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Differential expression of β-tubulin isotypes in different life stages of *Parascaris* spp after exposure to thiabendazole

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ABSTRACT

Anthelmintic resistance (AR) to macrocyclic lactones (ML) has been described in *Parascaris* of horses world-wide. In contrast, benzimidazoles (BZ) are still effective, although reduced efficacy to this drug class was recently reported. The mode of action of BZ is binding to β-tubulin, which prevents polymerisation of microtubules. In this study, β-tubulin gene expression of isotypes 1 and 2 was investigated at seven time points (0, 6, 24, 72, 96 and 120 h) during embryogenesis and in adult worms. In addition, an *in ovo* larval developmental test was developed to study β-tubulin gene expression of both isotypes in parasacaris eggs after exposure to different concentrations of thiabendazole (TBZ) for five days at 25 °C. A strong pattern of differential expression of β-tubulin and isotype 1 was observed in all stages, while isotype 2 expression was mainly found at an early phase of the embryogenesis. For isotype 1, a 5-fold increase was observed during the first 48 h, but gene expression gradually decreased after 72, 96 and 120 h. Isotype 2 was only expressed during the first 24 h, followed by a 130-fold decrease at (time points) 72, 96 and 120 h. The *in ovo* larval developmental test, in which we exposed initially unembryonated eggs to increased concentrations of TBZ, did affect isotype 1 gene expression but not isotype 2. This assumes that each isotype has specific functions in different life stages. This is in agreement with the 'multi-tubulin' hypothesis, which states that different tubulin isotypes are required for specialised microtubule functions. Isotype 1 is the most likely drug target for BZs, as isotype 2 was only expressed at very low levels later in development. Increasing concentrations of TBZ altered β-tubulin isotype 1 gene expression after exposure of the eggs for five days, but this was not seen for isotype 2.

1. Introduction

For many years the equine roundworm has been named *Parascaris equorum* in veterinary investigations. However, a recent study from U.S [1] observed that the dominant species infecting equines is most likely *Parascaris univalens* and not *P. equorum*. These species are identical apart from the fact that *P. univalens* has two chromosomes, and not four as in *P. equorum* [1]. However, concurrently we have shown with genetic tools that the global *Parascaris* spp population, including worms from the U.S later identified as *P. univalens*, is essentially homogeneous [2]. Thus, equine roundworms previously described as *P. equorum* in Sweden and in several other countries have most likely been misidentified and are probably identical to *P. univalens*.

Parascaris spp are an intestinal parasites of foals and yearlings where it is important due to its high prevalence and severe pathogenicity [3]. Since the advent of modern anthelmintics, *Parascaris* infection has been controlled by the strategic use of dewormers, including macrocyclic lactones (ML), benzimidazoles (BZ) and tetrahydropyrimidines (TH) [4]. Resistance to ML in *Parascaris* populations is spreading world-wide [5]. Furthermore, reduced efficacy of the TH pyrantel pamoate (PYR) has been reported in the U.S. and Australia [6–8]. Recently, an alarming report from Australia indicated for the first time treatment failure of fenbendazole (FBZ) on two out of four farms [8]. In most countries FBZ is the drug of choice for treatment of *Parascaris* infection, despite evidence suggesting that selection for anthelmintic resistance is associated with over-reliance on a single drug class (in this

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case BZ), especially in a dose-limiting parasite such as *Parascaris* spp [9].

Microtubules, the heterodimer of α-and β-subunits, are major constituents of the cytoskeleton and thus abundant in eukaryotic cells. They participate in a number of functions including cell division, intracellular transport of organelles, vesicles and other macromolecules. Most eukaryotic cells express multiple isotypes of related β-tubulins, which are encoded by different genes. The DNA sequences of the β-tubulin genes in several helminths have been extensively studied and it has been shown that BZ binds to β-tubulin and thereby prevents polymerisation of the α- and β-subunits to form heterodimers [10]. Structural change in the β-tubulin molecule is partly associated with single nucleotide polymorphisms (SNP), which alter the amino acid sequences, resulting in reduced binding of BZ and thus absence of an anthelmintic effect [11]. Recently, β-tubulin genes of isotypes 1 and 2 of *Parascaris* were sequenced and phylogenetic analysis of currently known isotypes has shown that Nematoda has more diversity of β-tubulin genes and that they are arranged in a more complex pattern than in Vertebrata [12]. There is a gap in knowledge about β-tubulin isotype genes in ascarids, especially concerning gene expression and function at different life stages.

The 'multi-tubulin' hypothesis allows functional expression of specific isotypes of specialised microtubules in different cells, tissues and/or stages during the life cycle. For example, the function of β-tubulin isotypes in axonemes of insects suggests that the tubulin structure can direct the architecture and supramolecular organization of microtubules through interactions with extrinsic proteins [13]. In other worms such as the free-living nematode *Caenorhabditis elegans* [14] and the trematode *Fasciola hepatica* [15], it has been shown that β-tubulin isotypes are differently expressed during life cycle stages, resulting in specialisation in microtubule function in diverse tissues. Little is known about expression of these genes in *Parascaris* spp. In this study we investigated the β-tubulin gene expression of isotypes 1 and 2 in life stages of *Parascaris* spp and at different time points during worm development. In addition, an *in ovo* larval developmental test was developed to evaluate the effect of increasing concentrations of thiabendazole (TBZ), a model substance of BZ, on β-tubulin gene expression.

2. Materials and Methods

2.1. Parasite material

Parascaris spp. eggs were collected from three horse farms previously included in a fecal egg count reduction test [16]. At that time there was no sign of reduced efficacy of treatment with FBZ at a dose of 7.5 mg per kg body weight. The samples were first soaked in water, then isolated from fecal matter by sieving with different mesh sizes (150–1000 μm) and finally collected in an 80 μm sieve. The eggs were further cleaned by centrifugal flotation in saturated salt, cleaned with tap water and stored for 3 weeks until use in ventilated cell culture flasks at 7 °C.

2.2. RNA extraction

Total RNA was isolated from unembryonated eggs, eggs with second/third stage larvae (L2/3) and the anterior part of adult females using Macherey-Nagel NucleoSpin RNA II columns with DNAse I. Washing buffers and rDNAse buffer were used to remove contaminating DNA. The integrity of the RNA was verified visually using 28s rRNA and 18S rRNA on a 1% agarose gel containing 18% formaldehyde at 60 V. RNA quantification of the pools was performed according to the Quant-iT RiboGreen protocol (Invitrogen) using a VICTOR^{2TM} 1420 Multilabel counter (software version 2.0). RNA was kept frozen at −80 °C for long-term storage.

2.3. Analysis of β-tubulin gene expression in different life stages

RNA was extracted from pools ($n = 4$) of ≈100 unembryonated eggs or from pools ($n = 4$) of ≈100 eggs with a developed L2/3. Adult worms ($n = 4$) naturally expelled from a naturally infected horse were immediately transported on ice to our laboratory (SLU, Uppsala) and stored at −80 °C.

2.4. β-tubulin gene expression during embryogenesis

Pools of ≈100 initially unembryonated eggs ($n = 4$) in 1 ml of tap water were incubated in the wells of a 48-well microtiter plate at 25 °C. Samples were collected at seven time points: at 0 h, 6 h and thereafter every 24 h up to 120 h. To track development, the thick brown outer coat of the eggs was washed off with Milton-2 (NaClO 2% (v/v) in NaCl 16.5%) solution at 1500 rpm for 3 min and the eggs were then carefully rinsed with cold water prior to analysis. Developmental stage of the eggs at sample collection was determined under microscope and photodocumented with a Nikon eclipse E600 camera (Nikon ACT-1 version 2.7) at 200× magnification. After five days (120 h), L2/3 had developed inside the shell. RNA was extracted (see section on RNA extraction) for comparison of gene expression at different time points during embryogenesis. Isolates collected from three different farms were analysed. From each time point, three biological replicates were prepared and analysed.

2.5. β-tubulin gene expression in L2/3 after exposure to TBZ

An *in ovo* larval developmental test (LDT) was developed for evaluation of the effect of TBZ exposure on expression of β-tubulin isotypes 1 and 2. Initially unembryonated eggs were exposed to increasing concentration of TBZ from 0–497 μM dissolved in 0.5% dimethyl sulphoxide (DMSO). TBZ-exposed eggs were incubated in a 48-well microtitre plate at 25 °C for 120 h (5 days). Unexposed eggs in water and in 0.5% DMSO served as controls. The LDT was repeated three times, with each TBZ concentration run in triplicate. After exposure, ≈100 eggs per well were counted under the microscope and the percentage of developed eggs were calculated. The eggs were classified into: eggs with L2/3 and eggs where larval development was inhibited. All eggs that did not contain larvae were considered dead. WinNonlin 4.0.1 was used to process the dose–response data. Data was first plotted to receive an overview and EC50 values were then calculated using a four parameter non-linear regression model based on the equation:

$$E = E_0 + \frac{E_{max} \times C^n}{EC_{50}^n + C^n}$$

The four analyzed parameters were E0, Emax, EC50 and n (Hills slope). After exposure to TBZ, the eggs were washed and RNA was extracted from pools of each replicate as described above.

2.6. Primer design and real-time PCR amplification

β-tubulin isotype-specific primers, isotype 1 (GenBank accession number JN034256) and 2 (GenBank accession number KC713798), were designed. Primer sequences for isotype 1 were: F 5'-AGATTCGTGAAGAATATCCC-3'; R 5'-TGGGATTTGTAAGCTTCAGAG-3' and for isotype 2: F 5'-AGATTCGTGAGGAGTATCCA-3'; R 5'-CTGGATTAGACAACTTCAGCG-3'. These primers were designed to amplify PCR products covering SNP in codon positions 167, 198

and 200. An intron is located between codon positions 167 and 198, which means that contamination of genomic DNA will give a product of about 500 bp, whereas the amplicon generated from RNA was about 201 bp.

Real-time quantitative polymerase chain reaction (qPCR) was performed to compare gene expression levels of β-tubulin isotype 1 and isotype 2. To calculate the real-time PCR efficiency (E), a four-fold dilution series was used for each isotype investigated, according to the equation $= 10\left[-1/slope\right]$ [17,18], where quantification cycle (C_q) number is plotted against logarithm RNA input. Cleaned PCR products (QIAquick PCR Purification Kit, Qiagen) of isotype 1 and isotype 2 with known DNA concentration were serially diluted to give a standard curve with five points ranging from 80 to 800 000 copies/μl. The copy numbers were calculated using the equation: Number of copies = $(A \times 6.022 \times 10^{23})/(B \times 10^9 \times 650)$, where A is template concentration (ng/μl) and B is amplicon length (bp). The data were analysed using Rotor-Gene 6 software. Onestep QuantiTect® PCR reactions with SYBR®green Master Mix (Qiagene) were performed according to the manufacturer's protocol using 1 ng of RNA as a template in total reaction volumes of 25 μl with 12.5 μl 2x Master Mix, 0.25 μl Quantitect RT enzyme, 0.5 μM each of forward and reverse primer specific for isotype 1 or 0.25 μM each of forward and reverse primers specific for isotype 2. Real-time PCR samples were run in a Rotor-Gene 3000 (Corbett) and the data analysed using Rotor-Gene 6.1.9 software. All amplification runs were performed under the following conditions: 50 °C for 30 min, 95 °C for 15 min, followed by 45 cycles of 94 °C for 15 s, optimal annealing temperature for 30 s and 72 °C for 30 s. Melt curve analyses were performed at the end of each PCR reaction to ensure specificity of the primers. Three technical replicates were run in the PCR.

2.7. Statistical analysis

Differences in gene expression between isotypes were tested with pairwise Wilcoxon test for each life stage whereas those in gene expression for both isotype 1 and isotype 2 with respect to life stages by Kruskal–Wallis test, since the data displayed skewness and heteroscedastic variation. Mixed linear models were used to analyze gene expression with respect to time and exposure, with technical replicate as a random effect. The response variable was log-transformed to reduce skewness and achieve better normality in the residuals. Tukey's method was used for pair-wise comparisons between time points and exposures based on the mixed linear models. All analyses were performed using R software (version 3.1.2) [19]. The significance level was set to $p < 0.05$.

3. Results

3.1. Data quality control of real-time PCR

The calibration curves of the transcript investigated showed high PCR efficiency, 1.95 for isotype 1 and 1.97 for isotype 2, with slope −4.4 and −4.3, respectively. The correlation coefficient (r^2) of the standard curve was 99.8% for isotype 1 and 99.7% for isotype 2.

3.2. β-tubulin gene expression in eggs, L2/3 and adult worms

Isotype 1 was significant higher expressed ($p = 0.0008$) at all three life stages compared to isotype 2. Significantly differential expression at selected life stages was observed for isotype 1 ($p = 0.023$) and isotype 2 ($p = 0.018$) (Fig. 1 A/B). Both isotypes were expressed at a similar level in unembryonated eggs (120 000 copies/ng RNA for isotype 1 and 87 000 copies/ng RNA for isotype 2), but in L2/3 and adults each isotype had a unique expression profile. There was a fourfold decrease in isotype 1 gene expression from the unembryonated stage to L2/3, and thereafter a twofold increase in expression level from L2/3 stage to adult worm (Fig. 1A). Moreover, expression of isotype 1 was also significantly higher than in isotype 2, both in eggs with larvae and in adult worms. However, high variation in expression level of isotype 1 was seen in the L2/3 stage (Fig. 1A). The expression profile for isotype 2 was clearly different from than for isotype 1, as there was a 250-fold decrease after embryogenesis of the eggs and thereafter another 80-fold decrease in the adult worms. In adults, the gene expression level of isotype 2 was very low, with only ≈10 copies/ng RNA (Fig. 1B).

3.3. β-tubulin gene expression during embryogenesis

Gene expression of β-tubulin isotype 1 and isotype 2 was observed on a time scale at 0, 6, 24, 48, 72, 96 and 120 h. The stages of embryogenesis at each time point for sample collection are shown in Fig. 2(A–L). At 0 h the developmental distribution was one-cell stage (88%) and two-cell stage (11%). After 6 h 84% of the eggs were at two-cell stage, 8% of the eggs were at three-cell stage and 4% of the eggs had exceeded the three cell stage. At 24 h 96% of the eggs had reached a morula stage with small blastomeres. At 48 h the cells becomes smaller and a line of separation appears (78%), some eggs had start to reach the "tad pole" stage (17%). After 72 h three developmental stages observed were; "short" tad pole (51%), long tad pole (41%), short larvae (2%). At 96 h two types of larvae were observed, one short (46%) and one long (48%). After 120 h 93% of the eggs were developed into larvae (L2/3) that had further increased in length. Unembryonated eggs, considered as dead, were 1% at 0 h, 4% at 6 h and 24 h, 5% at 48 h, 6% at 72 h and 96 h and 7% at 120 h at the different times point.

There were differing expression profiles of the two isotypes during embryogenesis apart from time 0, when both genes were evenly expressed, i.e., 120 000 copies/ng RNA for isotype 1 and 87 000 copies/ng RNA for isotype 2 (Fig. 3). For isotype 1 there was a fourfold increase in gene expression, from 140 000 copies/ng RNA to 530 00 copies/ng RNA, during the first 48 h of embryogenesis (Fig. 3). However, after 48 h a six-fold decline in gene expression was observed, i.e., to 90 000 copies/ng RNA at time point 120 h. Pairwise comparisons using Tukey's method showed a statistically significant increase in gene expression from time 0 to 6 and 48 h, but no significant difference was noted between time 0 and 24, 72, 96 and 120 h. In addition, gene expression of isotype 1 at 96 and 120 h of embryogenesis was significantly decreased from that at time points 6, 24, 48 and 72 h.

For isotype 2, a different gene expression profile than for isotype 1 was observed (Fig. 3B). For isotype 2, gene expression was significantly higher at time points 0, 6 and 24 h, with expression levels of about 90 000 copies/ng RNA. However, between 24 and 48 h there was a six fold decline in gene expression of isotype 2 and then it further decreased, by about three-fold, between 72 to 96 h to reach a final level of about 500 copies/ng RNA at 120 h. Interestingly, gene expression of isotype 2 decreased 130-fold during the first 24 h to the last time point at 120 h.

3.4. Dose–response relationship of TBZ and β-tubulin gene expression in L2/3 after exposure

An in ovo larval developmental test was performed with unembryoted eggs, which were exposed to gradually increasing concentrations of TBZ in the range 0–497 μM. According to our study design egg development at each concentration was investigate in biological triplicates and compared to negative (water) control samples ($n \approx 100$ eggs/well at each concentration). A dose–response relationship of TBZ could then be established (Fig. 4). At TBZ concentration of 55 μM 50% of the larvae were killed (EC_{50} of 55 μM) and maximum mortality (98%) was reach at 497 μM. The mean mortality in unexposed eggs in water and in

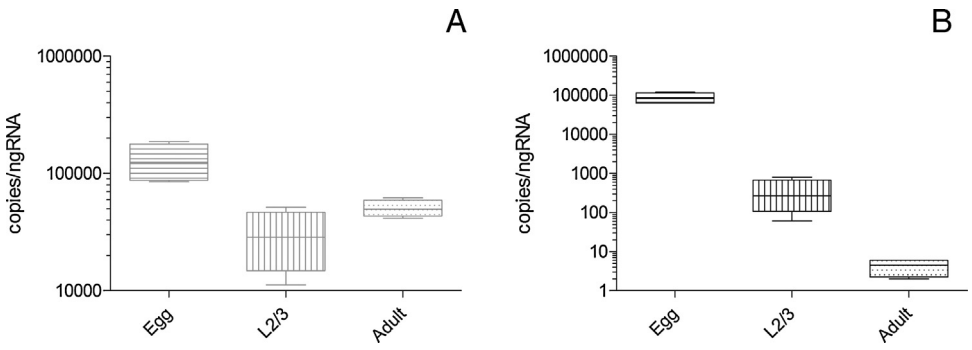

Fig. 1. β-tubulin gene expression (copies/ng RNA) of (A) isotope 1 and (B) isotope 2 at different life stages of *Parascaris* spp, visualised in a boxplot with a logarithmic y-axis. Gene expression was significantly different in unembryonated, L2/3 and adult worms in both cases.

0.5% DMSO was 9%. Gene expression of β-tubulin isotypes 1 and 2 was also evaluated after exposure of TBZ (0–397 μM) (Fig. 5). Gene expression of isotype 1 was significantly higher after exposure to 62, 150, 200 and 397 μM TBZ compared with the untreated negative controls. No significant differences were observed between the different TBZ concentrations (Fig. 5). Furthermore, TBZ exposure did not affect gene expression of isotype 2 (Fig. 5).

4. Discussion

The β-tubulin gene family is the binding site for BZ anthelmintics and it has been shown that point mutations in some of these genes of the target organism plays an essential role in the mechanism of developing resistance to this drug class [11]. Phylogenetic analysis of a total of 87 β-tubulin sequences has revealed great diversity among the nematode β-tubulin genes [12]. Few β-tubulin sequences have been identified in superfamily Ascaridoidea and the only two sequences that are known to date are derived from *Parascaris* spp. There are probably more isotypes to explore in this species after the genome sequencing project has been completed (www.sanger.ac.uk). It can be assumed that Ascaridoidea have a large number of isotypes in the β-tubulin family, since nine genes have been identified in the genome of *Ascaris suum*, a related roundworm of pigs [20]. There is a lack of knowledge about β-tubulin isotype genes in roundworms in general and about how different isotype genes are expressed and function at different life stages in particular.

This study shows that there is a pattern of differential expression of two β-tubulin gene isotypes during worm development. Initially, both isotypes were evenly expressed, while isotype 1 was expressed at high levels during all life stages. Isotype 2 was only expressed at high levels in eggs, and then decreased about 150-fold to very low levels during embryogenesis, leaving only a few copies/ng RNA in the adults. A similar pattern of differential expression of β-tubulin isotypes has been observed in the trematode *F. hepatica* [15]. However, the isotype expression pattern found in *F. hepatica* showed both differences and similarities to our observations in *Parascaris*. The β-tubulin in the common liver fluke *F. hepatica* consists of six isotypes that are all expressed in the adults. Isotype 1 is the dominant isotype of *F. hepatica*, as we observed for *Parascaris* in this study. In contrast, isotype 1 of *F. hepatica* is not expressed in the immature stages, whereas isotypes 2, 3 and 4 are expressed in all life stages [15]. Following assembly and annotation of the draft genome, four isotypes of the β-tubulin gene family have been identified in the sheep nematode *Haemonchus contortus* [21]. Thus two novel genes, namely isotype 3 (*Hco-tbb-iso-3*) and 4 (*Hco-*

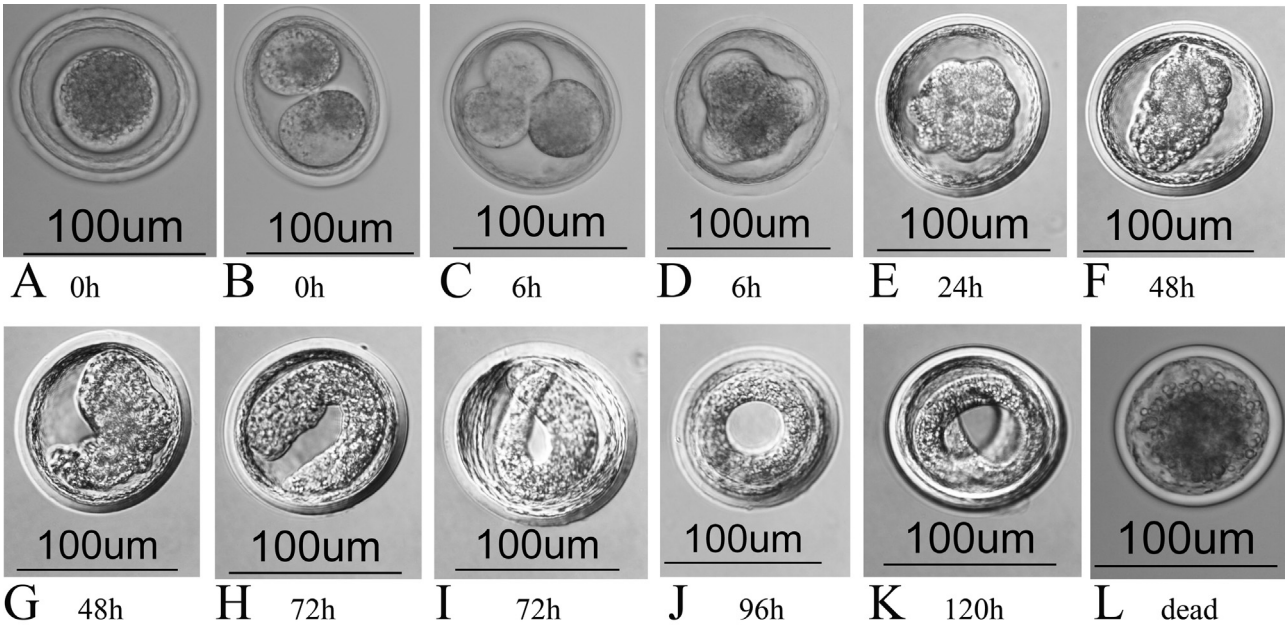

Fig. 2. Documentation of the different developmental phases of embryogenesis at the different time points (0, 6, 24, 48, 72, 96 and 120 h) used for investigation of β-tubulin gene expression of isotypes 1 and 2. Unembryonated eggs were washed in Milton-2 solution to enable visualisation. Image magnification 200×.

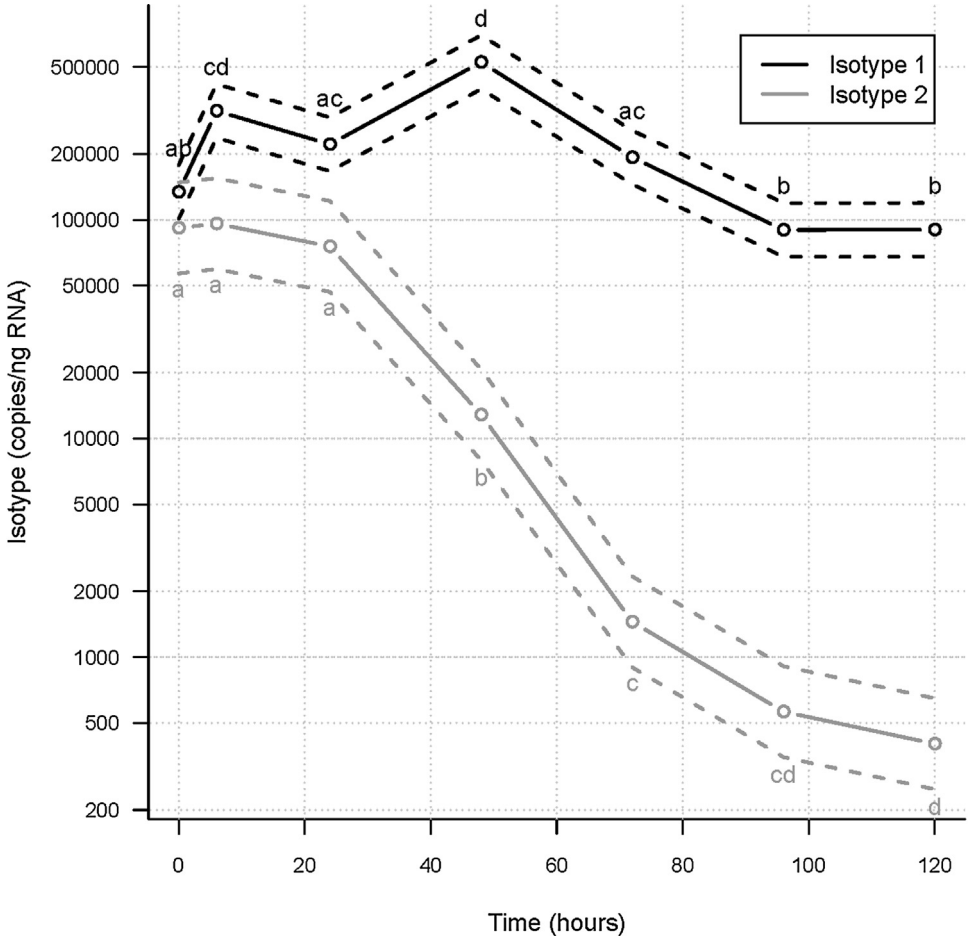

Fig. 3. β-tubulin gene expression (copies/ng RNA) of (A) isotype 1 and (B) isotype 2 at different time points during embryogenesis. Unfilled circles denote the time points for investigation of gene expression, filled lines represent the estimated mean of gene expression level and broken lines denote pointwise 95% confidence limit. At time point 0 h were 88% at one-cell stage, 11% at two-cell stage and 1% unembryonated eggs (considered as dead). At time point 6 h were 84% at two-cell stage, 8% at three-cell stage, 4% of the eggs had exceeded the three cell stage and 4% were considered as dead. At 24 h were 96% at morula stage with small blastomeres and 4% were considered as dead. At 48 h had line of separation appeared in 77% of the eggs, 17% were at the "tad pole" stage and 6% were considered as dead. At 72 h were 51% a "short" tad pole, 41% long tad pole, 2%short larvae and 6% were considered as dead. At 96 h were 46% a short larvae and 48% long larvae and 6% were considered as dead. At 120 h were 93% a larvae (L2/3) and 6% were considered as dead.

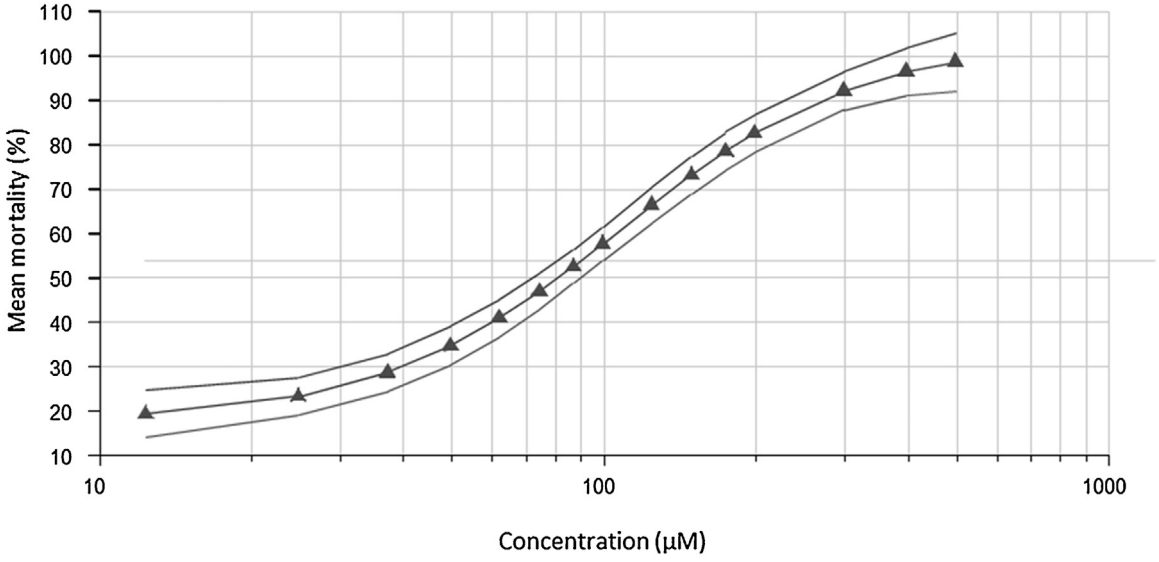

Fig. 4. Dose–response curve for TBZ (μM) obtained in the *in ovo* larval developmental test. The filled line with triangle represent the estimated mean ($n = 4$) of gene expression level and the two lines above and under denote pointwise 95% confidence limit.

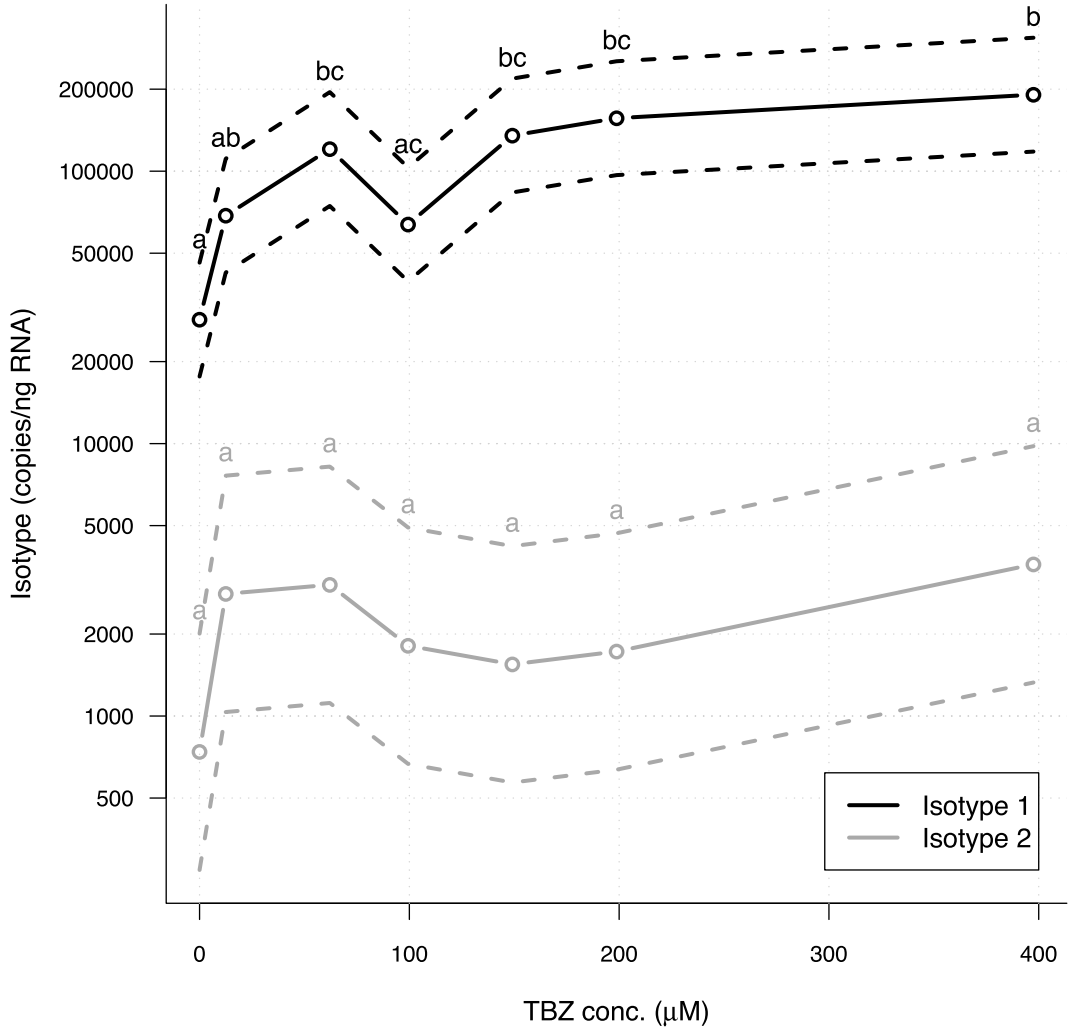

Fig. 5. β-tubulin gene expression (copies/ng RNA) of (A) isotype 1 and (B) isotype 2 (B) in L2/3 in unembryonated eggs exposed to increasing concentration of TBZ during embryogenesis. Gene expression was investigated after five days of exposure. Unfilled circles denote the time points for investigation of gene expression, filled lines represent the estimated mean of gene expression level and broken lines denote pointwise 95% confidence limit. Different letters indicate timepoints where signficant differences were seen. Timepoints that does not share any letter was significantly different in the pairwise Tukey comparisons.

tbb-iso-4), have recently been added to the *H. contortus* β-tubulin gene family. Relative expression of β-tubulin genes in adult *H. contortus* based on number of Illumina reads has shown that isotypes 1 and 2 are highly expressed, with 8909 and 4258 reads/mapped kb, respectively, whereas isotypes 3 and 4 are expressed with only 5.3 and 7.6 reads/mapped kb [21]. High expression of isotypes 1 and 2 in adult *H. contortus* is consistent with the observation in *F. hepatica* [15]. Also in the model nematode *C. elegans*, both isotypes 1 and 2 are highly expressed in the adult worms [22,23]. A slightly different pattern was seen here in adult *Parascaris*, with high expression of isotype 1 but very low expression of isotype 2. This indicates that these two β-tubulin isotypes are likely to play major roles in microtubule structure and function throughout the life cycle of *H. contortus, F. hepatica* and *C. elegans*, while only isotype 1 seems to matter later in life in *Parascaris*. However, again it needs to be emphasised that more isotypes will probably be explored and that there seems to be differences between isotypes in different helminth parasites.

Differential gene expression was observed during embryogenesis of *Parascaris* spp in this study. Both isotypes were expressed at the first time point (0 h) at cell division 1–2. The expression of isotype 1 increased in the beginning of embryogenesis, with a peak at 48 h. Thereafter it decreased to almost the same level as observed at 0 h. For isotype 2, gene expression was unchanged during the first 24 h but was subsequently almost turned off to very low expression levels. In a previous study [14] comparing expression of two β-tubulin isoforms, isotype 1 (*tbb-1*) and isotype 2 (*tbb-2*), during early embryo development of *C. elegans*, it was found that isotype 2 was required for centrosome stabilisation during the anaphase of the first cell division, suggesting that isotype 2 is specifically required for this purpose. Another study [22] indicated that isotype 2 is widely expressed throughout embryogenesis and even post-embryonically in both immature and adult *C. elegans*. However, neither of these genes seems to be essential, as deletion of each isotype has little effect on the viability of *C. elegans* [23,24]. To our knowledge, no previous study has compared gene expression of β-tubulin isotypes at embryogenesis timescale in other nematodes of veterinary importance. The differential expression of these two isotypes implies that they have specific functions in different life cycle stages. These specific functions are unknown in *Parascaris* spp. but it seems most likely that isotype 2 plays a central role in early stages of the life cycle and is similar to *tbb-2* in *C. elegans*.

The *in ovo* larval developmental test, in which we exposed initially unembryonated eggs to increased concentrations of TBZ, did affect isotype 1 gene expression after 62, 150, 200 and 397 μM TBZ exposure but not isotype 2. However, research into BZ resistance

has mainly focused on isotype 1, although it is still unknown which of the isotypes serves as the target for BZ in *Parascaris* spp. Our results indicate that it is most likely isotype 1, as isotype 2 was expressed only at very low levels in eggs with larvae and in the adult worms. There is clearly a need for further studies into the identity of currently unknown tubulin genes and isotypes but, more importantly, into their biological role.

5. Conclusions

Research on nematode β-tubulin families is warranted, as they are the target for BZs. The full β-tubulin family still needs to be described for many nematodes, but this study clearly shows that there is a pattern of differential expression of β-tubulin isotypes 1 and 2 in the life cycle stages of *Parascaris* spp, thereby highlighting the importance of further insights into microtubule diversity. In conclusion, the differential β-tubulin gene expression of isotypes in different life stages of *Parascaris* spp. observed in this study supports the hypothesis that different tubulin isotypes are required for specialised microtubule functions.

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