



Factors affecting colonisation success of the anecic earthworm *Lumbricus terrestris* (L.) in mesocosms on temperate pasture

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Abstract

Background & aims Changing precipitation patterns require climate adaptive measures to improve water regulation. Deep vertical earthworm burrows dug by the anecic species *Lumbricus terrestris* contribute to water infiltration rate and capacity, and deeper plant root growth. *L. terrestris* is considered a native species to western Europe, reaching its highest abundances in pastures. In pastures where the species is currently absent, water regulation could improve after inoculation with these earthworms. We conducted a field experiment to test the feasibility of introducing *L. terrestris*.

Methods Mesocosms were installed at two Dutch dairy farms. One farm had a resident *L. terrestris* population, the other did not. Subsequently, *L. terrestris* was introduced: half of the mesocosms received locally collected earthworms (NL), and the other half

received commercially imported inoculum from Canada (CA). Twelve months later, the mesocosms were harvested and all earthworms were counted.

Results The field experiment proved that *L. terrestris* can survive and produce offspring after introduction. At the location with a resident population, 15% of the *L. terrestris* introduced (tagged with Visible Implant Elastomer-tags) had survived, and at the *L. terrestris*-free site this was 26%. A hypothesised interspecific competitive relationship with *Lumbricus rubellus* (Hoffmeister) was not confirmed. Locally collected inoculum performed equal to or better than the commercial inoculum.

Conclusions Earthworm origin seems to influence chances of mesocosm colonisation success. VIE-tagging possibly interfered with survival. Future research could involve the role of pathogens and colonisation success in a non-enclosed set-up for a longer period of time.

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Introduction

The challenge of practising agriculture in a changing climate calls for climate adaptive strategies (Anderson et al. 2020). Ecological innovations can help to answer this call, for example with water regulation.

Precipitation patterns in Europe have shifted, resulting in increased incidence of peak rains (Santos et al. 2016), combined with prolonged periods of drought (Samaniego et al. 2018; Grillakis 2019). This shift can have a major impact on grasslands (Fitton et al. 2019), which represent one of the most prevalent land-use types of the continent. Agricultural grasslands cover over 48.0 million ha in Europe (Eurostat 2023—*Cropping Patterns*) and play an important role in both food production and water regulation. Rapid water infiltration is key to prevent flooding, runoff and associated nutrient loss during peak rains, also to replenishing groundwater for more resilience in periods of drought. Deep rooting makes grasslands and arable crops more resilient to prolonged periods of drought.

The anecic earthworm *L. terrestris* occurs in grasslands, and it can address all of the aforementioned issues (Edwards et al. 1990; Bouché & Al-Addan 1997; Spurgeon et al. 2013; Hallam & Hodson 2020). This species creates semi-permanent (Potvin & Lilleskov 2017) vertical burrows that can reach depths of up to two meters (Edwards & Bohlen 1996). These burrows connect the soil surface to deeper soil layers, allowing water to flow through, while also facilitating plants to extend their roots further down (Edwards & Lofty 1978). These features have been shown to improve the water infiltration rate and water infiltration capacity of grasslands (Edwards et al. 1990; Bouché & Al-Addan 1997; Spurgeon et al. 2013; Hallam & Hodson 2020). Introduction of *L. terrestris* into grasslands where it is absent could be an ecological innovation to improve the ecosystem service of water regulation.

In the Netherlands, 24% of the surface area is used as agricultural grassland (CBS website 2024), mainly associated with dairy production. *L. terrestris* is considered a native species, and it occurs throughout the country and in the three major soil types in the Netherlands (clay, peat and sand). However, at the level of individual grasslands, its occurrence and abundance are heterogenous and this diversity cannot be easily explained. Field inventories on grasslands reported population densities varying from 0 ind. m⁻² to over 400 ind. m⁻² (Van Eekeren et al. 2014; van de Logt et al. 2023a).

Low abundance or even absence of *L. terrestris* does not necessarily mean that the grassland in question is unsuitable to accommodate the species.

Previous research reports slow dispersal, with dispersion rates of 1.1 m year⁻¹ in arable heavy clay under boreal conditions (Nuutinen et al. 2006); 1.4 m year⁻¹ (Ligthart & Peek 1997) and 4.5 m year⁻¹ (Hoogerkamp et al. 1983), both in temperate Dutch pastures on a silt loam soil; 6 m year⁻¹ in an arable system on silt loam under a humid continental climate (Shuster et al. 2003). Moreover, a fragmented landscape where individual farms or grasslands are surrounded by roads and waterways can make it impossible for *L. terrestris* to move from one grassland to the next. In combination with the low dispersal rate, this fragmentation may simply prevent the species from reaching new territory. Soil disturbance in the form of tillage can be detrimental to anecic earthworms such as *L. terrestris* (Chan 2001; Van Eekeren et al. 2008; Fox et al. 2017) and could lead to local decimation or extinction of the population. Given the above, re-establishment can be difficult. Other specific factors may also have an effect on *L. terrestris* abundance. Correlative analysis of *L. terrestris* occurrence on sandy soil in relation to environmental factors indicated that the species is found more often and more abundantly in grasslands with a finer soil texture (Lindahl et al. 2009), i.e., loamier sand (Van de Logt & Van der Sluijs et al., 2023a). Loaminess possibly contributes to the water retention capacity of sandy soil. *L. terrestris* was found less in grasslands with a high groundwater level, probably to avoid the risk of flooding of its burrows (Hoogerkamp et al. 1983; Van de Logt & Van der Sluijs et al., 2023a). Additionally, interspecific competition for food with *Lumbricus rubellus* may play a role (Lowe & Butt 2002; Shuster et al. 2003; Van de Logt et al. 2023b). Partial overlap in the dietary niche of the two species in combination with the higher metabolic rate (Shipitalo et al. 1988) and higher reproduction rate of *L. rubellus* (Butt 2011; Klok & de Roos 1996) may limit population growth of *L. terrestris*.

The *L. terrestris* invasion in Canada (Addison 2009) and successful settlement of *L. terrestris* in the Dutch Flevopolder (Hoogerkamp et al. 1983) show that under suitable conditions, the species is able to disperse and establish itself. We expect that many grasslands have the potential to support *L. terrestris*, and the presence of *L. terrestris* is likely to improve the water-regulating capacity of these grasslands. Several research projects have explored the possibility of *L. terrestris* inoculation. Insights have been

gained into *L. terrestris* survival rate (Butt 1999; Andriuzzi et al. 2015; Forey et al. 2018), offspring production (Van de Logt et al. 2023a, b), dispersal (Hoogerkamp et al. 1983; Lighthart & Peek 1997), life history (Butt et al. 1994), the effect of management (Grigoropoulou & Butt 2010; Nuutinen et al. 2011; Kautz et al. 2014; Frazão et al. 2019) and experimental set-up (Butt 2008). It was shown that *L. terrestris* can survive for more than one year and also produce offspring after being introduced in a grassland mesocosm set-up (Van de Logt et al. 2023b). Despite the potential of inoculation, so far the survival and reproduction rate on sandy soils have not been high enough to conclude that *L. terrestris* inoculation of grasslands is a viable ecological innovation that can contribute to climate adaptation through improved water regulation. In this research we aimed to increase our understanding of which factors determine colonisation success of *L. terrestris*.

Building upon previous research, we designed and executed an *L. terrestris* inoculation experiment in mesocosms at two locations: unique about this set-up was that one location was selected where *L. terrestris* was already present and one where this species was absent. Also, both locally collected and commercially purchased earthworms were used, as to test for the effect of origin on colonisation success. The objective of the experiment was to test the following hypotheses: locally collected *L. terrestris* are better adapted to local field conditions and will therefore outperform imported Canadian *L. terrestris*, resulting in (I) higher survival rate and (II) higher reproductive output. Moreover, we hypothesised that *L. terrestris* will benefit from the absence of a competing resident earthworm population, resulting in (III) higher survival rate and (IV) higher reproductive output in the mesocosm with hand-sorted soil. In particular, we expected to find (V) a negative correlation between *L. terrestris* and *L. rubellus* abundance.

Materials and methods

Study area

In September 2021, a field experiment was set up in the south of the Netherlands. We selected two permanent grasslands on sandy soil at two organic dairy farms which were 8 km apart. Location A was

situated at 51°35'56.6"N 5°07'03.7"E and Location B at 51°38'31.4"N 5°12'25.3"E. Location A was selected because *L. terrestris* was already present and Location B was selected because *L. terrestris* was absent, as a baseline assessment indicated in the previous year. The baseline assessment was carried out following the same procedure at both farms: two 40*40*20 cm pits were dug at least 25 m apart and at least 10 m from the edge of the grassland. All soil from the pits was hand-sorted for earthworms and four litres of 0.01% allyl-isothiocyanate (AITC) solution was applied to the bottom of the pit to collect earthworms from deeper soil layers. All earthworms emerging at the pit surface within 20 min were collected, rinsed with water and stored in 70% ethanol for later species determination (see paragraph "[Earthworm extraction and identification at experimental end](#)" for details). Soil samples (0–10 cm and 30–40 cm depth) were taken in duplo and analysed by Eurofins (Table 1). The soil type at Location A can be classified as Typic Humaquept, with a higher silt content and groundwater table than Location B, which can be classified as Typic Haploquod (USDA classification) (Table 1). Both grasslands were dominated by perennial rye-grass (*Lolium perenne*) and white clover (*Trifolium repens*), management consisted of cutting and grazing at both farms (Table 1) with a total N-input of 170 kg N ha⁻¹ from slurry manure, and dung and urine from grazing. Because of its soil texture and groundwater table, Location B was more sensitive to drought and was irrigated during prolonged periods of drought (See Supp. Fig. A.2 for weather data and Supp. Table A.2 for soil temperature).

Installation of the mesocosm field experiment

We set out the plots and installed the mesocosms in both grasslands. With a crane, steel pipes (ø 61 cm, height 50 cm) were driven 40 cm into the soil, leaving a 10 cm ring above the soil surface (Supp. Fig. A.1). Pipes were installed in two rows of equal length, and pipes were spaced 90 cm apart. We assigned the mesocosms to six different treatments in a randomised block design (Table 2) with four replicates per treatment at Location A and three replicates per treatment at Location B.

At the time of installation, from half of the mesocosms (treatments 2, 4 and 6), the soil was removed in three layers: 0–20 cm, 20–40 cm, and 40–60 cm,

Table 1 Soil analysis and management of Locations A and B. Each value represents the mean of the two samples that were taken

Site	Location A	Location B
Chemical properties 0–10 cm		
pH-KCl	5.3	6.0
Organic matter (%)	5.2	4.4
N-total (g N/kg dry soil)	2210	2015
P-Al (mg P ₂ O ₅ /100 g)	63	71
% sand	65	81
% silt	27.5	13
% clay	2.5	1.5
Chemical properties 30–40 cm		
pH-KCl	5.2	5.6
Organic matter (%)	3.7	2.8
N-total (g N/kg dry soil)	1410	1115
P-Al (mg P ₂ O ₅ /100 g)	58	75
% sand	67	83
% silt	28	13
% lutum	2	1
Groundwater stage	VIIId*	VIIIo*
Management		
Cutting	Yes	Yes
Grazing	Yes	Yes
Irrigation	No	Yes
Fertilisation (total N kg ha ⁻¹)	170	170

*Groundwater stage VIIId refers to a mean highest groundwater level of 80–140 cm and mean lowest groundwater level > 180 cm; VIIIo refers a mean highest groundwater level of > 140 cm and mean lowest groundwater level > 180 cm

followed by mustard extraction at 60 cm. The soil removed was hand-sorted on site to remove all earthworms (cocoons were left in the soil). No *L. terrestris* was found at Location B, re-confirming the absence of the species from this grassland. After hand-sorting, the soil was returned to the mesocosm it had been removed from, with each layer in its original place, and subsequently grass-clover was seeded. The mesocosms under this treatment are referred to as ‘disturbed soil’.

Earthworm origin

The earthworms used as inoculum had two different origins. The first group of *L. terrestris* earthworms for the experiment was purchased commercially from a Dutch earthworm importer (<https://www.thedutchnighcrawlers.nl/>). The earthworms purchased originated from Ontario, Canada, where they had been collected from agricultural fields to serve the bait market (for more details, see Steckley 2020). These earthworms are referred to as ‘CA’ inoculum. The second group of *L. terrestris* was collected at Location A through mustard extraction. A rectangular, 15 cm deep pit was dug, and to this pit a mixture of water and mustard powder was applied (50 gr of Coleman’s mustard powder per 10 L of water, Grigoropoulou and Butt 2010). Earthworms that surfaced in the following 45 min were carefully collected, without pulling them, and immediately rinsed with tap water. Until the moment of introduction, the earthworms collected were kept in a bucket with soil, grass-clover

Table 2 Overview of all treatments that were applied to the mesocosms in the field experiment

Treatment	Soil	Earthworms
1. Undisturbed soil, control	Undisturbed permanent grassland	No earthworm inoculation
2. Disturbed soil, control	0–60 cm hand-sorted and returned to mesocosm, mustard extraction at 60 cm depth	Resident population removed
3. Undisturbed soil, Canadian <i>L. terrestris</i> (CA)	Undisturbed permanent grassland	Inoculated with 15 commercially purchased <i>L. terrestris</i> (51 m ⁻¹)
4. Disturbed soil, Canadian <i>L. terrestris</i> (CA)	0–60 cm hand-sorted and returned to mesocosm, mustard extraction at 60 cm depth	Resident population removed. Inoculated with 15 commercially purchased, <i>L. terrestris</i> (51 m ⁻¹)
5. Undisturbed soil, Dutch <i>L. terrestris</i> (NL)	Undisturbed permanent grassland	Inoculated with 15 locally collected <i>L. terrestris</i> (51 m ⁻¹)
6. Disturbed soil, Dutch <i>L. terrestris</i> (NL)	0–60 cm hand-sorted and returned to mesocosm, mustard extraction at 60 cm depth	Resident population removed. Inoculated with 15 locally collected <i>L. terrestris</i> (51 m ⁻¹)

cuttings and cow manure from the resident grassland. These earthworms are referred to as ‘NL inoculum’.

To distinguish between the introduced *L. terrestris* and resident *L. terrestris* at the end of the experiment, we tagged earthworms destined for inoculation at Location A. Visible Implant Elastomer (VIE) tags were prepared following the instructions from the supplier (Northwest Marine Technology, Inc.). A droplet of elastomer was injected behind the clitellum of all *L. terrestris* that were to be introduced at Location A (Butt & Lowe 2007).

Earthworm inoculation

Twenty holes (equivalent to \varnothing 0.4 cm, 5 cm in depth) were made in the soil surface of all mesocosms. These holes served as ‘easy access points’ for the *L. terrestris* that were to be introduced; for consistency, they were also made in the control mesocosms. Fifteen *L. terrestris* earthworms (51 m^{-2}) were introduced into the assigned mesocosms. Half of these mesocosms received CA *L. terrestris* (treatments 3 and 4) and half received NL *L. terrestris* (treatments 5 and 6). One bucket-full of autumn leaves (*Aesculus hippocastanum*) was spread on to the soil surface of each inoculated mesocosm before the surface was covered with mesh gauze. Control mesocosms also received one bucket-full of autumn leaves each, and then they too were covered with 1.5 mm mesh gauze to ensure that no earthworms would enter or leave the mesocosm, neither by themselves nor through predation. A fence was put up around the experimental plot to prevent cows from entering and potentially disturbing the mesocosms.

Maintenance of the field experiment

The field experiment was checked regularly and in January 2022, 800 g of farmyard manure from Location B was applied to each mesocosm by spreading it evenly over the soil surface. The fertilising value of the manure was analysed by Eurofins, Wageningen (Supp. Table A.1).

In April 2022, grass-clover was seeded again in the mesocosms with disturbed soil, as germination had been poor. From April onwards, the vegetation was cut regularly, both inside the mesocosms and in the rest of the plot. All mesocosms were provided with cuttings to ensure food supply for the earthworms.

In May, July and August, the farmer at Location B irrigated the grassland on which the experiment was situated. A total of four irrigations took place, 22 mm each time. In May and August, we watered the mesocosms at Location A with ~18 mm, as the soil was becoming very dry in the absence of rain. More detailed information about the weather conditions during the course of the experiment (temperature, rainfall and soil temperature) can be found in Supp. Table A.2.

Earthworm extraction and identification at experimental end

In September 2022, all mesocosms were harvested in the same way. The soil was carefully removed from each mesocosm in three layers (0–20 cm, 20–40 cm, 40–60 cm), using a garden fork to minimise the risk of damaging the earthworms. Each soil layer was hand-sorted to collect all the earthworms that it contained. Mustard extraction was used to also collect earthworms from deeper soil layers. Six L of water with mustard powder (see paragraph “[Earthworm origin](#)” for more details) was applied to the pit of each mesocosm (60 cm depth). The bottom of the pit was checked regularly for at least 30 min, and all earthworms that emerged were rinsed with tap water and processed like the rest of the earthworms. Earthworms were kept cool and taken to the laboratory, and the following day they were rinsed, weighed and put in 70% ethanol to store them until species determination.

Earthworms were identified to species level when possible. Individuals were categorised as adult if a clitellum was present or emerging, and they were categorised as juvenile if a clitellum was completely absent. Juveniles that belonged to either *L. rubellus* or *L. terrestris* were distinguished from each other by checking for considerable thickenings of any septa, which, as described by Stöp-Bowitz (1969), are absent for *L. rubellus*. No other species that could have been mistaken for *L. rubellus* or *L. terrestris* were present in the samples.

Statistical analysis

Prior to analysis, the collected data was checked for normality and transformed when needed. Rstudio version 2023.12.0+369 software (R Core Team 2023) was used to perform linear regression

analyses and ANOVA. Factors were *location* (A and B), *inoculum* (either locally collected inoculum [NL] or imported Canadian specimens [CA]; this was not applicable for Control [C] mesocosms as these were not inoculated), and *soil*

disturbance (undisturbed [U] and disturbed [D]). LSDs were used for the interpretation of two- and three-way interactions. Excel (Version 2409 Build 16.0.18025.20160) was used for a two sample t-test on data about tagged earthworms.

Table 3 Overview of the means (m^{-2}) of earthworm biomass and abundance per location, soil disturbance category, inoculation type and interactions between these. Significance is indicated as *, ** and *** when p-value was <0.05, <0.01 and <0.001 respectively; non-significant p-values are indicated with “ns”; p-values <0.05>0.1 are considered tendencies, these are indicated as p-values. ‘U’ is ‘undisturbed soil’, ‘D’ is disturbed soil from which the resident earthworm population was removed before the field experiment set off, ‘CA’ refers to inoculation with Canadian *L. terrestris*, ‘NL’ refers to inoculation with locally collected *L. terrestris*, ‘C’ refers to control treatment. When data was transformed to obtain normal distribution this is indicated. NB: only at Location A earthworms were tagged, n.a. = non applicable

	Unit	Means						
		Location (L)		Soil disturb. (S)		Inoculum (I)		
		A	B	U	D	CA	NL	C
Tot. Biomass	gr	158	164	142	179	159	171	153
Tot. Earthworms	#	550	345	405	519	457	477	453
Tot. Epigeics	#	34	147	77	87	57	101	87
Tot. Endogeics	#	385	185	246	353	323	286	290
Tot. Anecics	#	132	13	82	80	77	90	76
Tot. <i>L. terrestris</i>	#	128	13	81	77	76	87	73
Ad. <i>L. terrestris</i>	#	23	9,0	16	18	16	24	12
Juv. <i>L. terrestris</i>	#	105	4,0	64	59	60	63	62
tagged <i>L. terrestris</i>	#	7.9	n.a	5.6	11.0	5.1	10.7	n.a
Tot. <i>L. rubellus</i>	#	22	124	65	67	44	87	67
Ad. <i>L. rubellus</i>	#	0.7	47	18	24	17	19	25
Juv. <i>L. rubellus</i>	#	21	77	47	43	27	67	41
	<i>p</i> -value							
	Main effects				Interaction effects			
	L	S	I	L*S	L*I	S*I	L*S*I	Trans.
Tot. Biomass	ns	.057	ns	ns	ns	ns	ns	-
Tot. Earthworms	*	.077	ns	ns	ns	ns	ns	-
Tot. Epigeics	***	ns	**	ns	*	*	ns	-
Tot. Endogeics	*	*	ns	ns	ns	ns	ns	sqrt
Tot. Anecics	***	ns	*	ns	**	ns	ns	sqrt
Tot. <i>L. terrestris</i>	***	ns	*	ns	.085	ns	ns	sqrt
Ad. <i>L. terrestris</i>	***	ns	*	ns	ns	ns	ns	-
Juv. <i>L. terrestris</i>	***	ns	ns	ns	ns	ns	ns	sqrt
tagged <i>L. terrestris</i>	n.a.	ns	0.076	n.a.	n.a.	n.a.	n.a.	-
Tot. <i>L. rubellus</i>	***	ns	*	ns	.080	ns	ns	-
Ad. <i>L. rubellus</i>	***	ns	ns	ns	.080	ns	.053	sqrt
Juv. <i>L. rubellus</i>	***	ns	**	ns	ns	ns	ns	-

Results

Effects of location, soil disturbance and inoculation on *L. terrestris* abundance

Location had a significant effect on total *L. terrestris* abundance, adult *L. terrestris* abundance and juvenile *L. terrestris* abundance (Table 3). For this reason, the results are described separately per location in the following paragraphs. Inoculation of the mesocosms resulted in higher total *L. terrestris* abundance and higher adult *L. terrestris* abundance, but it did not affect juvenile *L. terrestris* abundance (Table 3).

L. terrestris survival and offspring at location A

At Location A, where *L. terrestris* already occurred, the introduction of *L. terrestris* did not lead to increased *L. terrestris* abundance, neither in the plots with undisturbed soil nor in the plots with disturbed soil from which the resident earthworm population had been removed. Neither the adult nor juvenile fraction of the *L. terrestris* population was affected by inoculation treatment (Fig. 1A). A total of 37 tagged earthworms was retrieved from inoculated mesocosms, which amounts to 15% of the original inoculum (Table 4 and Fig. 1A). There was a tendency towards higher NL than CA *L. terrestris* retrieval ($p=0.076$) among tagged specimens. The difference in the number of *L. terrestris* retrieved from disturbed and undisturbed mesocosms was not significant (Fig. 1A). At the level of each individual mesocosm (both inoculated and control), the number of juveniles per adult was variable and ranged from 1.5 to 13.7, with a mean of 4.93.

L. terrestris survival and offspring at location B

At Location B, where *L. terrestris* did not occur before inoculation, *L. terrestris* introduction resulted in a significantly higher *L. terrestris* abundance of both adults and juveniles, compared to controls (Fig. 1B). Overall, 26% of the *L. terrestris* introduced survived twelve months in the mesocosms, and they produced a mean of 0.44 juveniles per adult. Survival rates were comparable for NL and CA inoculum (27% and 25%, respectively) The highest mean survival rate – although non-significant – occurred in undisturbed mesocosms with NL

inoculum. This treatment yielded a mean population density of 30 *L. terrestris* m⁻² (51 ind. m⁻¹ had been introduced), with equal numbers of adults and juveniles (Fig. 1B).

At Location B, soil disturbance and the removal of earthworms did not have a significant effect on total and adult *L. terrestris* abundance in inoculated mesocosms (Fig. 1B). Among the juvenile fraction, there was a significant interaction effect between soil disturbance and inoculation treatment. In undisturbed mesocosms, NL exceeded CA in juvenile abundance (3.85 m⁻² and 0 m⁻², respectively), whereas in disturbed mesocosms there was no such difference between NL and CA. (Fig. 1B). At the level of each individual mesocosm, the number of juveniles per adult varied between 0.0 and 4.0 (mean of 0.7).

Relation between *L. terrestris* and resident earthworm community in general

At Location A, seven different earthworm species were identified whose average abundance was 550 ind. m⁻² (Supp. Table A.3). All three major ecological categories (epigeic, endogeic, anecic) were represented. Numerically, *Aporrectodea caliginosa* was the most dominant species, followed by *L. terrestris*; together they amounted to 70% of the total earthworm community. The juvenile fraction of the earthworm community was 0.68. At Location B, five different earthworm species were identified, among which no anecic species, apart from the introduced *L. terrestris* (Edwards & Arancon 2022). The average earthworm density was 345 ind. m⁻². Equal to Location A, *A. caliginosa* was the most abundant species, and *L. rubellus* was the second most common species; together these two amounted to 86% of all earthworms. The juvenile fraction of the earthworm community was 0.51.

Among the resident earthworms, four species significantly correlated to *L. terrestris* in abundance (Table 5). *L. rubellus* and *A. chlorotica* showed a significant positive correlation at Location B but not at Location A. *L. castaneus* was only present at Location B, where its abundance correlated significantly positively to that of *L. terrestris*. *A. nocturna* was only found at Location A, where its abundance correlated positively to that of *L. terrestris*.

Interaction between *L. terrestris* and *L. rubellus*

Considering both locations together, total *L. rubellus* abundance was lowest in the CA treatment. Highest *L. rubellus* juvenile abundance was found for the treatment of NL inoculation. These differences can be ascribed to the large juvenile *L. rubellus* fraction, as the adult fraction did not yield any significant relation to inoculum treatment. Control mesocosms hosted an intermediate *L. rubellus* abundance, which did not differ significantly from either the NL treatment or the CA treatment (Table 3).

The abundance and demography of *L. rubellus* differed between the two locations. At Location A,

there were hardly any adult *L. rubellus* (3%), whereas at Location B, each mesocosm contained both adults (38%) and juveniles (62%) (Fig. 2). At Location A, the population density of *L. rubellus* varied widely per mesocosm, ranging from 0 to 79 ind. m⁻²; no correlation was found between the abundance of *L. rubellus* and *L. terrestris* (Pearson's $r = -0.085$, $R^2 = 0.007$). A positive correlation between the number of *L. terrestris* and *L. rubellus* was found at Location B (Pearson's $r = 0.755$, $R^2 = 0.570$). Here, the population density of *L. rubellus* ranged from 17 to 232 ind. m⁻².

Fig. 1 **A** and **B** Bar graph based on ANOVA output. Adult (tagged: dark blue, untagged: light blue) and juvenile *Lumbricus terrestris* (ind. m⁻²) per inoculation treatment and soil disturbance treatment (for Location A (resident *L. terrestris* population present) and Location B (no resident *L. terrestris* population present)). NL = locally collected inoculum; CA = commercially purchased inoculum; C = control, no inoculation. Standard error bars refer to juveniles and adults separately; tagged adults and untagged adults are combined in one group

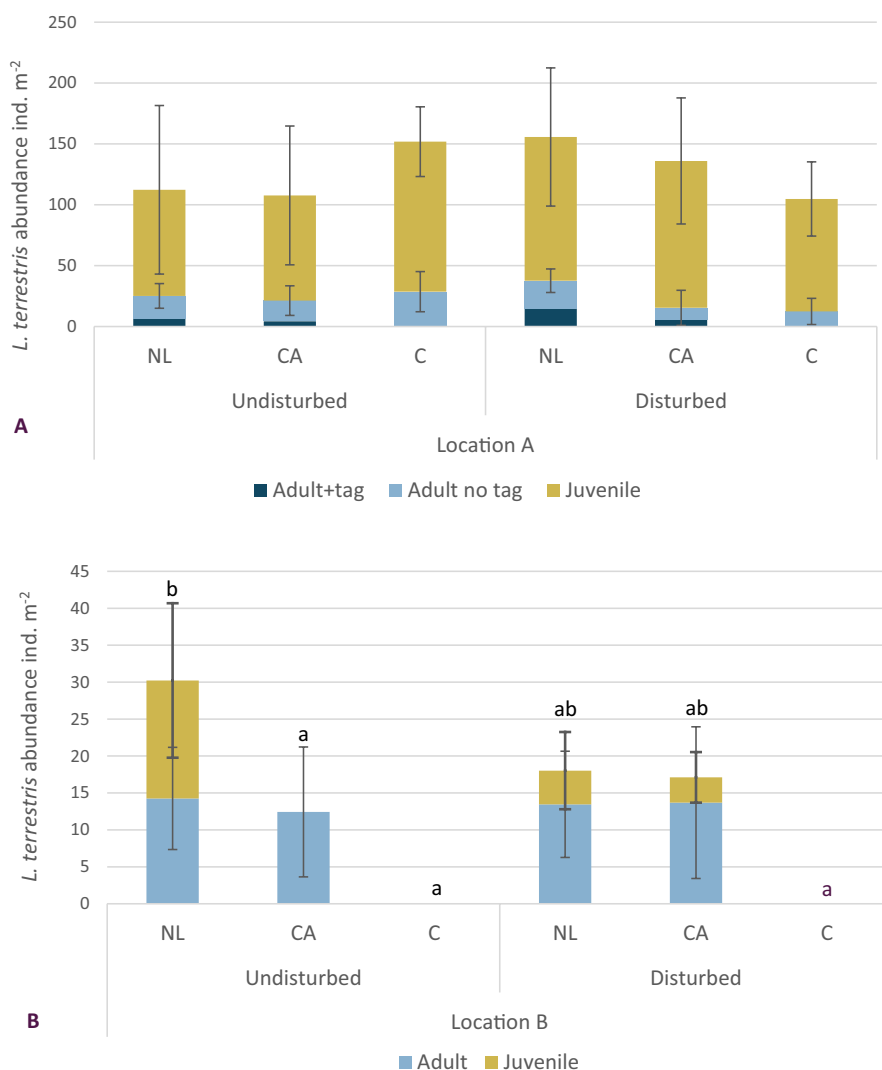


Table 4 Number of VIE-tagged *Lumbricus terrestris* earthworms recovered, in each treatment at Location A. NB: Numbers are absolute numbers

Treatment	Number of retrieved <i>L. terrestris</i> with tag	Number of retrieved untagged <i>L. terrestris</i>	Total number of retrieved <i>L. terrestris</i>	% Of total retrieved <i>L. terrestris</i> with tag
Undisturbed soil, NL	8	22	30	27
Undisturbed soil, CA		20	25	20
Disturbed soil, NL	17	27	44	39
Disturbed soil, CA	7	11	18	39
Total	37	80	117	32

Discussion

General *L. terrestris* survival and reproduction

In previous field studies, the reported *L. terrestris* retrieval ranges from as low as 0% to as high as 80% (15% and 26% in this study at location A and B respectively). In a field trial with a similar set-up that we executed in 2019—2020, we recorded 33% retrieval after eight months, with 0.17 juvenile per adult, and 6% survival after fifteen months, with 0.90 juvenile per adult (Van de Logt et al. 2023b). A mesocosm study on arable land by Andriuzzi et al. (2015) reported 40% retrieval from inoculated mesocosms after eight months. Also on arable land, Frazão et al. (2019) observed an estimated 14% recovery and 3.3 juveniles per adult after one year, but no recovery after two years. Forey et al. (2018) found at least 15% survival after 12 months on grassland, but as they did not sample deeper than 50 cm, it is possible that not all surviving earthworms were found. In a twelve-month field trial by Rajapaksha et al. (2014), mesocosms were inoculated with *L. terrestris* and 80% of the earthworms were recovered. Lab inoculation studies in a controlled environment resulted in *L. terrestris* survival rates of 68% (Butt 1991) and 100% after one year (Butt et al. 1992), along with 45% survival after more than two years (Svendsen et al. 2005).

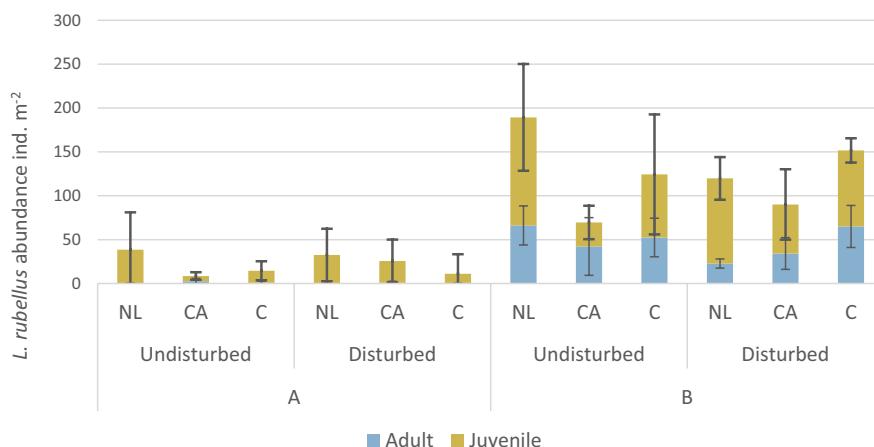
It is difficult to identify the cause of the high variability in survival rates among this and previous inoculation experiments. Andriuzzi et al. (2015) speculate that both mortality and inefficiency of the sampling method could explain limited retrieval. Forey et al. (2018) and Van de Logt et al. (2023b) suspect drought to have been fatal to the earthworms. Van de Logt et al. (2023b) also discuss the potentially detrimental effects of predation, of the life history of the

Table 5 Pearson's *r* correlation coefficient, R^2 and corresponding *p*-values for correlations between the abundance of *L. terrestris* and co-occurring species. For Location B, only inoculated mesocosms were included. NA Not applicable (due to absence of species); NS Non-significant, *, ** and *** refer to significance levels of >0.05, >0.01 and >0.001, respectively

Species	Location	Pearson's <i>r</i>	R^2	<i>p</i> -value
L.ter x L.cas	A	NA	NA	NA
	B	0.144	0.021	*
L.ter x L.rub	A	-0.085	0.007	NS
	B	0.755	0.570	**
L.ter x A.ros	A	-0.445	0.200	NS
	B	NA	NA	NA
L.ter x A.chl	A	0.522	0.272	NS
	B	0.013	0.000	***
L.ter x A.cal	A	0.679	0.461	NS
	B	0.579	0.335	NS
L.ter x A.lim	A	0.563	0.317	NS
	B	NA	NA	NA
L.ter x A.noc	A	0.134	0.021	*
	B	NA	NA	NA

L. terrestris introduced, and of competitive interaction with the resident earthworm population. In the set-up of our experiment, we aimed to rule out the risks of predation, drought, competition for food, unsuitable habitat and unfit life history (harvest, storage, transport process). To this end, we covered the mesocosms with nets, watered the plot in prolonged periods of drought, provided leaf litter, farmyard manure and grass cuttings, introduced *L. terrestris* into two different grasslands, one where the species already occurred (Location A) and one where it was absent (Location B), and introduced both commercially purchased *L. terrestris* from Canada (CA) and locally collected specimens (NL) for comparison.

Fig. 2 Adult and juvenile *L. rubellus* abundance (ind. m⁻²) per treatment, soil disturbance and location, no significant differences in either location. Standard error bars refer to juveniles (in bold) and adults (normal) separately



L. terrestris survival and reproduction at location A

Three remarkable observations that we made are discussed here. Firstly, we had expected that Location A would yield a higher survival rate than Location B, especially because the NL inoculum was introduced back into the same grassland that the earthworms had been collected from, and therefore we were certain to have introduced them into a suitable habitat. However, being removed, tagged, stored and returned to the grassland seems to have had a stronger negative effect on the earthworms at Location A than at Location B. We consider it unlikely that the difference can be ascribed to removal, storage or inoculation, as these procedures were identical for both locations. Tagging and the presence of resident *L. terrestris* are the most obvious differences between Locations A and B. It is possible that niche-saturation did not allow for an increase in *L. terrestris* population density. Potentially negative effects of VIE-tagging are discussed in more detail in the final paragraphs of the section “[Factors affecting *L. terrestris* persistence and survival](#)”.

The second observation was that the inoculation of undisturbed plots with *L. terrestris* did not lead to increased population density compared to controls after one year, possibly due to niche-saturation at Location A. Given the feeding regime, we consider it unlikely that food was a limiting resource, but perhaps competition for space played a role. Unfortunately, we cannot separate this from the impact that VIE-tagging might have had.

The third observation was that the total numbers of *L. terrestris* in undisturbed and disturbed control

mesocosms were more similar than expected. At the onset of the field trial, earthworms had been removed from these mesocosms by hand-sorting to a depth of 60 cm, combined with mustard extraction (see [Materials and methods](#), paragraph “[Earthworm origin](#)”). It was not expected that this would lead to complete absence of *L. terrestris* from disturbed control mesocosms, as cocoons were not removed. However, it was supposed that the effects of manual removal and soil disturbance would be at least comparable to intensive cultivation, which has been demonstrated to be able to severely decimate the *L. terrestris* population (Chan 2001; Pelosi et al. 2014; Frazão et al. 2019). However, the populations in the disturbed control mesocosms appeared fully recovered after 12 months (Fig. 1A). It is possible that the resource-rich and protective environment of the mesocosms and the loosened soil, combined with the removal of the vast majority of possible competitors, facilitated rapid population recovery from the cocoons. Most likely, also some juveniles that had gone unnoticed during the removal of the resident population contributed to recolonisation. What is interesting about this observation is that it suggests that under suitable conditions, population growth of *L. terrestris* can be rapid. This implicates that management interventions can contribute to stimulating the existing *L. terrestris* population in grasslands.

At Location A we retrieved a total of 37 from the 240 *L. terrestris* introduced (15%), which comprised 25 NL and 12 CA specimens. Among the *L. terrestris* retrieved, there were some interesting treatment effects (Tables 3 and 4). NL earthworms

outperformed CA earthworms, matching our hypothesis that locally collected *L. terrestris* have a higher survival rate than commercially purchased inoculum. We think this indicates that locally collected *L. terrestris* are more readily adapted to the environment. In the section about Location B, we elaborate on this. Another interesting difference was that, as we expected, more tagged *L. terrestris* were found in disturbed soil than in undisturbed soil. A possible explanation could be that intraspecific competition was very low at the moment of introduction, making it easy to settle. Additionally, the loosened soil likely facilitated easier burrowing, allowing the earthworms to allocate their resources to other processes important for survival. The absence of resident earthworms and loosened soil are confounding factors in this experimental set-up, making it impossible to distinguish between them.

Contrary to our hypothesis, no correlation between the abundance of *L. rubellus* and *L. terrestris* was found at Location A (Fig. 3), whereas previous studies seemed to suggest a negative interaction between *L. rubellus* and *L. terrestris* (Shuster et al. 2003; Van de Logt et al. 2023b; Van de Logt & Van der Sluijs et al., 2023a). Perhaps no correlation was found because the abundance of *L. rubellus* was low (on average 22 ind. m⁻²) and almost completely represented by juveniles, making competition for resources between the species under the circumstances as they were during the experiment highly unlikely.

L. terrestris survival and reproduction at location B

At Location B, the general recovery rate was higher than at Location A, it also exceeded the recovery rate that we found in our previous field trial (Van de Logt et al. 2023b). As mentioned earlier, the experimental set-up of the present field trial was based on the set-up that we used in a previous study (Van de Logt et al. 2023b), but with some adjustments (i.e. more irrigation, more food supply, and local inoculum). These adjustments may have improved the success of the introduction, and we will discuss them below.

First, more food was provided by leaving grass cuttings in the mesocosm instead of removing them. It was decided to do this as we considered resource availability a possible limiting factor in the previous study, mainly because of the seemingly negative interspecific interaction with *L. rubellus*, which feeds

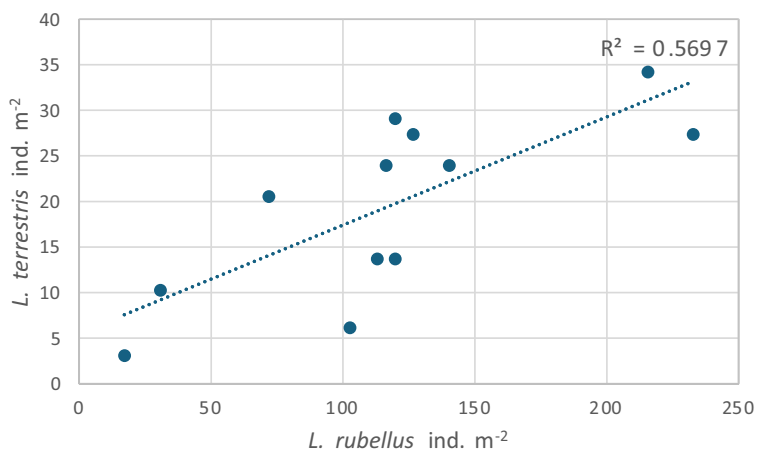
within the same niche. Contrary to previous findings (Van de Logt et al. 2023b; Van de Logt & Van der Sluijs et al., 2023a), we observed a positive correlation between *L. rubellus* and *L. terrestris* (Fig. 3). Thus, these two species do not necessarily compete. The grass cuttings may have ensured ample resource availability and prevented interspecific competition. Second, the grassland that hosted the field experiment was irrigated several times during the summer; if drought had been a problem in the previous experiment as had been suggested, it is safe to assume that irrigation contributed to increased *L. terrestris* survival. Third, no damage to the covering nets was inflicted by birds, eliminating the concerns about predation that we had in our previous experiment.

At Location B, we found a mean of 0.44 juveniles per adult, which seems too few to rejuvenate the population. However, cocoons were not collected and hence not included. Collecting and identifying cocoons is laborious but not impossible; for future research we would suggest including cocoon data. Furthermore, *L. terrestris* population abundance and demography fluctuate throughout the year (Potvin & Lilleskov 2017). Our harvest took place in September, after 12 months, but peaks in juvenile abundance are more likely to be found in November (Daniel 1992). Lastly, the fact that the number of juveniles per adult ranged from 0.0 (undisturbed plots with CA inoculum) to 4.0 (undisturbed plots with NL inoculum) demonstrates that in some mesocosms the *L. terrestris* community showed potential for rejuvenation and population growth. Long-term experiments are necessary to validate long-term population development.

Factors affecting *L. terrestris* persistence and survival

As is outlined by our results as well as by previous work by Rajapaksha et al. (2014), Andriuzzi et al. (2015), Forey et al. (2018), Frazão et al. (2019) and Van de Logt et al. (2023b), survival rates are highly variable. The question arises whether these results and studies report on *L. terrestris* populations that are dying out, or on populations that are going through a phase which is eventually part of long-term settlement? In the latter case, part of the inoculum may be unfit for the new territory and thus fail to survive, whereas the remaining earthworms are able to thrive

Fig. 3 Correlation between *L. rubellus* abundance and *L. terrestris* abundance at Location B



and reproduce, leaving the apt and their offspring to colonise the area in time to come. Unfortunately, the number of juveniles per adult is not mentioned in most studies (Rajapaksha et al. 2014; Andriuzzi et al. 2015; Forey et al. 2018), probably due to difficulty in species determination for juveniles. However, species determination for juvenile *L. terrestris* is possible in many cases (see also *Materials and methods*, paragraph “[Earthworm extraction and identification at experimental end](#)”). We think it is of vital importance to include juvenile abundance, since juveniles can make up a large part of the population (Fig. 1) and offspring production is simply indispensable for the persistence of the population. Consequently, monitoring of juveniles is a necessity in assessing whether a population is moving towards extinction or has the potential to rejuvenate itself.

Below, we try to elucidate factors that may have threatened *L. terrestris* population persistence in our experiment: life history of the earthworms introduced; the possibility of pathogen pressure; and possible trade-offs of the experimental set-up.

The life history of *L. terrestris* may have a strong influence on its performance after inoculation. Andriuzzi et al. (2015), Forey et al. (2018), Frazão et al. (2019), and Van de Logt et al. (2023b) all used commercially purchased *L. terrestris*, despite the remark by Grigoropoulou and Butt (2010) that persistence of locally collected *L. terrestris* was significantly greater than the persistence of commercially purchased specimens, probably because commercial purchase is cheap, easy and less labour-intensive. In contrast, Rajapaksha et al. (2014) used field-collected earthworms.

In our previous paper we suggested that it seems reasonable to assume that locally-collected earthworms experience a smaller disruption of their ‘normal life’ and therefore have a better starting position at the moment of introduction (Van de Logt et al. 2023b). Perhaps because they did not go through the post-harvest process of transportation, storage, artificial feeding conditions, indoor climate conditions and long-distance shipping to the Netherlands as described by Steckley (2020). Apart from logistics, the change in physico-chemical and microbial environment from heavily manured alfalfa fields in Ontario, Canada, to organic grasslands on sandy soil in the Netherlands may have severely challenged the imported *L. terrestris* that we introduced. Analysis of the genetic population differentiation between *L. terrestris* from a German meadow and *L. terrestris* from a Canadian reference population resulted in a considerable difference of 15.17% (Velavan et al. 2009). Part of the potential for adaptation to the environment could be embedded within this genetic variation. Through DNA barcoding, James et al. (2010) discovered cryptic diversity in *L. terrestris*, whether this could translate to colonisation success has not yet been elucidated by research.

It appeared that the difference in persistence between NL and CA *L. terrestris* was not the same for Locations A and B. At Location A, the majority (68%) of the retrieved *L. terrestris* were NL specimens, suggesting a ‘homefield-advantage’ for locally collected earthworms. At Location B, there was no significant difference in survival rate between NL and CA earthworms (27% and 25%, respectively). We speculate that this may mean that habitat adaptation

is very site-specific. In other words, for the inoculum, the difference between Location A and Location B was higher than the difference between their Canadian origin grounds and Location B; therefore, the ‘homefield-advantage’ of the NL *L. terrestris* was possibly lost and survival rates ended up similar. However, as our field experiment was small in scale and the survival rate at Location A was generally low, more research is required to validate this hypothesis.

Pathogen pressure could have played a role. The highest *L. terrestris* recovery rate in a field study in literature was 80% after one year. This was found in a study conducted on sterilised soil (Rajapaksha et al. 2014). All other studies were carried out on unsterilised soil. The sterilisation procedure may have killed pathogens that otherwise would have been harmful to *L. terrestris*. In current literature, interactions between *L. terrestris* and soil-borne pathogens are very rarely addressed. The effect of parasites on overall survival, growth and fecundity of *L. terrestris* is addressed even less (Edwards et al., 2022), neither from the perspective of earthworm introduction nor from earthworm invasion. However, *L. terrestris* interacts intimately with soil biota, as has been revealed by analyses of gut content, midden material and drilosphere (Tiunov & Dobrovolskaya 2002; Nechitaylo et al. 2010; Stromberger et al. 2012). Therefore, it seems reasonable to assume that pathogenic soil biota could have a strong effect on *L. terrestris* survival. It is possible that the inoculated *L. terrestris* (both NL and CA) in our mesocosm experiment suffered from interaction with pathogenic soil biota. Pathogen pressure affecting *L. terrestris* survival rate was also suggested in a paper based on a laboratory study, where mortality was observed to be unequally divided over batches and unrelated to treatment (Lowe & Butt 2007). We think that, with respect to *L. terrestris* colonisation, pathogen-pressure is an underexposed, but potentially very important, factor that deserves more scientific attention.

The experimental set-up that we used may have imposed some undesirable impacts on earthworm survival, in that light we will further discuss the trade-offs of mesocosms and VIE-tagging. In earthworm research, mesocosms are a valuable tool for carrying out semi-controlled experiments. A mesocosm allows testing the set-up under ‘normal’ climatic conditions and in virtually undisturbed soil profiles, without running the risks of escape and predation. Mesocosms cover a substantial part of the gap

between the laboratory and the field. However, the situation in a mesocosm does not exactly match field conditions; it remains a model. The physical presence of a mesocosm alters field conditions like predation, microclimate, air flow, temperature and freedom of movement and mate choice. Building upon our findings so far, the next step could be to move to field-scale and introduce *L. terrestris* into grassland where it is currently absent and monitor the development of the population for several years.

VIE-tagging is a well-established method for marking animals that would be otherwise hard to identify. It is commonly used in research on amphibians and fish (Pine et al. 2012; Hohn & Petrie-Hanson 2013). In earthworm research, VIE-tagging has also been documented multiple times as an accessible and reliable method for marking specimens without interfering with their potential for survival, maturation and reproduction (Gonzalez et al. 2006; Butt & Lowe 2007) up to 27 months after inserting the tag (Butt et al. 2009). We did not notice any abnormalities about the earthworms after tagging them. Nevertheless, incorrect administration of VIE tags could lead to increased mortality among the inoculum (Butt and Lowe 2007).

An inconvenience we discovered about VIE-tagging was that the VIE-tag inserted was often no longer visible from the outside. In most cases tags had migrated towards the posterior end of the body (Supp. Figs. A.3a-c and A.4a). Some of the tags had partly disintegrated (Supp. Figs. A.3a-b and A.4b). Consequently, all *L. terrestris* that were found at Location A needed to be dissected. Additionally, we had eleven earthworms with damaged tails, which brought about some uncertainty as it was impossible to be certain whether they had been tagged or not. To avoid overestimation, these are not included in the 15% retrieval.

Conclusions and future perspectives

This study aimed at gaining new insights into *L. terrestris* introduction into agricultural grasslands, mainly with respect to earthworm origin and suitability of the inoculated pasture. The results of the twelve-month mesocosm experiment at two organic dairy farms show that it was possible for *L. terrestris* to survive and to produce offspring after inoculation. Uniquely, we introduced earthworms of two origins (locally collected

and commercially purchased from Canada) into two grasslands (one where the species was already present and one where it was absent) with two soil treatments (undisturbed versus loosened soil with its residential earthworm population removed). The hypothesis that locally collected *L. terrestris* would outperform the Canadian specimens in survival, was only confirmed by the tagged earthworms at Location A. At Location B, the grassland without a resident *L. terrestris* population, there was no significant difference between the survival rates of NL and CA inoculum. In line with our hypothesis, more offspring was produced by NL than by CA inoculum, but only in undisturbed soil, there was no difference between offspring production of NL and CA in disturbed soil.

L. terrestris survival rate was not significantly affected by soil treatment (undisturbed versus loosened soil where the resident earthworm population was removed), nor did we find any significant effects of soil treatment on offspring production. Refuting our expectations, negative interactions between the abundance of residential species, *L. rubellus* in particular, and *L. terrestris* were absent. From this we conclude that introduced *L. terrestris* does not necessarily engage in interspecific competition when resources are abundantly available, allowing co-existence with the residential earthworm population.

Overall, we see potential for *L. terrestris* inoculations in agricultural grasslands where the species is absent or abundance is low. Such inoculation would take place preferably using locally collected or locally bred *L. terrestris*, as this reduces the environmental burden of transport and is likely to improve their chances of survival. Although our results were obtained under semi-controlled conditions, they cannot simply be extrapolated to normal field conditions. The next steps would be to gain more insight into the effect of pathogens by running controlled experiments with sterilised soil as well as by executing long-term experiments in non-enclosed plots. These experiments will be needed to elucidate whether and how *L. terrestris* inoculation can be used as an ecological tool by farmers to improve water regulation of their grasslands.

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Author contributions Roos van de Logt: conceptualisation, methodology, software, validation, formal analysis, investigation, data curation, writing – original draft, visualization. Nick van Eekeren: conceptualisation, methodology, validation, investigation, writing – review and editing, supervision, project administration, funding acquisition.

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Data availability The datasets generated and analysed in this study are available from the corresponding author on reasonable request.

Declarations

Competing interests 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.

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