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The horse nematode *Parascaris equorum*: in vitro experiments and evaluation of anthelmintic resistance

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Det finns inga dumma frågor, bara dumma svar.

— Proverbio svedese

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Abstract

Parascaris equorum is a worldwide-spread parasite which affects young horses and donkeys; although light infections are asymptomatic, symptoms such as coughing, greyish nasal discharge, poor growth rates, dull coats and lassitude may be observed in moderate to heavy infections. As the worms are large, their presence in huge numbers in the small intestine might give rise to intestinal occlusion with severe or fatal consequences such as rupture of the intestinal wall and peritonitis. Three different classes of anthelmintic drugs are commonly used to control horse parasites: Benzimidazoles (BM), Tetrahydropyrimidine (Pyrantel - PYR) and Macrocyclic Lactones (ML). Benzimidazoles are nowadays the preferred drug to treat *P. equorum* infections since this parasite has developed resistance to ML in multiple countries and to Pyrantel (described only in the USA). The development of anthelmintic resistance to ML is thought to be correlated with exclusive and/or excessively frequent use of these drugs in young foals; this is why constantly monitoring the effectiveness of these drugs is crucial meanwhile a new diagnostic method, which allows to detect early infections, is developed.

In this study, eggs of *P. equorum* were isolated from feces of contaminated foals, hatched and left develop into larvae, then exposed to Thiabendazole (TBZ) to investigate if the parasite was gaining resistance to this drug. After the exposure to TBZ was also analyzed the expression of two P-glycoproteins, p-gp 11 and p-gp 16, which seem to be responsible for the development of resistance in similar drugs.

Chapter 1

Introduction

1.1 Taxonomy: the class of Nematoda

Nematodes are also known as roundworms, because of their bilateral symmetry in cross-section.

A simplified classification is used for nematodes of veterinary interest: they have been grouped in sixteen superfamilies and these have been further divided into bursate and non-bursate. The bursa is an organ situated on the tail of male worms and it is used to hold the female during mating; the presence or absence of a male bursa enables a distinction between "bursate" and "non-bursate" worms. Ascaridoidea group, which *P. equorum* belongs to, is a non bursate group of nematodes. These worms are large and white, with a direct life cycle; well know parasites in this group, in addition to *P. equorum*, are *A. lumbricoides* (human), *A. suum* (pig) and *T. canis* (dog).

Most nematodes have a cylindrical unsegmented body, tapering at either end, and the body is covered by a colorless, somewhat translucent, layer: the cuticle.

The cuticle is a complex structure secreted by the underlying hypodermis, which

spreads into the body cavity forming two lateral cords (which incorporate the excretory canal), and a ventral and dorsal cord bringing the nerves. Body cavity contains fluids at high pressure, which allow the worms to maintain their body shape and turgidity.

The muscle cells take place between the hypodermis and the body cavity and are arranged longitudinally.

The digestive system is tubular and, unlike Plathelmints (flatworms), is fully developed and comprise a mouth and an anus. The mouth of many nematodes is a simple opening and may be surrounded by two or three lips; it leads directly into the oesophagus; nematodes generally obtain feeding from mucosal fluid, products of host digestion, cell debris and sometimes also blood. The oesophagus takes various forms and it is used for an introductory distinction between groups of worms; it is usually muscular and pumps food into the intestine. The intestine is tubular-shaped and its lumen is enclosed by a single layer of cells or by a syncytium. In female worms the intestine terminates in an anus while in males the cloaca perform as an anus.

Nematodes have separated sexes: the female organs (ovary, oviduct and uterus) end in a common vagina, while the male organs (a single continuous testis and a vas deferens) terminate in an ejaculatory duct in the cloaca. Males can help themselves with the transfer of sperm during mating protruding spicules, which are chitinous organs usually paired, from the cloaca.

Nematode eggs may vary widely in size and shape, and their shell, made up of three layers, may have variable thickness.

1.2 The horse nematode *Parascaris equorum*

The nematode *P. equorum* affects young horses and donkeys.
P. equorum is one of the rare nematodes which induces absolute acquired immunity. Most horses become immune during the first year of life, so patent Ascarid infections are rarely diagnosed in horses over two years of age (Reinemeyer, 2009). This parasite is large and rigid, with a pinkish-yellowish white color and cannot be confused with any other intestinal parasite of the equines. Males are 15-20cm long and females up to 40cm.
P. equorum, as all ascaridoidea, have a bulb-shaped oesophagus with a large posterior swelling while its spicules are long and stout; both of this characters are useful to differentiate *P. equorum* from other nematodes.
The eggs of *P. equorum* are almost spherical and have a brownish color. Eggs are between 90-100 microns in size and are unembryonated when passed into the environment; if conditions are favorable, an infective larva will develop inside each egg. Adult females are able to produce nearly 170.000 eggs daily (60.000.000/year).
It is difficult to diagnostic parascariosis using only clinical signs since they are unspecific and usually appear late (or never). Anyway, parascariosis should be considered if a young foal presents symptoms such as constipation alternate to foul-smelling diarrhea, coughing, fever and anorexia (Jacobs et al., 2015).

1.3 Epidemiology

The major features of the epidemiology of parascarosis are the extreme persistence of the infective stage in the environment and the predominance of infection in juvenile horses (Sellon and Long, 2013).
Female worms are prolific egg layers and, as a result, infected foals may pass mil-

lions of eggs daily in their feces. Larvated eggs are able to remain viable for more than 10 years and are resistant to freezing and drying, so one patent infection could affect the health of several future generations of foals.

Arrested larval development (also known as hypobiosis) has great epidemiological importance: it allows the survival of the nematode during periods of adversity and the subsequent maturation of arrested larvae increases the contamination of the environment. Hypobiosis influences also the control strategies against nematodes because during this phase fecal egg count may be negative even if the host is infested.

Periparturient rise in fecal egg count is an increase in the number of eggs of nematodes in the feces that has been noticed around parturition; this seems to be correlated to a temporary relaxation in immunity due to high levels of prolactin in the blood. Since *P. equorum* targets are young foals without mature immunity system, this phenomenon can lead to a higher rate of eggs excreted from the mare and to an increased risk of infection for the new born foal.

Foals became infected by ingesting larvated *Parascaris equorum* eggs from the environment; it is extremely rare to see patent infections in mature horses – older than 12 months – since they develop a highly effective immunity against this parasite. Experimental studies have shown that the immune response acts in the liver and lungs since infections of mature horses with large numbers of eggs produce significant lesions in livers and lungs but few larvae reach the small intestine.

Parascariosis is transmitted solely by equids, no different definitive hosts exist; this means there is absolutely no risk of infection in humans and other animals, even if eggs may hatch in rodents and undergo a limited migration (Johnstone, 1998).

1.4 Life cycle

During development, a nematode moults at intervals shedding its cuticle. In the complete life cycle there are four moults, which allow the larvae to develop from L1 to L5 stadium, the immature adult.

P. equorum has a direct life cycle, which means no intermediate host is needed: foals became infected by ingesting eggs containing second stadium larvae (L2). These hatch in the gut and larvae migrate to the liver through the wall of the small intestine via hepatic portal system (24 hours after infection). The molt from L2 to L3 appears to occur in this phase. Then larvae migrate on to the lungs via the heart and pulmonary arteries (7-14 days after infection). L3 break out of the alveolar capillaries into the alveoli and migrate up to trachea and pharynx, where they are coughed up and re-swallowed in the digerent system; the final two parasitic moults (from L3 to L4 and from L4 to immature adults) take place in the small intestine (Urquhart et al., 1996).

Otherwise, other nematodes may have indirect life cycle; this means that larvae need an intermediate host to develop into L3 and infection of the final host is either by the ingestion of the intermediate host or by inoculation of L3 if the intermediate host is a sucking insect. Anyhow, two more moults take place after infection to produce L5; a new life cycle will begin when young adults are able to copulate.

Development can occur entirely in the gut lumen or, as described for *P. equorum*, larvae may travel through the body before getting their final destination/development.

Depending on species, eggs may hatch in the environment or after ingestion. Outside hatching depends partially from the larvae and partially from environment (temperature, humidity ...) while after ingestion hatching only depends on digestive juices acidity.

1.5 Metabolism

The main food stock for preparasitic nematode larvae, whether inside the eggshell or free-living, is lipids, which may be seen as droplets in the lumen of the intestine.

The infectivity of these stages is often related to the amount of lipids present; larvae which have depleted their reserves are not as infective as those which still retain quantities of lipids.

Living first and second stage larvae of most nematodes eat bacteria but, once the infective stadium (L3) is reached, they are completely dependent on their stored reserve since they're struck in the second-stage cuticle and cannot feed; on the contrary, adults use to store energy disguised as glycogen in the lateral cords and muscles (*Veterinary parasitology. 2007*).

All stages of nematodes have an aerobic metabolism, however adults are also able to metabolize carbohydrate by glycolysis, method used only when the nematodes are too far from the intestinal mucosa and the oxygen tension is so low (<5mmHg) electron transport system can't operate (Walzer and Genta, 1988).

Hypobiosis may affect the normal parasitic life cycle of nematodes; it is a seasonal arrestment in larvae development: fourth stadium larvae became quiescent and remain sexually immature in the host, waiting for better conditions to protect their progeny. Apparently, there are three different circumstances which may initiate development arrest: environmental stimuli (such as temperature, humidity and photo-period), host-depending stimuli (immunity) and parasite-depending stimuli (self-control by the parasitic population to avoid overloading).

1.6 Pathogenesis and clinical signs

P. equorum infestations are most commonly characterized by nonspecific signs of weight loss or poor growth, rough hair coat and poor performance.
Respiratory symptoms may occur during migration through the lungs: these may be transient and mild — the so called "summer colds", characterized by intermittent coughing and slight nasal discharge — or may be more severe, with dyspnea, pyrexia and pallor of the mucous membranes. Resolving pulmonary lesions may be observed as subpleural nodular accumulations of lymphocytes up to 1 cm in diameter, and there may be residual lymphocytic cuffing of pulmonary vessels (Jubb, 1985).
Large worm burdens in the intestine may cause some inflammation and reduced rate of intestinal transit which occasionally may cause colic, obstruction, intussusception or, rarely, perforation of the gut.
Less serious effects include weight loss and ill thrift due to decreased food absorption, especially in 5 months old foals (with a range of 4 to 24 months).

1.7 Diagnosis

When a foal presents coughing with nasal discharge prepatent infection with *P. equorum* should be suspected even if diagnosis is dependent on finding the distinctive thick-shelled egg in the feces of horses with patent infections through coprological examination.
Coprological examinations involve examining the feces of animals to identify and count parasite eggs. It is nowadays considered the golden standard technique to diagnose parascariosis and almost all the most common parasitic diseases.
This kind of exam can be qualitative, whose aim is to identify if there are or

not eggs in the feces and which parasite they belong to, or quantitative, which is performed in order to quantify how many eggs are present in a definite amount of feces.

1.7.1 Qualitative coprological examination

Qualitative coprological examination is usually performed by fecal flotation and sedimentation to separate eggs from fecal matter.
This method consists in dissolving a few grams from the fecal sample into water, filtering it and centrifuging for 3 minutes at 1500-2000rpm; every time after centrifuging the fluid will be eliminated keeping only the deposit at the bottom of the tube. This operation — centrifuging and removing the fluids — needs to be repeated until the fluid is clean and transparent. Then the tube is filled with high specific gravity liquid until it forms a protruding meniscus from the upper edge of the tube; the high specific gravity liquid replaces the water and lets the deposit slowly float on the surface where it is possible to make it adhere to the microscopic slide simply putting it on top of the tube and waiting a few minutes.
The last step consists in taking the microscopic slide under the optic microscope and check if parasite eggs are present. This is usually done with low magnification, 10x, where *Parascaris equorum* eggs can be identified by their thick shell. They are about spherical and have a brownish color. Each egg contains a 1-celled zygote and are between 90-100 microns in size.

1.7.2 Quantitative coprological examination

Quantitative coprological examination contemplates the same steps done with the previous method but the suspension has to be prepared by weighting a precise aliquot of fecal material and diluting it into a well-know volume of flotation solution.

The examination of the results will be performed with a special microscopic slide, called the McMaster Egg Counting Chamber, which enables a known volume of fecal suspension (2 x 0,15ml) to be examine microscopically. It is important, differently from the previous method, to shake well the content in the tube so it is equally spread, otherwise the outcome could be biased. The possibly present eggs can be counted by the use of a grid (1cm x 1cm) and since the volume is known it is possible to go back to the number of eggs per gram (EPG).
This method can provide an estimate of worm burden, in addition to the identification of possible parasites.

Anyhow, it is important to remember that, although the outcome of these test is negative, it doesn't mean the animal is not infected but only that mature worms able to produce eggs were lacking at the time the sample was taken. This implies that is always recommended to perform coprological examination as a routine standard on all sensitive animals.

1.8 Available treatments

During the last two decades anthelmintic resistance in equine parasites has been found in the group of small strongyle species (cyathostomins) and in the Ascarid species *Parascaris equorum*. The ubiquitarious nature and possible severe consequences of disease with these nematodes make them the prime targets of current worm control programs in horses.
Traditional control strategies mainly rely on the strategic application of anthelmintics. Only three classes of anthelmintic drugs are allowed in equine medicine: Benzomidazoles (BM), Pyrimidine (PYR) and Macrocyclic Lactones (ML).

1.8.1 Benzimidazoles

Benzimidazoles are a large chemical family used to treat nematode and trematode infections in domestic animals. They are characterized by a broad spectrum of activity against roundworms, an ovicidal effect and a wide safety margin.
The BM target is beta-tubulin, they are able to bind with the tubulin of nematodes with a 25–400 times greater affinity compared with that of mammals. Tubulin is a protein subunit of the microtubules that is essential in the mechanism that allows the constitution of the mitotic spindle. On beta-tubulin there are some Benzimidazole-specific binding sites that, if activated, lead to the unfolding of the protein with abnormal conformation as a consequence; this inhibits further polymerization of alfa- and beta-tubulin subunits to form microtubules and in rapidly dividing cells, resulting in a lethal effect. At higher concentrations, BM have a variety of nonspecific effects on nematodes, e.g. the inhibition of fumarate reductase, which is necessary to transform glucose into energy, and decoupling of oxidative phosphorylation.
Because most benzimidazoles are hardly soluble in water, they are generally given PO. The most effective of the group are those with the longest half-life and their prodrugs, because they are not rapidly metabolized to inactive products; this is not extremely important in horses and cows since in these species Benzimidazoles appear to be more effective than in others because their rate of passage is slowed by the cecum and the rumen respectively.
Since the antiparasitic action of BM increase with prolongation of contact time, repeating the administration of the drug at full dose for 2-3 times at 12-hours intervals increases their efficacy, even against Benzimidazole-resistant worms. In addition, a reduced feed intake — which reduces the flow rate of digesta — or co administration with a fatty meal increases their availability.

In horses, BM are characterized by effective removal of almost all mature strongyles, but third- and fourth-stage larvae are more difficult to eliminate. High levels and repeated administration may be necessary for extra-intestinal migrating stages of large strongyles and for small-strongyle larvae embedded or encysted in the wall of the intestine.
Although there is a great deal of selectivity in the mode of action of the BM, particularly because of the poor systemic availability, rapidly dividing cells are at risk of toxicity if exposure is sufficient. Thus extremely elevated doses of BM are known to variously affect hematopoietic stem cells, intestinal epithelium and hair growth.

1.8.2 Pyrantel

Pyrantel pamoate or embonate (PYR) acts as a depolarizing neuromuscular blocking agent. It is a potent agonist of the acetylcholine receptors on the muscle cells of nematodes. Activation of these receptors induces a prolonged, spastic paralysis of the worms causing them to "lose their grip" on the intestinal wall and to be expelled from the horse's system by natural process; horses with a large number of worms may suffer an impaction: in this case it could be helpful to give a laxative prior to the administration of the dewormer. Outside the host's body the worm soon dies.
Only the worms in the intestine are vulnerable to PYR pamoate, this means that the administration should be repeated 2-4 weeks after the first deworming and possibly a third time 2 to 4 weeks after that to be fully effective. It is intended for use in horses as oral paste or granulates.
PYR pamoate is safe to use with foals over 4 weeks of age, and with pregnant and nursing mares, too. It should not be used in debilitated animals or foals less than 4 weeks old and its use is forbidden in horses intended for human consumption.

Pyrantel pamoate should not be used with organophosphate insecticides because of the danger of increased side effects.

1.8.3 Macrocyclic Lactones

The prototype Macrocyclic Lactone is Ivermectin; there are two different groups of ML: Avermectins and Milbemycins, which have a similar mechanism of action, but a longer half-life than the Avermectins. They are both products of fermentation by *Streptomices* species.
The Avermectins block the transmission of electrical activity in invertebrate nerve and muscle cells mostly by enhancing the effects of glutamate at the invertebrate-specific glutamate-gated chloride channel; this causes an influx of chloride ions into the cells, leading to hyperpolarisation and subsequent paralysis of invertebrate neuromuscular systems; comparable doses are not toxic for mammals because they do not possess glutamate-gated chloride channels.
These actions block the transmission of neuronal signals of the parasites, which are either paralyzed or expelled out of the body. They also affect the reproduction of some parasites by diminishing oviposition or inducing an abnormal oogenesis.

Typical parasite control practices for juvenile horses essentially constitute exclusive and/or excessively frequent use of a single dose class and thus select intensively for anthelmintic resistance.
Many farms use ML bimonthly in juvenile horses and since they are larvicidal against *P. equorum*, the refugia within a host is minimized each time an infected foal is dosed. This happens routinely whenever the interval between treatments is shorter than the prepatent period for *P. equorum*, usually 75 to 80 days (Reinemeyer, 2009).

1.9 Anthelmintic resistance

According to the World Health Organization, antimicrobial resistance occurs when microorganisms such as bacteria, viruses, fungi and parasites change in ways that make the medications used to cure the infections they cause ineffective. In particular, anthelmintic resistance is the ability of a parasite to survive a dose of anthelmintic which would normally be effective and a parasite is said resistant to a drug if it survives exposure to the standard recommended dose and can pass this ability to its offspring.

Antimicrobial resistance occurs naturally but is facilitated by the inappropriate use of medicines. Low-quality medicines, wrong prescriptions and poor infection prevention and control also encourage the development and spread of drug resistance.

Once a population has developed resistance, there are small possibilities it turns back to sensibility. No new anthelmintic drug have been developed in the last years and drug resistance is nowadays considered one of the most outstanding problems in veterinary medicine.

Adult females of *P. equorum* are able to produce nearly 170.000 eggs daily (60.000.000/year) which means there are great opportunities that resistance will spread out to the offspring and became widespread.

Drug resistance can arise in a limited number of ways: (i) a change in the molecular target, so that the drug no longer recognizes the target and is thus ineffective; (ii) a change in metabolism that inactivates or removes the drug, or that prevents its activation; (iii) a change in the distribution of the drug in the target organism that prevents the drug from accessing its site of action; or (iv) amplification of target genes to overcome drug action (Wolstenholme et al., 2004).

Benzimidazoles effect is linked to the polymerization of tubulin and resistance to

this group of drugs is clearly related to several different polymorphism in beta-tubulin genes that inhibit drug binding and/or reduce affinity for it.
Recent studies suggest that also modulation of the activity of the cell-membrane efflux pump P-glycoprotein (P-gp) could be involved in BM resistance in worms.

1.10 P-glycoprotein

P-glycoprotein (P-gp) is one of the first members of the ATP-binding cassette (ABC) transporter which acts as a physiological barrier by extruding toxins and xenobiotics out of cells (Amin, 2013).
P-gp is primarily found in epithelial cells which have excretory roles, including apical surface of epithelial cells lining the colon, small intestine, pancreatic ductules, bile ductules, kidney proximal tubules and the adrenal gland. The role of P-gp is likely to protect these susceptible organs from toxic compounds, preventing them to enter the cytosol and extrude them to the exterior. Thus, it also enhances the secretion of metabolites and xenobiotics into bile, urine and the lumen of gastrointestinal tract.
P-gps are located in the apical cell-membrane, act as ATP-dependent transporters for hydrophobic xenobiotics such as anthelmintics and decrease the concentration of the drugs at their target sites. This effect finally prohibits an efficient treatment (Janssen et al., 2013). In vitro and in vivo studies have demonstrated that P-glycoprotein plays a significant role in drug absorption and disposition (Lin and Yamazaki, 2003).
Unspecific resistance mechanisms involving transporters such as P-glycoproteins (P-gps) are expected to contribute to ML resistance in nematodes, as multiple studies have recently demonstrated an increased expression of P-gps is linked to a growing resistance against the anthelmintic drug Ivermectine in some well known

nematode parasites such as *H. contortus* and cyathostomins (Xu et al., 1998, Blackhall et al., 1998, Bourguinat et al., 2011) while other studies are now trying to understand in detail the already known correlation between resistance to BM and over expression of P-gps, too. (Blackhall et al., 1998, Bourguinat et al., 2011).

1.11 Aim of the project

This aim of this project is to establish a dose-response curve to Thiabendazole (TBZ) in *P. equorum* larvae.

Eggs of *P.equorum* has been isolated, hatched and kept alive. Though hatching *P. equorum* eggs is quite easy, there is no note protocol that guarantee an acceptable survival rate after hatching. An attempt with different kinds of media, following records successfully used with other nematodes such as *Toxocara cati*, was made but no one seemed particularly satisfying.

The obtained larvae were exposed to Thiabendazole in order to set up a dose-response curve to this drug and investigate expression of P-gp11 and P-gp16 genes in the parasite after 48 and 120 hours of exposure to this drug.

Nowadays no evidence of resistance to TBZ has been reported for *P. equorum*.

Chapter 2

Material and methods

2.1 Collection and preparation of fecal samples

Eggs were isolated from feces collected from foals naturally infected on pasture. Fecal samples were mixed with water and the resulting mixture was poured through three different mesh sieves (1000, 150 and 80 μm, respectively); this procedure allows to eliminate most of the impurities contained in the feces such as little stones, undigested material and other solids.

Eggs were collected and stored in PBS-filled Falcon tubes, in refrigerator at 7°C. Prior to the exposure, eggs contained in fecal samples have been washed using the following method: tubes were taken from the fridge and put in the centrifuge at 3000rpm x 3 min; then water in excess was aspired with vacuum pomp. To sterilize them was used Milton sterilizing fluid: it contains 1% sodium hypochlorite (NaClO) and 16.5% sodium chloride (NaCl; common salt); a 1:20 solution is isotonic with body fluids.

Every sample has been diluted with Milton's solution and divided into two or more tubes, subsequently filled with more Milton's solution: this was added with

the purpose of both remove the cuticle from the eggs and let them floating on the surface.

After being in the centrifuge at 1500rpm x 3 min, the top layer of every tube were removed with a pipette and put in a clean tube containing at least 25ml of tap water. Since the attempt was to preserve as many eggs as possible, this process has been repeated several times until there were few eggs left in the first tube, according to a verification with microscope.

When most of the eggs have been collected, the tubes containing eggs mixed with water were put in the centrifuge at 3000rpm x 3 min and then water in excess was dried with vacuum pomp (unlike Milton's solution, water let eggs lying in the bottom of the tubes). Every tube was refilled at least ten times with water and the whole process was repeated in order to eliminate all the chlorine from the water; indeed solutions with high concentrations of chlorine cause osmosis which give rise to cell death.

Eggs were stored in the fridge with about 10ml of Phosphate-buffered saline (PBS) solution.

2.2 Hatching

Unembryonated *P. equorum* eggs were incubated at 24°C to develop into second stage larvae (L2) and were checked every day at the microscope to verify their development. An average of 10 days was required to reach the infective stage.

When zygotes in the eggs have reached the larva stadium, eggs were smashed with a little pestle directly in the tube in order to release the larvae; their vitality was checked and then they were divided into wells. With this method only 10-15% of the eggs was successfully smashed, but almost all the larvae which were released from the eggs had survived.

By changing hatching method, using a bigger pestle which fits perfectly in its container and creates vacuum when PBS containing larvae was pulled up and down, 95% successful hatching was reached.
The larvae obtained with this procedure were stored with pure water only, PBS only and PBS plus four different concentrations of Roswell Park Memorial Institute 1640 (RPMI), a medium originally developed to culture human leukemic cells in suspension but which has been found suitable for a variety of animal cells.
Living larvae were checked every day at the microscope, counting as alive only the ones which were moving.
The tested concentrations were:

(A) 1 ml PBS + 150 μl RPMI(130,5 μl/ml)

(B) 1 ml PBS + 300 μl RPMI (230,8 μl/ml)

(C) 1 ml PBS + 500 μl RPMI (333,33 μl/ml)

(D) 1 ml PBS + 750 μl RPMI (428,5 μl/ml)

2.3 Thiabendazole exposure

The main intention of this study was to verify if *P. equorum* was gaining resistance to Thiabendazole, as it already gained for Macrocyclic Lactones and Pyrimidines.
In order to test larvae resistance to Thiabendazole a stock of drug was created solving 0,05g of TBZ in 10ml of Dimethyl Sulfoxide (DMSO), which is a solvent needed because TBZ can't be melted in water; according to this the solution used has a concentration of 5μg/μl.
Different amounts of the stock were added to the wells (each one of these contained 1970μl of PBS + 10μl PBS solution containing at least 50 moving larvae)

(a) 0 μl of stock + 20 μl of DMSO (negative control)

(b) 6 μl of stock + 14 μl of DMSO $\longrightarrow$ final concentration 15 μg/ml

(c) 10 μl of stock + 10 μl of DMSO $\longrightarrow$ final concentration 25 μg/ml

(d) 14 μl of stock + 6 μl of DMSO $\longrightarrow$ final concentration 35 μg/ml

Before setting the plate in the incubator at 24°C all the moving larvae were counted (moving larvae at start point).
Larvae have been checked 24 and 48 hours after exposure, counting them each time. After 48 hours all the liquid in each well was collected in Eppendorf tubes which were then stored in freezer. Since there was no significant difference between treated and untreated larvae, with >70% of the untreated larvae died in 5 days, a new exposure experiment was started using the most vital larvae only: they were selected filtering hatched larvae using a multiple layer cotton gauze.
A funnel with closable bottom was filled with PBS an multiple layered gauze was placed on top; after addition of the larvae it was put in the incubator at 37°C and let rest overnight. In this way, larvae, attracted by clean water, could swam from the top to the bottom. Everything has passed through the filter was collected in tubes and centrifuged at 1500rpm x 3min in order to eliminate all the PBS in excess; after that, larvae were divided into wells as done in the previous experiment and counted every day.

2.4 RNA extraction

The samples collected after having exposed larvae to TBZ were used to extract RNA in order to analyze gene expression.
Samples were unfrozen and centrifuged at 14000g x 2min since a pallet was obtained and water could have been discharged, then pellets were frozen at -80°C

waiting for RNA-extraction.
To isolate RNA from the samples Nucleospin RNA pro kit was used, following the instructions of the manufacturer. It consist in 8 different steps:

- Homogenize sample
- Lyse cells adding 350l of buffer RA1 and 3,5μl of β-mercaptoethanol, then vortex vigorously
- Adjust RNA binding conditions by adding 350μl 70% ethanol and mix by pipetting up and down for 5 times
- Bind RNA by loading lysate in a Nucleospin RNA column placed in a collection tube; centrifuge at 11,000g x 30s then place the column in a new collection tube
- Desalt silica membrane adding 350μl Membrane Desalting Buffer (MDB) and centrifuge at 11,000g x 1min to dry the membrane
- Digest DNA: prepare DNase reaction mixture in a sterile 1,5ml microcentrifuge tube and, for each isolation, add 10μl reconstituted rDNase to 90μl Reaction Buffer for rDNase; mix by flicking the tube then apply 95μl DNase reaction mixture onto the center of the silica membrane of the column. Incubate at room temperature for 15min
- Wash and dry silica membrane:
  - First wash adding 200μl Buffer RAW2 and centrifuge at 11,000g x 30s, then place the column into a new collection tube
  - Second wash adding 600μl Buffer RA3 and centrifuge at 11,000g x 30s, then discard flow-through and place the column back into the collection tube

  - Third wash adding 250μl Buffer RA3 and centrifuge at 11,000g x 2min to dry the membrane completely then place the column into a nuclease-free collection tube

- Elute RNA in 60μl RNase-free H2O and centrifuge at 11,000g x 1 min

2.5 RNA quantification

The concentration of RNA within the samples was calculated by using Ribogreen Reagent and Kit, according to the instructions from the manufacturer.
Ribogreen buffer was diluted 20 times by mixing 50ml of the original buffer with 9,5ml of Milli-Q water while RNA standard was diluted, in order to obtain a 100ng/ml solution from a 2μl/ml one, by mixing 475μl of diluted buffer with 25μl of RNA standard provided from the kit.
Control wells contained 0ng/ml (blank well), 1ng/ml, 5ng/ml, 25ng/ml, 50ng/ml and 100ng/ml and were created by mixing different amounts of the two previous solutions while other wells contained 1μl of sample plus 99μl of diluted buffer. To each well 100μl of a solution made up of 16μl of Ribogreen dye melted in 3184μl of buffer was added so the end each well contained 200μl of volume.
The real amount of RNA contained in each well was estimated by taking away from each value the average between the two blank wells; then a line with control concentration on the Y-axis and deviation from the mean on the X-axis was draw; it was used to predict all the other values. Each value has been multiplied by 200 (the amount of each well) and divided by 1000 (to switch ng/ml into ng/μl).
The extracted RNA was stored at -80°C until the RT-PCR was carried out. Approximately 15ng RNA was used for the PCR assay.

2.6 Primer design RT-PCR

Primers targeting the two candidate genes P-gp 11 and P-gp 16 and the reference gene glyceraldehyde 3-phosphate dehydrogenase (gpd-1) were used in this study (Table 1). The primers were taken from a published study (Janssen et al., 2013) and they were provided by Eurofins Genomics (Ebersberg, Germany).

Gene	Direction	Primer sequence 5'- 3'	Accession number	Product size (bp)
Pgp-11	Forward	gtc atc gga aga ggg cat t	JX308230	137
Pgp-11	Reverse	gtg aaa tct ggc gat acg gt	JX308230	137
Pgp-16	Forward	gaa aag cga caa cat cac ga	JX308231	120
Pgp-16	Reverse	gag cat aga gtg gag ccg tc	JX308231	120
gpd-1	Forward	atc ggt tgt cga tct tac gg	Unpublished data	152
gpd-1	Reverse	gac ttt gtt ggt gat tcg ca	Unpublished data	152

Table 1: Primer sequences for Real-Time PCR

2.7 Real-Time One Step RT-PCR on larvae exposed to Thiabendazole

RT-PCR was performed in selected samples to test gene expression of the two candidate genes P-gp 11 and P-gp 16 as a consequence of the exposure to different concentrations of Thiabendazole.

The samples chosen to run the PCR had different concentration and the lowest one used was 1,5ng/μl so all the other samples were diluted, according to the following formula (C1xV1=C2xV2), in order to reach the same concentration in each sample, otherwise it would have been difficult to compare the results.

Each PCR reaction was in a total volume of 25μl, consisted of 15μl of a solution composed by 12.5μl 2xMasterMix, 1,125μl of each primer (F and R) and 0,25μl RTmix plus 10μl template.

The RT-PCR was conducted on a Rotor-Gene 3000 and the program was set with a 10 min long reverse transcription at 55degrees followed by a PCR initial activation step lasted for 5 min at 95°C to activate the HotStarTaq Polymerase. The amplification part contained 40 cycles of denaturation at 95°C for 5s and combined annealing/extension at 50°C for 10s; during this last part fluorescence data collection take place.

The software used to obtain RNA quantification and melt curve analysis was Rotor-Gene Q; the gained data for the cycle thresholds (Ct) were exported and fold expression was calculated using the gdp-1 gene as normalization; the final value was calculated with the following formula: $2^{-\Delta Ct}$.

Chapter 3

Results

3.1 Hatching

Hatched larvae were kept in pure water only, PBS only and RPMI in different concentrations (obtained diluting RPMI with PBS) in order to find out a way to make them survive longer.
As evident in fig. 3.3, after 10 days of observation there seems to be no difference in survival rate between keeping larvae in PBS only (light blue) and keeping them in low concentrated RPMI solutions (13%, in green), while it's pretty evident high concentrated RPMI solutions (>40%, in orange) and pure water (in purple) let them die faster.
An attempt to add some DMSO to the PBS was tried, since larvae are reported to survive mainly thanks to their lipid storage in first stages of their lives and negative controls from the drug-exposure experiment - which were kept in 1% DMSO solution - have shown good viability through the length of the experiment; low concentrated DMSO solutions (in fuchsia - 2.5% - and in blue - 5%) appear to help larvae to survive better than other tested media but still are not enough

efficient since larvae starts to die quickly anyway around 8 days after hatching. It is also evident that larvae need time to adapt to their media: since the only way used to differentiate between living larvae and dead larvae is their moving and since it is impossible they had reproduced in the media, the only explanation for a marked fall in the first days followed by a new rise is that they weren't moving but they were alive and as soon as they adapted themselves to the new media they started moving again.

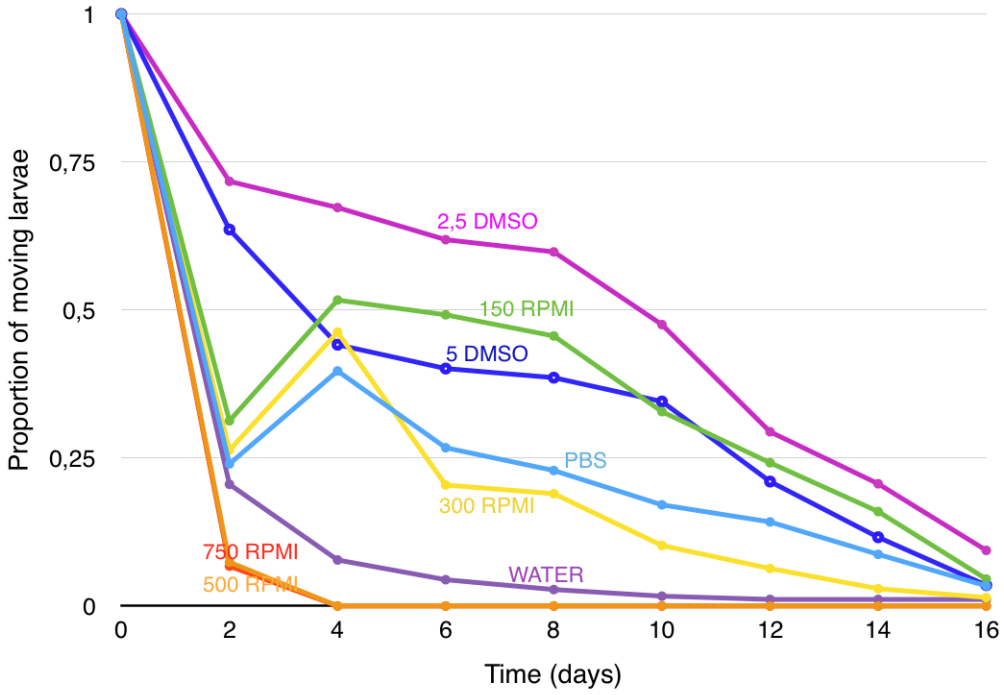

Figure 3.1: Percentage of living filtered larvae after 16 days in different media.

3.2 Thiabendazole dose-response curve

P. equorum larvae were exposed to three different Thiabendazole concentrations: 15 μg/ml, 25 μg/ml and 35 μg/ml for 48h. Since not all the larvae have died after 48 hours of exposure to the drug, another test was started with the intention of watch them for 120 hours. In this study effectiveness of Thiabendazole was set at 90% of dead larvae/sample according to other studies on anthelmintic resistance to this drug, though those were conducted on field and not in vitro (Matthews, 2014).

In all the performed tests TBZ seems to be effective against *P. equorum* larvae with some differences between concentrations: after 48 hours >95% of larvae exposed to 35 μg/ml drug were dead, while 15 μg/ml and 25 μg/ml drug concentrations killed about 80% and 85% respectively.

24hours exposure to the drug is clearly insufficient in order to obtain a good drug effect and also 48 hours of exposure didn't seem the best solution, especially using low drug concentrations. For this reason exposure time has been extended to 120 hours (5 days).

After a 5-days exposure to the drug, this test basically confirmed the results obtained in the previous, giving some news about time exposure; 72hours exposure to the drug is enough to obtain a significant percentage of dead larvae (near to 100%) in all three concentrations, indeed after this exposure time the percentage of dead larvae basically doesn't change.

Unfortunately there was no significant difference neither between treated and untreated larvae, since >70% of the untreated larvae has died during the experiment. This is why a new exposure experiment was started using the most vital larvae only: they were selected filtering hatched larvae using a multiple layer cotton gauze. Regarding exposed larvae the result was the same (>95% dead larvae) but after 5

days there were still >50% of untreated larvae moving.

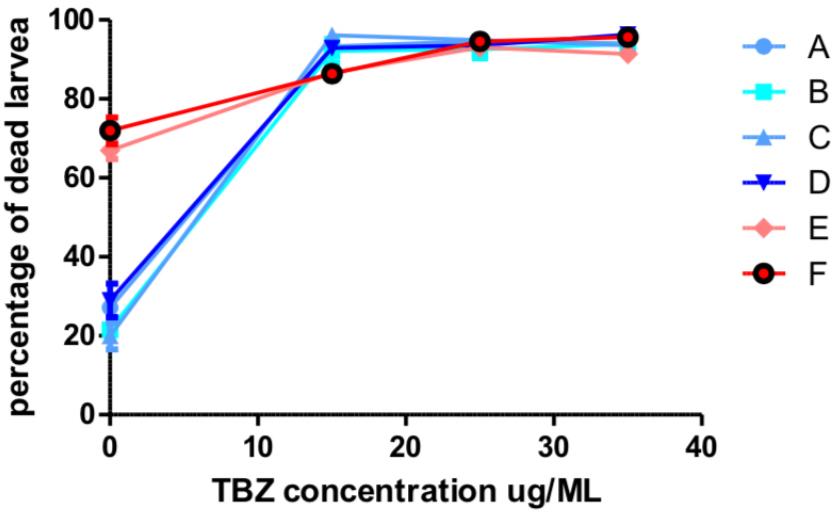

Figure 3.2: TBZ dose-response curve after 72-hours exposure in filtered and unfiltered larvae.

The picture 3.2 shows the difference between the samples containing unfiltered larvae (in red) and those in which the most living larvae were selected by the use of a cotton gauze.

Thiabendazole, when present in the solution, killed about 98% of the exposed larvae in all the samples with no significant difference between filtered and unfiltered samples, regardless of its concentration.

In negative controls the difference between the use of unfiltered and filtered larvae is pretty evident: in the first case (samples E to F, in red) most of the larvae (about 85% of them) died within the considered period of time, compromising the reliability of the entire experiment, while in the second case (samples A to D, in blue) the percentage of death larvae dropped to 25-30% which, considered the difficulties in keeping the larvae alive in any media and the absence of a protocol, was considered a success.

According to the result of this plot, from now on, only the experiments conducted on filtered larvae will be considered to define a dose-response curve to the drug of choice.

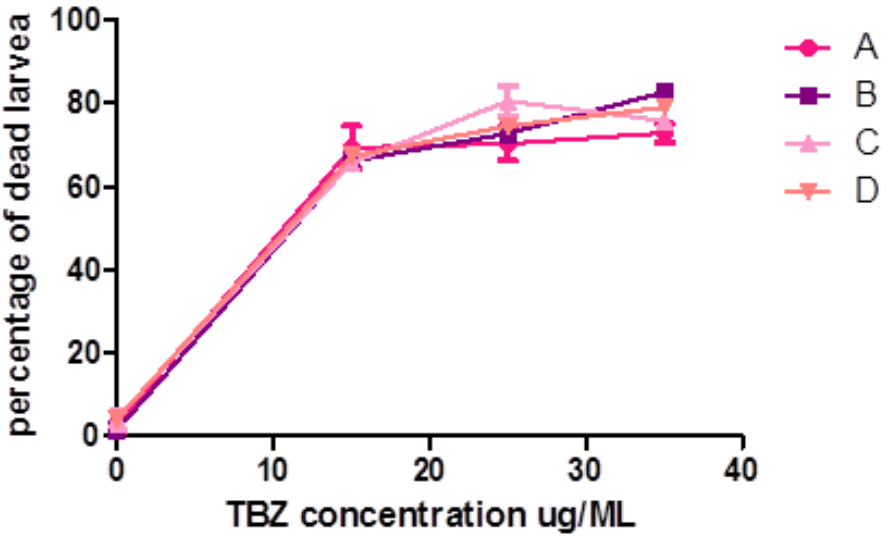

Figure 3.3: Dose-response curve to TBZ after 24-hours exposure.

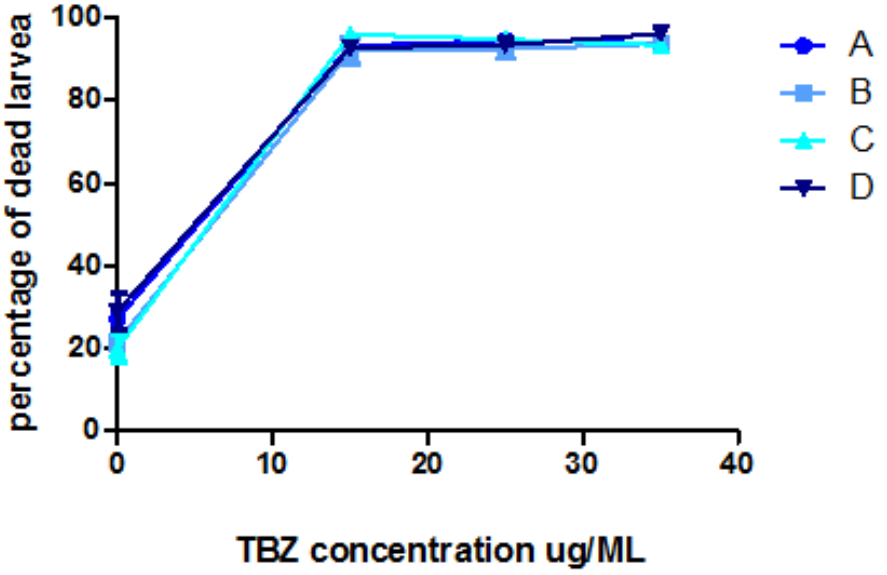

Figure 3.4: Dose-response curve to TBZ after 72-hours exposure.

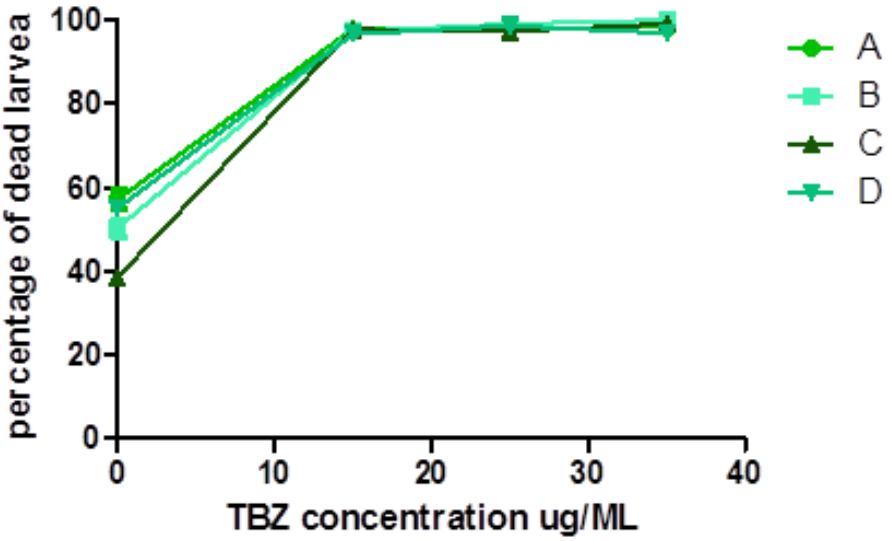

Figure 3.5: Dose-response curve to TBZ after 120-hours exposure.

Figures 3.3, 3.4 and 3.5 show different times (24, 72 and 120 hours of exposure) of the same experiment in which filtered larvae has been exposed to different concentrations of Thiabendazole.

In particular, fig. 3.3 shows how, after a 24-hour exposure, the highest concentration of the drug seems to be a little bit more effective than the others, killing about 75-80% of the larvae, even though no one of the tested concentrations has reached a satisfying level of effectiveness. Almost all larvae in negative control were still alive (97% of them) proving once again the results obtained in the previous experiment were reliable.

Fig. 3.4 represents the situation after 72 hours of exposure. All the tested concentrations of the drug have reached a satisfying level of effectiveness (>90% of dead larvae), even if it could be noted a small gap in effectiveness between the lowest and the highest concentration. Unexposed larvae have started dying but a remarkable difference in the percentage of dead larvae is still visible between treated and untreated ones (negative control). From this plot it's evident TBZ has proven to be still effective on *P. equorum* larvae. In fig. 3.5 there is no significant

difference in drug effectiveness compared to the previous plot. Unexposed larvae kept on dying, reaching 55%, therefore optimal time exposure in experiments involving *P. equorum* larvae appears to be 72 hours: extending the exposure time doesn't influence the efficacy of the drug but compromise the results since also most of the negative control would die.

3.3 Gene expression of Pgp-11 and Pgp-16 in Thiabendazole exposed larvae

Real-Time RT-PCR was used to study the expression of the two candidate genes P-gp 11 and P-gp 16 in *P. equorum* larvae as a response to Thiabendazole exposure using a protocol developed for an unpublished study conducted on *P. equorum* eggs exposed to the same drug at SLU university in Uppsala, Sweden. The reference gene gpd-1 was used as normalization in order to calculate the fold change.

The fold expression in both cases doesn't seem to show high differences between treated and untreated larvae; as seen in fig. 3.6 there is no significant change in gene expression for any of the genes when exposed to TBZ. Nevertheless, it looks like there is a tendency to increase in fold change for Pgp-16 in relation to increasing concentration of TBZ.

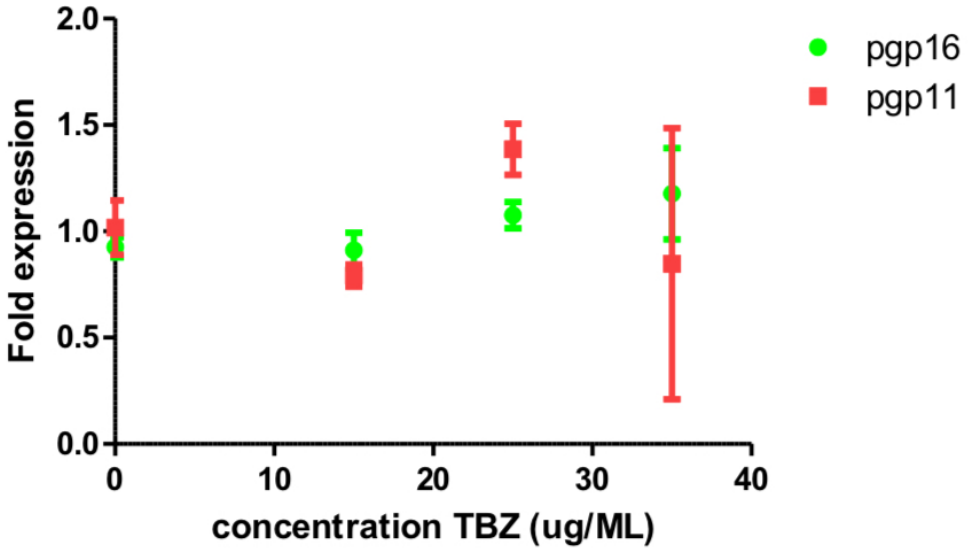

Figure 3.6: Fold change expression in relation to the house keeping gene after exposure to different concentrations of Thiabendazole in gene Pgp-11 and Pgp-16.

Chapter 4

Discussion

P. equorum is considered, together with cyathostomins, the most important parasite of horses.

Fecal Egg Count is the only method available for field use to verify if foals are infested or not, but eggs are released in the feces only after 79 to 110 days since the infestation and meanwhile the migrant young larvae can cause several symptoms such as pneumonia, colic and intestinal disturbance (Schimdt et al., 2000). This is why, due to the relatively low cost compared to the potential benefit and the quest for maximizing productivity and performance, frequent and consistent use of anthelmintic drugs has become common in horses.

Anthelmintic resistance among equine nematodes is usually detected by using the Fecal Egg Count Reduction Test (FECRT), which is essentially the ratio of the means of eggs found in feces before and after deworming. A genetic-level research is needed to deeply understand how anthelmintic resistance occur (Gilleard, 2006).

4.1 Hatching

All the attempts to hatch *P. equorum* larvae has been successful, however the problem of a high spontaneous mortality of the larvae persist. It is possible that this is due to the fact recreating the optimal living condition for them failed; indeed eggs store energy adsorbing lipids while they're in the intestine waiting to be released in the soil. The energy stored is absolutely necessary for the larvae to survive and develop into third stadium larvae, indeed during their molt between second and third stadium they're struck in their cuticle and can't feed (*Veterinary parasitology. 2007*).

Since all the eggs used to hatch larvae to expose to the drug were kept in PBS solution it is possible there was a lack of energy and that's the reason why they died so fast even without exposing them to Thiabendazole. Evidence supporting this explanation is given by the fact larvae died less quickly when kept in a mixture of PBS and DMSO, even of course if this is not their ideal source of lipids.

However it can't be excluded that other factors have led larvae to death, such as non visible damage during hatching or unsuited temperature in the incubator or more easily the fact that eggs of *P. equorum* do not hatch outside the host and this make their survival difficult.

4.2 Exposure to Thiabendazole

In all the experiments *P. equorum* was found sensible to Thiabendazole exposure so, at the moment, there is no evident sign of anthelmintic resistence in *P. equorum* against this drug.

Anyway it is necessary to develop a new detecting method which can identify infected foals before they start releasing eggs in the soil. This could be helpful to

both drastically reduce the number of treatments required (expanding refugia) and preserve the effectiveness of the drug without causing any damage or loss to the foals and their owners.

4.3 Gene-response after exposure to the drug

Based on the result from this study there is no significant change in the expression of P-gp 11 and P-gp 16 after exposure to Thiabendazole.
However this experiment is not concluded because there are a few factors to improve: first of all the samples ran in PCR were not filtered, which means not exclusively RNA from larvae was analyzed; then the protocol used to set up the RT-PCR was taken from a similar experiment carried on eggs of *P. equorum* but melt curves and Agar gel running showed some changes were needed in this protocol to obtain best results; last but not least all the sample used for the PCR were diluted and had very low concentrations of RNA if compared to the golden standard recommended from instructions of the manufacturer.
The tendency to increase in fold change for P-gp 16 in relation to increasing concentrations of the drug was already reported in a previous study executed on *P. equorum* eggs.

4.4 Final comment

At the beginning this study was meant to investigate whether *P. equorum* was showing resistance to Thiabendazole and if there was any change in its gene expression for two target genes, P-gp 11 and P-gp 16, which seem to be correlated to the appearence of drug resistance in other anthelmintic drugs.
It was necessary to start from the basis since no one have ever tried before to

hatch larvae of *P. equorum*; the hatching has been successful however the question about which media is better to keep the hatched larvae alive still remains since this study has only demonstrated which media are definitely not usable and which ones should be considered for further studies, without giving any specific guideline. The in vitro drug resistance test had quite clear results: drug exposure was tested in six different attempts and each one included from 2 to 12 replicates. No one of these tests has highlighted resistance to TBZ but it is important to notice that rarely totality of exposed larvae has dead and that the administration of lower amounts of anthelmintic than required is ineffective on a relatively high percentage of larvae and could lead to faster resistant worms selection. This is why this drug should only be administrated in full dose and a shift to a target treatment program is needed to decrease the risk of resistant worms selection.

This study also tried to investigate the possibility of a connection between an increment in fold expression of two genes codifying for P-gp 11 and P-gp 16 which are membrane pumps and are correlated with drug resistance in similar anthelmintic molecules. No significant increase in fold change expression in relation to the housekeeping gene has been pointed out. This could be explained by the fact that all the larvae exposed were still susceptible to the drug and gene expression increase is clear only when also drug resistance is. However this test has been ran only one time with no excellent material (RNA was isolated from non filtered larvae) and further studies are needed before sentence about correlation between increase expression of P-glycoproteins and drug resistance to TBZ in *P. equorum* is possible.

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