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Research paper

Species composition of larvae cultured after anthelmintic treatment indicates reduced moxidectin susceptibility of immature *Cylicocyclus* species in horses

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ABSTRACT

For the control of cyathostomins in horses, the macrocyclic lactones (MLs), moxidectin (MOX) and ivermectin (IVM) are the most commonly used anthelmintics. However, reduced activity, observed as shortening of the egg reappearance period (ERP) has been described. Shortening of the ERP may be caused by a decreased susceptibility of immature worms for MLs. Alternatively, immature worms may develop faster into egg producing adults as a result of repeated ML treatments. The species composition of the larval cultures obtained shortly after ML and pyrantel (PYR) treatment can confirm the hypothesis of decreased ML susceptibility, as this is often class-specific, whereas faster development would also occur after treatment with anthelmintics with a different mode of action. From 3 farms with a known history of shortened ERP, 8 horses per farm were selected and divided into 2 groups. The MOX-PYR-MOX group was treated twice with MOX (day 0 and 126) and once with PYR (day 84) and the IVM-PYR-IVM group was treated twice with IVM (day 0 and 98) and once with PYR (day 56). Cultured infective larvae (L3s) were counted and differentiated with the reverse line blot on pooled samples. Per cyathostomin species, the number of larvae per gram was calculated. The efficacy of all ML treatments was 100% and a shortened ERP was found on all 3 farms. The species composition of the larval cultures after ML treatment did not differ significantly from that after PYR treatment in the IVM-PYR-IVM group, but it did differ in the MOX-PYR-MOX group. The larval cultures obtained after MOX treatment consisted mostly of *Cylicocyclus nassatus*, while after PYR treatment *Cylicostephanus longibursatus* was the most abundant species. In the cultures from 42 days after MOX treatment 6 cyathostomin species from 3 genera were found on the farm with the lowest activity (farm 1), while on the farm with the highest activity (farm 3) only 3 species from one genus were found in the same number of examined L3s. The high numbers of L3s of *Cylicocyclus* species 42 days after MOX treatment and the low numbers 42 days after PYR treatment can be explained by reduced susceptibility of the immature worms to MOX, but not by a faster development. In conclusion, shortening of the ERP following MOX treatment is most likely a process in which an increasing number of immature worms from an increasing number of species is becoming less susceptible to the active compound.

1. Introduction

Cyathostomins are the most prevalent nematodes in equids worldwide. Macrocyclic lactones (MLs) are currently the most used anthelmintics, of which ivermectin (IVM) and moxidectin (MOX) are registered for the control of cyathostomins in horses (Reinemeyer and Rohrbach, 1990; Nielsen et al., 2014).

The egg reappearance period (ERP) is the time between treatment and the reappearance of eggs in the feces. The ERP for IVM and MOX at the time of introduction has been reported to be 8–9 weeks (Borgsteede et al., 1993; Parry et al., 1993; Boersema et al., 1996, 1998) and 12–25 weeks (Demeulenaere et al., 1997; DiPietro et al., 1997; Boersema et al., 1998), respectively. However, worldwide reduced ERP has been reported both for IVM and MOX (von

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Samson-Himmelstjerna et al., 2007; Lyons et al., 2011b; Molento et al., 2012; Geurden et al., 2014).

Several mechanisms can explain the shortening of the ERP. After ML treatment, the ERP is often shorter than the shortest pre-patent period (PPP) of 57 days for cyathostomins (Lyons et al., 2011a) and shortening of the ERP has also been observed in housed horses (van Doorn et al., 2014). This indicates that larvae already present in the host at the time of treatment must be responsible for the shortening of the ERP. Therefore, a reduced ERP is regarded as an early sign of development of resistance (Lyons et al., 2009). A shift within the cyathostomin population towards species with a shorter development time is not a likely cause, as horses with and without a shortened ERP showed a similar species composition before treatment (van Doorn et al., 2014). Furthermore, 20 years of ML treatment did not result in a change in the cyathostomin species composition within the same herds (Chapman et al., 2002). Therefore, shortening of the ERP is most likely caused by decreased susceptibility of immature stages to MLs or by a faster pace of development of immature stages into egg producing adults. In a critical test described by Lyons et al. (2010), the efficacy of MOX against luminal larval stages in one horse was only 82%, while the efficacy against adult cyathostomins was still 100% in the same horse. Critical tests performed by the same group demonstrated a effectivity of IVM against L4s of 36–80% (Lyons et al., 2009) and 3 years later the effectivity against L4s has dropped to only 0–16% (Lyons and Tolliver, 2013). The effectivity against adults was in both cases higher than 99%. The authors suggested that this was caused by reduced efficacy of MLs against the immature luminal stages, but they could not exclude the possibility of a fast development of mucosal stages into luminal stages in the 6 days between ML treatment and necropsy.

Experimentally, it may be possible to distinguish faster development from decreased susceptibility for MLs because a faster pace of development from L4 into egg laying adult should also occur after treatment with an anthelmintic outside the ML group e.g. pyrantel (PYR). A faster development can be advantageous for the parasite. It increases the chance to reproduce in between 2 subsequent treatments. Plasticity of the development time in nematodes has been demonstrated before, for example in *Anguillicola crassus*, a parasitic nematode of the Japanese and European eel (Weclawski et al., 2013).

A shortened ERP does not necessarily involve all present cyathostomin species. One species may be more prone to develop resistance or may have a higher level of plasticity than another. Although differentiation of the eggs or cultured L3s by morphological means is not possible, they can be differentiated by molecular methods (Traversa et al., 2007; van Doorn et al., 2010, 2014; Cwiklinski et al., 2012). These studies used the reverse line blot (RLB), a method based on the hybridization of species specific probes with an amplified fragment of the intergenic spacer (IGS) region. Individual L3s can be differentiated into 21 of the most common cyathostomin species (Cwiklinski et al., 2012). Recently, a RLB on pooled L3s was described for the differentiation of larval cultures allowing an estimation of the larvae per gram (LPG) per cyathostomin species (Kooyman et al., 2016).

The aims of this study were to determine which species are involved in the shortening of the ERP and to determine the cause of shortening of the ERP: reduced susceptibility to MLs or a faster development of the species involved.

2. Material and methods

2.1. Farms and horses

This study was designed as a field study in the Netherlands with horses naturally infected with cyathostomins and lasted from February to September 2013. Three farms were selected based on the presence of a shortened ERP in 2012 after ML treatment (Geurden et al., 2014). Farms NEO1, NEO2 and NEO3 from that study correspond to farm 1–3, respectively in the present study. The 3 farms practiced regular deworming (at least twice a year). On each farm, 8 horses (2–3 years old) with ≥150 eggs per gram (EPG) were randomly allocated to one of the two treatment groups. The IVM-PYR-IVM group was treated with IVM (Eqvalan® oral paste, Merial at 0.2 mg/kg bodyweight) at day 0 and day 98 and in between (day 56) with PYR (Strongid-P® paste, Pfizer at 19 mg/kg bodyweight). The MOX-PYR-MOX group was treated with MOX (Equest® oral gel, Pfizer at 0.4 mg/kg bodyweight) at day 0 and day 126 and in between (day 84) with PYR. The PYR treatments in between the ML treatments were timed according to the regular ERP of the respective ML products. Before each treatment the bodyweight was estimated with a girth tape. In order to minimize under-dosing, the dose was based on 110% of the estimated bodyweight. Fecal samples were collected at day −5 to day −2 from both groups and at day 14, 42, 56, 70, 98, 112, 140, 154 from the IVM-PYR-IVM group and at day 14, 42, 56, 84, 98, 126, 140, 168, 182 and 210 from the MOX-PYR-MOX group. All fecal samples were used for egg counts and larval cultures and all larval cultures were counted and differentiated.

2.2. Egg counts and larval cultures

Egg counts and culturing of L3s were performed as described (van Doorn et al., 2014) with a detection limit of 25 EPG and 0.4 larvae per g (LPG). The recovery of the L3s (100% × LPG/EPG) was calculated for all samples with positive EPG and positive LPG. The mean recovery of a group is the mean of the recoveries of all individual samples from that group.

2.3. Drugs activity and egg reappearance period (ERP)

There are no formal guidelines for obtaining one clear estimate for the activity of anthelmintics by fecal egg count reduction test (FECRT) in horses (Vidyashankar et al., 2012). For the present study the activity of a drug for the herd was determined by taking the mean of the arcsine transformed activity of the individual horses (Pook et al., 2002; Relf et al., 2014). For small groups of horses, it is an advantage that no control group is needed when using the individual activity. The activity at day 14 after treatment is defined as the efficacy (EFF) and determines whether there is resistance or not. A mean EFF–2 SE ≤95% at day 14 after ML treatment was defined as resistance. ERP is defined here as the period between treatment and the moment that the EPG increased to ≥10% of the pre-treatment EPG (Borgsteede et al., 1993; von Samson-Himmelstjerna et al., 2007; van Doorn et al., 2014).

2.4. Polymerase chain reaction (PCR) and reverse line blot (RLB)

PCR and RLB on pooled L3s as well as the transformation of RLB score into a frequency and a LPG per cyathostomin species was performed as described (Kooyman et al., 2016). In brief, for the differentiation of one culture, 4 pools with 10 L3s each were subjected to PCR and RLB with 19 species specific probes allowing the differentiation into 17 cyathostomin species belonging to 7 genera. For convenience, the genera were abbreviated to 3 or 4 letters, instead of the official 1 letter. Differentiated species were: *Cylicocyclus (Cyc. ashworthi, Cyc. insigne, Cyc. nassatus, Cyc. leptostomum), Cylicostephanus (Cys. calicatus, Cys. goldi, Cys. longibursatus, Cys. minutus var. A, Cys. minutus var. B), Cyathostomum (Cya. catinatum, Cya. pateratum, Cya. tetracanthum), Coronocyclus (Cor. coronatus, Cor. labiatus, Cor. labratus), Cylicodonthophorus (Cyd. bicoronatus) Parapoteriostomum (Para. mettami)* and *Poteriostomum (Pot. imparidentatum)*. The presence of a species within a pool was determined

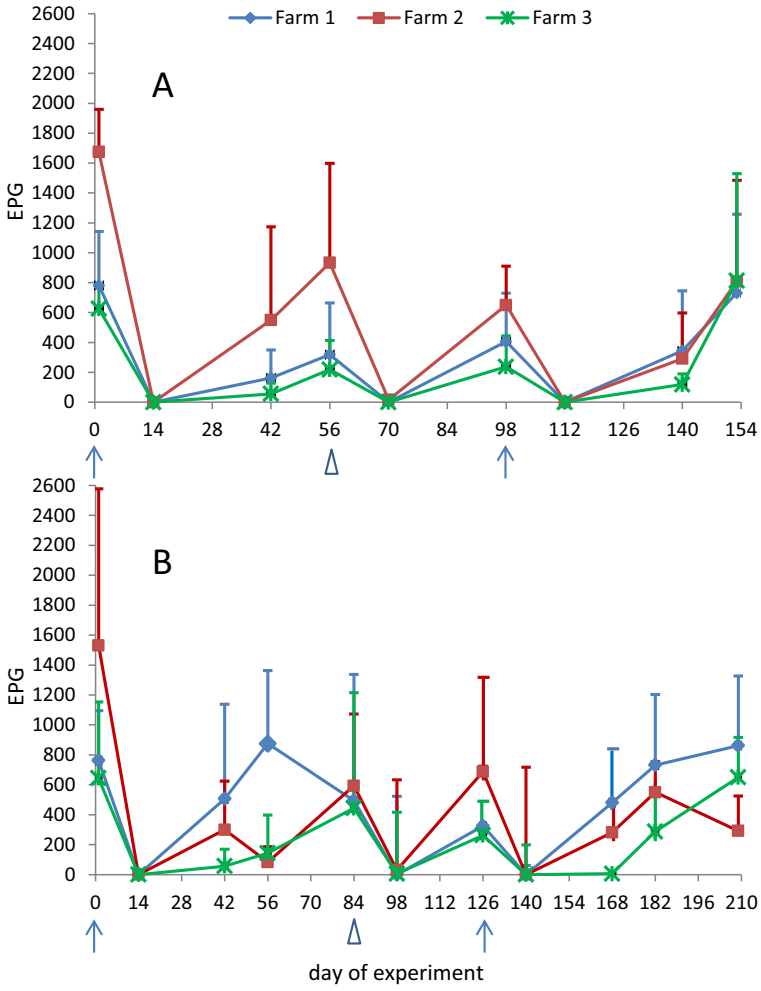

Fig. 1. Mean eggs per gram (EPG) + SD from the IVM-PYR-IVM (A) and the MOX-PYR-MOX (B) treatment group from farm 1 (blue diamonds), farm 2 (red squares) and farm 3 (green asterisk). ML treatments (arrow) and PYR treatment (arrowhead) were given on the indicated days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by hybridization of the homologues probe. For every pool in which a species was identified, the species scored 1 point. The scores of a species (0–3 or 4 points) is related to the frequency in which that species was present in the larval culture (Kooyman et al., 2016). Scores for a species of 0–3 or 4 points corresponds to the median frequency for that species of 0.014, 0.04, 0.08, 0.16 or 0.59, respectively. The frequency multiplied with the LPG obtained from culture counts results in a semi-quantitative estimation of the LPG per species.

2.5. Statistical analysis

Linear correlation between EPG and LPG was measured with Pearson's correlation coefficient (r). The effect of the ML and PYR treatments on the LPGs per species was tested using mixed-effects negative binomial regression models, which are suited to model parasitological count data like LPGs (Wilson and Grenfell, 1997; Stancampiano et al., 2010) and have been used previously to model similar dependent variables (Lello et al., 2004; Stancampiano et al., 2010; Mughini Gras et al., 2011). In these models, a random effect was included to account for clustering of observations at the level of individual horse, whereas two categorical variables, one defining the farm of origin and the other one defining the days post treatment, were included as fixed effects. The same modelling approach was also used for comparing the LPGs of different species with each other. Results were expressed as rate ratios (RR) and 95% confidence intervals (95%CI). Statistical analysis was performed using STATA v.13 (StataCorp, College Station, USA).

3. Results

3.1. Egg counts and activity

One horse from the IVM-PYR-IVM group from farm 2 was removed from the experiment for reasons not related with parasites and was not replaced. No data were generated from this horse. The EPGs from the individual horses are shown in the Supplementary data (Table S1) and the mean EPGs per farm per treatment group are shown in Fig. 1. The activities of the MLs and ERPs are given in Table 1. The activity of all ML treatments 14 days after treatment (efficacy) was 100%. The ERP after all ML treatments was shortened to between 14 and 42 days in both groups from farms 1 and 2 and after the 2nd IVM treatment on farm 3. The ERP on farm 3 after the 1 st MOX and 1st IVM treatments was not shortened and after the 2nd MOX treatment the ERP was shortened to between 42 and 56 days. The mean efficacy of PYR at day 14 ranged from 98 to 100% in all groups. The decrease in egg counts in the MOX-PYR-MOX group of farm 2 at day 56 after the 1st MOX treatment was caused by the missing value of horse 2 and an unexplained

Table 1
Mean activities of the 1st and 2nd ML (IVM or MOX) and PYR treatments at day 14, 42 and 56 after treatment. For the MLs only the days before the regular ERP are given (before day 56 for IVM and before day 84 for MOX). Activities ≤0.90 indicate shortening of the ERP and are given in bold.

Farm	Group	1st ML			PYR		2nd ML		
		D14	D42	D56	D14	D42	D14	D42	D56
1	IVM-PYR-IVM	1.00	**0.89**		1.00	0.10	1.00	**0.48**	
	MOX-PYR-MOX	1.00	**0.51**	**0.14**	1.00	0.19	1.00	**0.25**	**0.00**
2	IVM-PYR-IVM	1.00	**0.73**		0.98	0.21	1.00	**0.67**	
	MOX-PYR-MOX	1.00	**0.80**	**0.96**	0.98	0.47	1.00	**0.56**	**0.24**
3	IVM-PYR-IVM	1.00	0.94		1.00	0.13	1.00	**0.27**	
	MOX-PYR-MOX	1.00	0.99	0.92	1.00	0.33	1.00	1.00	**0.15**

decrease in egg count of the horse with highest egg counts 14 days earlier (horse 5).

3.2. Larval cultures

The mean larval recovery was 67%. The LPG correlates significantly with the EPG ($r^2 = 0.635$, $p < 0.01$) and was similar for all farms (Fig. 2) and treatment groups (not shown).

3.3. RLB on pooled samples

3.3.1. Species composition of larval cultures and LPG per species

The LPGs per species and the summation of the LPGs per species are given in Table S2 of the Supplementary data, together with the total LPGs resulting from the larval counts. Because *Cyc. leptostomum* and *Cyc. ashworthi* were both recognized by the LEP2 probe, the scores for the *Cyc. ashworthi* specific probe (ASH2) were not included in the summation (Kooyman et al., 2016). The LPGs for the 6 most abundant species or combination of species (*Cyc. nassatus, Cyc. leptostomum/ashworthi, Cyc. ashworthi, Cys. longibursatus, Cya. catinatum* and *Cya. pateratum*) representing the 3 most abundant genera (*Cylicocyclus*, *Cylicostephanus* and *Cyathostomum*) are given in Fig. 3.

The LPGs for these 6 species within the IVM-PYR-IVM group all varied in a similar way in response to the treatments, although the levels of the LPGs were different for the different species (Fig. 3, left column). The *Cylicocyclus* species were the most abundant, followed by *Cylicostephanus* and *Cyathostomum* species. The species composition 42 days after the IVM treatments (day 42 and day 140) was not significantly different from the one 42 days after PYR treatment (day 98). On farm 3, the LPGs for the individual species were lower after the treatments than on the 2 other farms, corresponding to the less shortened ERP at that farm (Table 1).

In cultures from the MOX-PYR-MOX groups (Fig. 3, right column), there was a change in the species composition after treatment. The LPGs of species from the genus *Cylicocyclus*, especially *Cyc. nassatus,* were high at 42 days after the 1st and 2nd MOX treatment (day 42 and day 168, respectively), but much lower at 42 days after PYR treatment (day 126). Farm 1 with the lowest activity of MOX showed at 42 days after MOX treatment the highest LPGs and the largest variety of cyathostomin species (*Cyc. insigne, Cyc. nassatus*, *Cyc. ashworthi*, *Cyc. leptostomum*, *Cys. goldi, Cys. longibursatus* and *Cor. coronatus),* while at the same day from the farm with the highest activity (farm 3) only low LPGs of *Cyc. nassatus, Cys. leptostomum and Cys. insigne* were found in the same number of differentiated L3s. The *Cys. longibursatus* LPGs showed a completely different pattern than the LPGs of the *Cylicocyclus* species, being absent or low at 42 days after MOX treatments and high after PYR treatment in all 3 farms. Therefore, the LPGs of *Cyc. nassatus* and *Cys. longibursatus* at 42 days after the treatments were further analyzed as a measure for the differential effect of the PYR and MOX treatment on the different species. While adjusting for the effect of the farm of origin and accounting for the individual horse variability, *Cyc. nassatus* LPGs at 42 days after the 1 st (RR 3.95, 95%CI 2.44–6.43, $p < 0.001$) and 2nd (RR 4.44, 95%CI 2.47–7.98, $p < 0.001$) MOX treatments were significantly higher than those at 42 days after the PYR treatment. In contrast, no significant differences existed in the *Cyc. nassatus* LPGs between the 1 st and 2nd post MOX treatments ($p = 0.665$). The *Cyc. nassatus* LPGs 42 days after the 1 st and 2nd MOX treatments were the highest (mean LPG 124 and 176, respectively) on the farm with the lowest activity (farm 1) and the lowest (mean LPG 47 and 0.5 LPG) on the farm with the highest activity (farm 3). Regarding *Cys. longibursatus,* its LPGs at 42 days after the 1 st (RR 0.12, 95%CI 0.06–0.26, $p < 0.001$) and 2nd (RR 0.15, 95%CI 0.07–0.32, $p < 0.001$) MOX treatments were significantly lower than those at 42 days after the PYR treatment, with no significant differences in *Cys. longibursatus* LPGs between the 1 st and 2nd MOX treatment ($p = 0.628$).

When comparing the LPGs of *Cyc. nassatus* and *Cys. longibursatus,* it was found that at the beginning of the experiment (pre-treatment), the LPGs did not differ significantly from one another, but after the treatments they did. The *Cyc. nassatus* LPGs 42 days after the 1 st (RR 5.47, 95%CI 2.64–11.37, $p < 0.001$) and 2nd (RR 4.95, 95%CI 2.34–10.49, $p < 0.001$) MOX treatments were significantly higher than those of *Cys. longibursatus,* whereas after the PYR treatment, the *Cys. longibursatus* LPGs were significantly higher than those of *Cyc. nassatus* (RR 2.65, 95%CI 1.42–4.95, $p = 0.002$). The species from the genus *Cyathostomum* had low LPGs on all farms before treatment and remained low throughout the experiment.

3.3.2. The reliability of the differentiation by RLB on pooled samples

The LPG per treatment group obtained by larval counts and by summation of the LPGs per cyathostomin species were similar and gave a similar pattern in time for all 3 farms (Supplementary data, Fig. S1).

4. Discussion

The cultured L3s obtained after ML and after PYR treatment from farms with a shortened ERP were counted and differentiated with RLB on pooled samples. Differentiation of the excreted eggs and/or cultured L3s obtained early after the different treatments can provide more insight into the potential causes of a shorter ERP. Because egg and larval counts correlated linearly it was decided to differentiate the L3s, because they can be harvested and stored more easily than eggs. The species composition of the cultured L3s was estimated with RLB on pooled samples. Cwiklinski et al. (2012) described the differentiation of 21 cyathostomin species with RLB, but for 3 of those species the specificity of the probes was not verified with homologous differentiated adults. Kooyman et al. (2016) demonstrated that of the 18 remaining species the probe for *Cyc. radiatus* could not be used in RLB on pooled samples because of putative cross-hybridization. Therefore, in the present study probes for 17 species were used and only of the species *Cyd. bicoronatus*, *Pot. imparidentatum*, *Cya. tetracanthum* and *Cor. labratus* no L3s

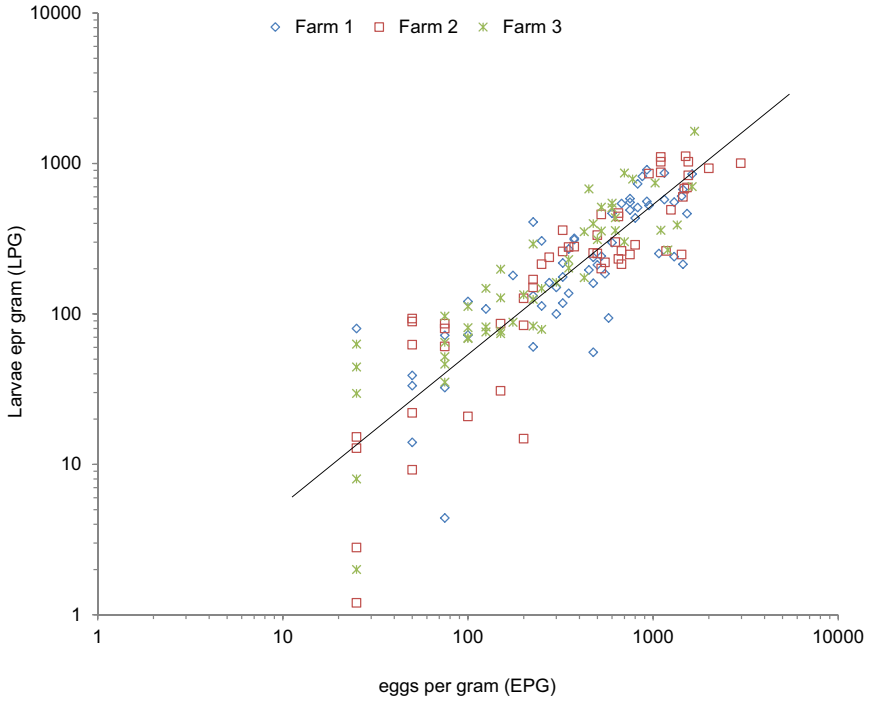

Fig. 2. Linear correlation between eggs per gram (EPG) and larvae per gram (LPG). All samples with a positive EPG and positive LPG from farm 1 (blue diamonds), farm 2 (red squares) and farm 3 (green asterix) are given. The linear correlation ($r^2 = 0.635$), was highly significant ($p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were found. The LPGs obtained directly from culture larval counts were similar to the summation of all LPGs per species obtained from the pooled samples in the RLB. This implies that the latter provides reliable estimations of the LPGs per species.

On all 3 farms, the LPG per cyathostomin species and the species composition of the larval culture changed in response to the different treatments in the MOX-PYR-MOX groups, but not in the IVM-PYR-IVM group. At introduction of the drug the ERP for MOX was much longer than for IVM, most likely because of the larvicidal effect of MOX. However, the activity of the IVM and MOX treatments at the same day after treatment were in this study comparable. Therefore, the relative shortening of the ERP after MOX treatments was more pronounced than after IVM treatments. This may explain the rather small differences in species composition before and after IVM treatment, while there were larger differences before and after MOX treatment. It is expected that more *Cylicocyclus* will be found when the sampling is done earlier after IVM treatment. This is in agreement with van Doorn et al. (2014). They differentiated L3s obtained earlier after IVM treatment (day 28 and 35) and relatively more *Cylicocyclus* species were found on those days than on days 42 or 56 after treatment. An important difference between the present study and van Doorn et al. (2014) was the use of 2 the same ML treatments with a PYR treatment in between in the present study. That offered the opportunity to compare the species composition of the cultured L3s obtained 42 days after ML treatment with those obtained from the same horse 42 days after PYR treatment. Furthermore, the influence of the season can be evaluated because the 2 ML treatments were separated in time (98 days for IVM and 126 days for MOX treatments) with the PYR treatment in between. The EPGs and the species composition of the L3 cultures obtained after 1st and 2nd ML treatment were comparable. This indicates that there was no clear seasonal effect. More importantly, possible differences in the effect of the PYR and ML treatments can therefore not be attributed to seasonal effects.

The species composition of the larval cultures obtained 42 days after MOX and PYR treatment were compared. In comparison to *Cyc. nassatus, Cys. longibursatus* was more abundant after PYR than after MOX treatment. *Cys. longibursatus* is a much smaller worm than *Cyc. nassatus* with a shorter PPP, 57 versus 71 days (Lyons et al., 2011a), and it is therefore likely that the development time from mucosal stage to adult worm is also shorter in *Cys. longibursatus* than in *Cyc. nassatus.* MOX has activity against mucosal stages, whereas PYR activity is restricted to adults and to a lesser extent other luminal stages (Chapman et al., 1996; Lind et al., 2003). This can explain why the *Cys. longibursatus* LPG was higher at 42 days after PYR treatment than after MOX treatment and also that *Cys. longibursatus* LPG was higher than *Cyc. nassatus* LPGs at day 42 after PYR. Resistance of *Cys. longibursatus* against PYR could also have explained the high LPGs of this worm after PYR, but seems unlikely because of the very high efficacy (99%) of PYR in the present study, similar to that found in previous studies (Boersema et al., 1991; Eysker et al., 1991; Lyons, 1974). And even when there is PYR resistance in *Cys. longibursatus*, it cannot explain the low *Cyc. nassatus* LPG after PYR treatment.

In contrast, the *Cylicocyclus* species, especially *Cyc. nassatus,* were more abundant after MOX treatment than after PYR treatment. This can only be explained by the presence of worms that developed at a faster pace from L4 to adult or by worms less susceptible for MOX. A faster pace of development will be independent of the anthelmintic used and therefore the same should have been seen after PYR treatment. However, higher *Cylicocyclus* LPGs were found at 42 days after MOX than at the same day after PYR treatment. This suggests that the shortening of the ERP is not caused by a faster development of *Cylicocyclus* species. Furthermore, despite the fact that the *Cylicocyclus* LPGs on farm 1 and farm 3 were similar at pre-treatment, they were higher on farm 1, with the lowest MOX activity than on farm 3, with the highest MOX activity, after both MOX treatments. If this would have been caused by a faster development, the same was expected after PYR treatment. However, we found similar *Cylicocyclus* LPGs for farm 1 and 3 after PYR treatment. Therefore, it was concluded that shortening of the ERP after MOX treatment is most probably caused by reduced susceptibility of the (mainly *Cylicocyclus*) immature worms and not by

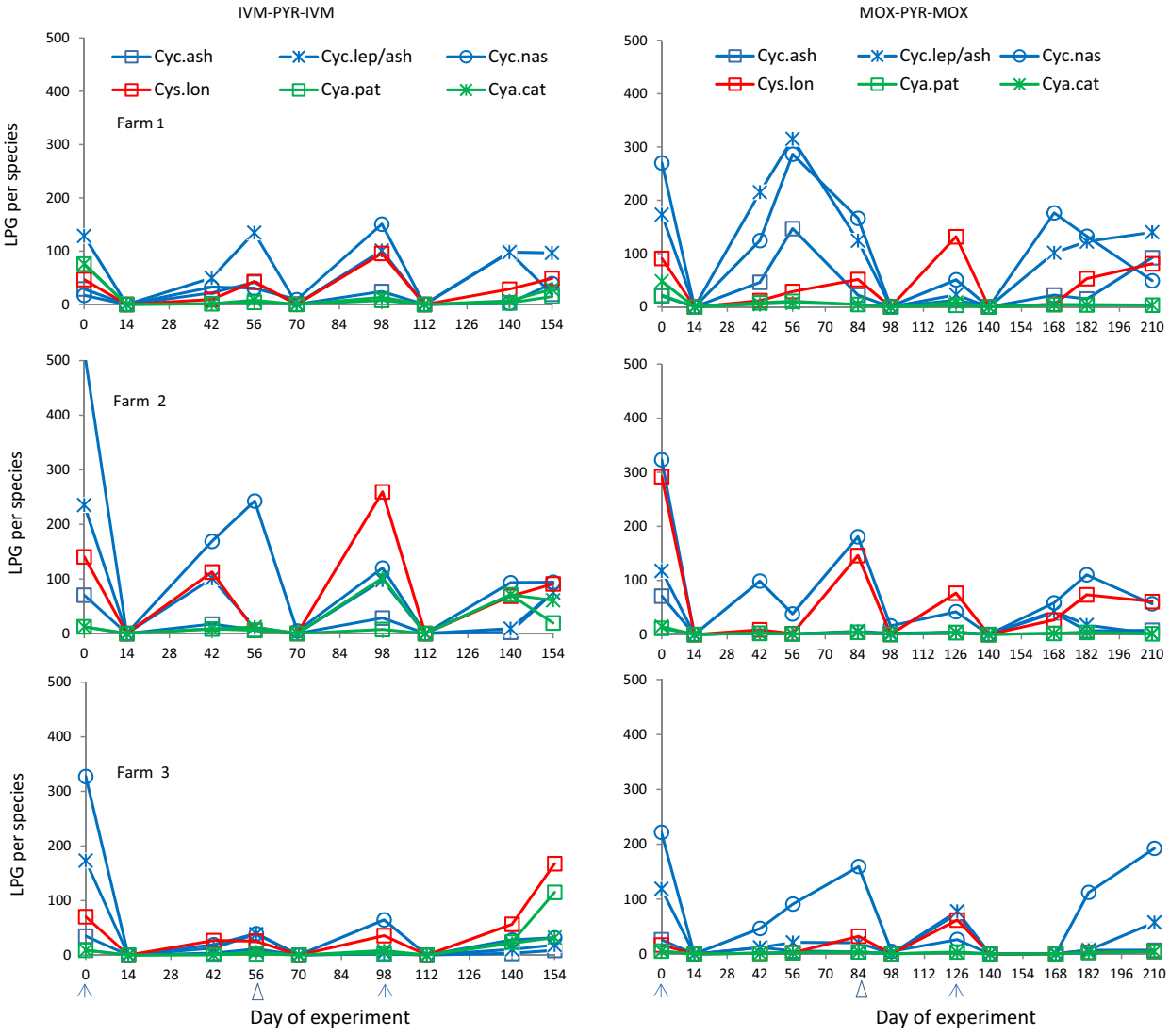

Fig. 3. The mean larvae per gram (LPG) of the 6 most abundant species (*Cyc. nassatus, Cyc. ashworthi, Cyc. leptostomum, Cys. longibursatus, Cya. catinatum* and *Cya. pateratum*) from the 3 most abundant genera (*Cylicocyclus, Cylicostephanus and Cyathostomum*) are given for all sample days for farm 1 (top row), farm 2 (middle row) and farm 3 (bottom row). The IVM-PYR-IVM groups are given in the left column, the MOX-PYR-MOX groups are given in the right column. The ML treatments (arrows) and the PYR treatments (arrowheads) were given on the indicated days.

a faster development from immature to adult. Cross-resistance is unlikely to be at play here, because the species that returned early after MOX treatment were different from those that returned early after PYR treatment.

The fact that reduced susceptibility and not faster development is the most likely cause for ERP shortening has most likely implications for the possibility to reverse development of MOX resistance, or more precisely the restoration of the original ERP. It is to be expected that in case of faster development both slow and faster developing immature worms will be killed by MOX, as they will have similar susceptibility to MOX. Alternatively, in case of reduced larval susceptibility to MOX, MOX will predominantly kill the susceptible individuals. At the time MOX was first marketed, the majority of larvae was still developing at 84 days post MOX treatment when the first eggs re-appeared. Consequently, the majority of larvae with a regular slow rate of development or with a regular susceptibility will not reach the adult stage at day 84 after MOX. This implies that a PYR treatment given at day 84 after MOX, will predominantly kill individuals with either a faster development or a reduced MOX susceptibility responsible for the shortened ERP. Therefore, the net result (combined effect of MOX followed by PYR at day 84) will have little effect on restoring a shortened ERP in case of reduced MOX susceptibility, because MOX will kill the susceptible individuals whereas PYR, given 84 days later, will mostly kill the resistant individuals responsible for the shortened ERP. Therefore, the ratio between susceptible and less susceptible individuals may be expected to remain largely the same. In case shortened ERP is caused by a faster development, the net result would have been selection against individuals with a faster development, because MOX will kill both slow and fast developing individuals, while PYR would predominantly kill the fast developing worms. Consequently, the ratio between slow and fast developing individuals would change in favor of the slower developing individuals. This implies that in this case it might be more feasible to reverse a shortened ERP back to the original ERP with a PYR treatment 84 days after a MOX treatment compared to the situation of reduced larval susceptibility to MOX. If there would be cross-resistance between MOX and PYR, the different effects resulting from reduced susceptibility versus faster development would even be more pronounced, as in such a case PYR would be less able to kill MOX resistant individuals.

LPGs at day 42 after MOX treatment were low on the farm with the least reduced ERP (farm 3), whereas LPGs were high on the farm with the strongest reduced ERP (farm 1). The species composition also differed between these 2 farms; on farm 3 only *Cylicocyclus* species (mainly *Cyc. nassatus*) were found in cultures after both MOX treatments, while on farm 1, other *Cylicocyclus* species were also abundant as well as that species from other genera were found (*Cylicostephanus*, *Cyathostomum* and *Coronocyclus)*. This suggests that in the early stages of shortening of ERP *Cylicocyclus* species (mainly *Cyc. nassatus*) dominate, or in other words, appear to be the first species in which a lesser susceptibility becomes apparent. In later stages, when shortening of ERP has progressed, species composition becomes more diverse as other species also become less susceptible. A similar pattern was found by van Doorn et al. (2014). Canever et al. (2013) and Traversa et al. (2009) already demonstrated egg reappearance at 14 days after ML treatment. To our knowledge, nothing was reported about the species composition of these early excreted eggs.

In conclusion, this study provides evidence that a shortening of the ERP is a process in which an increasing number of immature worms from an increasing number of cyathostomin species are becoming less susceptible to MOX and possibly IVM, although this was not clear in the present study. Monitoring shortening of the ERP as an early sign of resistance development can help in evaluating old and new treatment strategies in order to be able to use the still effective MLs for an extended period of time.

Conflict of interests

The study was supported by Zoetis. Thomas Geurden is an employee of Zoetis and was involved in the study design and reporting. He was not involved in the sampling and the data analysis.

Animal welfare statement

The study was approved by the Zoetis Ethical Review Committee.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2016.07.029.

References

Boersema, J.H., Borgsteede, F.H., Eysker, M., Elema, T.E., Gaasenbeek, C.P., van der Burg, W.P., 1991. The prevalence of anthelmintic resistance of horse strongyles in the Netherlands. Vet. Q. 13, 209–217.

Boersema, J.H., Eysker, M., Maas, J., van der Aar, W.M., 1996. Comparison of the reappearance of strongyle eggs on foals, yearlings and adult horses after treatment with ivermectin or pyrantel. Vet. Q. 18, 7–9.

Boersema, J.H., Eysker, M., van der Aar, W.M., 1998. The reappearance of strongyle eggs in the faeces of horses after treatment with moxidectin. Vet. Q. 20, 15–17.

Borgsteede, F.H., Boersma, J.H., Gaasenbeek, C.P., van der Burg, W.P., 1993. The reappearance of eggs in faeces of horses after treatment with ivermectin. Vet. Q. 15, 24–26.

Canever, R.J., Braga, P.R., Boeckh, A., Grycajuck, M., Bier, D., Molento, M.B., 2013. Lack of cyathostomin sp reduction after anthelmintic treatment in horses in Brazil. Vet. Parasitol. 194, 35–39.

Chapman, M.R., French, D.D., Monahan, C.M., Klei, T.R., 1996. Identification and characterization of a pyrantel pamoate resistant cyathostome population. Vet. Parasitol. 66, 205–212.

Chapman, M.R., French, D.D., Klei, T.R., 2002. Gastrointestinal helminths of ponies in Louisiana: a comparison of species currently prevalent with those present 20 years ago. J. Parasitol. 88, 1130–1124.

Cwiklinski, K., Kooyman, F.N., Van Doorn, D.C., Matthews, J.B., Hodgkinson, J.E., 2012. New insights into sequence variation in the IGS region of 21 cyathostomin species and the implication for molecular identification. Parasitology 139, 1063–1073.

Demeulenaere, D., Vercruysse, J., Dorny, P., Claerebout, E., 1997. Comparative studies of ivermectin and moxidectin in the control of naturally acquired cyathostome infections in horses. Vet. Rec. 141, 383–386.

DiPietro, J.A., Hutchens, D.E., Lock, T.F., Walker, K., Paul, A.J., Shipley, C., Rulli, D., 1997. Clinical trial of moxidectin oral gel in horses. Vet. Parasitol. 72, 167–177.

Eysker, M., Boersema, J.H., Kooyman, F.N.J., 1991. Effect of early season ivermectin and pyrantel treatments on strongylid infections in young shetland ponies in the Netherlands. Vet. Parasitol. 38, 33–39.

Geurden, T., van Doorn, D., Claerebout, E., Kooyman, F., De Keersmaecker, S., Vercruysse, J., Besognet, B., Vanimisetti, B., di Regalbono, A.F., Beraldo, P., Di Cesare, A., Traversa, D., 2014. Decreased strongyle egg re-appearance period after treatment with ivermectin and moxidectin in horses in Belgium, Italy and the Netherlands. Vet. Parasitol. 204, 291–296.

Kooyman, F.N.J., van Doorn, D.C.K., Geurden, T., Wagenaar, J.A., 2016. Semi-quantitative differentiation of cyathostomin larval cultures by reverse line blot. Vet. Parasitol. 216, 59–65.

Lello, J., Boag, B., Fenton, A., Stevenson, I.R., Hudson, P.J., 2004. Competition and mutualism among the gut helminths of a mammalian host. Nature 428, 840–844.

Lind, E.O., Eysker, M., Nilsson, O., Uggla, A., Höglund, J., 2003. Expulsion of small strongyle nematodes (cyathostomin spp) following deworming of horses on a stud farm in Sweden. Vet. Parasitol. 115, 289–299.

Lyons, E.T., Tolliver, S.C., 2013. Further indication of lowered acitivity of ivermectin on immature small strongyles in the intestinal lumen of horses on a farm in Central Kentucky. Parasitol. Res. 112, 889–891.

Lyons, E.T., Tolliver, S.C., Collins, S.S., 2009. Probable reason why small strongyle EPG counts are returning early after ivermectin treatment of horses on a farm in central Kentucky. Parasitol. Res. 104, 569–574.

Lyons, E.T., Tolliver, S.C., Kuzmina, T.A., Collins, S.S., 2010. Critical tests evaluating efficacy of moxidectin against small strongyles in horses from a herd for which reduced activity had been found in field tests in central Kentucky. Parasitol. Res. 107, 1495–1498.

Lyons, E.T., Kuzmina, T.A., Tolliver, S.C., Collins, S.S., 2011a. Observations on development of natural infection and species composition of small strongyles in young equids in Kentucky. Parasitol. Res. 109, 1529–1535.

Lyons, E.T., Tolliver, S.C., Collins, S.S., Ionita, M., Kuzmina, T.A., Rossano, M., 2011b. Field tests demonstrating reduced activity of ivermectin and moxidectin against small strongyles in horses on 14 farms in central Kentucky in 2007–2009. Parasitol. Res. 108, 355–360.

Lyons, E.T., 1974. Critical test of three salts of pyrantel against internal parasites of the horse. Am. J. Vet. Res. 35, 1515–1522.

Molento, M.B., Nielsen, M.K., Kaplan, R.M., 2012. Resistance to avermectin/milbemycin anthelmintics in equine cyathostomins – current situation. Vet. Parasitol. 185, 16–24.

Mughini Gras, L., Usai, F., Stancampiano, L., 2011. Strongylosis in horses slaughtered in Italy for meat production: epidemiology, influence of the horse origin and evidence of parasite self-regulation. Vet. Parasitol. 179, 167–174.

Nielsen, M.K., Reist, M., Kaplan, R.M., Pfister, K., van Doorn, D.C., Becher, A., 2014. Equine parasite control under prescription-only conditions in Denmark – awareness, knowledge, perception and strategies applied. Vet. Parasitol. 204, 64–72.

Parry, J.M., Fisher, M.A., Grimshaw, W.T., Jacobs, D.E., 1993. Anthelmintic dosing intervals for horses: comparison of three chemical groups. Vet. Rec. 133, 346–347.

Pook, J.F., Power, M.L., Sangster, N.C., Hodgson, J.L., Hodgson, D.R., 2002. Evaluation of tests for anthelmintic resistance in cyathostomes. Vet. Parasitol. 106, 331–343.

Reinemeyer, C.R., Rohrbach, B.W., 1990. A survey of equine parasite control practices in Tennessee. J. Am. Vet. Med. Assoc. 196, 712–716.

Relf, V.E., Lester, H.E., Morgan, E.R., Hodgkinson, J.E., Matthews, J.B., 2014. Anthelmintic efficacy on UK thoroughbred stud farms. Int. J. Parasitol. 44, 507–514.

Stancampiano, L., Mughini Gras, L., Poglayen, G., 2010. Spatial niche competition among helminth parasites in horse's large intestine. Vet. Parasitol. 170, 88–95.

Traversa, D., Iorio, R., Klei, T.R., Kharchenko, V.A., Gawor, J., Otranto, D., Sparagano, O.A., 2007. New method for simultaneous species-specific identification of equine strongyles (nematoda, strongylida) by reverse line blot hybridization. J. Clin. Microbiol. 45, 2937–2942.

Traversa, D., von Samson-Himmelstjerna, G., Demeler, J., Milillo, P., Schurmann, S., Barnes, H., Otranto, D., Perrucci, S., di Regalbono, A.F., Beraldo, P., Boeckh, A., Cobb, R., 2009. Anthelmintic resistance in cyathostomin populations from horse yards in Italy, United Kingdom and Germany. Parasit. Vectors 2 (Suppl. 2), S2,3305-2-S2-S2.

van Doorn, D.C., Kooyman, F.N., Eysker, M., Hodgkinson, J.E., Wagenaar, J.A., Ploeger, H.W., 2010. In vitro selection and differentiation of ivermectin resistant cyathostomin larvae. Vet. Parasitol. 174, 292–299.

van Doorn, D.C., Ploeger, H.W., Eysker, M., Geurden, T., Wagenaar, J.A., Kooyman, F.N., 2014. *Cylicocyclus* species predominate during shortened egg

reappearance period in horses after treatment with ivermectin and moxidectin. Vet. Parasitol. 206, 246–252.
Vidyashankar, A.N., Hanlon, B.M., Kaplan, R.M., 2012. Statistical and biological considerations in evaluating drug efficacy in equine strongyle parasites using fecal egg count data. Vet. Parasitol. 185, 45–56.
von Samson-Himmelstjerna, G., Fritzen, B., Demeler, J., Schurmann, S., Rohn, K., Schnieder, T., Epe, C., 2007. Cases of reduced cyathostomin egg-reappearance period and failure of *Parascaris equorum* egg count reduction following ivermectin treatment as well as survey on pyrantel efficacy on German horse farms. Vet. Parasitol. 144, 74–80.
Weclawski, U., Heitlinger, E.G., Baust, T., Klar, B., Petney, T., Han, Y.S., Taraschewski, H., 2013. Evolutionary divergence of the swim bladder nematode *Anguillicola crassus* after colonization of a novel host, *Anguilla anguilla*. BMC Evol. Biol. 13 (78) (2148-13-78).
Wilson, K., Grenfell, B.T., 1997. Generalized linear modelling for parasitologists. Parasitol. Today 13, 33–38.