaris spp. have a direct lifecycle where foals ingest eggs containing infective larvae from the environment (Figure 1). The egg hatch in the small intestine, the larvae penetrate the intestinal wall and are transported to the liver via lymphatic or blood vessels. Larvae then migrate through the hepatic parenchyma for approximately one week before being found in the lungs around two weeks post-infection. The larvae migrate out into the airways and

up the trachea to the pharynx where they are swallowed and end up back in the small intestine within three weeks post-infection. In the small intestine the larvae mature to adult worms that reproduce. Patency is established 10 - 15 weeks post-infection and the female worm release large numbers of eggs (Lyons *et al.* 1976; Clayton & Duncan 1979b; Reinemeyer & Nielsen 2018). The eggs are passed with the foals faeces and if conditions are favourable, infective larvae develop inside the egg within two weeks (Reinemeyer & Nielsen 2018).

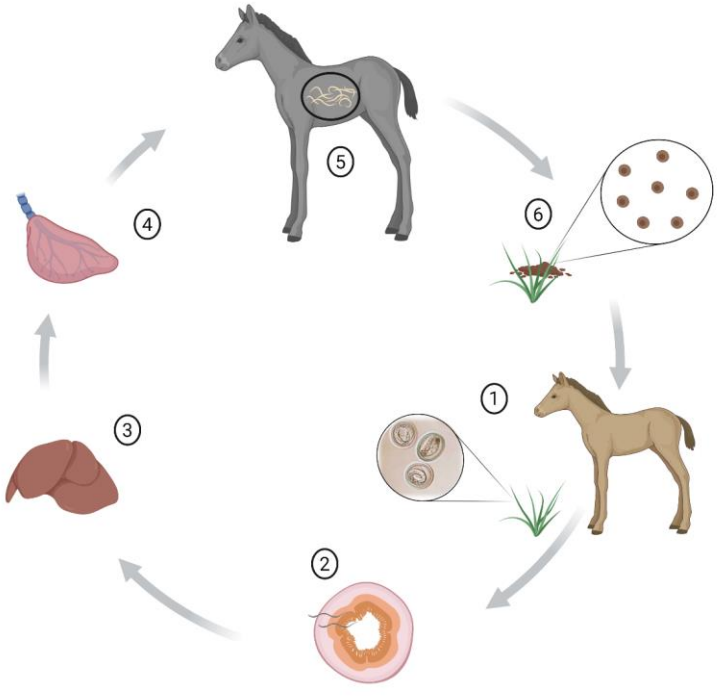

Figure 1. Lifecycle of *Parascaris* spp. 1. Eggs containing infective larvae are ingested by the foal. 2. Eggs hatch in the small intestine and larvae penetrate the intestinal wall. 3. Approximately one week post-infection larvae migrate through the liver. 4. Larvae migrate from the liver to the lungs and airways, approximately two weeks post-infection. 5. Larvae migrate through the trachea and pharynx and are then swallowed. Back in the small intestine, the larvae develop into adult worms that reproduce. 6. Patency is reached approximately 10-15 weeks post-infection and eggs are deposited in the environment with the foals faeces. Illustration created with BioRender.

Heavily infected foals can shed millions of *Parascaris* spp. eggs every day, and the infection pressure on stud farms is therefore usually high (Clayton & Duncan 1979b). In addition, the infective eggs are considered to be resistant to weather conditions and survive for more than 18 months in the environment, ready to infect the next generation of foals (Lindgren *et al.* 2008; Lindgren & Höglund 2010).

2.1.2 Immunity

In experimental infections of two to four week old foals, approximately 50 % of the larvae returned to the small intestine after the hepato-tracheal migration. Once present in the small intestine the number of mature worms decreased steadily during several months, but whether this was due to overcrowding as the worms increased in size or immunological reactions have not been elucidated (Clayton & Duncan 1979a).

Foals around 6 months old develop immunity to the parasite and infection of adult horses is therefore uncommon with reported prevalence of 3-4 % (Fritzen *et al.* 2010; Relf *et al.* 2013). The exact mechanism of the immune reaction is unknown, but it is suggested to occur in the liver and/or lungs and to be age related rather than depend on previous exposure to the parasite (Clayton & Duncan 1979a). In tropical areas prevalence as high as 15 % has been observed in working horses over four years of age. These high infection rates in adult horses are suggested to be caused by reduced immunity due to hard work in combination with poor nutritional status (Getachew *et al.* 2008). In addition, a study showed that the prevalence of *Parascaris* spp. in two-year old Finnish Standardbred trotting horses was 20 %, suggested to be caused by the stress of hard training of the young horses (Hautala *et al.* 2019).

2.1.3 Impact on the host

Clinical signs of infection may include mild respiratory symptoms such as coughing and bi-lateral nasal discharge due to larval migration in the lungs two to four weeks post-infection (Figure 2a) (Clayton & Duncan 1978). The larval migration through the liver does not normally cause clinical signs of liver disease even though both macroscopic and microscopic lesions and signs of inflammation have been observed (Brown & Clayton 1979). Signs such as lethargy, weight loss and impaired growth are suggested to be caused by adult worms competing for space and nutrients in the small intestine (Clayton *et al.* 1980). In severe cases, large worm burdens in the small

intestine can cause impaction and even rupture of the intestinal wall, which may be lethal (Figure 2b). The incidence of small intestine impaction is unknown and there are few reports available. In a study of 621 foals < 1 year of age admitted for colic surgery at a Canadian horse clinic, only 0.4 % were diagnosed with *Parascaris* spp. impaction (Cribb *et al.* 2006). The long-term survival after surgery varied between 27 % and 60 % in different studies (Cribb *et al.* 2006; Tatz *et al.* 2012). It is often debated whether anthelmintic treatment is a risk factor for small intestine impaction of *Parascaris* spp. A majority of horses undergoing surgery for *Parascaris* spp. induced impaction had been treated with anthelmintic drugs, mainly pyrantel or ivermectin, within 24 h prior to the onset of colic (Cribb *et al.* 2006; Tatz *et al.* 2012).

a b

Figure 2. Clinical signs and consequence of *Parascaris* spp. infection: a) Nasal discharge (Photo: Frida Martin) b) intestinal impaction due to heavy *Parascaris* spp. burden (Photo: Elin Svonni).

2.2 Diagnosis

Patent *Parascaris* spp. infection can be diagnosed by examination of faecal samples. However, due to the long pre-patency period, worms may be present in the small intestine even though faecal egg counts (FECs) are negative. The egg output does also seem to vary, with a peak in FECs around four months post infection and a possible second peak in egg shedding around nine months post infection (Clayton & Duncan 1979b; Donoghue *et*

al. 2015). It should also be noted that the FEC does not correlate to the number of roundworms present in the small intestine (Nielsen *et al.* 2010).

The presence of ascarid worms in the small intestine may be diagnosed by transabdominal ultrasound (Nielsen *et al.* 2016b). Since this method requires specialized equipment and trained operators it may however not be applicable for routine diagnosis.

2.3 Treatment and prevention

Due to the long pre-patency period and the high infection pressure of *Parascaris* spp. on most stud farms, foals are normally treated with anthelmintic drugs at regular intervals without previous faecal examination.

In Sweden, the current recommendations are to treat foals routinely at 8-10 weeks and 16-18 weeks with any further treatments depending on the results of faecal examination (SVA 2020). Three classes of anthelmintic drugs are the most commonly used for treatment of roundworm infection: macrocyclic lactones, tetrahydropyrimidines and benzimidazoles.

2.3.1 Macrocyclic lactones

Macrocyclic lactones are considered to be the antiparasitic drugs used most widely in veterinary medicine (Lanusse *et al.* 2010b). Ivermectin, belonging to the avermectin sub-group and moxidectin, belonging to the milbemycin sub-group are both registered for oral use in horses in Sweden (FASS 2021).

When ivermectin was approved for use in horses in the 1980s it was introduced as a solution for intramuscular injection. This formula was later withdrawn due to reports of inflammation and infection at the injection sites (Campbell *et al.* 1989). This formulation is however sometimes used off label in horses but are then generally administered subcutaneously (Paulrud *et al.* 1997).

The macrocyclic lactones are large, hydrophobic molecules that are easily absorbed and distributed throughout tissues such as liver and lungs, resulting in effect against migrating larval stages. They are poorly metabolised and mainly excreted with bile and faeces in unchanged form (Lanusse *et al.* 2010b).

Macrocyclic lactones act by binding to parasite-specific glutamate-gated chloride channel receptors and gamma-aminobutyric acid (GABA) receptors located in the membrane of nerve and muscle cells (Lanusse *et al.* 2010b).

The glutamate receptors are suggested to be the primary target as they are sensitive to lower concentrations of macrocyclic lactones compared to the GABA receptors (Beech *et al.* 2010). Both glutamate and GABA receptors are pentameric and the subunit composition of the receptors has been suggested to affect the sensitivity to the ligands (Accardi *et al.* 2012; Sieghart *et al.* 1999). Four glutamate receptor subunits have been identified in *P. univalens* (Lamassiaude *et al.* 2020).

Receptor binding causes an influx of chloride ions into the cell (hyperpolarisation), leading to inhibition of neurotransmission and thereby flaccid paralysis of the parasite, resulting in expulsion from the host (Martin *et al.* 2002). Macrocyclic lactone treatment also results in reduced pharyngeal pumping, and thereby starvation, due to binding to receptors in the pharynx (Geary *et al.* 1999). In addition, macrocyclic lactones have been suggested to affect the egg output from parasites since GABA and glutamate receptors are responsible for ovipositioning (Fellowes *et al.* 2000).

2.3.2 Tetrahydropyrimidines

The tetrahydropyrimidine pyrantel was introduced in 1966 and is available associated with different salts (Lanusse *et al.* 2010a). Pyrantel embonate is registered for use in horses in Sweden as oral paste or powder formulation at a dosage of 19 mg/kg, equal to 6.6 mg/kg of pyrantel base (FASS 2021).

Pyrantel embonate is insoluble in water and therefore poorly absorbed across the intestinal wall resulting in low plasma concentration and hence no activity against migrating larval stages (Lanusse *et al.* 2010a).

Tetrahydropyrimidines act as agonists to the L-type nicotine acetylcholine gated ion channels (nAChR) present at the neuromuscular junction of somatic and pharyngeal muscle cells in the parasite. The nAChR consists of five subunits that can vary in composition and the combination of subunits has been shown to affect the susceptibility to anthelmintic drugs (Williamson *et al.* 2009). Binding of pyrantel opens the channel, allowing an influx of cations, leading to depolarisation of muscle cells and spastic paralysis of the worm. The parasite is then expelled from the host by peristalsis (Martin & Robertson 2007).

2.3.3 Benzimidazoles

The first benzimidazole drug was introduced in 1961 (Brown *et al.* 1961). Since then several more benzimidazole substances have been developed,

however only fenbendazole paste is registered for use in horses in Sweden (FASS 2021).

Benzimidazoles have limited solubility in water, resulting in poor absorption from the intestinal tract (Lanusse *et al.* 2010a). This results in activity against luminal parasites while the larvicidal effect of a single treatment is poor. Studies have however shown that treatment with fenbendazole for five consecutive days is highly effective against migrating *Parascaris* spp. larvae (Reinemeyer *et al.* 2010).

Benzimidazole drugs act by binding to β-tubulin molecules, causing a conformational change and thereby hindering the polymerization of α- and β-tubulins in the construction of microtubules and other essential cellular components. This results in disruption of several essential functions of the cell, leading to starvation and death of the parasite (Lacey 1990). Most nematodes have several β-tubulin genes, often referred to as isotypes. For example, the model organism *Caenorhabditis elegans* has six β-tubulin genes (Gogonea *et al.* 1999), though only one of these, *ben-1*, is a drug target for benzimidazoles (Driscoll *et al.* 1989). The ovine strongyle parasite *H. contortus* has four β-tubulin genes of which isotype 1 and isotype 2 have been shown to be involved in benzimidazole resistance (Saunders *et al.* 2013). Previous to this thesis two β-tubulin genes have been described in *Parascaris* spp. (Tyden *et al.* 2013a).

2.3.4 Management practices

The use of management practices to lower the infection pressure on farms has not yet been thoroughly investigated for *Parascaris* spp. It has however been shown that *Parascaris* spp. eggs from decaying faecal matter are transferred to the soil approximately six weeks after deposition of faeces on the pasture. The infective eggs persisted in the soil for at least 18 months (Lindgren & Höglund 2010). It has also been shown that the use of horse manure as fertilizer of fields for grazing and housing foals on deep litter bedding increased the probability for *Parascaris* spp. positive egg counts (Fritzen *et al.* 2010). In addition, several studies show that the strongyle contamination on pasture can be controlled by removal of faeces twice weekly (Herd 1986; Corbett *et al.* 2014). Together these results suggest that regular removal of faeces from the pasture could be a way of reducing the infection pressure of *Parascaris* spp.

2.4 Anthelmintic resistance

Anthelmintic resistance means that parasites have acquired the ability to survive drug treatment at a dose that would normally be lethal. This ability is heritable and once established it is usually non-reversible (Sangster 1999).

Resistance in *Parascaris* spp. was first reported 2002 in the Netherlands, where several foals continued to shed *Parascaris* spp. eggs despite treatment with ivermectin and moxidectin (Boersema *et al.* 2002). Since then resistance to macrocyclic lactone drugs has been reported from all continents (Reinemeyer 2009). Previous to our studies resistance to pyrantel had been reported from the USA and Australia (Lyons *et al.* 2008; Armstrong *et al.* 2014) and resistance to fenbendazole from Australia and Saudi Arabia (Armstrong *et al.* 2014; Alanazi *et al.* 2017).

2.4.1 Resistance status in Sweden and Iceland

In 2008 the first case of ivermectin resistance was reported in Sweden (Lindgren *et al.* 2008). Further studies have shown widespread resistance to ivermectin on Swedish stud farms, while both pyrantel and fenbendazole have shown the expected efficacy against *Parascaris* spp. (Lindgren *et al.* 2008; Osterman Lind & Christensson 2009; Tyden *et al.* 2014).

The efficacy of anthelmintic drugs against *Parascaris* spp. has not been studied previously in Iceland. The Icelandic *Parascaris* spp. population is of interest to study due to the long isolation of the Icelandic horses, and hence their parasites (Adalsteinsson 1981). In addition, the resistance status was of interests to us since we had the opportunity to collected adult *Parascaris* spp. worms from foals slaughtered at an Icelandic abattoir.

2.4.2 Diagnosis of anthelmintic resistance

The Faecal Egg Count Reduction Test (FECRT) is the only current method available for regular screening of anthelmintic resistance in *Parascaris* spp. on farms (Nielsen *et al*. 2019). Guidelines from the World Association for the Advancement of Veterinary Parasitology (WAAVP) and the American Association for Equine Practitioners (AAEP) suggest to include at least six horses with a minimum of 150 eggs per gram faeces (EPG) in each test group. The number of *Parascaris* spp. eggs are counted in paired faecal samples before and around 14 days after treatment with the anthelmintic

drug. The mean egg reduction in the group is calculated and equals the efficacy of the drug (Coles *et al.* 2006; Nielsen *et al.* 2019).

There are currently no specific guidelines available for the interpretation of *Parascari*s spp. FECRT results, but the AAEP has published suggested cut-off levels for interpreting FECRT results for strongyle parasites (Table 1) (Nielsen *et al.* 2019).

Table 1. Cut-off levels suggested by the American Association for Equine Practitioners for interpretation of faecal egg count reduction test results.

Anthelmintic	Expected efficacy	Suspected resistance	Resistant
Ivermectin	99.9 %	95-98 %	< 95 %
Pyrantel	94-99 %	85-90 %	< 85%
Fenbendazole	99 %	90-95 %	< 90 %

Many factors apart from the efficacy of the drug may affect the results of a FECRT, such as age and nutritional status of foals, possible under-dosing of the drug and variation in laboratory techniques or egg dispersion (Vidyashankar *et al.* 2012; Carstensen *et al.* 2013). These factors need to be adjusted for in the planning, performance and statistical analysis of the experiments.

For several gastrointestinal nematodes with free-living larval stages *in vitro* methods such as egg hatch tests and larval development assays are available to study the efficacy of anthelmintic drugs (Coles *et al.* 2006). There are however currently no validated *in vitro* methods available for diagnosis of anthelmintic resistance in *Parascaris* spp. since ascarid larva do not hatch outside their host.

Molecular methods such as PCR-screening for mutations have been developed with the aim to diagnose resistance in several strongyle gastrointestinal nematodes (Coles *et al.* 2006; Charlier *et al.* 2018). However, to develop such methods for ascarids, the mechanisms behind anthelmintic resistance first need to be elucidated.

2.4.3 Mechanisms behind anthelmintic resistance

The mechanisms behind anthelmintic resistance have been suggested to be multigenic and include changes of the drug target or changes in the metabolism or efflux of the drug (Figure 3) (James *et al.* 2009; Whittaker *et al.* 2017). Mutations in genes coding for the drug target can lead to conformational changes that result in reduced affinity of the drug. Changes

in expression levels of drug targets may result in reduced numbers of receptors available for drug binding or assembly of receptor subunits resulting in receptors with lower affinity for the drug (Whittaker *et al.* 2017). Increased drug metabolism or increased drug efflux results in lower concentration of the drug at the site of action (Matouskova *et al.* 2016).

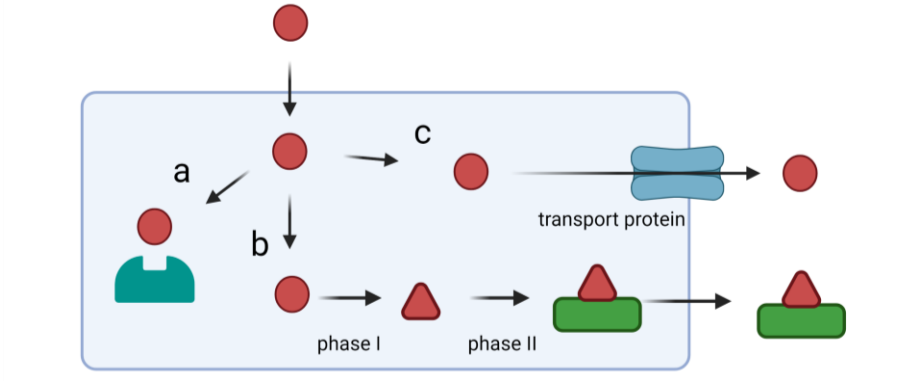

Figure 3. Overview of xenobiotic metabolism and possible resistance mechanisms in the parasite cell. a) Conformational changes or lower affinity of drug target, b) increased drug metabolism and c) increased drug efflux by transport proteins. Illustration created with BioRender.

Changes in the drug target

There is compelling evidence that single nucleotide polymorphisms (SNPs) in β-tubulin genes, coding for the benzimidazole drug target, are involved in resistance in strongyle nematodes as well as in the model organism *C. elegans* (Driscoll *et al.* 1989; von Samson-Himmelstjerna *et al.* 2007). Single nucleotide polymorphisms resulting in amino acid substitutions at position 167 (phenylalanine to tyrosine), 198 (glutamic acid to alanine or leucine) or 200 (phenylalanine to tyrosine) in the β-tubulin isotype 1 gene have been correlated to benzimidazole-resistant phenotypes in *H. contortus* and other parasitic nematodes (Kwa *et al.* 1994; Silvestre & Cabaret 2002; Ghisi *et al.* 2007; Redman *et al.* 2015; Avramenko *et al.* 2019). In addition, SNPs in the *H. contortus* β-tubulin isotype 2 gene have been suggested to be involved in benzimidazole resistance in field strains (Kwa *et al.* 1993a; Kwa *et al.* 1993b).

The β-tubulin family of ascarid parasites has not been as thoroughly investigated as those of strongyle nematodes and the target for benzimidazole drugs is still unknown. In addition, very few cases of reduced

efficacy of anthelmintic drugs have been observed in ascarids. Resistance causing SNPs are suggested to be present in parasite populations even before resistance is diagnosed (Sangster 1999). However, none of the polymorphisms described above were found in the two previously described β-tubulin genes of benzimidazole-susceptible *Parascaris* spp. (Tyden *et al.* 2013a; Tyden *et al.* 2014) or in a β-tubulin gene of benzimidazole-susceptible *Ascaridia galli* (Tarbiat *et al.* 2017). Several studies with ambiguous results have been made in *A. lumbricoides*: No polymorphisms were detected at any of the sites of four β-tubulin genes in a population with reduced efficacy of the benzimidazole albendazole (Krucken *et al.* 2017). However, SNPs were found in a β-tubulin gene at position 167 in a benzimidazole-susceptible population and at position 200 in a population with unknown resistance status (Diawara *et al.* 2009; Furtado *et al.* 2019).

Polymorphisms in the GABA receptor subunit gene *lgc-37* have been suggested to be connected to decreased sensitivity to macrocyclic lactones in *H. contortus* (Feng *et al.* 2002; Beech *et al.* 2010). In addition, reduced expression of the glutamate gated chloride channel subunit *avr-14* was described in ivermectin-resistant isolates of the cattle parasites *Cooperia oncophora* and *Ostertagia ostertagi* (El-Abdellati *et al.* 2011). These results indicate that both polymorphisms and changes in expression of receptor subunits can be involved in ivermectin resistance. Similarly, differences in expression of subunits or mutations leading to dysfunctional subunits have been proposed to be causing modified nAChRs with lower affinity to pyrantel and related nicotinic agonists (Whittaker *et al.* 2017).

Metabolism

Drug metabolism is usually divided into two phases. In the first phase (phase I), oxidation, reduction or hydrolysis converts the drug to a more reactive compound that can be conjugated with an endogenous molecule such as glutathione or glucuronic acid in the second phase (phase II). This results in a soluble, inactive drug that can easily be removed from the cell (James *et al.* 2009; Matouskova *et al.* 2016).

Phase I enzymes include the cytochrome P450 family (CYP), short-chain dehydrogenase/reductase (SDR) superfamily and flavin containing monooxygenase (FMO) superfamily. The *C. elegans* genome contains over 80 CYP genes, of which several have been shown to be inducible by xenobiotic substances including benzimidazoles (Menzel *et al.* 2001; Menzel *et al.* 2005; Laing *et al.* 2010; Stasiuk *et al.* 2019). Several CYP genes have

also been identified in *H. contortus* (Laing *et al.* 2015), and a slight elevation of the expression of a CYP gene was found in a highly benzimidazole-resistant isolate of *H. contortus* in comparison to isolates susceptible and with moderate resistance to benzimidazoles (Yilmaz *et al.* 2017). The expression of CYP genes has also been found to be inducible by albendazole in *H. contortus* and by ivermectin and the benzimidazole oxibendazole in *P. univalens* (Kellerova *et al.* 2020; Scare *et al.* 2020). Taken together these results suggest that increased activity of phase I enzymes such as CYPs may contribute to anthelmintic resistance.

The SDR superfamily is a large group of enzymes that metabolize endogenous and xenobiotic substances (Kavanagh *et al.* 2008). These could be of interest in regards to anthelmintic resistance since SDR genes were found to be upregulated in response to benzimidazole exposure in *C. elegans* (Stasiuk *et al.* 2019). An increased activity of SDR enzymes has also been suggested to be involved in resistance to chemotherapeutic drugs in human cancer treatment (Soldan *et al.* 1996).

In addition, increased activity of FMO enzymes has been shown to be involved in the metabolism of albendazole in the liver fluke *Fasciola Hepatica* (Alvarez *et al.* 2005), indicating that the FMO superfamily may also be of interest to study in regards to anthelmintic resistance.

Increased activity of phase II enzymes of the uridine 5'-diphospho-glucuronosyltransferase-glucosyltransferases (UGTs) and glutathione S-transferase (GST) families have also been suggested to be involved in drug resistance (Matouskova *et al.* 2016). Several UGTs and GSTs were upregulated in response to *in vitro* exposure of *C. elegans* to several benzimidazole drugs (Stasiuk *et al.* 2019) and in *H. contortus* after *in vitro* exposure to albendazole (Kellerova *et al.* 2020). A UGT was also found to be constitutively upregulated in a benzimidazole-resistant *H. contortus* strain compared to a susceptible strain (Matouskova *et al.* 2018).

Transport proteins

P-glycoproteins (Pgps) are membrane bound ATP-binding cassette (ABC) transport proteins involved in protecting cells against xenobiotic substances (Schinkel & Jonker 2003). Nematodes express numerous Pgps and increased efflux of drugs by Pgps has been suggested to be involved in resistance to anthelmintic drugs, mainly macrocyclic lactones (Xu *et al.* 1998; Dicker *et al.* 2011; Raza *et al.* 2016b) but also benzimidazoles (Blackhall *et al.* 2008).

The Pgp family in *P. univalens* has been thoroughly investigated and found to contain ten genes, of which at least two, *pgp-2* and *pgp-9*, can interact with ivermectin (Gerhard *et al.* 2020). In addition, increased expression of *pgp-11.1* has been correlated to reduced sensitivity to ivermectin in *Parascari*s spp. and transgenic expression of *Parascaris pgp-11.1* in *C. elegans* led to reduced ivermectin susceptibility, strengthening the theory that this efflux pump is involved in ivermectin resistance (Janssen *et al.* 2013; Janssen *et al.* 2015).

In addition to the Pgps, other transport proteins are involved in drug transport and resistance in other organisms. The solute carrier superfamily (SLC) include the major facilitator superfamily (MFS) and is a large group of membrane transporters involved in the transport of endogenous substances and drugs across membranes in prokaryotes and eukaryotes (Paulsen *et al.* 1996; Hoglund *et al.* 2011). It has been proposed that SLCs are involved in uptake of chemotherapeutic drugs in human cancer treatment and a downregulation of these transporters may result in drug resistant cancer cells (Huang 2007). Upregulation of MFS transporter genes have been shown to be involved in drug efflux and resistance in bacteria (Fluman & Bibi 2009). To our knowledge the roles of these transporters have not been investigated in regards to anthelmintic drug metabolism and resistance.

2.4.4 Additional factors affecting resistance development

It has long been known that an overuse of anthelmintic drugs contributes to anthelmintic resistance (Sangster 1999). In addition, a modelling study recently suggested that the combination of numbers of treatments, type of drug used and the timing of treatments may affect the time of resistance development (Leathwick *et al.* 2017). The study showed that the development of resistance could be faster if a single fenbendazole treatment were administered to three or four month old foals compared to at any other time during the first year. Similarly, ivermectin treatment of two, three or four month old foals resulted in faster development of ivermectin resistance. In addition, two treatments of fenbendazole or pyrantel administered to two and five month old foals would result in slower development of resistance compared to treatments at either two and four months or three and five months (Leathwick *et al.* 2017).

The term refugia refers to a part of the parasite population not reached by the anthelmintic treatment. The population in refugia is suggested to dilute

the occurrence of resistance alleles selected for in the treated population and slow down the development of resistance (Hodgkinson et al. 2019). Whether refugia is important in the development of resistance in *Parascaris* spp. is uncertain since resistance has developed despite the infective larvae surviving at least 18 months on the pasture (Lindgren & Höglund 2010).

2.5 One health aspects

Parascaris spp. is closely related to the human roundworm *A. lumbricoides*, a soil transmitted neglected tropical helminth infecting around 819 million people, mainly children in third world countries (Pullan *et al.* 2014). The World Health Organization (WHO) recommends that these children should be administered preventive treatment with benzimidazole drugs once or twice yearly (WHO 2006). Recently the first cases of reduced efficacy of anthelmintic drugs have been observed in *A. lumbricoides* (Krucken *et al.* 2017), a development that may affect the health of millions of people. Due to this development, there is an urgent need to understand the mechanisms involved in anthelmintic resistance and to develop novel methods for diagnosing resistant ascarid parasites. Research performed in *P. univalens* could possibly be extrapolated to the closely related human roundworm.

3. Aims of the thesis

Emergence of resistance to all anthelmintic substances registered for use in horses makes regular monitoring of drug efficacy increasingly important to ensure correct treatment of foals. Despite the serious situation, little is so far known about the molecular mechanisms underlying resistance in *P. univalens*.

The overall aims of this thesis were therefore to establish the current status of anthelmintic resistance in *P. univalens* on stud farms and to develop laboratory methods to study mechanisms and pathways associated with resistance.

The specific aims of this thesis were:

- To investigate the efficacy of the commonly used anthelmintic substances in Swedish and Icelandic *P. univalens* populations.
- To develop *in vitro* methods based on *P. univalens* larvae to facilitate research into resistance mechanisms.
- To investigate possible mechanisms behind the development of anthelmintic resistance in *P. univalens*.

4. Materials and Methods

This chapter provides a summary of materials and methods used in this thesis. Detailed descriptions are presented in the respective papers.

4.1 Study populations

In **study I** farms located across Sweden with at least four foals ≤ 12 months old and excreting at least 150 EPG were included in FECRTs in 2016 and 2017. The efficacy of pyrantel was tested on 97 foals on nine farms. Two of the farms included two groups of foals resulting in a total of 11 groups (group 1-11). The efficacy of fenbendazole was tested on 50 foals on six farms. Each farm included one group of foals resulting in six groups (group 12-17).

In **study II** the efficacy of ivermectin was studied on farms in the north and west of Iceland. Farms with a minimum of four foals between three and five months old and excreting at least 100 EPG were included, resulting in a total of 85 foals on eight farms. In addition, six foals from one farm were included as an untreated control group.

In **study V** the efficacy of fenbendazole was studied on a farm that showed reduced efficacy of this drug in **study I**. Personnel on the farm suspected continuous treatment failure of the drug and FECRTs were therefore repeated. In 2019, 16 foals between five and nine months old were included (group A) and in 2020 35 foals (group B) and 11 foals (group C) between three and six months of age were included. All foals excreted at least 100 EPG at the pre-treatment sampling. In addition, parasite eggs were collected post-treatment from these foals for amplicon sequencing of β-tubulin genes (**study V**). A group of four foals between five and six months of age from a farm with a documented fenbendazole efficacy of 100 % (group D) was also included in the 2020 FECRT and *P. univalens* eggs were

collected pre-treatment to be used as controls in the amplicon sequencing of β-tubulin genes.

For *in vitro* exposure in **study III** and **IV**, adult *P. univalens* worms were collected in collaboration with the University of Iceland from foals slaughtered at an abattoir in Sellfoss, Iceland. The worms were collected on two different occasions from the intestines of foals originating from three farms in the south of Iceland. The foals were around six months old and had never been treated with anthelmintic drugs.

For the larval exposure in **study IV** *P. univalens* eggs were collected from the adult parasites described above and from pooled faecal material collected in connection with the FECRT in **study II**.

4.2 Evaluation of anthelmintic efficacy

In **study I**, **II** and **V** FECRTs were used to assess the efficacies of three different anthelmintic drugs. Paired faecal samples were collected before and 10-20 days after treatment. Foals were treated with pyrantel embonate oral paste (Banminth, Pharmaxim) at a dosage of 19 mg/kg (6.6 mg of pyrantel base), fenbendazole oral paste (Axilur, Intervet) at a dosage of 7.5 mg/kg or ivermectin (Ivomec vet 10 mg/ml (Boehringer Ingelheim) or Noromectin 1 % (Norbrook Laboratories)) at a dosage of 0.2 mg/kg administered as a subcutaneous injection.

In **study I** and **V** 3 g of faeces were mixed with 42 ml of water resulting in a minimum detection level of 50 EPG (Coles *et al.* 1992). In **study II** 4 g of faeces were mixed with 56 ml of water and two McMaster slides were examined for each sample, resulting in a minimum detection level of 7.5 EPG. The faeces were dissolved in the water and strained through a 150 µm sieve. The flow through was centrifuged and the pellet dissolved in flotation media (saturated sodium chloride solution in **study I** and **V**, saturated magnesium sulphate solution in **study II**). Determination of the number of eggs was done in McMaster chambers using a light microscope.

Egg reductions were analysed using a Bayesian model for paired design included in the "eggCounts" package in R (version 2.0 in **study I** and 2.3 in **study II**) and the shiny-eggCounts web interface (**study V**) (Torgerson *et al.* 2014; Wang *et al.* 2018). Since it was recently suggested to include an untreated control group to account for natural changes in egg shedding (Morris *et al.* 2019), the efficacy was also calculated according to the

Presidente formula in **study II** (Presidente 1985). Results from all three studies were interpreted according to parasite control guidelines from the AAEP where an efficacy below 95 % indicates resistance to ivermectin, below 90 % resistance to fenbendazole and below 85 % resistance to pyrantel (Table 1) (Nielsen *et al.* 2019).

4.3 Karyotyping

In **study I**, **II** and **III** karyotyping was performed to establish the species of *Parascaris* present in the study populations.

Parasite eggs were isolated from the faeces then decorticated and washed using 2 % sodium hypochlorite in 16.5 % sodium chloride before incubation at 37 °C for 1-3 h for the first or second embryonic division to occur. Eggs were stained and the number of chromosomes in each egg was examined using a fluorescent microscope (Nielsen *et al.* 2014).

4.4 *In vitro* exposure

To investigate parasite response to anthelmintic drugs *P. univalens* were exposed to anthelmintic drugs *in vitro*, followed by examination of changes in gene expression.

To facilitate further research into the molecular background of anthelmintic resistance in *P. univalens* we aimed to develop and evaluate a novel, larval-based *in vitro* model for drug exposure.

4.4.1 Larval development and hatching

A protocol for hatching *P. univalens* eggs was developed in **study IV**. Decorticated eggs as above were incubated at 28 °C for 21 days, allowing larvae to develop inside the eggs. The eggs were hatched by six slow strokes in a Kimble Kontes 15 ml glass homogeniser, using a pestle leaving 0.16 mm clearance. Larvae were then cultured in tissue culture media at 37 °C, 5 % CO_2.

4.4.2 Anthelmintic substances and *in vitro* exposure

Larvae and adult worms were cultured in tissue culture media with the addition of anthelmintic drugs. Since fenbendazole and pyrantel embonate are insoluble in water, the more soluble compounds thiabendazole and

pyrantel citrate were used for the *in vitro* incubations (Wishart *et al.* 2018). All anthelmintic drugs were dissolved in dimethyl sulfoxide (DMSO) (SVA), with a 0.1 % final concentration in the exposure solution. In **study III** ivermectin (Sigma-Aldrich) was used at 10^{-9} M, 10^{-11} M and 10^{-13} M, pyrantel citrate (Santa Cruz Biotechnology) at 10^{-6} M, 10^{-8} M and 10^{-10} M and thiabendazole (Sigma-Aldrich) at 10^{-5} M, 10^{-7} M and 10^{-5} M. Drug concentrations used were based on similar studies in adult *P. univalens* and *Ascaris suum* larvae (Janssen *et al.* 2013; Zhao *et al.* 2017). In **study IV** only the highest concentration of each drug was used for exposure of larvae and adult worms, based on previous results. In **study III** the worms were exposed for 24 h at 37 °C. In **study IV** adult worms were exposed for 3 h, 10 h and 24 h at 37 °C to investigate if expression varied between different time points. Larvae in **study IV** were only exposed for 24 h at 37 °C, 5 % CO_2 due to low amounts of parasite eggs and based on results of adult worm exposure. In all experiments, controls incubated in tissue culture media with the addition of 0.1 % DMSO were included. In **study III** additional controls were used: one group incubated in tissue culture media without addition of DMSO for 24 h and one group of non-incubated worms (0 h).

In **study III** the viability of the adult worms was observed after exposure and scored between 2 (movement only when stimulated with forceps) and 6 (seven or more spontaneous whole body movements during 15 s) (Scare *et al.* 2018). After the exposure adult worms were dissected and stored in RNAlater (Invitrogen) while larvae were flash frozen in liquid nitrogen after addition of Trizol (Invitrogen).

4.5 Molecular methods and bioinformatics

4.5.1 Identification of candidate genes and primer design

Candidate genes for putative involvement in drug efflux and anthelmintic resistance in *P. univalens* (**study IV**) were identified based on previously published data (Janssen *et al.* 2013; Gerhard *et al.* 2020) and results from **study III**. Primer sites for qPCR were chosen to flank intron sequences for identification of any contaminating genomic DNA. Primer specificity was confirmed by PCR, gel electrophoresis, Sanger sequencing and nucleotide searches in WormBase ParaSite (Howe *et al.* 2017). Primers for qPCR were optimised regarding annealing temperature and concentration, then validated

to 92.9 % - 99.9 % efficiency. Melt curve data confirmed single products for each primer pair.

In **study V** *P. univalens* β-tubulin genes were identified from the functional annotation in **study III** and verified by BLAST searches in WormBase ParaSite. Unique primer sites for amplicon sequencing of each β-tubulin gene were picked flanking the codons 167, 198 and 200. Primers were then designed and confirmed as above and unique barcodes to identify genes and groups were added. Due to differences in intron length between the exons containing the sites, the expected amplicon sizes ranged from 559 to 1834 bp.

4.5.2 RNA isolation

RNA was isolated using the NucleoSpin® RNA Plus kit (Macherey Nagel) for adult worm tissues in **study III** and **IV**. In **study IV** NucleoSpin® RNA XS kit (Macherey Nagel) was used to isolate RNA from larvae. Initial steps of Trizol and chloroform (Sigma-Aldrich) was performed before use of the kits for both larvae and adult worm tissues.

4.5.3 RNA-Seq

To study the impact of anthelmintic exposure on gene expression of adult worms exposed to different concentrations of ivermectin, pyrantel and thiabendazol as well as controls, the entire transcriptomes were investigated by RNA-Seq in **study III**.

For each condition described in 4.4.2 the anterior end from each of three biological replicates, in total 36 individual worms, were sequenced using Illumina NovaSeq.

Filtered reads were mapped against the *P. univalens* transcriptome available in WormBase ParaSite. Transcripts were quantified using Salmon and differential expression analysis was performed by the DeSeq2 package in R (Love *et al.* 2014). Genes with a log2 fold change of ≥ 1 or ≤ -1 and an adjusted *P*-value < 0.05 were considered differentially expressed. Functional annotation of differentially expressed genes were identified by searching protein sequences against the Swiss-Prot database (UniProt 2019).

4.5.4 Quantitative PCR

In **study IV** the constitutive expression of six transport protein genes was investigated in adult worms and larvae. In addition, the expression of these genes were investigated after *in vitro* exposure to ivermectin, pyrantel and thiabendazol.

Coding DNA for qPCR was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). Reactions for qPCR were set up using QuantiTect SYBR® Green PCR kit (Qiagen), run on BioRad CFX Opus 96 and analysed using BioRad CFX Maestro. Samples were run in duplicates or triplicates with no template controls and inter plate calibrator. Relative expression was calculated using the ΔΔCt-method (Livak & Schmittgen 2001). Differences in gene expression were analysed using either unpaired *t*-test, one-way ANOVAs or two-way ANOVAs with Tukey's multiple comparisons test in GraphPad Prism 9.1.0.

4.5.5 Amplicon sequencing

In **study V** the presence of SNPs at position 167, 198 and 200 in the seven β-tubulin genes were investigated in a *P. univalens* population with reduced sensitivity to fenbendazole.

Genomic DNA for amplicon sequencing of β-tubulin genes was extracted from parasite eggs with resistant (three groups) and susceptible (one group) phenotypes using NucleoSpin® Tissue kit (Macherey Nagel). Two DNA reactions were performed for each group and each of these was used for an individual PCR reaction to minimise the risk for PCR bias. Amplicons were generated using 24 cycles of PCR with Phusion Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher Scientific). To get a similar number of amplicons for each gene in the multiplex library the amount of each amplicon was calculated based on amplicon size and concentration. Amplicons were then pooled and sequenced using the PacBio Sequel Technology Platform at Uppsala Genome Center.

Circular consensus (CCS) reads were generated and mapped to the genes of interest in the *P. univalens* reference genome (PRJNA386823) (Wang *et al.* 2017) available in WormBase Parasite. After demultiplexing, variants were called on reads from each sample group.

4.5.6 Phylogenetic analysis of β-tubulin genes

In **study V** the seven β-tubulin genes of *P. univalens* were compared to full length β-tubulin sequences from clade III and V parasitic nematodes *A. lumbricoides, A. suum, Toxocara canis, Brugia malayi, H. contortus, Necator americanus, Onchocerca volvulus* and the model organism *C. elegans*. *Saccharomyces cerevisiae* β-tubulin mRNA was used as outgroup. The phylogenetic analysis was performed using a maximum likelihood method on the Phylogeny.fr platform, advanced mode with default settings (Dereeper *et al.* 2008; Dereeper *et al.* 2010). The tree was visualized using the R package ggtree (Yu 2020).

5. Main results and discussion

The following chapter is a summary of the main findings from **study I**-**V**. Detailed results are presented in the respective papers.

5.1 Evaluation of anthelmintic efficacy

5.1.1 Efficacy testing on Swedish farms

The efficacy of pyrantel and fenbendazole was studied on Swedish stud farms in 2016 and 2017 (**study I**). Of the 11 groups treated with pyrantel only four groups showed expected efficacies according to the AAEP guidelines (Table 1, Figure 4a). An additional four groups showed efficacy levels below the 85 % level for resistance (Figure 4a) and as many as 43 % of the foals treated with pyrantel excreted eggs after the treatment. Four of the six groups treated with fenbendazole showed the expected efficacy, while one group had an efficacy of 89 %, which is classed as resistant, and one group 94 %, classed as suspected resistant (Figure 4b). Of the 50 foals treated with fenbendazole only three individuals (6 %) excreted eggs at the post-treatment sampling, indicating that the results of reduced efficacy may be due to treatment failure of a few individuals rather than emerging resistance.

On the farm that had a FECR of 89 % after fenbendazole treatment in 2017 (**study I**), the personnel noted continuous reduced efficacy of fenbendazole at their monthly monitoring of FECs and therefore additional efficacy studies were performed on this farm in 2019 and 2020 (**study V**). The results showed efficacies of 78 %, 73 % and 88 % in the three tested groups, all below the 90 % threshold for fenbendazole resistance (Figure 4c). These results confirm that there are fenbendazole resistant *P. univalens* on the farm, which was suspected in **study I**. In addition, the proportion of foals

on the farm excreting eggs post treatment increased from 15 % in the first study to 55 % in the second study, supporting that the proportion of fenbendazole resistant *P. univalens* had increased on the farm. Foals on the control farm (group D) showed an efficacy of 100 %, confirming the populations susceptibility to fenbendazole (Figure 4c).

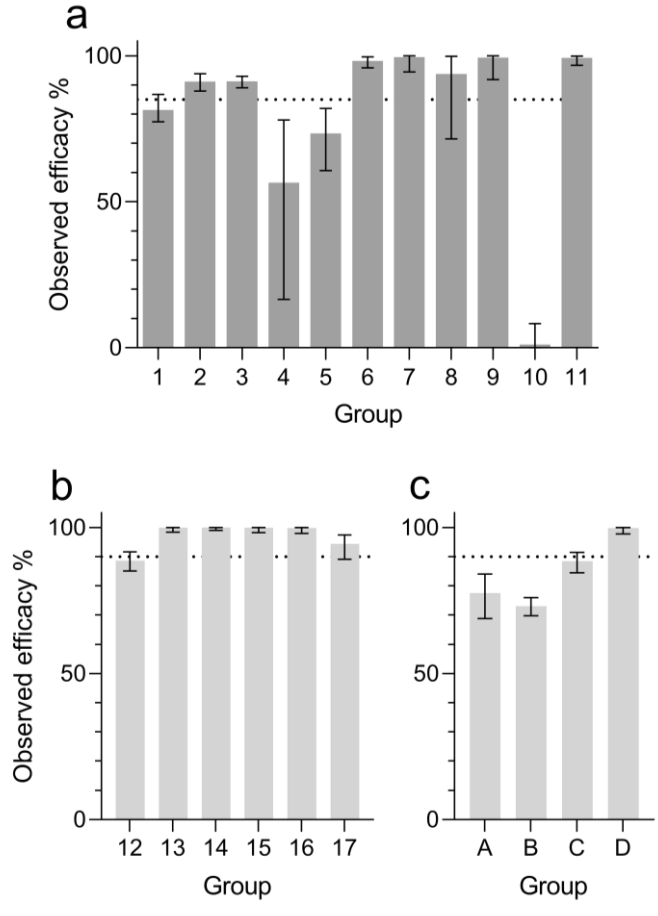

Figure 4. Results from faecal egg count reduction tests with 95 % confidence intervals. Observed efficacy of a) pyrantel on Swedish stud farms 2016 and 2017, b) fenbendazole on Swedish stud farms 2017 and c) fenbendazole on two Swedish stud farms 2019 and 2020. The dotted lines corresponds to the resistance levels suggested by the American Association of Equine Practitioners, 85 % for pyrantel and 90 % for fenbendazole. Groups with efficacies below these levels are classed as resistant.

These are the first cases of reduced efficacy of pyrantel and fenbendazole in *P. univalens* in Sweden and Europe. In combination with similar results from across the world (Lyons *et al.* 2008; Armstrong *et al.* 2014; Alanazi *et al.* 2017) these results suggest that anthelmintic resistance in *Parascaris* spp. is emerging for all available drug classes.

5.1.2 Efficacy testing on Icelandic farms

The efficacy of injectable ivermectin was investigated on eight Icelandic farms in **study II**. Results showed the presence of ivermectin-resistant parasites on all farms with efficacies calculated by the "eggCounts" package in R ranging from 0 % to 81 % (Table 2), well below the 95 % resistance level for ivermectin suggested by the AAEP (Table 1). In total, 91 % of the foals excreted *P. univalens* eggs after treatment and on four of the eight farms the mean egg counts were higher post-treatment than the pre-treatment. To account for natural variations in shedding of parasite eggs, results were also calculated in relation to an untreated control group (Presidente 1985). According to these results efficacies on the farms were between -132 % and 71 % (Table 2). Since the mean EPG of the foals in the control group was lower at the second sampling compared to the first sampling several farms showed negative efficacy values.

Table 2. Results of faecal egg count reduction tests performed on Icelandic farms in 2019. Observed efficacies of ivermectin calculated by two different methods: the "eggCounts" package in R and the Presidente formula.

Farm	% Efficacy "eggCounts"	% Efficacy Presidente
1	0	-67
2	34	0
3	0	-84
4	0	-132
5	81	71
6	6	-42
7	56	34
8	0	-84
Untreated control group	33	-

The injectable form of ivermectin is not registered for use in horses but is regularly used for the treatment of horses in Iceland rather than the oral paste (Paulrud *et al.* 1997). Recent studies suggest that parasites in the intestinal

lumen are exposed to a higher dose of drug after oral administration than after injection since the efficacy of ivermectin against small strongyles was lower after intramuscular injection than after treatment with oral paste (Saumell *et al.* 2017). In addition, the ivermectin concentration in *H. contortus* in lambs was 15 times higher after intraruminal administration than subcutaneous administration (Lloberas *et al.* 2012). However, both the oral paste and injectable ivermectin showed 100 % effect against adult stages of *Parascaris* spp. at a dose of 0.2 mg/kg at the time of registration, indicating that the lack of efficacy observed in this study is due to emerging resistance to ivermectin (Yazwinski *et al.* 1982; Campbell *et al.* 1989).

In **study II**, the foals were likely administered some additional ivermectin as the mares were treated at the same time as the foals and ivermectin is known to accumulate in milk (Gokbulut *et al.* 2016). A recent study however showed that the plasma levels of ivermectin in foals suckling ivermectin treated mares were below the active concentration of the drug. Even though the strongyle egg counts in the foal faeces were not affected, the authors suggest that the exposure to low levels of ivermectin during several days may contribute to development of ivermectin resistance (Mayinda *et al.* 2021).

Since the Icelandic horse population has been isolated long before the introduction of anthelmintic drugs (Adalsteinsson 1981), resistance has most likely not been introduced from populations outside Iceland, but developed independently. The development of ivermectin resistance in the Icelandic *P. univalens* population is rather surprising as foals in Iceland often receive few anthelmintic treatments and roam large areas of grazing, suggesting a lower selection pressure for anthelmintic resistance genes. They do however often share pasture with foals from several other farms, which may facilitate the spread of any resistance alleles.

5.1.3 Methodological considerations

The FECRT is currently the only available method to test anthelmintic efficacy *in vivo* on stud farms. The method does however have some drawbacks. Results may be affected by foal related factors such as the age and health of foals which can affect the immunity and thereby the worm burden. Testing of foals older than six months could result in false high efficacies due to onset of age related immunity. However, in many cases it is difficult to include young foals in the FECRT as they are often treated routinely against *P. univalens* infection twice with 8 weeks intervals,

resulting in the first egg shedding around six months due to the long patency period of the parasite. Mistakes in estimation of foal weights and administration of the drug may lead to under-dosing and thereby treatment failure of the drug. In **study I** and **V** the anthelmintic drug was dosed for the estimated weight and an additional 50 kg to avoid under-dosing. In **study II**, the weight of all foals was estimated by an experienced veterinarian who also administered the drug to minimize under-dosing.

Method related factors that may affect results include uneven dispersion of eggs in the sample and counting of few eggs, which may result in lower specificity. However, the statistical methods included in the "eggCounts" software used for all three studies correct for these factors to some extent (Torgerson *et al.* 2014; Wang *et al.* 2018).

In **study I** and **II** several groups included only four or five foals, despite recommendations to include at least six horses in each group (Coles *et al.* 2006). The WAAVP guidelines are however written for efficacy control of strongyle parasites in adult horses and may not be applicable for studs as many are small, family owned farms with only a few foals. In a small group the treatment failure of one foal may affect the group result to a higher extent than in a larger group, which should be considered when interpreting the results. Since there are currently no guidelines available for efficacy testing of *P. univalens* in foals the guidelines available for strongyle parasites from WAAVP and AAEP have been used in this thesis.

5.2 Karyotyping

Karyotyping performed in **study I**, **II** and **III** revealed that *P. univalens* was the parasite species present in the study populations in both Sweden and Iceland. These results are in line with other studies where *P. univalens* was detected in samples from the USA and Europe (Jabbar *et al.* 2014; Nielsen *et al.* 2014; von Samson-Himmelstjerna *et al.* 2021). In addition, a population genetics study comparing *Parascaris* spp. from six different countries, including Iceland, showed that the investigated populations were genetically homogenous (Tydén *et al.* 2013b). It is however somewhat surprising that *P. univalens* is the exclusive species present in Iceland since the horse population and hence their parasites have been isolated on the island for well over 100 years (Adalsteinsson 1981).

In an older study performed in the 1980s, both *P. univalens* and *P. equorum* as well as hybrids between the two species were detected (Goday & Pimpinelli 1986), indicating that *P. equorum* has diminished fairly recently. A recent study suggests that many of the sequences deposited as *P. equorum* in GeneBank may actually be *P. univalens* (von Samson-Himmelstjerna *et al*. 2021).

5.3 Transcriptional responses to *in vitro* exposure

To explore transcriptional responses to anthelmintic exposure in an unbiased way, changes in expression across the entire transcriptomes of adult worms incubated at different conditions were investigated in **study III**. Adult worms were exposed to three different concentrations of the anthelmintic drugs ivermectin, pyrantel and thiabendazole for 24 h. The total gene expression of these worms were compared to three sets of controls: non-incubated control (0 h), control worms incubated in tissue culture media (24 h^{-DMSO}) and control worms incubated in tissue culture media with the addition of 0.1 % DMSO (24 h^{+DMSO}). All worms survived the incubation, but worms incubated at the highest concentration of each drug were visibly less viable than worms incubated at lower concentrations or in the absence of anthelmintic drugs (all controls).

The largest number of differentially expressed genes was observed between the non-incubated control (0 h) and all worms incubated for 24 h, both controls and those exposed to anthelmintic drugs (Figure 5). This suggests that the stress caused by removal of the worms from the host and incubation in tissue culture media affects the gene expression more than the exposure to anthelmintic drugs. The variation between individual worms incubated under the same conditions was also high in some cases, affecting the transcriptional response for the group. These findings need to be considered when interpreting results from studies where gene expression is studied after *in vitro* incubation.

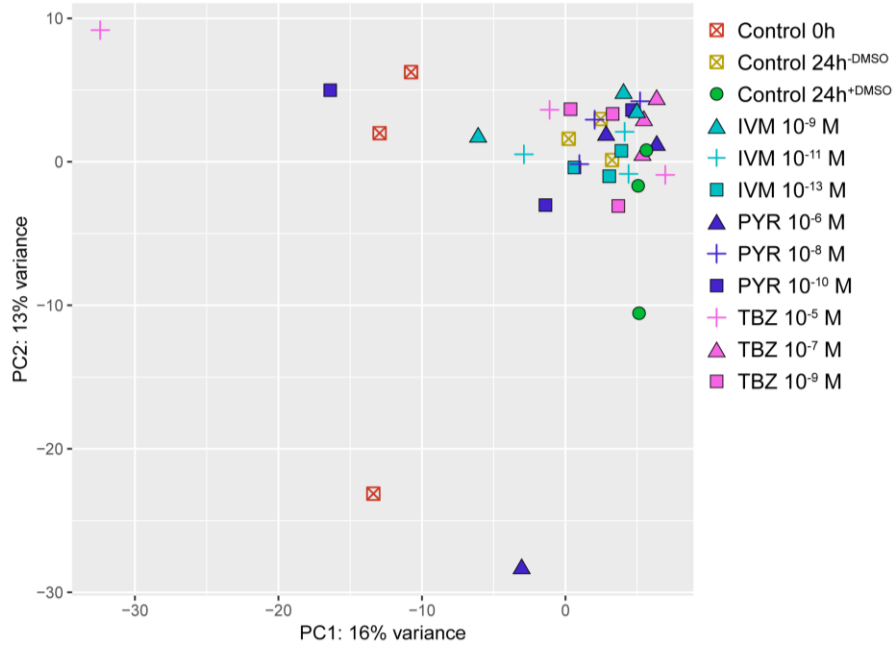

Figure 5. Principal components analysis plot showing the variation in gene expression of *Parascaris univalens* incubated under different conditions. The largest variation in gene expression was observed between the non-incubated worms (control 0 h) and all worms incubated *in vitro*. Each symbol corresponds to an individual worm. The anthelmintic drugs ivermectin (IVM), pyrantel citrate (PYR) and thiabendazole (TBZ) were used in the experiment (Martin *et al.* 2020).

Several genes with possible involvement in drug metabolism, drug transportation and coding for putative drug targets were differentially expressed after *in vitro* drug exposure. An interesting finding was the upregulation of a GABA receptor subunit homologous to *lgc-37*, after exposure to high concentration (10^{-9} M) of ivermectin. Mutations in the *lgc-37* gene in *H. contortus* have been shown to decrease the sensitivity to macrocyclic lactones, suggesting that the gene may contribute to resistance (Feng *et al.* 2002; Beech *et al.* 2010). The upregulation of *lgc-37* after exposure to ivermectin suggests that this gene may be an interesting candidate in the mechanism of ivermectin resistance and should be further investigated.

Several transcripts homologous to phase I enzymes belonging to the SDR and FMO superfamilies were differentially expressed after drug exposure.

Two SDR genes were upregulated after exposure to different concentrations of all three drugs, whereas two SDR genes were downregulated in response to ivermectin exposure. Previous studies suggest that members of the SDR superfamily may participate in the xenobiotic metabolism in *C. elegans* and *H. contortus* (Cvilink *et al*. 2008; Stasiuk *et al*. 2019). Two FMO genes were upregulated in response to drug exposure, one in response to ivermectin and one in response to pyrantel and thiabendazole. A third FMO gene was downregulated in response to various concentrations of all three drugs. Members of the FMO superfamily are involved in biotransformation of xenobiotic compounds in many organisms (Cashman 2018) including metabolism of benzimidazoles in *F. hepatica* (Alvarez *et al.* 2005). Genes belonging to both of these phase I enzyme families may therefore be of interest as novel candidate genes for anthelmintic resistance research. Genes belonging to the CYP family have been given much attention regarding their possible role in *C. elegans* and *H. contortus* drug metabolism and resistance (Menzel *et al.* 2001; Laing *et al.* 2010; Stasiuk *et al.* 2019). However, in **study III** only one CYP gene was differentially expressed after exposure to ivermectin.

The involvement of transport proteins in anthelmintic resistance, mainly the Pgp family, has been supported in studies of *H. contortus* (Xu *et al.* 1998; Blackhall *et al.* 2008; Raza *et al.* 2016a; Raza *et al.* 2016b). In **study III** no Pgp genes were differentially expressed after anthelmintic exposure, which is in accordance with other studies where the expression of Pgps have been studied in *P. univalens* after *in vitro* exposure to anthelmintic drugs (Janssen *et al.* 2013; Gerhard *et al.* 2020; Scare *et al.* 2020). Despite the lack of transcriptional response of Pgps to anthelmintic drugs, there have been support for their involvement in anthelmintic resistance through constitutive upregulation in an ivermectin-resistant population of *P. univalens* (Janssen *et al.* 2013). In addition, transgenic expression of a *P. univalens* Pgp in *C. elegans* led to reduced susceptibility to ivermectin, further supporting the involvement of Pgps in anthelmintic resistance in *P. univalens* (Janssen *et al.* 2015; Gerhard *et al.* 2021). Transcripts belonging to the MFS superfamily were upregulated after exposure to ivermectin and pyrantel and an SLC gene after exposure to ivermectin. The involvement of these transport proteins in xenobiotic response of nematodes has not yet been investigated, but they might be of interest since MFS transporters have been shown to be involved in antibiotic resistance in bacteria (Fluman & Bibi 2009), and

downregulation of SLC transporters may cause decreased drug uptake and resistance to cancer drugs in human medicine (Huang 2007). It would therefore be of interest to investigate the function of these genes further in *P. univalens.*

In addition to the results above, many uncharacterized genes were highly upregulated after exposure to anthelmintic drugs. The possible role of these genes in anthelmintic metabolism and resistance needs to be examined in future studies.

5.4 Development and evaluation of a larval model

A novel protocol for fast and easy hatching of *P. univalens* eggs was developed in **study IV**. The method resulted in a hatching rate of 92 % with fully viable larvae that could be cultured in tissue culture media and used for *in vitro* studies. To evaluate the use of a larval model in anthelmintic resistance research, the expression of six transport protein genes was compared between larvae and different tissues of adult worms. The transcriptional response of these genes in response to *in vitro* drug exposure was also evaluated in larvae and compared to adult worms.

The difference in constitutive expression of four Pgp genes (*pgp-2, pgp-9, pgp-11.1* and *pgp-16.1*) and two MSF genes (PgR006_g137 and PgR015_g078) was investigated between larvae and tissues from the anterior end and intestine of adult worms. Results showed that most genes were expressed at lower levels in larvae than in both adult tissues (Figure 6). In adult tissues the expression was higher in the intestine than in the anterior end for all genes except *pgp-11.1* which was expressed at similar levels in the two tissues (Figure 6). In previous studies of *P. univalens* where Pgp expression have been compared between the intestine and other tissues results have varied, but the majority of Pgps are expressed at high levels in the intestine, which is suggested to be a major site for efflux of xenobiotics (Janssen *et al.* 2013; Gerhard *et al.* 2020).

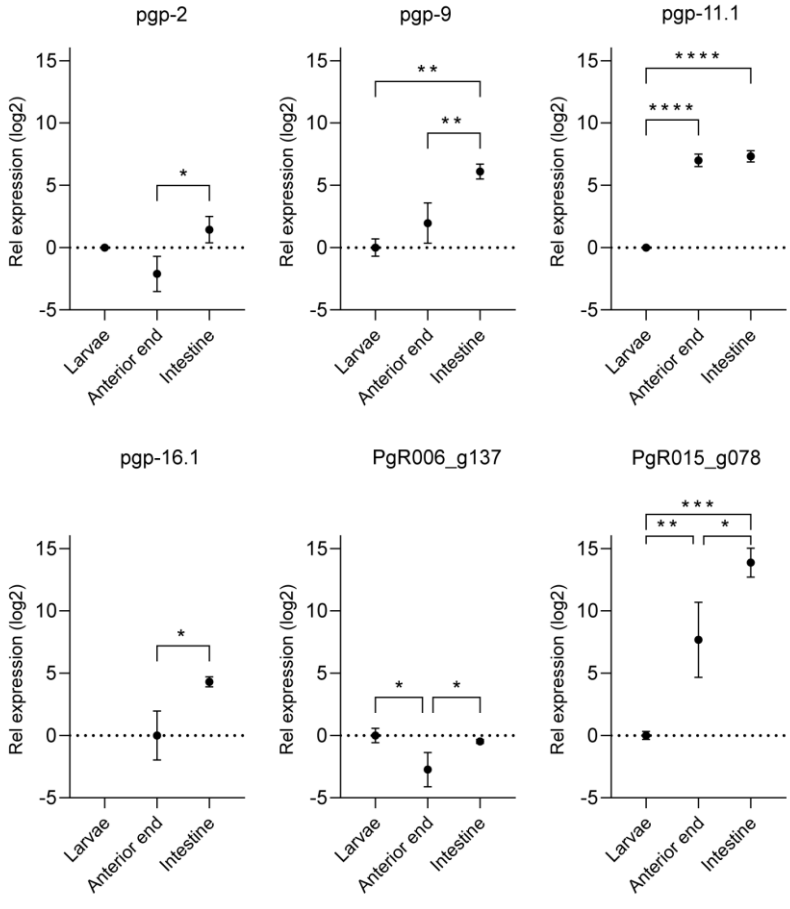

Figure 6. Expression levels of transport protein genes in tissues from the anterior end and intestine of individual adult *Parascaris univalens* (n=3) and pools of 12.000 larvae (n=3). Gene expression was normalised to the reference gene *gpd-1* and expression levels of *pgp-2*, *pgp-9*, *pgp-11.1* and MFS genes PgR006_g137 and PgR015_g078 are shown relative to the mean expression in larvae. A one-way ANOVA with Tukey's multiple comparisons test was performed to identify differences in expression between tissues. Expression of *pgp-16.1* in larvae was too low to be detected, therefore gene expression in adult tissues is shown relative to the mean expression in the anterior end. An unpaired t-test was performed to identify differences in expression between anterior end and intestine. Significant differences are marked with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or **** ($p > 0.0001$) (Martin *et al.* 2021a).

The transcriptional responses in larvae, as well as in adult anterior end and intestinal tissues were investigated after *in vitro* exposure to ivermectin, pyrantel and thiabendazole. All adult worms and larvae were alive after 24 h incubation in tissue culture media with DMSO and with addition of anthelmintic drugs. There was no significant upregulation of Pgp or MFS genes in the adult tissues after 3 h, 10 h or 24 h *in vitro* drug exposure, which is in accordance with previous studies (Janssen *et al.* 2013; Gerhard *et al.* 2020; Martin *et al.* 2020; Scare *et al.* 2020). Expression of *pgp-9* was however significantly increased in larvae after *in vitro* exposure to pyrantel and thiabendazole (Figure 7). Benzimidazole drugs have been suggested to interact with Pgps in *H. contortus* (Beugnet *et al.* 1997), but so far there are no studies showing the interaction between pyrantel and Pgp expression. However, other anthelmintic drugs such as monepantel and levamisole have been shown to alter Pgp expression in multidrug-resistant *H. contortus* (Raza *et al.* 2016a; Raza *et al.* 2016b), indicating that Pgp expression might be influenced by several different drugs. Surprisingly, the larvae showed no transcriptional changes of any of the investigated transporters in response to ivermectin exposure (Figure 7). Ivermectin is a well-known substrate for Pgp transport, and both *pgp-2* and *pgp-9* have been shown to interact with ivermectin in *P. univalens* (Gerhard *et al.* 2020). The lack of response after ivermectin exposure may depend on the drug concentration used as expressional response of Pgps have been shown to be dose dependent in both mammalian cells and *H. contortus* larvae (Menez *et al.* 2012; Raza *et al.* 2016a). The highest ivermectin dose (10^{-9} M) used in **study III** and **IV** has previously been shown to be the highest non-lethal dose for *in vitro* incubation of adult *Parascaris* spp. (Janssen *et al.* 2013). To be able to compare results the same drug concentrations were used for exposure of adult worms and larvae in **study IV.**

Despite differences in gene expression between larvae and adult worm tissues the larval model should prove useful in future research. The collection of *P. univalens* eggs from foal faeces enables use of larger study populations in comparison to the restricted parasite populations available in slaughtered foals. The model should also prove useful to compare differences in gene expression between *P. univalens* populations with different resistance phenotypes.

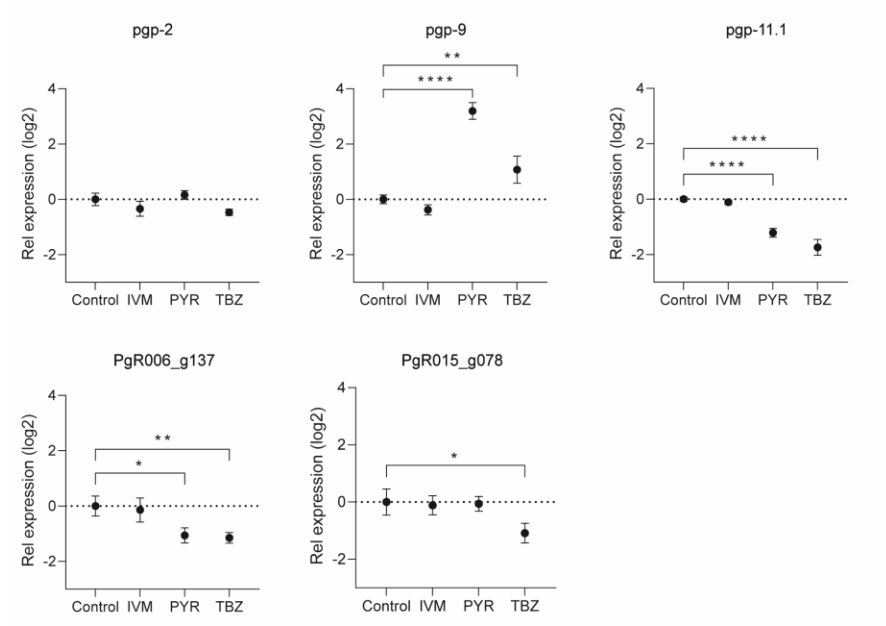

Figure 7. Expressional responses of transport protein genes in pools of 4.000 *Parascaris univalens* larvae (n=3) *in vitro* exposed to ivermectin (IVM) 10^{-9} M, pyrantel citrate (PYR) 10^{-6} M and thiabendazole (TBZ) 10^{-5} M in media with 0.1 % DMSO as well as in media containing 0.1 % DMSO only (control) for 24 h. Gene expression was normalised to reference genes *gpd-1* and *actin* and related to the mean of the control. Significant differences between exposed larvae and controls were identified by one-way ANOVA with Tukey's multiple comparisons test and marked with * ($p < 0.05$), ** ($p < 0.01$) or **** ($p > 0.0001$) (Martin *et al.* 2021a).

5.5 The β-tubulin family of *Parascaris univalens*

Resistance to benzimidazole drugs is rare in *P. univalens* and the mechanism behind benzimidazole resistance is unknown in ascarid worms. The unique benzimidazole-resistant population identified in **study V** was therefore used to investigate whether the SNPs at position 167, 198 or 200 of the β-tubulin isotype 1 gene in strongyles were present in any of the β-tubulin genes of fenbendazole-resistant *P. univalens*.

In addition to the two previously published β-tubulin genes (Tyden *et al.* 2013a), five novel β-tubulin genes were identified. The phylogenetic relationship between these seven genes as well as β-tubulin genes from other parasitic nematodes in clade III and V and the model organism *C. elegans* was investigated (Figure 8). Results show that the β-tubulin genes form four

clusters and that none of the ascarid β-tubulin genes cluster with the *C. elegans* benzimidazole drug target *Cel-ben-1* or the *H. contortus* isotype 1 (*Hco-tbb-iso-1*) or isotype 2 (*Hco-tbb-iso-2*) genes associated with benzimidazole resistance. Five of the *P. univalens* β-tubulin genes form a separate cluster with β-tubulin genes from other clade III nematodes. To avoid confusion with the isotype 1 and isotype 2 genes of strongyle parasites these five *P. univalens* β-tubulins were named *Pun-tbb-5* (previously isotype 1), *Pun-tbb-6* (previously isotype 2), *Pun-tbb-7*, *Pun-tbb-8* and *Pun-tbb-9* (Figure 8). The remaining two ascarid β-tubulin genes cluster with *Hco-tbb-iso-3* and *Hco-tbb-iso-4* respectively and were hence named *Pun-tbb-3* and *Pun-tbb-4* (Figure 8).

Functional studies in *C. elegans* have shown that *mec-7* and *tbb-4* are expressed exclusively in specific sensory neurons, and are therefore not considered to be involved in benzimidazole binding or resistance (Savage *et al.* 1989; Hurd *et al.* 2010). In addition, *Cel-tbb-1* and *Cel-tbb-2* are also suggested not to be targets for benzimidazoles as they express a tyrosine in position 200 rather than the phenylalanine required for benzimidazole-binding (Saunders *et al*. 2013). Since *Pun-tbb-3* and *Pun-tbb-4* cluster with *mec-7* and *tbb-4* respectively we suggest that they have similar functions. In addition, *Pun-tbb-7* has a tyrosine at position 200 in the *P. univalens* reference genome as well as in the samples sequenced in **study V**. Based on this and similar findings in *H. contortus* (Saunders *et al*. 2013), we suggest that *Pun-tbb-3*, *Pun-tbb-4* and *Pun-tbb-7* are most likely not benzimidazole targets in *P. univalens*. However, since *Pun-tbb-5*, *Pun-tbb-6*, *Pun-tbb-8* and *Pun-tbb-9* express a phenylalanine at position 200 and are equally related to *Cel-ben-1* we suggest that they may be targets for benzimidazole drugs in *P. univalens*.

Amplicon sequencing covering position 167, 198 and 200 of the seven *P. univalens* β-tubulin genes did not identify any SNPs at these positions in resistant or susceptible parasites. These results are in accordance with a previous study where no SNPs were found in any of four examined β-tubulin genes in *A. lumbricoides* with reduced efficacy of benzimidazoles (Krucken *et al.* 2017). Taken together these results suggest that the mechanism behind benzimidazole resistance in ascarids is different from the β-tubulin polymorphisms in strongyle parasites.

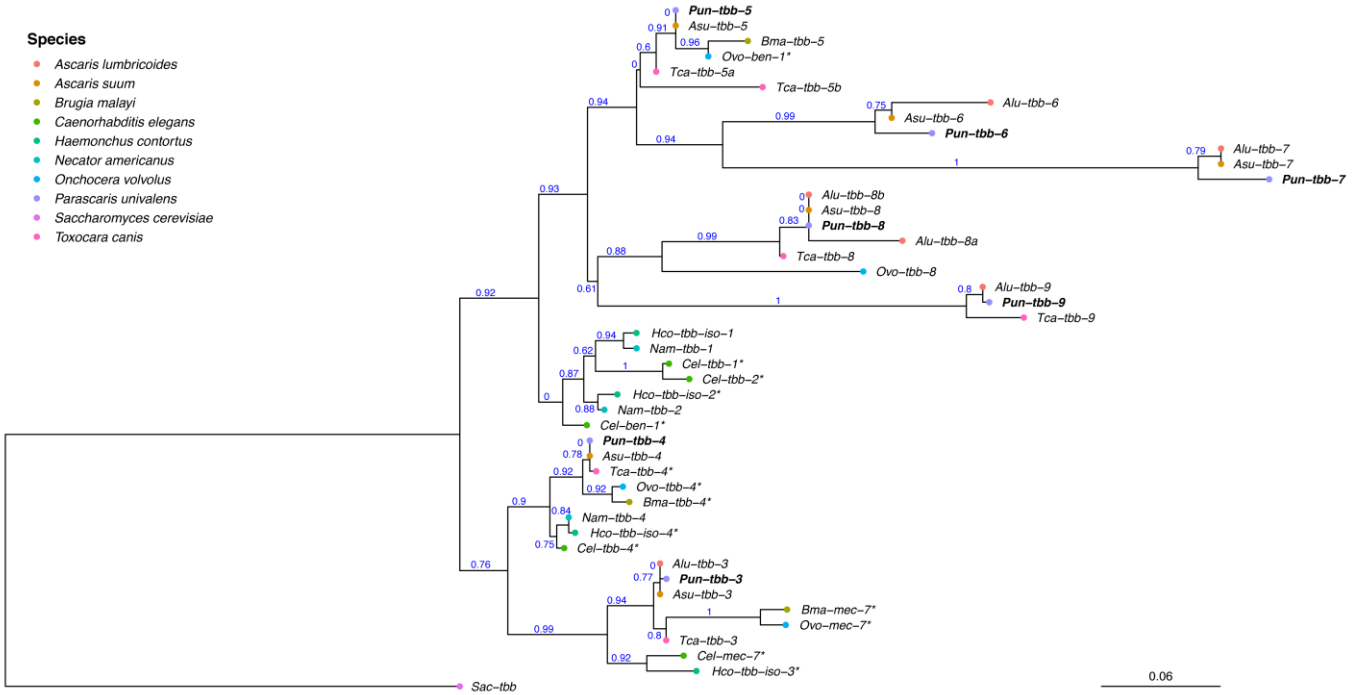

Figure 8. Phylogenetic tree of β-tubulin genes. The tree was constructed on the Phylogeny.fr platform, using a maximum likelihood based analysis of seven *Parascaris univalens* β-tubulin genes and their full length homologues in *Ascaris lumbricoides*, *Ascaris suum*, *Toxocara canis*, *Brugia malayi*, *Haemonchus contortus*, *Necator americanus* and *Onchocerca volvulus* retrieved from WormBase ParaSite and *Caenorhabditis elegans* obtained from WormBase. *Saccharomyces cerevisiae* was used as outgroup for the phylogenetic analysis. * Gene name from WormBase, all other gene names are putative (Martin *et al*. 2021b).

6. Concluding remarks

In this thesis we have updated the resistance status on Swedish and Icelandic stud farms, developed a novel research model and investigated possible genetic mechanisms involved in drug metabolism and anthelmintic resistance in *P. univalens*.

- Novel findings of emerging resistance to pyrantel and fenbendazole in *P. univalens* on Swedish farms were presented. In addition to previously reported ivermectin resistance, this implies that roundworm populations resistant to all drug classes available for treatment of horses may be present on some Swedish stud farms. This is a major problem for horse owners since heavy worm burdens can result in severe colic and even intestinal rupture and death.
- The first *P. univalens* efficacy study was performed on Iceland where ivermectin is commonly used to treat foals. Results showed that ivermectin-resistant *P. univalens* were present on all investigated farms. Due to the long isolation of the Icelandic horse population, resistance has most probably developed independently without transfer of resistance alleles from populations outside the island.
- Karyotyping showed that *P. univalens* is the main ascarid species infecting horses in Sweden and Iceland, further supporting the theory that this is the dominating species infecting horses throughout the world.
- A number of novel candidate genes such as the GABA receptor subunit gene *lgc-37*, genes coding for phase I enzymes belonging to the SDR and FMO superfamilies and genes coding for transport proteins belonging to SLC and MFS families were differentially

expressed after *in vitro* drug exposure of adult worms. The possible involvement of these genes in anthelmintic metabolism or resistance needs to be further studied.

- The development of a functional *in vitro* model based on *P. univalens* larvae is a major step forward in ascarid research as parasite eggs can easily be collected from naturally infected foals in a non-invasive way.
- The *P. univalens* genome contains seven β-tubulin genes, of which four are suggested as possible drug targets for benzimidazole drugs.
- No polymorphisms could be detected at position 167, 198 or 200 in any of the seven β-tubulin genes in fenbendazole-resistant *P. univalens*, indicating that benzimidazole resistance in ascarid parasites is likely caused by a different mechanism than in strongyle parasites.

7. Future perspectives

The results from this thesis, showing emerging resistance to all anthelmintic drugs currently registered for horses, is alarming. Due to the potential severity of *P. univalens* infection, it is important that foals receive the correct anthelmintic treatment. There is also an urgent need to elucidate the mechanisms behind resistance development to be able to develop methods for fast and sensitive diagnosis of anthelmintic resistance. As a continuation of this thesis, the following points would be interesting to consider for future research:

- Continuous monitoring of the resistance status on Swedish stud farms. As we could see in this thesis, anthelmintic resistance can emerge rapidly and it is important that an effective drug is used for treatment of *P. univalens* infections.
- To be able to tackle the problem with increasing anthelmintic resistance, novel recommendations for biosecurity and hygiene measures are needed to lower the infection pressure and avoid the spread of resistance alleles.
- Development of rapid and easy methods for diagnosis of anthelmintic resistance. In collaboration with the French Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), work has started to adapt an automated larval migration assay (ALMA) for detection of resistant *P. univalens* populations.
- Identification of the benzimidazole drug target in *P. univalens* and further investigation of the mechanism behind benzimidazole resistance in *P. univalens*.

- Further investigation of *lgc-37* and other candidate genes identified in **study III** regarding their involvement in anthelmintic resistance.
- Use of the larval model developed in **study IV** to compare expression of candidate genes between *P. univalens* populations with different resistance phenotypes to identify genes that may be involved in anthelmintic resistance.

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Popular science summary

The equine roundworm is a gastrointestinal parasite of foals around the world. The foals get infected by swallowing eggs containing infective larvae present on the pasture and in the environment. The eggs hatch in the stomach and the larvae migrate thorough the liver and lungs before returning to the small intestine where they develop into adult worms that reproduce. The female worm produce large numbers of eggs that are shed in the foal faeces and survive in the environment for at least 18 months.

Common symptoms of infection are reduced growth, coughing and nasal discharge, but severe infections can cause impaction of worms in the intestine, leading to colic and even death.

The species of roundworm infecting horses have traditionally been referred to as *Parascaris equorum*, but in recent years several studies have shown that the related species *Parascaris univalens* are more frequently found. Since the two species are indistinguishable by eye the number of chromosomes in the parasite egg needs to be examined to tell them apart.

Due to the potential severity of roundworm infection foals are usually dewormed at regular intervals during their first year of life. Three classes of anthelmintic drugs (dewormers) are commonly used for treatment of roundworm infections: benzimidazoles (such as fenbendazole), macrocyclic lactones (such as ivermectin) and tetrahydropyrimidines (such as pyrantel). The frequent deworming has contributed to the development of resistance to several anthelmintic drugs in the equine roundworm, meaning that the parasite survives drug treatment at a dose that would normally be lethal.

In 2002 the first case of ivermectin resistance in the equine roundworm was reported in Europe. Since then resistance to macrocyclic lactones have spread across the world and sporadic cases of treatment failure of pyrantel and fenbendazole have also been reported. In 2008, the first case of

ivermectin resistance was reported in roundworms on Swedish stud farms, while the effect of pyrantel and fenbendazole was satisfactory at that time.

Despite the scenario of multidrug resistance in the equine roundworm, the causes of resistance are still unknown. However, knowledge from other parasites suggest possible mechanisms such as increased effect of enzymes and proteins involved in the metabolism and secretion of drugs, resulting in less active drug available at the parasite target. In addition, mutations, i.e. changes in the genetic code, have been shown to be involved in resistance to certain anthelmintic drugs in other parasitic worms. These mutations can change the shape of the target molecule, making it impossible for the drug to bind and resulting in lack of anti-parasitic effect.

Due to the complex lifecycle requiring the host to be completed, there are no laboratory strains available of the equine roundworm. Therefore, adult worms for experiments need to be collected from the small intestine at slaughter of infected foals. The development of a roundworm laboratory model would greatly facilitate future research into resistance mechanisms.

The aim of this thesis was to examine the efficacy of anthelmintic drugs on stud farms as well as the species of roundworm infecting foals. To understand the development of resistance in the equine roundworm we have explored potential genetic mechanisms that may be involved in anthelmintic resistance. We have also developed methods to hatch roundworm eggs and culture larvae for use in experiments.

The efficacy of pyrantel and fenbendazole was investigated on Swedish stud farms in 2016 and 2017. Results showed that resistance against pyrantel were present on several farms, while fenbendazole was effective on all farms but one. Since ivermectin resistance is considered to be widespread in Sweden, this means that there are now roundworms resistant to both ivermectin and pyrantel on Swedish stud farms, and possible developing fenbendazole resistance. Since a large part of the molecular studies in this thesis was performed on Icelandic roundworms, the efficacy of ivermectin was evaluated on Icelandic farms in 2019. The results showed that ivermectin-resistant roundworms were present on all examined farms. In both Sweden and Iceland *P. univalens* was exclusively identified in all samples, supporting the theory that this is the main species of roundworm infecting horses across the world.

In collaboration with University of Iceland we collected roundworms at an Icelandic abattoir where foals are slaughtered on a regular basis. We did

an unbiased examination of the gene expression in the worms after laboratory exposure to the different drug classes. Results showed that the expression of a drug target molecule and a number of genes possibly involved in metabolism and secretion of drugs were changed in response to drug exposure. The results support the theories that changes in these mechanisms may be involved in resistance to anthelmintic drugs.

A system for hatching and culturing of roundworm larvae was developed to simplify studies of roundworm resistance mechanisms. Parasite eggs can then be isolated from foal faeces and used in experiments, instead of collecting live adult worms at slaughter of foals. The expression of a number of genes was compared between adult worms and larvae, and results showed that there were some differences between the life stages. Despite this, the larval system will be an important tool in the research of resistance mechanisms and to compare differences between parasite groups.

In 2019 and 2020 a resistance study was repeated on the farm where reduced efficacy of fenbendazole was observed in 2017. Results showed that the efficacy of fenbendazole had been further reduced since the previous trial. These results are alarming since it means that resistance to all three available drug classes are present in Swedish roundworm populations. Since benzimidazole resistance is rare in roundworms, this unique population was used to study the benzimidazole resistance mechanism. In many other parasitic worms mutations at three positions in a gene coding for β-tubulin, the target molecule for benzimidazole drugs, are known to cause benzimidazole resistance. A total of seven β-tubulin genes were identified in the roundworm genome. DNA sequencing of the three mutation sites showed that no changes of the genetic code were present at these positions in any of the genes from the resistant worms. Hence a different mechanism is likely responsible for benzimidazole resistance in roundworms.

The work performed in this thesis has given us knowledge about the current resistance status on Swedish stud farms and shows the need for continuous monitoring of the resistance situation. We have also achieved some insights into the genetics behind resistance development that can be used as a basis for elucidating the resistance mechanisms of the equine roundworm in future research.

Populärvetenskaplig sammanfattning

Hästens spolmask är en inälvsparasit som infekterar föl och unghästar över hela världen. Fölen smittas med infektiösa ägg som finns på betet och i omgivningen. Äggen kläcks i fölets tunntarm och larverna vandrar genom lever och lungor innan de återvänder till tunntarmen där de utvecklas till vuxna maskar som parar sig. Spolmaskhonorna producerar ägg som utsöndras med fölens träck och överlever minst 18 månader på betet.

De vanligaste symptomen vid spolmaskinfektion är dålig tillväxt, hosta och näsflöde. Vid allvarliga infektioner kan dock fölets tunntarm packas full av spolmaskar vilket kan leda till livshotande kolik.

Det har sedan länge varit känt att spolmaskarten *Parascaris equorum* infekterar hästar, men under senare år har flera studier visat att den närbesläktade arten *Parascaris univalens* är betydligt vanligare. De två arterna kan inte skiljas åt med blotta ögat, utan antalet kromosomer i parasitäggets kärna måste studeras för artbestämning.

På grund av de svåra skador som spolmaskinfektion kan orsaka så behandlas föl rutinmässigt med avmaskningsmedel under sitt första levnadsår. Tre läkemedelsklasser är registrerade för användning till häst i Sverige: benzimidazoler (t. ex. fenbendazol), makrocykliska laktoner (t. ex. ivermectin) och tetrahydropyrimidiner (t. ex. pyrantel). Överdriven användning av avmaskningsmedlen har gjort att parasiten har utvecklat resistens, vilket betyder att parasiterna överlever behandling med en läkemedelsdos som normalt varit dödlig.

År 2002 rapporterades det första fallet av resistens mot ivermektin hos hästens spolmask i Europa. Sedan dess har resistens mot makrocykliska laktoner spridit sig över världen och även sporadiska fall av resistens mot pyrantel och fenbendazol har rapporterats. År 2008 påvisades för första

gången resistens mot ivermektin hos spolmask på svenska stuterier, medan effekten av pyrantel och fenbendazol har varit god vid tidigare studier.

Trots det allvarliga läget så är mekanismerna som orsakar resistens hos spolmasken fortfarande okända. Studier av andra parasiter har dock visat att möjliga mekanismer kan inkludera ökad aktivitet hos enzymer och proteiner som bryter ner och utsöndrar läkemedel, vilket leder till mindre mängd aktivt läkemedel i parasiten. Mutationer, dvs. förändringar i arvsmassan, har också visats orsaka resistens hos flera parasiter. Mutationerna leder bland annat till förändrad bindningsyta hos målmolekylen och gör att läkemedlet inte kan binda och utöva sin effekt.

Forskning om resistensmekanismer hos hästens spolmask försvåras av dess komplexa livscykel som kräver sitt värddjur för att fullbordas. Det innebär att parasiter för forskning i dagsläget endast kan samlas in från föl som avlivas och utveckling av en laboratoriemodell för spolmaskforskning skulle därför vara av stort värde.

Syftet med avhandlingen var att undersöka effekten av avmaskningsmedel på stuterier och fastställa vilken art av spolmask som infekterar fölen. Vi har även utvecklat metoder för kläckning av spolmaskägg och odling av larver för användning i experiment, samt undersökt mekanismer som kan orsaka resistens mot avmaskningsmedel.

Effekten av pyrantel och fenbendazol undersöktes på svenska stuterier 2016 och 2017. Resultaten visade att det förekom resistens mot pyrantel på flera av de svenska gårdarna, medan fenbendazol fortfarande hade effekt på alla gårdar utom en. Eftersom ivermektinresistens är utbredd på svenska gårdar innebär dessa resultat att det finns spolmaskar resistenta mot både ivermektin och pyrantel på svenska gårdar, samt misstanke om en begynnande resistens mot fenbendazol.

Eftersom stora delar av de molekylära studierna utfördes med isländska spolmaskar undersöktes förekomsten av ivermektinresistens på isländska gårdar 2019. Resultaten visade att det fanns spolmaskar på alla undersökta gårdar som var resistenta mot ivermektin. Enbart *P. univalens* identifierades i prover från både Sverige och Island, vilket stödjer teorierna om att detta numera är den huvudsakliga spolmaskarten hos häst världen över.

I samarbete med Islands universitet samlades spolmaskar in på ett Isländskt slakteri där föl slaktas regelbundet. Vi gjorde en förutsättningslös undersökning av genuttrycket i maskarna efter att de i laboratoriet behandlats med de olika läkemedelsklasserna. Resultaten visade bland annat ett ökat

uttryck av en potentiell målmolekyl för läkemedel, samt av flera gener som kodar för enzymer och proteiner inblandade i nedbrytning och transport av läkemedel. Dessa resultat stödjer teorier att förändringar i dessa mekanismer är inblandade i resistens mot antiparasitära läkemedel.

Ett protokoll för kläckning och odling av spolmasklarver utvecklades för att förenkla studier av resistensmekanismer. Uttryck av ett antal gener jämfördes mellan larver och vuxna maskar och resultaten visade att det finns vissa skillnader i genuttryck mellan livsstadierna. Trots detta kan larvmodellen vara ett användbart verktyg för forskning om resistensmekanismer hos hästens spolmask och för att jämföra skillnader mellan olika grupper. Användning av larvmodellen innebär också att spolmaskägg från fölträck kan användas i försök istället för insamling av levande maskar från avlivade av föl.

År 2019 och 2020 gjordes en ny studie på den gård där nedsatt effekt av fenbendazol observerats 2017. Resultaten visade att effekten av fenbendazol minskat ytterligare sedan den tidigare studien. Dessa resultat är mycket oroande eftersom det innebär att det förekommer resistens mot alla avmaskningsmedel tillgängliga för häst i Sverige.

Då resistens mot läkemedelsgruppen benzimidazoler är ovanlig hos spolmask så användes den här unika spolmaskgruppen för att undersöka mekanismen för benzimidazolresistens. Hos många andra parasiter beror benzimidazolresistens på mutationer i någon av tre positioner i en gen som kodar för β-tubulin, målmolekylen för benzimidazoler. Sju β-tubulingener identifierades i spolmaskens arvsmassa. Vid DNA-sekvensering av de tre mutationspositionerna kunde inga förändringar identifieras i någon av generna, vilket innebär att en annan mekanism orsakar benzimidazolresistens hos spolmasken.

Sammanfattningsvis har vi uppdaterat kunskapen om resistensläget hos hästens spolmask i Sverige, något som kontinuerligt behöver undersökas då det ständigt förändras. Vi har även utforskat genetiska mekanismer som tros orsaka läkemedelsresistens hos hästens spolmask och som även kan vara intressanta för forskning om läkemedelsresistens hos spolmaskar som infekterar till exempel människa, höns och gris.

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The common foal parasite *Parascaris univalens* has developed resistance to anthelmintic drugs, which is a threat to equine health and welfare. In this thesis, parasite populations with emerging resistance to pyrantel and fenbendazole were identified on Swedish stud farms and ivermectin-resistant populations on Icelandic farms. In addition, a novel research model was developed and genetic mechanisms potentially involved in drug metabolism and anthelmintic resistance were studied.

Frida Martin received her graduate education at the Department of Biomedical Sciences and Veterinary Public Health. Her undergraduate degrees in veterinary medicine and biology were obtained at the Swedish University of Agricultural Science, Uppsala, Sweden.

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