CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF BIOCHEMICAL SCIENCES

THESIS

Gene expression analysis of selected UDP-glucosyltransferases from *Haemonchus contortus*

Supervisor: Ing. Petra Matoušková, PhD.

Head of department: Prof. Ing. Vladimír Wsól, Ph.D.

Hradec Králové, 2016

Mgr. Michal Janura

STATEMENT

“I declare that I have developed and written this thesis completely by myself and I have not received any unauthorized assistance in its completion. All the exploited literature sources have been properly cited in the text and supplied with bibliographical section. The thesis was not used to achieve same or any other academic degree”

In Hradec Králové, 26.1.2016 signature:

I devote my word of thanks to Ing. Petra Matoušková, PhD. for all her effort, helpfulness and professional assistance, she afforded to me by processing of this thesis.

I thank also to the whole department of biochemical sciences for help and pleasant collaboration.

ABSTRACT

Charles University in Prague
Faculty of Pharmacy in Hradec Králové
Department of Biochemical Sciences

Candidate: Mgr. Michal Janura
Supervisor: Ing. Petra Matoušková, PhD.
Title of thesis: Gene expression analysis of selected UDP-glucosyltransferases from *Haemonchus contortus*

Haemonchosis is one of the most important parasitical diseases in small ruminants caused by *Haemonchus contortus*. Resistance to anthelmintics causes several problems in therapy of the disease and it increases costs of the therapy. With an effective therapy of resistant parasites the economical situation in agriculture would improve. Resistance mechanisms in *H. contortus* are still insufficiently understood. The resistant *H. contortus* isolate forms significantly more glucose conjugates of benzimidazole anthelmintic albendazole (ABZ) than the susceptible one. More than 40 UDP-glucosyl transferases (UGT) genes were identified in the genome of *H. contortus*, which displays a great variety in this family of enzymes. This thesis is a part of a systematic research focused on studying UGT enzymes, by which ABZ is detoxified. To investigate, which one of the UGTs is responsible for the increased metabolism of ABZ, mRNA levels of eight selected genes were analyzed by real-time PCR in adults of resistant isolate and compared with susceptible isolate. There was observed a higher expression of the UGT8N enzyme (GenBank ID: HCISE00244800) in resistant isolate. However, the difference was quite small and did not fully reflect higher amount of glucose conjugates of ABZ. Therefore we have analyzed inducible changes of selected UGTs upon ABZ treatment.

ABSTRAKT

Univerzita Karlova v Praze
Farmaceutická fakulta v Hradci Králové
Katedra biochemických věd

Kandidát: Mgr. Michal Janura
Školitel: Ing. Petra Matoušková, PhD.
Název rigorózní práce: Stanovení exprese mRNA vybraných UDP-glukosyltransferas u parazita *Haemonchus contortus*

Hemonchóza je jednou z nejzávažnějších parazitárních onemocnění u malých přežvýkavců způsobena parazitem *Haemonchus contortus* (vlasovka slézová). Rezistence na anthelmintika způsobuje problémy v léčbě této nemoci a zvyšuje náklady na její léčbu. Při účinné terapii proti rezistentním parazitům by se zlepšila ekonomická situace v zemědělství. Mechanizmy způsobující rezistenci u *H. contortus* jsou pořád nedostatečně známé. Rezistentní *H. contortus* je schopen produkovat mnohem víc glukózových konjugátů benzimidazolového anthelmintika albendazolu (ABZ) než citliví jedinci. V genomu *H. contortus* bylo identifikováno více než 40 genů pro UDP-glukosyltransferázy (UGT), které disponují širokou rozmanitostí v této rodině enzymů. Táto práce je část systematického výzkumu zaměřeného na stadium UGT enzymů, kterými je ABZ detoxifikován. Pro zjištění, který z UGT enzymů způsobuje zvýšený metabolismus ABZ se analyzovaly mRNA hladiny 8 vybraných genů z dospělých jedinců rezistentního kmene *H. contortus* pomocí real-time PCR, které se následně porovnávaly s mRNA hladinami u citlivých kmenů. Byla zjištěna zvýšená exprese enzymu UGT8N (GenBank ID: HCISE00244800). Zjištěný rozdíl je ale relativně malý a nevyjadřuje zvýšené množství glukózových konjugátů s ABZ, proto jsme analyzovaly indukované změny vybraných UGT enzymů po expozici vzorků ABZ.

CONTENT

1. INTRODUCTION 8
2. THEORETICAL PART 10
2.1. NEMATODES 11
2.1.1. Taxonomy 11
2.1.2. General characteristics 11
2.1.3. Muscle system 12
2.1.4. Nervous system 12
2.1.5. Digestive system and breathing 13
2.1.6. Reproductive system 13
2.2. HAEMONCHUS CONTORTUS 15
2.2.1. General overview 15
2.2.2. Development 15
2.2.3. Pathogenesis 16
2.2.4. Diagnosis 17
2.3. THERAPY OF HELMINTHIASES 18
2.3.1. General background of therapy 18
2.3.2. Anthelmintics 19
2.3.2.1 Albendazole 19
2.4. RESISTANCE 20
2.5. METABOLISM OF XENOBIOTICS 22
2.5.1. General 22
2.5.2. Enzymes of 1st biotransformation phase 23
2.5.3. Enzymes of 2nd biotransformation phase 25
3. AIM OF STUDY 28
4. EXPERIMENTAL PART 30
4.1. BIOLOGICAL MATERIAL, LABORATORY DEVICES AND CHEMICAL REAGENTS 31
4.1.1. Acquiring of the biological material 31
4.1.2. Chemical reagents 31
4.1.3. Laboratory devices 32
4.2. PRACTICAL PROCEDURE 32
4.2.1. Processing of biological samples and RNA isolation 32
4.2.2. DNase treatment 33
4.2.3. Reverse transcription 33
4.2.4. Real-time PCR 34
4.2.4.1 Verification of primers and the efficiency of the primers 34
4.2.4.2. Constitutive gene expression analysis. 37
4.2.4.3. Inducible gene expression analysis. 38
4.2.5. Statistical analysis 39

5. RESULTS 41
5.1. VERIFICATION OF PRIMERS 43
5.2. EFFICIENCY 45
5.3. CONSTITUTIVE GENE EXSPRESSION ANALYSIS 46
5.4. INDUCIBLE GENE EXPRESSION ANALYSIS. 48
6. DISCUSSION 51
7.CONCLUSION 55
ABBRAVIATIONS 57
6. REFERENCES 58

1. INTRODUCTION

Parasitic helminths are significant group of endoparasites, which are widely expanded in our ecosystem. Diseases inflicted by parasitical helminths cause several health problems in human and animal organism. This aspect is very important for domesticated and especially agriculture animals. Helminthiases severely affect economic situation in agriculture. One of the most dangerous and widespread parasite of small ruminants is *Haemonchus contortus* from family Trichostrongylidae. It is a hematophagous gastrointestinal parasite, which is extremely detrimental to animal health and productivity. Infected animals are commonly treated against helminthiases with anthelmintic drugs. Some species of helminths dispose of certain resistance to the medicaments. This resistance is rapidly rising in the last years, what represents a difficult question for future of treating helminthiases.[1,2,3]

There are more possible mechanisms of the resistance in helminths. Despite of intensive research in the area of resistance, these mechanisms are insufficiently understood. Several studies[4,5] found out the direct association between drug resistance and detoxifying enzymes (UDP-glucosyltransferases, UGTs) in *H. contortus*. Based on the facts, the study focuses on the detoxification mechanisms of susceptible and resistant isolates and differences between them.

This thesis is part of a large research, which focuses on expression of UGTs in drug-sensitive and resistant nematode *H. contortus*. The study compares constitutive expression of the specific UGTs between drug-sensitive and resistant individuals as well as inducible expression upon albendazole (ABZ) treatment.

2. THEORETICAL PART

2.1. NEMATODES

2.1.1. Taxonomy

The origin of the word „nematode" comes from Ancient Greek (*nêmatos* as a thread and the suffix *-eidēs* as a species). In the taxonomy are nematodes a subset of the clade protostomia, family Trichostrongylidae. The nematodes comprise numerous taxonomical units in the animal kingdom. Until now, more than 25 000 species in this phylum have been described.[6] Phylum nematoda, together with the phylum platyhelminths with its classes (especially cestoda and trematoda) are the point of interest in the parasitology.[7]

2.1.2. General characteristics

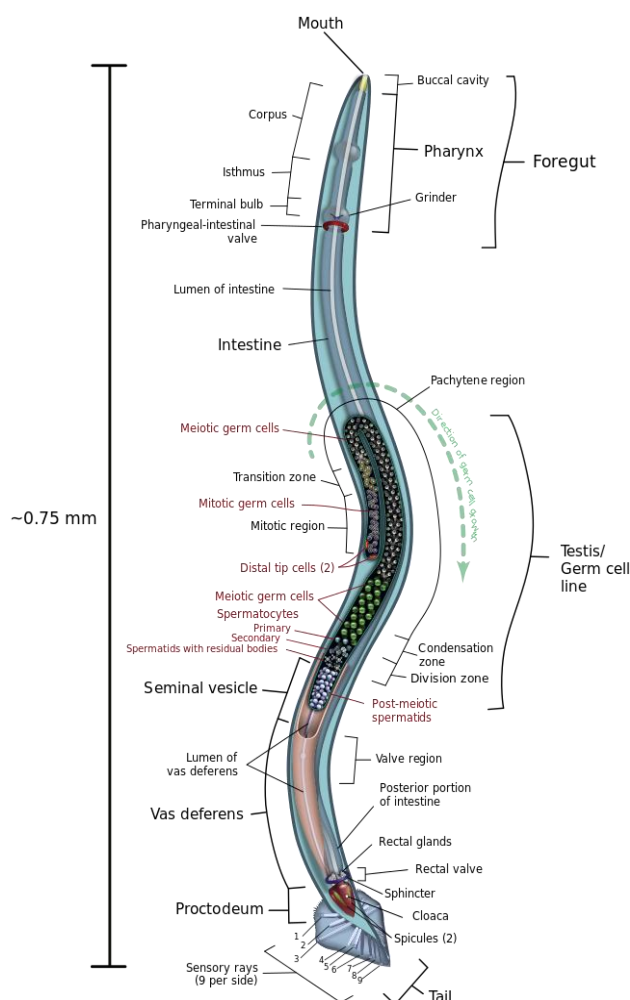


Fig. 1: Basic anatomy of male *Caenorhabditis elegans*, the model organism.[8]

Nematodes are relatively small, colorless, slender thread-like worms. Average individuals grow to 100 µm thick and to 2.5 mm long (some species several meters). Males are usually smaller than females. Their body is bilaterally symmetrical and it has typical worm shape (long and round in cut), but without any segmentation. Ventricle is a narrow pseudocoel as in other similar organisms. The mouth is located in the front part of the body (Fig. 1)[8], often equipped with an extensions assimilated for food intake. Anus is usually placed in the posterior or mid part of the body.[7]

The surface of its primitive skin consists of a specific epidermis – the mass of cells and cell materials without any membranes. Some of the cells contain more nuclei – syncytium.[7]

The skin is made from 4 layers: lipid layer, cortical/collagen layer, matrix layer and fiber layer. This skin is covered with multi-level collagenous cuticle, which forms a flexible exoskeleton and protects the worm against dehydration, mechanical stress and other undesirable factors as digestive fluids for parasitic individuals.[7]

Nematodes are relatively adaptable to different surrounding conditions and occupy almost every environment – marine water, fresh water and also soil and other animal biotopes from tropic to polar regions. Their quantity in the ecosystem is very high, together are taking 80 % of all individual animals in the world. More than half of them are animal-, plant- or human- parasites (such as *Ascaris lumbricoides, Enterobius vermicularis* etc.).[7]

2.1.3. Muscle system

Nematodes use for the locomotion longitudinal muscles, which are situated along its body from the "head" to the "tail". Muscle system is covered with the skin. Muscles together with the skin and the cuticle press visceral tissues and pseudocoel liquid and evolve pressure from 70 to 210 mmHg. This system creates hydroskelet, which is important for the homeostasis.[7]

2.1.4. Nervous system

The nervous system of nematodes is very simply developed. In the front part of the body, there is the main part of nervous system – circumpharyngeal ring or circumoesophageal ring, which surrounds worm's pharynx (Fig. 1). Cephalic sensory threads are outgoing from the circumpharyngeal ring. The sensation is performed through bristles, papillae on the surface and two amphids in the frontal part. The ring is further associated with lateral, dorsal and ventral main threads directing to the rear part of the body. The dorsal nerve controls locomotion, whereas lateral nerve possesses a sensitive function. The ventral one is the biggest and it is responsible

for control of both them. Nematodes are able to apperceive incentives from environment. The nerve threads are in a direct contact with muscle cells and cytoplasm. The connections between muscle and nerve are in these organisms very specific in comparison with the most of other organisms with a muscle system. Nerve fibers are normally spread to attach to muscle cells. In nematodes muscle cells are spread to the nerve threads.[7]

2.1.5. Digestive system and breathing

In the head, there is a small sense organ for choosing a right orientation and one relative big oral cavity with pharynx. Around the oral cavity extensions as ridges, "teeth", stylet and others are often present, which help to feed, drain, taste or penetrate into prey's body. The pharynx is connected to the intestine through pharyngeo-intestinal valve (Fig. 1). Food is after intake processed with strong muscles and then it is transferred to the intestine, where digestive glands producing enzymes are also located. The digestion continues with assistance of endosymbiotic microorganisms (bacteria and fungi). Afterwards nutrients from the food infiltrate surrounding tissues, because nematodes don't dispose of any vascular system to spread the nutrients through the body.[7]

Undigested rest is transported to the anus and further expelled, but some products as ammonia are excreted through the body wall. An intestine-rectal valve is at the junction of the intestine and rectum (Fig. 1). The movement of the whole digestive system is dependent on the body motion. The alimentation depends from the species; germs, algae, fungi, carrion, feces, life animal tissues and liquids are preferred. They have no breathing organ. The oxygen is received through the skin.[7]

2.1.6. Reproductive system

The majority of nematodes exhibit sexual dimorphism. Several species are hermaphrodites. *C. elegans* has two sexes: male – determined on the 6th chromosome pair with XO, and hermaphrodites determined on the 6th chromosome pair with XX. Hermaphrodites are able to self-fertilize or mate with male, but not with another hermaphrodite.[9]

The female reproductive organs are composed of a primitive uterus, one or two ovaries and genital pore (referred to as vulva) approximately in the middle of the body's ventral side (Fig. 2).[10] Parasitic females have enlarged reproductive organs, what enables an increased egg production. The males dispose of cloaca at the end of the body, where it embraces ejaculatory duct, rectum and spicularapparat (Fig. 1). It is a copulatory organ typical for nematodes, which is composed of indurated hook-shaped and chitinized spicula lying in cuticle bag. This organ is able to move out of the cloaca.[7] Spicula do not serve for transport of semen, but as an anchor to the wall of the female vulva. Then is the sperm able to transfer directly from the tubular gonads through sperm duct and ejaculatory duct into the vulva. After the fertilization, eggs are excreted, from which afterwards develop larvae. After the hatch, the larvae of some species ingest their parent.[7]

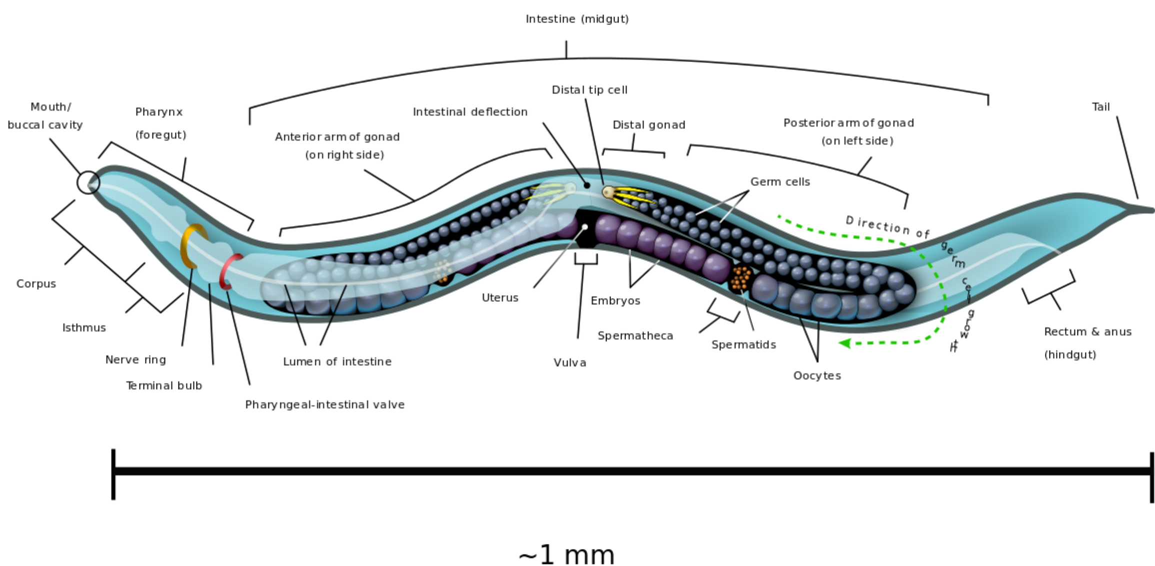


Fig. 2: Basic anatomy of hermaphrodite *Caenorhabditis elegans*, the model organism.[10]

2.2. HAEMONCHUS CONTORTUS

2.2.1. General overview

H. contortus, called the barber's pole worm or twisted stomach worm, is one of the most expanded pathogenic gastrointestinal parasites worldwide. In the 1990s, a rising resistance of this species to common anthelmintics was documented in Australia, South and East Asia, America, Africa and Europe. The most affected region is the South America, where various resistances (to benzimidazole, levamisole, salicylanilide, ivermectine etc.) were described in the last century. In the central Europe, the anthelmintic resistance is one of the biggest therapeutic and agricultural problems.[11,12]

The parasitic worm has a common nematode morphology. Females grow to 20 – 30mm in length with typical barber pole coloration (Fig. 3),[13] which appears with blood-filled intestine and conspicuous ovaries coiled around the intestine. Males are generally thinner and smaller. They grow only to the length of circa 20 mm. In males bursa copulatrix with copulatory organs is observable.[11,14]

Fig. 3: *H. contortus* female with its typical "barber pole" coloration.[13]

2.2.2. Development

Larvae grow through 4 stages (denoted as L_1 – L_4). The L_1 – L_3 stages are outside of a host organism. L_1 – L_2 are not infectious and live in environment or in feces. After 4 days evolves from L_2 stage an infectious L_3 individual with cuticle. L_3 larvae are orally ingested by a host ruminant. Afterwards begins the internal development and the parasite has to pass through 3 stomachs to reach the abomasum (part of stomach). The developmental stage L_4 evolves in the mucus of abomasum. Prepatent period takes 12 – 15 days in lambs and 16 – 24 days in sheep. This evolution from L_3 to L_4 is dependent on surrounding conditions such as humidity, temperature and sufficient

oxygen supply. Therefore *H. contortus* has better conditions in tropics and subtropics, but it is also assimilated to European conditions and more tolerant to colder temperatures. In negative circumstances during the L_3 stage, they do not enter the L_4 stage, but a latent stage – hypobiosis. This stage could be triggered also by other surrounding parameters such daylight length as well climatic conditions, annual rainfall etc. Hypobiotic stage is more resilient and less sensitive to these conditions. Parasites can persist several years in this stage. In our geographical latitudes *H. contortus* occurs in hypobiosis especially in the winter months.[11,14]

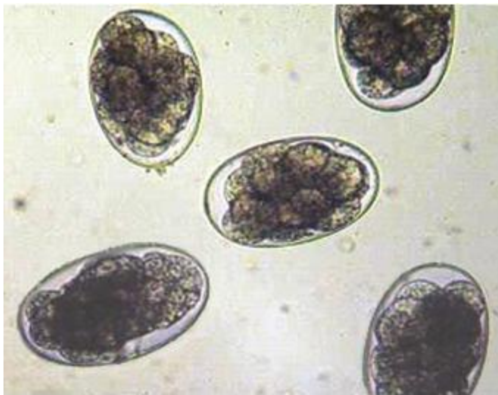


Fig. 4: *H. contortus* eggs.[15]

Females are able to produce eggs after 18 days. Adult female oviposits 5000 –10 000 eggs per day. More eggs are produced, when the host ruminant is pregnant or breast-feeding. Eggs have approximately 80 µm diameter (Fig. 4).[11,14]

2.2.3. Pathogenesis

H. contortus is a bloodsucking parasite, infiltrating gastrointestinal tract and causing disease called haemonchosis. Severe anemia, edema and excessive damages of stomach tissue are characteristic for the infection. An adult *Haemonchus* consumes big amount of blood – 50 µl per worm per day. While parasitizing, they produce anticoagulants, what promotes the blood drain and affects the health condition, resulting in gastritis. Haemonchosis is followed by anemia, modified permeability of mucus membranes, secretion and motility of the stomach, what results in weight loss, lower milk and wool production, tachycardia, diarrhea (mostly melena, because of upper gastrointestinal bleeding), alimentation difficulties, dehydration and lethargy with consequences of mortality. Edema in submandibular tissue called bottle jaw, but also in thorax and abdomen often arises in infected animals. The infected tissues are infiltrated with immunocytes as an immune reaction. Around the inflammation and damaged parietal cells in the stomach wall the pH value is increased and concentration

of Na^+ and HCO_3^-, while K^+ and Cl^- are decreased. This electric dysbalance degenerates the stomach permeability. With growing pH value, lowers the pO_2, what locally changes microflora and impacts pepsinogen activation, gastrin secretion and whole alimentation process. Due to anemia a host organism has highly increased erythropoesis, what is burdensome for the metabolism and requires higher intake of iron and other nutrients. The parasite infects mainly sheep and goats. In the Middle Europe *H. contortus* infects 50 – 75 % of small ruminants. [11,14,16,17]

2.2.4. Diagnosis

The infection is prognosticable from its clinical symptoms such as bottle jaw, weight loss, lower milk and wool production, diarrhea, lethargy and edema. It could be also diagnosed providing other diagnostic methods – fecal flotation, FAMACHA© test, blood examination, biopsy, autopsy and many other.[18,19]

Blood depletion in mucus membranes is typical sign for anemia. This phenomenon is well observable on eye mucus membrane. The more intensive the anemia is, the paler is the eye mucus membrane. The FAMACHA© card assesses the intensity of anemia considering to the appearance of the mucus membrane. It grades to 5 categories regarding to the color (Fig. 5).[17,18,19]

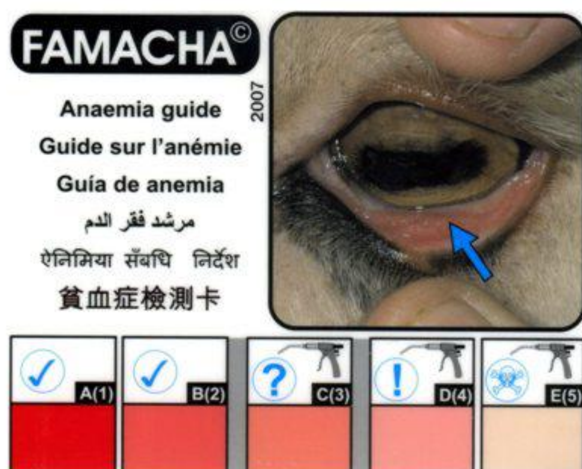


Fig. 5: FAMACHA© card, A(1) – Optimal B(2) – Acceptable
C(3) – Borderline D(4) – Dangerous E(5) – Fatal
C(3), D(4), E(5) – medication needed

2.3. THERAPY OF HELMINTHIASES

2.3.1. General background of therapy

Anthelmintic drugs are medicaments to treat helmintic infections. These medicaments were in the last decade improved regarding their effectiveness, specificity and safety. Drugs are active in gastrointestinal tract, but also in the rest of cured organism and they can dispose of a wide spectrum of activity and affect variable stages of parasites' development. Wide-spectral anthelmintic drugs exhibit toxicity to more genera, families or even classes or phyla. An anthelmintics with a narrow spectrum of activity suppress only smaller taxonomical units such genus or family. These medicaments are applied after an exact diagnosis and are generally more effective and acceptable for cured subject. A correct diagnose plays the key role in every anthelmintic therapy to choose the right medicaments, dosage form and perhaps a localization. Intestinal parasitic infections are treated with drugs with lower absorption, because of side effects, but the unresorbable drugs are also frequently applied to treat specific infections caused by parasites evolving in intestine. When it is necessary, drugs with systematic effect are administrated. The most problematic is presence of larvae in hypobiotic stage, which are usually more resistant to anthelmintics. The anthelmintics possess a selective toxicity to helminthic parasites and influence the metabolism or neuromuscular neurotransmission. Common concepts of the processes are following:

- Exhaustion of energy reserves. For example by decoupling the oxidative phosphorylation, mitochondrial enzymes or inhibition of glucose resorption
- Interruption of microtubules assembly
- Tegument damaging
- Spastic paralysis based on inhibition of acetylcholinesterase or direct cholinergic effect
- Neuroparalysis through inhibitory neurotransmitters as GABA

Medicaments based on the last two mechanisms paralyze parasites, but do not kill them. Subsequently they are excreted to environment and are potentially infectious.[19,20,21,22]

2.3.2. Anthelmintics

Anthelmintics are classified into 3 groups depending on infection – anticestodal, antitrematodal and antinematodal drugs. There are a lot of substances to treat nematodal infections. These medicaments are administrated not only for the therapy but even as prevention after birth, during grazing season and suckling period to avoid infection during lactation. Between the most used structures are derivates of macrocyclic lactone, benzimidazole, imidazothiazole and oxantel, pyrantel and nitroscanate. This thesis is focused on ABZ. Its fundamental structure is benzimidazole. Derivation of this molecule changes its pharmacodynamic and pharmacokinetic properties such solubility, resorption, activity, plasmatic half-life time etc.[18,19,20,21,22]

2.3.2.1 Albendazole

Albendazole (ABZ) (Fig. 6) interrupts the assembly of tubulin to microtubules, thereby are damaged important structural and functional units, such cytoskeleton, spindle building by mitosis and intracellular transport. It consequents to lower glucose intake, reduction of mitochondrial processes, for example: inhibition of fumarate reductase, decrease of ATP production etc. The time until onset is relatively long. The drug needs to hold relatively long contact with parasite and frequent administration to achieve exhaustion of parasite's energy reserves. After 2 – 3 days parasites die. ABZ possesses also ovicidal activity, which onsets after 8 hours. Due to lipophilic substitutions in ABZ structure, it has beneficial pharmacokinetic properties. The medicament is absorbed from intestine after p.o. administration (>45 %) and it has also quite large distribution volume, therefore it is active against extraintestinal parasites. It reaches therapeutic concentration also in lungs and other tissues. Maximal plasma concentration is

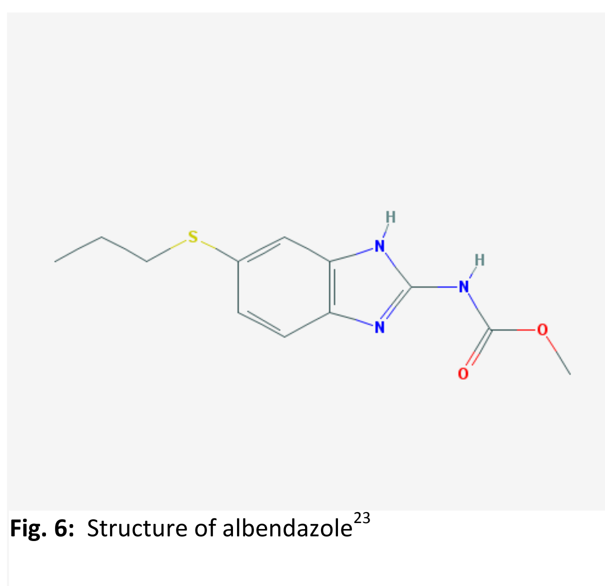


Fig. 6: Structure of albendazole[23]

reached after 6 – 30 hours. The p.o. resorption is higher by monogastric animals. ABZ is a prodrug and it is metabolized in liver by two enzymatic systems cytochrome P450 (CYPs) and flavin containing monooxygenase (FMO) to its active form (sulfoxide- and then sulfone derivate), which is through enterohepatic circulation returned into intestine. Afterwards it remains in animal organism for several weeks. Therefore the animals cannot be used for consume purposes until the end of withdrawal time. 47 % of ABZ is excreted by kidney within 9 days (28 % in first 24 hours). ABZ possesses activity against many parasitic species (also cestoda and some trematoda) and is highly active (>95 %) against adult and juvenile stages of nematodes living in gastrointestinal tract, but it is also active against larvae and extraintestinal parasites. The dose of ABZ against nematode infection at sheep is 3.8 – 5 mg/kg. ABZ is relatively well tolerated, except in pregnancy, as this drug is strong teratogenic and embryotoxic substance already in low doses.[19,20,21,22]

2.4. RESISTANCE

A resistant individual is no more sensitive to therapeutic or higher concentrations of a compound, which harms a susceptible individual. This property is inherited. Resistance to a single compound is rare. It is a matter of resistance to a whole group of medicaments with an identical base chemical structure. Some infectious species developed resistance to more independent classes of medicaments and it is called MDR – multi drug resistance. This property is persistent and any loss of the resistance has never been proved. Resistance occurs as result of genetic mutation(s) influenced by presence of a medicament or a random mutation. Higher rate of resistance allele could occur after therapy as result of selection. After exposure to an agent as a selection factor, susceptible individuals are killed, but the resistant individuals survive, thus the frequency of a resistant allele is bigger and disperses through the population.[1]

The nematodes can develop a resistance against variable groups of anthelmintics. Time of development is in comparison with bacteria quite long and

takes 9 – 10 generations. The most problematic are *Haemonchus*, *Trichostrongylus*, and *Ostertagia* spp. The resistance is result of selection of individuals with a better adaptation factor. The genetic diversity leads to variety of reactions in drug response. It is possible that some nematodes develop multi resistance to a whole medicament group, with a same fundamental chemical structure, whereas other chemical structures could be effective. In therapy, it is important to consider the dosage, frequency, season, drug and character of a parasite. The resistance can be prevented with a proper administration and changing structures or using 2 or more structures in parallel.[21,22]

There are several methods to decrease the selection pressure and to prevent formation of resistance.[1]

- Preference of short-acting drugs to avoid exposure of sub-therapeutic concentrations.
- Ensure dose is satisfactory and the treated object is not under-dosed.
- Administration in proper season, time and frequency.
- Usage of an alternative or more anthelmintics together.
- Not to use medicaments as prevention.
- Monitoring of therapy.[1]

Resistance can be theoretically caused by change of biochemical processes, which detoxify or activate drug; displace or change a drug target molecules so, drug has no binding activity; induce of metabolic changes restraining drug's activity; or change of pharmacokinetics through distribution parameters. There are a lot of possible modifications in helminth organism to induce resistance. A typical is for example the resistance to benzimidazoles. At some resistant *H. contortus* loci F200Y and F167Y are mutated, which encode β-tubulin. These mutations are responsible for phe-tyr polymorphisms in codon 200 for β-tubulin isotype 1 and 167 for β-tubulin 2. These abnormities modify the primary and secondary conformation of the β-tubulin; consequently, the benzimidazoles are unable to bind to this structure. The study refers about the F200Y and F167Y mutation: „The best studied mutations and probably the most important. 200Y seems to be the most important mutation in *Haemonchus contortus*, but this might not be true for all species."[1] Previous study in our laboratory[24] detected a higher activity of UGTs at White River (WR) *H. contortus*

isolate, what might play an important role in the resistance and should be thoroughly investigated. It is still unknown, which one of the UGT enzymes is responsible for the resistance. Previous part of this research[25] refers, the induced enzyme metabolizing the ABZ might be UGT7 (GenBank ID: HCISE00058000).[25]

Measuring of resistance

There are more options to test and quantify the resistance. Tests are provided *in vivo* or *in vitro.* Very popular and simple *in vivo* test is FECRT – fecal egg count reduction test. This type is widely used and it is suitable for all anthelmintics and its metabolites. The test is based on the comparison between count of fecal eggs obtained during pre-treatment phase of the test and count of fecal eggs obtained during/after treatment phase. The length of the phases is dependent on a drug; for benzimidazoles it is 8 – 10 days after the treatment. When the reduction is <95% (in sheep), then resistance is present. This method is capable of measuring the efficacy of a certain dose, concentration needed to kill 50 % of parasites and proportion of farm resistance specification.[1,26]

Egg hatch test – EHT is the most used *in vitro* test to detect resistance to benzimidazole. This test is based on measuring ovicidal activity of a benzimidazole. Undeveloped eggs are exposed to standard effective dose of benzimidazole. If the eggs survive and hatch after LD99, then they are resistant. When the EHT is performed in parallel with more decreasing concentrations, it is possible to calculate LD50 and compare with a standard. There are also other tests using larvae or adult parasites. To more precise identification biochemical, genetic and molecular techniques are used.[26]

2.5. METABOLISM OF XENOBIOTICS

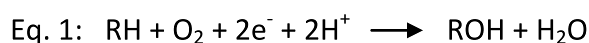
2.5.1. General

The metabolism in living organisms is very important not only for alimentary processing or other important life-keeping biochemical mechanisms, but it serves also for detoxifying of xenobiotics, especially in individuals resistant to medicaments.

The metabolism of *H. contortus* and its resistant forms have never been described in important and responsible details for resistance. Most of the reactions, xenobiotics undergo, are catalyzed by enzymes and only in exceptional cases are xenobiotics changed nonenzymaticly. Some xenobiotics possess certain similarity to eubiotics and could be metabolized as endogenous substrates. The others are metabolized by detoxification enzymes. Some substances are metabolized by both of the enzyme types. Detoxification process can be divided into three phases: modification, conjugation and excretion; involving oxidation, reduction and hydrolytic enzymes, such as CYPs and short chain dehydrogenase/reductase (SDRs) in the 1st phase; conjugation enzymes such as UDP-glycosyl transferases (UGTs) and glutathione S-transferases (GSTs) in the 2nd phase and transport proteins (mainly ATP-binding cassettes – ABCs) in the 3rd phase of metabolism, which carry substances across the membranes. Transport proteins have a lot of properties and regulations mechanisms shared with the enzymes. All these enzyme systems could be responsible for resistance.[5,27,28,29]

2.5.2. Enzymes of 1st biotransformation phase

CYPs are a wide family of hemoproteins. These enzymes are very important for metabolism of eo- and xenobiotics. CYPs are exceptional not only for their significance, but also for amount of possible substrates, catalytic activities and inducibility. They occur in all studied prokaryotic and eukaryotic organisms in a wide variety of isoforms. The superfamily CYP is differentiated into families, subfamilies and then into certain isoforms; which have different structure, different affinity to substrate(s) and other properties compared to one another. These enzymes are situated mostly in microsomes and mitochondrial membrane. Its structure consists of apoprotein assembled from a polypeptide chain and protoporphyrin IX containing Fe^{+3} in the center of the molecule. Most of these enzymes are important for their oxidative activity; CYP is also known as monooxygenase. It breaks oxygen from air to single oxygen atom, incorporates it into a substrate structure (chemical equation 1 below). The enzyme cooperates with enzyme NADPH-CYP-reductase. CYP has also other functions.[29]



Eq. 1: $RH + O_2 + 2e^- + 2H^+ \longrightarrow ROH + H_2O$

Genome of the model organism *C. elegans* encodes 80 different CYPs. Their functions are mostly unexplored. A lot of them were induced after exposure to xenobiotics. ABZ induces CYP 35A2, CYP 35A5 and CYP 35C1 in *C. elegans*. It seems that *H. contortus* produces the same metabolites of ABZ, but it must not mean both of them dispose of the same enzyme systems and biotransformation pathways. In *H. contortus* 73 partial CYP sequences were found. The enzyme induction seems to be more complicated, because there are more factors influencing the induction, which changes also within live stages, environment, gender, feed etc.[27]

There are also other enzymes important for the 1st phase of biotransformation:
FMO – flavine monooxygenases; monomer structures containing one molecule of flavine coenzyme FAD (flavine adenine dinucleotide), localized in endoplasmic reticulum. The enzymes cooperate with NADPH.[29] FMOs and CYPs enzymes catalyze sulfoxidation of ABZ in mammals. In nematodes these metabolic processes are still unknown, but any dependence in *H. contortus* between these enzymes and sulfoxidation it has not been proved. Parasites resistant to benzimidazoles presented significantly lower concentrations of ABZ-SO. The multi-resistant sample in comparison with the susceptible one exhibits similar or lower concentrations.[5,27]

Short chain dehydrogenases/reductases (SDRs) belong to the most extensive group of enzymes, present in all living organisms. SDRs enzymes are NAD(P)(H)-dependent oxidoreductases. They are important for physiological processes and metabolism of xenobiotics.[29]

Peroxidases – hemoproteins important for intracellular antioxidant activity, located mostly in erythrocytes, leucocytes and trombocytes. Some peroxidases are also capable to oxidize xenobiotic structures.[29] Peroxidase concentrations were in some organelles from *H. contortus* resistant isolates relatively higher. Unlike peroxidase, catalase concentrations were lower in resistant helminths. [30,31]

Further: Alcoholdehydrogenases, aldehydedehydrogenases, aldehydeoxidases, xanthinoxidases, monoaminooxidases, diaminoxidases, reductases, and other.[29]

2.5.3. Enzymes of 2nd biotransformation phase

Metabolic products of 1st phase react with a certain endogenous substance (conjugation agent) to form a conjugate. Conjugates are more hydrophilic and easier to eliminate out of an organism. These substances normally possess also smaller activity as well as smaller toxicity. Most of the conjugation enzymes are located in cytosol or microsomes. There are several possible conjugation reactions, such acetylation, sulfonation, methylation, and conjugations with sugars, glutathione and amino acids.[29]

GSTs belong to a superfamily of multifunctional eu- and xenobiotic-metabolizing enzymes which play a crucial role in 2nd phase of detoxification. The main activity of GSTs is the conjugation of compounds with electrophilic centers to the tripeptide glutathione (GSH), but many other activities have now been associated with GSTs.[32] In helminths, GSTs participate in the detoxification of lipid hydroperoxides and carbonyl compounds produced by oxidative stress. Moreover, helminth GSTs are also able to conjugate GSH to xenobiotic compounds or to bind to anthelmintic drugs.[33,34] GSTs, in helminths, apart from conjugation with glutathione, are capable of isomerase activity, peroxidase activity as well as transport and binding of various substances. Nevertheless, in *H. contortus* any differences in activity of the enzyme between susceptible and resistant strains have not been observed, but an increased activity of GST has been found in triclabendazole resistant *Fasciola hepatica*. [27,35]

UDP-glycosyltransferases (UGTs)

UDP-glycosyl transferases (UGTs), group of enzymes involved in the 2nd phase of xenobiotic metabolism, catalyze the covalent attachment of hexose moieties to lipophilic chemicals, thereby enhancing their hydrophilicity and availability for influx and efflux transporters. Activated sugar donors in the form of the uridine diphosphate (UDP) sugars, e.g. UDP-glucuronic acid, UDP-glucose, UDP-xylose and UDP-N-acetylglucosamine are conjugated by UGTs with the hydroxyl, carboxyl, thiol, amine and carbonyl functional groups of various chemicals. Glucuronidation and glucosylation are prevailing reactions catalyzed by UGTs. The overwhelming role of glucuronidation, a major pathway for the elimination of hundreds of lipophilic

by-products of metabolism and xenobiotics (e.g. drugs), in mammals was recently reviewed.[36,37] Although glucosidation is an unusual conjugation reaction in vertebrates, it is prevalent in plants and bacteria. Glucosidation exclusively was reported to be the deactivation pathway for benzimidazole anthelmintics such as ABZ and flubendazole (FLU) in *C. elegans*[38] and *H. contortus*.[4,5] The glucosidation of the model substrate p-nitrophenol was also described in *H. contortus in vitro*. UGTs in helminths, have been studied to a relatively lesser extent.[4]

However, it is important to investigate these enzymes, because there were found an associations between drug resistance and these enzymes. There is a study comparing biotransformation of FLU (drug from benzimidazole family similar to the ABZ) between susceptible and resistant *H. contortus.*[26] According to these results, the parasites are able to detoxify the drug via glucose conjugates formation with UDP-G (UDP-glucose). In the 1st phase of the biotransformation FLU carbonyl group is reduced (FLU-R) and in the 2nd phase FLU and FLU-R are both conjugated with glucose. It has been proved, that the amount of all glucose conjugates was significantly higher in resistant individuals than in susceptible. The ABZ is drug from the same group, but is processed via different metabolic pathway. The ABZ is first S-oxidized unlike the FLU, and then further metabolized by UGTs into three possible glucose derivates (Fig. 7).[4,5] In addition, the study found differences in biotransformation of ABZ in specific cell organelles of *H. contortus* between amount of ABZ-SO metabolized by susceptible-, resistant- and multi-resistant organelles of the parasite after the 1st metabolic phase. The biotransformation was more intensive in mitochondrial fraction than in microsmal fraction.[5]

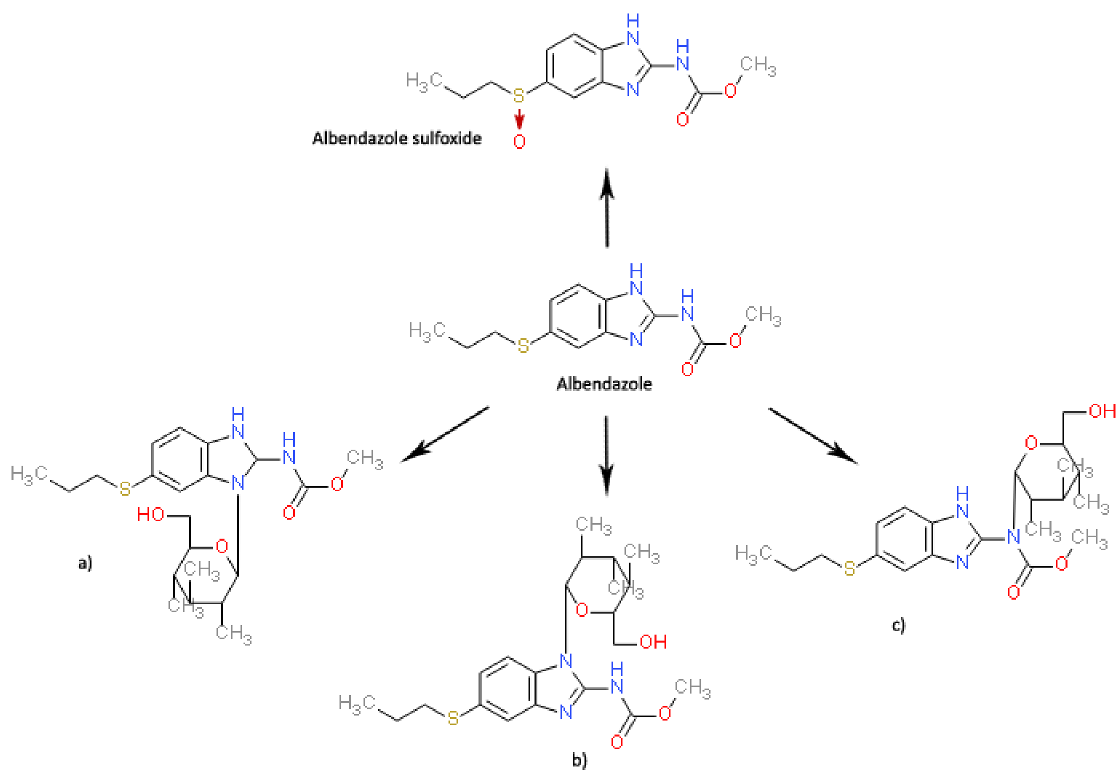


Fig. 7: Metabolism of the ABZ in *H. contortus*; a), b), c) – *N*-glucose conjugates of ABZ. Enzymes responsible for the biotransformations are still unidentified.

3. AIM OF STUDY

This study aims to continue the process of resolving and understanding the resistance to anthelmintics in *H. contortus* species, especially with regard to UGT enzymes. To obtain the conclusion it was necessary to:

- Process the helminth samples (exposed and unexposed to the ABZ) and isolate total RNA
- Detect and compare constitutive gene expression of selected UGTs between male and female adults of *H. contortus*
- Compare constitutive gene expression of selected UGTs between susceptible and resistant isolates of *H. contortus.*
- Compare inducible gene expression of selected UGTs upon ABZ treatment in susceptible and resistant *H. contortus* isolate.
- Analyze the results and draw conclusion.

4. EXPERIMENTAL PART

4.1. BIOLOGICAL MATERIAL, LABORATORY DEVICES AND CHEMICAL REAGENTS

4.1.1. Acquiring of the biological material

Healthy lamb individuals (2 – 3 months old) were infected with L_3 larval stage of *H. contortus*. To every lamb peroral suspension containing approx. 5000 – 6000 L_3 infectious individuals of *H. contortus* was administrated. Two different isolates of the helminth were used: the "inbred susceptible Edinburgh" (ISE) and the multidrug-resistant strain – "white river" (WR). These lambs were kept in boxes, without cattle-run and isolated from environment, to prevent them from an infection of other gastrointestinal parasites. The lambs were killed after 6 weeks and transported to our laboratory. All the animals were treated in accordance with the Czech guidelines for the care and use of farm and experimental animals. Adult nematodes were removed from sheep abomasum using agar method as previously described.[39] Afterwards the parasites were sexed based on morphology, 3 times rinsed with physiological solution and immediately transferred to TRI reagent and frozen to -80 °C. For the *in vitro* ABZ-exposure experiments, batches of ten males and batches of ten females (three biological replicates) were cultured in RPMI 1640 media at 37 °C. The media was supplemented with ABZ dissolved in 0.1% DMSO (10 µM ABZ) or 0.1% DMSO only for control samples. After 12 or 24 h, worms were transferred directly to TRI reagent and frozen in −80 °C for later use.

4.1.2. Chemical reagents

RPMI 1640 medium – Sigma-Aldrich

Albendazole – Sigma-Aldrich

Dimethyl sulfoxide (DMSO) – Serva

TRI reagent – Molecular Research centre

Chloroform – Chemapol

Isopropanol – Penta

Ethanol, absolute – Penta

Diethylpyrocarbonate (DEPC) – Sigma-Aldrich

RNase-free DNase I – New England biolabs

ProtoScript© reverse transcriptase – New England biolabs

dNTPs (2.5 mM each) – Eurogentec

qPCR Core kit for SYBR Green I – Eurogentec

Primers (designed by Ing. Petra Matoušková, Ph.D. and synthesized by Generi Biotech)

4.1.3. Laboratory devices

Automatic pipettes – Research plus, Eppendorf

Centrifuge – Biofuge stratus, Heraeus

Incubator – Hera cell, Heraeus

Flow box – UV-caninet for PCR preparations, UVC/T-AR, Biosan

Microhomogenizators – p-lab

Minicentrifuge – Sprout, Heathrow scientific

Spectrophotometer – Nanodrop 1100, Thermo scientific

Termocycler – MJ mini, BioRad

Termocycler – Quant studio 6 flex, Life technologies

Vortex – V-1 plus, BioSan

4.2. PRACTICAL PROCEDURE

4.2.1. Processing of biological samples and RNA isolation

The biological samples, batches of ten males or females of *H. contortus* adults in 500 µl of TRI reagent were taken out of freezer, thawed and homogenized with a microhomogenizators in laboratory flow box to prevent a contamination and to keep the samples safe from RNases. The samples were re-frozen in dry ice and mechanically homogenized again with the microhomogenizators to achieve a better homogeneity. Afterwards 500 µl of TRIreagent was added and after 5 minutes at room temperature 200 µl of chloroform was added. This mixture was shaken and incubated for 2–3 min at

room temperature, then the samples were centrifuged at 4 °C (15 min, 12 000 g). After division of the phases, the upper aqueous phase, containing RNA, was transferred to new eppendorf tube. Next, 500 µl of isopropanol was added, vigorously shaken and the RNA was let to precipitate for 10 minutes at room temperature. The precipitated RNA was isolated by centrifugation at 4 °C (10min, 12 000 g). The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol centrifuged at 4 °C (5 min, 7 500 g). The supernatant was again removed and the obtained RNA pallet was air-dried for 5–10 minutes and dissolved in the water (treated with DEPC) (20 – 30 µl). The RNA concentration was measured with NanoDrop™ spectrophotometer. The purity of the RNA was checked by the ratio of 260 nm/280 nm, all isolated RNA samples had the optimal ratio of 1.8–2.0. These samples were again frozen in -80 °C for later use.

4.2.2. DNase treatment

The isolated RNA was necessary to purify from the possible contaminant DNA. 10 µg of the RNA was mixed with the buffer (supplied with DNase I) and 1µl of DNase I was added and incubated at 37 °C for 25 minutes. Afterwards 1.5 µl of 0.1M EDTA was added and the samples were incubated at 75 °C for 10 minutes. Then 18.5 µl of DEPC water was added to obtain total RNA of defined concentration (0.2 µg/µl). These samples were stored in the freezer at -80 °C.

4.2.3. Reverse transcription

In this step the isolated RNA was reverse-transcribed to complementary DNA (cDNA) using the enzyme reverse transcriptase (RT). 5 µl of the treated RNA (1 µg) and 1 µl of random hexamers were incubated in PCR cycler at 65 °C for 5 minutes. Then the samples were cooled on ice, shortly centrifuged, and then 14 µl of the master-mix containing 4 µl buffer, 2 µl DTT (dithiothreitol), 4 µl dNTPs, 0.5 µl RT and 3.5 µl H_2O was added. Alongside parallel samples were similarly treated where RT was replace by respective amount of water (noRT control). Afterwards the samples were incubated at 25 °C for 10 min, 42 °C for 50 min, and heated up to 80 °C for 5 minutes for RT denaturatation. After the synthesis the cDNA samples were diluted using 80 µl H_2O and stored in -20 °C.

4.2.4. Real-time PCR

To quantify the expression of the selected genes the real-time PCR was used, using fluorescent SYBR green dye. The samples were amplified with a specific PCR program. The PCR program for each part was used as described in Fig. 8:

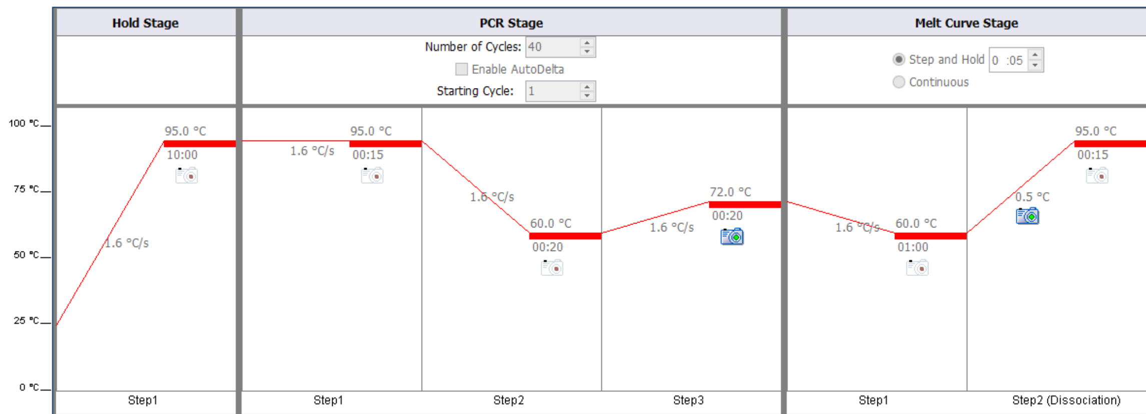


Fig. 8: The temperature program of the real-time PCR. In the initial Hold Stage the PCR device heats up to the 95 °C for 10 minutes to activate DNA-polymerase. The PCR Stage is a regular cycling program (40 cycles) divided into denaturation step (95 °C for 15 sec); annealing step (60 °C for 20 sec); and elongation step (72 °C for 1 min) with the fluorescence reading after each cycle. In the third Melt Curve Stage the temperature rises stepwise (0.5 °C /5 sec) along with fluorescence reading and the melting curve analysis is performed.

4.2.4.1 Verification of primers and the efficiency of the primers

At the beginning a mastermix was prepared into a lock tube, what is mixture of substances necessary for the PCR (Tab. 1).

Tab. 1: Composition of the mastermix per sample

Substance	µl
Water	9.30
10 X buffer +ROX	2.00
50mM MgCl2	1.40
5mM dNTP	0.80
SYBR diluted	0.60
For Primer (5uM)	0.40
Rev Primer (5uM)	0.40
Hotgoldstar enzyme	0.10
cDNA (1:4 Diluted)	5.0
Total	20.0

All the PCR samples were performed in duplicates. Mastermix was prepared in sufficient amount for all samples run in one experiment; 15 µl were pipetted into PCR-specific tubes and 5 µl of diluted cDNA was added into each sample. For every primer set tested no template control (NTC) was analyzed in parallel, where, instead of cDNA, water was used. All the samples were labeled, mixed and briefly centrifuged and analyzed in the real-time PCR device. Then, the C_t values of duplicates averaged to obtain an optimal data. All the tested primers are listed in Tab. 2:

Tab. 2: Verified primers

Primer	Sequence	Amplicon (bp)
F_UGT26	TCATTCGGTTCAGCAATCAAGG	70
R_UGT26	CGAAGACTTCCAGAAAATTCCTCT	
F_UGT27	TTCACAAAACCATCCCGCTC	149
R_UGT27	CGGCATGTAGATTGATTTAGCCA	
F_UGT28	TCCAAGTTATGTTCCAGGGCAT	177
R_UGT28	AGTTCTTCATAATCCTTGAACCGC	
F_UGT17N	TCCGCTGAGTCACTTAAAACT	142
R_UGT17N	AGATTGAACAGTCCACACAGT	
F_UGT1N2	ACGACGATGAGCTAATGAAACA	133
R_UGT1N2	ACCGCTGAGAATACACCAATTG	
F_UGT10N	CTCGTCGTTTGGGTATCGCT	99
R_UGT10N	CGAAGCTGGTGTCCGTAAGT	
F_UGT8N	ACGTACACCTATTCCAATGGCT	60
R_UGT8N	CCTTGGTTTTGGGGTGTTGAAG	
F_UGT20A	TCGAGCTGATTGGTGTGAAGA	96
R_UGT20A	AGCTGGGATTCGTGGAAACT	
F_UGT20B	CTGGGAACGGCAACTCTGG	126
R_UGT20B	CGAGGCGTTTCTGTGTATTCG	
F_GAPDH	TGCACCACCAACTGCTTAGC	87
R_GAPDH	GGCATGGACTGTGGTCATGAG	

After the verification, It was necessary to verify the efficiency of amplification of the used primers. To specify the efficiency, the samples with decreasing initial concentration were analyzed by PCR and obtained results were processed with mathematical methods. A concentration series of a 5x diluted cDNA was prepared. All samples were prepared and analyzed by PCR as described previously.

Each concentration (together 5 different concentrations) was run in duplicate and there was also prepared one negative control to every analyzed primer set (Fig. 9).

Fig. 9: Schematic design of positions of samples for efficiency determination with real-time PCR. Each color represents a certain concentration. In the 1st concentration bar is the highest concentration – 1000 relative units, 2nd – 200, 3rd – 40, 4th – 8 and in the 5th was 1.6 relative units. Each line (labeled A, B, C, D) contained a verified R/F primer. **NTC** – no template control.

The selection of the primers tested for specifying their amplification efficiency was based on previous results.

All the data from the real-time PCR were analyzed to specify the efficiency of the samples. The C_T values of a certain concentration were averaged and with the corresponding relative logarithmic concentration values were used for generation of a standard curve. The linear tradeline was used to determine the slope of the standard curve (Eq. 2):

Eq. 2: $y = kx + q$

Where the y is C_T; k is slope; x is concentration and q is intercept.

The slope was used for the efficiency calculation using standard equation (Eq. 3).

Eq.3: $\% \text{ Efficiency} = 10^{-1/k} - 1 \times 100$

Where the k is the slope.[40]

4.2.4.2. Constitutive gene expression analysis.

Quantitative real-time PCR was used to study differences in gene expression. To acquire suitable and comparable results, the comparative C_t method was chosen. The PCR analysis of all samples were run as described previously. 5µl of diluted cDNAs, prepared by reverse transcription as described above for each biological sample, were analyzed by qPCR with verified sets of primers (Fig. 10).

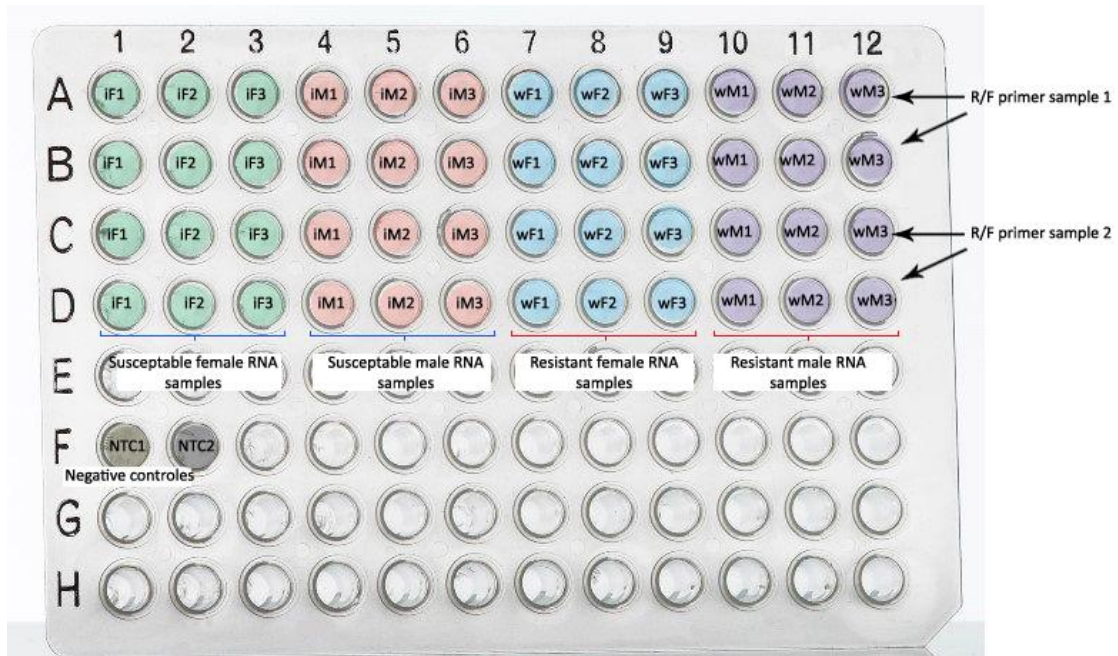


Fig. 10: Schematic design of sample positions on the PCR plate for quantifying of constitutive gene expression. Columns 1–6 samples of susceptible *H. contortus;* columns 7–12 samples of resistant *H.* contortus. **iF1 – iF3** stands for three biological replicates of susceptible female helminths; **iM1 – iM3** for susceptible male helminths; **wF1 – wF3** for resistant female helminths; and **wM1 – wM3** for resistant male helminths. **NTC** – no template control.

After the PCR run all the data and graphs were processed and analyzed in a spreadsheet. The duplicates with difference in C_T values higher than 0.5 were not further processed. The other C_T values were averaged and used for the relative gene expression calculation using the equation 4:[41]

Eq. 4: Fold change = $2^{-\Delta\Delta C_T}$

$\Delta\Delta C_T$ = [(C_T gene of interest – C_T internal control)sample A – (C_T gene of interest – C_T internal control)sample B]

Where the C_T is threshold cycle.[41]

In this relative quantification all the genes of interest are normalized to reference gene, which should be totally independent of tested factor(s) and exhibits no differences after exposure to these factors. In this case primers for GAPDH gene was used as the reference gene, which proved to be stable under tested conditions.[42]

As relative gene expression was used the average of the iM1–3 samples were set to one and all other samples (iFs, wMs and wFs) were relatively compared. The results were compared to one another and turned into bar charts with its standard deviations and also analyzed in the GraphPad for statistically significant results using Students´ t-test.

4.2.4.3. Inducible gene expression analysis.

Based on the results from the constitutive expression analysis, genes to be analyzed also upon ABZ treatment were chosen to assess possible inducibility of these genes.

The technique to quantify and compare the gene expression between the control samples and treated samples is based on the same concept as in the previous chapter.

The cDNA samples from susceptible and resistant helminths unexposed to ABZ (control samples) and the samples of susceptible and resistant helminths exposed to the ABZ we prepared and tested by qPCR. All the samples were run in duplicates; see the schematic design of the PCR plate (Fig. 11).

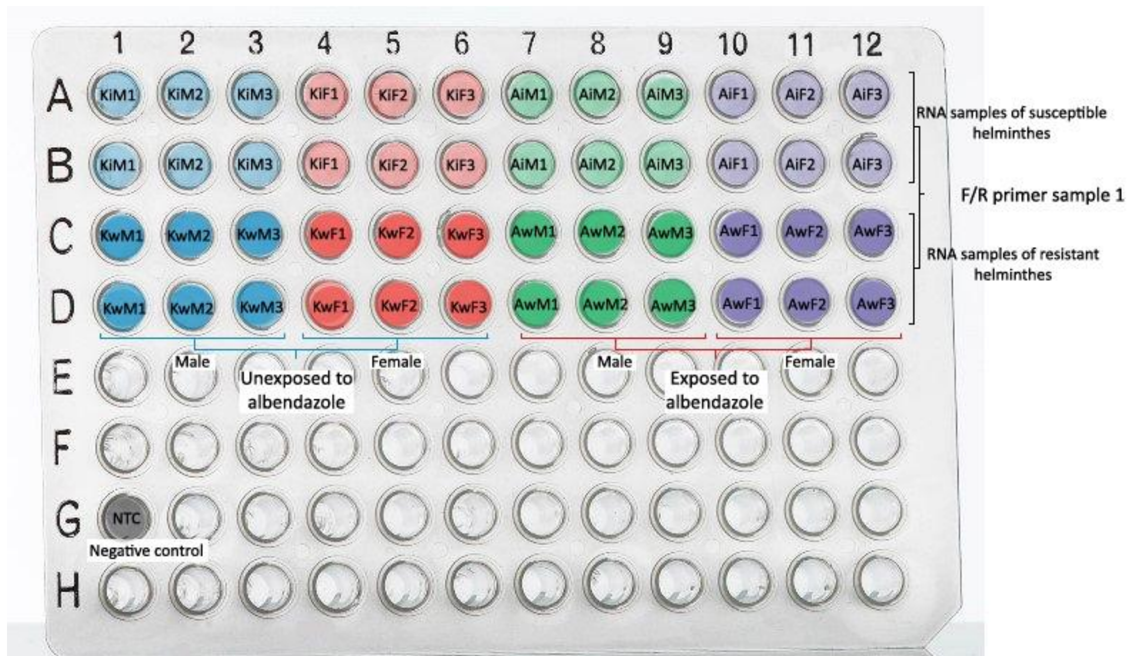


Fig. 11: Schematic design of sample positions on the PCR plate for quantifying of gene expression at susceptible and resistant individuals exposed and unexposed to the ABZ. On the left half were samples from *H. contortus* unexposed to the ABZ (under the numbers 1–6; **K**- samples) and on the right side were samples from *H.* contortus exposed to the ABZ (under the numbers 7–12; **A**- samples). The PCR lock tubes were labeled as: **iM1 – iM3** for susceptible male helminths and **iF1 – iF3** for susceptible female helminths (on the upper side); **wM1 – wM3** for resistant male helminths and **wF1 – wF3** for resistant female helminths (on the downer side). These samples were tested for one gene. **NTC** – no template control (without cDNA).

After the run, all the data were stored in spreadsheet and further analyzed. The C_T values of sample duplicates were averaged. The data were analyzed using the equation 4 as previously described; the data obtained with the primers for GAPDH gene served as the reference gene for normalization, and the sample B stands for control samples to obtain fold change differences of ABZ treated samples over respective controls. All control samples were set to 1 and respective samples exposed to ABZ represented calculated fold change of the relative expression levels of analyzed genes. Standard deviation was calculated for each group. The values were compared and presented as a bar chart with their standard deviations and statistically analyzed in GraphPad. This data were assessed and used to draw conclusions.

4.2.5. Statistical analysis

All data are presented as arithmetic mean of biological replicates ± SD. All calculations were done using Microsoft Excel and GraphPad Prism 6.0. Unpaired, two-sided

Student's t-test was used for the statistical evaluation of differences between tested groups. The differences were considered significant at $P < 0.05$.

5. RESULTS

In this study were verified several UGTs: UGT1N2, UGT8N, UGT10N, UGT17N, UGT20A, UGT20B, UGT26, UGT27 and UGT28. The investigated genes were chosen from all known UGT genes of *H. contortus*. The fig. 12 represents the available genes and relation between them.

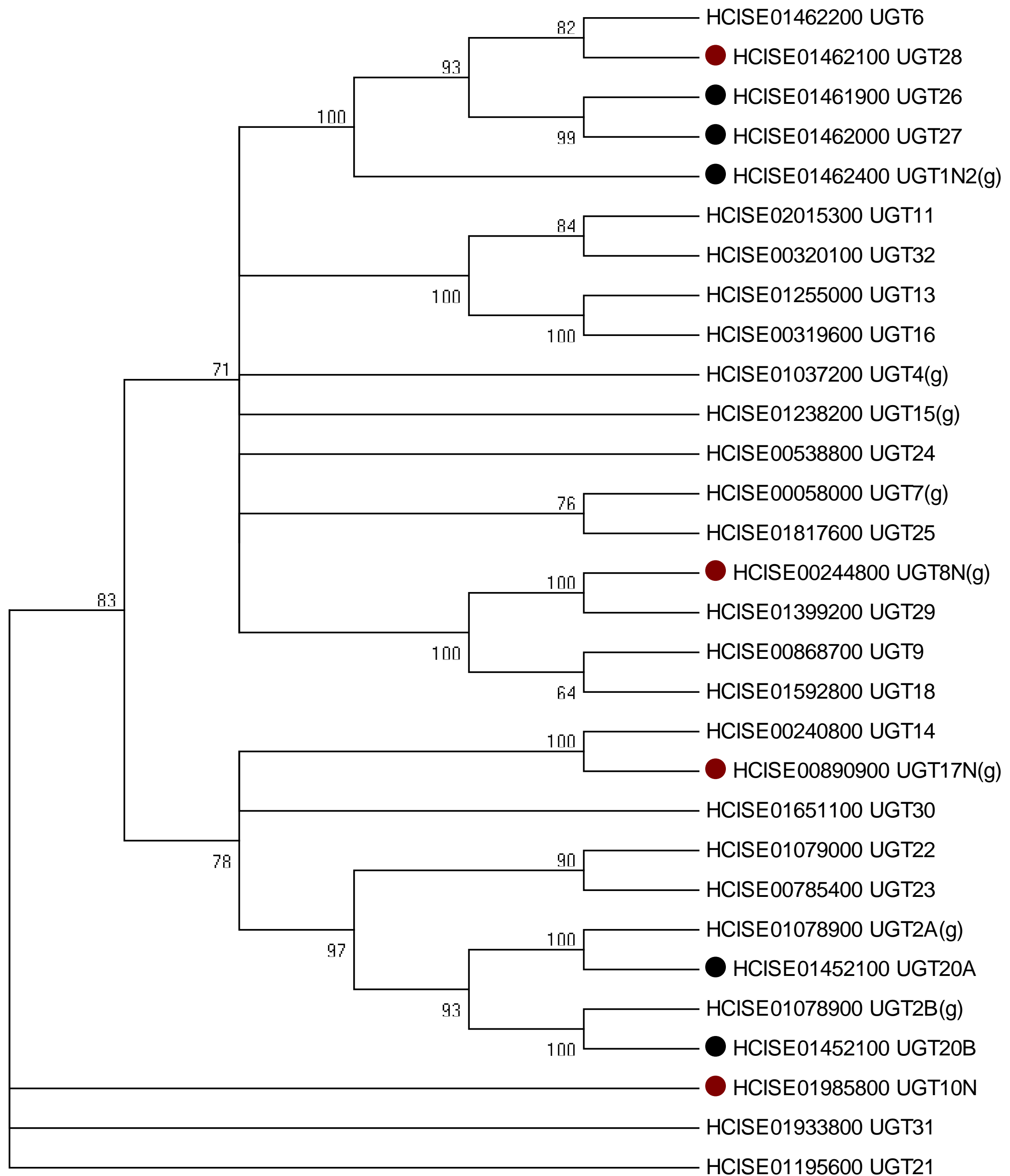


Fig. 12: Phylogeny of the UGT genes of *Haemonchus contortus*.
The computer program MEGA6 was used to reconstruct the tree using the Maximum-Likelihood method. Numbers along the branches indicate bootstrap support from 500 replicates with collapsed nodes under 50. Genes analyzed in this thesis are marked with dots, genes with red dots were analyzed also upon ALB treatment. The name of the UGTs consists of GenBank ID and a assigned abbrevitaion.

Phylogenetic analysis was done using the computer program MEGA6.[43,44] At first, MEGA Caption Expert found GTR+G (General Time Reversible + discrete Gamma distribution) as the best-fit substitution model for phylogeny reconstruction. The reliability of the tree was tested by bootstraping 500 replicates and nodes supported by less than 50 were collapsed.

To acquire the desired results, selected UGTs gene expression, it was necessary to perform elementary steps that are divided into 4 parts. At first, functions of the analyzed primers were verified. In the second step efficiencies of the primer sets were analyzed. Then constitutive expressions of genes encoding selected UDP-glucosyltransferases, using verified primers, were compared in male and female adults and also the expression in susceptible and resistant isolates (both genders) were compared. From these results "interesting" genes were chosen, which were further analyzed in samples of helminths exposed to ABZ.

5.1. VERIFICATION OF PRIMERS

After the first analyzes, it was decided, if the tested primers are suitable for further procedures. The requirements were as follows; ideally the NTC control should be undetectable, in case some primer dimers appearance, the difference between C_T values of a sample and the NTC should be more than 5 and the melting curve analyses has to show only one peak. As example are used amplification plot and melting curve of the UGT27 (Fig. 13 and 14).

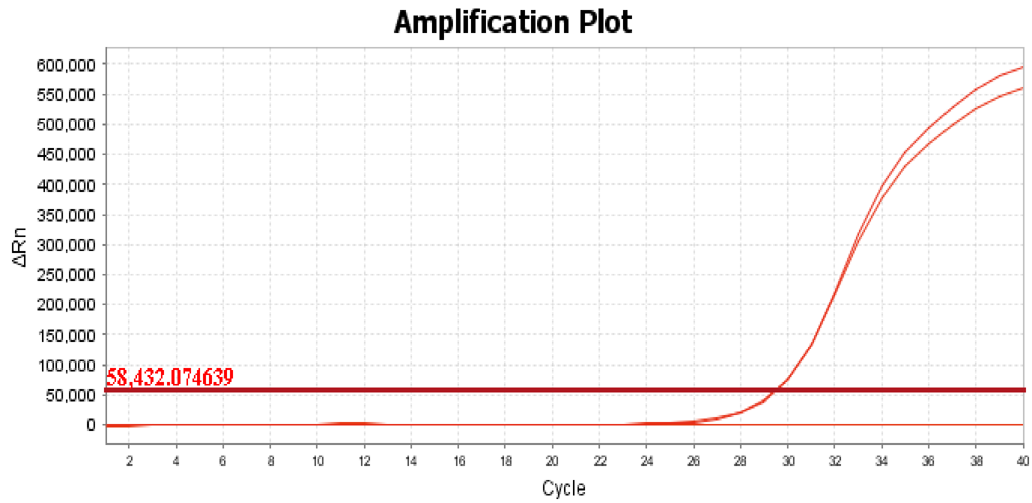


Fig. 13: Amplification plot of the UGT27 analysis.
ΔRn – normalized reporter value or normalized fluorescent signal. On the x axis is the number of cycles. The 2 sigmoid curves represent the amplification of the duplicate samples in UGT27 gene analysis, the non-raising curve is the negative control. The highlighted straight line represents threshold for "Cycle quantification" and the red number above is value of fluorescence of the threshold.

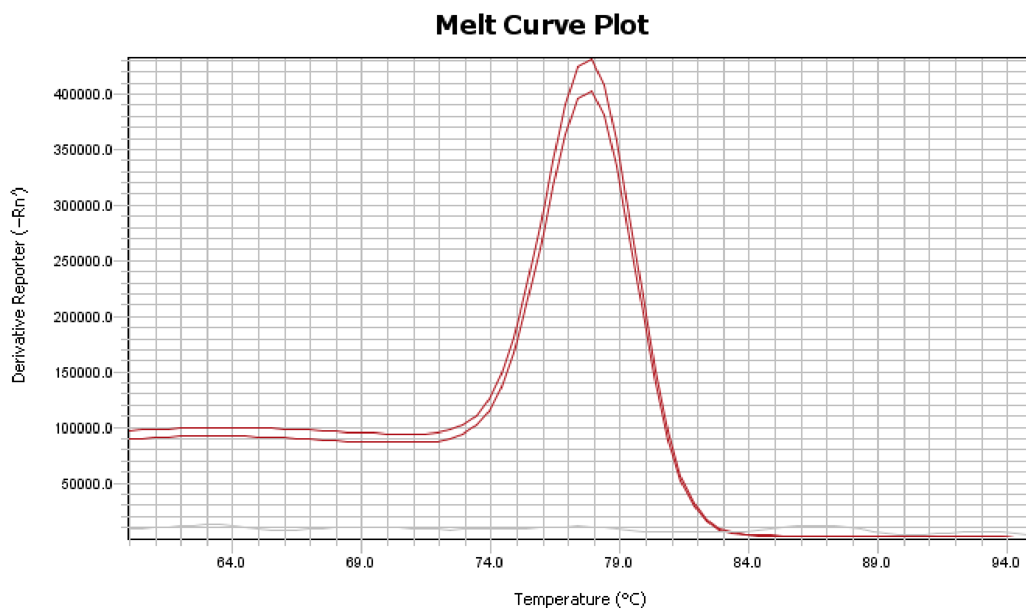


Fig. 14: Derivation of melting curve of the UGT27 analysis.
The graph shows the relation between change of fluorescence and temperature. The curve determines the melting temperature of the product(s).

5.2. EFFICIENCY

All the primers underwent the efficiency test, to prove their comparability and to obtain relevant results. The efficiency test was performed as described in methods. As an example point graph of the UGT8N is used (Fig. 15).

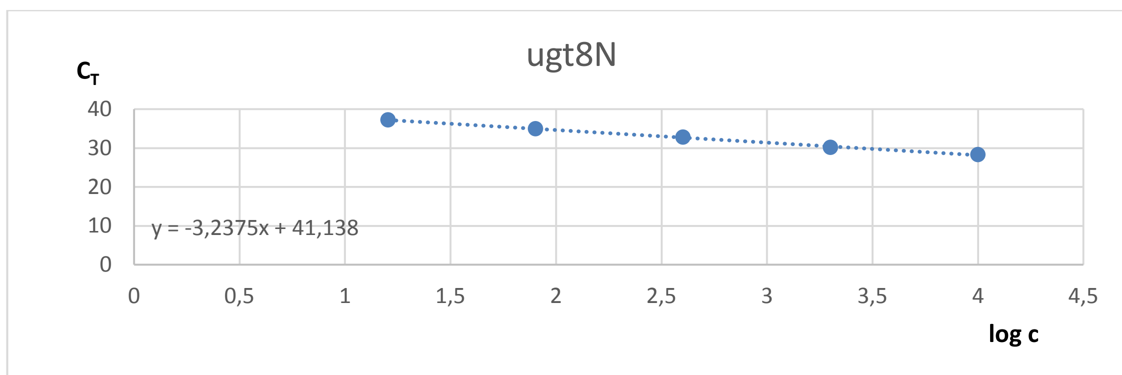


Fig. 15: Point graph of UGT8N with linear function, where C_T = threshold cycle, log c = logarithm of cDNA concentration.

The table 3 contains the assigned names of tested UGT genes , GenBank IDs and obtained amplification efficiency for respective primer set. The optimal efficiency of the primer sets is between 90 % and 110 %. All tested primer sets had appropriate efficiency (Tab. 3). The efficiency of UGT20A and UGT20B was not tested in this study. Their efficiencies were analyzed previously by Ing. Petra Matoušková, Ph.D.

Tab. 3: Efficiency of the analyzed F/R primers.

Primer	GenBank ID	Efficiency
UGT26	HCISE01461900	100 %
UGT27	HCISE01462000	99 %
UGT28	HCISE01462100	94 %
UGT17N	HCISE01462100	99 %
UGT1N2	HCISE01462400	96 %
UGT10N	HCISE01985800	109 %
UGT8N	HCISE00244800	104 %
UGT20A	HCISE01462000	101 %
UGT20B		103 %
UGT4N2	HCISE01037200	109 %
UGT24	HCISE00538800	100 %
UGT17	HCISE00240800/HCISE00890900	99 %

5.3. CONSTITUTIVE GENE EXPRESSION ANALYSIS

Quantitative real-time PCR was applied for quantification of the relative gene expression in susceptible and resistant *H. contortus* isolates. Expression of several genes encoding UDP-glucosyltransferases (UGT26, UGT27, UGT28, UGT17N, UGT1N2, UGT10N, UGT8N, UGT20A, UGT20B, UGT4N2, UGT24 and UGT14/17) was analyzed. The results of the gender comparison in susceptible strain are present in Fig. 16. Male samples (iM) were set to 1 and female samples (iF) represent the fold change difference. All the tested genes, but UGT20A, display statistically significant differences between the genders. Surprisingly, only two tested genes (UGT26 and UGT17N) were more expressed in females. All the other tested genes had higher expression in males (Fig 16).

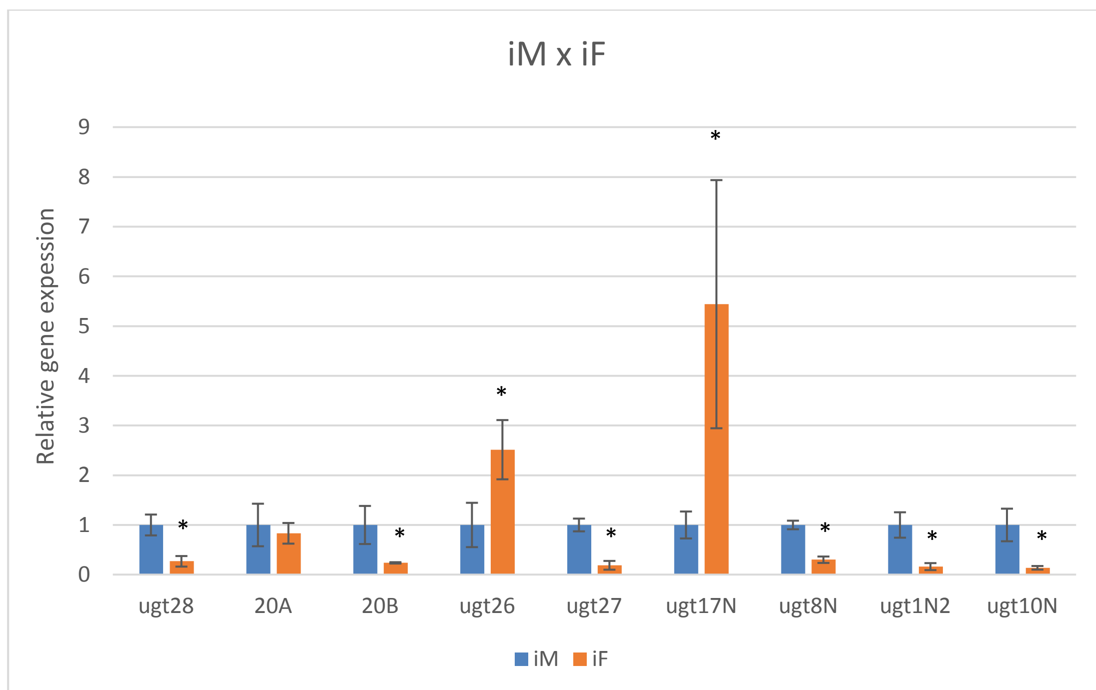


Fig. 16: Column graph demonstrating the differences between genders of susceptible *H. contortus* isolate (iM x iF) in gene expressions of the UGT genes. The expressions are presented in a relative scale, with their standard deviations calculated from biological replicates. The relative gene expressions of male samples set to 1 are referent for the female samples. * – Statistically significant result, P<0.05.

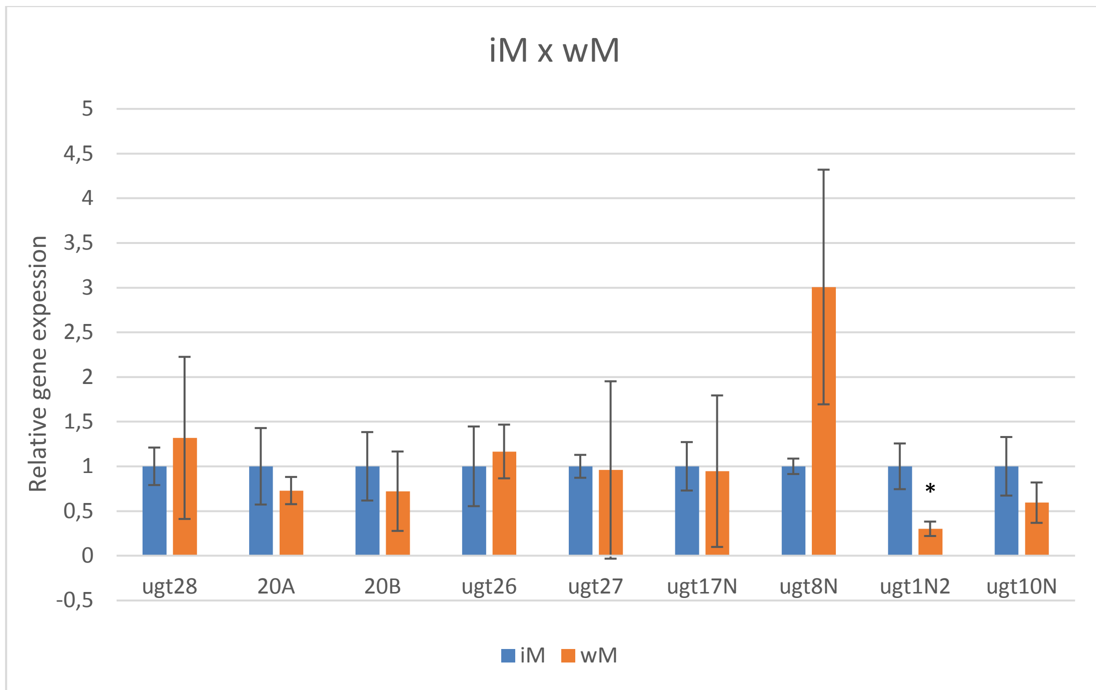


Fig. 17: Column graph demonstrating the differences between susceptible and resistant *H. contortus* male (iM x wM) in gene expressions of the UGT genes. The expressions are expressed in a relative scale, with their standard deviations calculated from biological replicates. The relative gene expressions of samples of susceptible isolate set to 1 are referent for the samples of resistant parasites. * – Statistically important result, P<0.05.

The main aim of the constitutive analysis was to assess possible differences between susceptible and resistant *H. contortus* isolates. The obtained data are presented for each gender separately, because the gender differences proved to be significant (as mentioned previously, Fig.16). This data were further processed in Graphpad, to obtain an objective and statistically significant results as marked by asterisk in figures 17 and 18. In males, no UGT gene displayed higher constitutive expression in resistant isolate. Surprisingly, the only statistically significant result in males was lower expression of UGT1N2 in resistant isolate. For unknown reason some UGT genes displayed high variability between biological replicates, especially in resistant isolate (e.g. UGT27, UGT17N), which might be interesting for further studies.

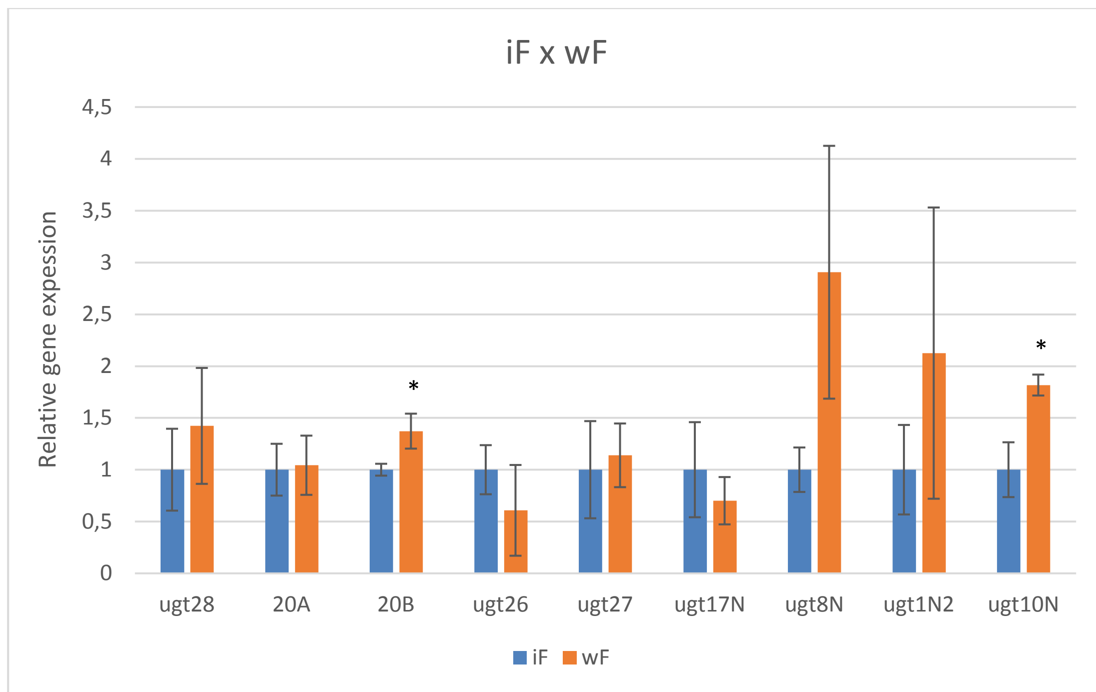


Fig. 18: Column graph demonstrating the differences between susceptible and resistant *H. contortus* female (iF x wF) in gene expressions of the UGT genes. The expressions are expressed in a relative scale, with its standard deviations calculated from biological replicates. The relative gene expressions of samples of susceptible parasite are referent to the samples of resistant parasites. * – Statistically important result, $P<0.05$.

In female samples several UGT genes had higher constitutive expression in resistant isolate, however, only UGT20B and UGT10N were significantly higher. The genes, where differences in transcription between isolates were observed were further investigated upon exposure to ABZ (UGT8N, UGT10N, UGT17N and UGT28).

5.4. INDUCIBLE GENE EXPRESSION ANALYSIS.

To assess whether selected UGTs are inducible upon anthelmintic treatment in *H. contortus* adults, the isolated worm (sexed) were cultivated with or without addition of sublethal concentration of ABZ (10µM) to medium for 24h. The qPCR analysis was used to analyze differences in gene expression of exposed and unexposed parasites (susceptible and resistant isolates) to ABZ.

In this part genes, which exhibited a statistically important values in the first part were analyzed (UGT8N, UGT10N, UGT17N and UGT28), and GAPDH was used as

the reference gene for data normalization. All the treated samples were compared to respective control group. Figures 19 – 22 show results for each assessed gene.

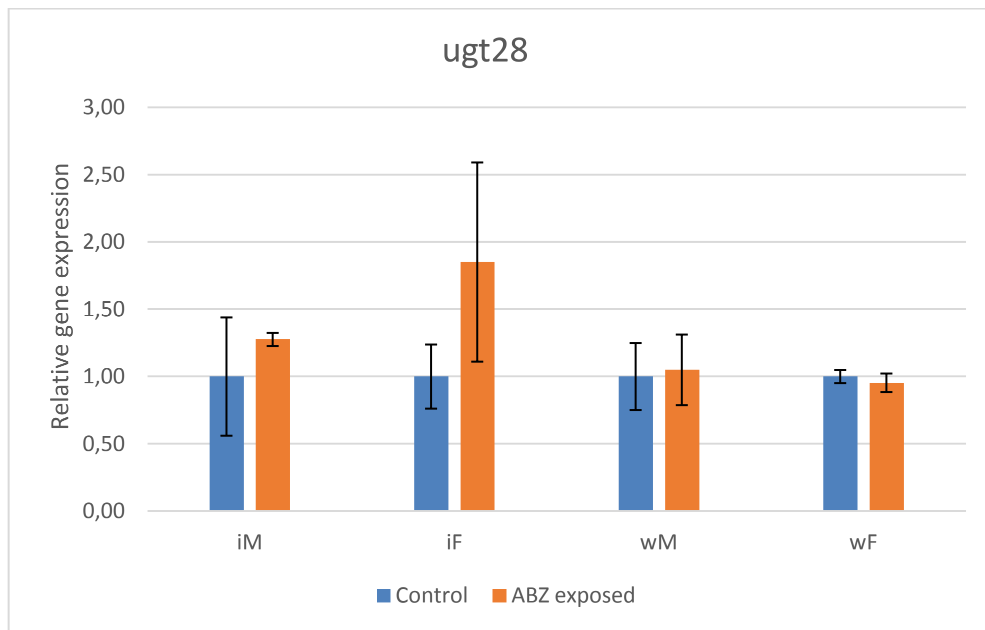


Fig. 19: Column graph demonstrating the gene expression of the UGT28 gene in susceptible and resistant *H. contortus* exposed to ABZ. A control sample unexposed to the ABZ was prepared in parallel for every treated sample. All the values are expressed in a relative scale with corresponding standard deviations.
i – susceptible, **w** – resistant, **F** – female, **M** – male

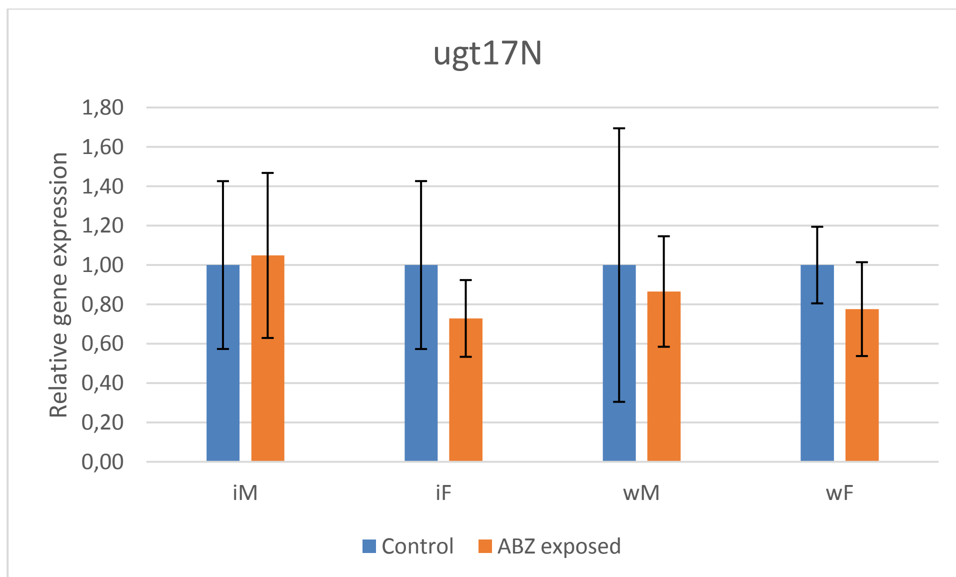


Fig. 20: Column graph demonstrating the gene expression of the UGT17N gene in susceptible and resistant *H. contortus* exposed to ABZ. A control sample unexposed to the ABZ was prepared in parallel for every treated sample. All the values are expressed in a relative scale with corresponding standard deviations. **i** – susceptible, **w** – resistant, **F** – female, **M** – male

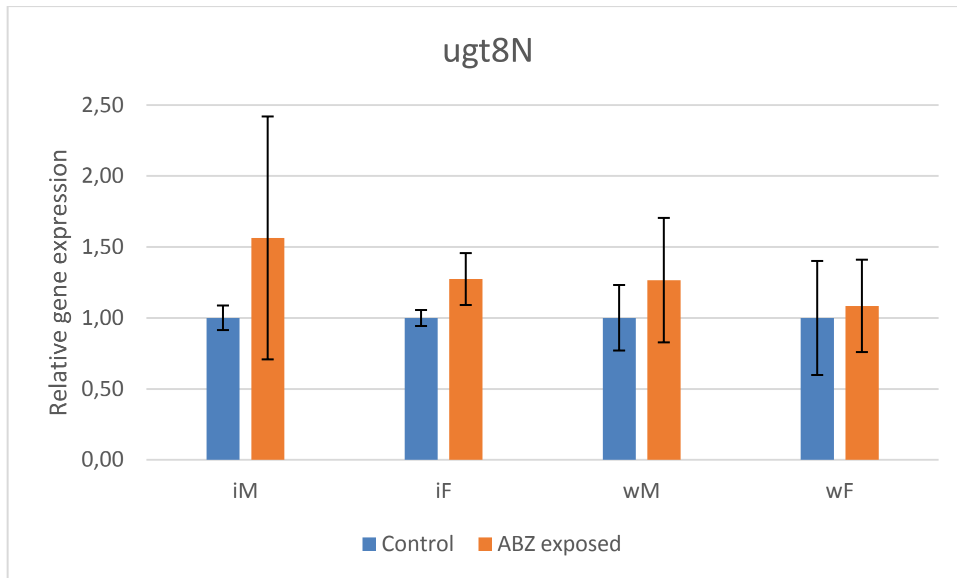


Fig. 21: Column graph demonstrating the gene expression of the UGT8N gene in susceptible and resistant *H. contortus* exposed to ABZ. A control sample unexposed to the ABZ was prepared in parallel for every treated sample. All the values are expressed in a relative scale with corresponding standard deviations.
i – susceptible, **w** – resistant, **F** – female, **M** – male

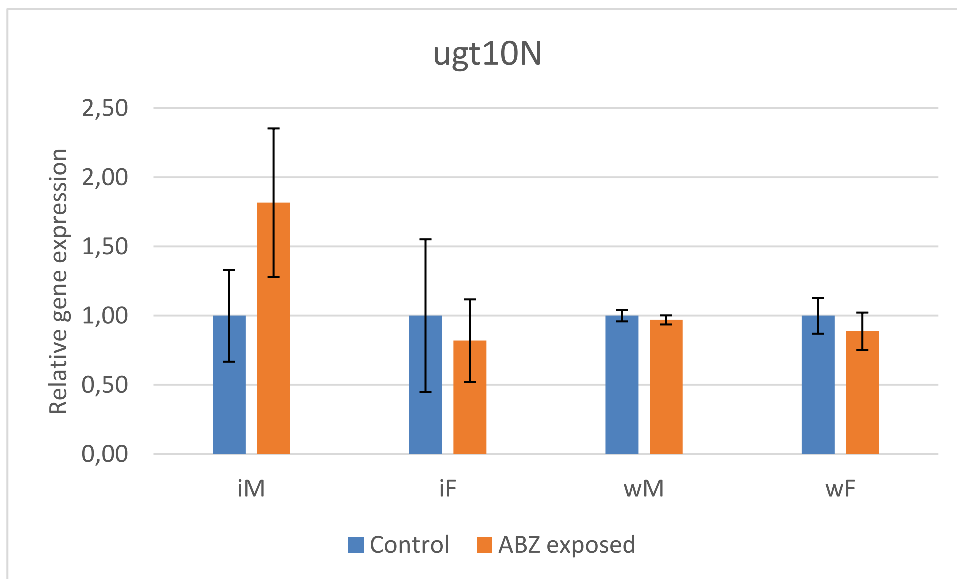


Fig. 22: Column graph demonstrating the gene expression of the UGT10N gene in susceptible and resistant *H. contortus* exposed to ABZ. A control sample unexposed to the ABZ was prepared in parallel for every treated sample. All the values are expressed in a relative scale with corresponding standard deviations.
i – susceptible, **w** – resistant, **F** – female, **M** – male

These results were also analyzed in GraphPad for statistical significance. The GraphPad did not labeled any difference in gene expression between control and ABZ exposed samples as statistically important.

6. DISCUSSION

H. contortus, one of the most pathogenic parasite of small ruminants*,* which belongs to endoparasitic helminths is widely expanded worldwide. *H. contortus* is a hematophagous gastrointestinal parasite, extremely detrimental to animal health and to their productivity. This aspect markedly influences domesticated and especially agriculture animals. Haemonchosis severely affects economic situation in agriculture. Infected animals are commonly treated with anthelmintic drugs and some species of helminths dispose of resistance to those drugs. The resistance rapidly rises in the last years, what represents a difficult problem for future of treating helminthiases. In the 1990s, it was documented in Australia, south and east Asia, America, Africa and Europe a rising resistance of this species to common anthelmintics. The most affected region is the South America and the anthelmintic resistance is in the central Europe one of the biggest therapeutic and agricultural problems.[1,2,3,11,12]

To improve medication and therapy of helminthiases it is necessary to discover and apprehend the resistance of helminths. It would lead to decrease of costs in agriculture stock raising and bringing more productivity from breeding (meat, milk, wool etc.). There are several possible mechanisms of resistance in *H. contortus*. For example regarding the resistance to benzimidazoles; resistant *H. contortus,* possess polymorphism in F200Y or F167Y locus, which encode β-tubulin isotype 1 and isotype 2, what leads to conformation change in β-tubulin, therefore benzimidazoles are unable to bind to this structure.[1] Other defensive strategies of resistant helminths might be caused by another modification of drug pharmacodynamics or pharmacokinetics.[1]

Glucosidation is a conjugation process catalyzed by UGTs. Several studies has proven a presence of UGTs in nematodes.[45,24] This thesis is directed to study the resistance in *H. contortus* influenced by UGTs enzymes, through glucosidation of anthelmintics. Since previously it was described that resistant *H. contortus* isolate is able to produce more glucosyl derivates of ABZ.

From cca 40 UGT genes present in *H. contortus* 10 UGT genes were analyzed in this theses. The gender-specific differences where described in susceptible isolate. All but one tested UGTs were expressed differently, where the UGT26 and UGT17N reach significantly higher levels at females, whereas the other enzymes are more produced at male individuals. It seems that the differences in enzyme productions are gender specific and hence the metabolic pathways could be different in each gender. Previous results, where the detection of more ABZ glucose metabolites were observed, however, were performed only in non-sexed pool of worms.[5] Therefore it requires more thorough analysis whether the higher ABZ-glu production is relevant for both genders.

In the comparison between susceptible and resistant males (iM and wM) only one statistically important difference in the transcription of tested UGTs genes was detected (UGT1N2). However, the expression of the enzyme UGT1N2 is surprisingly higher at susceptible male *H.* contortus. The lower transcription of UGT1N2 in the WR strain might be substituted by another enzyme with higher expression in males, which wasn´t analyzed in this theses. The UGT8N seems to be more induced in resistant males, but the result has high standard deviation and the result is not statistically important. From this comparison it is presumable, that enzymes tested in this theses are probably not responsible for higher production of ABZ glucose derivates and are probably are not responsible for the resistance of *H. contortus*.

In the comparison of gene production in females (iF and wF), two genes are produced in higher amounts at resistant females of *H. contortus* (UGT20B and UGT10N). These differences are not present at resistant males. It is worth noting, the production of UGT8N seems to reach significantly higher levels at the WR sample than at the ISE sample, but this result has also higher standard deviation, so the result is not trustworthy and was not labeled as statistical important. Regarding to the fact, the UGT8N seems to be more produced also at resistant male of *H. contortus,* these correlating data may lead to more thorough research of the enzyme, to obtain accurate data.

Since the differences in formation of ABZ-glucose conjugates might be caused by different inducibility of the genes between isolates, this hypothesis was also tested. Four genes (UGT8N, UGT10N, UGT17N, UGT28) were further tested upon exposure of the *H. contortus* to ABZ. But no statistically important results in induction/supression of UGTs were detected. However, to sufficiently understand, how the enzymes in *Haemonchus* are induced and to discover their purpose, it is necessary to provide further research.

7.CONCLUSION

In this thesis were reached following objectives:

- RNA from the helminth samples was successfully isolated with the described method in required quantity and quality, suitable for constitutive gene expression analysis.
- The gene expression of selected UGTs between susceptible and resistant (male and female) isolates of *H. contortus* was compared. In males the expression of UGT1N2 was higher in susceptible isolate, whereas in females UGT20B and UGT10N were higher in resistant isolate. The gene expression of selected UGTs upon ABZ treatment in susceptible and resistant *H. contortus* isolate was compared. No significant change in expression was detected.

ABBRAVIATIONS

ABC	ATP-binding cassette
ABZ	albendazole
ABZ-SO	albendazole sulfoxide
cDNA	complementary deoxyribonucleic acid
C_T	threshold cycle
CYP	cytochrome P450
ddH_2O	double destiled water
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
ds-DNA	double stranded deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EHT	egg hatch test
F/R	forward/reverse
FAD	flavin adenine dinucleotide
FAMACHA	faffa malan chart – a diagnostic method of parasites
FECRT	fecal egg count reduction
FLU	flubendazole
FMO	flavin monooxygenase
GA	glucuronic acid
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GTR+G	General Time Reversible + discrete Gamma distribution
FECRT	fecal egg reduction test
GST	glutathione S-transferase
ISE	inbred susceptible Edinburgh (susceptible *H. contortus* isolate)
L_{1-4}	larval stage
MDR	multi drug resistance
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NTC	no template control
NTP	nucleosidetriphosphate
PCR	polymerase chain reaction
Phe	phenylalanine
qPCR	quantitative real-time PCR
RT	reverse transcriptase
SDR	short chain dehydrogenase/reductase
Tyr	tyrosine
UDP	uridine diphosphate
UDP-GA	UDP glucuronic acid
UGT	UDP- glycosyltransferase
WR	white river (resistant *H. contortus* isolate)

6. REFERENCES

1. FAIRWEATHER I., et al.: Drug resistance in veterinary helminths. Tr. Parasitol., *20*(10), 469–476 (2004).

2. GILLEARD J.S.: Haemonchus contortus as a paradigm and model to study anthelmintic drug resistance. Parasitol., *140*(12), 1506–1522 (2013).

3. PETER J.W., CHANDRAWATHANI P.: Haemonchus contortus: parasite problem No. 1 from tropics - Polar Circle. Problems and prospects for control based on epidemiology. Trop. Biomed., *22*(2), 131–7 (2005).

4. VOKŘÁL I., et al.: The metabolism of flubendazole and the activities of selected biotransformation enzymes in *Haemonchus contortus* strains susceptible and resistant to anthelmintics. Parasitol., *139*(10), 1309–1316 (2012).

5. VOKŘÁL I. et al.: Biotransformation of albendazole and activities of selected detoxification enzymes in *Haemonchus contortus* strains susceptible and resistant to anthelmintics. Vet. Parasitol., *196*(3–4), 373–381 (2013).

6. BONGERS T., FERRIS H.: Nematode community structure as a bioindicator in environmental monitoring. Trends Ecol. Evol., *14*(6), 224–228 (1999).

7. LEE D.L.: *The biology of nematodes*. 1. ed., London: Taylor & Francis, 2010, ISBN 978-0415272117.

8. [online] (27.9.2015) available from: http://www.wormatlas.org/male/introduction/Introframeset.html

9. JONATHAN H., CHISHOLM A., SHEN M.M.: Major sex-determining genes and the control of sexual dimorphism in *Caenorhabditis elegans*. Genom., *31*(2), 625–637 (2011).

10. [online] (27.9.2015) available from: http://www.wormatlas.org/hermaphrodite/introduction/Introframeset.html

11. HOFELE A.: Disertační práce: Untersuchung zur Rezistenz der Rassen Merinolandschaf und Rhönschaf gegenüber *Haemonchus contortus* unter besonderer berücksichtigung von Kreuzungseffekten. (2008).

12. WALLER P. J., CHANDRAWANTHI P., et. al.: *Haemonchus contortus*: Parasite problem No. 1 from tropics – polar circle. Problems and prospects for control based on epidemiology. Trop. Biomed., *22*(2), 131–137 (2005).

13. [online] (29.9.2015) available from: http://veterinaryrecord.bmj.com/content/166/1/5.1/F1/graphic-1.large.jpg

14. BATH G.F.: Non-pharmaceutical control of endoparasitic infections in sheep. Vet. Clin. Food Anim. Pract., *27*(1), 157–162 (2011).

15. [online] (2.10.2015) available from: https://www.studyblue.com/notes/note/n/lab-practical-1/deck/9610242

16. SCHNIEDER T.: *Veterinärmedizinische Parasitologie*. 6. Auflage, Stuttgart: Parey, 2006, ISBN 978-3-8304-4135-9.

17. DEPLAZES P., et al.: *Lehrbuch der Parasitologie für die Tiermedizin*. 3. Auflage, Stuttgart: Enke, 2013, ISBN 978-3-8304-1135-2.

18. ZAJAC A.: Gastrointestinal nematodes of small ruminants: lifecycle, anthelmintics, and diagnosis. Vet. Clin. Food Anim. Pract., *22*(3), 529–541 (2006).

19. LÖSCHER W., RICHTER A., HEIDRUN P.: *Pharmakoterapie bei Haus- und Nutztieren*. 9. Auflage, Stuttgart: Enke, 2014, ISBN 978-3-8304-1250-2.

20. LAMKA J., DUCHÁČEK L.: *Veterinární léčiva pro posluchače farmacie*. 4. vyd., Praha: Karolinum, 2014, ISBN 978-80-246-1790-8.

21. LAMKA J., DUCHÁČEK L.: *Veterinární vademecum pro farmaceuty*. 2. vyd., Praha: Karolinum, 2014, ISBN 978-80-246-2792-2.

22. HANS-HASSO F., WOLFGANG L.: *Lehrbuch der Pharmakologie und Toxikologie für die Veterinärmedizin*. 3. Auflage, Stuttgart: Enke, 2009, ISBN 978-3830410799.

23. [online] (7.10.2015) available from: https://pubchem.ncbi.nlm.nih.gov/image/imagefly.cgi?cid=2082&width=500&height=500

24. LASOTOVÁ S.: Diplomová práce: Porovnání metabolismu anthelmintik a aktivit biotransformačních enzymů u citlivých a rezistentních kmenů vlasovky slézové. (2011).

25. DĚDKOVÁ T.: Diplomová práce: Navození rezistence hlístic na albendazol. (2015).

26. PAPADOPOULOS E.: Anthelmintic resistance in sheep nematodes. Small Rum. Res., *76*(1-2), 99–103 (2008).

27. LAING R., et al.: The cytochrome P450 family in the parasitic nematode *Haemonchus contortus*. Intern. Journ. Parasit., *45*(4), 243–251 (2015).

28. LAING R., et al.: The genome and transcriptome of *Haemonchus contortus*, a key model parasite for drug and vaccine discovery. Gen. Biol., *14*(8), R88 (2013).

29. SKÁLKOVÁ L., BOUŠOVÁ I., et. al.: *Metabolismus léčiv a jiných xenobiotic*. 1. vyd., Praha: Karolinum, 2011, ISBN 978-80-246-1917-0.

30. KOTZE A.C.: Catalase induction protects *Haemonchus contortus* against hydrogen peroxide *in vitro*. Int. J. Parasitol., *33*(4), 393–400 (2003).

31. KOTZE A.C., MCCLURE S.J.: *Haemonchus contortus* utilizes catalase in defense against exogenous hydrogen peroxide *in vitro*. Int. J. Parasitol., *31*(14), 1563–1571 (2001).

32. OAKLEY A. J.: Glutathione transferases: a structural perspective. Drug Met. Rev., *43*(2), 138–151 (2011).

33. CVILINK V., LAMKA J., SKALKOVA L.: Xenobiotic metabolizing enzymes and metabolism of anthelminthics in helminths. Drug Met. Rev., *41*(1), 8–26 (2009).

34. AHMAD R., SRIVASTAVA A.: Inhibition of Filarial Glutathione-S-transferase by various classes of compounds and their evaluation as novel antifilarial agents. Helminth., *45*(3), 114–120 (2008).

35. SCARELLA S., et. al.: Expression differential of microsomal and cytosolic S-transferases in *Fasciola hepatica* resistant triclabendazole. Mol. Biochem. Parasitol., *181*(1), 37–39 (2012).

36. MEECH R., et al.: The glycosidation of xenobiotics and endogenous compounds: Versatility and redundancy in the UDP glycosyltransferase superfamily. Pharmacol. Therap., *134*(2), 200–218 (2012).

37. ROWLAND A., MINERS J.O., MACKENZIE P.I.: The UDP-glucuronosyltransferases: Their role in drug metabolism and detoxification. Intern. Journ. of Bioch. Cell Biol., *45*(6), 1121–1132 (2013).

38. LAING S.T., et al.: Characterization of the xenobiotic response of *Caenorhabditis elegans* to the anthelmintic drug albendazole and the identification of novel drug glucoside metabolites. Bioch. Journ., *432*(3), 505–514 (2010).

39. VANWYK J.A., GERBER H.M., GROENEVELT H.T.: A technique for the recovery of nematodes from ruminants by migration from gastro-intestinal ingesta gelled in agar: large-scale application. Onderst. Journ. Vet. Res., *47*(3), 147–158 (1980).

40. [online] (16.1.2016) available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2291683/

41. SCHMITTGEN T.D., KENNETH J.L.: Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc., *3*(6), 1101–1108 (2008).

42. LECOVÁ L., et al.: Reliable reference gene selection for quantitative real time PCR in *Haemonchus contortus*. Mol. Biochem. Parasitol., *201*(2), 123–127 (2015).

43. TAMURA K., et al.: MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol., *30*(12), 2725–2729 (2013).

44. HALL B.G., et al.: Building phylogenetic trees from molecular data with MEGA. Mol. Biol. Evol., *30*(5), 1229–1235 (2013).

45. O´HANLON G. M., et. al.: Metabolism and fate of ecdysteroids in the nematodes *Ascaris suum* and *Parascaris equorum*. Mol. Biochem. Parasitol., *47*(2), 179–187 (1991).