



Original article

The anecic earthworm *Lumbricus terrestris* can persist after introduction into permanent grassland on sandy soil

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ABSTRACT

Grasslands are important water-regulating agro-ecosystems. Their ability to store and retain water is of vital importance under the current trend of increasing peak rains and droughts, which are events that can result in economic damage to infrastructure and crops. The presence of the anecic earthworm *Lumbricus terrestris* has a positive effect on the water regulation of the soil. This earthworm creates semi-permanent burrows with a depth of up to 2 m, which increase the water infiltration rate and capacity of the soil. The burrows can also facilitate plant root penetration into deeper soil layers, thus increasing drought resistance. The objective of this research was to explore the ability of *L. terrestris* to survive and reproduce after introduction into grasslands on sandy soils where they had been absent.

We introduced *L. terrestris* into mesocosms on sandy soil under permanent grassland at two dairy farms in the Netherlands. Results showed that while *L. terrestris* was able to survive and produce cocoons, survival rate was low (32% after seven months, 6% after fifteen months) and the number of juveniles was low (2.6 and 2.7 ind. m⁻² after seven and fifteen months, respectively). The causes of this low survival rate may be related to the life history of the earthworms introduced, the effects of soil moisture, interspecific competition for food with the native population of epigeic earthworms, and the risk of predation.

We conclude that *L. terrestris* can survive and produce cocoons after introduction into permanent grassland on sandy soil, but only further experimental trials over greater time spans and in non-enclosed plots will determine whether there is realistic potential for *L. terrestris* to develop a stable population.

1. Introduction

Peak rains and droughts [1–4] have detrimental effects on both agriculture and society as a whole. If the water infiltration capacity of the soil does not match the amount of precipitation, waterlogging and surface runoff can occur, which result in economic damage to infrastructure and crops. Drought creates problems for drinking water supply, and in recent years it has had negative effects on European crop and grass production [5–7]. A trend of decreasing soil moisture in agricultural soils is expected to continue [8,9]. The higher sandy soils in the Netherlands are sensitive to drought as they fail to retain water, which subsequently increases problems in lower areas that are sensitive to flooding. The different stakeholders in these areas, such as farmers, drinking water companies and water boards, are looking for measures to increase water infiltration, to enhance water-holding capacity and to decrease drought sensitivity.

Earthworms are known to provide water-regulating ecosystem

services. They can increase the hydraulic conductivity, water infiltration capacity and water-holding capacity of the soil [10–19]. Three major ecological groups of earthworms can be distinguished: epigeic, endogeic and anecic [20]. The anecic species *Lumbricus terrestris* is particularly suited for the objective of water regulation because of its ability to penetrate compacted soil layers [15] and to create semi-permanent [21] vertical burrows that can reach a depth of >2 m [22], facilitating both water flow and root growth into deeper soil layers [23].

Lumbricus terrestris has been found in abundance in grasslands in several parts of the world [24–27]. A review by Eijsackers showed that *L. terrestris* dispersal was highest in grassland on fertile mineral soil [28]. However, a national inventory among Dutch dairy farms revealed the presence of anecic earthworms in the grasslands of only 21% of the Dutch dairy farms studied. These earthworms belonged to two species, namely *Aporrectodea longa* and *L. terrestris*, the latter being more common [29]. The low prevalence of anecic earthworms was somewhat surprising and may result from the frequent grassland renewal of the last

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40 years, combined with arable crop rotation and the associated intensive soil cultivation, such as ploughing. Anecic earthworms are known to be sensitive to soil cultivation and rotation of grassland with arable crops [30–33].

Since the natural dispersal of *L. terrestris* is slow [13,34,35], a number of research projects investigated whether it is possible to inoculate soils with this species. Matters like survival rate [36–38], dispersal [10,13,39], the effect of management [35,39–41] and experimental set-up [42] were addressed. Various lessons can be learned from these studies. Butt [43] compared different methods for introduction and introduced the Earthworms Inoculation Unit (EIU). Grigoropoulou and Butt [39] found that individuals that were collected on-site had a higher chance of survival than commercially-bought *L. terrestris*. Hoogerkamp et al. [10] studied introduction and dispersal in Dutch polders with clayey soil, and they found that *L. terrestris* prefers a low water table, as flooding can damage its burrow and leave it without oxygen. Both interspecific and intraspecific competition have been reported to negatively affect the survival and growth of *L. terrestris*, underlining the importance of interaction with the native earthworm population [44–47]. Nuutinen et al. [35] emphasised the importance of field margins and long-term monitoring. The species was found to be able to burrow through compact soil layers [48], although it displayed a preference for less compacted soil [49,50].

However, some questions remain unresolved. For example, the requirements for *L. terrestris* to survive introduction and to start reproducing are quite poorly understood, high earthworm mortality in field experiments is regularly reported [36–38], and establishment and dispersal rates can be exceedingly low [13,34,35]. Furthermore, research on *L. terrestris* introduction has mainly focused on clay and loamy soils. We argue that *L. terrestris* introduction in grasslands on sandy soils needs attention, as it is a common soil type, which is sensitive to both peak rains and drought. *L. terrestris* is known to already occur in some sandy soils [29,51], and this work aimed to explore the possibility of introducing this earthworm into grasslands where they are currently absent.

To this end, we introduced *L. terrestris* into mesocosms in permanent grasslands on sandy soils on two dairy farms: a conventional farm with a history of artificial fertiliser and slurry application, and an organic farm with a history of farmyard manure use. We harvested half of the mesocosms after seven months and re-inoculated them, and after fifteen months all the mesocosms were harvested. The re-inoculated mesocosms served as an *explorative experiment* to see how *L. terrestris* would react to less compact disturbed soil without a resident earthworm population. Our aim was to determine whether introduced *L. terrestris* would be able to survive and reproduce in the protective and semi-controlled environment of this mesocosm set-up. We intended to provide a proof of principle, not to mimic actual field conditions. We hypothesised (i) that *L. terrestris* is able to survive introduction into permanent grassland on sandy soil, and (ii) that *L. terrestris* is able to reproduce after introduction into permanent grassland on sandy soil. In our explorative experiment we expected to find (i) that *L. terrestris* would be able to survive and reproduce on disturbed soil without a resident population, (ii) that *L. terrestris* would dig deeper burrows in disturbed (less compact) soil, and (iii) that *L. terrestris* would settle more successfully in the absence of a competing resident earthworm population.

2. Material and methods

2.1. Study area

On the 5th of April 2019, a field experiment was installed in two permanent grasslands on sandy soils (Haploquod [USDA]) in the Netherlands. One grassland was located on a conventional farm (location A, 52°32'05.0"N 6°30'19.7"E) while the second was located on an organic farm (location B, 52°05'25.6"N 5°35'33.3"E). Both grasslands were grazed by dairy cows and were dominated by *Lolium perenne*. A

baseline assessment of the selected grasslands had previously demonstrated absence of *L. terrestris*. On the 3rd of May 2018 (Location B; this location was sampled by Salánki and De Goede [52]) and the 11th of September 2018 (location A), four soil blocks of 20 × 20 × 20 cm were dug out and carefully hand-sorted. Samples were taken at representative and evenly distributed locations within these grasslands. After each soil block was dug out, 1–2 l of AITC (allyl isothiocyanate) solution was applied to the pit and for 30 min it was checked whether earthworms surfaced. On no occasion was *L. terrestris* detected. Soil organic matter was higher at location B, whereas other soil chemical properties and texture were comparable (Table 1). In the 10–40 cm soil layer, soil penetration resistance was higher at location B, whereas in the 40–50 cm soil layer, penetration resistance was higher at location A (Supplementary data S1).

2.2. *Lumbricus terrestris* introduction into permanent grassland (Experiment 1)

Twenty mesocosms were installed per location. Before installation, grass was mowed to a height of 4 cm. With a crane, 20 steel pipes (ø 61 cm, height 50 cm) were driven 40 cm into the soil, leaving a 10 cm ring above the soil surface. Pipes were spaced 90 cm apart (supplementary data S2). Ten mesocosms were assigned to be harvested after seven months and ten mesocosms were assigned to be harvested after fifteen months. In both groups, five mesocosms received 15 adult *L. terrestris* individuals each, 51 ind. m⁻², which is the same inoculation density as used by Forey et al. [38]. We chose this rather high density as we expected that not all earthworms would survive. The other five mesocosms served as controls, and no earthworms were added to these. Earthworms were purchased from a commercial supplier (<https://www.thedutchnightcrawlers.nl/>) that had imported them from Canada, and individuals with a clitellum were considered adult. The average weight per earthworm at the moment of introduction was 4.03 g.

To increase the chances of successful introduction, each mesocosm received 3 mm of water. An auger was used to loosen the top 2 cm of the soil in three places, so as to facilitate earthworm burrowing without damaging the whole grass sod. Each mesocosm received 50 g of freshly cut grass from the site and was then covered with a moist burlap cloth which was removed after five days. All mesocosms were covered with 1.5 mm mesh gauze to prevent the earthworms from escaping above-ground and to protect them against predators (supplementary data S2). Escape belowground was considered highly unlikely, as *L. terrestris* burrows vertically and disperses over the soil surface [53].

On the 23rd of April 2019, the grass in and around the pipes was mowed to a height of 4 cm, and 10 mm of water and 300 g of farmyard manure (collected at location B) was applied to the mesocosms (316 g

Table 1

Soil properties in the 0–10 cm soil layer and management for location A and location B.

	Location A	Location B
Soil type (USDA)	Haploquod	Haploquod
Chemical properties		
pH	5.9	5.5
Organic Matter (%)	4.3	7.4
N-total (g N/kg dry soil)	2.3	4.4
P-Al (mg P2O5/100 g)	55	55
Soil texture		
% Sand	88	84
% Clay	<1	1
% Silt	8	8
Management		
Cutting	Yes	No
Grazing	Yes	Yes
Fertilisation	Yes	Yes
	Slurry manure + artificial fertiliser	Farmyard manure

dry matter kg^{-1} ; 6.6 g total N kg^{-1} ; 0.9 g $\text{NH}_3\text{-N}$ kg^{-1} ; 5.8 g N-org kg^{-1}). Subsequently, the sites were mown in and around the pipes every four weeks, to prevent the grass from growing into the mesh gauze covers. After mowing, the grass cuttings were removed from the mesocosms. In July 2019, all mesocosms received 20 mm of water because of the exceptionally dry summer. In addition to the water we applied, the farmer at location A irrigated the grassland three times with 22.5 mm of water during the growing season of 2019.

In November 2019, seven months after inoculation, five inoculated mesocosms and five controls were harvested at each location, so as to determine whether the *L. terrestris* introduced had been able to survive and produce cocoons in the short term. The harvesting procedure was as follows: in situ, the soil was removed from the mesocosms with a spade. This was done in three layers (0–20 cm, 20–40 cm and 40–60 cm depth), and each soil layer was collected in a separate container. It is important to note that *L. terrestris* can move rapidly through its burrows, especially when disturbed, like during the harvesting process. The depth at which we found the earthworms was therefore considered the *minimum* depth that they had reached since inoculation. The soil layers were hand-sorted on-site, to collect all earthworms. The hand-sorted soil layers were returned to the mesocosms in their original order. The collected earthworms were rinsed, weighed and preserved in alcohol. The earthworms were classified as adults or juveniles and identified to species level if possible [54,55]. The other mesocosms were left untouched. In February 2020, 600 g of farmyard manure was applied to each of the unharvested mesocosms, at both locations. In July 2020, 15 months after inoculation, all mesocosms were harvested following the same procedure as in November 2019.

2.3. *Lumbricus terrestris* introduction into disturbed soil without resident earthworms population (Experiment 2)

After sorting in November 2019 (Experiment 1), the soil was restored in the mesocosms. All three layers (0–20, 20–40 and 40–60 cm) were put back in their original order. Subsequently, the five mesocosms previously allotted to inoculation during Experiment 1 once again received 15 *L. terrestris* (51 ind. m^{-2}) each, following the same procedure as in April 2019. The five control mesocosms again served as controls in Experiment 2, to ensure that no *L. terrestris* cocoons were present. Perennial ryegrass (*Lolium perenne*) was seeded and a layer of leaf litter (mainly *Aesculus hippocastanum*) was added to the mesocosms to provide a food source and a protective layer for the earthworms. In February 2020, the remaining leaf litter was removed and 900 g of compost and 30 g of concentrates (composed of maize, barley, wheat, nutricell, pulp

and minerals [900 g dry matter kg^{-1} ; 12.8 g total N kg^{-1}]) was applied to the surface. These concentrates were provided as an additional protein source to support the earthworms (personal communication with the Dutch Nightcrawlers, earthworm supplier). Harvesting took place after eight months in July 2020 (Fig. 1), using the same procedure as in November 2019 and July 2020 for Experiment 1.

2.4. Weather data

Soil temperature data was obtained from the nearest weather stations and can be found in supplementary data T1 [56] and T2 [57], and monthly rainfall and air temperature per location can be found in supplementary data S3 and S4. Generally, 2019 and 2020 were relatively dry, especially July (2019 and 2020) and August (2019). April and May of 2020 were exceptionally dry, with little precipitation for two months. Temperatures were close to the long-term average, with January and February 2020 being slightly warmer than average.

2.5. Statistical analysis

GenStat software was used to perform a three-way ANOVA on the data collected. Factors were location (A and B), treatment (control and *L. terrestris*) and harvest (November 2019 and July 2020). Experiment 1 and Experiment 2 were treated as two separate experiments. LSDs were determined for the interpretation of two- and three-way interactions.

3. Results

3.1. Introduction into undisturbed soil (Experiment 1)

3.1.1. Anecic earthworms in undisturbed soil (Experiment 1)

Anecic species were represented by *L. terrestris* (22%) and *Aporrectodea nocturna* (78%) at location A and by *L. terrestris* (100%) at location B. The number of adult *L. terrestris* (m^{-2}) was significantly affected by treatment and harvesting date, but not by location (Table 2). Seven months after introduction (November 2019), *L. terrestris* abundance was significantly higher than it was after fifteen months (July 2020) (Table 2; Fig. 2). After seven months, 32% of the density initially introduced was recovered, and after fifteen months 6% was recovered.

The total *L. terrestris* biomass (g m^{-2}) was significantly affected by both treatment and harvesting date, but not by location. The interaction effect of harvesting date and treatment was the only one yielding significance (Table 2). We measured 48 g m^{-2} of *L. terrestris* biomass in inoculated mesocosms in November 2019 (after seven months), and 12

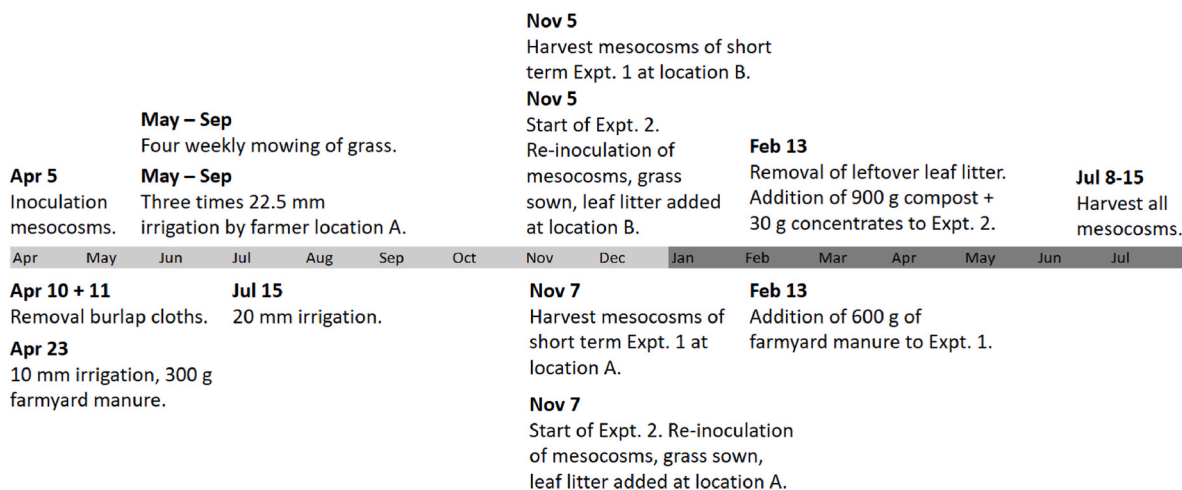


Fig. 1. Timeline of the field experiment. Light grey: 2019, dark grey: 2020. When location is not mentioned explicitly, the intervention was executed at both locations.

Table 2

Earthworm abundance (ind. m⁻²) and biomass (g m⁻²) for *Lumbricus terrestris*, epigeic and endogeic in undisturbed soil (Experiment 1). Location A = conventional dairy farm; location B = organic dairy farm; Harvest 1 = November 2019; Harvest 2 = July 2020; Treatment C = control; Treatment Lt = inoculation with 15 *L. terrestris* (51 ind. m⁻²). HxT is the interaction effect between harvesting date and treatment. Other interactions (H x L, H x L and H x L x T) were not significant. Numbers in **bold** indicate significance. P-values: *p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant.

	Means						P-value			
	Treatment (T)		Location (L)		Harvest (H)		T	L	H	H x T
	C	Lt	A	B	2019	2020				
Abundance (ind. m⁻²) in 0–60 cm soil layer										
Total	619	588	587	620	465	742	ns	ns	***	ns
Epigeic	177	153	121	208	136	194	ns	**	*	ns
Endogeic	416	395	422	388	295	515	ns	ns	***	ns
Anecic	30	14	30	14	22	22	**	**	ns	ns
<i>L. terrestris</i>	1.8	12	8.5	5.7	11	3.4	***	ns	***	**
Adults	0.8	9.5	5.9	4.5	8.5	1.8	***	ns	**	**
Juveniles	1.0	2.7	2.5	1.2	2.2	1.5	ns	ns	ns	ns
Biomass (g m⁻²) in 0–60 cm soil layer										
Total	134	162	151	145	147	149	*	ns	ns	ns
<i>L. terrestris</i>	2.5	30	19	13	26	6.6	***	ns	**	**
Adults	1.9	27	17	12	24	4.2	***	ns	**	**
Juveniles	0.6	3.3	2.1	1.7	1.5	2.4	**	ns	ns	ns
Non- <i>L. terrestris</i>	132	133	132	133	122	143	ns	ns	ns	ns

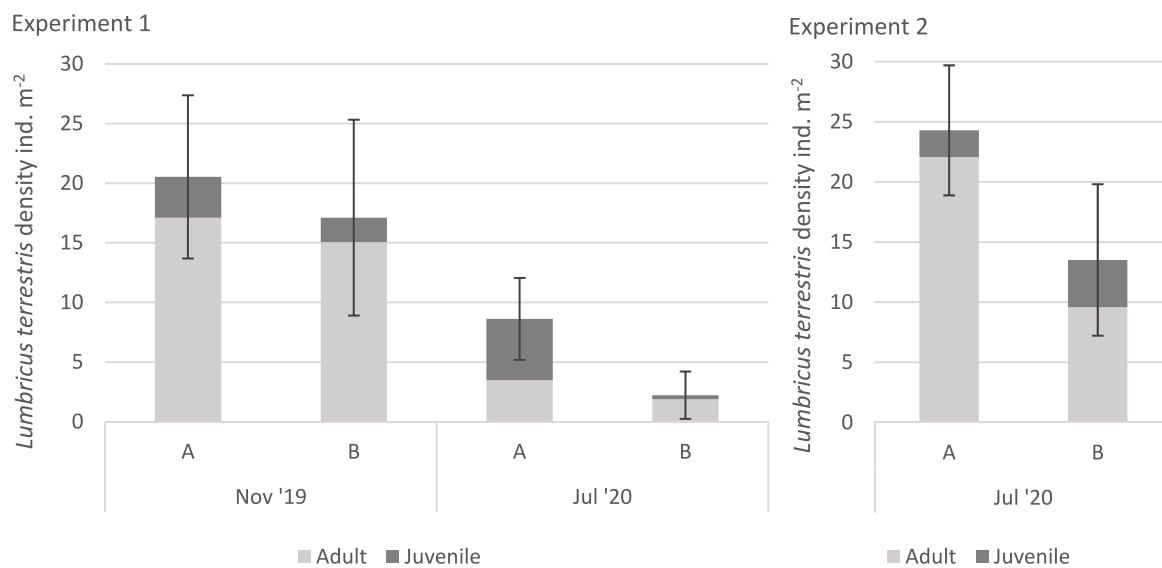


Fig. 2. Experiment 1, undisturbed soil; Experiment 2, disturbed soil. Density of *Lumbricus terrestris* at locations A and B in inoculated mesocosms. Harvested in November 2019 and July 2020. Bars indicate average deviation of total number *L. terrestris* (adult and juvenile combined).

g m⁻² of *L. terrestris* biomass in July 2020 (after fifteen months). The average weight per *L. terrestris* that was harvested intact was 2.8 g in November 2019 and 4.2 g in July 2020.

The vertical distribution of *L. terrestris* over the three different soil layers at the first harvest indicated a discrepancy between locations A and B. At location A, 77% of the *L. terrestris* were found in the upper 20 cm of the soil, whereas this was only 44% at location B. After fifteen months, the proportion of *L. terrestris* found in the 20–40 and 40–60 layers had increased at both locations (Fig. 3).

3.1.2. Non-anecic earthworms in undisturbed soil (Experiment 1)

Total earthworm abundance was significantly higher in July 2020 than it was in November 2019 (means of 742 and 465 m⁻², respectively) (Table 2). The total earthworm abundance was affected neither by location nor by treatment.

The inoculated mesocosms yielded a significantly higher total earthworm biomass (g m⁻²) than the controls (162 g versus 134 g m⁻²) (Table 2) as a consequence of the added *L. terrestris*. The non-*L. terrestris*

biomass (g m⁻²) was not significantly affected by treatment, location or harvest (Table 2).

Across all treatments, epigeic species were represented by *Lumbricus rubellus* (98.9%), *Lumbricus castaneus* (0.8%) and *Dendrobaena octaedra* (0.3%) at location A, and by *L. rubellus* (83.6%), *L. castaneus* (13.5%), *Dendrobaena rubida* (2.6%) and *D. octaedra* (0.3%) at location B. Epigeic earthworm abundance was significantly affected by location, but not by treatment or harvesting date. The difference was non-significant, but on average, the inoculated mesocosms contained fewer epigeic worms than the control mesocosms at both locations and both harvesting dates (Fig. 4). The largest numerical difference between the number of epigeic earthworms in inoculated mesocosms and control mesocosms was observed in November at location B, with means of 136 and 194 ind. m⁻², respectively.

Endogeic species were represented by *Allolobophora chlorotica* (97.3%) and *Aporrectodea caliginosa* (2.7%) at location A and *A. caliginosa* (100%) at location B. Endogeic earthworm abundance was affected by harvesting date, but not by treatment or location (Table 2).

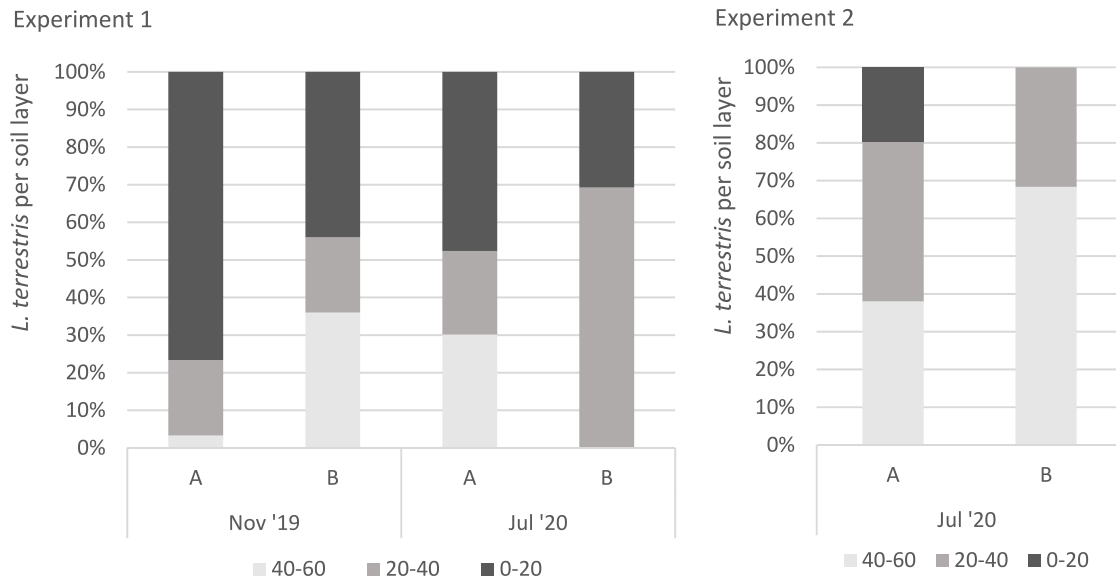


Fig. 3. Experiment 1, undisturbed soil; Experiment 2, disturbed soil. Distribution of *Lumbricus terrestris* in inoculated mesocosms over three soil layers (0–20; 20–40 and 40–60 cm) at locations A and B, harvested in November 2019 and July 2020.

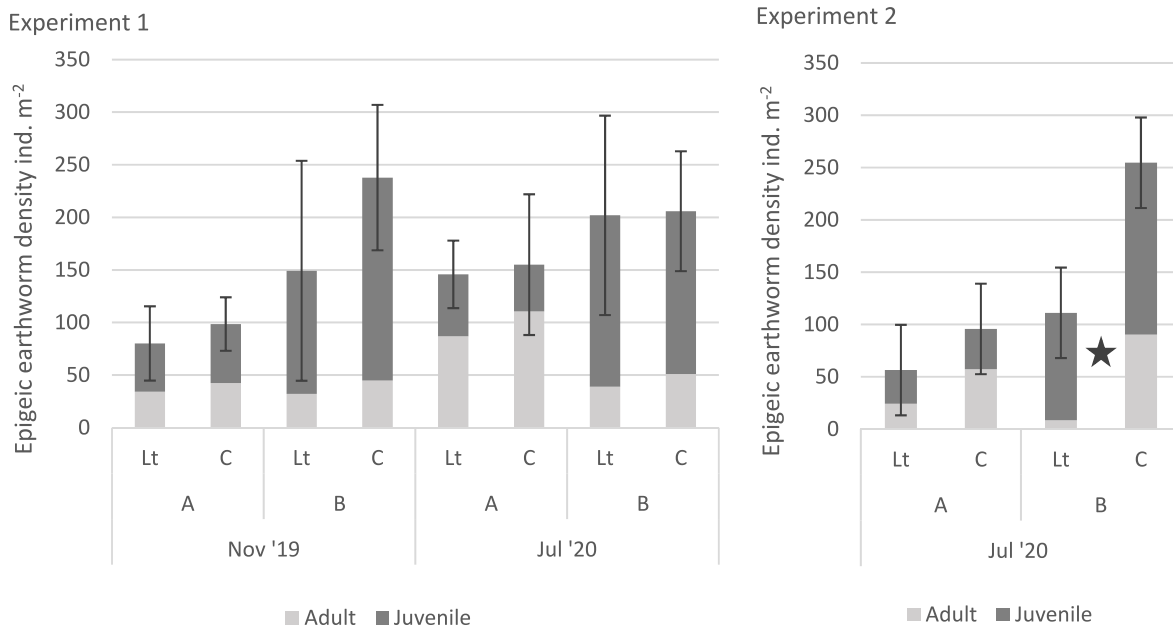


Fig. 4. Experiment 1, undisturbed soil; experiment 2, disturbed soil. Mean epigeic earthworms density at locations A and B. C = control; Lt = inoculated with 15 *Lumbricus terrestris* (51 ind. m⁻²). Bars indicate average deviation of total number epigeic earthworms (adult and juvenile combined). The star indicates a significant difference between the number of epigeic worms in C and Lt mesocosms at location B.

3.2. Introduction into disturbed soil without resident earthworm population (Experiment 2)

3.2.1. Anecic earthworms in disturbed soil (Experiment 2)

Anecic species were represented by *L. terrestris* (47%) and *A. nocturna* (53%) at location A and *L. terrestris* (100%) at location B. We recovered 33% of the *L. terrestris* introduced (Table 3). Both treatment and location had a significant effect on *L. terrestris* abundance. Inoculation resulted in an average of 19.4 *L. terrestris* m⁻² after eight months, mostly adults (84%). The interaction effect of location and treatment yielded

significant differences. More *L. terrestris* were found in inoculated mesocosms at location A than at location B, namely 26 m⁻² versus 12.8 m⁻² (Fig. 2). The two locations differed significantly in abundance for adult *L. terrestris*, but not for juveniles. In inoculated mesocosms at location A, 23.4 m⁻² adults and 2.6 m⁻² juveniles were found, meaning that 11% of the population was juvenile. At location B, 10.4 m⁻² adults and 2.4 m⁻² juveniles were found, which means that 23% of the population was juvenile.

The total *L. terrestris* biomass (g m⁻²) was significantly affected by treatment, but not location (Table 3). The average weight of the

Table 3

Earthworm abundance (ind. m⁻²) and biomass (g m⁻²) *Lumbricus terrestris*, epigeic and endogeic in Experiment 2 (disturbed soil); Location A = conventional dairy farm; location B = organic dairy farm; Treatment C = control (no added *L. terrestris*); Treatment LT = 15 added *L. terrestris* (51 ind. m⁻²). Significant values at the confidence interval of P < 0.05 in bold. P-values: *p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant.

	Means				P-value		
	Treatment		Location		T	L	T x L
	C	LT	A	B			
Abundance (ind. m⁻²) in 0–60 cm soil layer							
Total	525	390	541	374	*	**	*
Epigeic	178	106	92	191	*	**	ns
Endogeic	332	264	432	165	ns	***	ns
Anecic	14	21	21	14	ns	ns	*
<i>L. terrestris</i>	0.3	19	13	6.7	***	*	*
Adults	0	17	12	5.2	***	*	*
Juveniles	0.3	2.5	1.3	1.5	*	ns	ns
Biomass (g m⁻²) in 0–60 cm soil layer							
Total	112	133	129	117	ns	ns	ns

recovered *L. terrestris* that were harvested intact was 3.9 g per individual.

The distribution of *L. terrestris* over the three different soil layers appeared to differ between the locations. At location A, 20% of the *L. terrestris* was found in the 0–20 cm soil layer, whereas at location B, 100% of the *L. terrestris* was found in the lower 20–60 cm of the soil (Fig. 3).

3.2.2. Non-anecic earthworms in disturbed soil (Experiment 2)

Treatment, in addition to location, had a significant effect on the total earthworm abundance (Table 3). Control mesocosms yielded significantly more earthworms than inoculated mesocosms (means of 525 and 390 m⁻², respectively) (Table 3). The total earthworm abundance was significantly higher at location A than at location B (means of 541 and 374 ind. m⁻², respectively) (Table 3).

The presence of *L. terrestris* had a significant impact on the biomass dynamics of the other earthworms. The non-*L. terrestris* part of the earthworm biomass was almost halved under the inoculation treatment (means of 112 g m⁻² for controls and 60 g m⁻² under inoculation treatment) (Table 3). The contribution of *L. terrestris* to the total earthworm biomass in the inoculated mesocosms masked the distinction in total biomass between inoculated and control treatments.

Epigeic species were represented by *L. rubellus* (99.5%) and *L. castaneus* (0.5%) at location A and *L. rubellus* (95.6%) and *D. rubida* (4.4%) at location B. Treatment as well as location had a significant effect on epigeic earthworm abundance. Inoculated mesocosms contained significantly fewer epigeics than the controls (means of 106 and 178 m⁻², respectively) (Table 3; Fig. 4). Location A harboured significantly fewer epigeics than location B (means of 92 and 191 m⁻², respectively) (Table 3; Fig. 4).

Endogeic species were represented by *A. chlorotica* (97.3%) and *A. caliginosa* (2.7%) at location A, and by *A. caliginosa* (100%) at location B. The number of endogeic earthworms was significantly affected by location, but unlike the epigeic earthworms it was not significantly affected by treatment. More endogeics were found at location A than at location B (means of 432 m⁻² and 165 m⁻², respectively) (Table 3).

4. Discussion

4.1. *Lumbricus terrestris* survival

We hypothesised that *L. terrestris* is able to survive introduction into a permanent grassland on sandy soil where it is currently absent. Indeed, we retrieved adult *L. terrestris* individuals from the mesocosms at both locations, which shows that they can survive after introduction. As it can

take *L. terrestris* over one year to mature under field conditions [25], we assume that the adult *L. terrestris* earthworms that we retrieved were the earthworms that we had initially introduced and not a second generation.

Unexpectedly, we also encountered some *L. terrestris* individuals in control mesocosms. It might be that somehow earthworms managed to escape and colonise a neighbouring mesocosm. Another explanation could be that the morphological species determination was inaccurate for these individuals. Although the determination was carried out by an expert, earthworms can be affected from the harvesting process or display some degree of phenotypic plasticity [58] which complicates morphological species determination.

Overall, the survival rate was lower than we had expected. From the undisturbed mesocosms of Experiment 1, we retrieved 32% of the *L. terrestris* earthworms after seven months and only 6% after fifteen months. From the disturbed soil mesocosms of Experiment 2, we retrieved 33% after eight months. This is in contrast to our prediction that disturbed soil – which is less compact and easier to burrow through – and the absence of a resident earthworm population would promote *L. terrestris* survival rate.

The survival rate we observed is in line with the findings of Andriuzzi et al. [37]. They retrieved 40%, eight months after *L. terrestris* introduction into mesocosms on a conventionally managed arable field on sandy-loamy soil. Possible explanations for the low survival rate could be the life history of the earthworms introduced, soil moisture, interspecific competition, and predation; these are discussed in the following sections.

4.1.1. Life history of the introduced *Lumbricus terrestris*

L. terrestris mortality may have been related to their origin and life history. As mentioned before, the earthworms were harvested and collected in Ontario, Canada. Soils in this area are classified as grey-brown luvisols or orthic and albic luvisols. Alfalfa is the most prevalent crop, and fields are generally fertilised with liquid cow manure [27]. After harvesting, earthworms were stored and subsequently sent to the Netherlands. They were kept for 1–2 weeks in a mixture of compost and were fed a specially composed concentrate (personal communication with ‘The Dutch Nightcrawlers’) until we received them. We selected mature individuals, i.e. having secondary sexual characteristics [59]. The exact age of the earthworms was unknown.

We cannot know the precise effects of the harvest, travel, storage and introduction procedure on the earthworms. Grigoropoulou and Butt [39] reported that *L. terrestris* earthworms that were collected on-site performed better than commercially purchased individuals, but they give no explanation for this observation. In accordance with this, Vos et al. [60] reported that in their experiment on sandy soil, *L. terrestris* from Canada showed limited survival, whereas locally collected *A. longa* performed better. However, we cannot exclude that this difference relates more to differences between the suitability of the species than to differences between the life history of the earthworms.

The sudden and repeated change of environment may have weakened the Canadian earthworms. The presence of pathogens or parasites (new to these earthworms) may have been lethal.

4.1.2. Soil moisture

The spring of 2020, one year into the experiment, was particularly dry and warm, causing a risk of death through desiccation. Aestivation is very rarely observed for *L. terrestris* [61,62]. In general, the species remains active during periods of drought [63] and applies different strategies to avoid desiccation, such as habitat choice, deep burrowing and possibly cocoon incubation time. Deep burrowing allows the earthworms to retreat to the more stable moisture and temperature regimes of deeper soil layers in times of adverse conditions in the top soil. We think some of the earthworms introduced used this strategy, which may explain why we found a larger proportion of *L. terrestris* in deeper soil layers at location B, where the farmer did not apply irrigation.

4.1.3. Interspecific competition

We expected that competition with resident earthworms would negatively affect *L. terrestris* establishment. Interspecific competition between earthworms is not uncommon [64–66]. *L. rubellus* (which represented close to 99% of the resident epigeic population in our research) and *L. terrestris* share a similar dietary niche [67]. Shuster et al. [68] introduced *L. terrestris* into an arable field. They noticed that under one treatment, *L. terrestris* establishment was very modest (7 ind. m⁻²) and apparently happened at the expense of *L. rubellus*, with numbers of the latter declining to one-quarter of the original. Under the treatment of higher resource availability and variety, they did not observe this effect, which led them to conclude that scarcity in resources can cause interspecific competition between *L. terrestris* and *L. rubellus*. In our experiment, food may have been a limiting factor, leading to competition between species, which may have limited population growth and maturation of the earthworms. Preferably, weight change of *L. terrestris* inoculated in relation to *L. rubellus* abundance would have been calculated. But unfortunately, only very few *L. terrestris* were harvested intact. The average biomass per individual is thus based on too few animals to draw conclusions about weight gain or loss after inoculation.

Interspecific competition may also explain the difference in *L. terrestris* survival rate between the two locations. At location B, *L. rubellus* was more abundant than at location A (means of 159 and 110 *L. rubellus* m⁻², respectively, possibly because farm B had a history of using farmyard manure, whereas farm A had a history of slurry application. Farmyard manure is known to stimulate epigeic earthworms [69]. The higher abundance of epigeic earthworms may have led to more intense competition for resources and thus to lower *L. terrestris* survival. In the inoculated mesocosms of Experiment 1, we found on average 146 *L. rubellus* m⁻² at location B and 101 m⁻² at location A (non-significant difference).

During the first harvest, all earthworms were removed from the mesocosm but the cocoons remained. As location B harboured more *L. rubellus* than location A, it is most likely that more *L. rubellus* cocoons also remained in the soil. In this case, *L. terrestris* possibly suffered from more intense interspecific competition for food, which could explain why we found significantly fewer *L. terrestris* at location B in Experiment 2.

4.1.4. Predation

We suspect that predation by rooks (*Corvus frugilegus*) may have influenced the survival rate of the *L. terrestris* in the mesocosms at location B. Although the mesocosms were covered completely with nets, shortly after the establishment of the experiment, rooks made small holes in the nets. Rooks are known to include *L. terrestris* in their diet [70]. Afterwards, we replaced the damaged nets and set up a scarecrow. The bird damage reduced, but we cannot completely exclude predation. In contrast, at location A we found hardly any traces left by birds trying to reach into the mesocosms. Predation by rooks at location B could also explain why *L. terrestris* was generally found deeper in the soil than at location A; the *L. terrestris* in the 0–20 cm layer may have been predated on or the earthworms may have burrowed deeper to escape from the rooks.

4.2. *Lumbricus terrestris* reproduction

Our second hypothesis was that *L. terrestris* is able to reproduce after introduction into a grassland on sandy soil. The discovery of juveniles confirmed that *L. terrestris* produced cocoons after introduction (Fig. 2). Unfortunately, due to the relatively short time span of the field experiment, we cannot be sure that the earthworms mated after inoculation. *L. terrestris* can store sperm and has been observed to produce viable cocoons six months [71] or even 17 months [72] after mating had taken place.

We expected that the benefits of a less compacted soil which is devoid of earthworms would lead to increased reproductive output in

the mesocosms with disturbed soil without a resident population (Experiment 2). Earthworms that need less time and energy for burrowing invest more in foraging and reproduction [73]. However, this was not apparent from the results of the explorative study. Experiment 2 did not run simultaneously with Experiment 1, hence we only consider these comparisons as an indication. The density of *L. terrestris* juveniles in inoculated mesocosms was very similar across both experiments and harvesting dates. However, when we compare our findings to previous observations in the laboratory (Butt [74] 25 ind. year⁻¹; Butt [75] 40 ind. year⁻¹; Lakhani and Satchell [76] 3–13 ind. year⁻¹), the number of juveniles that we found was extremely modest (0.2 juvenile per adult in the first harvest of Experiment 1; 1 juvenile per adult in the second harvest of Experiment 1; 0.2 juvenile per adult in Experiment 2).

Several factors may have affected the *L. terrestris* reproductive output. First, drought may have played a role, especially at location B since no irrigation took place there. Previous research has confirmed that soil moisture content influences *L. terrestris* reproduction output, with too little moisture having adverse effects on reproduction [74, 77–79]. In addition, *L. terrestris* mating takes place on the soil surface. Edwards and Bohlen [22] describe that *L. terrestris* surfaces at night during periods of considerable rain, although not exclusively under these circumstances [80]. The dry summer of 2019 and dry spring of 2020 may have prevented *L. terrestris* from exploring the soil surface and encountering a partner. Adverse conditions may have caused a preference for cocoon production through the use of previously (pre-experiment) stored sperm over mating in situ.

Second, the age of the earthworms introduced may have played a role. Unfortunately, we could not know their age, as they were ‘wild earthworms’. Previous research indicates that reproduction output decreases as *L. terrestris* ages [77,81]. It is also possible that senescence of the worms limited cocoon output.

Third, considerable variety in the incubation time of *L. terrestris* cocoons has been observed under artificial conditions. Several months is no exception [74], and in extreme cases, incubation can take almost two years, as was demonstrated in a lab experiment [50,72]. Prolonged incubation time can be used as a strategy to avoid drought for the offspring. This makes it even more complex to quantify reproductive output in the field. We only collected juveniles and not cocoons, as it was practically impossible to collect and identify *L. terrestris* cocoons by hand-sorting the soil from the mesocosms. The earthworms had either produced few cocoons, or the dry conditions during the experiment had extended cocoon incubation time and influenced the detectable reproductive output of the individuals that we had introduced.

Overall, the low number of juveniles could result from low cocoon production, low hatching success, low juvenile survival, extended incubation time, or a combination of these.

4.3. Distribution over soil layers

In agreement with our expectations, a larger proportion of *L. terrestris* was found in deeper soil layers in disturbed soil compared to undisturbed soil (Fig. 3). However, as the experiments did not run simultaneously, we consider these results as merely an indication. Possibly, less compact soil facilitates deeper burrowing. *L. terrestris* is able to burrow through compacted soil [82], but burrowing activity is known to be negatively correlated to soil bulk density [21], and deeper burrows were found in less compacted soil [48,50].

More *L. terrestris* were found deeper than 20 cm at location B than at location A (Fig. 3). We think this may be due to soil moisture, as location A was irrigated and location B was not. The upper part of the soil is more susceptible to drought, which may have killed earthworms in the top 20 cm layer or forced them to move to deeper soil layers. Moreover, the predation pressure of rooks at location B may have played a role.

5. Conclusion

This mesocosm field study demonstrated that it is possible for *L. terrestris* to survive and produce cocoons after introduction into permanent grassland on sandy soil. It showed that *L. terrestris* can survive and produce cocoons after introduction into disturbed sandy soil from which the resident earthworm population has been removed. However, these results should be interpreted with care, as the field experiment lasted only 15 months and both survival rate and number of juveniles were low. Our observations indicate that life history, soil moisture, predation, as well as food availability and interspecific competition with epigeics could have led to limited survival and reproduction. Based on our results, we think there is still some potential for *L. terrestris* inoculation into grasslands on sandy soil. However, only further experimental trials over greater timespans and in non-enclosed plots will determine whether *L. terrestris* inoculation can lead to the development of a stable population in permanent grassland on sandy soil. Evidently, reproduction has to be greater than loss if the population is to persist and disperse. We make the following recommendations for future research: collect *L. terrestris* from nearby grasslands that resemble field conditions at the experimental site; monitor soil moisture and soil temperature at the experimental site; keep the individuals from the Canadian inoculum under perfect lab conditions – parallel to the field experiment to monitor survival; provide sufficient suitable food to prevent competition; and run a disturbed and undisturbed soil experiment simultaneously, for better comparison between these treatments.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejsobi.2023.103536>.

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