



Dose-dependent in vivo effects of formulated moxidectin on seedling emergence of temperate grassland species

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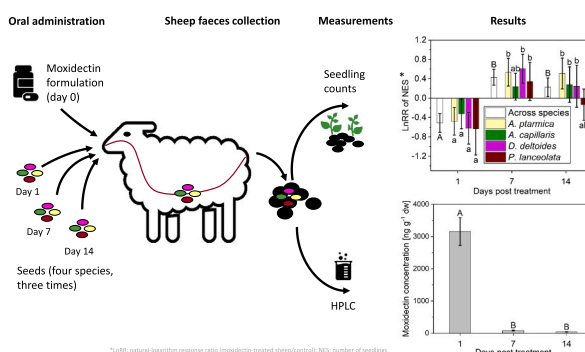
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HIGHLIGHTS

- Sheep have the capacity to disperse seeds of many grassland species via faeces.
- A feeding experiment showed that moxidectin can affect this ecological function.
- Seedling emergence from faeces of treated sheep showed hormesis-like response patterns.
- Even two weeks after treatment, there were still effects on ingested seeds.
- These effects might alter population growth of grassland species.

GRAPHICAL ABSTRACT



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ABSTRACT

Sheep function as effective endozoochorous seed vectors in grasslands. Recent laboratory-based studies showed that this important function can be impaired by macrocyclic lactone anthelmintics, which are used to control parasites and enter into the environment mainly via faeces; however, there is a lack of in vivo studies. We conducted a seed-feeding experiment with sheep that included four temperate grassland species from four different families (*Achillea ptarmica*, Asteraceae; *Agrostis capillaris*, Poaceae; *Dianthus deltoides*, Caryophyllaceae; *Plantago lanceolata*, Plantaginaceae). A series of three feeding trials was carried out after one of two groups of sheep received a single administration of a common oral formulation of the macrocyclic lactone moxidectin. Faeces were collected to determine seedling emergence rate and emergence timing as well as moxidectin concentration via HPLC. Seedling emergence differed significantly between the anthelmintic-treated sheep and the control group. This impact depended on time of seed uptake after anthelmintic administration. Number of emerging seedlings was significantly reduced (27.1 %) when faeces moxidectin concentrations were high (on average 3153 ng g⁻¹; 1 d post treatment) and significantly increased (up to 68.8 %) when moxidectin concentrations were low (≤ 86 ng g⁻¹; 7, 14 d pt). Mean emergence time was significantly lowered at low moxidectin

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concentrations. These results demonstrate dose-related effects of deworming on seedling emergence which might affect endozoochory and eventually plant population dynamics in grasslands.

1. Introduction

Endo- and ectoparasites such as nematodes and mites are a major factor for illness and poor productivity of livestock (Sutherland and Scott, 2010). To control these pests and guarantee animal welfare, anthelmintic macrocyclic lactones (MLs), such as moxidectin (MOX), are used on a large scale worldwide (Lanusse et al., 1997; Prichard et al., 2012). They kill internal and external invertebrate parasites of target animals by affecting invertebrate-specific chloride channels localised in membranes of nerve and muscle cells (Adler et al., 2016; Lumaret et al., 2012). MLs enter the environment mainly via faeces of treated animals and affect non-target organisms such as coprophagous beetles and flies, with detrimental impact on ecosystem services, such as dung removal and nutrient cycling (Afzal et al., 1994; Ambrožová et al., 2021; Baena-Díaz et al., 2018; Gokbulut et al., 2001; Kolar et al., 2006; Suárez et al., 2009). While the detrimental effects on arthropods are demonstrated in a large body of publications (reviewed in Floate et al., 2005; Lumaret et al., 2012; Junco et al., 2021), the impact on other non-target organisms, such as plants, have only recently drawn attention (Bártíková et al., 2016; Eichberg et al., 2016, 2022).

In the life cycle of plants, successful germination and seedling establishment are decisive stages for later growth and reproduction success; thus, any changes in these stages might have significant effects on persistence of plant populations (Grubb, 1977; Kitajima and Fenner, 2000). In this context, an increase in the number of emergent seedlings, as can be caused by a range of environmental factors, almost always indicates higher fitness. In contrast, environmental-related changes in temporal response of a seed cohort, measured as mean time of emergence or synchrony of emergence, are not monotonically linked to recruitment success and the benefits of a changed emergence time (i.e., lower or higher emergence time) or altered temporal spread of emergence (i.e., higher or lower synchrony) may vary with environmental context.

The few studies that addressed effects of MLs on seed germination or seedling development are under laboratory conditions and predominantly found suppressing, but also some stimulating effects, depending on plant species, ML drug and concentration of ML solution (Eichberg et al., 2016, 2022; Laber et al., 2023; Vokřál et al., 2019). Among tested MLs, formulated MOX appeared to have the strongest effects on seeds and generally led to reductions in germination percentage, increases in mean germination time and reductions in synchrony of germination (Eichberg et al., 2016). To our knowledge, only one study has so far tested ML impacts on seeds in vivo, conducting a sheep-feeding experiment that included *Plantago lanceolata* seeds, a key grassland species, and formulated MOX (Eichberg et al., 2016). This study found a strong reduction in the number of seedlings emerging out of sheep faeces. However, in this initial experiment, concentrations of the active ingredient in faeces were not determined and only one time point of seed uptake after deworming was tested. Results of a multi-species laboratory experiment showed that pure MLs enhance germination rates across species compared to controls, including low concentrations (0.1 mg l^{-1} ; Laber et al., 2023). At the species level, both increasing and decreasing effects along a series of ML concentrations have been observed in this experiment (Laber et al., 2023). In concert, the initial results on ML effects on seed germination suggest that MLs may also exert non-linear effects, which are commonly occurring in living organisms (Coenders and González, 2014). A particularly widespread non-linear relationship in living organisms is the hormetic relationship which is given when a chemical substance induces suppressing effects to an organism at higher concentrations, but stimulating effects at lower concentrations (Agathokleous et al., 2018; Mattson and Calabrese, 2010).

There is a pronounced time course of faecal ML elimination (Prichard et al., 2012) indicating that seeds eaten are prone to varying ML concentrations depending on the time point of ingestion after deworming. After subcutaneous administration of a single standard dose (0.2 mg kg^{-1} bodyweight) of a ML drug, peak residue levels occurred 2–3 days post treatment in sheep faeces and reached values in the ng g^{-1} to $\mu\text{g g}^{-1}$ dw (dry weight) range (Hentz et al., 2019; Kolar et al., 2006; Vokřál et al., 2019). In a study of Hentz et al. (2019), MOX faecal excretion of subcutaneously-dosed lambs continues in the low ng g^{-1} range over the whole measurement period of 42 days. Analysing blood plasma samples, Alvinerie et al. (1998) were able to show for an even longer study period (60 d) that MOX is present in the treated animal for a long time after both subcutaneous and oral administration to sheep. Generally, MOX shows much longer elimination half-life in ungulate tissues than the closely related ivermectin. For instance, in blood plasma of orally treated sheep, Molento et al. (2004) found a 7-fold higher elimination half-life in MOX (15.4 d) than ivermectin (2.1 d). MLs are lipophilic substances (log K_{OW}, MOX: 4.8; Mooney et al., 2021; K_{OW}: n-octanol-water partition coefficient, a measure of the relationship between lipophilicity and hydrophilicity of a substance). They oscillate between fat tissues and the lumen of the gastro-intestinal tract, which explains their long-lasting efficacy (Coop et al., 2002). The particularly high persistence of MOX activity in host plasma and tissues is related to its higher in vivo stability and lower affinity to P-glycoprotein efflux transporters as compared to other MLs (Ballent et al., 2013; Cobb and Boeckh, 2009; Prichard et al., 2012). In contrast to MOX, its main co-formulants in oral formulations (e.g. benzyl alcohol, butylated hydroxytoluene) do not accumulate in tissues, are rapidly metabolised in the digestive tract of mammals and are predominantly or to a considerable extent excreted via the urine pathway (Johnson et al., 2017; Nair, 2001; Witschi et al., 1989). Nair (2001) reports that the major fraction of the metabolites of benzyl alcohol was excreted by mammals via urine within 6 h. Because of the very different residence times of MOX and its co-formulants in the host body, MOX probably plays the most important influencing role in the interaction of seeds with orally applied MOX formulations.

Here, we focus on sheep because of their unique role in maintaining or generating a high phytodiversity in grasslands, if kept in large migratory flocks (Plachter and Hampicke, 2010; Rosenthal et al., 2012). Especially responsible for this is their potential to foster long-distance seed dispersal and post-dispersal establishment success. It has been shown that roaming sheep flocks can transport viable seeds of >70 species via their faeces (process of endozoochory; Kuiters and Huiskes, 2010) and functionally connect plant populations of fragmented grasslands via seed dispersal (Honnay et al., 2006; Rico et al., 2014; Wagner et al., 2013). Grassland fragmentation is widespread in the European landscape and coupled with isolation of plant populations due to low rates of seed exchange (Auffret et al., 2017). Since grassland seeds can come into contact with MLs in the digestive tract of recently dewormed domestic ungulates, endozoochory is potentially impaired by ML use.

Against this background, we set up a seed-feeding experiment with sheep that includes four endozoochorously dispersed plant species native to temperate grasslands in central Europe and a series of three seed-feeding trials after a single oral administration of formulated MOX and aimed at answering the following questions:

- Does formulated moxidectin administered orally to sheep affect seedling emergence of ingested seeds?
 - We expected that the anthelmintic drug tested leads to a decrease of seedling emergence, an increase in mean emergence time and a lower synchrony of emergence of ingested seeds.

- Do the effects of formulated moxidectin depend on when the seeds are ingested by the treated animal after deworming?
 - o We expected a clear impact of timing of seed ingestion on the tested emergence parameters, which coincides with concentration of moxidectin in sheep faeces.

2. Materials and methods

2.1. Test species and seed material

We tested seedling emergence of four species with perennial life strategies belonging to four plant families: *Achillea ptarmica* (Asteraceae), *Agrostis capillaris* (Poaceae), *Dianthus deltooides* (Caryophyllaceae) and *Plantago lanceolata* (Plantaginaceae). Their seeds have shown sensitivity to macrocyclic lactone (ML) anthelmintics in previous experiments (Eichberg et al., 2016, 2022; Laber et al., 2023). While in *A. capillaris*, *D. deltooides* and *P. lanceolata* the survival of the passage through the gastrointestinal tract of sheep is well documented (Kuiters and Huiskes, 2010; Benthien et al., 2016), in case of *A. ptarmica* survival of the passage can be assumed from findings of the close relative *Achillea millefolium* (Kuiters and Huiskes, 2010). Seeds were purchased from Rieger-Hofmann GmbH, Blaufen, Germany. Their germination potential before the start of the feeding experiment was in the typical range of wild plant species (means \pm SE): *A. ptarmica* 29.1 \pm 9.1 %, *A. capillaris*: 70.6 \pm 4.3 %, *D. deltooides*: 63.4 \pm 4.8 %, *P. lanceolata*: 34.0 \pm 3.1 % (Laber et al., 2023).

2.2. Test substance

We tested the anthelmintic formulation Cydectin® 0.1 % oral drench for the application in sheep (Zoetis Deutschland GmbH, Berlin, Germany; ingredient list: see Appendix A, Table A.1). The active ingredient of Cydectin is moxidectin (MOX), a globally available broad-spectrum anthelmintic from the milbemycin sub-class of MLs (Cobb and Boeckh, 2009). As in other MLs, the mode of action of MOX is binding to glutamate-gated chloride channels and thereby triggering an irreversible increase of membrane conductance that leads to paralysis and death of parasites, such as nematodes (Prichard et al., 2012; Shoop et al., 1995). Since vertebrates do not possess such type of channels, they are insensitive to this mode of action (Cotureau et al., 2003).

2.3. Experimental design

To test the effects of formulated MOX on plant regeneration by seed under in vivo conditions, a sheep feeding experiment was carried out comprising two animal groups and four plant species. The two sheep groups received one of either treatment, 1. A single administration of the anthelmintic drug (treatment group) or 2. No deworming treatment (control group). Then, in a series of trials, sheep of both groups were fed with a mix of seeds of the four study species a day, a week and two weeks after administration of the anthelmintics. Faeces were collected for two days after each feeding trial.

Each animal group consisted of eleven sheep, which were provided by the animal park Arche Warder (Warder, Schleswig-Holstein, Germany). All animals in our study were healthy, adult males of different land sheep breeds. Within the same breed, the experimental animals had roughly the same age and bodyweight and both tested sheep groups comprised the same number of animals per breed. Sheep groups were kept separated in two areas of a stable for the whole duration of the experiment. To minimise seed contamination, all sheep were brought from a pasture into the stable five days before first faeces sampling. For the whole experimental phase, sheep were provided food (hay and common sheep pellet food) and water ad libitum.

On June 7th 2021, Cydectin oral drench was administered to the animals of the treatment group in accordance to the regular treatment cycles and dosed as recommended by the producer (0.2 mg MOX per kg

bodyweight). The last pre-experimental anthelmintic treatment of the animals, i.e. sheep of both treatment and control group, was with a drench formulation of the benzimidazole albendazole (Valbazen) and took place four months before the start of the experiment. The presence of residues of albendazole, its major metabolites and major co-formulants in the sheep at the time of the start of the experiment can be excluded (EFSA, 2015; Johnson et al., 2017; Marriner et al., 1981).

Following the deworming of the treatment group, in a series of three trials, mixtures containing 15,000 seeds of each of the four test species were individually administered to the experimental animals of both sheep groups. The trials were conducted one day (June 8th 2021), seven days (June 14th 2021) and 14 days (June 21st 2021), respectively, after drug administration by means of opened syringes to ensure complete consumption of freshly moistened seed mixtures. We chose a 1-wk interval between trials to ensure trial independence in terms of seed lots fed. This assumption is based on data from previous sheep feeding trials on fed seeds with regard to the faecal excretion peak (approx. 1–2 d post ingestion) and the mean retention time (approx. 1.5–2.5 d) as well as the fact that after 4 d only small amounts of seeds, if any, could be detected in the analysed faeces (Oveisi et al., 2021; Ramos et al., 2006; Russi et al., 1992; Wang et al., 2017). At three collection times (24, 30 and 48 h) after each feeding trial, faeces samples were taken directly from the rectum of each individual animal of both groups by a veterinarian. Animal treatment was in accordance with the German Animal Welfare Legislation Act (TierSchG, TierSchVersV). The present feeding experiment did not need approval by an ethical committee or by the government since this type of investigations did not induce pain, suffering or harm to the animals.

Each faeces sample was weighed. For later chemical analysis, we separated approximately 5 g faeces (fresh weight) from each of the two largest faeces samples per collection time and froze (-32°C) these sub-samples immediately. The remaining material of each faeces sample (16.4 ± 0.6 g fresh weight; mean \pm SE) was used to determine the germinable seed content. For this purpose, the seedling emergence method was applied that is commonly used in endozoochory studies (Malo and Suárez, 1995). Accordingly, we crumbled the faeces material and mixed it with seed-free potting soil in a 1:1 ratio, and spread this mixture as a 0.3 cm thin layer on top of a 4 cm thick layer of potting soil in Styrofoam boxes ($29.0\text{ cm} \times 19.5\text{ cm} \times 5.5\text{ cm}$). The prepared boxes were exposed outdoors on a large, open-spaced area completely paved with flagstones at Kiel University (Bremerskamp 133, 24118, Kiel, Germany). They were placed in randomized groups directly on the flagstones and covered with exclusion cages. Constructions consisted of a wooden frame with a hinged lid, which was covered with a layer of gauze to ensure sufficient sunlight exposure and ventilation as well as protection against airborne seeds. In boxes with soil only (contamination control), no seedlings of the test species emerged. In the rare case that seedlings of other than the test species emerged in control boxes or boxes with faeces they were removed during the regular seedling censuses. Emerging seedlings [appearance of cotyledons (dicots) or first true leaf (*A. capillaris*)] were identified, counted and removed twice a week. All boxes were watered regularly to keep the samples continuously moist. The experiment was terminated on November 1st 2021, when no seeds had emerged for more than two weeks resulting in a total sample exposure time of five months.

2.4. Determination of moxidectin residues in sheep faeces

From the sheep faeces sub-samples described above, 3–4 g (fresh weight) were used to extract MOX residues. The extraction procedure and MOX quantification were based on a protocol by Wohde et al. (2016). To determine faeces water content, homogenized aliquots of each sample were dried for 24 h at 105°C . Mean \pm SD water content was 75.1 ± 2.1 % ($n = 19$). Before extraction, fresh samples were fortified with an internal surrogate standard (ivermectin, LGC-Standards, DRE-CA14488000) and mean \pm SD ivermectin recovery was 94.0 ± 7.8 % (n

= 22). A stock solution with 2000 ng ml⁻¹ ivermectin and MOX (Sigma-Aldrich, PHR1827) was prepared in acetonitrile (gradient grade for HPLC, 20060.320, VWR International) and diluted to the following calibration series: 1, 5, 10, 50, 100, and 2000 µg l⁻¹. To facilitate MOX extraction, fresh samples were fortified with purified water in a 15 ml polypropylene vial to increase the water content to 85–89 %. Samples were left to sit overnight, before the internal standard was spiked. For extraction, 25 ml acetonitrile were added. Before each following step, vials were manually shaken for 5 s. Vials were then sonicated for 20 min, horizontally shaken (350 rpm) for 40 min, and sonicated for a further 20 min. After this, phases were separated by centrifugation at 1970 g for 30 min at 20 °C. For clean-up and concentration, a solid phase extraction step was performed as reported by Nieman et al. (2018) using Strata C18-E cartridges (500 mg, 6 ml, Phenomenex); the routine for quantification with fluorescence detection on an Agilent 1200 HPLC system was also adopted from this protocol. Derivatization before quantification was performed as described by Wohde et al. (2016). Excitation wavelength of the fluorescence detector was 364 nm and the emission wavelength 463 nm. Column temperature was 30 °C on a Dionex Acclaim PolarAdvantage II C18-Column (3 µm, 2.1 × 150 mm). The gradient elution was performed with purified water (A) and acetonitrile (B) at 0.3 ml/min flow rate with the gradient: 0 min to 10 min, 88 % to 100 % B; 10 min to 11 min 100 % B. The injection volume was 40 µl and the retention time of the main MOX peak at 6.9 min.

2.5. Data analysis

For each replicate, we calculated final number of emerged seedlings (NES) standardised to 1 g faeces (fresh weight), mean emergence time (MET; days) and synchrony of emergence (Z; dimensionless) as dependent variables. MET and Z were calculated as proposed by Ranal et al. (2009) with regard to the process of seed germination. The three faeces collection times after each trial were merged before calculation of variables. Seeds of different plant species vary in germination behaviour, which in addition can change over time due to seed- or environment-driven processes (Baskin and Baskin, 2014). Furthermore, in seed-feeding experiments, germination varies at the level of animal individuals. Thus, differences in absolute seedling numbers caused by these processes might obscure relative response patterns. Therefore, we used the raw data (cf. Appendix A, Tables A2–A4) to calculate a ratio between the seedling number emerging from faeces of treated sheep and of the control group for each trial-data set separately, which was natural-logarithmised to meet the assumptions of the applied inferential statistics (Goldberg et al., 1999; Vogt et al., 2010). Thus, this natural-logarithm response ratio (LnRR) is defined as $\text{LnRR} = \ln(x_{\text{trt}}/x_{\text{c}})$, where x_{trt} is the seedling number emerging from faeces of treated sheep and x_{c} is the mean value of the control. An effect of an independent variable was considered significant, when the 95 % confidence interval did not overlap with zero (Koorem et al., 2011).

The overall effects of the between-subject factor (species identity) and the within-subject factor (trial) on NES, MET and Z were subjected to repeated measurement analyses. These consisted of a factorial ANOVA for the between-subject factors and a MANOVA based on Wilks lambda for the within-subject factor since the assumptions of 'circularity' and 'compound symmetry' were not fulfilled (von Ende, 1998). This procedure was employed across species including the independent variables plant species ($k = 4$; *A. ptarmica*, *A. capillaris*, *D. deltooides*, *P. lanceolata*) and seed-feeding trial ($k = 3$; 1, 7, 14 d) for the three dependent variables (NES, MET, Z). Across species and on species level, significance of differences between trials were assessed by means of Tukey HSD test.

Statistical analysis was conducted using Statistica™ 13.3 (StatSoft Inc., Tulsa, Oklahoma, USA) and graphs were created using ORIGIN® (ORIGINLab Corporation, Northampton, Massachusetts, USA).

3. Results

Analysis across species revealed significant main effects of trial on all response variables (NES, MET, Z; Table 1). Only in Z, analysis revealed additionally a significant main effect of species as well as a significant species × trial interaction (Table 1).

Across species, NES of treated sheep differed significantly from the control in all three trials (Fig. 1a). Interestingly, there was a change in direction of the effect between trials, i.e. while NES was lowered by 27.1 % compared to the control at trial 1, it was increased by 68.8 % and 45.2 % compared to the control in trial 2 and trial 3, respectively (Fig. 1a, Appendix A, Table A.2). This general pattern of change in effect direction also held true at the species level with two exceptions: NES of *Agrostis capillaris* (trial 2) and *Plantago lanceolata* (trial 3) were not significantly different from NES in trial 1. In addition, only in *Achillea ptarmica* and *Dianthus deltooides* NES was significantly different from the control in trial 2 and 3 or only in trial 2, respectively (Fig. 1a).

With respect to MET, there were no significant differences of treated and non-treated sheep in trial 1, both across species and on species level (Fig. 1b). In trial 2, MET was significantly lowered across species (−1.7 d/8.2 %), but only the response of *A. capillaris* (−2.4 d/10.7 %) and *D. deltooides* (−2.9 d/20.4 %) showed a congruent significant pattern (Appendix A, Table A.3). In contrast to NES, no significant differences occurred between trial 1 and 2. This changed in trial 3, where MET across species was significantly lowered by 22.0 % (−3.6 d) compared to the control, which was caused by a significant reduction of MET in all species, except *P. lanceolata*. While the latter was also the only species with no significant different MET between trials, the other species had significantly lower MET in trial 3 compared to trial 1, but only in *A. capillaris* MET was also significantly lowered in trial 3 compared to trial 2 (Fig. 1b).

Z showed a less clear pattern than NES and MET due to high variation within and between species. In the across-species analysis, there was no significant difference between the treatment and the control at any trial, and the trials did not differ from one another (Fig. 1c; Appendix A, Table A.4). Only in two cases, species × trial combinations differed significantly from the control, i.e. *P. lanceolata* showed a significant decrease of Z (−6.7 %) in trial 2 and *A. ptarmica* a significant increase of Z (+21.1 %) in trial 3. And in only one case was there a significant difference between trials at the species level, i.e. in *A. ptarmica*, Z was significantly higher in trial 3 than trial 1 (Fig. 1c; Appendix A, Table A.4).

Mean ± SE MOX concentration in sheep faeces decreased strongly over time with significant difference between trial 1 (3153.2 ± 417.5 ng g⁻¹ dw) and trial 2 (86.4 ± 10.8 ng g⁻¹ dw), but no further significant decrease from trial 2 to trial 3 (45.5 ± 4.9 ng g⁻¹ dw, Fig. 1d). From trial 1 to trial 3, concentration of MOX dropped by 99.0 %. In faeces from the control group, no MOX was detected (0.0 ± 0.0 ng g⁻¹ dw).

4. Discussion

4.1. Formulated moxidectin administered orally affected seedling emergence

In our in vivo experiment, we found a clear impact of formulated moxidectin (MOX) on the quantity and timing of seedlings that emerged from seeds ingested by sheep and released viable embedded in faeces substrate. This means, that individual or all components of the tested anthelmintic formulation interacted with the seeds in the sheep digestive tract and/or with the emergent seedlings in the faeces substrate. On its way through a mammalian digestive tract, pharmaceutical mixtures are de-formulated, i.e. they are broken down into its components and these components are metabolised to varying degrees (Backhaus, 2016). Thus, only those seeds that have been eaten by treated animals immediately before or after deworming are exposed to the original formulation. Seeds eaten later will be exposed to diverse mixtures of residues. As stated before, we expect the active ingredient MOX to play the central

Table 1

Effects of between-subject (BS) and within-subject (WS) factors on natural-logarithm response ratio of seedling emergence (NES), mean emergence time (MET) and synchrony of emergence (Z).

			NES			MET			Z				
Source of variation			MQ	F	p	MQ	F	p	MQ	F	p		
BS	Species (S)	3	0.62	0.83	0.484	0.22	2.39	0.083	0.31	3.97	0.014		
	Error	40	0.74			0.09			0.08				
Source of variation			DF _{num}	DF _{den}	Wilks	F	p	Wilks	F	p	Wilks	F	p
WS	Trial (T)	2	39	0.32	42.36	≤0.001	0.62	11.82	≤0.001	0.85	3.39	0.044	
	S × T	6	78	0.80	1.55	0.173	0.75	2.02	0.073	0.72	2.28	0.044	

Between-subject effects were determined by ANOVA, while within-subject effects were determined by MANOVA. DF: degrees of freedom, DF_{num}: degrees of freedom numerator, DF_{den}: degrees of freedom denominator, Wilks = Wilks λ , MQ = mean sum of squares, F = F value, p = p value. Significant results ($p \leq 0.05$) are shown in bold.

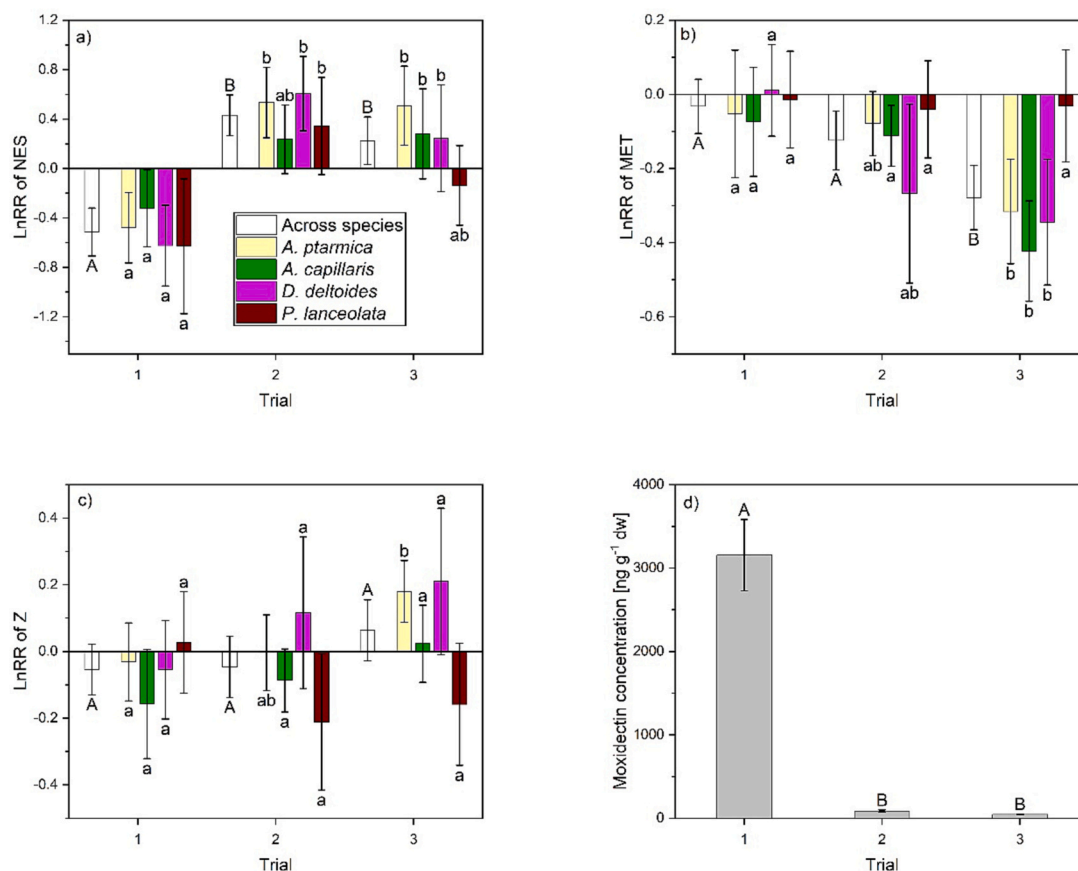


Fig. 1. Mean ($\pm 95\%$ confidence interval) natural-logarithm response ratios (LnRR) of (a) number of emerged seedlings (NES), (b) mean emergence time (MET) and (c) synchrony of emergence (Z) for the three consecutive seed-feeding trials carried out 1, 7 and 14 d, respectively, after the experimental sheep have been treated with the anthelmintic formulation Cydectin (moxidectin 0.1 %). Effect of formulated moxidectin was considered significant, i.e. different from the control, when the 95 % confidence interval did not overlap with zero. Different letters indicate significant differences between trials on across-species level (capitals) and on species level (lower case), respectively ($p \leq 0.05$, Tukey HSD test). (d) Mean (\pm SE) moxidectin concentrations in sheep faeces of the three trials ($n = 6$ per trial). Different letters indicate significant differences between trials ($p \leq 0.05$, Tukey HSD test).

role in causing the observed effects, because it is less readily metabolised in mammalian digestive tracts than its co-formulants (Johnson et al., 2017; Molento et al., 2004; Witschi et al., 1989) and mainly excreted via the faeces pathway (Afzal et al., 1994).

Ecologically, in our experiment we focused on the endozoochorous seed transport including the first stage of seedling recruitment. This multi-step section of the life cycle of seed plants includes the passage of a seed through the digestive tract of an animal, its excretion together with faeces substrate, and its germination and seedling emergence out of

faeces. At each step, macrocyclic lactone (ML) drugs may affect seed and seedling fate. There are different potential processes how MLs might affect seeds and/or seedlings. If the seed coat is penetrable for MLs, which to our knowledge has not yet been tested, embryos could be affected by MOX already at the quiescent or dormant seed stage in the chyme and/or faeces substrate. As eaten seeds remain in the digestive tract of sheep for approximately two days before they are excreted (Cosyns et al., 2005), the uptake of water (and chemical substances dissolved in it), the first step in the germination process, already starts

during passage of the digestive tract (Baskin and Baskin, 2014). However, after being ingested by a recently dewormed sheep, seeds are not only influenced by anthelmintic substances but in the course of ruminating also regularly and repeatedly by physical (mastication) and chemical (stomach acid, digestive enzymes) factors (Manzano et al., 2005; Ramos et al., 2006; D'hondt and Hoffmann, 2011; Mancilla-Leytón et al., 2011). These factors could potentially scarify the seed coat so that MOX would more readily reach the embryo.

Once contact has been made with the quiescent, dormant or germinating embryo, MOX could affect chloride channels in cells, leading to a change in membrane conductance (McKellar and Gokbulut, 2012). Even though this mode of action has been proven in animal cells, it is theoretically also conceivable in plant cells because they have homologous channel genes (Elter et al., 2007; Ward et al., 2009). Another possibility might be the hindrance of protein synthesis, where MOX molecules could dock on multiple active sites of protein-substructures/subunits, preventing them from forming correctly and therefore functioning correctly or at all. This process was reported for the ML ivermectin and viral proteins, which were then not able to connect with each other thus stopping viral reproduction (Lehrer and Rheinstein, 2020; Bello, 2021).

4.2. Effects of formulated moxidectin depended on timing of seed ingestion

In line with our expectations, the feeding experiment revealed a strong time-dependency of effects of formulated MOX on ingested seeds. We found a hormetic-like response pattern for the quantity of emergence and for the average time of emergence, but not for the distribution of emergence over time, i.e. synchrony, which showed no general pattern.

Related to the number of emerging seedlings (NES), only the first seed-application trial showed a lowering effect coinciding with high MOX concentrations in the faeces, while the other two trials showed enhancing effects that coincide with low MOX concentrations. On the species level, this general pattern holds true for two test species (*Achillea ptarmica*, *Dianthus deltoides*). However, in all test species, NES was significantly reduced in trial 1 compared to the control and all species showed at least trends of enhancements of NES in later trials. The result for trial 1 is comparable to and consistent with a previous feeding experiment, where sheep received formulated moxidectin immediately after administration of *Plantago lanceolata* seeds (Eichberg et al., 2016). Although mean emergence time (MET) was not affected in the first trial, the significant reduction of MET compared to control in the second and/or third trial in three species, corroborated the patterns found in NES, which again indicated a stimulating effect on emergence at low MOX concentrations. It may surprise that this pattern did not show up in NES when looking at the raw data, which suggest changes in seedling emergence over time to occur rather in controls than in treatment samples. However, one has to keep in mind that not only plant species vary in germination capacity but that this capacity also varies over time due to seed- or environment-driven processes (Baskin and Baskin, 2014). Against this backdrop, a focus on relative differences between treatment and independent control groups, which we ensured by calculating the log-response ratio, is more appropriate.

An explanation for the widespread phenomenon of stimulation in the lower concentration range of a substance is upregulation of adaptive cellular stress response pathways (ACSRPs; Mattson and Cheng, 2006; Mattson, 2008). These enhance the expression of genes encoding cytoprotective proteins and growth factors when specific receptors in the cell membrane are triggered (Mattson, 2008; Mattson and Calabrese, 2010). Receptors could be triggered by the effects of reactive oxygen species (ROS; Mattson and Calabrese, 2010) on the cell. ROS are present in higher concentrations in plants when in prolonged contact with MLs, such as ivermectin (Navrátilová et al., 2020). In plant species, hormetic effects are well known and have been described for several plant parts, including seeds, with research on organic environmental contaminants so far focusing mainly on herbicides (Belz et al., 2011; Belz and Duke,

2014; Cedergreen, 2008) and antibiotics (Agathokleous et al., 2018; Migliore et al., 2010; Pan and Chu, 2016; Xie et al., 2011). To our knowledge, the present study is the first to find evidence to suggest hormetic effects of anthelmintics on non-target organisms, i.e. plants.

Even when low MOX concentrations were present in the faeces analysed here (14 d after deworming), all emergence variables were affected at least in some species. This finding is in line with other studies that have highlighted the importance of low concentrations of organic environmental contaminants in affecting wild plant populations (Minden et al., 2017). Reported values for ML contents in faeces cover a broad range with the highest reported value for MOX concentration after oral administration to sheep being $3390 \text{ ng g}^{-1} \text{ 1 d pt}$ (Steel, 1998), which is in line with our measurements. The route of administration greatly influences the amount of ML excreted and the time period an ML is excreted in relevant/detectable amounts (Suárez et al., 2009). After a single subcutaneous MOX administration to sheep, Hentz et al. (2019) found a maximum MOX concentration of 22.7 ng g^{-1} in dry faeces at 1.7 d post treatment which is lower than the minimum of 45.5 ng g^{-1} that we measured in the present study 14 days after oral MOX administration in sheep faeces.

4.3. Formulated moxidectin has larger ecological relevance than previously assumed

Our study shows that the treatment of sheep with anthelmintics can change emergence patterns of seedlings. Since sheep are important seed dispersers in fragmented landscapes (Albert et al., 2015; Eichberg et al., 2007; Kuiters and Huiskes, 2010), this impact might have consequences for assembly and diversity of grazed plant communities. Sheep influence not only the spatial distribution of seeds but also their post-dispersal fate (Rosenthal et al., 2012). Without the influence of MLs, emergence of seedlings from sheep faeces is enhanced by sheep trampling because cracking of faeces pellets and mixing with soil material promotes seedling recruitment (Faust et al., 2011). These turbation events are common on sheep pastures, especially in dry grasslands, because grazing animals, which are kept in large migratory herds, can develop high movement and trampling dynamics (Hillenbrand et al., 2019; Plachter and Hampicke, 2010).

However, trampling may not have advantages for seeds in all circumstances. For example, trampling on ML-contaminated faeces might lead to a quicker and more complete incorporation of MLs into the soil, potentially harming even seeds in the soil seed bank. We conclude that the risk MLs pose to grazed habitats concerns not only coprophilous arthropods (Lumaret et al., 2012) but also vascular plants. Also, due to their long elimination half-lives, not only single habitats but habitat networks are affected (i.e., metapopulations), if dewormed sheep are kept in large roaming flocks.

Changes in number and timing of seedling emergence can have opposite effects on a plant population. While more seedlings emerging earlier might give species an interspecific competitive advantage leading to a higher abundance of a species in a plant community, higher seedling densities might induce increased seedling mortality due to higher intraspecific competition (Fenner and Thompson, 2005). Several studies showed that a reduced emergence time fosters plant growth faster and flower production per plant (de Luis et al., 2008; Orrock and Christopher, 2010; Verdú and Traveset, 2005). Still, in the long run, stimulation of emergence through changes in the seed's environment may lead to a depletion of the soil seed bank that species developed as a bet-hedging strategy against short- and long-term environmental stochasticities (Hölzel and Otte, 2004), which are particularly given with grazing (Fenner and Thompson, 2005).

We still know too little about the effects of MLs on seeds and need more research in this field. But it is not too early to recommend that domestic ungulates freshly treated with ML anthelmintics should not be placed on pasture grassland to avoid unwanted side effects on plant populations. This is in line with risk avoidance recommendations for

animal non-target organisms, such as dung beetles, that have been given for some time (Floate et al., 2005).

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CRediT authorship contribution statement

Lars Laber: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Anabell Jan-dowsky:** Methodology, Validation, Investigation, Resources, Writing – review & editing. **Kai Frölich:** Methodology, Resources, Writing – review & editing. **Andre P. Heinrich:** Methodology, Writing – original draft, Writing – review & editing. **Rolf-Alexander Düring:** Methodology, Resources, Writing – review & editing, Funding acquisition. **Tobias W. Donath:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Carsten Eichberg:** Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data regarding this publication is now available online at the eDal-Data repository under the DOI: <http://dx.doi.org/10.5447/ipk/2023/23>.

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Appendix A. Supplementary data

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