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RESEARCH ARTICLE

Effects of macrocyclic lactone anthelmintics on seed germination of temperate grassland species

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ABSTRACT

- Macrocyclic lactone anthelmintics are widely used to control invertebrate pests in livestock, such as sheep. While anthelmintic effects on non-target animals, such as dung-dwelling insects, are well studied, effects on seed germination are largely unknown. Seeds can come into contact with anthelmintics either during passage through the gastro-intestinal tract of grazing animals or when anthelmintics are excreted with their dung into the environment, which may result in changed germination patterns.
- We used four commonly applied macrocyclic lactones to assess their effects on germination: moxidectin, ivermectin, abamectin and doramectin as pure substances; moxidectin and ivermectin also in formulated form. We tested these pharmaceuticals on 17 different temperate grassland species from five plant families. Seeds were exposed to three concentrations of macrocyclic lactones (0.1, 1.0 and 10.0 $mg \cdot l^{-1}$) under controlled conditions, and germination was assessed over a 6-week period. From these data, we calculated germination percentage, mean germination time and germination synchrony.
- Most of the tested species were significantly affected in germination percentage and/or mean germination time by at least one of the tested pharmaceuticals, with formulated moxidectin having the largest impact. In general, the effects found were species- and pharmaceutical-specific. While formulated substances generally reduced germination percentage and increased mean germination time, pure substances increased germination percentage. Synchrony showed less clear patterns in all pharmaceuticals.
- Although effect size and sign varied between species, our study shows that non-target effects of macrocyclic lactones commonly occur in terrestrial plants. This may impede successful seed exchange between habitats *via* sheep, and even translate into profound changes to grazed ecosystems.

INTRODUCTION

Anthelmintics are widely used around the world to rid livestock of parasites, which are a main cause of reduced livestock productivity and welfare. A particular potent and common group of anthelmintics are macrocyclic lactones (MLs), including commonly used representatives such as moxidectin (MOX), ivermectin (IVM), abamectin (ABA) and doramectin (DOR) (Vercruysse & Rew 2002). Target organisms of MLs are common parasites that live in animals (*e.g.* their intestines), such as nematodes and tapeworms (Gokbulut *et al.* 2010). From a medical point of view, MLs are ideal anthelmintics, since their mechanism affects glutamate-gated chloride channels, which are absent from almost all vertebrates, making them insensitive to these substances (Vercruysse & Rew 2002). Since MLs are only weakly metabolized, weakly absorbed in the body and bind preferentially to solid particles due to their high lipophilicity, a large part of the administered active substance is excreted *via* dung (Lifschitz *et al.* 2000; Junco *et al.* 2021).

Many studies have shown that the concentrations of ML anthelmintics in livestock dung are sufficient to kill non-target organisms, in particular dung-decomposing arthropods, such as dung beetles (Lumaret *et al.* 2012; Blanckenhorn *et al.* 2013). However, the effects of ML anthelmintics on other invertebrate groups (*e.g.* earthworms, springtails), on the soil microbiome and on terrestrial vascular plants are largely unknown. Organisms can also be indirectly affected by ML anthelmintics. For instance, a lack of dung-decomposing arthropods can reduce germination success of excreted and viable seeds or nutrient return into the soil (Bang *et al.* 2005; Milotić *et al.* 2019; deCastro-Arrazola *et al.* 2020). While the uptake of MLs from soil by plants has been shown in several studies (Iglesias *et al.* 2018; Navrátilová *et al.* 2020a; Navrátilová *et al.* 2020b), the effects of MLs on plant reproduction are not well studied.

Our research focuses on seed germination, since this development stage is a critical stage in the life of plant individuals and determines survival and persistence of plant populations (Grubb 1977; Fenner 2000). Most species require specific environmental conditions for successful germination (Baskin & Baskin 2014). First evidence that ML anthelmintics can influence seed germination was found by Eichberg *et al.* (2016). They tested three temperate grassland species and showed that formulated as well as

pure MOX can decrease germination success and alter the timing of germination with the formulation revealing stronger effects compared to the pure substance. Vokřál *et al.* (2019) found IVM solutions reduced *Sinapis alba* seedling root length. To our knowledge, no study has yet assessed effects of ABA or DOR on seed germination parameters.

Seeds potentially come into contact with MLs as they pass through the gastro-intestinal tract of treated domestic ungulates, and may remain in contact with MLs when collectively excreted and embedded in dung heaps. Concentrations of MLs are much higher in dung than in digestive contents. As such, ingested seeds are in contact with the highest ML concentration for the longest time (Chiu *et al.* 1990; Fernandez *et al.* 2009). In general, in grasslands, internal seed dispersal (endozoochory) by ungulate species plays an important role for plant population dynamics (Bartuszevige & Endress 2008; Albert *et al.* 2015a) and also ensures establishment of new and maintenance of already existing plant populations in grazed grasslands (Rosenthal *et al.* 2012).

Sheep, for example, are not only effective seed dispersers, but they also recruit seedlings (Rosenthal *et al.* 2012; Eichberg & Donath 2018). Especially in fragmented cultural landscapes, where dispersal limitation is a major hindrance to the restoration of species-rich grasslands, sheep are often moved between habitat islands of the same vegetation type; this coupled function, is important for maintenance of a high phytodiversity (Rosenthal *et al.* 2012). Considering the abundance of livestock regularly treated with MLs, the large numbers of seeds consumed by livestock (Kuiters & Huiskes 2010) and long elimination half-life of MLs in the gastro-intestinal tract (IVM 2 days, MOX 15 days; Molento *et al.* 2004), a considerable number of seeds could come into contact with anthelmintic substances. Therefore, any effects on the effectiveness of seed germination after ingestion by livestock will be relevant for nature conservation. If ML anthelmintics influence germination, this could lead to changes in plant population sizes and, ultimately, to shifts in plant species compositions of grazed habitats.

Besides a reduction of germination percentage, a shift in the timing of germination can also hamper successful establishment of seedlings, if germination occurs at inopportune times (Ross & Harper 1972; Dyer *et al.* 2000; Rühl *et al.* 2016). Therefore, the number of seeds germinating is not sufficient to assess the effects of environmental conditions on seed germination (Ranal & de Santana 2006; Ranal *et al.* 2009). Additional parameters of germination behaviour, such as mean germination time or synchrony of germination, allow for a more specific evaluation of effects on seed germination (Ranal & de Santana 2006; Ranal *et al.* 2009). Perennial plant species may rely less on temporally spreading emergence risk because they are less dependent on environmental variation in time than annuals, due to their iteroparous reproduction (Rees 1996). However, in herbaceous perennials, early emergence is often related to higher fitness and fecundity in terms of seedling survival, seedling height/biomass and number of flowers (Verdú & Traveset 2005).

To determine the extent to which ML effects on seed germination of plant species of temperate grasslands can be generalized, we carried out a climate cabinet experiment. We tested the effects of four common MLs as pure substances (MOX, IVM, ABA, DOR) at three concentrations, as well as MOX and IVM as pharmaceutical formulations (Cydectin®, MOX; Oramec®, IVM) on 17 grassland plant species belonging to five families. All active ingredients are currently used in sheep husbandry.

We addressed the following questions:

- How general are responses of seeds of grassland species to macrocyclic lactone anthelmintics in terms of germination?
- Do higher concentrations of the active ingredients (pure or formulated) lead to stronger effects on seed germination?
- Are there differences in the effects caused by the pure active ingredient or its formulation?

MATERIALS AND METHODS

Test species and seed material

We tested seed germination of 17 species with predominantly perennial life strategies belonging to five plant families (Asteraceae, Caryophyllaceae, Fabaceae, Plantaginaceae and Poaceae; Table 1). The test species are typical representatives of temperate grasslands in Central Europe and cover a broad spectrum of soil conditions (Ellenberg indicator values for moisture: 3–7, soil reaction: 3–8 and nitrogen: 2–7; Ellenberg & Leuschner 2009). For most of the test species references exist for natural spread of their seeds by sheep *via* passage through the gastro-intestinal tract in viable condition (Mitlacher *et al.* 2002; Albert *et al.* 2015b; Benthien *et al.* 2016). A meta-analysis showed that the ecological filtering effect of endozoochorous seed transport is comparatively low, suggesting that more species are dispersed endozoochorously than have been recorded so far (Albert *et al.* 2015a). Seeds were obtained from a commercial supplier (Rieger-Hofmann, Blaufelden-Raboldshausen, Germany). Nomenclature of plant species follows Müller *et al.* (2021).

Test substances

We tested the four active ingredients moxidectin (MOX, $C_{37}H_{53}NO_8$, 640 g·mol^{-1}), ivermectin (IVM, $C_{48}H_{74}O_{14}$, 875 g·mol^{-1}), abamectin (ABA, $C_{49}H_{75}O_{14}$, 873 g·mol^{-1}) and

Table 1. Tested plant species and information on plant family and germination percentage (GP; mean ± SE) for the blank control treatment (C_W).

Family	Species	Blank control (C_W) GP [%]
Asteraceae	*Achillea ptarmica* L.	29.1 ± 9.1
	Centaurea jacea L.	25.7 ± 2.5
	Hypochaeris radicata L.	92.0 ± 2.0
Caryophyllaceae	*Cerastium holosteoides* Fr.	47.1 ± 2.7
	Dianthus deltoides L.	63.4 ± 4.8
	Lychnis flos-cuculi L.	55.7 ± 3.9
Fabaceae	*Hippocrepis comosa* L.	22.3 ± 2.5
	Medicago lupulina L.	25.4 ± 4.7
	Trifolium aureum POLLICH	71.4 ± 3.3
	Trifolium pratense L.	81.4 ± 2.6
Plantaginaceae	*Plantago lanceolata* L.	34.0 ± 3.1
	Plantago media L.	22.0 ± 3.2
	Veronica officinalis L.	5.4 ± 0.9
Poaceae	*Agrostis capillaris* L.	70.6 ± 4.3
	Cynosurus cristatus L.	78.3 ± 4.1
	Lolium perenne L.	53.7 ± 5.5
	Phleum phleoides (L.) H. KARST	7.4 ± 0.8

doramectin (DOR, $C_{50}H_{74}O_{14}$, 899 g·mol^{-1}), which were obtained from SigmaAldrich (Taufkirchen, Germany; purities of all substances ≥95%). For MOX and IVM, we also used common formulated veterinary products for application on sheep: Cydectin® 0.1% oral drench (MOX, Zoetis Deutschland, Berlin, Germany) and Oramec® 0.08% oral drench (IVM, Boehringer Ingelheim Vetmedica, Ingelheim, Germany). Oral administration is the typical application route of macrocyclic lactone (ML) formulations in small ruminants (Prichard *et al.* 2012).

As MLs, the tested active ingredients have a three-part pharmacophore in common, consisting of a 16-membered macrocycle with fused benzofuran and spiroketal units (McKellar & Gokbulut 2012). There are two ML sub-classes, differing structurally mainly by presence (avermectins) or absence (milbemycins) of a disaccharide substituent at C-13 (McKellar & Gokbulut 2012). We studied three avermectins (IVM, ABA and DOR) and one milbemycin (MOX). Besides absence of a sugar moiety, MOX differs structurally from avermectins by the presence of a methoxime moiety (contains N) at C-23 and an olefinic group at C-25 (Prichard *et al.* 2012). In addition, it has a much lower molecular weight. The study avermectins differ only slightly structurally from each other; specifically, in the bonds at C-22,23 and/or substituents at C-25 (Vercruysse & Rew 2002). MLs essentially exert the same mode of action: In invertebrates, they bind to glutamate-gated chloride channels leading to an irreversible influx of chloride ions followed by membrane hyperpolarization and muscle paralysis (Köhler 2001). However, the precise mode of action has not yet been fully clarified, and the structural differences, especially in MOX *versus* avermectins, lead to different interactions of MLs with chloride channels and pharmacodynamic behaviours (Prichard *et al.* 2012).

Anthelmintic formulations contain further ingredients in addition to the respective active ingredient (these are summarized here under the term "co-formulants"; Table S1). Nevertheless, in order to improve readability, we also refer to the concentration of the active ingredient in relation to effects of formulations.

We studied active ingredient concentrations in the range of 0.1–10.0 mg·l^{-1} for all six test drugs. These values are within the range of concentrations of MLs reported for substrates (digestive contents, dung) that are relevant for a seed endozoochorously dispersed by sheep. The comparability of values of ML concentrations in environmental matrices related to sheep is complicated by the fact that there are few published values and methods and units vary. After intraruminal administration of single formulated MLs (MOX, IVM, ABA) to lambs, Lloberas *et al.* (2013) found mean concentrations for digestive contents in the range of 452 ng·g^{-1} (0.5 days post treatment (p.t.); equivalent to 0.45 mg·l^{-1}) to 32 ng·g^{-1} (2 days p.t.; 0.03 mg·l^{-1}). In their study, the mean concentrations of the three tested MLs were similar at 0.5 days p.t. Generally, dung had much higher ML concentrations than digestive contents, but reported values cover a broad range between 23 ng·g^{-1} (MOX, 40 h p.t., subcutaneous administration, (Hentz *et al.* 2019), equivalent to 0.02 mg·l^{-1}) and 13,615 ng·g^{-1} (IVM, 1 day p.t., oral administration, Lang 1996; 13.62 mg·l^{-1}). The following values for sheep dung were found within this range in other studies: 0.93 µg·g^{-1} (IVM, 2 days p.t., subcutaneous administration; Vokřál *et al.* 2019), 1277 ng·g^{-1} (ABA, 3 days p.t., subcutaneous administration; Kolar *et al.* 2006), 2186 ng·g^{-1} (DOR, 2 days p.t., subcutaneous administration; Kolar *et al.* 2006) and 3390 ppb (MOX, 1 day p.t., oral administration; Steel 1998).

Experimental design

We prepared aqueous solutions of the six anthelmintic drugs in three concentrations of the active ingredient: 0.1, 1.0 and 10.0 mg·l^{-1}. In a first step, we prepared stock solutions of 0.04, 0.4 and 4.0 mg·ml^{-1} in ethanol (purity: 99%, Carl Roth), since MLs are highly lipophilic (Hennessy 1997). From each of these stock solutions, 0.5 ml was taken and added to 200 ml ddH_2O to create the treatment solutions. In case of the formulated MLs, we added the appropriate amount of the anthelmintic drug directly to ddH_2O to achieve the same anthelmintic concentrations as used with the pure chemical. In addition, two types of control were established: a blank control (C_W) with ddH_2O only, and an alcohol control with an ethanol concentration of 2.5 µl·ml^{-1} (C_E, solvent: ddH_2O). C_E was used as a control for the pure substances, since they were dissolved in the same amount of alcohol. C_W was used as a control for the formulated anthelmintics, since these formulations are aqueous-based solutions. Throughout the lab work, we used glass materials for the preparation of the solutions as MLs are known to adhere to plastics surfaces (Cerkvenik *et al.* 2002).

We used glass Petri dishes with a diameter of 9 cm and fitted them with two filter papers each, so that the whole dish and a part of the rim was covered to prevent seeds from slipping under the papers. Each dish contained 50 seeds of one plant species and 5 ml of the respective treatment solution. Seven replicates were generated for each treatment combination (fixed factors: *anthelmintic*, *concentration* and *species*) and for the two control types, resulting in a total of 2,800 experimental units. To reduce evaporation, five Petri dishes were stacked in one transparent plastic bag. Each bag contained only dishes with the same anthelmintic drug and concentration, but different seed species to avoid cross-treatment effects and ensure randomization. Dishes were kept in three identical climate cabinets.

Prior to the germination census period, all dishes were exposed to a phase of stratification for 1 week in which cabinets were set to 4 °C and complete darkness to improve seed germination (Baskin & Baskin 2014). After this initial cold-wet stratification phase, the dishes were exposed to a day/night cycle of 16 /8 h and 15/5 °C. Twice per week and at equal intervals germinated seeds (appearance of radicle) were counted and removed from the dishes. The positions of the bags in the cabinets and the positions of the dishes in the bags were randomly changed after every census. The experiment was terminated after 6 weeks.

Data analysis

Three response variables were calculated from the data obtained. First, we calculated final germination percentage (GP, %) for each replicate, *i.e.* the percentage of germinated seeds from the initial number of seeds (Ranal *et al.* 2009).

Second, mean germination time (MGT) was calculated using the formula:

$$\mathrm{MGT\ [d]} = \frac{\sum_{i=1}^{k} n_i t_i}{\sum_{i=1}^{k} n_i}, \quad (1)$$

where i is the day number, t_i is time from day 1 to day i, n_i is number of germinated seeds on day i and k is the final time of germination (Ranal *et al.* 2009). MGT indicates the average number of days the seeds of one replicate needed to germinate.

Third, the synchrony of germination (Z, dimensionless) was calculated from the formulae:

$$Z = \frac{\sum_{i=1}^{k} C_{n_i,2}}{C_{\sum n_i,2}} \quad (2)$$

$$C_{n_i,2} = \frac{n_i(n_i-1)}{2} \quad (3)$$

where C_{n_i} is the two-by-two combination of the seeds that germinated at day i, and n_i is the number of germinated seeds at day i. Z represents how synchronous a species germinated during the study period and generally ranges from 0 to 1. Increasing values indicate increasing synchrony of germination (Ranal *et al.* 2009).

We started our experiment with 20 plant species (four species from each of the five plant families), but three species (*Arenaria serpyllifolia*, Caryophyllaceae; *Serratula tinctoria*, Asteraceae; *Veronica teucrium*, Plantaginaceae) did not show sufficient germination success for statistical testing and were excluded from analyses.

Univariate three-way ANOVA was applied to analyse effects of *species*, *family* and *anthelmintic concentration* on the dependent variables GP, MGT and Z. For this analysis, *species* was nested in *family*. Although we have chosen only four species per family, which is too few species to assess phylogenetic effects, we included *family* in our model, since we expected it to explain some of the variance. This analysis will be referred to as "across species" from this point onward. One-way ANOVA was used for analyses of the effects of concentration at the species level, which will be referred to as "at species level". Post-hoc Tukey HSD test was used if significant effects were revealed. Before employing inferential statistics, data were visually checked using QQ plots and scatterplots of observed values against non-standardized residuals whether necessary requirements were met, and we employed Box-Cox transformation to meet normality and homogeneity of variance in every case.

Statistical analysis was done with Statistica™ 13.3 (StatSoft, Tulsa, OK, USA) and graphs were created using ORIGIN® (ORIGINLab, Northampton, MA, USA).

RESULTS

The ANOVAs across species revealed that the general response patterns of germination parameters caused by the pure or formulated anthelmintics showed large variations (Table 2). Only the factors *species*(*family*) and *family* were significant for all germination parameters and anthelmintics. In contrast, the significance patterns of the factor *concentration* and its interactions with other factors, as the main interest of our study, were often quite inconsistent. However, with the exception of *Medicago lupulina*, the germination behaviours of all plant species were influenced by at least one pure macrocyclic lactone (ML) substance or formulation. Interestingly, the comparison of germination in the two controls (C_E *versus* C_W) revealed that the solubilizer ethanol, used to dissolve pure MLs in water, also itself had an impact on germination of some species: decreasing in germination percentage (GP) and increasing in mean germination time (MGT) (Table 1, Table S2).

Effects of pure and formulated moxidectin on germination

The ANOVA across species for pure MOX treatments revealed a significant main effect for *concentration* and a *family* × *concentration* interaction in GP, as well as a significant *species* (*family*) × *concentration* interaction in synchrony (Z) (Table 2). Across species, GP was lowest in the control (35.4%; ethanol control: C_E), and differed significantly from all concentration levels (38.3–38.6%) (Fig. 1a). Analysis at species level showed that for GP only in four species was the control significantly different from at least one concentration level (Table S3). In all four species, GP was enhanced for most concentration levels compared to the control. GP of *Centaurea jacea* was enhanced up to 81.5% (10.0 $mg \cdot l^{-1}$), GP of *Hypochaeris radicata* up to 5.8% (1.0 $mg \cdot l^{-1}$) and GP of *Plantago lanceolata* up to 84.0% (10.0 $mg \cdot l^{-1}$) compared to the control (C_E). For MGT, in contrast to the across-species analysis (Table 2), analysis at species level did reveal concentration effects. In three species (*H. radicata*, *Hippocrepis comosa* and *Agrostis capillaris*), MGT of MOX-treated seeds differed significantly from the control (C_E) for at least one concentration level, while for one species (*Lolium perenne*), we found significant differences between concentration levels but not compared to the control

Table 2. ANOVA results for effects of *species* (nested within *family*), *family* and *concentration* of all tested macrocyclic lactone drugs on germination percentage (GP), mean germination time (MGT) and synchrony of germination (Z) ($P > 0.05$ = –, $0.05 \geq P > 0.01$ = *, $0.01 \geq P > 0.001$ = **, $P \leq 0.001$ = ***). For detailed information, see Tables S21–S26.

	Moxidectin			Cydectin			Ivermectin			Oramec			Abamectin			Doramectin		
	GP	MGT	Z	GP	MGT	Z	GP	MGT	Z	GP	MGT	Z	GP	MGT	Z	GP	MGT	Z
Species (Family) [S(F)]	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
Family [F]	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
Concentration [C]	***	–	–	***	***	*	***	–	–	***	***	–	***	***	–	*	–	**
S(F) × C	–	–	**	***	***	***	–	***	–	***	***	***	***	***	–	**	–	–
F × C	*	–	–	***	***	***	–	***	–	*	**	***	***	***	–	–	–	*

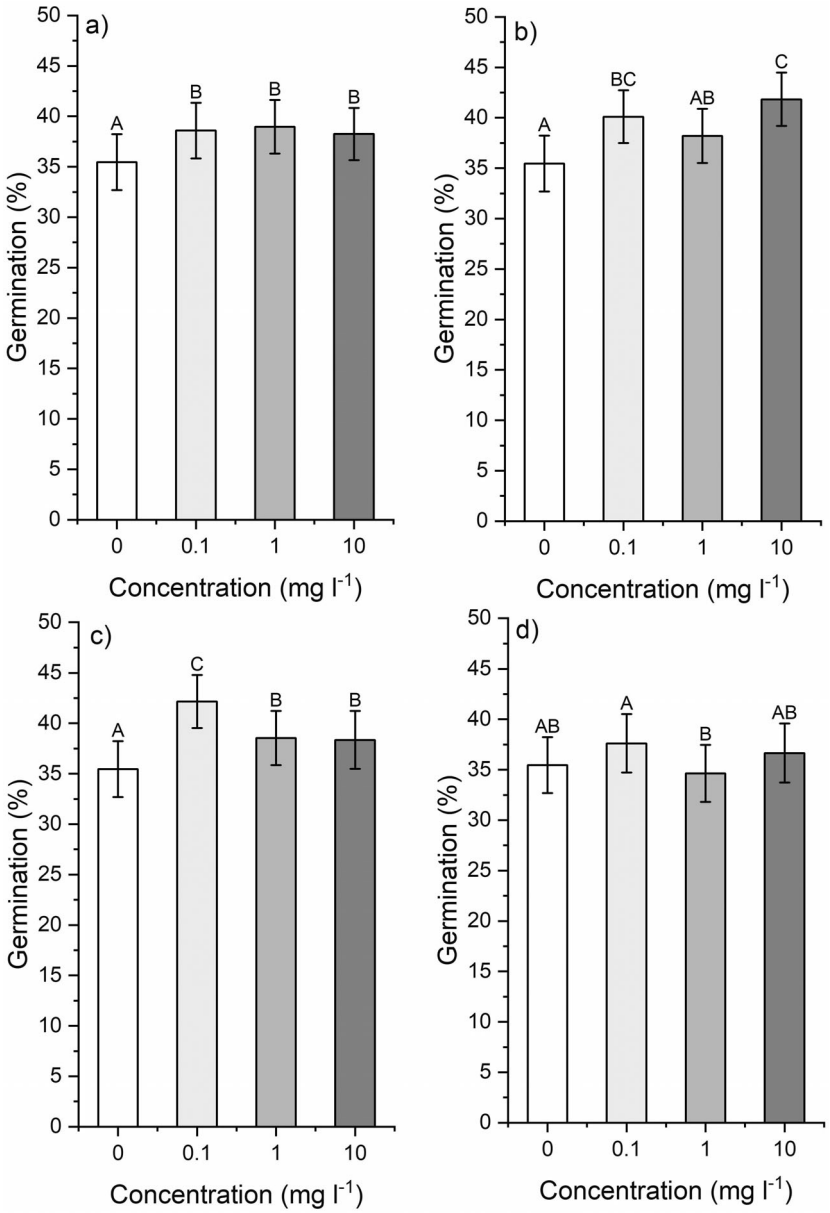

Fig. 1. Mean germination percentage ± SE across species at different concentrations of the pure macrocyclic lactones moxidectin (a), ivermectin (b), abamectin (c) and doramectin (d). Means and errors were calculated with untransformed data. Box-Cox transformed data were used to test for significance ($P \leq 0.05$; Tukey HSD test). Different letters above bars indicate significant differences between concentration levels.

(Table S4). In *H. radicata*, MGT was significantly increased at the 0.1 mg·l^{-1} concentration (+2.4 days) and 1.0 mg·l^{-1} (+2.3 days) level compared to the (C_E) while in the other two species, MGT was significantly reduced (−2.4 days, *A. capillaris*, 1.0 mg·l^{-1}; −4.1 days, *H. comosa*, 0.1 mg·l^{-1}). For Z, analysis at species level revealed no significant differences in the post-hoc tests in any species (Table S5).

In contrast to pure MOX, formulated MOX (Cydectin) analysis across species showed significant main effects for all factors and their interactions on all response variables (Table 2). Across species, blank control (C_W) had the highest GP and differed significantly from all concentration levels, which decreased significantly with increasing concentration (Fig. 2a). In terms of the number of species significantly affected, formulated MOX showed more effects than pure MOX: GP of 12 species was changed by formulated MOX compared to the control (C_W) (Table S6). Eight of these species showed reductions of GP >80% at the highest MOX concentration (10.0 mg l^{-1}). In contrast, effects of lower concentrations were less pronounced (Fig. 2a). In nine species, significant differences between concentration levels within the same species were found (Table S6).

While in pure MOX treatments, analysis across species revealed no significant differences between concentration levels for MGT compared to the control (C_E) (not shown), in formulated MOX treatments, analysis across species revealed

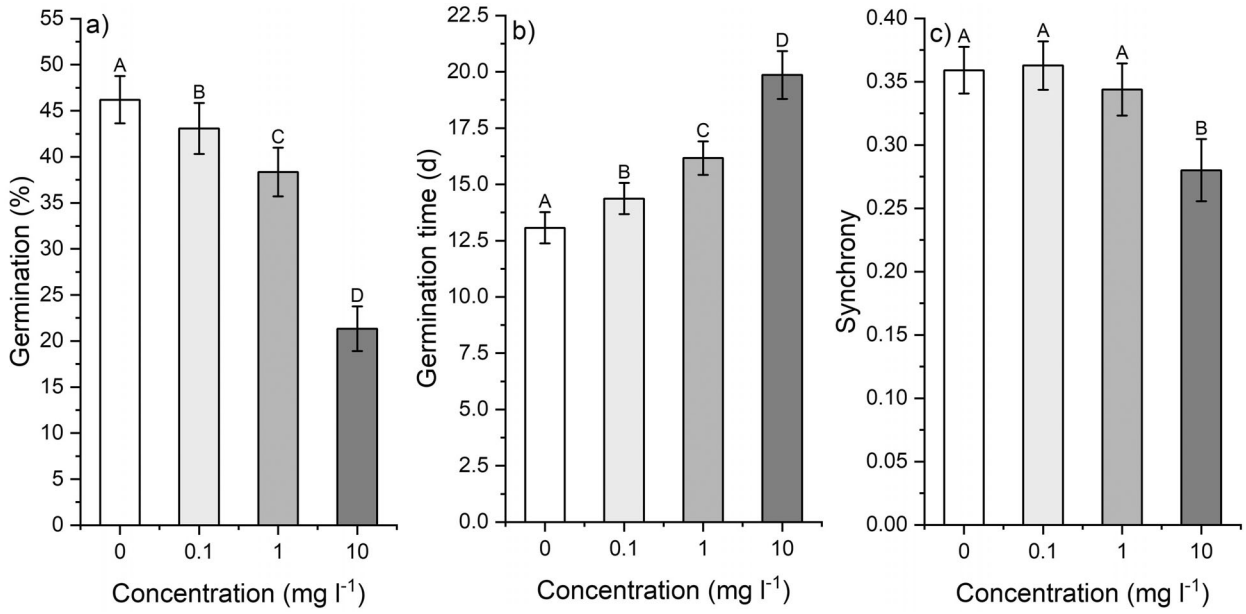

Fig. 2. Effects of different Cydectin (formulated moxidectin) concentrations on mean germination percentage ± SE (a), mean germination time ± SE (b) and mean synchrony of germination ± SE (c) (across-species analysis). Means and errors were calculated with untransformed data. Box-Cox transformed data were used to test for significance ($P \leq 0.05$; Tukey HSD test). Different letters above bars indicate significant differences between concentration levels.

significant differences between all concentration levels (including control C_W), with MGT rising significantly with concentration (Fig. 2b). In 12 species, MGT increased significantly with formulated MOX compared to the control (C_W) (Table S7). In formulated MOX, mean increase of MGT across species and concentration levels was around 3.7 days, and the highest increase for a single species and concentration was 27.3 days (*Dianthus deltoides* at 10.0 mg·l^{-1}). *H. comosa* (+3.8 days, 10.0 mg·l^{-1}), *Trifolium pratense* (+5.3 days, 10.0 mg·l^{-1}) and *L. perenne* (+7.7 days, 10.0 mg·l^{-1}), in which GP was not significantly affected, differed significantly from the control (C_W) in regard to MGT. In nine species, significant differences between concentration levels were revealed by analysis at species level (Table S7).

Across species, Z was significantly reduced at the highest concentration level of formulated MOX (10.0 mg·l^{-1}; Fig. 2c). Only in four test species were significant differences between at least one concentration level and control (C_W) found in Z (Table S8).

In summary, we found contrary response patterns between pure and formulated MOX: Compared to the respective controls (C_W, C_E) and across all other factors, formulated MOX significantly decreased GP (−25.9%) and significantly increased MGT (+26.5%), whereas pure MOX significantly increased GP (+8.9%) and decreased MGT (−0.4%). Both ML drugs significantly but weakly affected Z (Cydectin: −0.6%, MOX: +0.4%).

Effects of pure and formulated ivermectin on germination

Across species, pure IVM caused effects related to *concentration* on GP (main effect) and MGT (interactions), whereas Z was not affected (Table 2). Analysis across species showed that GP was enhanced with the control (C_E), differing significantly between the 0.1 and 10.0 mg·l^{-1} levels (Fig. 1b). Among concentration levels, 10.0 mg·l^{-1} differed significantly from 1.0 mg·l^{-1} (Fig. 1b). Analysis at species level revealed GP was significantly raised with pure IVM at least at one concentration compared to the control (C_E) only in two species (*C. jacea*, +79.6%, 10.0 mg·l^{-1}; *P. lanceolata*, +68.0%, 10.0 mg·l^{-1}) (Table S9).

While analysis across species for MGT revealed no significant differences between concentration levels and compared to the control (C_E) (not shown), analysis at species level revealed that four species were significantly affected by pure IVM at least at one concentration compared to the control (C_E), but these effects were inconsistent in sign and size (Table S10). The four species significantly affected were *H. radicata* (+3.1 days, 0.1 mg·l^{-1}), *H. comosa* (−4.1 days, 1.0 mg·l^{-1}), *T. pratense* (−2.2 days, 0.1 mg·l^{-1}) and *Cynosurus cristatus* (+3.6 days, 0.1 mg·l^{-1}). For Z, only in *T. pratense* (−38.5%, 10.0 mg·l^{-1}) were there significant differences between concentration levels and control (C_E) (Table S11).

Analysis across species with formulated IVM revealed significant effects of almost all main factors and their interactions on GP, MGT and Z (Table 2; exception: no main effect for *concentration* in Z). For GP, the 1.0 mg·l^{-1} (41.6%) and 10.0 mg·l^{-1} (40.5%) treatments differed significantly from the control (46.2%; C_W), while the 0.1 mg·l^{-1} level did not differ from the control and the 1.0 mg·l^{-1} level, which also did not differ from the 10.0 mg·l^{-1} level (Fig. 3a). Analysis at species level showed significant decreasing effects on GP in four species: *Cerastium holosteoides* (−45.5%, 10.0 mg·l^{-1}), *Lychnis flos-cuculi* (up to −49.7%, 0.1 mg·l^{-1}), *Plantago media* (up to −76.6%, 1.0 and 10.0 mg·l^{-1}) and *Phleum phleoides* (up to −61.5%, 10.0 mg·l^{-1}) (Table S12).

Analysis across species in MGT revealed a small yet significant increasing effect of *concentration* for formulated IVM,

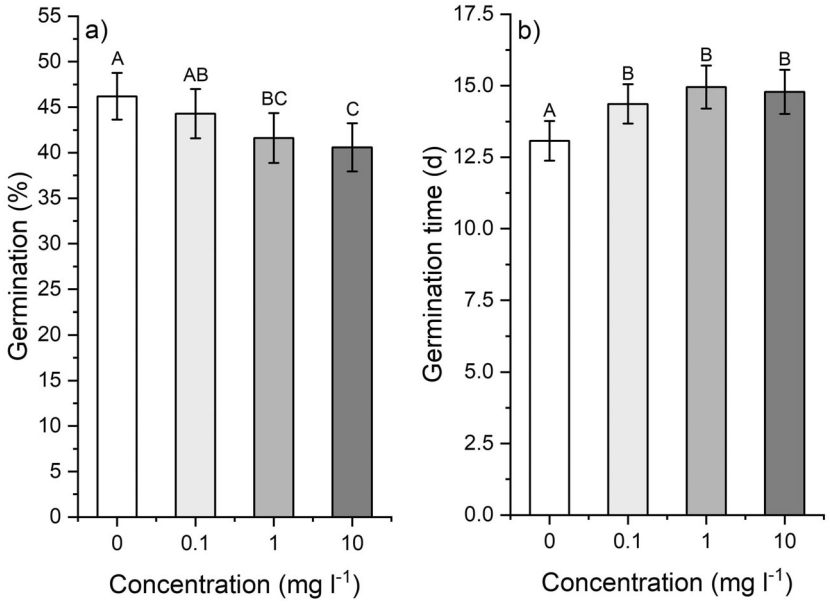

Fig. 3. Effects of different Oramec (formulated ivermectin) concentrations on mean germination percentage ± SE (a) and mean germination time ± SE (b) (across-species analysis). Means and errors were calculated with untransformed data. Box-Cox transformed data were used to test for significance ($P \leq 0.05$; Tukey HSD test). Different letters above bars indicate significant differences between concentration levels.

with all concentration levels differing from the control (C_W), but not from each other (Fig. 3b). Analysis of *concentration* effects for formulated IVM at species level revealed a significant difference from controls in five species: *C. jacea* up to −6.0 days (10.0 mg·l^{-1}), *H. radicata* up to −2.7 days (0.1 mg·l^{-1}), *C. holosteoides* up to +10.1 days (1.0 mg·l^{-1}), *T. pratense* up to +1.9 days (10.0 mg·l^{-1}) and *L. perenne* up to +1.0 day (1.0 mg·l^{-1}) (Table S13).

Despite the strong main effects and interactions (excluding *concentration*) for Z revealed by analysis across species (Table 2), only a few species were affected by formulated IVM, *i.e.* only *A. capillaris* (up to −35.2%, 1.0 and 10.0 mg·l^{-1}) and *D. deltoides* (up to −46.3%, 1.0 mg·l^{-1}), with a significant deviation from the control (C_W) (Table S14).

Comparing the differences to the respective controls (C_W resp. C_E) across all species and concentrations, formulated IVM significantly reduced GP (−8.7%), while pure IVM significantly enhanced GP (+12.9%) (Figs 1b and 3a). MGT was, compared to the control (C_W), only significantly affected by formulated IVM (+1.6 days; Fig. 3b). Only in Z, both pure and formulated IVM had no significant effects compared to control (C_W to C_E) across all species (Table 2).

Comparison of effects of formulated moxidectin and formulated ivermectin

Across species, both ML formulations led to similar response patterns in GP (regular decrease with concentration) and MGT (regular increase), with stronger effects in formulated MOX than in formulated IVM (GP across the three concentration levels: MOX −25.9%, IVM −8.7%; MGT across the three concentration levels: MOX +26.5%, IVM +12.5%; Figs 2a and b and 3a and b). Formulated MOX (16 species) also significantly influenced more species than formulated IVM (11 sp.) when taking all germination parameters into account (Tables S6–S8, S12–S14).

Effects of pure abamectin on germination

Analysis across species revealed significant interaction and main effects of pure ABA for all fixed factors in GP and MGT, while Z only showed significant general effects for *species(family)* and *family* (Table 2). Across species, GP was highest at 0.1 mg·l^{-1} (42.2%) differing significantly from the control (35.4%; C_E) and the other concentration levels (38.4% and 38.5%, 1.0 and 10.0 mg·l^{-1}, respectively; Fig. 1c). Analysis at species level for GP revealed significant increases compared to the control (C_E) for five species, with maxima for each species at the lowest concentration (0.1 mg·l^{-1}; *C. jacea* + 105.5%, *L. flos-cuculi* + 304.5%, *P. lanceolata* + 60.0%, *P. media* + 137.2%, *Veronica officinalis* + 587.8%; Table S15).

While in MGT seven species showed a significant difference compared to the control (C_E), effects were more inconsistent than in GP (Table S16). Across all species, MGT was highest at 10.0 mg·l^{-1} (19.1 days), differing significantly from the 0.1 mg·l^{-1} level (16.4 days), but not from the control (18.3 days) or the 1.0 mg·l^{-1} level (18.0 days) (Fig. 4). Only 0.1 mg·l^{-1} differed significantly from the control (C_E). For Z, no species showed significant differences between control (C_E) and concentration level or levels among each other (Table S17).

Effects of pure doramectin on germination

Analysis across species for pure DOR showed that GP was highest at the 0.1 mg·l^{-1} level (37.6%), which differed significantly from the 1.0 mg·l^{-1} level (34.6%) (Fig. 1d). In contrast, MGT and Z showed no significant differences between concentrations for DOR across species (not shown). Analysis

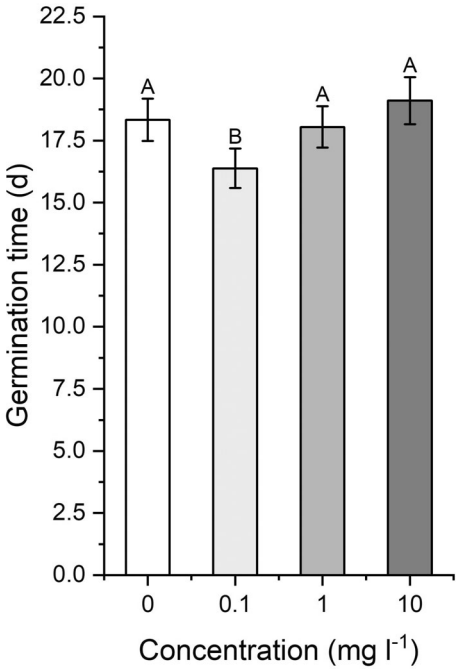

Fig. 4. Mean germination time ± SE across species at different concentrations of pure abamectin. Means and errors were calculated with untransformed data. Box-Cox transformed data were used to test for significance ($P \leq 0.05$; Tukey HSD test). Different letters above bars indicate significant differences between concentration levels.

at species level revealed that only *P. lanceolata* (+52.0%, 0.1 mg·l^{-1}) was significantly affected by DOR compared to the control (C_E; Table S18). MGT was significantly affected in three species (*D. deltoides*, *A. capillaris* and *C. cristatus*) compared to the control (C_E), but effects were mostly weak and inconsistent with respect to concentration (Table S19). In GP and Z, analysis across species revealed a main and interaction *concentration* effect [Table 2, *species(family)* × *concentration* for GP, *family* × *concentration* for Z], but whe analysed at species level, there were no significant effects for Z (Table S20).

Comparison of effects of pure anthelmintics

For GP, the number of species in which the control (C_E) was significantly different from at least one concentration level of a pure ML decreases in the following order: ABA (5 species) > MOX (4) > IVM (2) > DOR (1) (Tables S3, S9, S15 and S18). *P. lanceolata* was the only species in which all four pure substances affected GP (in all cases, with an increase in GP). The same species did not respond significantly to any pure ML in timing variables (MGT, Z). For MGT, the following ranking in the number of species with significant differences between control (C_E) and treatment was found: ABA (6) > IVM (4) > MOX (3), DOR (3) (Tables S4, S10, S16 and S19). Of all pure MLs used in this experiment, only DOR increased MGT across species and concentrations, while all others decreased MGT compared to the control (C_E). In Z, with the exception of *T. pratense* in pure IVM, no species was affected by any of the pure substances used (Tables S5, S11, S17 and S20).

DISCUSSION

How general are responses of seeds of grassland species to macrocyclic lactone anthelmintics in terms of germination?

Our study is the first multi-species study concerned with effects of macrocyclic lactones (MLs) on seed germination and showed that, with the exception of *Medicago lupulina*, all test species were influenced in their germination behaviour by at least one ML drug. However, we found large variations in ML effects on seed germination and inconsistent patterns within and among species. We suggest two possible mechanisms through which MLs could affect seed germination. One possibility would be MLs seeping into the seed through the testa and then changing seed dormancy and germination behaviour before it becomes visible in the form of an emerged radicle. Thus, the variations we observed could be the result of differing seed coat traits, such as thickness, chemical composition and special structural elements, like micropores. In addition, the specific factors to break seed dormancy vary between species, so the seeds could be affected at different stages of development, meaning the MLs might not affect all species to the same extent or on the same molecular process (Baskin & Baskin 2004; Soltani *et al.* 2018). A second possibility would be that the tested MLs cannot pass through the testa and first come into contact with embryo tissue after the testa has been cracked by the growing radicle.

Not only did the effects of a specific ML on different species vary, effects of different MLs on the same species also varied. Since MLs are very similar in their molecular structure and biological activity, this result is surprising. Moxidectin (MOX) and the avermectins show the largest structural differences (Shoop *et al.* 1995). Differences in molecular structure can have various effects (Prichard *et al.* 2012), *e.g.* improving or hindering entrance into cells *via* protein pathways, impeding or enhancing export of ML molecules, which impact retention times (Shoop *et al.* 1995). Possibly, the slight differences of MLs in molecular structure impact interactions with other molecules, *e.g.* interaction with P-glycoproteins, to which ivermectin (IVM) and MOX have vastly different affinities (Shoop *et al.* 1995;Griffin *et al.* 2005; McKellar & Gokbulut 2012; Lloberas *et al.* 2013). In animals, this leads to differing excretion rates, since P-glycoproteins are responsible for transporting MLs out of cells back into the intestinal lumen (Griffin *et al.* 2005; McKellar & Gokbulut 2012; Lloberas *et al.* 2013).

Another possible binding site of MLs in seed embryos are glutamate-gated chloride channels, which are the sites of action in invertebrates. Plant cells have similar channels, so MLs could have an effect on those (Elter *et al.* 2007; Ward *et al.* 2009). Also, the disruption of proteins inside plant cells by ML attachment to protein binding sites might be a relevant process. Studies have shown the capacity of IVM to bind to several viral proteins, rendering them inert and stopping reproduction, as well as binding to the transport proteins IMP α/β1, preventing transport into the nucleus (Caly *et al.* 2020; Heidary & Gharebaghi 2020; Lehrer & Rheinstein 2020; Bello 2022).

Most likely, the substance- and species-specific responses observed result from a combination of the factors mentioned above: differences in testa structure, number and structure of chloride channels and P-glycoproteins and differences in molecular structure of the MLs probably affect their ability to

pass through the testa and cell membranes and to bind to cell structures. In addition, still unknown mechanisms might play a role.

Do higher concentrations of the active ingredients or formulations lead to stronger effects on seed germination?

In formulated MOX (Cydectin), we found the effects on seed germination, especially on germination percentage (GP) and mean germination time (MGT), increase in strength with increasing concentrations for most species, which is in accordance with previous studies (Eichberg *et al.* 2016, 2022). Across all species, this pattern was similarly observed for formulated IVM (Oramec), but not for the four pure substances, where we found no regularly increasing or decreasing concentration-specific response patterns. At species level, in all six tested ML pharmaceuticals, non-linear and species-specific response patterns were found, which is also in accordance with previous studies (Eichberg *et al.* 2016, 2022). We found that the highest concentration of MLs (10.0 $mg \cdot l^{-1}$) affected seeds of most test species, whereas at lower concentrations (0.1, 1.0 $mg \cdot l^{-1}$) effects were less frequent and less consistent (except for pure abamectin).

With each concentration tested, the number of molecules increased tenfold, but the effect did not, *e.g.* the differences from the control in affected species did not increase at the same level as the ML concentration (Tables S3–S20). This might indicate the existence of a threshold in at least some species, below which MLs have close to no effect, and above which they can affect germination. Studies have shown that ML overdoses can negatively affect vertebrates, because they cross the blood–brain barrier and affect neural cells (Martin *et al.* 2021). This could be attributed to MLs overwhelming P-glycoproteins, since dogs with genetically caused deficiencies in these proteins are susceptible to IVM side effects (Mealey *et al.* 2002; Griffin *et al.* 2005). Since plants have homologous P-glycoproteins, we suggest that a sufficiently high number of ML molecules would also overwhelm P-glycoproteins, thus affecting intracellular processes and therefore germination (Terasaka *et al.* 2005; Blakeslee *et al.* 2007). These thresholds could vary between species, even in the same family, which would also explain why the effects are so inconsistent between species. Higher concentrations of MLs could then negate any effects caused by the lower concentrations. These effects occurred only with pure substances and predominantly affected MGT.

Interestingly, in contrast to Eichberg *et al.* (2016), where effects of pure MOX on GP were neutral, in the current study GP of most species was enhanced by the pure ML solutions, including MOX (Fig. 1). In general, the increase in GP might be attributed to the ML molecules causing intracellular stress and as a response to a higher metabolic activity, as found in multiple studies in regard to other typically phytotoxic substances applied in small doses (Migliore *et al.* 2000, 2003, 2010; Christou *et al.* 2018). This assumption is supported by studies showing that MLs, when taken up by plants, trigger stress response mechanisms (Navrátilová *et al.* 2020b; Langhansová *et al.* 2021).

Are there differences between the pure active pharmaceutical ingredient and formulations?

Formulated MOX affected far more species and to a much larger extent than any of the other five MLs tested. Since we were not able to produce controls with all other co-formulants of formulated MOX or IVM, which differ in their ingredients, we cannot rule out the possibility that the larger effect was caused by single co-formulants or their combinations. Until now, ecotoxicological research has focused strongly on analysis of the active ingredients of agrochemical formulations (Backhaus *et al.* 2008). However, many active ingredients of veterinary drugs are applied in formulated form. This is because these substances need to be protected from chemical degradation, *e.g.* by oxidation or hydrolysis, and/or facilitated by co-formulants, *e.g.* by improving solubility or permeability, in order to be fully effective (Awasthi *et al.* 2013).

Nevertheless, despite their omnipresence, the ecotoxicological study of co-formulants has been largely neglected and there are few studies on the effects of these substances on living organisms, especially on terrestrial vascular plants. The study of effects of chemical substances occurring in mixtures, *e.g.* formulations, has also been neglected (Backhaus *et al.* 2008; Christou *et al.* 2018). Consequently, the scientific as well as the political community is increasingly finding that toxic substances in the environment occur in mixtures and not individually, and that a risk assessment of agrochemical formulations is only realistic if ingredient mixtures are studied (Nagy *et al.* 2020; Authority (EFSA) 2022).

There is evidence that synergistic or additive effects occur when mixtures, rather than single substances, act on plants (Christou *et al.* 2018). One example would be the stabilization of ML molecules by co-formulants, *e.g.* protection from degradation by UV light or temperature. If ML molecules in formulation remain stable for longer than their pure counterparts, they can affect seeds or processes in seeds for far longer and to a far larger extent, which would explain the differences. It is reasonable to assume that pure MLs degraded relatively quickly in our experiment, having a half-life of around 7 days under daylight conditions and moderate temperatures (Halley *et al.* 1989, 1993; Bloom & Matheson 1993; Adler *et al.* 2016). In water, such degradation could be even faster, as little as 12 h according to Halley *et al.* (1993). This would reduce the length of contact of MLs with the seeds to potentially far less than the formulated MLs, assuming co-formulants stabilize the active ingredients in these conditions.

The solubility-enhancing effect of some co-formulants on lipophilic MLs in aqueous formulations (Shoop *et al.* 1995) could allow MLs to seep into seeds, which might not have been possible without such assistance. This would explain why some species were affected only by formulations, not by pure MLs. Furthermore, there is also evidence that co-formulants can have an independent toxicity. For instance, for benzyl alcohol (a solving and conservative substance) and butylated hydroxytoluene (an antioxidant), which are both particularly frequent and abundant in Cydectin (Table S1), it has been reported that they directly affect seed germination (Taylorson 1988, 1989; Zamyatnina *et al.* 2003; Lu *et al.* 2013; Tang *et al.* 2013; Zhao *et al.* 2019).

Similar to co-formulants, ethanol, which was used as a solubilizer to dissolve the lipophilic pure MLs in water in the present study, affected some test species. GP tended to be reduced and MGT increased in the ethanol control (C_E) compared to the blank control (C_W). This is not surprising, since species-specific effects of ethanol on germination are known. While at concentrations similar to the ethanol concentration used in the

present study (0.25%), no effects on germination percentage were found in previous studies (Foley & Chao 2008; Salehi *et al.* 2008; Eichberg *et al.* 2016), effect size increased significantly at higher ethanol concentration (≥0.60%) (Foley & Chao 2008; Salehi *et al.* 2008; Afzal *et al.* 2013). However, we do not consider this alcohol effect on germination affects the general validity of our findings, since the controls, *i.e.* C_E and C_W, were systematically compared only to the pure and formulated macrocyclic lactones, respectively. In general, from a methodological viewpoint, it remains unresolved which might be the optimal solubilizer for non-polar chemicals in germination experiments.

Ecological relevance

While the ecological consequences of decreased GP are straightforward, *i.e.* lower germination directly translates into lower seedling establishment and subsequently lower number of adult plants (Grubb 1977), changes in MGT and synchrony (Z) might be more ambivalent. In general, seeds of a certain species usually germinate when environmental conditions are ideal; thus, both accelerated and delayed germination can have negative effects on post-germination fate. While the two ML formulations used in our experiment led to increased MGT, pure MLs generally decreased MGT (with the exception of doramectin). A decreased MGT could cause the plant to germinate and grow before the optimal climate conditions, like a longer rainy period. Conversely, an increase in MGT is usually detrimental to growth, because the plant is then substantially disadvantaged in the race for resources, particularly sunlight and water (Ross & Harper 1972; Dyer *et al.* 2000). This can cause a significant reduction in fitness and plant biomass (Rühl *et al.* 2016). Similarly, while less synchronous germination, *i.e.* lower Z, indicates a spread of germination into potentially less suitable periods, more synchronous germination, *i.e.* higher Z, incapacitates the spread of germination over a certain time period as a bet-hedging strategy against environmental stochasticity (Ludewig *et al.* 2014).

Studies show that, depending on substance and application, MLs are excreted by livestock for weeks or even months and in measureable amounts (Chiu *et al.* 1990; Kolar *et al.* 2006; Fernandez *et al.* 2009; Vokřál *et al.* 2019) and, therefore, could influence plant germination for a long period throughout the year. Reports suggest IVM persistence in soil varies between 7 and 240 days (Halley *et al.* 1993; Alvinerie *et al.* 1999; Mougin *et al.* 2003). These timeframes are highly dependent on soil composition and temperature; Bloom & Matheson (1993) measured the half-life in winter conditions as up to to 52 weeks. Within this timeframe, it is reasonable to assume that animals would undergo a second anthelmintic treatment, leading to the accumulating of MLs in soil over time (Wohde *et al.* 2016). Accumulation of MLs on or in pastures would then become a serious problem for fauna and flora alike, as our study has shown the effects these substances can have on multiple germination factors.

Although, the mode of action of MLs in plants and the ecological significance of our findings need to be further explored in future studies, our results support the notion that, in general, the germination of most species is affected by MLs. Despite the variability in sign and size of the response of the addressed germination parameters, ecological relevance is very likely since MLs are excreted into the environment largely unchanged and in comparatively large amounts after administration to livestock (Suárez *et al.* 2009; Beynon 2012). In addition, large numbers of seeds potentially come into contact with MLs (and co-formulants) in the course of phytomass consumption and passage of eaten seeds through the gastrointestinal tract of freshly dewormed sheep or other herbivores (Rosenthal *et al.* 2012). In the long run, there might be effects on the biodiversity of larger areas, especially when plant species rely on endozoochory to spread and bridge larger distances (Albert *et al.* 2015a,b) and plant populations are exposed to ML influences over a longer period of time (Mougin *et al.* 2003).

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CONFLICT OF INTEREST

The Authors declare none.

AUTHOR CONTRIBUTIONS

L. Laber, C. Eichberg and T. W. Donath designed the experiment. L. Laber and A. Zimmerbeutel performed the experiments. L. Laber, C. Eichberg and T. W. Donath analysed and interpreted the data and wrote the main part of the manuscript. All authors discussed the results and reviewed and edited the manuscript.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Ingredients of the tested anthelmintic formulations.

Tables S2–S20. ANOVA and post-hoc Tukey HSD results for all tested anthelmintic drugs at species level; mean values of ethanol controls.

Tables S21–S26. ANOVA results for all tested anthelmintic drugs across species.

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