Surviving the summer as the climate changes: Investigating drivers and costs of aestivation in earthworms

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Abstract

Earthworms are vital ecosystem engineers, with important implications for soil structure, fertility and productivity. However, their reliance on soil water makes them vulnerable to the increasing frequency and severity of droughts predicted under climate change. A key survival strategy employed by some species is aestivation, a state of reduced metabolic activity during which earthworms coil up and seal themselves into chambers until favourable conditions return. Despite its ecological importance, the environmental thresholds that induce aestivation and the consequences for earthworm fitness and community dynamics remain poorly understood. This thesis investigates the drivers, costs and ecological implications of aestivation through controlled laboratory experiments involving *Allolobophora chlorotica* (Savigny, 1826), one of the most common UK earthworms, and year-long field surveys with molecular analyses. Laboratory studies demonstrated that aestivation is environmentally induced, triggered between ~18-13 wt% soil moisture, with 100 % mortality below ~10 wt%. Soil type modulated responses, with water potential (pF) a stronger predictor for the onset of aestivation than gravimetric moisture. While aestivation protected individuals against desiccation, repeated droughts reduced body mass, induced regression of reproductive characters, and diminished cocoon production and viability. Nonetheless, mass losses were largely transient, with rapid rehydration and compensatory growth enabling recovery once favourable conditions returned. Field surveys confirmed that aestivation was common both during dry (<25-30 vol%) and saturated (>50 vol%) conditions, highlighting its role as a general stress response. During drought, earthworm communities became less diverse and were dominated by a few aestivating, burrowing species, while litter-dwellers largely disappeared. Populations demonstrated resilience, likely through migration and drought-resistant cocoons, but recovery potential is constrained by land use and the frequency of climatic extremes. Overall, this thesis establishes soil moisture as the primary driver of earthworm aestivation, identifies thresholds critical for survival and reproduction, and demonstrates both the resilience and vulnerability of earthworm populations to climate change.

Acknowledgements

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I owe particular thanks to my dad (**Fig. 1**), who generously dedicated much of his time to assist me with field sampling over several years. I am also grateful to Andy Krupa for his help in sourcing equipment and for providing manure from his sheep, which supplied an essential food source for maintaining my experimental earthworms.

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Finally, a special thanks go to my family for all their support: my mum for her help with proofreading, my sister for illustrating the earthworms that appear on the final page, and my cat (**Fig. 1**) for keeping me company during the writing process.

Figure 1. My dad (left) assisting with fieldwork and my cat (right) helping with thesis writing.

Declaration

I, Roberta Bray, confirm that this thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means (https://www.sheffield.ac.uk/study-skills/assessment/academic-integrity/academic-integrity). This work has not previously been presented for an award at this, or any other, university.

This is a publication format thesis. Each data chapter (2, 3, 4 and 5) is presented as a stand-alone research article. At the time of submission, none of these chapters have been published.

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Chapter 1

General introduction

1.1 Earthworm diversity, distribution and ecological functions

Soil contains ~59 (± 15) % of known species on earth (Anthony *et al.*, 2023), and so harbours a great biodiversity of organisms on which we depend. Excluding nematodes, earthworms often make up the largest biomass contribution in many terrestrial ecosystems (Edwards and Lofty, 1977; Lavelle and Spain, 2001). Earthworms occupy all continents except Antarctica, and around half of Earth's terrestrial surface is thought to permit their activity (Ruiz *et al.*, 2021) (**Fig. 1.1**). To date, over 5,700 earthworm species have been described, although estimates suggest this number may represent only one-fifth of the actual species diversity, with as many as 30,000 species expected globally (Misirlioğlu *et al.*, 2023; Decaëns *et al.*, 2024). The majority (~2,800 species) are tropical, whereas temperate regions host a more modest diversity (Decaëns *et al.*, 2024), with only 29 species known to occur in Great Britain (Ashwood *et al.*, 2024).

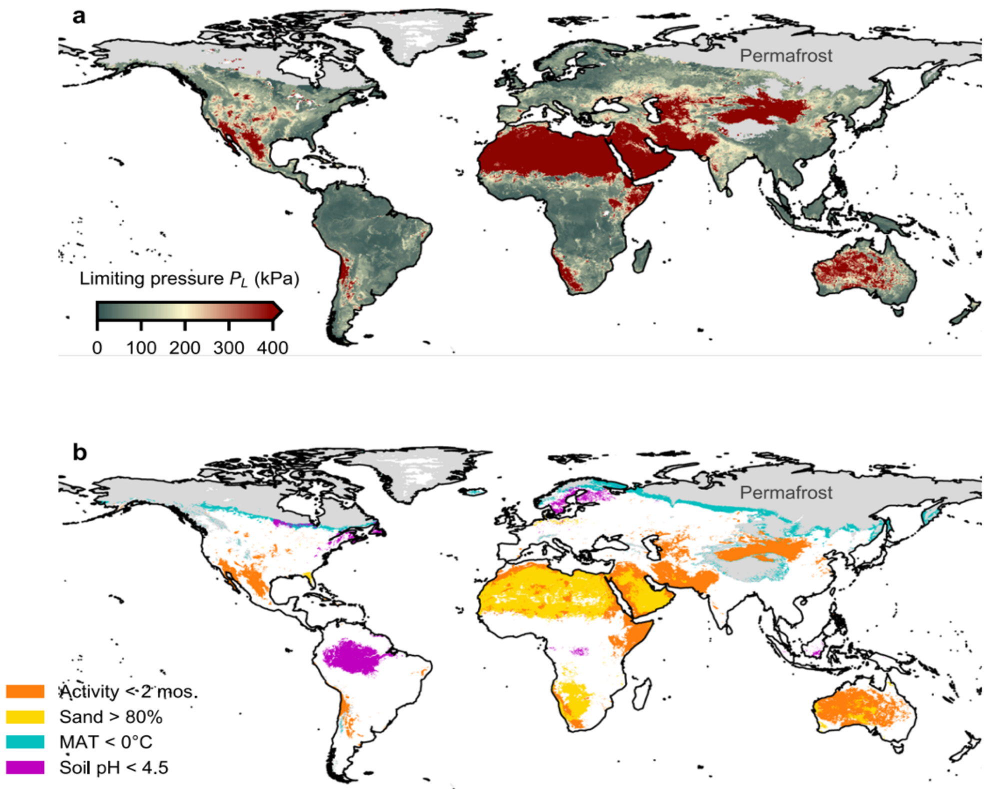

Figure 1.1. Hospitable global climatic regions supporting earthworm bioturbation. (a) Regions permitting activity (green) with mean annual pressures lower than the earthworms hydrostatic pressure limit (<200 kPa). (b) Other factors constraining earthworm activity including; sub-zero mean annual temperatures (MATs, blue), soil pH < 4.5 (purple), sand content > 80 % (yellow), and regions with fewer than two consecutive months where mechanical properties permit cavity expansion (orange). Taken from Ruiz *et al.* (2021).

Earthworms are commonly classified as either anecic, epigeic or endogeic (Bouché, 1977), mainly based on their anatomical-morphological features such as body length and skin pigmentation, but also their physiology and feeding behaviour (Adomako *et al.*, 2025; Bottinelli *et al.*, 2020). Epigeic earthworms are surface-dwelling species that feed on leaf litter and other organic matter on the soil surface, tending to be most abundant in woodland habitats (Natural England, 2014). They are small and pigmented (usually a gradient of red; Bottinelli *et al.*, 2020), and because of the niche they occupy and lack of ability to move to deeper soil depths, are subject to more continuous predation at all stages unlike endogeic and anecic species (Brown and James, 2007). Anecic earthworms are large, pigmented with a brown colour gradient, and often have flattened caudal segments (Bottinelli *et al.*, 2020). They inhabit semi-permanent vertical burrows, which for species such as *Lumbricus terrestris* (Linnaeus, 1758) have been found to extend over 1 metre deep (Potvin and Lilleskov, 2017), and feed on a combination of surface and soil organic matter (Bouché, 1977). Endogeic species tend to be medium-sized and mostly non-pigmented, giving them a pink colouration (Bottinelli *et al.*, 2020). They are adapted to feeding on soil organic matter, forming horizontal burrows within the upper 50 cm of soil, typically in disturbed soils with higher pH such as pastures, arable land and field margins (Natural England, 2014).

The three-group distinction is increasingly understood to be oversimplified. Many species display behaviours or morphologies consistent with more than one of the three categories with Bouché (1977) himself describing endogeic, anecic and epigeic as three main poles in a triangular continuous distribution and thus some species fall between groups as intermediates. Furthermore, some species were not categorised by Bouché (1977) and a lack of clear rules set out for classification makes it difficult to assign new species to groups (Bottinelli *et al.*, 2020). Bottinelli *et al.* (2020) suggested earthworms could be classified based on their percentage membership to each of the three groups rather than to a distinct category. More recently, efforts have been made to group earthworms based on their functional roles in the soil. Hsu *et al*. (2023) propose that stable isotopes of carbon and nitrogen (^{13}C and ^{15}N) could be incorporated as functional traits when categorising earthworm species. Their analysis of 10 earthworm species revealed that these species could be grouped into nine isotopic niches, forming a continuum that spans from species feeding primarily on leaf litter to those consuming microbially-processed soil organic matter (Hsu *et al*. 2023). Moreover, Capowiez *et al.* (2024) describe six new functional groups based on bioturbation behaviour: intense tunnelers, burrowers, shallow bioturbators, deep bioturbators, litter dwellers and intermediates. However, such classifications are currently limited by a lack of ecological data in the literature for certain earthworm species, highlighting the need for further studies into earthworm behaviour and ecology.

As ecosystem engineers, earthworms have profound effects on soil function and are thus often used as bioindicators of healthy and productive soils (Paoletti, 1999). Earthworms alter soil physical properties through the formation of biogenic structures such as stable aggregates, soil pores and mucilage-lined burrows which act as macropores, improving soil aeration, water infiltration, nutrient transport, facilitating carbon sequestration and supporting a wide range of microorganisms (De Oliveira *et al.*, 2012; Coleman *et al.*, 2004; Perreault and Whalen, 2006; Zhang *et al.*, 2013; Lavelle *et al.*, 1997; Brown *et al.*, 1999, 2000; Blouin *et al.*, 2013; Angst *et al.*, 2022). Anecic species, like *L. terrestris,* form deep burrows which remain stable in space and can persist for years (>7 years) (Potvin and Lilleskov, 2017), even after the earthworm has died, given their expected lifespan of 1-4 years (Lee, 1985). Therefore, burrows are an important structural component of the soil and act as a useful resource patch and accumulation of organic matter, which facilitates long-term soil fertility (Brown *et al.*, 2000; Andriuzzi *et al.*, 2013; Nieminen *et al.*, 2015; Hoang *et al.*, 2016). Additionally, by ingesting and decomposing soil organic matter and excreting nutrient-rich casts, earthworms form zones of increased carbon and nitrogen availability which creates hotspots of microbial activity (Tiunov and Scheu, 1999; Kuzyakov and Blagodatskaya, 2015). This in turn stimulates plant growth, promoting increases in aboveground biomass and crop yields (Lavelle *et al.*, 1997; McDaniel *et al.*, 2013b; Van Groenigen *et al.*, 2014). Recent meta-analyses show that earthworms can enhance macronutrient availability by 29-51 %, significantly boost soil respiration by 68 %, and increase microorganism biomass by 45 % (Wu *et al.*, 2025). As such, earthworms contribute significantly to global food production, increasing plant biomass by 20-23 %, with the strongest effects seen in nitrogen-limited regions (Van Groenigen *et al.,* 2014; Wu *et al.*, 2025). Estimates suggest that earthworms account for approximately 6.5 % of global grain and 2.3 % of global legume production annually (Fonte *et al.*, 2023). Moreover, through their activities, earthworms can ameliorate soils and aid ecosystem recovery, with Hallam *et al.* (2020) finding improvements in the quality and functioning of agricultural land converted to grass-clover leys were enhanced by the presence of earthworms. Given the numerous crucial functional roles of earthworms, it is important to consider factors that determine their distribution. In addition, because these functions are tightly coupled with soil physical, chemical and biological properties, earthworms are useful indicators of soil health. For instance, earthworm abundance, biomass and species composition (such as the balance of anecic, epigeic and endogeic groups) reflect land use intensity and are good indicators of soil contamination, with populations responding to tillage, compaction, pesticide use, pH and organic matter inputs (Paoletti, 1999; Pérès *et al*., 2011; Pelosi *et al.*, 2014).

1.2 Earthworms in a changing climate

Globally, earthworm distribution is in part mediated by climatic factors such as temperature and precipitation (Rutgers *et al.*, 2016; Johnston, 2019; Phillips *et al.*, 2019, **Fig. 1.2**). The influence of these factors is expected to intensify as climate change exacerbates the frequency and severity of extreme weather events throughout the 21st century (Seneviratne *et al.*, 2012). Despite this, most climate change research has focussed on aboveground biodiversity, leaving the impacts on soil taxa and belowground communities less well understood (Veresoglou *et al.,* 2015). Model projections applied to French earthworm communities suggest a decline in species and functional diversity under future climate change scenarios, potentially affecting the provision of ecosystem services both in natural and agricultural systems (Fourcade and Vercauteren, 2022). A recent assessment of climate change stressors found that while temperature change and increases in carbon dioxide are detrimental to soil biodiversity, the greatest (and only significant) negative effects were from drought (Phillips *et al.*, 2024). Similarly, species distribution models have found that seasonal changes in precipitation and mean annual temperatures (MATs) were the main environmental drivers of earthworm distribution and species richness across Europe (Zeiss *et al.*, 2024).

a)

b)

c)

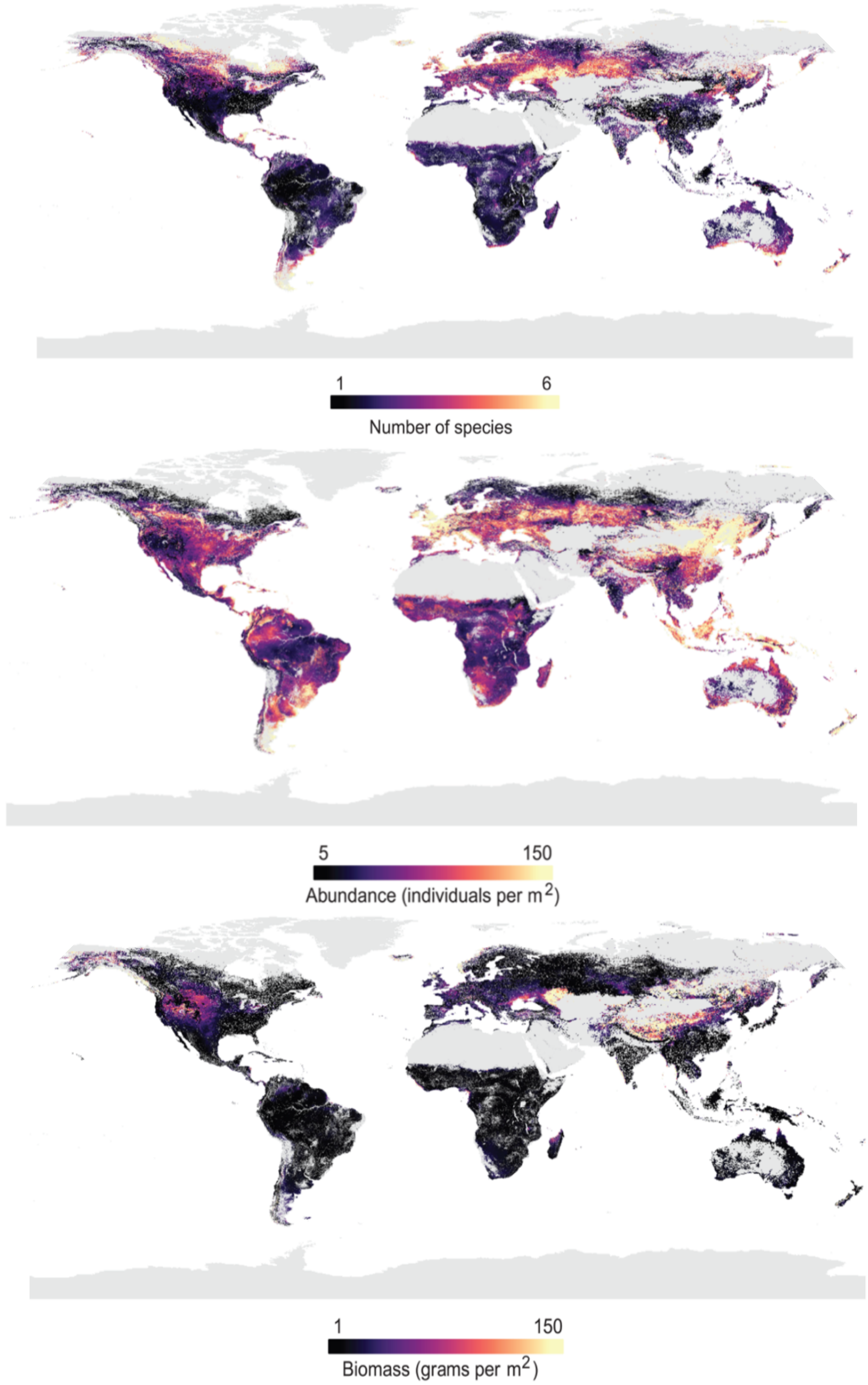

Figure 1.2. Predicted global distribution of earthworms in terms of species richness (a), abundance (b) and biomass (c), based on environmental drivers using data from 180 datasets. Taken from Phillips *et al*. (2019).

Although soils can buffer temperature changes to some extent (Peng *et al.*, 2022), by 2100 it has been projected that soils may increase in temperature by 4.5 °C to depths of 1 metre (Soong *et al.*, 2020). The impact of rising soil temperatures on earthworms is context-dependent and often modulated by soil moisture levels. Higher temperatures typically exacerbate soil water loss through evaporation (Kardol *et al.*, 2011), which leaves earthworms vulnerable to desiccation (Hogben and Kirk, 1944). For instance, Eisenhauer *et al.* (2014) found that warming reduced earthworm biomass at low water contents, whereas effects were neutral or positive at higher soil moisture levels (>21 wt%). Consequently, in moist environments such as temperate zones, the effect of increasing temperature on earthworm populations may be positive (Berman and Meshcheryakova, 2013), while in more arid regions with seasonal dry periods, a combination of increased temperatures with reduced rainfall may be detrimental for earthworms (Hughes *et al.*, 2019). The interaction between temperature and soil moisture also influences earthworm growth. In their study, Wever *et al.* (2001) observed that growth rates of *Aporrectodea tuberculata* (Eisen, 1874) exposed to 10 °C began to decline after 4 weeks in 20 wt% moisture, whereas growth increased at 25 % moisture. Additionally, juvenile *Ap. tuberculata* grew much faster at 20 °C than at 10 °C when moisture levels were maintained at 25 %. Moreover, low temperatures can reduce metabolic rates (Edwards and Lofty, 1972) and influence the vertical distribution of earthworms. For example, Potvin and Lilleskov (2017) found that most *L. terrestris* and *Aporrectodea caliginosa* (Savigny, 1826) were located below 20 cm during winter, when soils neared freezing.

Even under ambitious climate change mitigation targets, an increased frequency (5-10 times) of droughts is predicted for many regions (Naumann *et al.*, 2018). Soil moisture is expected to decline sharply in the top 10 cm of soil by the end of the 21st century in many regions, including Europe, southern Africa, most of the Americas and Australia (Dai, 2013, **Fig. 1.3**), posing significant risks to earthworm survival and their associated ecosystem functions. Drought directly impacts the soil environment by reducing soil water films

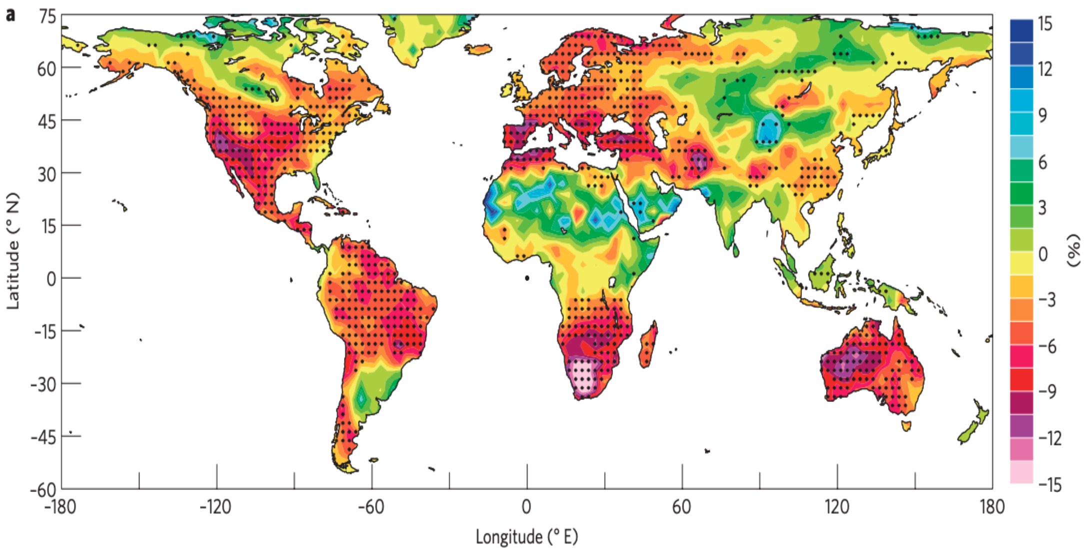

Figure 1.3. Changes in mean soil moisture content (%) in the top 10 cm simulated by 11 models under the RCP4.5 intermediate emissions scenario from 1980-1999 to 2080-2099. Stippling indicates at least 9/11 models agree on the sign of change (Taken from Dai, 2013).

(Coleman *et al.*, 2004), and through structural changes due to shrinkage and fracturing of soil aggregates (Gimbel *et al.*, 2016). Together these effects reduce the plasticity of soil and increase the mechanical stresses and energy required for movement (Coyle *et al.*, 2017; Kretzschmar, 1991) which makes feeding more difficult for detritivores (Thakur *et al.*, 2018). Drought conditions can also influence earthworms indirectly (Coyle *et al.*, 2017) through reductions in aboveground plant biomass (Wu *et al.*, 2011; Li *et al.*, 2021) which modify the soil surface microclimate.

Earthworms are particularly sensitive to soil drying given their limited capacity to control the release of their body water (Carley, 1978). This is problematic as among other uses, earthworms require free water to facilitate oxygen uptake for respiration (Holmstrup, 2001). For instance, in *Eisenia fetida* (Savigny, 1826) earthworms, even slight reductions in soil moisture reduce the availability of oxygen for percutaneous uptake (Diehl and Williams, 1992; Williams and Diehl, 1992). Water is also used to form coelomic fluid which acts as a hydrostatic skeleton, keeping earthworms turgid and allowing movement through the soil (Kretzschmar and Bruchou, 1991; Ramsay, 1949; Whalen *et al.*, 2000). At higher moisture levels it is easier for earthworms to extract water from soil, which allows them to regulate their body water content and allocate more energy to their development (Wever *et al.*, 2001). Accordingly, soil moisture level is important for their growth and reproduction (Zorn *et al.*, 2008) and is the primary factor limiting earthworm survival (Wever *et al.*, 2001). Subsequently, on a local scale, soil moisture is the key determinant limiting the abundance, biomass, distribution and activity of earthworms (Lee, 1985; Curry, 2004; Perreault and Whalen, 2006). Therefore, ecosystems with high soil moisture and moderate temperatures in the upper soil layers and litter are believed to be most favourable for earthworms (Singh *et al.*, 2019). However, too much water is also suboptimal as, although many earthworms can survive in submerged soil for a period, their tolerances and responses are species-specific (Zorn *et al.*, 2008; Singh *et al.*, 2019; Kiss *et al.*, 2021). Generally, flooding has been found to reduce earthworm abundance, diversity and biomass (Plum, 2005), mostly due to associated anaerobic conditions (Singh *et al.*, 2019).

Furthermore, disruptive human activities and land management practices have long been known to adversely affect earthworm populations (Curry, 2004). Conventional agricultural practices like ploughing and tillage significantly reduce soil biodiversity, including earthworms (Phillips *et al.*, 2024), through both direct mortality and indirect effects like structural damage to burrows, reducing their longevity in the soil and bringing earthworms to the surface where they are more exposed to dry conditions and predation (Roger-Estrade *et al.*, 2010; Briones and Schmidt, 2017). Soil compaction from machinery or livestock can reduce pore space, and so lower soil water-holding capacity and further reduce earthworm abundance (Piearce, 1984). The addition of synthetic fertilisers, herbicides and pesticides can also directly kill earthworms or their food resources e.g. by altering the plant community composition (Paoletti, 1999; Beumelle *et al.*, 2023). Often, degraded agricultural ecosystems also have reduced resilience and are thus more vulnerable to climatic perturbations (Siebert *et al.*, 2019).

1.3 Aestivation

Strategies such as aestivation, hibernation, torpor and diapause can be described as periods of dormancy, used to permit survival in suboptimal conditions (Jiang *et al.*, 2023). Aestivation refers to an ecophysiological strategy (Morgan and Winters, 1991) involving reversible metabolic inhibition (Jiang *et al.*, 2023) and is initiated in some organisms to survive a period of adverse conditions incompatible with active life (Hiong *et al.*, 2005). It is derived from 'aestas', the Latin word for summer (or heat, 'aestus') (Storey and Storey, 2012). Unlike obligatory diapause, which is seasonally programmed (e.g. hibernation) or happens at a specific developmental stage (Jiang *et al.*, 2023) and persists regardless of external factors, aestivation can be described as facultative diapause, which terminates once environmental conditions become favourable again (Díaz Cosín *et al.*, 2006; Storey and Storey, 2012).

Many organisms are capable of aestivation, for instance, giant African snails (*Achatina fulica*) secrete a white epiphragm (temporary structure) to seal their operculum and minimise water loss when food and water are lacking (Hiong *et al.*, 2005, Umezurike and Iheanacho, 1983). African lungfish (*Protopterus sp.*) form a mucus cocoon and live in it to survive dry summer months (Heimroth *et al.*, 2021, Jiang *et al.*, 2023), and freshwater tubificid worms such as *Rhyacodrilus hiemalis Ohraka* aestivate to avoid unfavourable summer conditions (Narita, 2006). In earthworms, aestivation typically involves coiling into a spherical knot to reduce their surface area exposed to the surrounding soil (McDaniel *et al.*, 2013a), secreting a protective layer of mucus and gut contents (Holmstrup, 2001, Holmstrup *et al.*, 2016) and constructing a sealed chamber (Bayley *et al.*, 2010) (**Fig. 1.4**). They remain in the protective environment of their chamber until favourable conditions return (McDaniel *et al.*, 2013a), so minimising water loss (Friis *et al.*, 2004). The term 'quiescence' is sometimes also used to refer to a period of low activity and suspended feeding influenced by environmental factors but does not usually involve the formation of a chamber (Díaz Cosín *et al.*, 2006). Fossil evidence from the Pleistocene shows spherical aestivation chambers with knobbly surfaces and meniscate pellet patterns which indicates that they have a characteristic morphology and suggests this behaviour is an ancient trait that has been evolutionarily conserved among earthworm species (Verde *et al.*, 2007; Genise *et al.*, 2013; Storey and Storey, 2012).

Figure 1.4. Earthworms in aestivation recorded at the field site and an empty aestivation chamber (far right, ~15 mm diameter).

Physiologically, aestivation is marked by a significant reduction in metabolic activity (Jiménez *et al.*, 2000; Lee, 1985). For example, Abe and Buck (1985) found a 78 % decrease in gaseous exchange in the giant earthworm *Glossoscolex paulistus* during aestivation.

Similarly, Bayley *et al.* (2010) reported that in *Ap. caliginosa*, gaseous exchange dropped by ~50 % after one day at 97 % relative humidity (RH) (short-term aestivation), and by ~75-80 % after 30 days of dehydration (long-term aestivation). Notably, normal metabolic function was resumed only in earthworms subject to short-term aestivation on release from their chambers, while those subject to long-term aestivation remained metabolically suppressed (Bayley *et al.*, 2010), suggesting that the physiological cost of recovery is linked to the duration of stress exposure.

Water and solute balance play a central role in earthworm survival during aestivation. As soil dries, the force at which water is held (the water potential) increases (pF and kPa values become more positive) meaning that water becomes less available for uptake (Lee, 1985; Kretzschmar and Bruchou, 1991; McDaniel *et al.*, 2013a). It has been suggested that when soil water potential increases above ~400 kPa (~pF 3.6), the osmotic pressure of earthworm body fluids becomes greater than that of the surrounding soil, leading to water loss across their skin surface via osmosis (Bayley *et al.*, 2010; McDaniel *et al.*, 2013a; Holmstrup *et al.*, 2016). As water is lost, earthworms experience osmotic stress caused by increasing concentrations of inorganic ions such as Cl^- and Na^+ (Holmstrup *et al.*, 2016). For instance, *Ap. caliginosa* exhibited a threefold increase in body fluid osmolality (to 560 mOsmol kg^{-1}, equivalent to 1340 kPa) within one day of exposure to 97 % RH (Bayley *et al.*, 2010). Similarly, Friis *et al.* (2004) found that drought-exposed *Ap. caliginosa* increased their body fluid osmolality and solute concentrations as their body water content decreased.

To mitigate osmotic stress, earthworms synthesise osmolytes (small organic molecules like glucose, sorbitol, and free amino acids (FAAs) such as alanine), which help dilute harmful ion concentrations and maintain cellular function (Yancey, 2005; Storey, 1997). For example, Bayley *et al.* (2010) found alanine levels in adult *Allolobophora chlorotica* (Savigny, 1826) increased to over 80 mmol l^{-1} during long-term aestivation (30-day exposure to 97% RH). Likewise, Holmstrup *et al.* (2016) observed that *Aporrectodea icterica* (Savigny, 1826), *Aporrectodea longa* (Ude, 1885), *Ap. tuberculata* and *Lumbricus rubellus* (Hoffmeister, 1845) all roughly doubled their FAA concentration under drought-stress (2000 kPa), especially alanine, which correlated significantly with declining body water content in *Ap. tuberculata*. Even earthworm cocoons have been found to display this adaptive mechanism, and accumulate sorbitol and alanine during gradual dehydration (Petersen *et al.*, 2008), suggesting that resistance to osmotic stress begins early in development. Osmolytes like alanine have also been suggested to help counter protein denaturation and enzyme inhibition caused by low water availability (Holmstrup *et al.*, 2016).

By increasing their body fluid osmolality, earthworms reduce the pressure gradient between their internal fluids and the surrounding soil, thereby promoting passive water diffusion and minimising desiccation risk (Bayley *et al.*, 2010; Holmstrup *et al.*, 2016). Friis *et al.* (2004) found that in severe drought (pF 4-4.5), water content was up to 30 % higher in *Ap. caliginosa* that formed aestivation chambers compared to those that did not. Similarly, Bayley *et al.* (2010) reported a 20 % increase in *Ap. caliginosa* body water content when forming aestivation chambers. This highlights the effectiveness of this protective behaviour in conserving moisture and is further supported by survival data. Mortality in *Ap. caliginosa* was found to be significantly higher in individuals that did not form aestivation chambers (McDaniel *et al.*, 2013a). Similarly, Friis *et al.* (2004) found mortality was significantly higher in individuals that did not form aestivation cells, except at the highest drought level (pF 4.5-5) where all earthworms died, suggesting there are limits to the protective capacity of aestivation. However, not all earthworms rely on aestivation for survival. Friis *et al.* (2004) found 43 % survival of non-aestivating earthworms even in drought levels up to pF 4.5, exceeding the permanent wilting point (pF 4.2). Holmstrup *et al.* (2016) found no difference in the ability of aestivating *Aporrectodea* to conserve body water than non-aestivating *L. rubellus* when exposed to the same drought severity. This suggests that some species may be just as capable at preventing water loss through physiological mechanisms such as alanine accumulation, without needing to induce a behavioural response (Holmstrup *et al.*, 2016).

Furthermore, alanine appears to play a crucial role in nitrogen metabolism, which is significantly impacted by the lack of activity and food consumption associated with aestivation (Jiang *et al.*, 2023). Prento (1989) suggested earthworms switch to being ureotelic during dehydration rather than their normal ammonotelic state in hydrated conditions (Cohen and Lewis, 1949). Under severe dehydration and prolonged fasting, *Ap. caliginosa* reduced or ceased urine production, leading to an accumulation of nitrogenous waste, specifically urea, which increased from 0.3 to 1 µmol g^{-1} dry mass during 30 days of aestivation (Bayley *et al.*, 2010). Since urea excretion is suppressed, Bayley *et al.* (2010) proposed that alanine may act as a temporary nitrogen reservoir, incorporating excess ammonium until normal excretory functions can resume. This detoxification strategy is consistent with findings in other aestivating invertebrates. For example, Hiong *et al.* (2005) found that the Giant African snail (*Achatina fulica*) detoxified ammonia by converting it to urea, especially during early aestivation (the first four days).

At the genetic level, earthworms undergo broad transcriptional changes during aestivation. In *Carpetania matritensis* (Marchán, 2020), prolonged aestivation is associated with downregulation of many genes, particularly those involved in metabolism, digestion, protein turnover, transcription and translation (Tilikj *et al.*, 2023). This repression reflects the dormancy of metabolic and digestive processes characteristic of aestivation. Importantly, on arousal from aestivation, gene expression rapidly resumed, returning to levels seen in non-aestivating earthworms (Tilikj *et al.*, 2023). Genes involved in abiotic stress mitigation, immune function, and cellular maintenance also show notable changes. For example, with increasing aestivation duration, genes controlling antibacterial and antiviral responses were progressively downregulated (Tilikj *et al.*, 2023). Instead, transcriptional responses shift from maintenance of immune response towards preventing damage, with upregulation of genes related to reactive oxygen species (ROS) scavengers, DNA repair and telomere maintenance (Tilikj *et al.*, 2023). As the duration of unfavourable conditions is unpredictable, adaptations that enhance longevity, stress resistance and reprioritise adenosine triphosphate (ATP) use to vital functions, allow a state of prolonged aestivation and are critical for increasing the chances of survival until conditions improve (Tilikj *et al.*, 2023; Storey and Storey, 2012).

Given that aestivation is a reversible and environmentally-cued response, it is essential to understand the external factors that induce this state. In earthworms, exposure to drought stress is thought to be the most important factor influencing aestivation, with many species entering this phase of suspended development when soil moisture levels fall below critical thresholds (Jiménez *et al.*, 2000; Díaz Cosín *et al.*, 2006). In general, earthworms remain active in soils where moisture levels exceed 20 wt% (Díaz Cosín *et al.*, 2006; Tilikj and Novo, 2022, **Table 1.1**). Water potential is also an important factor influencing earthworm activity given its association with body osmolality and water loss mentioned previously (**Table 1.1)**. Consequently, earthworm activity is thought to be constrained in soils with water potential values above pF 3 to 3.3 (Gerard, 1967; Kretzschmar and Bruchou, 1991; Nordström, 1975; Rundgren, 1975; Baker *et al.*, 1993).

Earthworm drought tolerance is likely shaped by adaptations to local soil conditions (Holmstrup, 2001; McDaniel *et al.*, 2013a). Environmental factors such as water-holding capacity and organic matter content contribute to the variable responses of earthworms to drought (Plum and Filser, 2005). Additionally, shifts in plant community compositions resulting from drought can affect resource availability, influencing earthworm activity. For instance, Potvin and Lilleskov (2017) found organic matter availability, driven by plant community dynamics, accounted for differences in aestivation rates. Earthworms also exhibit species-specific moisture preferences, which further modulate their responses to drought (Eisenhauer *et al.*, 2014; Curry, 2004). Grant (1955) observed that *Pheretima hupeiensis* (now *Amynthas hypeiensis*) preferred wetter conditions, with a minimum of 66 % of the individuals found in soil layers with moisture contents between 20-30 wt% and none found below 18 wt% moisture, indicating a clear threshold below which activity is greatly reduced. In contrast, *Ap. caliginosa* showed greater tolerance to drier conditions, with a maximum of 53.3 % of the individuals found in the wettest zones (Grant, 1955). Doube and Styan (1996) further explored moisture preferences, showing that *Aporrectodea rosea* (Savigny, 1826) avoided soils with less than 5-10 wt% moisture, while *Aporrectodea trapezoides* (Dugès, 1828) was even more moisture sensitive, avoiding soils below 15 wt%.

Table 1.1. The water contents given as gravimetric % on a dry mass basis, or water potential (pF) at which earthworms have been recorded active or in aestivation.

Earthworms	Aestivation	Active	Reference
Hormogaster elisae (Álvarez, 1977)	10 wt% moisture	20 wt% moisture	Díaz Cosín *et al.* (2006)
Carpetania matritensis (Marchán, 2020)	5 wt% moisture	>20 wt% moisture	Tilikj and Novo (2022)
Australian lumbricids		150 kPa (~pF 3.18)	Baker *et al.* (1993)
Aporrectodea caliginosa (Savigny, 1826)	>pF 2.79-3.28; >pF 2.3-2.6 (20-40 kPa, RH 99.96 %); >pF 3.2		McDaniel *et al.* (2013a); Holmstrup (2001); Gerard (1967)
Aporrectodea longa (Ude, 1885)	167-620 kPa (>pF 3.22)	60 kPa (~pF 2.8)	Kretzschmar and Bruouchou (1991)

Under high temperature conditions, earthworms may respond by burrowing deeper (Perreault and Whalen, 2006) or entering aestivation especially when high temperatures are associated with limited soil moisture availability (Jiménez *et al.*, 2000). For instance, Díaz Cosín *et al.* (2006) found that the proportion of *Hormogaster elisae* (Álvarez, 1977) entering aestivation significantly increased with both higher temperatures and lower soil moisture, though temperature had the greatest effect on aestivation in the driest treatment (10 wt% moisture). Similarly, Wever *et al.* (2001) reported that the proportion of *Aporrectodea tuberculata* entering aestivation rose with increasing temperature, particularly at 10 and 15 wt% moisture. In contrast, in very cold conditions, water may not be available for uptake by earthworms, and while some species are thought to be freeze-tolerant, others may become inactive or enter aestivation (Rundgren, 1975; Garnsey, 1994; Holmstrup, 2001; Nuutinen and Butt, 2009; Nordström, 1975). It is evident that the precise conditions restricting earthworm activity depend not only on species-specific tolerance ranges but also on local environmental contexts.

The onset of aestivation and mortality in earthworms also depends on the severity and duration of unfavourable conditions. For instance, after two weeks of exposure to 10 wt% moisture some *H. elisae* remained active, but after four months at this moisture level, all the earthworms had entered aestivation (Díaz Cosín *et al.*, 2006). Similarly, McDaniel *et al.* (2013a) found a clear positive correlation between the length of drought stress and the increasing number of aestivating earthworms. The strategy earthworms adopt and the duration they spend in aestivation are likely influenced by the conditions they experience (Morgan and Winters, 1991). For example, a prolonged state of aestivation may be beneficial during extended dry spells, whereas in regions with more variable moisture conditions (i.e. intermittent precipitation), short-term aestivation might be possible (Morgan and Winters, 1991). The impact of aestivation duration on survival likely depends on the tolerance range of individual species (Jiang *et al.*, 2023). For instance, Tilikj *et al.* (2023) found that *C. matritensis* could survive in a state of aestivation for up to a year in soils with 5 wt% moisture. In contrast, while *Ap. caliginosa* recovered from 3-week drought cycles lasting up to 63 days, mortality was significantly greater in 3-week drought stress periods than 2-weeks, though 86 % still survived (McDaniel *et al.*, 2013a).

Furthermore, there is evidence to suggest that certain factors, including toxic compounds, may act synergistically with soil moisture resulting in detrimental impacts for earthworms at levels that if present independently would be tolerated. Friis *et al.* (2004) found that copper-exposed *Ap. caliginosa* were less likely to form aestivation chambers in response to drought conditions and had higher mortality rates compared to non-copper-exposed individuals. They suggest that the reduced activity of copper-exposed earthworms limits their soil consumption and thus ability to construct aestivation chambers (Friis *et al.*, 2004). Therefore, when studying earthworm populations, it is important to consider multiple interactions that may be occurring between stresses.

1.4 Overall research aims and implications

Aestivation remains a largely understudied phenomenon in earthworms despite being recognised as an important self-preservation strategy. Several key aspects of this behaviour are still poorly understood, with Kiss *et al.* (2021) acknowledging the need for more research into the environmental triggers of aestivation. Moreover, the associated costs to the earthworm itself, as well as to the broader soil ecosystem, warrant further investigation. Earthworms play a vital role as ecosystem engineers, contributing to soil functions like nutrient cycling, organic matter decomposition, and soil structure maintenance. Reducing activity during aestivation, including the cessation of feeding, burrowing and reproduction, may disrupt these processes. For instance, Bayley *et al.* (2010) highlight that nitrogen inputs into the soil may be significantly disrupted during droughts when earthworm activity is halted due to periods of aestivation, resulting in altered soil fertility and nutrient availability for plants. Understanding the factors that drive aestivation, particularly moisture tolerances, will provide valuable insights into how climate change may affect earthworm activity, soil biota, and overall soil health. This knowledge is crucial for predicting the impacts of global change on soil ecosystems and for developing strategies to mitigate potential negative effects on soil productivity and ecosystem services.

In addition, while this thesis mainly focuses on the impacts of aestivation on earthworms themselves, the broader ecological implications must also be considered. Earthworms are an important food source for many species, and changes in their availability could have cascading effects on food webs. For example, climate-induced shifts in earthworm activity and their distribution throughout the soil profile could impact species like meadow birds (Onrust and Piersma, 2017; Singh *et al.*, 2019). This is especially relevant during the early summer breeding season, when earthworms provide an important food resource for many birds, a time that will likely overlap with periods of aridity when earthworms are less available (Martey and Pearce-Higgins, 2018).

Chapters 2-4 cover behavioural experiments carried out using *Allolobophora chlorotica* (Savigny, 1826), chosen as it is one of the most common earthworms in UK farmland (Ashwood *et al.*, 2024) and is known to use aestivation as a survival strategy (Edwards and Lofty, 1977). This species is typically classified as endogeic, although there is some discrepancy within the literature, with some considering it epi-endogeic (Le Couteulx *et al.*, 2015), or intermediate (Bouché, 1977; Bottinelli *et al.*, 2020). More recently, assessments of burrowing behaviour have placed it within the functional group of shallow bioturbators, which represents small species that make shallow galleries in the soil (Capowiez *et al.*, 2024). As such, *Al. chlorotica*, is primarily found in the upper 10 cm of soil and in close association with plant roots (Le Couteulx *et al.*, 2015). There are two morphs: a green morph containing porphyrins and a biliverdin-like substance and a pink morph lacking some of these pigments (Satchell, 1967). The taxonomic status of *Al. chlorotica* is still under investigation, but it is known to be a complex of cryptic species with several highly divergent mitochondrial lineages (King *et al.*, 2008, Dupont *et al.*, 2011). The green morph is believed to be a single taxon composed of two divergent lineages (L2 and L3), while the pink morph has three lineages (L1, L4 and L5) which may represent distinct taxa (Dupont *et al.*, 2011; Dupont *et al.*, 2016). Although they can be found together, pink and green morphs are thought to have unique moisture preferences, which may limit inter-morphic breeding under extreme conditions (Lowe and Butt, 2007). For instance, the green morph dominates in wetter conditions in which they have a faster growth rate and higher fecundity than the pink morph, giving them a competitive advantage and potentially restricting the distribution of the pink morph to drier soil conditions (Lowe and Butt, 2007). Moreover, in pink x green pairings, individuals of both morphs produced cocoons, but the viability of those produced by green individuals was much lower (6% vs 59% for pink) (Lowe and Butt, 2008). When inter-morphic bred offspring matured and were paired, all cocoons produced were non-viable, suggesting genetic divergence of the two morphs (Lowe and Butt, 2008). In this thesis, experiments focus on the green morph (**Fig. 1.5**), which was abundant in high numbers and easily distinguishable from other species at the sampling site (Spen farm, Tadcaster 53°52'25.9"N 1°19'33.4"W) due to its pigmentation.

Figure 1.5. *Allolobophora chlorotica* (~5-8 cm in length).

Chapter 2 explores the response of *Allolobophora chlorotica* to gradual soil drying. The first experiment investigates the soil moisture conditions that induce aestivation in *Al. chlorotica* and assesses the extent of soil drying they can tolerate. Additionally, changes in *Al. chlorotica* mass during and after exposure to drying conditions were investigated, specifically whether individuals can recover to pre-drought masses once returned to favourable conditions.

Chapter 3 builds on these findings by exploring the impact of repeated drought and aestivation on the reproductive output of *Al. chlorotica*. While questions remain regarding the duration of aestivation and how many times earthworms can aestivate throughout their lifetime (Tilikj *et al.*, 2023), it is clear that repeated aestivation could have significant implications for earthworm populations. In particular, understanding the effects on earthworm fecundity is crucial, as it directly impacts the viability of earthworm populations and their ability to recover from perturbations. This chapter assesses the reproductive output of *Al. chlorotica* after exposure to one, two, or three bouts of aestivation, focussing on cocoon production, cocoon mass, viability and hatching time once the earthworms are returned to optimal soil moisture conditions. Changes in *Al. chlorotica* mass are also evaluated to provide insight into the physiological effects of repeated drought and aestivation on earthworms.

Chapter 4 examines the response of *Al. chlorotica* to drought stress across four different soil types. Soils differ in their pore space and structure, which influence their hydrological properties and ability to retain water. This chapter investigates how soil type affects the onset of aestivation and changes in earthworm mass during drought exposure. Earthworms were exposed to different gravimetric water contents and water potentials (pF) to better understand the relative importance of water availability in governing the response of *Al. chlorotica* to drought stress.

Chapter 5 shifts focus to field-based research, investigating the behaviour and community composition of earthworms in two UK farms over the course of a year. While controlled experiments offer valuable insights, studying earthworm behaviour in natural settings is necessary to gain a more comprehensive understanding of their responses to changing environmental conditions. This is essential as earthworm populations are already facing substantial losses. For instance, Barnes *et al.* (2023) reported declines in earthworm populations, most apparent in woodland and pasture and grassland in farmlands, with annual declines of 1.6-2.1 %, equating to a 33-41 % loss over 25 years. Covering 72 % of the UK's total land (FAOSTAT, 2023), agricultural ecosystems represent an important area to study. This chapter aims to identify species that use aestivation as a survival strategy and determine the soil conditions that induce it. To achieve this, DNA metabarcoding was used with a novel non-destructive swabbing method to identify earthworms to the species level. This research also examines how changes in soil properties such as moisture content, soil organic matter (SOM), pH, bulk density and temperature, affect earthworm densities and species richness. Additionally, the age structure and composition of earthworm communities are explored to gain deeper insights into the vulnerability of different species and life stages to changing soil conditions.

The thesis concludes with a chapter that draws together the key research findings, considers their broader implications and outlines directions for future research.

A conceptual framework is outlined in **Figure 1.6,** which illustrates the main themes of this thesis and the links between components explored in each research chapter.

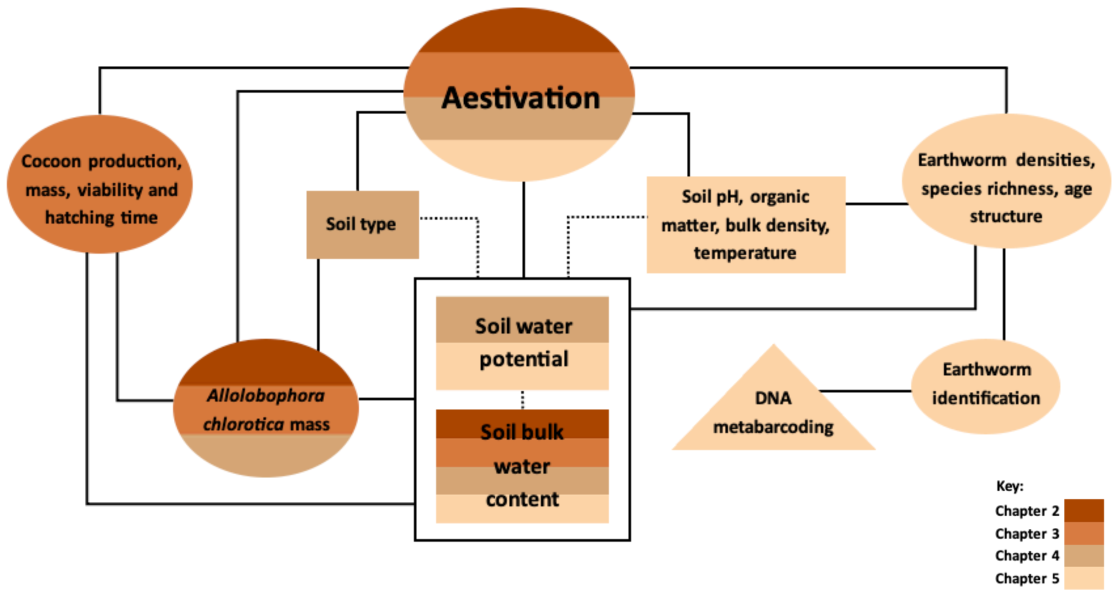

Figure 1.6. Conceptual framework of the thesis, illustrating the interrelations between key themes and research components (soil properties = rectangles, earthworm parameters = ovals, method = triangle). The key shows chapter colour-coding.

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Chapter 2

The response of *Allolobophora chlorotica* to gradual soil drying

2.1 Abstract

Earthworms are key ecosystem engineers, crucial to the functioning of healthy and productive soil, yet their reliance on water leaves them threatened by the increased frequency and severity of droughts expected with climate change. To prevent desiccation, some earthworms enter aestivation whereby they curl into a knot to reduce their surface area exposed to the dry soil, cover themselves in mucus and remain in a state of low metabolic activity. The precise conditions that induce aestivation in earthworms remain largely unknown. Therefore, adult *Allolobophora chlorotica* (Savigny, 1826) of known mass were placed into soil with initial gravimetric moisture contents of either 30 wt% or 25 wt% and subjected to gradual drying to investigate which soil moisture contents induce aestivation. The state and fresh mass of earthworms were determined at 18 wt%, 13 wt% and 8 wt% moisture, and survivors were transferred to soil substrates with optimal moisture conditions to undergo a period of recovery (of between 19 - 30 days duration), before being weighed once more. Soil moisture significantly affected the proportion of aestivating earthworms with all *Al. chlorotica* active at 18 wt% moisture, while at 13 wt% moisture 83 % were found in aestivation. Survival decreased below 13 wt% moisture, with 100 % mortality at 8 wt% moisture, and all but one individual deceased in a visible state of aestivation. Contrary to expectations, all surviving *Al. chlorotica* gained mass from the start to the end of the experiment. However, the extent of these increases depended on the degree of drying experienced and whether they aestivated or not, with those that remained active increasing in mass significantly more. Overall, smaller increases in mass during the period of soil drying were compensated for by greater increases during subsequent exposure to optimal conditions, resulting in no significant differences in net changes in mass between treatments. These results support evidence that aestivation is induced by specific moisture conditions, in this case between 18 wt% and 13 wt% for *Al. chlorotica*, rather than it being an obligatory diapause occurring at a certain time of year. Moreover, changes in *Al. chlorotica* mass due to aestivation and exposure to drying conditions appear transient, with any losses recovered when earthworms were subsequently transferred to soil substrates with more optimal moisture conditions.

2.2 Introduction

Earthworms are key ecosystem engineers (Eisenhauer, 2010), vital to the functioning of healthy and productive soils with important roles in the provision of numerous ecosystem services, many of which have implications for agriculture. For instance, by mineralising soil organic matter through decomposition, earthworms can improve the availability of macronutrients, leading to increases in aboveground plant biomass, microbial biomass and soil respiration (McDaniel *et al.*, 2013b; Van Groenigen *et al.*, 2014; Wu *et al.*, 2025). Earthworms increase the porosity of soil through their burrowing behaviour and formation of biogenic structures, which in turn improves aeration, water infiltration and storage (Delgado-Baquerizo *et al.*, 2025). Indeed, the presence of earthworms can increase soil moisture content by 13 % (Wu *et al.*, 2025). Soil moisture is generally considered to be the most important environmental variable for the functioning of earthworms (Lavelle, 1983), with even slight deviations from optimal moisture conditions believed to influence their physiology (Diehl and Williams, 1992). Among other functions, earthworms rely on water in the soil to keep their body turgid and act as a hydrostatic skeleton, allowing movement within the soil (Kretzschmar and Bruchou, 1991; Edwards and Bohlen, 1996), and facilitating oxygen uptake for respiration (Holmstrup, 2001). Consequently, earthworms are vulnerable to drought conditions (Lavelle, 1983) and so are threatened by the increased frequency and severity of extreme climatic events projected under climate change (Senevirantne *et al.*, 2012).

When the soil moisture becomes too low, some earthworms enter a state of suspended development called aestivation (Jiménez *et al.*, 2000). During aestivation, earthworms form a knot to reduce their surface area exposed to the soil (McDaniel *et al.*, 2013a), cover themselves with mucus and gut contents (Holmstrup, 2001), and seal themselves into a chamber (Bayley *et al.*, 2010). They remain in a state of low metabolic activity, unable to carry out their usual ecological functions until conditions improve (Gerard, 1967; Jiménez *et al.*, 2000; McDaniel *et al.*, 2013b). Therefore, prolonged periods spent in aestivation are likely to have detrimental consequences for the soil environment. Despite this, little is known about the drivers of aestivation in earthworms, particularly at which moisture content the soil becomes too dry for earthworms to function. Research investigating the specific conditions that induce aestivation in earthworms will allow us to consider the potential costs of droughts on soil ecosystems and how we could implement strategies to mitigate them.

This study investigated which soil moisture conditions induce aestivation in the green morph of *Al. chlorotica*, one of the most common earthworm species in the UK, particularly in farmland (Ashwood *et al.*, 2024). Soil moisture contents between 25 - 30 wt% have been suggested as optimal conditions for *Al. chlorotica* (Lowe and Butt, 2005), therefore as the moisture content decreases below this range and the availability of soil water becomes more limiting, the proportion of earthworms entering aestivation was expected to increase as they try to avoid desiccation. The onset of aestivation may be triggered by a specific moisture content, or earthworms may be able to detect the loss of soil moisture and thus enter aestivation after experiencing a period of time in drying conditions without the addition of water. Drying conditions starting from a higher initial moisture content may allow earthworms more time to detect the gradual decrease in water and thus provide greater opportunity to form aestivation chambers. Therefore, at each target moisture content sampled, a higher proportion of *Al. chlorotica* were predicted to be aestivating in the group that started drying from a higher moisture content. Finally, changes in *Al. chlorotica* mass were assessed after exposure to drying conditions and again following a period of optimal moisture conditions. Diehl and Williams (1992) suggest earthworm growth is affected by the reduced oxygen availability and thus depressed aerobic metabolism associated with low soil moisture conditions. Therefore, *Al. chlorotica* weighed after exposure to drying conditions and a period of aestivation were predicted to have decreased in mass and be smaller than those kept in substrates where water and thus oxygen availability was not a limiting factor to their growth. Moreover, Díaz Cosín *et al.* (2006) recorded the weight of *Hormogaster elisae* (Álvarez, 1977) as they exited their chambers and found aestivating earthworms decreased in mass by an average of 41.6 % but recovered back to their initial body mass after approximately one week (6.5 ± 3.6 days) in 20 wt% moisture. Consequently, no net change in earthworm mass was predicted after rehydration following drought exposure, with any losses regained when returned to optimal moisture conditions.

2.3 Methods

2.3.1 Field site and earthworm extraction

Earthworms were collected from Spen farm, Tadcaster (53°52'25.9"N 1°19'33.4"W), part of the University of Leeds Research Farm. Four sampling trips took place during spring 2022 (March - May) in Warren Paddock, a silage field with a well-drained, calcareous loam soil (Holden *et al.*, 2019), that had been in permanent pasture since 2012. *Al. chlorotica* earthworms of the green morph (**Fig. 2.1**) were collected by digging soil pits (18 cm x 18 cm at the surface and 20 cm deep) and hand-sorting the soil. After collection, *Al. chlorotica* were transferred to a controlled temperature room at the University of Sheffield and cultured in field soil in 750 ml containers with perforated lids ('Nationwide Paper' brand rectangular reusable plastic food containers with lids, purchased from Amazon) at 15 ± 1 °C and 24-hour darkness until use in experiments.

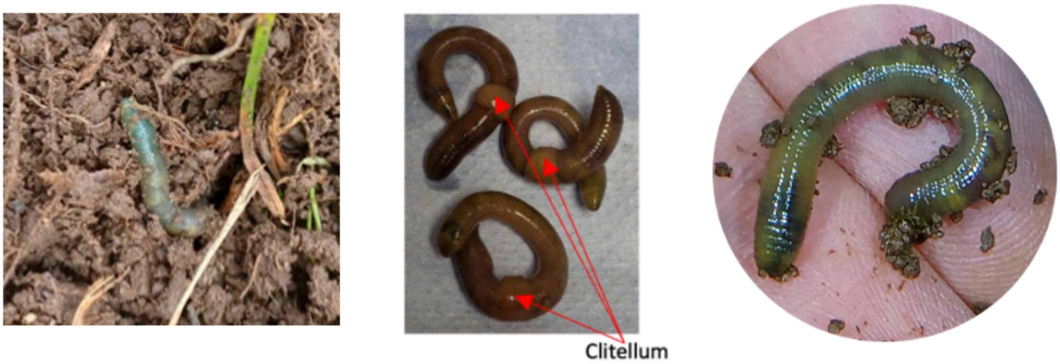

Figure 2.1. Individuals of the green morph of *Allolobophora chlorotica* (~5-8 cm in length), varying in pigmentation intensity. Central image shows adults each with a fully developed clitellum.

2.3.2 Acclimation period

To account for differences in the size of earthworms collected at different time points, *Al. chlorotica* from all collection dates were combined and 96 adults (mean mass of 0. 224 ± 0.051 g) with a fully developed clitellum (**Fig. 2.1)** were randomly selected and transferred into experimental substrates consisting of commercially available Kettering loam soil (purchased online from Agrigem, Lincoln LN1 2FU) mixed with dried sheep manure (~0.5 g per earthworm/week) as a food source, and moistened with deionised distilled water (DIW) to approximately 25 wt% gravimetric moisture for a minimum of 7 days to acclimatise to the new conditions. Sheep manure was obtained from sheep that had been reared without the use of anti-helminthic drugs. Such acclimation periods of at least one week's duration have been recommended by Fründ *et al.* (2010) when transitioning to experimental conditions as they help reduce the likelihood of any stress responses resulting from sudden changes from field to laboratory conditions that could interfere with the response of earthworms to drought exposure (Holmstrup, 2001).

2.3.3 Depuration and fresh mass determination

Earthworms were removed from the acclimation substrates 1 day prior to the start of the experiment. They were placed into Petri dishes containing filter paper moistened with DIW and left for 24 hours to depurate (void their gut contents). After 24 hours they were rinsed with DIW, gently blotted dry with a paper towel to remove excess moisture and reduce them to their basal mass, and weighed on a top pan balance to three decimal places to determine their soil-free starting fresh mass. Fresh mass was measured as an indicator of earthworm health and to allow changes in mass to be calculated following exposure to drought conditions i.e. to determine whether aestivation causes decreases in fresh mass. Each of the 96 earthworms were then randomly assigned to one of four treatments: drought exposure with an initial moisture content of 30 wt% (D30), drought exposure with an initial moisture content of 25 wt% (D25), control conditions with constant 30 wt% moisture (C30), or control conditions with constant 25 wt% moisture (C25). There was no significant difference in the initial mass of earthworms in each treatment (**Fig. S2.1**).

2.3.4 Soil substrate preparation

Soil substrates consisting of ~400 g (weighed to two decimal places) air-dried Kettering loam soil and ~10 g (weighed to two decimal places) dried ground sheep manure sieved to 2 mm were prepared in 750 ml plastic containers and mixed to homogenise. Appropriate volumes of DIW were added to each of the containers to give 48 substrates made to 25 wt% gravimetric moisture on a dry mass basis (0.25 ml water per 1 g dry substrate) and 48 substrates at 30 wt% moisture (0.3 ml water per 1 g dry substrate). A single earthworm was added to the surface of each of the 96 substrates in containers which were covered with nylon mesh (100 % polyamide 15 Denier tights purchased from Matalan and cut into smaller strips) to allow evaporation but prevent the earthworms from escaping and kept in a controlled temperature room at 15 ± 1 °C and 24-hour darkness. *Al. chlorotica* in the control treatments (C30 n=24 and C25 n=24) were kept in constant moisture contents maintained through regular weighing of the substrates to determine water loss and addition of DIW to the substrate surface when necessary, using a spray bottle for even application. The remaining *Al. chlorotica* were exposed to drought treatments (D30 n=24 and D25 n=24), in which substrates were left to gradually air dry with no further additions of water. As opposed to acute exposure to drought stress, drying was gradual in this experiment to simulate natural conditions in which soils lose water over a period of days and weeks (Petersen *et al.*, 2008), with the aim to produce more ecologically relevant results. The temperature, soil moisture conditions and food quantity were selected based on suggested optimal conditions for the culture of *Al. chlorotica* (Lowe and Butt, 2005) and another endogeic earthworm species, *Aporrectodea tuberculata* (Eisen, 1874) (Wever *et al.*, 2001).

2.3.5 Destructive sampling, determination of state and fresh mass

Substrates (n=6 for D30 and n=6 for D25) were destructively sampled when they reached moisture contents of 18 wt%, 13 wt% and 8 wt%, determined gravimetrically through daily weighing and calculations of substrate water loss. At each target moisture content, 6 substrates from the corresponding group kept in constant optimal moisture conditions (C30 and C25) were also sampled as controls. All individual *Al. chlorotica* were sacrificial replicates and once sampled they were removed from the drying conditions to avoid pseudoreplication. On destructive sampling, substrates were carefully removed from the containers and hand-sorted to identify the state of individual earthworms as being either active (outstretched body, moving), aestivating (coiled within an aestivation cell), deceased (dried out, loose in the substrate), or deceased in aestivation (dried out, coiled in an aestivation chamber) (**Fig. 2.2**). On removal from their aestivation chambers, surviving *Al. chlorotica* became active when handled, as was the case for *Aporrectodea caliginosa* (Savigny, 1826) earthworms in the experiments of Friis *et al.* (2004). At 8 % moisture, all earthworms were found to be deceased in the D25 group, and so the decision was made to sample and gain information on the activity status of the D30 group at an additional higher moisture content (10 wt%). The D25 group was sampled at 6 wt% moisture to confirm 100 % mortality below 8 wt%. All surviving earthworms were placed onto filter paper moistened with DIW for 24 hours, before gently being blotted dry with tissue paper and weighed to determine their fresh mass.

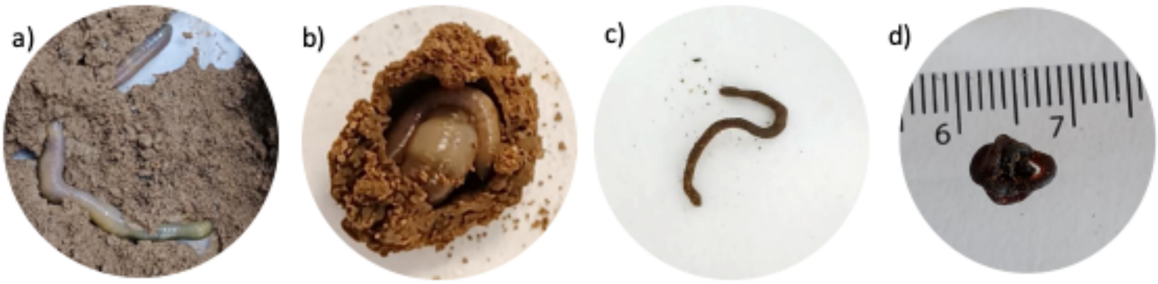

Figure 2.2. The state of earthworms when extracted from soil substrates during destructive sampling; a) active (~5-8 cm length), b) aestivating (chamber ~12 mm diameter), c) deceased and d) deceased in aestivation (~7 mm diameter).

2.3.6 Recovery period

After determination of their fresh mass, surviving earthworms were returned to their experimental containers in which the substrate had been re-wetted back to the initial starting 'optimal' moisture content (i.e. either 30 wt% or 25 wt%) to undergo a period of recovery. Moisture contents were kept constant through regular weighing and addition of DIW to the substrate surface using a spray bottle, and individuals remained in these conditions until the final substrates had been destructively sampled. Consequently, the time individuals spent in this period of recovery differed depending on the degree of drying they had been exposed to, with those sampled at 18 wt% moisture experiencing longer in the period of recovery/optimal moisture conditions (27 - 30 days) than those that were sampled at 10 wt% moisture (19 days). Once all containers had been destructively sampled, surviving individuals were once more placed onto wet filter paper for 24 hours then weighed after being blotted dry, to get a final measure of fresh mass.

2.3.7 Statistical analyses

The programming language of R (version 4.4.1, R Core Team, 2024) was used for all analyses. Differences in the proportion of earthworms in aestivation across moisture treatments were analysed using binomial generalised linear models (GLM) with a logit link function. The numbers of aestivating and active individuals were modelled as a binomial response, with sampled moisture, starting moisture and their interaction as fixed effects. Model significance was assessed using likelihood ratio tests. For analyses involving *Al.*

chlorotica mass, only data for surviving earthworms were included as fresh masses could not be calculated for deceased worms. One individual from the control treatment was found dead at the end of the experiment and so its final mass could not be recorded. Model assumptions of normality and homogeneity of variance were checked using diagnostic plots. Changes in relative earthworm mass over time (compared to their initial mass) were analysed using a linear mixed-effects model. Relative mass was log transformed to improve residual normality. Day of the experiment was included as a fixed effect, and container identity (each earthworm) was included as a random effect to account for repeated measurements. Differences in growth from the start to the first sampling were analysed using a two-way ANOVA, with earthworm state (active or aestivating) and treatment (constant control or drying) as fixed factors. The interaction between state and treatment was included to test whether the effect of earthworm state on their mass depended on treatment. Linear models with fixed and interactive effects of treatment (control or drying), sampling point and soil moisture content were fitted to assess differences in earthworm mass and changes in mass (measured relative to the start of each sampling period) both as a percentage and on a gram per day basis depending on time spent in each period. Posthoc Tukey tests were conducted to determine which treatments differed significantly.

2.4 Results

2.4.1 Earthworm aestivation and mortality

All *Al. chlorotica* in the control conditions with constant moisture conditions were found active when sampled at each time point. The rate of water loss (**Fig. S2.2**) was similar for those in the D30 and D25 groups with substrates starting at 30 wt% moisture taking 3 days longer to reach each target moisture content and thus experiencing 3 more days without any addition of water compared to those starting at 25 wt% moisture. Despite this, the proportion of *Al. chlorotica* found aestivating at each target moisture content in the D30 treatment did not differ significantly from those in the D25 treatment (Binomial distribution glm: $X^2 = 0.394$, df = 1, 2, $p = 0.5304$, **Fig. 2.3**).

Given this lack of statistically significant difference data from the D30 and D25 treatment groups were pooled for the following analysis. As expected, there was a significant main effect of soil moisture on the proportion of aestivating earthworms (Binomial distribution glm: $X^2 = 100.35$, df = 1,5, $p < 0.001$). Down to and including 18 wt% moisture content, all earthworms were found to be active on destructive sampling (**Fig. 2.4**). When sampled at a moisture content of 13 wt%, 83 % the earthworms had formed aestivation cells, suggesting that in this soil type, the onset of aestivation occurs between 18 and 13 wt% for *Al. chlorotica* (**Fig. 2.4**). Below 13 wt% moisture content, *Al. chlorotica* survival decreased, with only half of the individuals sampled at 10 wt% surviving and 100 % mortality below this moisture content (**Fig. 2.4**).

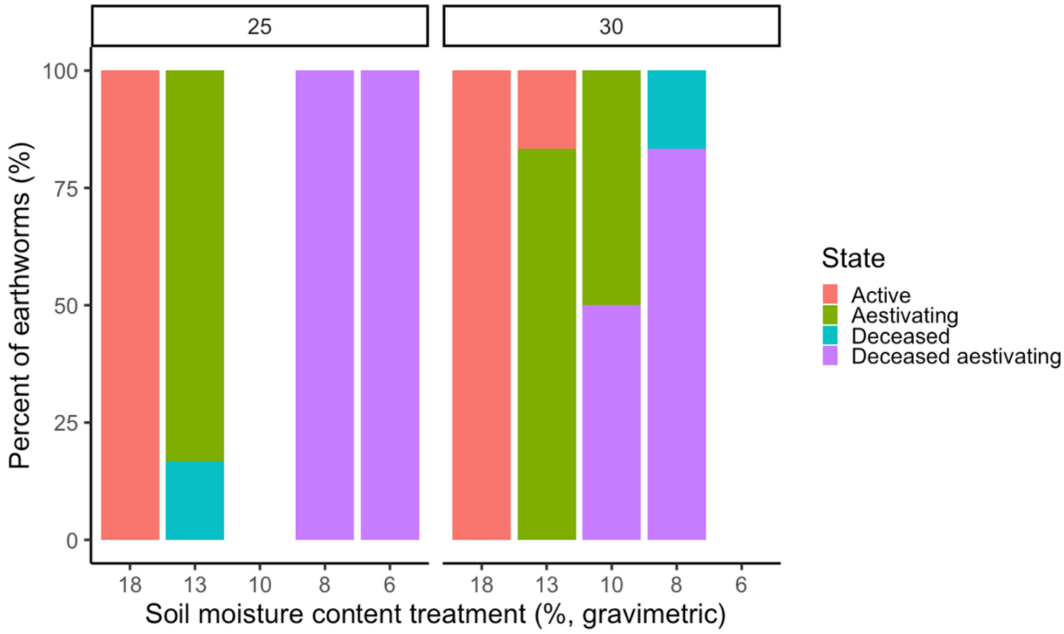

Figure 2.3. The proportion of *Al. chlorotica* (n = 6) found in each state; active=red, aestivating=green, deceased=blue, deceased in aestivation=purple, when destructively sampled at each moisture content, grouped according to the initial substrate moisture content (25 wt% and 30 wt%).

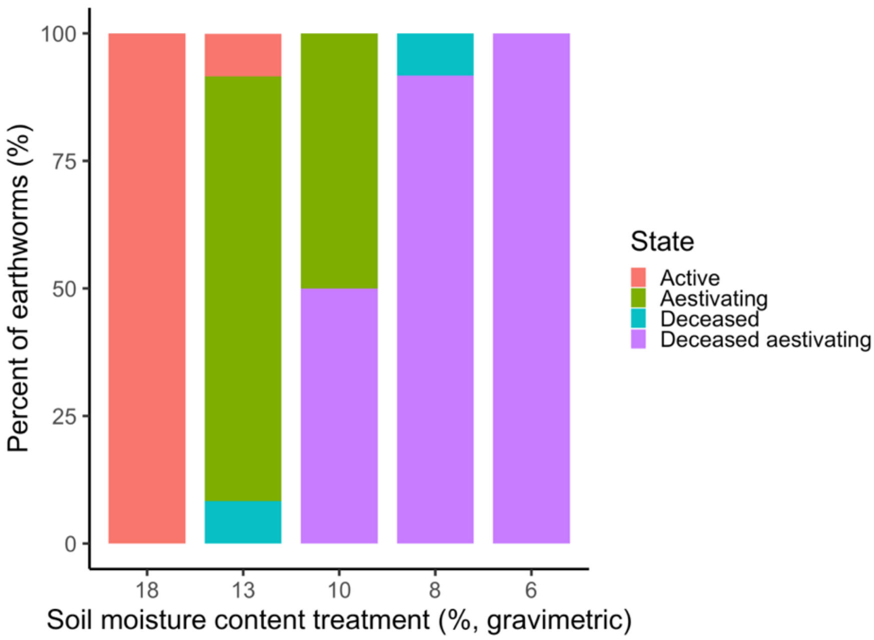

Figure 2.4. The proportion of *Al. chlorotica* in each state; red = active, green = aestivating, blue = deceased and purple = deceased in aestivation, when substrates were destructively sampled at each target moisture content. For 18 wt%, 13 wt% and 8 wt% moisture n = 12, for 10 wt% and 6 wt% n = 6.

2.4.2 Fresh mass of Al. chlorotica

Overall, there was a statistically significant positive association between time spent in the experiment and the fresh mass of surviving *Al. chlorotica* when weighed after depuration relative to their initial mass at the start of the experiment (LMM, $F = 1454.4$, $df = 1, 151$, $p < 0.001$). Contrary to the prediction, when weighed at each target moisture content after depuration, surviving *Al. chlorotica* had increased in mass since the start of the experiment (**Fig. 2.5**). The extent of these increases in mass at the point of destructive sampling after exposure to drying or constant control conditions depended on the state of *Al. chlorotica*, with those found active gaining more mass than those found in aestivation. This difference was statistically significantly for those found active in the control conditions compared to those aestivating in the drying treatment (with increases of ~49 % and ~23 % mass respectively) (ANOVA: $F = 9.592$, $df = 1, 71$, $p < 0.01$) (**Fig. 2.6**).

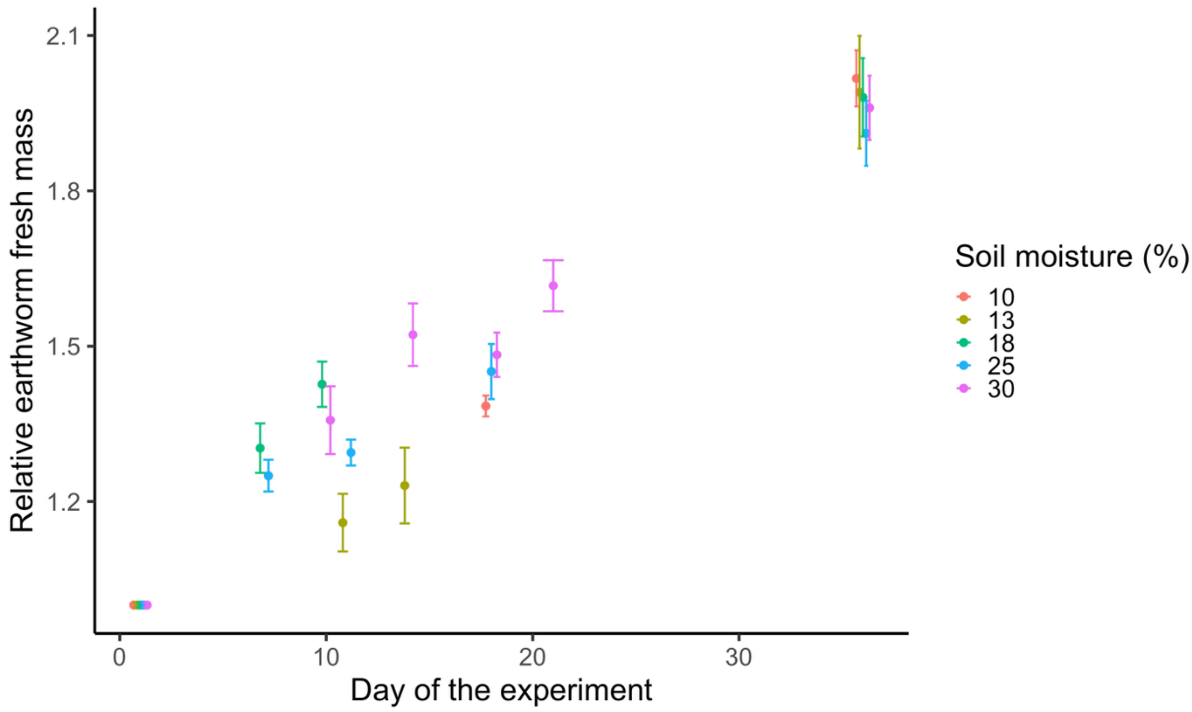

Figure 2.5. The relationship between the mean fresh mass of *Al. chlorotica* (relative to their starting mass, n = 6) and time in the experiment. Different colours represent the lowest moisture content (wt%) *Al. chlorotica* were exposed to in the drying period.

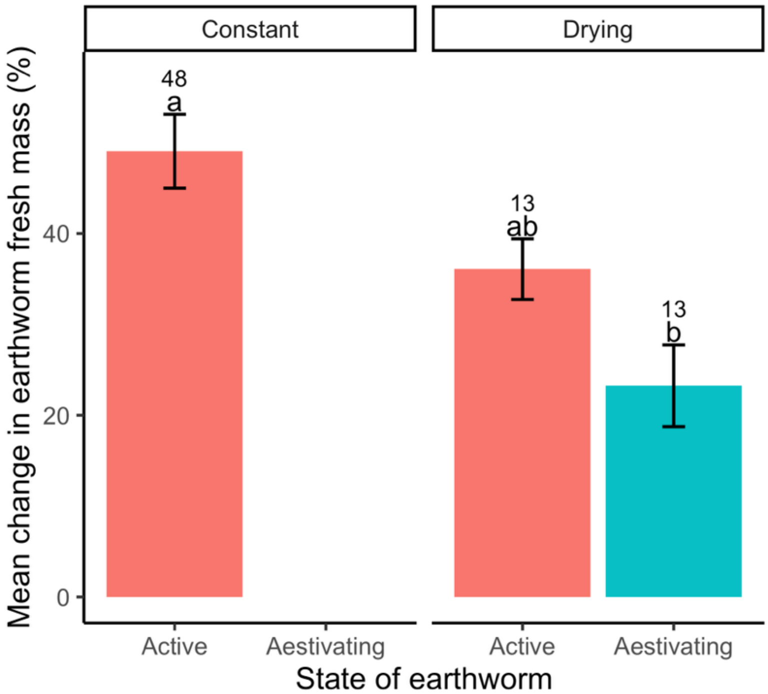

Figure 2.6. The mean change in *Al. chlorotica* fresh mass from the start of the experiment to the point of destructive sampling (following 24 hours on wet filter paper) for earthworms found either active or in aestivation, split by exposure to constant control or drying conditions. Error bars show standard errors. Treatments with different letters differ to a statistically significant degree (Tukey, $p < 0.05$). Numbers above bars represent live earthworms in each treatment.

To further investigate when these changes in mass were taking place, the fresh mass of *Al. chlorotica* was compared at three time points: before earthworms were placed into experimental conditions, on destructive sampling after the period of drying (or constant control conditions), and following the recovery period spent in optimal conditions (**Fig. 2.7**). The extent of drying (ANOVA: $F = 3.52$, $df = 2, 148$, $p < 0.05$) and the experimental period at which they were weighed (ANOVA: $F = 138.27$, $df = 2, 148$, $p < 0.001$) had a statistically significant effect on the mass of *Al. chlorotica*. Posthoc treatment comparisons revealed no significant differences between the mean mass of *Al. chlorotica* in any of the treatment groups on the first day of the experiment, with earthworms weighing between 0.214-0.242 g on average (Tukey test $p<0.05$, **Fig. 2.7, group A**). When weighed following depuration after the period of drying (or constant control conditions), all surviving *Al. chlorotica* had increased in mass from the start of the experiment. *Al. chlorotica* exposed to drying conditions were lighter than their corresponding control group, but not to a statistically significant degree (**Fig. 2.7, group B**). After the recovery period spent in optimal moisture conditions (**Fig. 2.7, group C**), all *Al. chlorotica* had gained further mass since destructive sampling after the drying conditions. These increases were statistically significant for those that had been exposed to drying down to moisture contents of 18 wt% and 13 wt% (**Fig. 2.7, group C**). Furthermore, all *Al. chlorotica* had grown significantly since the start of the experiment and to a similar mass (0.418 - 0.475 g), irrespective of the extent of drying they had been exposed to.

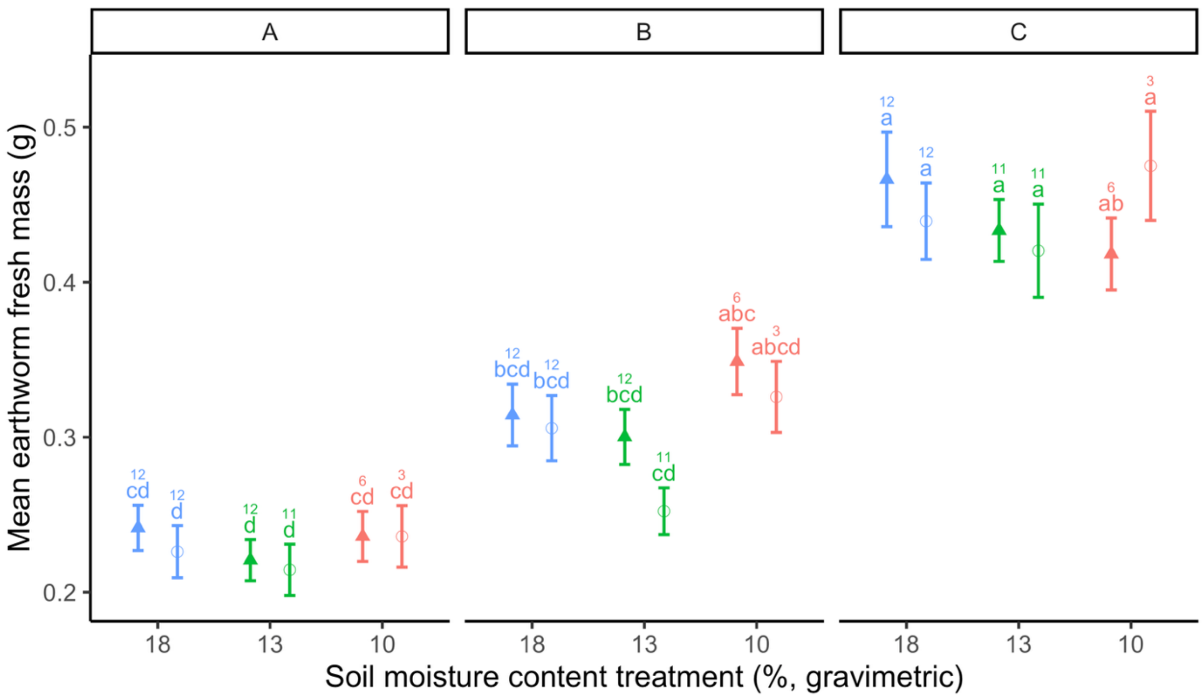

Figure 2.7. The mean fresh mass of surviving earthworms weighed at three time points: (A) on day 1 of the experiment, (B) on destructive sampling following drying (or constant control conditions), and (C) on removal from the recovery phase. Colours represent the lowest moisture content *Al. chlorotica* experienced during drying conditions (hollow circles) and the corresponding control groups (filled triangles) sampled at the same time. Error bars show standard error. Treatments with the same letter do not differ to a significantly significant degree (Tukey, p > 0.05). Numbers above points represent live earthworms in each treatment.

Changes in *Al. chlorotica* mass were also calculated as a percentage of their fresh mass at the start of each measurement period (**Fig. 2.8**). There were significant differences in the extent of changes in mass between the different experimental periods (ANOVA: F= 136.57, df = 2, 148, p<0.001). There was also a significant interaction between experimental period and the moisture content *Al. chlorotica* were destructively sampled at on the observed changes in earthworm mass (ANOVA: F = 3.431, df = 4, 148, p < 0.05), and a significant interaction between moisture content, weighing period and treatment group on changes in mass (ANOVA: F = 2.542, df = 4, 148, p <0.05). From the start of the experiment to destructive sampling following the drying period (A to B), individuals exposed to 13 wt% moisture showed the smallest increase in mass (~20 %), while those in constant moisture conditions in the control group sampled at the same time as the earthworms exposed to 10 wt% moisture increased the most (~48 %), but these differences were not statistically significant (Tukey test, p > 0.05). In contrast, during the recovery period of optimal conditions from the destructive sampling to the end of the experiment (B to C), *Al. chlorotica* that had been previously exposed to the 13 wt% moisture treatment increased by the most mass (~67 %). This increase was significantly greater than that of those from the control treatment sampled at the same time as *Al. chlorotica* exposed to 10 wt% moisture (Tukey test, p < 0.05). Over the whole experimental period (A to C), all individuals increased in mass by a similar magnitude (between ~79 to ~104 %), irrespective of the degree of drying they had been exposed to.

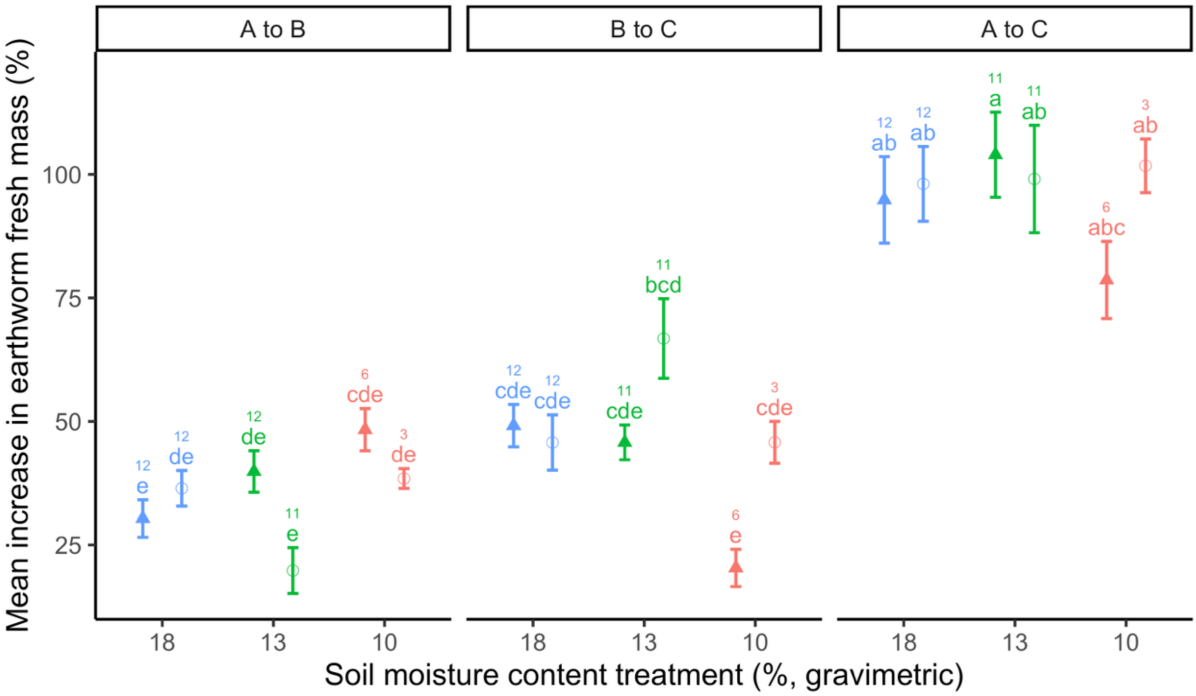

Figure 2.8. The mean percentage increase in *Al. chlorotica* fresh mass (relative to mass at the start of each period), split into three time periods: A to B = pre-experiment to destructive sampling after drying, B to C = destructive sampling after drying to post-recovery phase, and A to C = pre-experiment to post-recovery. Colours represent the lowest moisture content *Al. chlorotica* were exposed to during the drying phase. Shapes represent the control (filled triangles) and drying (open circles) treatments. Error bars show standard error. Treatments with the same letter do not differ to a significantly significant degree (Tukey, $p > 0.05$). Numbers above points represent live earthworms in each treatment.

To account for the different durations *Al. chlorotica* spent in each period of the experiment, which were dependent on the specific moisture content they had been destructively sampled at, increases in mass were measured on a gram per day basis (**Fig. 2.9**). There were significant effects of experimental time period (ANOVA: $F = 25.27$, df = 2, 148, $p < 0.001$), degree of drying experienced (ANOVA: $F = 10.34$, df = 2, 148, $p < 0.001$) and the interaction between moisture content, treatment group and weighing period (ANOVA: $F = 4.46$, df = 4, 148, $p < 0.001$) on the per day increases in *Al. chlorotica* mass. In the initial experimental period (A to B), the rate of mass gain was slowest for *Al. chlorotica* sampled at moisture contents of 13 wt% and 10 wt%, and significantly greater for those sampled at 18 wt% (Tukey test, $p<0.05$). The opposite was true for the period in optimal conditions after sampling (B to C) whereby *Al. chlorotica* which had been sampled at the driest moisture contents gained mass at a quicker rate than those that had been sampled at 18 wt% moisture during the drying period. Consequently, over the whole experimental period (A to C), the rate of mass gain was very similar (0.0052 - 0.0068 g/day) irrespective of the moisture treatment individuals had been exposed to.

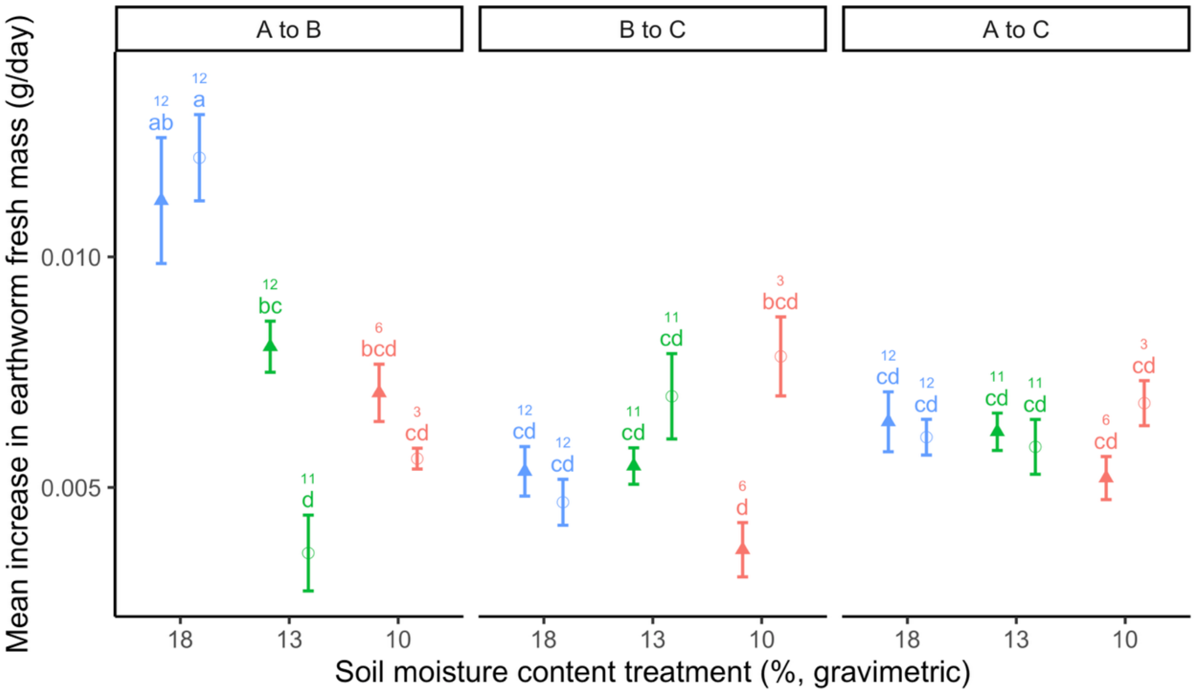

Figure 2.9. The mean increase in *Al. chlorotica* fresh mass relative to time spent in each of the experimental time periods (grams per day). Different colours represent the lowest moisture conditions *Al. chlorotica* were exposed to during drying and the corresponding control groups sampled at the same time. Shapes represent the control (filled triangles) and drying (open circles) treatments. Error bars show standard error. Treatments with the same letter do not differ to a significantly significant degree ($p > 0.05$). Numbers above points represent live earthworms in each treatment.

2.5 Discussion

All earthworms in the control conditions remained active throughout the experiment and no aestivation was observed at 18 wt% moisture in the drying conditions. This supports Díaz Cosín *et al.* (2006) finding that very few earthworms aestivated during summer in moisture contents of 20 wt%, leading them to conclude that earthworm aestivation is not driven by an 'internal clock'. Instead, consistent with the finding of increased aestivation with increased moisture stress in *Ap. caliginosa* (McDaniel *et al.*, 2013a), as the soil dried to 13 wt% moisture in the present study, ~83 % of the *Al. chlorotica* were found aestivating. This suggests that the onset of aestivation in this soil type occurs between 18 and 13 wt% moisture. Similarly, Walsh *et al.* (2019) observed aestivation in field conditions of 14.4 % (± 5.5, vol) moisture and 15.3 °C (+-3.5) soil temperature. Most earthworms were aestivating in substrates below 13 wt% moisture, however many were no longer alive, with 100 % mortality observed below 10 wt% moisture. This is consistent with Wever *et al.* (2001) who found the greatest mortality of *Aporrectodea tuberculata* at soil moisture contents less than 10 wt%. This suggests that the protective ability of aestivation is limited when conditions become too dry.

Earthworms have limited physiological ability to reduce water loss, which occurs through osmosis across their skin cuticle (Carley, 1978) and results in desiccation at a critical threshold when the osmotic pressure of their body fluids is greater than that of the surrounding soil (Bayley *et al.*, 2010; McDaniel *et al.*, 2013a; Holmstrup, 2001; Holmstrup *et al*., 2016). It is likely that the duration of exposure to drying conditions is an important factor influencing the onset of aestivation and mortality in earthworms. In this experiment, the rate of water loss was similar for substrates starting at 30 wt% and 25 wt% moisture. Consequently, while substrates in the D30 group took 3 days longer to reach each target moisture content, the additional time was spent in optimal higher moisture conditions and they would have spent the same duration exposed to conditions with limited water availability, hence their similar responses at each sampling point. Therefore, the influence of duration spent in drying conditions is likely dependent on the severity of drying conditions with conditions only detrimental once the availability of water becomes limiting. For instance, laboratory experiments found some *H. elisae* were able to maintain an active state after two weeks exposure to 10 wt% moisture, whereas after four months at 10 % moisture, 100 % were aestivating (Díaz Cosín *et al.*, 2006). Further studies exposing earthworms to substrates maintained at constant low water contents (rather than gradually drying) would be beneficial to provide greater insight into the effect of drought duration on onset of aestivation and mortality e.g. to determine whether it takes less time for earthworms to enter aestivation at higher degrees of water limitation.

Here a single earthworm species, *Al. chlorotica*, was studied, however drought tolerance is likely to be species-specific and dependent on the population studied and the conditions they are locally adapted to such as the average soil water content (Holmstrup, 2001; McDaniel *et al.*, 2013a). For instance, Liu *et al.* (2025) found *Al. chlorotica* declined in abundance under experimentally induced future climate conditions, while four other earthworm species showed no such pattern, supporting the notion that some species are more vulnerable than others to changing moisture and temperature levels. In addition, within-species differences in habitat preferences have been reported which may also influence their response to drought conditions. For instance, Satchell (1967) reported that the pink morph of *Al. chlorotica* dominated substrates below 25 wt% moisture, whereas wetter substrates above 40 % moisture were dominated by the green morph. Moreover, the present study was conducted in a single soil type. However, the force at which water is held in the soil and thus how available it is for uptake depends on the water potential which is influenced by physical properties such as pore space, organic matter content and particle size (Collis-George, 1959). Therefore, to get a broader understanding of the response of earthworms to drought in different environmental conditions, further research should investigate the onset of aestivation in a variety of soil types, with a focus on the response of earthworms to different water potentials.

We predicted that *Al. chlorotica* exposed to drying conditions would decrease in mass due to desiccation, but that after a period of recovery in optimal conditions they would rehydrate, resulting in no net change in mass. However, no decreases in mass resulting from aestivation or exposure to drying conditions were recorded. Instead, a positive association was found between earthworm mass and time spent in the experiment, with surviving *Al. chlorotica* increasing in mass relative to the start of the experiment at every time point measured. Similarly, McDaniel *et al.* (2013a) found no effect of drought on the fresh mass of *Ap. caliginosa* earthworms exposed to 2- or 3-week drought stress. However, in both McDaniel *et al.* (2013a) experiment and ours, earthworms were placed onto wet filter paper to depurate before being weighed to determine their fresh mass free from gut contents. Therefore, earthworms exposed to drying conditions were able to rehydrate (McDaniel *et al.*, 2013a), at least partially, and so any changes due to differences in body water mass would not be detected in measurements. It would be beneficial for future studies to weigh earthworms both immediately on extraction from soil substrates and again after a period on wet filter paper, to see if the expected changes in mass due to water loss are evident. Furthermore, calculating earthworm dry mass would be useful to determine how much of their mass could be attributed to tissue water, however, this could only be done at the end of an experiment.

We cannot be sure that the *Al. chlorotica* were fully hydrated at the start of the experiment, despite experiencing optimal moisture conditions in the acclimation period followed by 24 hours on wet filter paper prior to entering experimental conditions. However, *Al. chlorotica* experienced the same duration on wet filter paper prior to each weighing occasion throughout the experiment, so changes in mass would still be relative even if fresh mass measurements were not reflective of their maximum hydrated masses. It is more likely that the *Al. chlorotica* were not at their maximum body size at the start of the experiment, even though they were all adults, as determined by the presence of a developed clitellum. Other studies have reported that mature *Al. chlorotica* weigh between 0.212-0.41 g on average (Roots, 1956; Lowe and Butt, 2003; Osman and van Noort, 2004; Lowe and Butt, 2007; Brami *et al.*, 2017), so with a mean mass of 0.224 g at the start of the experiment, adults in the present study were at the lower end of the range. It has also been recorded that densities above one individual per litre can significantly reduce growth rates both in *Ap. caliginosa* and *L. terrestris* (Eriksen-Hamel and Whalen, 2007). Therefore, the increases in mass observed even for *Al. chlorotica* exposed to drying conditions in the present study could be explained by the greater organic matter availability resulting from the provision of excess food and absence of competition for resources in experimental conditions compared to the culture and acclimation periods.

All surviving *Al. chlorotica* had significantly increased in mass from the start to the end of the experiment, but at the weighing point after the initial period of drying (or optimal control conditions), the extent of these increases varied depending on the state earthworms were found in. When weighed after depuration on wet filter paper, active *Al. chlorotica* had increased by twice as much mass as those found aestivating, suggesting some negative effect of aestivation, possibly because feeding is suspended during this state (Díaz Cosín *et al.*, 2006). Assuming all earthworms were fully hydrated and their guts were completely voided after 24 hours on the wet filter paper, the differences in mass between different states is unlikely to be due to differing body water contents, but rather a result of differing tissue mass, indicating a negative influence of aestivation on *Al. chlorotica*. The degree of drying *Al. chlorotica* were exposed to also influenced their mass when sampled at the end of that period. In general, *Al. chlorotica* exposed to drying conditions were lower in mass than those exposed to constant optimal conditions, and earthworms sampled at 18 wt% moisture were greater in mass than those sampled at 13 wt%, but not greater than those sampled at 10 wt% moisture. Given *Al. chlorotica* sampled at 10 wt% were exposed to greater desiccation, it might be expected that they would be lower in mass than earthworms exposed to a lesser degree of drying (or optimal moisture conditions in the controls). However, as fresh mass could not be determined for deceased *Al. chlorotica*, data for earthworm masses at 10 wt% moisture were limited to the three surviving individuals that were found aestivating on sampling. The larger mass of these three surviving *Al. chlorotica* could indicate a greater desiccation resistance of larger earthworms in aestivation if their smaller surface area to volume ratio results in a smaller proportion of their skin in contact with walls of the dry soil chamber. Díaz Cosín *et al.* (2006) found that the body weight of the earthworm *H. elisae* had no influence on the onset of aestivation, however, the relationship between earthworm body size and the onset of mortality in drying conditions merits further investigation.

Increases in mass for *Al. chlorotica* exposed to drying conditions down to 10 and 13 wt% moisture mostly occurred during the recovery period where water was more readily available. In contrast, *Al. chlorotica* kept in optimal conditions (at 25 wt% and 30 wt%) and those dried down to 18 wt% moisture experienced most of their growth in the initial 'drying' period. A similar pattern was observed when the rate of mass increase (g/day) was calculated to account for the different durations of time *Al. chlorotica* spent in each period. In the initial drying period, *Al. chlorotica* exposed to the driest substrates had the slowest rate of mass gain, while in the period of optimal conditions, they gained mass faster than those that had previously experienced the more optimal, wetter moisture conditions. These increases in *Al. chlorotica* mass during more favourable conditions following exposure to harsh conditions align with compensatory responses in growth rate such as those found by Aira *et al.* (2007) for juvenile *E. fetida* earthworms following experimental stress conditions. In addition, evidence suggests it is easier for earthworms to ingest food in soils with higher moisture conditions (Holmstrup, 2001), therefore, *Al. chlorotica* coming from drying conditions would have had greater ability to consume food in the recovery period. Suspended feeding associated with aestivation would have also likely resulted in greater food availability in the recovery period compared to those in the control group and drying conditions that remained active and could thus ingest food throughout the whole period. Moreover, soil microbial activity tends to scale linearly with increasing soil moisture conditions as soil water facilitates respiration (Cook and Orchard, 2008; Liu *et al.*, 2022), potentially further reducing the quantity and/or quality of food available in the recovery period for *Al. chlorotica* coming from substrates exposed to higher moisture contents. Considering this, the rate of *Al. chlorotica* mass gain may have decreased with increasing time spent in the recovery period as the food became less abundant, hence smaller increases in mass were observed in B to C for the 18 wt% group who spent longer in this period. In addition, there is likely to be an upper limit to the mass of adult *Al. chlorotica,* and their growth rate may slow as they get closer to this threshold/equilibrium. Consequently, *Al. chlorotica* that experienced the more optimal moisture contents may have reached their maximum size sooner than those that experienced the drier conditions, resulting in smaller residual increases in mass during the recovery period. In the present study, percentage changes in mass represented total increases from the start to end of each period, and g/day rates of mass gain were averages based on the total duration spent in each period. Therefore, it was not possible to detect more intricate (e.g. day-to-day) changes in mass. To get a more detailed insight into the influence of different moisture conditions on changes in earthworm growth, future studies could record the mass of earthworms at multiple time points during each period of drying or optimal conditions.

Over the whole experiment, small changes in mass during the drying conditions were compensated for by large increases during the subsequent period of optimal conditions and vice versa. Consequently, when the final measurements were taken at the end of the experiment, there were no longer any significant differences in *Al. chlorotica* mass, regardless of the drying conditions they had been exposed to. All groups experienced similar percentage increases in mass (~79 - 104 %) and at a similar rate (0.0052 to 0.0068 g/day) since the start of the experiment. These results suggest that the availability of food was not a limiting factor and indicate that for surviving earthworms, the potential negative effects of exposure to drying conditions and aestivation on fresh mass were transient.

2.6 Conclusion

As this experiment took place over the summer, the presence of active earthworms in the constant optimal moisture conditions provides supporting evidence that the onset of aestivation in *Al. chlorotica* is driven by specific moisture contents rather than being an obligatory diapause. All earthworms were active in substrates down to 18 wt% moisture, began to aestivate at 13 wt%, and could no longer survive below 10 wt% moisture. This suggests the threshold for inducing aestivation in this species and soil type is between 18 and 13 wt% moisture. Once rehydrated following destructive sampling, surviving *Al. chlorotica* increased in mass over the course of the experiment, regardless of the moisture contents they were exposed to, and whether they underwent aestivation or remained active. However, the magnitude of these increases was dependent on the state of *Al. chlorotica* during the drying period and the degree of drying they had been exposed to. Assuming earthworms were fully hydrated after 24 hours exposure to high moisture conditions, the residual observed differences in earthworm mass were likely a result of differences in tissue deposition rather than differences in hydration. Despite this, any negative effects on *Al. chlorotica* mass were transient because on return to more favourable soil moisture conditions, earthworms were able to reach masses which ultimately did not differ significantly from those that had remained in optimal conditions throughout the whole experiment. Overall, these results suggest *Al. chlorotica* are resilient to drought conditions and that aestivation serves as a beneficial behavioural strategy for this species to aid survival in harsh conditions. However, the effectiveness of aestivation in protecting against desiccation depends on the severity of drought experienced, with mortality induced when the soil moisture gets too low. To get a more comprehensive understanding of the potential impacts of future climate change on earthworm populations, the exact conditions that drive earthworm aestivation and restrict their activity and survival warrant further study.

This study provided valuable methodological insights, with findings used to inform the experimental design of subsequent experiments. For instance, later studies were designed to reduce mortality of *Al. chlorotica* by preventing soils from drying to moisture contents below 10 wt%. In the following experiments earthworms were also weighed both immediately on extraction from the soil and again after a period of depuration on wet filter paper, to allow differentiation between changes in mass resulting from water loss versus tissue loss.

2.7 References

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2.8 Supplementary materials

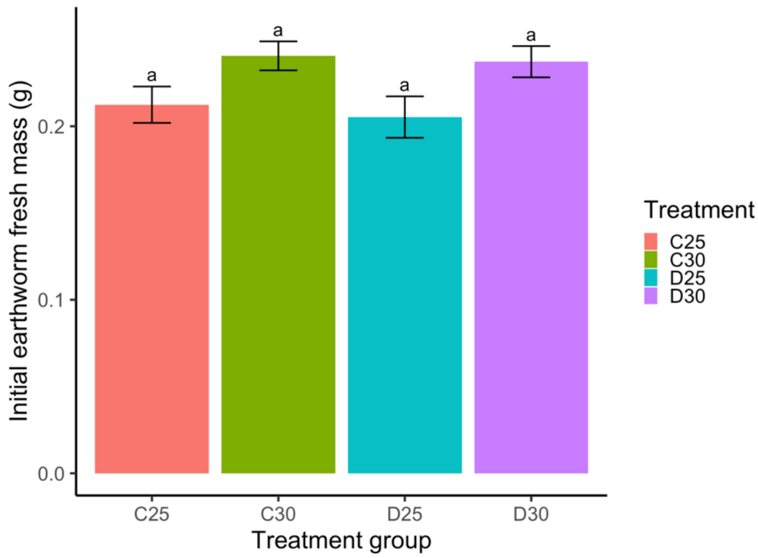

Figure S2.1. The mean initial mass (g) of *Al. chlorotica* (n = 24) allocated to each of the treatment groups. Error bars show standard error. Treatments with the same letter do not differ to a statistically significant degree (Tukey, $p > 0.05$).

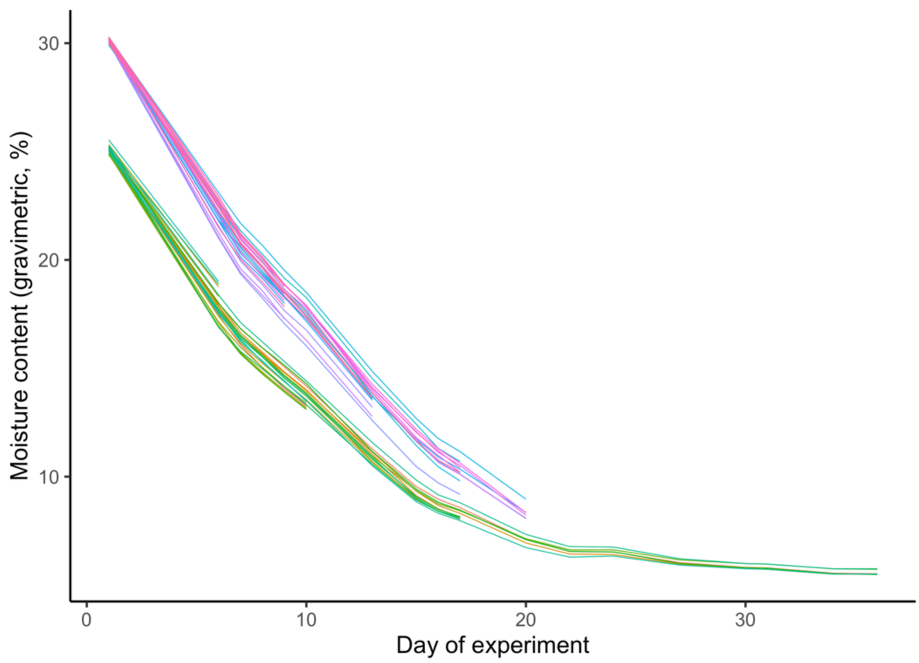

Figure S2.2. Changes in the soil gravimetric moisture content with time (days) in the experiment for substrates starting from either 30 wt% or 25 wt% moisture.

Chapter 3

The effect of repeated periods of drought and aestivation on *Allolobophora chlorotica* reproductive output

3.1 Abstract

Increasing drought frequency under climate change is expected to intensify periods of suboptimal soil moisture, negatively affecting earthworms and other soil organisms. To survive desiccation, some earthworms enter aestivation, a state of reduced metabolic activity during which reproduction and ecosystem service provision are suspended. Understanding how repeated droughts and aestivation affect earthworm reproductive output is therefore critical for predicting the viability of future earthworm populations and their capacity to function as ecosystem engineers. In this study, adult *Allolobophora chlorotica* (Savigny, 1826), one of the most common UK earthworm species, were exposed to one, two or three 14-day drying periods in loamy soil to gravimetric moisture contents of ~11-13 wt%. Each drought exposure was followed by three days in favourable soil moisture conditions. After their final bout of aestivation, earthworms were placed in groups of four into soils under optimal moisture conditions for 50 days to reproduce. Cocoon number, mass, viability and hatching time were measured as indicators of reproductive success. Cocoon production declined significantly with increasing bouts of aestivation ($p < 0.001$). Cocoon production was lowest during Days 1-10 of recovery (~0-0.02 cocoons/earthworm/day), peaked between Days 20-30 (~0.08-0.16 cocoons/earthworm/day) and declined over the final 20 days (~0.08-0.12 cocoons/earthworm/day). Unexpectedly, earthworms previously exposed to drying and aestivation produced more (226 vs 171 cocoons, $p < 0.05$) and heavier (6.701 ± 1.205 vs 6.036 ± 1.256 mg, $p < 0.05$) cocoons than those that remained active in the constant higher moisture control conditions, possibly reflecting food limitation and soil compaction in controls. Cocoon viability and hatching time did not differ significantly between treatments. Across treatments, earthworm mass was a strong predictor of fecundity. Heavier individuals produced more ($p < 0.001$) and heavier ($p <0.001$) cocoons, and cocoon mass was positively correlated with hatching success ($p < 0.001$). Overall, *Al. chlorotica* showed reduced reproductive output following repeated aestivation, but also demonstrated resilience through compensatory responses once favourable conditions returned.

3.2 Introduction

The frequency and severity of climatic extremes is expected to increase with climate change (Seneviratne *et al.*, 2021). Models based on intermediate greenhouse gas emission scenarios predict widespread droughts and decreases in soil moisture in the top 10 cm of soil for many land areas over the 21st century (Dai, 2013), posing a threat to important soil organisms like earthworms that rely on soil water to function. As a survival strategy to limit desiccation during drought, some earthworm species enter aestivation whereby they coil into a knot and remain in a low metabolic state until soil conditions improve (Gerard, 1967; Jiménez *et al.*, 2000; McDaniel *et al.*, 2013a; McDaniel *et al.*, 2013b). Increased periods of aestivation and inactivity will likely have detrimental impacts for the wider soil ecosystem given the important functional role of earthworms in the provision of numerous ecosystem services (Blouin *et al.*, 2013). Moreover, the survival and success of earthworm populations depend on their ability to reproduce and expand spatially, which is constrained by the time they spend in aestivation, the associated energy costs, and thus the duration and frequency of drought periods (McDaniel *et al.*, 2013a).

Earthworms are simultaneous hermaphrodites containing both male and female structures and during reproduction there is a mutual exchange of sperm and seminal fluids from the male pores to the spermathecae of the other partner (Porto *et al.*, 2012; Christyraj *et al.*, 2025). Sperm and an egg are released into a band of mucus produced by the clitellum (Christyraj *et al.*, 2025) which hardens to form an ellipsoid cocoon (Sherlock, 2018). The rate and size of cocoons produced by earthworms depends on the species, but also the environmental conditions they are exposed to. Lower reproductive rates can occur due to poor body condition resulting from indirect or direct effects on growth and behaviour (Reinecke and Reinecke, 2007). Consistent with this, there is evidence of a trade-off between growth and reproduction in some earthworms during periods of stress. For instance, West *et al.* (2003) found *Lumbricus rubellus* (Hoffmeister, 1845) altered their resource allocation by reducing their cocoon production in response to low soil calcium levels. Similarly, *Esenia andrei* (Bouché, 1972) maintained growth over reproduction, producing fewer cocoons under environmental stress (soil pH > 7, at and below 15 °C, and at 30 °C) (van Gestel *et al.*, 1992). Moreover, Aira *et al.* (2007) induced stress in *Esenia fetida* (Savigny, 1826) via mechanical manipulation and found they maintained growth at the expense of their current reproduction, producing fewer and lighter cocoons. In terms of drought stress, some studies have investigated the reproductive output of earthworms in response to different soil water potentials which represents the force at which water is held in the soil and thus how available it is for uptake. Holmstrup (2001) found *Aporrectodea caliginosa* (Savigny, 1826) cocoon production was optimal at water potentials lower than ~pF 2 (10 kPa), reduced at those above ~pF 2.08 (12 kPa) and completely inhibited above ~pF 2.6 (40 kPa). Similarly, Evans and Guild (1948) found *Al. chlorotica* cocoon production was optimal at around pF 2 but inhibited at pF 3.

Cocoon development and hatching times are also influenced by environmental conditions, particularly the soil moisture content (Jensen and Holmstrup, 1997). This is important given that cocoons tend to be produced in the upper soil layers which are more exposed to fluctuating conditions and lose water at a faster rate than deeper layers (Nepstad *et al.*, 2002). For instance, Butt (1997) found that out of 336 *Al. chlorotica* cocoons, all were within the top 10 cm and over half were within the top 5 cm of the soil. There is some evidence to suggest that once formed, cocoons are resistant to harsh conditions. Holmstrup (1994) found that earthworm cocoons exhibit a degree of cold hardiness, tolerating at least 80 % water loss when exposed to -2 °C, a result of dehydration lowering the super-cooling point (SCP) and preventing the embryo from freezing. For instance, the SCP of *Al. chlorotica* cocoons was -7.8 ± 1.8 °C when fully hydrated compared to -11.6 ± 2.8 °C when partially dehydrated (Holmstrup, 1994). Moreover, Petersen *et al.* (2008) found cocoons of the surface-dwelling earthworm *Dendrobaena octaedra* were incredibly tolerant to desiccation and able to survive losses of ~95 % of their water content when exposed to gradual dehydration. In contrast, in acutely exposed cocoons, 100 % cocoon mortality occurred below 89 % RH, highlighting that survival is dependent on both the extent of desiccation and the rate at which water loss occurs (Petersen *et al.*, 2008). Such adaptations enable earthworm communities to recover from climatic perturbations when conditions improve, even in cases where adults and juveniles may not have survived. The temporal period of favourable conditions required to support earthworm populations where soil pressures are non-limiting is unknown, but ~4-6 weeks has been suggested as the minimum time required to complete a full earthworm reproductive cycle and allow time for hatchlings to build up biomass (Edwards and Bohlen, 1996; Ruiz *et al.*, 2021). This suggests that periods of drought occurring more frequently than every 4-6 weeks may not be conducive to the survival of earthworm populations.

While the effects of drought conditions on cocoon production and survival are becoming better understood, the lasting impact of aestivation and repeated drought events on subsequent reproductive output once optimal conditions return remains largely understudied. This information is important to determine the period of favourable conditions required for earthworm populations to recover from exposure to drought conditions. Existing research from Holmstrup (2001) has demonstrated that the negative effects of drought exposure can be quite long-lasting, depending on the degree of drying experienced, as *Ap. caliginosa* took between two weeks to two months to resume normal rates of cocoon production following 14-day bouts of drying. The present study investigated how repeated bouts of drying and concurrent aestivation, followed by a return to optimal conditions, affected the mass and reproductive output of *Al. chlorotica*. Specifically, it was assessed whether there are lasting detrimental effects on cocoon numbers, mass, hatching success (viability) and development time. Fewer and lighter cocoons were predicted to be produced with increasing bouts of drying and accompanying aestivation. In addition, when transferred to more favourable soil moisture conditions, the initial rate of cocoon production was predicted to be low but increase with time spent in these conditions.

3.3 Methods

3.3.1 Earthworm collection and culturing

Adult *Al. chlorotica* (indicated by the presence of a developed clitellum and tubercula pubertatis) of the green morph were collected in October 2024 by hand sorting soil from pits (18 cm x 18 cm at the surface to 20 cm depth) dug in the margins of Warren Paddock, Spen farm, Tadcaster (53°52'25.9"N, 1°19'33.4"W) which has a well-drained, loamy and calcareous soil (Holden *et al.*, 2019). *Al. chlorotica* were kept at 15 °C ± 1 °C and 24-hour darkness in a controlled temperature room at the University of Sheffield in field soil maintained at 25 to 30 wt% (gravimetric) through addition of distilled deionised water (DIW). Temperature, light and moisture conditions were selected based on Lowe and Butt (2005). Earthworms were fed *ad libitum* with sheep manure (from sheep raised without anti-helminthic drugs) that had been oven dried at 105 °C to kill harmful organisms and milled to < 2 mm.

3.3.2 Initial acclimation

Eleven groups of 15 earthworms (n = 165) were randomly selected from the culture substrates and placed into containers of ~4 kg of Kettering loam (Boughton Loam, Kettering, UK purchased online from Agrigem). Sheep manure was added into the soil as a food source (0.5 g per earthworm), mixed thoroughly to homogenise and DIW added to reach a moisture content of 25 wt%. *Al. chlorotica* were kept in these conditions for one week based on Fründ *et al.* (2010) recommendations for acclimation. After one week, individual

Al. chlorotica were removed from the soil and placed onto filter paper moistened with DIW for 24 hours to depurate, before being gently blotted dry with tissue and weighed on a balance.

3.3.3 Drought exposures to induce aestivation

After determination of their fresh mass, individual *Al. chlorotica* were randomly assigned to one of four treatments: a drought-exposed group (n = 105) subjected to either one, two or three two-week periods of gradual substrate drying to induce aestivation (**Fig. 3.1**), or a control group (n = 60) kept in constant optimal moisture conditions. The mean earthworm starting mass was 0.287 ± 0.039 g and did not differ significantly between the drought or control groups (ANOVA: F = 0.009, df = 1, 114, p = 0.925), or between those assigned to one, two, and three exposure groups (ANOVA: F = 1.118, df =1, 114, p = 0.33) (**Fig. S3.1**). All *Al. chlorotica* were placed individually into 300 ml plastic cups containing soil packed to an air-dried density of ~1.13 g/cm^3 (170 g of Kettering loam and 0.5 g of sheep manure milled to <2 mm). Control soils were kept at a gravimetric moisture content of 25 wt% as it has been suggested to be within the optimal range of moisture conditions for *Al. chlorotica* (Lowe and Butt, 2005) and successfully prevented aestivation in a previous experiment (chapter 2) as well as those of Tilikj and Novo (2022) with *Carpetania matritensis* (Marchán, 2020) earthworms. Instead of immediate exposure to water-limiting conditions, the drought-exposed treatments started at an intermediate gravimetric moisture content of 20 wt% to reduce the stress of transitioning from optimal conditions. Plastic cups were covered with nylon mesh (100 % polyamide 15 Denier tights purchased from Matalan and cut into smaller strips) and secured with an elastic band to prevent earthworms from escaping but also to allow evaporation. Soils in the drought-exposed treatment were left to air dry gradually with no addition of water to reflect natural conditions whereby desiccation stress would increase over a period of days or weeks (Petersen *et al.*, 2008).

After a 14-day period of drying, soils were destructively sampled and the state of *Al. chlorotica* was determined as either active (outstretched body, mobile) or aestivating (coiled into a knot, usually within a soil chamber) before they were weighed on a balance to five decimal places. Earthworms that had not entered aestivation in the drying conditions at the end of each 14-day period were removed from the experiment. Of the earthworms found aestivating, 20 were randomly selected to undergo a recovery period in soil kept at optimal moisture conditions, the rest were placed into soils at 25 wt% moisture for 3 days before being transferred to fresh soils at 20 wt% moisture for a second 14-day period of drying conditions. This was done to replicate conditions expected to occur in a natural context whereby earthworms are subject to intermittent periods of drought and more favourable moisture conditions resulting in transitions between active and aestivating states. After the second period of drying the above process was repeated. Earthworms found aestivating on extraction were weighed before 20 were selected at random to be transferred to the recovery period. The remaining earthworms that had aestivated were placed into soils at 25 wt% moisture for three days before moving to fresh soils at 20 wt% moisture for a third and final period of drying (**Fig. 3.1)**. The soil moisture content was maintained at ~25 wt% throughout the control conditions through daily weighing and addition of DIW to the soil surface using a spray bottle where necessary. Individual earthworms in the controls were transferred to fresh soils at the same time as those in groups exposed to drying conditions to keep handling consistent.

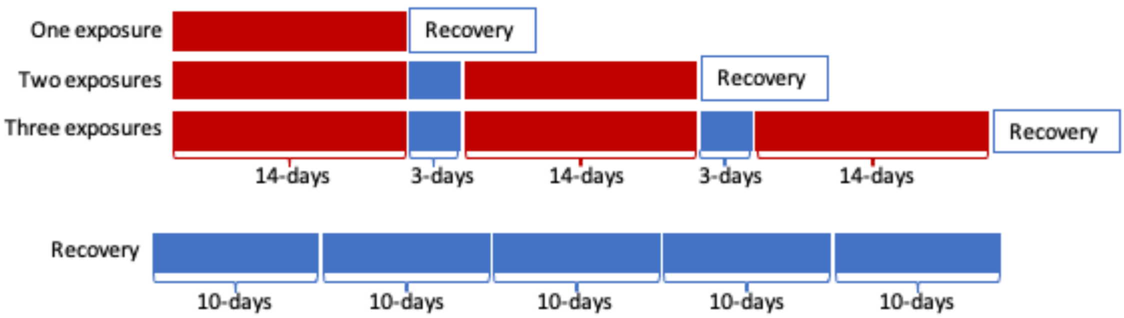

Figure 3.1. Summary of experimental design. *Al. chlorotica* were subject to 1, 2 or 3 bouts of drying (red), broken up by 3-day periods of optimal moisture conditions (blue) and a 50-day recovery period in which reproductive output was assessed every 10 days.

The rate of water loss from the soil increased slightly with each bout of air drying, resulting in a lower minimum moisture content after each 14-day period, despite starting from the same moisture content (**Fig. 3.2**). The average moisture contents were 12.85 %, 11.39 % and 10.58 % after one, two and three bouts respectively.

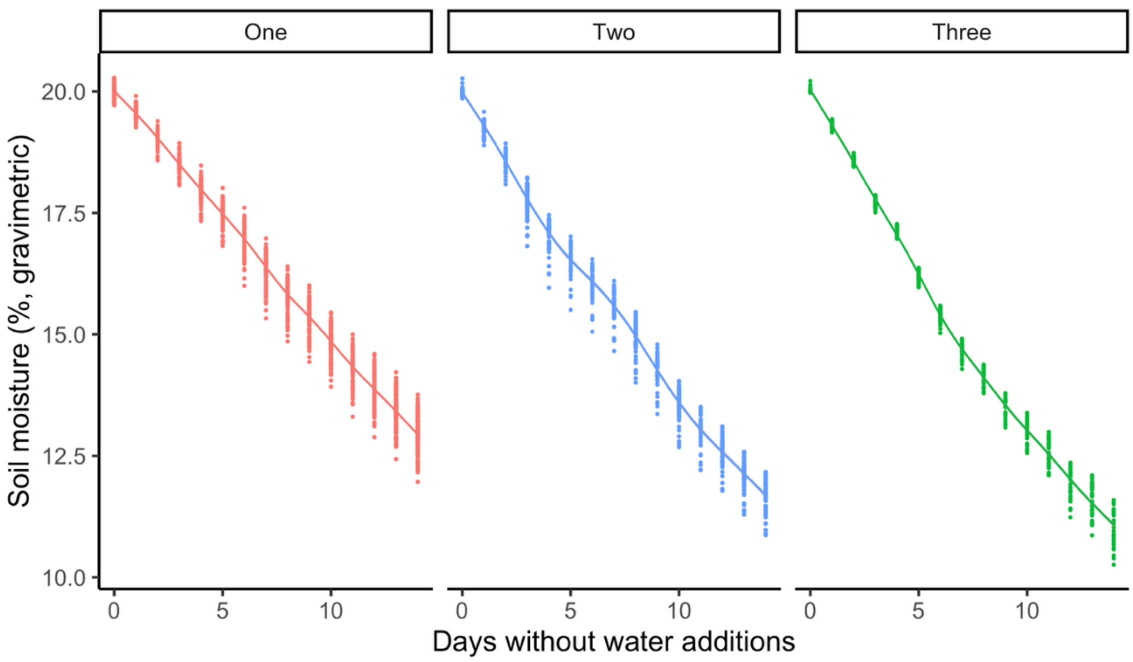

Figure 3.2. The rate of soil water loss for each of the three drying exposures. Individual data points are plotted. Red = one exposure (y = -0.51x + 20.02), blue = two exposures (y = -0.59x + 19.7) and green = three exposures (y = -0.65x + 19.67).

3.3.4 Recovery period and reproductive output

After experiencing the predetermined number of drying periods, *Al. chlorotica* were removed from the soil. Excess soil was gently removed from their skin with a paper towel and the mass of individual earthworms was determined. Individual *Al. chlorotica* were then placed onto filter paper moistened with DIW for 24 hours to depurate, before being blotted dry and weighed again to get their fresh mass. *Al. chlorotica* were split according to the number of exposures they had experienced and randomly assigned into groups of four to undergo a recovery period of 50 days in 750 ml plastic containers with perforated lids ('Nationwide Paper' brand rectangular reusable plastic food containers with lids, purchased from Amazon) and soil (400 g dry soil mixed with 2.8 g dried sheep manure ground to <2 mm), moistened to 25 % moisture with DIW. Every ten days, each group of four earthworms was extracted from the soil, excess soil was removed from their skin and they were weighed individually on a balance to five decimal places before being placed together into fresh soil (**Fig. 3.1**). After 50 days in the recovery period, earthworms were weighed pre- and post-hydration consisting of 24 hours on filter paper moistened with DIW. Soils were wet sieved through a 1 mm metal mesh to extract cocoons (Bart *et al.*, 2018). Cocoons from the two and three exposure groups were blotted dry, weighed on a balance to five decimal places, and then placed onto filter paper in Petri dishes moistened with DIW (Butt, 1997; Petersen *et al.*, 2008) and incubated in a controlled temperature room under the same conditions they had been produced in (15 °C ± 1 °C, complete darkness) (**Fig. 3.3**). Petri dishes were kept moist with DIW and inspected daily for hatchlings, recording the date of hatching to calculate incubation time. The experiment was terminated when no more cocoons had hatched over a period of two months.

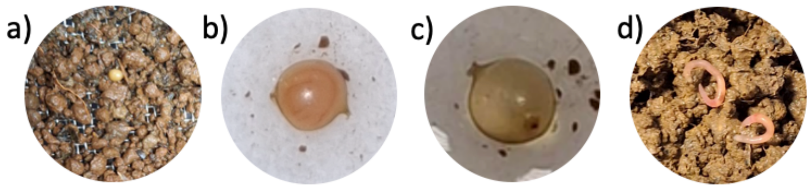

Figure 3.3. (a) A yellow *Al. chlorotica* cocoon (~ 4 mm at longest point) seen on a 1 mm sieve after wet sieving, (b) Cocoon on moist filter paper with visible earthworm embryo developing, (c) Empty cocoon, translucent after hatching, (d) Two earthworm hatchlings (~ 15 mm in length).

3.3.5 Statistical analyses

The programming language R (version 4.4.1, R Core Team, 2024) was used to conduct all statistical analyses. As earthworm masses were measured per group in each container, a linear mixed-effects model was initially fitted including group ID as a random factor to account for non-independence. However, the random effect variance was estimated as zero, indicating that group-to-group variation did not contribute meaningfully to the response. Consequently, changes in earthworm mass (% relative to initial mass) over time were analysed using a standard linear model. Time (days in the experiment) was treated as a categorical factor to account for the non-linear, fluctuating pattern of mass changes. Fixed factors included treatment (control vs. drying) and number of bouts (one, two, three) as well as their interactions. Assumptions of linearity, normality, and equal variance were checked by inspecting residual plots before ANOVAs were carried out. Post-hoc treatment contrasts were carried out using Tukey HSD tests to identify where statistical differences were occurring. Four individuals in the control treatment exposed to three bouts of constant moisture conditions were found aestivating after the third 14-day period and so were excluded from the analyses. No mortality was observed during the initial exposures to drying or control conditions, however a total of 3 individuals died during the 50-day recovery period of optimal conditions and so were excluded from calculations of mean cocoon production. One was found dead on Day 50 from the group exposed to two bouts of drying and two were found dead from the group exposed to three bouts of control conditions (one on Day 40 and one on Day 50). Regression of the clitellum was observed among some individuals during the experiment and so cocoon production was calculated on a per clitellate worm per day basis for each of the 10-day intervals in the recovery period. Cocoon masses and hatching success were calculated as averages for each replicate group. A three-way ANOVA was conducted to compare the mean rate of cocoon production for earthworms exposed to one, two and three drying periods. Non-parametric Spearman's rank tests were used to assess linear relationships between earthworm mass, cocoon production, mass and hatching success. As the data for hatching success did not meet the assumption of equal variance, an arcsine square root transformation was first applied before a linear regression and ANOVA were carried out. An ANOVA was also performed to analyse the differences between the time taken for hatchlings to emerge from cocoons produced during each of the 10-day intervals in the recovery period.

3.4 Results

3.4.1 Changes in Al. chlorotica mass prior to reproduction

Earthworm mass changed significantly over time ($p < 0.001$), and drought-exposed individuals differed significantly from those in the control conditions ($p < 0.001$) (**Table 3.1**). Temporal patterns of mass change also differed by treatment ($p < 0.001$). Within the first 14 days, *Al. chlorotica* exposed to drying conditions lost mass relative to their initial values, whereas control individuals maintained under optimal moisture gained mass (**Fig. 3.4**). The number of bouts of drying or control conditions had no significant main effect on changes in earthworm mass, although there was a significant interaction between treatment and bouts ($p < 0.05$). During the 3-day recovery periods under optimal moisture between drying periods, drought-exposed earthworms consistently regained mass (~22-24 % per recovery). However, subsequent drying periods caused greater losses than the preceding gains, resulting in progressively larger overall mass reductions with each additional drying bout. In contrast, control earthworms under constant optimal moisture initially gained mass during the first 14 days but later lost mass, returning to or dropping below their starting values after the second 14-day period.

Table 3.1. ANOVA results for percent change in *Al. chlorotica* mass. The model included days in the experiment (factor), treatment (control vs drought), and number of bouts of drying or constant moisture (one, two, three) as fixed effects, as well as their interactions. Significant effects are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Term	Df	F-value	p-value
Days (in the experiment)	5	71.16	p < 0.001 (***)
Treatment (control vs drought)	1	37.54	p < 0.001 (***)
Bouts (one, two, three)	2	0.94	0.395
Days:Treatment	5	29.96	p < 0.001 (***)
Days:Bouts	4	1.07	0.378
Treatment:Bouts	2	3.69	p < 0.05 (*)
Days:Treatment:Bouts	4	0.94	0.447
Residuals	90		

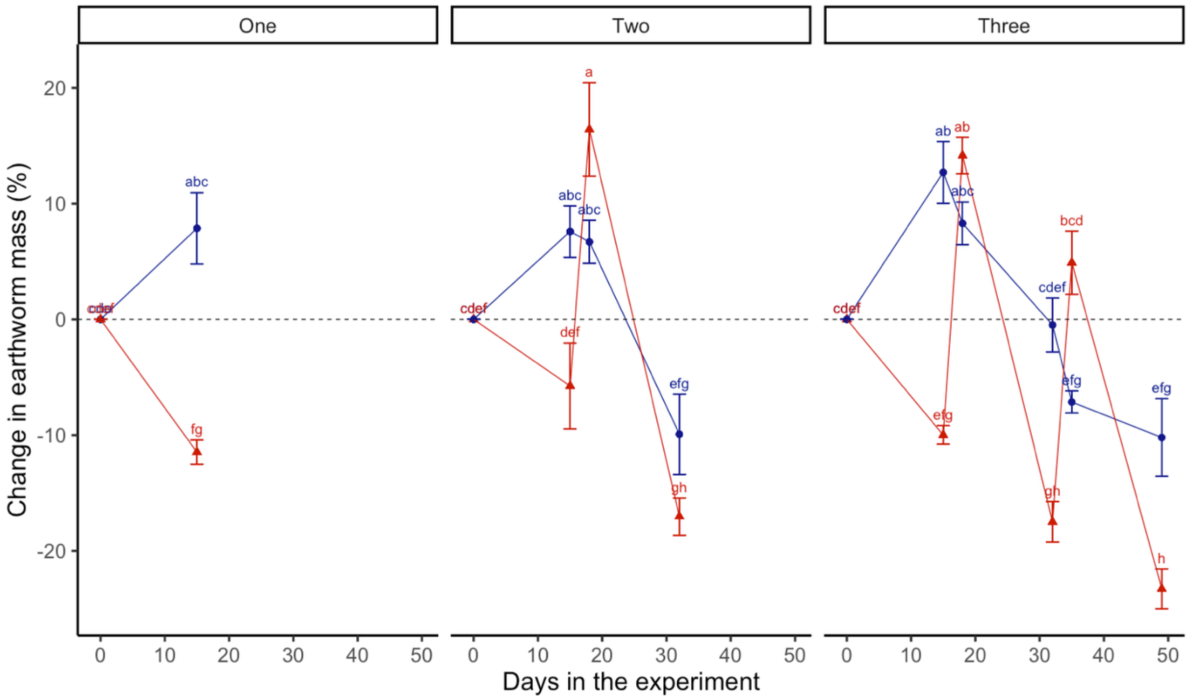

Figure 3.4. The change in mean (n = 20) earthworm mass relative to their starting mass, measured after each 14-day period of drying (red triangles) or constant moisture (blue circles) conditions and subsequent 3-day period of optimal moisture conditions. Treatments with a different letter differ to a statistically significant degree (Tukey, $p < 0.05$)

3.4.2 Cocoon production

Overall, total cocoon production was significantly influenced by the number of drying (or constant control) bouts experienced by *Al. chlorotica* (ANOVA: $F = 12.874$, $df = 2, 139$, $p < 0.001$, **Fig. S3.2**). Earthworms exposed to a single bout produced significantly more cocoons than those exposed to three (Tukey, $p < 0.5$). Earthworms subjected to two bouts also produced fewer cocoons than those with only a single exposure, but this difference was significant only under constant control conditions (Tukey, $p < 0.5$, **Fig. S3.2**).

Temporal patterns of cocoon production also differed among treatments. Between Days 10-20, significantly more cocoons were produced by earthworms that had been exposed to one drying bout compared to three (Tukey, $p < 0.05$, **Fig. 3.5**). Moreover, the duration *Al. chlorotica* spent in the recovery period also had a significant effect on cocoon production (ANOVA: $F = 23.945$, $df = 1, 133$, $p < 0.001$). As expected, cocoon production was initially low (Days 1-10) following aestivation but increased thereafter, peaking at Days 20-30 (**Fig. 3.5**). Relative to Days 1-10, cocoon production was significantly higher in Days 10-20 for earthworms exposed to a single bout of drying and in Days 20-30 for those exposed to two bouts. In contrast, earthworms exposed to three drying periods consistently produced fewer cocoons, with no significant differences across sampling intervals. After ~30 days in the recovery period, cocoon production generally declined, although rates remained higher than during the initial 10 days for most treatment groups. Across all treatments, earthworms subjected to drying produced significantly more cocoons than those from the constant control conditions (ANOVA: F = 4.484, df = 1, 133, $p < 0.05$). These treatment differences were most evident in earthworms that had been subjected to multiple drying or control periods, though not statistically significant within each sampling point (Tukey, $p > 0.05$).

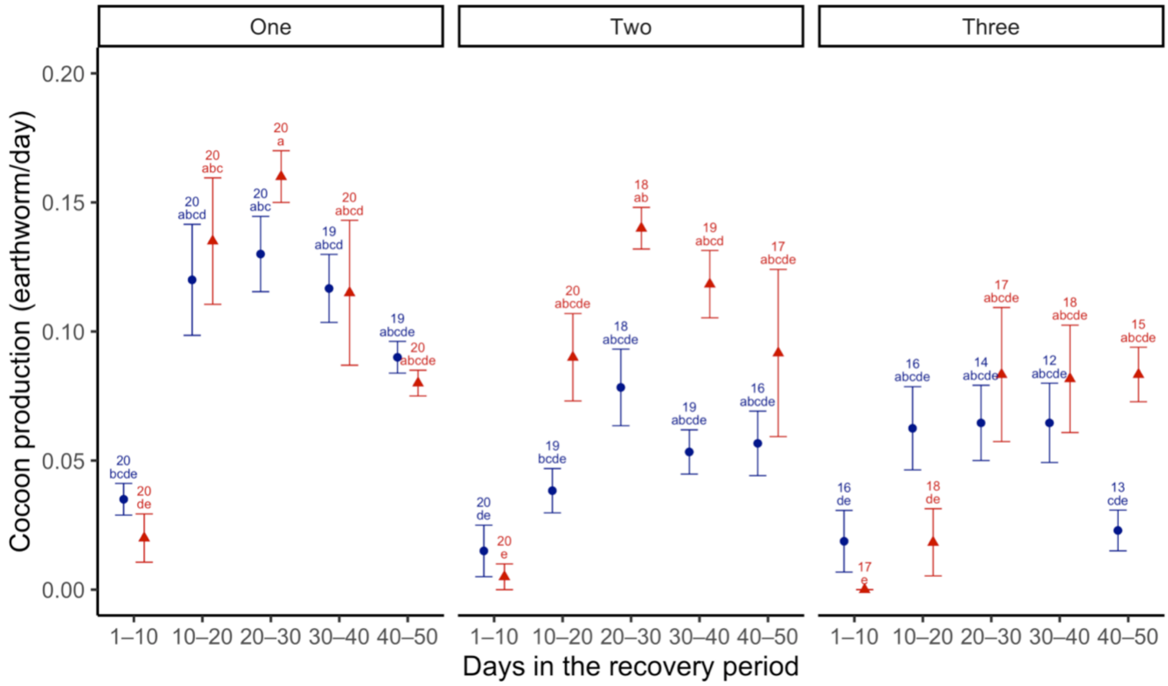

Figure 3.5. The mean cocoon production (per clitellate earthworm per day) recorded every 10 days in the recovery period from those that had been exposed to either one, two or three bouts of drying (red triangles, n = 5) or constant control conditions (blue circle, n = 5 except three bouts where n = 4). Numbers represent live clitellate earthworms. Treatments with the same letter do not differ to a statistically significant degree ($p > 0.05$).

3.4.3 Cocoon mass

Overall, cocoon mass was significantly influenced by treatment, with earthworms exposed to drying conditions producing heavier cocoons than those maintained under constant optimal conditions (ANOVA: F = 8.54, df = 1, 68, $p < 0.05$). Similar to rates of cocoon production, cocoon mass was lowest during the first 10 days of the reproductive period and increased thereafter, reaching maximum size between Days 20-40. However, these temporal differences were not statistically significant (ANOVA: F = 0.27, df = 1, 68, p = 0.605) (**Fig. 3.6**). Cocoon mass did not differ significantly between earthworms that had previously experienced two or three periods of drying (or the equivalent duration of constant control conditions) (ANOVA: F = 0.0004, df = 1, 68, p = 0.984).

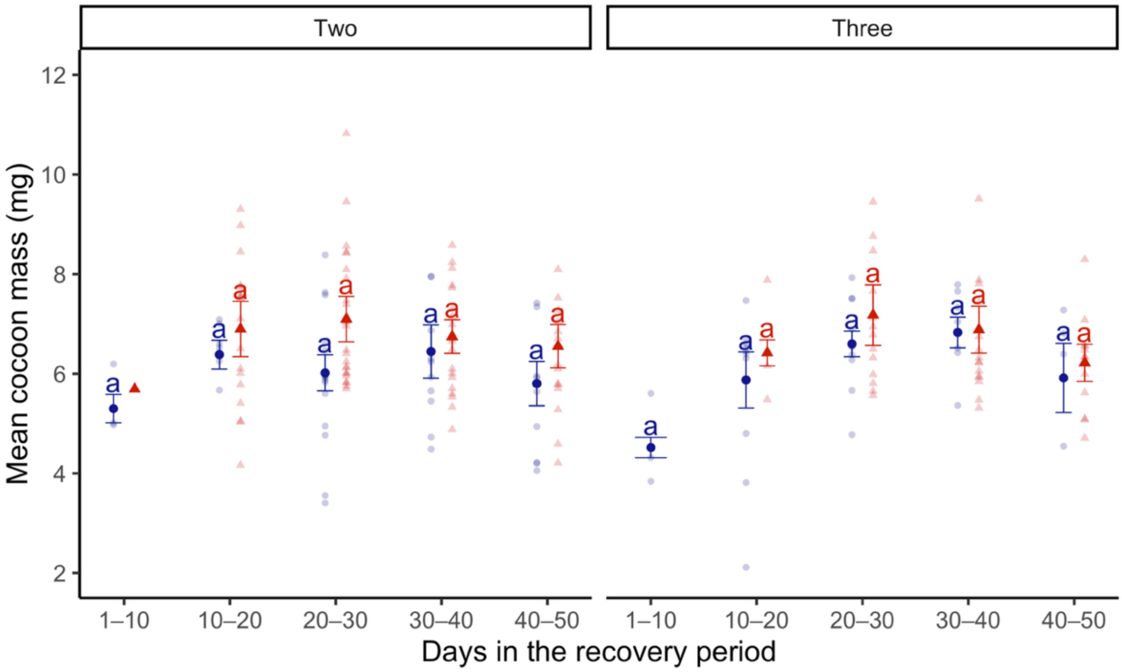

Figure 3.6. The individual (pale points) and mean (bold points) mass of cocoons produced per group for each of the five 10-day intervals in the recovery period following exposure to constant optimal (blue circle, n = 5 except three bouts where n = 4) or drought (red triangle, n = 5) conditions, grouped by number of 14-day bouts of drying (or control) conditions experienced. Cocoon mass was not measured for earthworms that experienced a single bout of drying or control conditions. Error bars show standard errors. Treatments with the same letter do not differ to a statistically significant degree ($p > 0.05$).

3.4.4 Al. chlorotica mass and its relationship with cocoon production and mass

The number of drying bouts (or constant conditions) significantly influenced the mass of clitellate *Al. chlorotica* during the recovery period, with individuals exposed to a single bout of drying (or constant) conditions being significantly heavier than those subjected to two or three bouts (ANOVA: $F = 11.53$, $df = 2, 133$, $p < 0.001$). Recovery period duration also had a significant effect on clitellate earthworm mass (ANOVA: $F = 8.266$, $df = 1, 133$, $p < 0.01$), following a similar temporal trend to cocoon production and cocoon mass. Clitellate *Al. chlorotica* mass increased from Day 10 to Day 20, peaked at Days 20-30, and declined over days 30 to 50 (**Fig. 3.7**). For earthworms subjected to an equal number of bouts, mass differences across the recovery period were generally not significant, except for those that had experienced three bouts of drying whose mass at Day 10 was significantly greater than at Day 50 (Tukey, $p < 0.05$). Overall, clitellate earthworms that had experienced drying conditions were significantly heavier than those maintained under constant control conditions throughout (ANOVA: $F = 65.363$, $df = 1, 133$, $p < 0.001$). These differences were smallest in individuals that had undergone only a single 14-day bout. Notably, after 10 days in the recovery period, the mean mass of *Al. chlorotica* subjected to three drying bouts was significantly higher than that of earthworms from the constant control conditions (Tukey, $p < 0.05$).

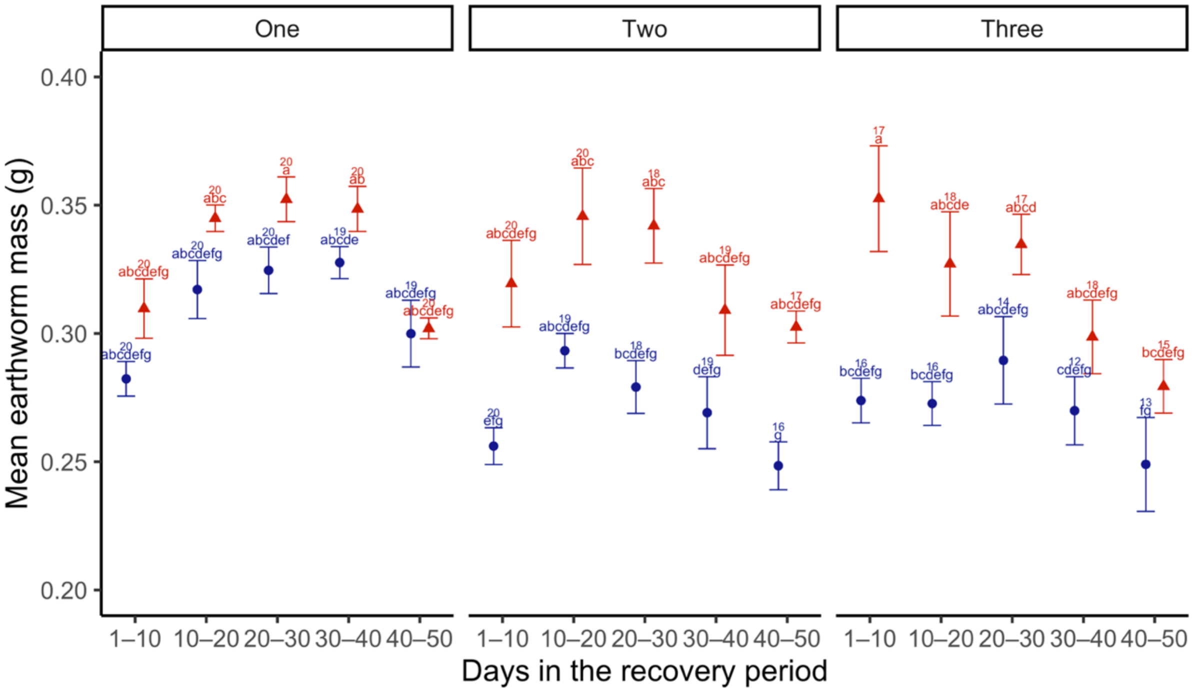

Figure 3.7. The mean mass of clitellate earthworms (per group) at each of the 10-day sampling points during the recovery period for earthworms exposed to one, two, or three bouts of drying (red triangle, n = 5) or constant moisture conditions (blue circle, n = 5 except three bouts where n = 4). Treatments with different letters differ to a statistically significant degree ($p < 0.05$). Numbers represent live clitellate earthworms.

Overall, the mean mass of clitellate *Al. chlorotica* was positively correlated with the mean number of cocoons produced (**Fig. 3.8**). This relationship was significant for earthworms from the constant control conditions (Spearman's rho = 0.699, n = 76, $p < 0.01$) and marginally significant for those previously exposed to drying (Spearman's rho = 0.225, n = 76, $p = 0.052$). Clitellate earthworm mass was also positively associated with cocoon mass. Significant correlations were found both in earthworms from drying treatments (Spearman's rho = 0.592, n = 76, $p < 0.01$) and from control conditions (Spearman's rho = 0.331, n = 76, $p < 0.05$) (**Fig. 3.9**).

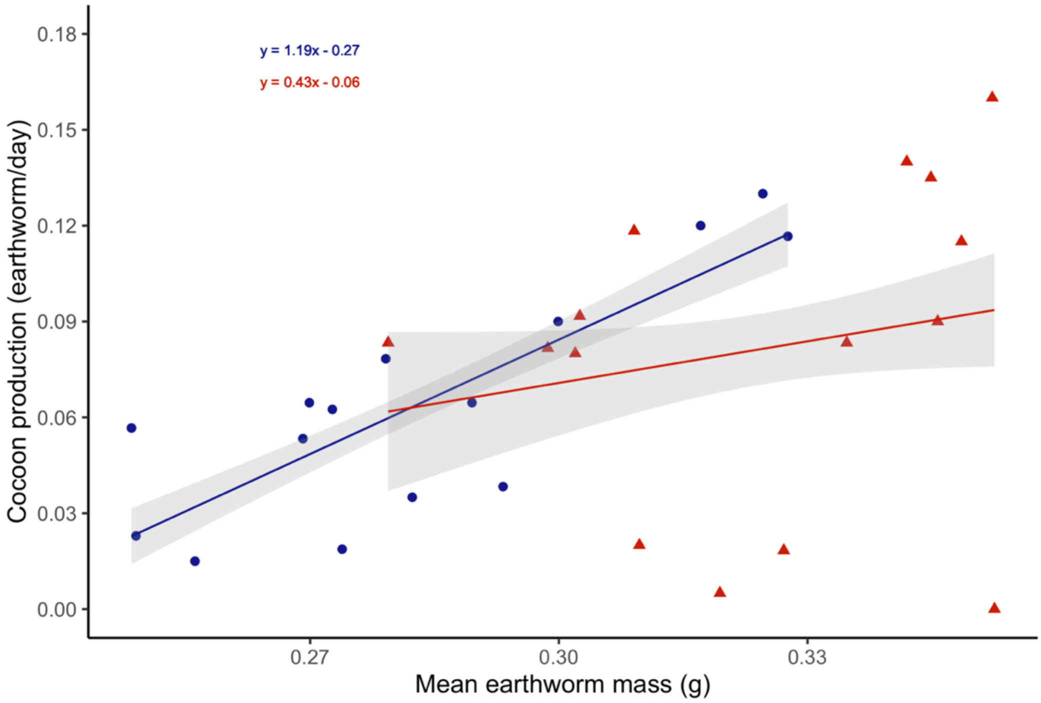

Figure 3.8. The relationship between the mean mass of clitellate earthworms (per group) and the number of cocoons produced (per earthworm per day) in the recovery period following exposure to constant optimal (blue circle) or drying (red triangle) conditions. Lines are fitted using the linear model method with standard error ribbons and equations given for each treatment.

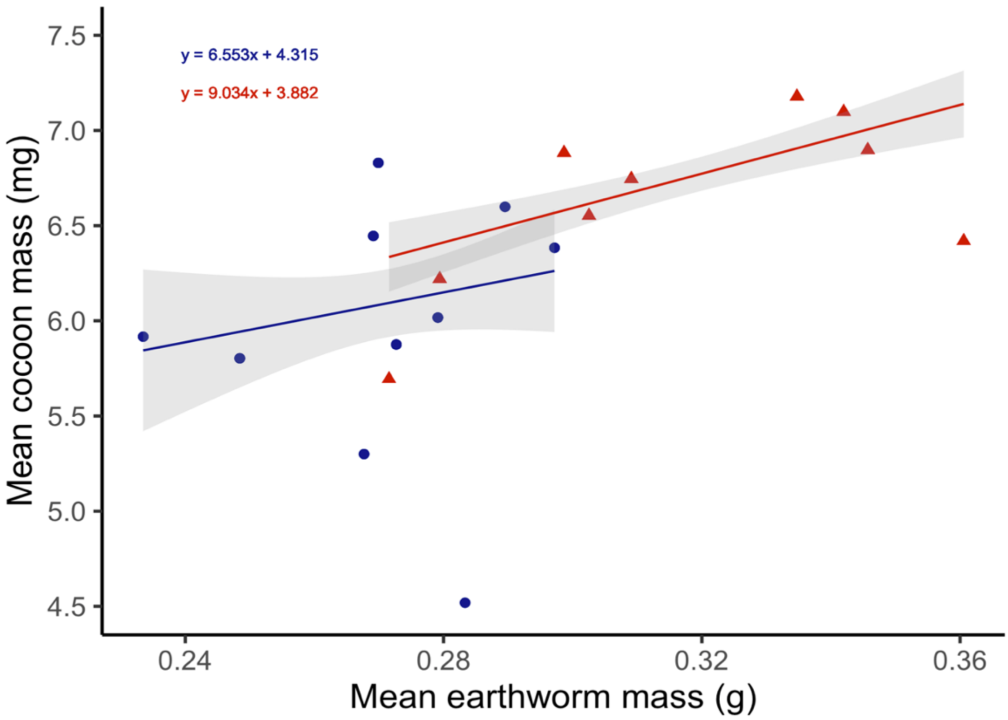

Figure 3.9. The relationship between the mean mass of clitellate earthworms (g) and the mean mass of cocoons (mg) they produced in the recovery period following exposure to constant optimal (blue circle) or drying (red triangle) conditions. Lines are fitted using the linear model method with standard error ribbons and equations given for each treatment.

3.4.5 Cocoon viability and development time

Cocoon viability and incubation time were significantly affected by the number of drying or constant control bouts experienced by the parent earthworms. Overall, *Al. chlorotica* that had undergone three bouts produced cocoons with a higher hatching success (ANOVA: F = 4.897, df = 1, 57, p < 0.05) and shorter incubation time (ANOVA: F = 57/733, df = 1, 75, p < 0.001) compared to those produced by earthworms subjected to two bouts. The timing of cocoon production within the recovery period also influenced outcomes. Both hatching success (ANOVA: F = 5.81, df = 4, 57, p < 0.01) and incubation time (ANOVA: F = 13.409, df = 1, 75, p < 0.001) varied significantly with recovery duration. Cocoons produced during the first 20 days had relatively low viability and longer development times, whereas the highest hatching success (**Fig. 3.10**) and fastest hatching (**Fig. 3.11**) occurred in cocoons produced on Days 30-40 (two bouts) and Days 20-40 (three bouts). No significant differences were detected in hatching success (ANOVA: F = 0.08, df = 1, 57, p = 0.778) or incubation time (ANOVA: F = 2.749, df = 1, 75, p = 0.102) between cocoons produced by *Al. chlorotica* that had previously been exposed to drying versus constant control conditions. Cocoon mass was positively associated with hatching success. Heavier cocoons had a greater likelihood of successful development, both from earthworms that had undergone drying (Linear regression: F = 15.669, df = 1, 36, p < 0.001) and those from constant control conditions (Linear regression: F = 12.156, df = 1, 36, p < 0.01) (**Fig. 3.12**).

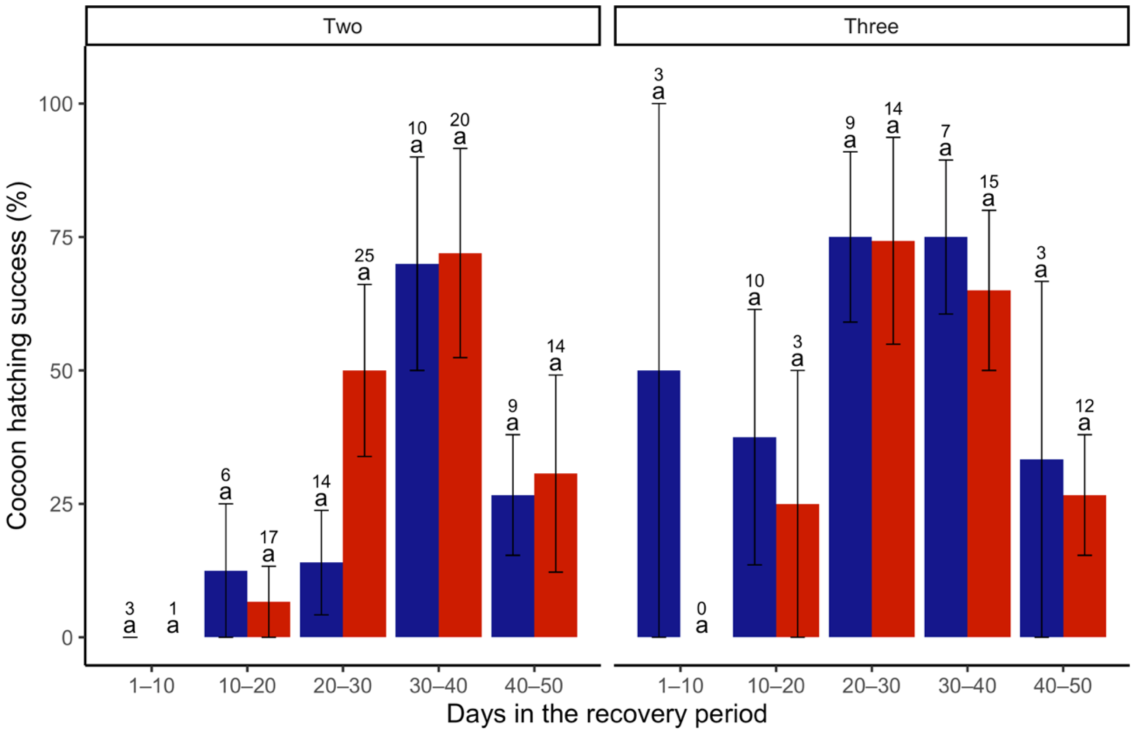

Figure 3.10. The mean hatching success (percentage hatched) of cocoons produced by replicate groups in each of the 10-day intervals of the recovery period from earthworms previously subjected to either two or three 14-day bouts of constant control (blue) or drying (red) moisture conditions. Error bars show standard error, numbers above bars indicate total numbers of cocoons produced (hatched and unhatched). Treatments with the same letter do not differ to a statistically significant degree (Tukey, p > 0.05).

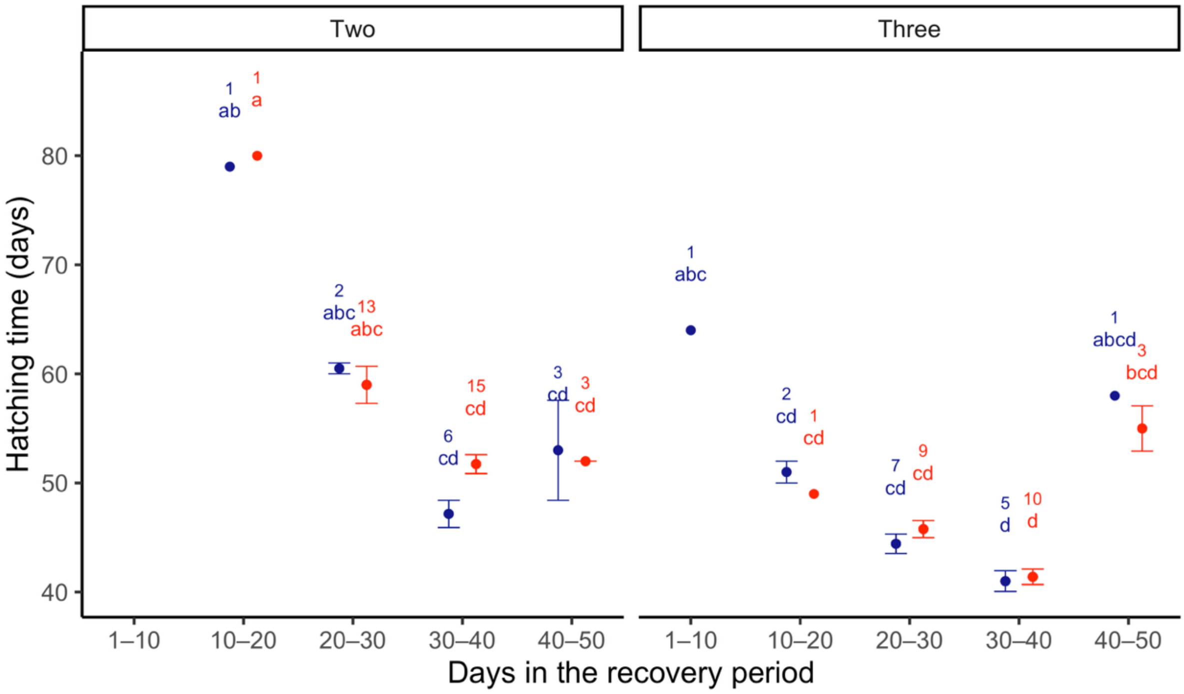

Figure 3.11. The mean time (days since removal from the experiment) taken for hatchlings to emerge from cocoons produced at each of the 10-day intervals during the recovery period, grouped by those subjected to two or three bouts of constant control (blue) or drying (red) conditions. Error bars represent standard error. Treatments with different letters differ to a statistically significant degree (Tukey, $p < 0.05$). Numbers represent hatched cocoons.

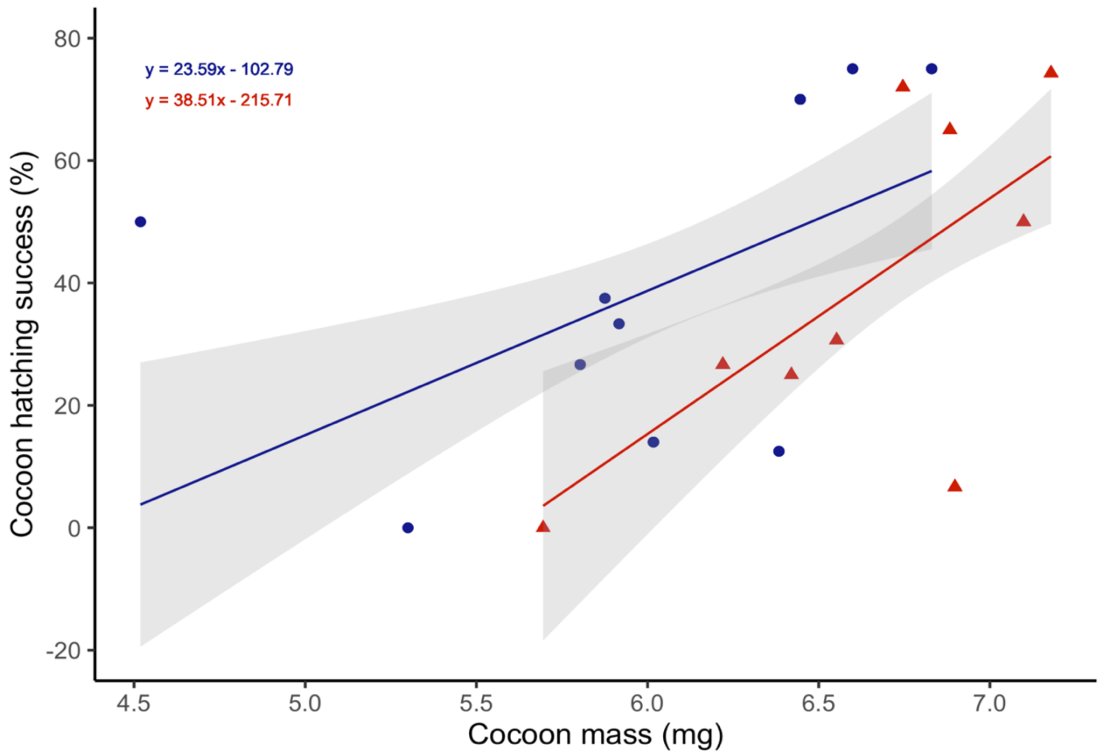

Figure 3.12. The relationship between the mean mass and the mean hatching success (%) of cocoons produced by replicate groups in the recovery period following exposure to constant optimal (blue circle) or drying (red triangle) conditions. Lines are fitted using the linear model method with standard error ribbons, and equations given for each treatment.

3.5 Discussion

As predicted, there was a significant negative effect of the number of drying exposures and accompanying periods of aestivation on the reproductive output of *Al. chlorotica.* Earthworms that experienced a single bout of drying, and thus one period of aestivation, were significantly heavier and produced significantly more cocoons than those that had been subjected to two or three bouts. This indicates that *Al. chlorotica* may reduce their reproductive output under repeated stressful conditions. Similar responses have been observed in other earthworms, for example Aira *et al.* (2007) reported that *Eisenia fetida* (Savigny, 1826) exposed to stress via experimentally induced contractile movements produced ~25 % fewer and ~30 % lighter cocoons.

Consistent with expectations, the initial rate of cocoon production was low, with fewer and lighter cocoons produced during Days 1-10 of the recovery period. Production then increased in Days 10-20 and 20-30 for earthworms that had been exposed to one or two bouts of aestivation, respectively. No cocoons were produced in Days 1-10 by earthworms that had experienced three bouts of drying. This pattern aligns with Holmstrup (2001), who found that *Ap. caliginosa* did not immediately resume cocoon production on return to optimal moisture conditions. Negative effects persisted for 2-4 weeks after exposure to water contents of ~13.2 ± 0.2 wt% (~12 kPa) to ~11.1 ± 0.05 wt% (~22 kPa), despite the earthworms recovering their fresh weight in this time (Holmstrup, 2001). At more extreme desiccation (~6.9 ± 0.2 wt%, ~330 kPa), cocoon production was still reduced even after two months in optimal conditions (Holmstrup, 2001). In contrast, *Al. chlorotica* in the present study showed no such prolonged suspension of reproduction, suggesting that drought conditions were less severe in terms of water availability. Alternatively, species-specific sensitivity to soil moisture and desiccation tolerance may explain the observed differences. For instance, Roots (1956) reported that *Lumbricus terrestris* (Linnaeus, 1758) were able to survive losses of up to 70 % of their body water, whereas *Al. chlorotica* survived losses up to 75 %, suggesting they are more tolerant to desiccation. The response of earthworms to drying conditions is also likely in part dependent on the conditions they are locally adapted to. Considering this, Holmstrup (2001) acknowledged that the abrupt water potential shift *Ap. caliginosa* experienced in their experiment may have elicited stronger negative effects than would occur under the gradual soil drying typical in natural systems. The rate of desiccation has been found to significantly influence the survival of earthworm cocoons with Petersen *et al.* (2008) suggesting that studies using acute drought exposure may underestimate the tolerance of cocoons to drought conditions. A similar effect on tolerance may also apply for adult earthworms, emphasising the importance of simulating drying rates more reflective of natural conditions. In the present study, soil moisture was reduced gradually from 25 wt% (acclimation) to 20 wt%, and then to ~11-13 %. However, *Al. chlorotica* were manually removed from their chambers after each drying period and thus their period of aestivation was abruptly terminated. In a natural context, earthworms would only resume an active state when conditions became more optimal i.e. when they absorb water from the environment (Jiang *et al.*, 2023). A more gradual exit from aestivation could allow earthworms time to return to a pre-aestivation state (e.g. increase their metabolic rate), potentially altering reproductive outcomes.

Despite differences in soil moisture treatments, patterns of reproductive output were broadly similar for *Al. chlorotica* from both the drying and constant control conditions. For instance, earthworms exposed to a single bout of control conditions produced more cocoons than those exposed to two or three bouts, and there were no significant differences in cocoon number, mass, or hatching success between drying and control treatments at any point in the recovery period. This suggests that the initially low cocoon production observed in Days 1-10 of recovery was not driven by prior exposure to drying and aestivation but was instead more likely due to limited opportunities to mate with conspecifics during this early phase. Moreover, previous work has shown that earthworm reproductive success can be enhanced by multiple matings. For instance, *Al. chlorotica* produced more juveniles (Dupont *et al.*, 2022) and the hatching success of *Eisenia andrei* (Bouché, 1972) cocoons increased (Porto *et al.*, 2012) when earthworms received sperm from multiple partners. Consequently, the initially low cocoon production and hatching success may reflect fewer occurrences of multiple matings. The reduction in reproductive output in the final 10 days could reflect both energetic costs and a reduced availability of clitellate conspecifics at this point as some individuals had regressed their sexual characters.

Contrary to expectations, *Al. chlorotica* that had experienced drying were overall significantly heavier and produced more, heavier cocoons than earthworms maintained under constant control moisture conditions, although cocoon viability did not differ between treatments. Similarly, Aira *et al.* (2007) found that juvenile *E. fetida* exposed to stress subsequently grew faster than controls, while their cocoons had a similar viability, suggesting no effect of stress on cocoon quality. This pattern may reflect a compensatory response by *Al. chlorotica* once returned to higher moisture conditions, where ingestion of food is easier (Holmstrup, 2001), leading to higher masses and reproductive outputs. This was evident to some extent in the initial exposure periods as although *Al. chlorotica* decreased in mass during each 14-day drying bout, they were able to recover this mass during the following 3-days of optimal moisture conditions, even exceeding their starting mass.

Alternatively, the overall lower mass and reproductive output of clitellate earthworms in the constant control conditions may indicate that the conditions were suboptimal in some way. For instance, the mean mass of cocoons produced by *Al. chlorotica* that had been exposed to the drying and control treatments were 6.701 ± 1.205 mg (n = 74) and 6.036 ± 1.256 mg (n = 121) respectively. Both values are lower than published averages for *Al. chlorotica* cocoons of 7 mg (in experimental conditions with a 8:5 ratio of soil to separated cattle solids at 10-20 °C, soil moisture content not reported, Butt, 1997), ~9 to 16 mg (in experiments with field soils kept at ~25 and 43 % moisture respectively, exposed variations in temperature from 10 to 8 °C, Evans and Guild, 1948) and 11 ± 1 mg (for earthworms kept at 15 °C in soil with moist cow dung, soil moisture content not specified, Holmstrup, 1994). These discrepancies may therefore reflect differences in soil conditions including the source and quantity of supplemented organic matter, which could contribute to varying earthworm masses. Single hatchlings emerged from individual cocoons, consistent with previous findings (Evans and Guild, 1948; Butt, 1997; Butt *et al.*, 1997). Moreover, overall hatching success was relatively low in both treatments, at 45.5 % (55/121) for the drying treatment and 37.8 % (28/74) for the control group. However, variable hatching success has been reported for *Al. chlorotica,* ranging from 34 % to ~90 % depending on the conditions that the cocoons were reared under (Pedersen and Bjerne, 1991; Jensen and Holmstrup, 1997; Butt, 1997; Butt *et al.*, 1997). The observation that four individuals entered aestivation during the third exposure to constant control conditions further supports the view that the control conditions were suboptimal. Potvin and Lilleskov (2017) noted that earthworms can aestivate to avoid starvation in the absence of appropriate food availability, therefore, limited organic matter could be a contributing factor. Consistent with this interpretation, earthworms in the control conditions began to lose mass after the first 14-day period and eventually fell below their starting mass. Thus, although the moisture conditions (~25 wt%) may have been sufficient to support activity (for the most part), other limiting factors constrained their growth. Curry (2004) emphasises that when physiochemical environmental factors are otherwise favourable, food quality and quantity are the main constrains on earthworm populations. Similarly, Diehl and Williams (1992) found that both soil moisture and food availability significantly affected the growth rate and activity of *E. fetida*.

While the soil organic matter content was supplemented with sheep manure, this was incorporated into the soils prior to the introduction of earthworms and not replenished while they were inhabiting the soil. Consequently, the organic matter content would have declined over time until the earthworms were transferred to fresh soils. In the control treatments, earthworms likely consumed food continuously throughout each 14-day period, whereas those in the drying conditions would have ceased consumption when they entered aestivation (Díaz Cosín *et al.*, 2006). As a result, the quantity of food available would be reduced in the control compared to the drying conditions. The higher soil moisture in the control conditions and recovery period may have also created more hospitable conditions for soil microbes (Cook and Orchard, 2008; Liu *et al.*, 2022), accelerating the decomposition of organic matter and potentially leaving food of lower quality or reduced digestibility for earthworms. However, if food availability had been the limiting factor, an increase in earthworm mass might have been expected following transfers to fresh soils where food was more abundant i.e. in the 3-day transitions between each 14-day period and every 10 days in the recovery period. This was not observed, instead changes in mass progressively declined with increasing time spent in the recovery period, even for earthworms previously exposed to drying (**Fig. S3.3**). In addition, organic matter remained visible in soils at the end of each exposure period, suggesting that food remained available, although it may have been reduced quality or indigestible. Future studies should ensure that food is supplied in excess to avoid organic matter becoming a limiting factor.

A gravimetric soil moisture content of 25 wt% was chosen as optimal for the control treatment based on recommended culture conditions (Lowe and Butt, 2005) and a prior experiment (chapter 2) in which all earthworms gained mass at this moisture content. Despite this, there is some evidence to suggest that the green morph of *Al. chlorotica* may prefer and perform better at higher moisture levels, with significantly greater growth and maturation rates observed for hatchlings in soils at 29 wt% compared to 21 wt% moisture (Lowe and Butt, 2007). This preference for wetter conditions is also reflected by their distribution as Satchell (1967) reported most sites below 25 % moisture were dominated (>90 %) by the pink morph, while sites above 40 % moisture predominantly consisted on the green morph (>90 %). Thus, 25 wt% moisture represent the lower end of the green morph's tolerance range and so, whilst sufficient for activity, may be suboptimal for their water requirements. In contrast, although earthworms in the drying treatments were exposed to more severe water limitation, they may have largely avoided its costs by entering aestivation, in which gas exchange is depressed to conserve energy and body fluid osmolality increases to prevent water loss (Bayley *et al.*, 2010). On emerging from aestivation, individuals may therefore have had higher residual fitness than controls that remained active under suboptimal conditions. In addition, repeated surface additions of DIW in the control soils led to compaction, likely due to the breakdown of weaker soil aggregates. Increased bulk density reduces pore space and raises mechanical resistance, constraining burrowing and increasing energy costs in earthworms such as *Ap. longa* and *Ap. caliginosa* (Arrázola-Vásquez *et al.*, 2022). Structural changes in the control soils resulting in reduced pore spaces may therefore have further contributed to their suboptimal nature.

Overall, earthworm mass clearly influenced reproductive output. Cocoon number, cocoon mass, and hatching success all followed parabolic trends with earthworm body mass, peaking between Days 20-40 of the recovery period when earthworms were heaviest. This strong positive association between earthworm size and fecundity is consistent with findings from Teferedegn and Ayele (2024), who found cocoon production in three epigeic species increased initially but declined after ~13 to ~17 days, coinciding with progressive weight loss of the earthworms. Similar positive associations between earthworm mass and cocoon production have been reported for *E. andrei* (Van Gestel *et al.*, 1992) and for earthworm and cocoon mass in eight tropical earthworm species (Chaudhuri and Bhattacharjee, 2011). Water balance also plays a role, with rates of cocoon production declining at water potentials where adults remained active but lost mass (Holmstrup, 2001). In contrast to our findings, Pedersen and Bjerre (1991) found no relationship between cocoon mass and hatching success of *Al. chlorotica*, *Ap. longa*, *Ap. caliginosa* or *L. terrestris* cocoons, although they did find a significant positive correlation with hatchling mass.

In the present study, the mean incubation time was 50.61 (± 8.45) days, consistent with previous reports of 50 (Gerard, 1967), 59 (Holmstrup *et al.*, 1991) and 51.4 (Butt, 1997) days at the same temperature used here (15 °C). However, there was a significant effect of time in the recovery period as cocoons produced in the first 20 days of the recovery period were slower to hatch than those produced on Days 30-40. This along with their smaller mass and lower viability suggests reduced reproductive quality immediately following exposure to harsh conditions. This implies that at least ~ 50 days of optimal moisture conditions may be required for successful *Al. chlorotica* cocoon development, although this will likely depend on the soil, namely temperature. For instance, Holmstrup *et al*. (1991) found that *Al. chlorotica* cocoons took 34-38 days to develop at 20 °C, while at 5 °C incubation exceeded 400 days. Future work should examine whether aestivation has any negative effects on hatchling growth rates to better understand the duration of favourable conditions required for earthworm populations to recover from exposure to stressful conditions.

Finally, the regression and reabsorption of secondary sexual structures/tissues has been reported to be a key feature of aestivation (Olive and Clark, 1978; Jiménez *et al.*, 2000). These changes in sexual mating characteristics resulting from exposure to stressful conditions are temporary and functions are usually regained on return to favourable conditions (Christyraj *et al.*, 2025). This allows allocation of resources for survival to be prioritised over current reproduction in the hopes of survival and reproduction in future improved conditions. Evidence of such a trade-off was observed in this experiment, as some adult *Al. chlorotica* regressed their clitellum, usually associated with individuals below 0.2 g in mass. Interestingly, this occurred not only in drying treatments but also in active earthworms in the constant optimal moisture conditions, particularly during the 50-day recovery period, again indicating that despite not being water limited, control conditions were stressful. Similarly, Butt (1997) showed that the reproductive state of *Al. chlorotica* was strongly influenced by environmental conditions, with only 23.3 % of the adults remaining clitellate after one year at 10 °C, compared to 60 % at 15 °C and 86.7 % at 20 °C. While reproductive output in the present study was adjusted to account for the reduction of clitellate individuals, some individuals regained their fully developed state during the recovery period, indicating plasticity. Further research is needed to determine whether regression and re-development of the clitellum has lasting detrimental impacts on cocoon production or viability.

3.6 Conclusion

Consistent with existing studies, *Al. chlorotica* exposed to multiple bouts of drying and aestivation produced fewer and lighter cocoons than those exposed to a single bout, reflecting reduced reproductive output under stress. However, contrary to expectations, earthworms from drying treatments often recovered mass more effectively and produced more and heavier cocoons than those maintained under constant control conditions. This highlights the adaptive benefit of aestivation as a protective strategy and suggests that subsequent recovery under favourable conditions can stimulate compensatory responses. The lower mass and reproductive output of control earthworms indicate that, while not water-limited, control conditions were suboptimal, likely due to depletion of organic matter and increased soil compaction constraining growth and movement. This highlights the importance of considering soil quality and structure alongside moisture availability. Across treatments, earthworm body mass strongly predicted fecundity, with maximum cocoon production, cocoon mass, and hatching success occurring when earthworms were heaviest. Nevertheless, cocoon quality and incubation time varied over the recovery period, with reduced performance in the first 20 days suggesting a lag before full reproductive recovery. Evidence of clitellum regression further indicates that both drying and constant-moisture conditions imposed stress that temporarily shifted resource allocation away from reproduction. Overall, these findings demonstrate that *Al. chlorotica* are resilient to short-term intermittent drought through aestivation, but reproductive output is sensitive to the combined effects of soil moisture, food availability and soil structure. More frequent and prolonged droughts may lead to stronger negative impacts. For populations to persist under fluctuating environmental conditions, sufficient periods of favourable conditions are required to enable recovery of both adult condition and cocoon viability. Further research could investigate the long-term reproductive consequences of aestivation, as well as the vulnerability of earthworm species that lack this strategy.

3.7 References

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3.8 Supplementary materials

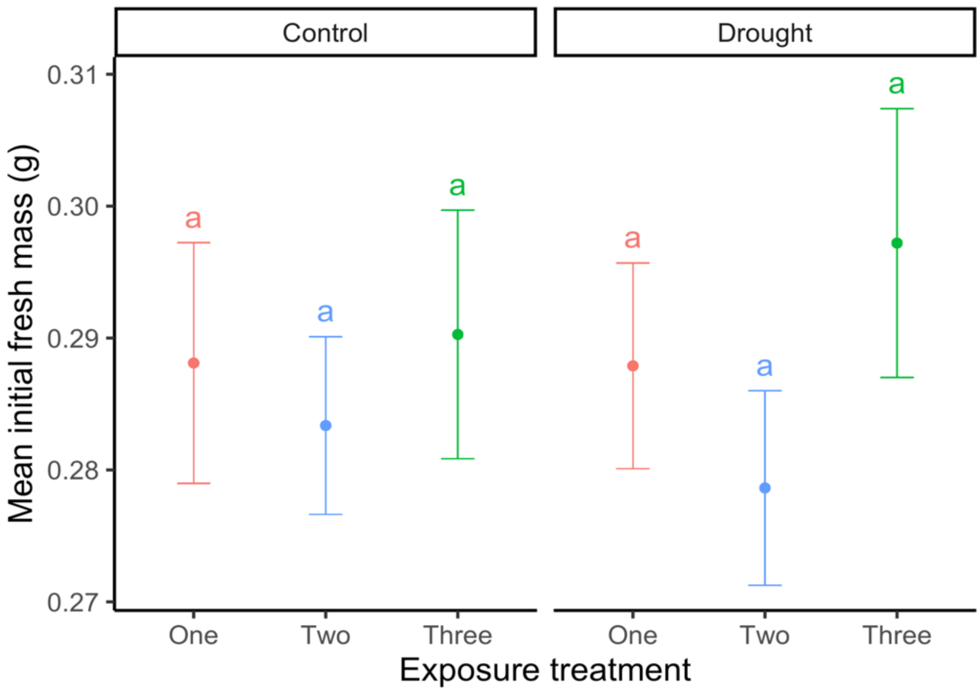

Figure S3.1. The mean (n = 20) initial mass of *Al. chlorotica* assigned to experience either one (red), two (blue) or three (green) bouts of control or drying conditions. Error bars show standard error. Treatments with the same letter do not differ to a statistically significant degree ($p > 0.05$, Tukey test) .

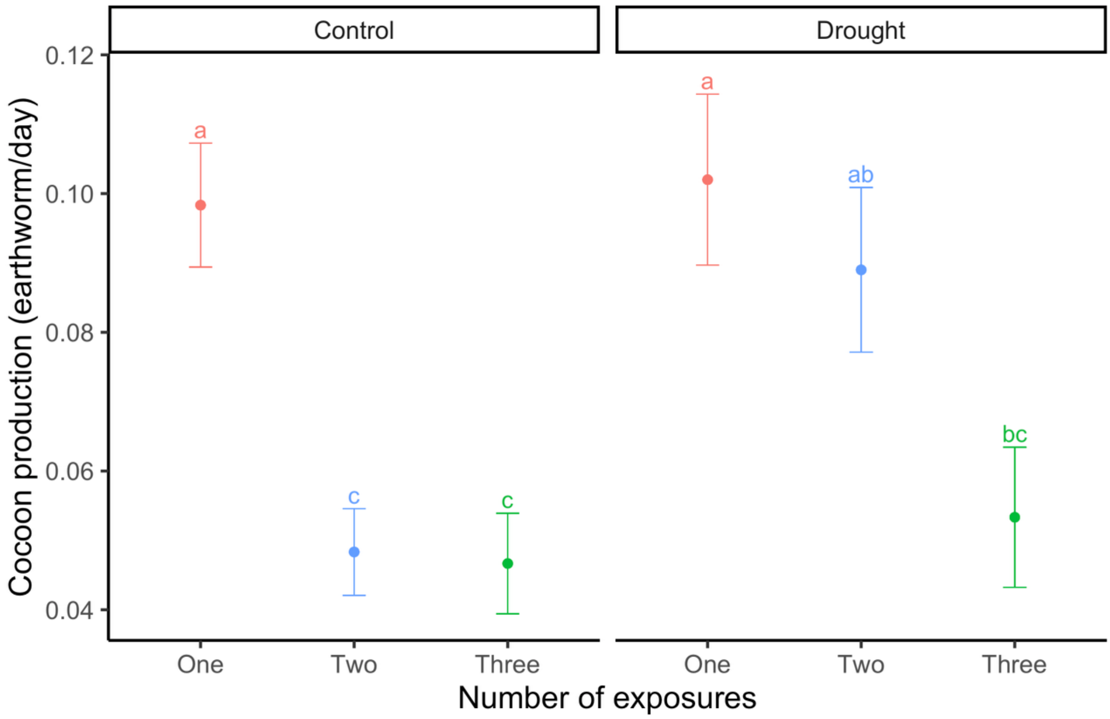

Figure S3.2. The mean overall cocoon production (per earthworm per day) of *Al. chlorotica* assigned to experience either one (red), two (blue) or three (green) bouts of control or drying conditions. Error bars show standard error. Treatments with the same letter do not differ to a statistically significant degree (p > 0.05, Tukey test).

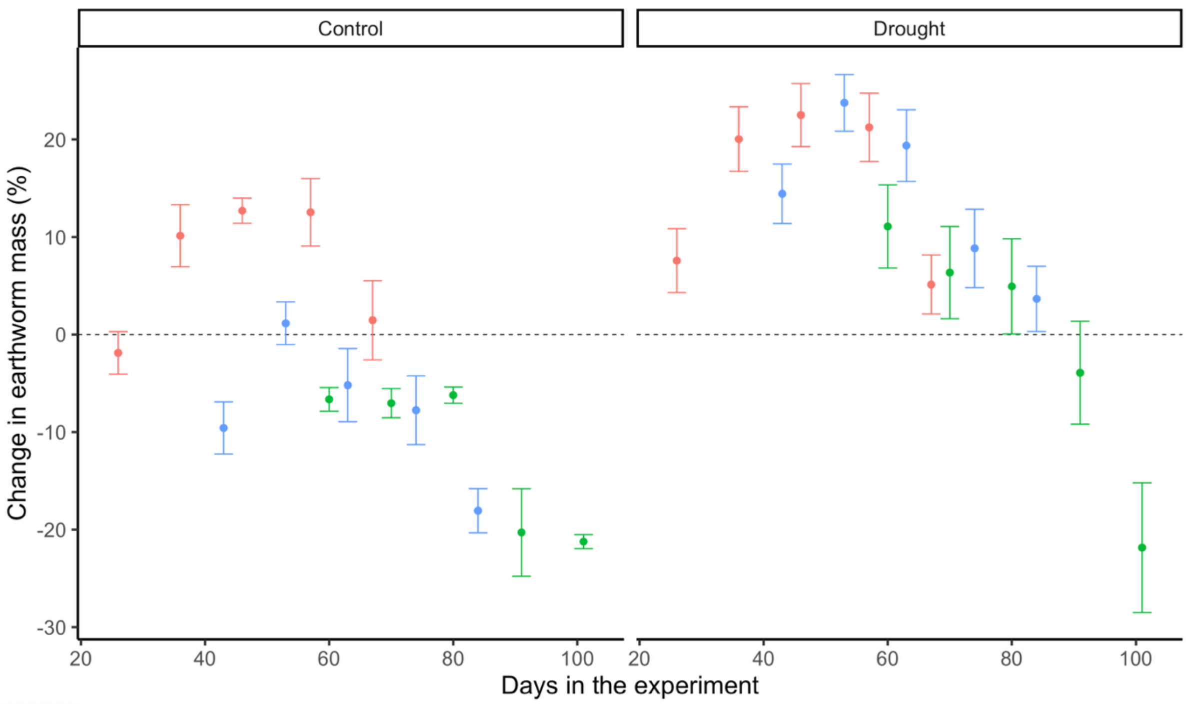

Figure S3.3. The mean change in mass (n = 5) during the recovery period relative to the starting mass of earthworms subjected to one (red), two (blue) or three (green) bouts of control or drought conditions. This data includes both clitellate and aclitellate earthworms.

Chapter 4

The response of *Allolobophora chlorotica* to drought stress in four soil types

4.1 Abstract

Earthworms are key contributors to healthy and productive soils, yet their reliance on water makes them vulnerable to the increased frequency and severity of droughts predicted under climate change. To avoid desiccation, some earthworms induce aestivation, a period of reduced metabolism during which they coil up and seal themselves into a chamber until conditions improve. However, the environmental conditions that trigger aestivation remain poorly understood. Here the responses of *Allolobophora chlorotica* (Savigny, 1826), a common UK earthworm, to gradual air drying (at 15 ± 1 °C) in four soils were examined. Earthworms were identified as active or aestivating at three water contents (~19.7, 15.55 and 12.39 wt%) and three water potentials (~pF 1.59, 2.92 and 4.05). Changes in earthworm mass relative to initial values were recorded at each sampling point and following 24-hours of hydration. Soil type significantly influenced earthworm responses to drying. At the highest water availability (~pF 1.59), all earthworms remained active and gained ~36-53 % mass. At the lowest water availability (~pF 4.05), 100 % aestivated in the sandy loam, loam, and clay soil, with mass losses up to ~26 %. Responses were more variable across soils when soil water was expressed as gravimetric moisture. In clay soil, all earthworms aestivated and lost mass at each of the three water contents, with reductions up to ~45 % at the intermediate moisture (~15.55 wt%). In contrast, earthworms in sand and sandy loam soil gained mass and did not aestivate except in the lowest of the three moisture contents (~12.30 wt%), where some still remained active. Changes in *Al. chlorotica* mass were largely transient and attributable to water loss as after 24 hours of hydration, all individuals had increased relative to their pre-experiment mass. However, control earthworms generally gained more than those previously subjected to drying, particularly at intermediate and least available water potentials, suggesting differences in tissue mass or earthworm fitness. Differences in mass were less consistent across soils when expressed as gravimetric moisture. Overall, these results suggest that water availability, rather than bulk soil water content, is the primary driver of aestivation, although soil type also modulates responses. Moreover, *Al. chlorotica* demonstrated significant desiccation resistance, recovering from mass losses of nearly 50 %, highlighting the effectiveness of aestivation as a survival strategy.

4.2 Introduction

One consequence of climate change, already documented and predicted to increase, is the frequency and severity of droughts (Seneviratne *et al.*, 2021). This presents a significant challenge for soil organisms such as earthworms, which require free water for critical functions such as facilitating oxygen uptake for respiration (Holmstrup, 2001), and forming coelomic fluid, which acts as a hydraulic skeleton to maintain turgor and permit movement through the soil (Kretzschmar and Bruchou, 1991; Ramsay, 1949; Whalen *et al.*, 2000). Under water-limiting conditions, some earthworms enter aestivation, a period of suspended development (Jiménez *et al.*, 2000) during which they coil into a knot to reduce their surface area exposed to the soil (McDaniel *et al.*, 2013a), expel their gut contents (Holmstrup, 2001), and construct a chamber in which they enclose themselves (Bayley *et al.*, 2010). In this state, metabolic activity is reduced and their role in the provision of ecosystem services as key ecosystem engineers is halted until conditions improve (Gerard, 1967; Jiménez *et al.*, 2000; McDaniel *et al.*, 2013b).

Soil water is commonly described using either gravimetric or volumetric moisture content, which quantify the amount of water in soil relative to its dry mass or volume of dry soil (Weil and Brady, 2017). However, water potential is often considered to be a more biologically relevant measure of soil water availability, as it refers to the force at which water is held in the soil and therefore the energy required to extract it (Lee, 1985; Kretzschmar and Bruchou, 1991; McDaniel *et al.*, 2013a). Water potential can be expressed in hectopascals (hPa), kilopascals (kPa) or as a pF value where pF = log(hPa). The field capacity of a soil represents the maximum amount of water held by the soil after excess has drained away and typically occurs at pF 2 (10 kPa) (Weil and Brady, 2017). As soils dry, the water potential increases, meaning that a greater force is required to extract water. Above pF 3.5, free water is typically no longer readily available in soil (Friis *et al.*, 2004), and at pF 4.2 (1,500 kPa), the permanent wilting point is reached, beyond which plants cannot recover their turgidity, a common occurrence in temperate regions during summer (Friis *et al.*, 2004; Weil and Brady, 2017). Importantly, the relationship between water content and water potential depends on soil texture meaning soils can differ substantially in their water availability despite having the same water content (Kretzschmar and Bruchou, 1991; Collis-George, 1959). Generally, at the same moisture content and mass of dry soil, finer-textured soils such as clays have a larger surface area and hold water more tightly than coarser soils like sands and so have a lower water availability (Potvin and Lilleskov, 2017; Weil and Brady, 2017).

Determining the soil water thresholds that influence earthworm behaviour is critical to predicting how droughts may affect their survival, activity and subsequently the ecosystem services they support. Current evidence suggests that earthworms remain active above 20 wt% soil moisture content (Díaz Cosín *et al.*, 2006; Tilikj and Novo, 2022), and that their activity is constrained at water potentials above pF 3 to 3.3 (Gerard, 1967; Kretzschmar and Bruchou, 1991; Nordström, 1975; Rundgren, 1975; Baker *et al.*, 1993). However, available data is relatively scarce, tolerances are likely to be species-specific, and further work is required to improve our understanding of the precise conditions that induce aestivation.

This study investigated how soil moisture and water potential influence the responses of *Allolobophora chlorotica* (Savigny, 1826), one of the most common UK earthworms (Ashwood *et al.*, 2024), to drought stress in four soils of differing texture. Earthworms were kept in soils that were allowed to air dry gradually and were sampled at the same three moisture contents (measured gravimetrically) and the same three water potential values (derived from water retention curves generated for each soil) for all four soils. At each sampling point, earthworms were identified as either active or aestivating and changes in mass were recorded to assess physiological responses to drought conditions and identify when conditions became limiting (Kretzschmar and Bruchou, 1991). Predictions were that (1) earthworms would exhibit a higher incidence of aestivation and greater mass loss as soil water loss increased, and (2) aestivation and earthworm mass loss would occur at higher gravimetric water contents in clay-rich soils due to their lower water availability at a given moisture content. If the availability of water for uptake governs the response of earthworms to drought, earthworms in different soils were expected to show similar responses at equivalent water potentials.

4.3 Methods

4.3.1 Soil types and properties

Four standard soils (LUFA 2.1, 2.2, 2.4 and 6S) were obtained from LUFA Speyer (Speyer, Germany). These soils are routinely used in soil ecology studies and were obtained in 2022 from fields under agricultural management that had been free from pesticide, biocidal fertiliser and organic manure application for at least five years (LUFA, Speyer 2022). Soils were air dried and sieved to 2 mm; subsamples were oven dried at 105 °C to constant mass to calculate their residual moisture contents. Loss on ignition as a proxy for soil organic matter (SOM) was determined by burning oven-dried soil at 400 °C in a muffle furnace for 4 hours (following Jensen *et al.*, 2018, but with reduced temperature to minimise structural water loss). Soil pH was measured from a suspension of 10 g air dried soil in 25 ml deionised distilled water (DIW), mixed in an end over end shaker working at 30 rpm for 15 minutes (The Analysis of Agricultural Materials – Ministry of Agriculture, Fisheries and Food) using a SciQuip 930 Precision pH/Ion meter calibrated with standards of pH 10, 7 and 4. pH and SOM were measured both for soils with and without sheep manure added as a food source (**Table 4.1**). Additional details of the soil compositions and properties can be found in **Table 4.1** and **Fig. S4.1**.

Table 4.1. The particle size distribution (mm) % based on USDA data given in 'Chemical and physical characteristics of standard soils' (LUFA Speyer, 2022). Means with standard deviations given in brackets. The soil pH and SOM values are given both with (n = 50) and without (n = 3) sheep manure added as a food source. Saturated moisture content (vol%) taken from HYPROP measurements.

USDA Soil type	2.1 Sand	2.2 Sandy loam	2.4 Loam	6S Clay
Clay <0.002 (%)	3.5 (± 0.9)	10.8 (± 1.7)	23.7 (± 1.4)	42.5 (± 3.0)
Silt 0.002 - 0.05 (%)	8.4 (± 1.1)	15.7 (± 1.1)	42.2 (± 1.4)	35.1 (± 1.1)
Sand 0.05 - 2 (%)	88.2 (± 0.6)	73.5 (± 1.8)	33.1 (± 1.7)	22.4 (± 2.5)
SOM without food (%)	1.7 (± 0.1)	3.6 (± 0.1)	5.9 (± 0.2)	3.8 (± 0.4)
SOM with food (%)	3.9 (± 0.7)	5.4 (± 0.5)	7.0 (± 0.4)	5.1 (± 0.4)
pH without food	5.44 (± 0.02)	5.27 (± 0.03)	7.52 (± 0.03)	7.74 (± 0.05)
pH with food	6.48 (± 0.20)	6.07 (± 0.14)	7.47 (± 0.14)	7.47 (± 0.16)
Saturated moisture (%)	38.76	30.73	50.34	50.55

4.3.2 Water retention curves

Water retention curves (**Fig. 4.1**) were generated using HYPROP 2 instruments (following the HYPROP manual UMS 2015, **Fig. S4.2**) which use tensiometers to measure changes in the mass and water potential as saturated soil samples air dry (details of the procedure are provided in the supplementary materials). As the HYPROP reports volumetric soil moisture values, these values were converted to gravimetric moisture contents (based on the density of each soil sample) to allow direct measurement of moisture content during the experiment.

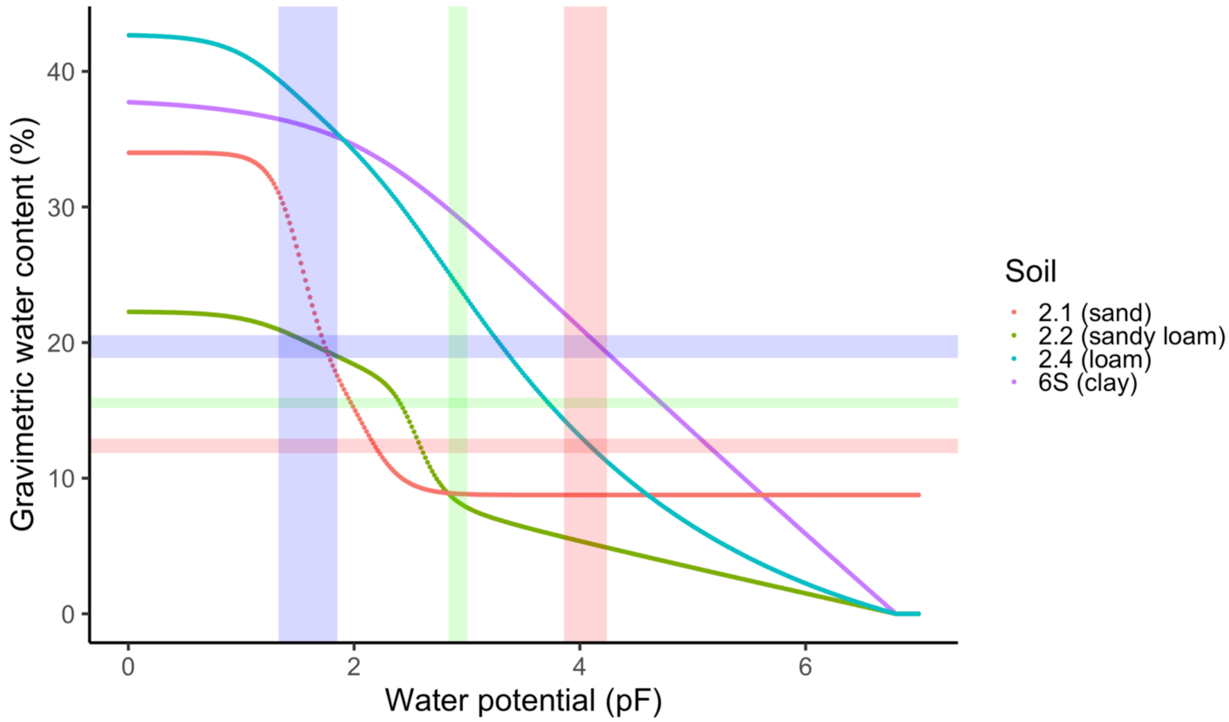

Figure 4.1. Four water retention curves showing the relationship between water potential (pF) and moisture content (wt%) for each of the standard soils 2.1, 2.2, 2.4 and 6S. Data is generated from the HYPROP values with curves fitted based on the PDI-variant of the binomial constrained van Genuchten model (m = 1 - 1/n). Shaded bands represent pF (vertical) and gravimetric water content (horizontal) sampling ranges (means ± standard error) determined post-sampling (blue = highest wt% and most available pF, green = intermediate sampling points, red = lowest wt% and least available pF).

4.3.3 Earthworm collection and culturing

Adult (i.e. clitellate) *Al. chlorotica* were collected in Spring 2024 by hand-sorting soil from pits (~18 cm x 18 cm at the surface to 20 cm depth) dug in the field margins of Warren Paddock, a silage field at the University of Leeds Research Farm, Tadcaster (53°52'25.9"N 1°19'33.4"W) which was in permanent pasture from 2012 until it was sown with oilseed rape in Autumn 2023. Earthworms were cultured in a controlled temperature room at the University of Sheffield at ~25 wt% moisture, 15 ± 1 °C and in complete darkness in field soil, a well-drained, calcareous loam (Holden *et al.*, 2019) with a pH of ~6.9 and SOM (soil organic matter content) of ~8.2 % (determined in the laboratory following the same procedures noted previously), and fed *ad-libitum* with sheep manure until the start of the experiment.

4.3.4 Experimental setup and sampling point selection

Sheep manure (from sheep reared without the use of anti-helminthic drugs) was collected from a farm in Sheffield, oven-dried, ground to <2 mm and added to the experimental soils as a food source. The manure was ground as evidence suggests smaller particle sizes of organic matter are easier for earthworms to ingest; Lowe and Butt (2003) found that *Al.*

chlorotica attained a significantly higher mass (185 % greater) when provided with separated cattle solids that had been milled compared to unmilled. Approximately 3.5 g of dry sheep manure per earthworm was mixed into the soil used in each exposure replicate to provide enough food for 7 weeks (based on Lowe and Butt, 2005, suggestion of 0.5 g/earthworm/week). Soil together with the added sheep manure was placed in 300 ml plastic cups, mixed and levelled to the 150 ml marker to achieve pre-determined densities consistent with the density of the soil cores in the Hyprop measurements: 2.1 = 1.14 g/cm^3 (171 g dry mass), 2.2 = 1.38 g/cm^3 (207 g dry mass), 2.4 = 1.18 g/cm^3 (177 g dry mass), and 6S = 1.34 g/cm^3 (201 g dry mass). Soils 2.1 and 2.2 were made to gravimetric moisture contents of 22 %, soil 2.4 to 38 % and soil 6S to 37 % (equivalent to water potentials between pF 0.8-1.6) through the addition of distilled deionised water (DIW) using a spray bottle and mixed to homogenise. These initial moisture contents were selected so that soil water was wetter than the first sampling point but below saturation to ensure high water availability whilst also retaining the soil's structural integrity. Soils in the control treatments were kept at these respective moisture contents throughout the experiment.

Al. chlorotica (n = 200) were placed onto filter paper moistened with DIW for 24 hours to depurate. After gently blotting dry, individual fresh masses were measured on a top pan balance to 3 decimal places. Half of the *Al. chlorotica* (n = 100, n = 25 per soil type) were randomly assigned to drying treatments, while the other half (n = 100, n = 25 per soil type) were kept in constant, high moisture conditions throughout the experiment as controls. Mean earthworm fresh mass was 0.291 g (± 0.074 g) and did not differ significantly between those in the drying and constant treatments (ANOVA: F = 2.448, df = 1, 192, p = 0.119), or in each soil type (ANOVA: F = 1.066, df = 3, 192, p = 0.365) (**Fig. S4.3**). One earthworm was placed onto the soil surface of each cup and the total mass was recorded to allow monitoring of water loss and addition of DIW to maintain water content in controls when needed. Cups were covered with nylon mesh (100 % polyamide 15 Denier tights purchased from Matalan and cut into smaller strips), secured with an elastic band to allow evaporation but prevent earthworm escape. They were then placed into a controlled temperature room at 15 ± 1 °C and kept in complete darkness throughout the experiment. For the first 48 hours, soils were maintained at constant moisture to allow *Al. chlorotica* to acclimatise. After 48 hours, soils in the drying treatment received no further additions of DIW for the duration of the experiment and were allowed to gradually lose water.

For each soil, earthworms in the drying treatment were sampled at the same three gravimetric moisture contents (~19.7 wt%, 15.55 wt% and ~12.39 wt%) and at the same three water potentials (pF ~1.59, ~2.92 and ~4.05) (**Fig. 4.1, Table 4.2**). These gravimetric moisture contents were selected to confirm findings from an initial range finding experiment (chapter 2) in which all *Al. chlorotica* remained active down to 18 wt% moisture, but most had entered aestivation by 13 wt%. Water potentials were chosen to be close to field capacity, permanent wilting point and an intermediate water availability between the two. As each soil had a different water retention curve, when compared at equal gravimetric moisture contents, their corresponding water availability (pF) was unique. In the same way, when measured at the same water potential, each soil had a different water content (wt%). In each soil type one of the pF levels corresponded to a gravimetric water content at one of the gravimetric sampling points (**Fig. 4.1**) so that the total number of sampling points per soil was five rather than six.

Table 4.2. Mean and standard error value (n =20) for each of the a) gravimetric and b) water potential values determined after sampling.

a)

Gravimetric sampling points	Relative water content
19.7 (± 0.84) wt%	Highest
15.55 (± 0.38) wt %	Intermediate
12.39 (± 0.53) wt %	Lowest

b)

Water potential sampling points	Relative water availability
pF 1.59 (± 0.26)	Most
pF 2.92 (± 0.08)	Intermediate
pF 4.05 (± 0.19)	Least

When the soils had dried to each of the pre-determined sampling points, five replicate cups were randomly selected to be destructively sampled, along with five from the corresponding soil's control group. Soils were hand-sorted in layers and the state of *Al. chlorotica* was determined as active (outstretched body, moving) or aestivating (curled into a knot within a soil chamber). Excess soil was removed by gently wiping earthworms with tissue before they were individually weighed to determine their pre-hydration fresh mass. *Al. chlorotica* were then placed onto filter paper moistened with DIW for 24 hours to depurate/hydrate. After this period, earthworms were gently blotted dry and re-weighed to get their post-hydration mass.

4.3.5 Statistical analyses

All statistical analyses were performed in R (version 4.4.1, R Core Team, 2024). Diagnostic plots were created to confirm that the assumptions of linearity, constant variance, and normality were met. Data for initial earthworm masses were not normally distributed and so were log transformed before an ANOVA was carried out to test for statistical differences. The effects of water content (pF or wt%) and soil type on earthworm state were analysed using generalised linear models (GLMs) with binomial error distributions and logit link functions. The response variable (state) was specified as a two-column matrix representing the number of active and aestivating earthworms. Soil water content (either pF or wt%) and soil type were included as fixed effects, and their interaction was tested to assess whether the effect of water content differed among soil types. Changes in earthworm mass were calculated as a percentage increase or decrease relative to their initial mass at the start of the experiment. After log transforming earthworm masses to meet assumptions of normality, differences between those found active and aestivating were assessed using linear models. Earthworm state (active or aestivating), soil type, and treatment group (control or drying) were included as fixed effects. Three-way interactions among state, soil type and treatment were included in the model. The same model was fitted separately to earthworm mass measured pre- and post-hydration. For analyses of changes in mass of earthworms in the drying treatment (both pre- and post-hydration), two-way ANOVAs were conducted with main effects and interactions of soil type and water content (expressed as either wt% or pF). Three-way ANOVAs were then conducted with treatment group as an additional factor to allow comparison between masses of earthworms from the drying treatment with their corresponding control group sampled at the same time (but not at the same water content). To assess the relationships between continuous soil water content and changes in earthworm mass, generalised additive models (GAMs) were fitted. The response variable was mass change and soil type was included as a fixed factor. Smooth terms for soil water content were fitted separately for each soil type using the 'by' argument, allowing non-linear relationships to differ among soils. Analyses were conducted separately for pre-hydration and post-hydration measurements, and for soil water expressed as either pF or gravimetric wt%, resulting in four GAMs in total. For all analyses involving ANOVAs, post-hoc treatment contrasts were performed using Tukey tests to identify which groups differed significantly.

4.4 Results

4.4.1 State of Al. chlorotica on sampling

Overall, across all soil types, 99 % of the *Al. chlorotica* in the control conditions under constant optimal moisture conditions remained active throughout the whole experiment. In contrast, for the earthworms subjected to drying conditions, there was a significant effect of water potential on the proportion of *Al. chlorotica* aestivating (Binomial distribution glm: $X^2 = 30.468$, df = 2, 9, $p < 0.001$) and this varied with soil type (Binomial distribution glm: $X^2 = 21.299$, df = 3, 6, $p < 0.001$). All earthworms were active at the water potential at which water was most available (~pF 1.59). At the intermediate water availability (~pF 2.92), *Al. chlorotica* were found aestivating in soils with a higher sand content (2.1 and 2.2), whereas no individuals were found aestivating in the loam (2.4) and clay (6S) soils until the water potential where water was least available (~pF 4.05) (**Fig. 4.2**). The proportion of earthworms found aestivating was only slightly significantly influenced by gravimetric moisture content (Binomial distribution glm: $X^2 = 5.858$, df = 2, 9, $p = 0.053$), but once again significantly varied with soil type (Binomial distribution glm: $X^2 = 50.332$, df = 3, 6, $p < 0.001$).

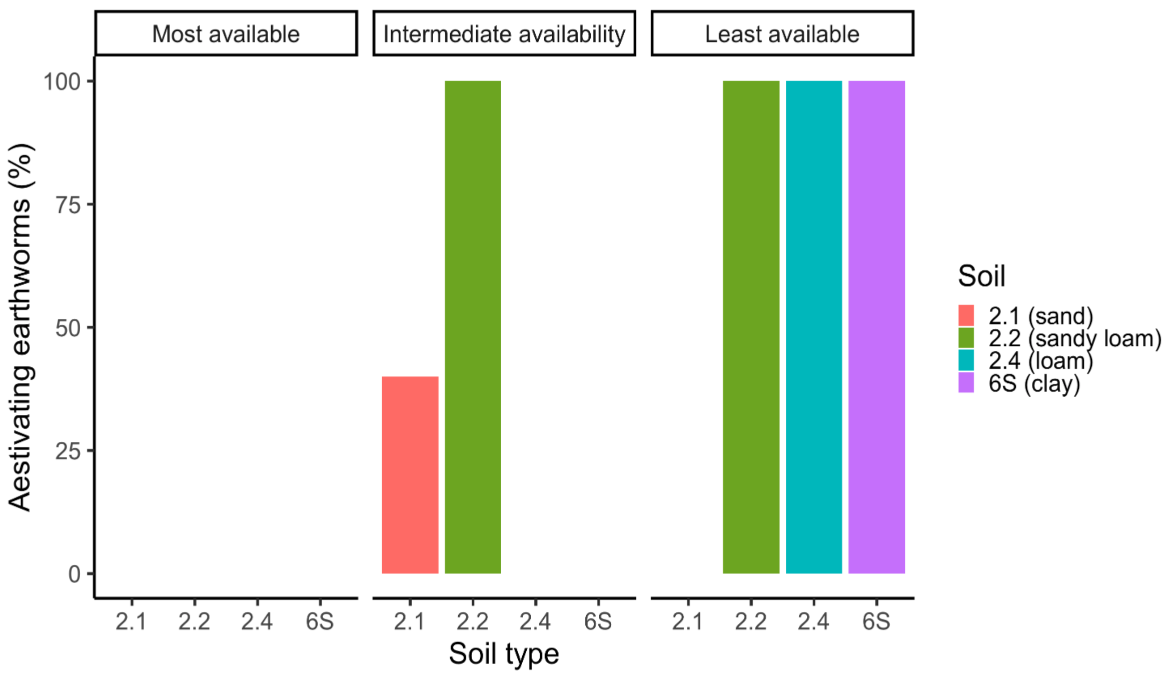

Figure 4.2. The percentage of *Al. chlorotica* (n = 5) found aestivating at each water potential sampling point (most available = pF ~1.59, intermediate availability = pF ~2.92 and least available = pF ~4.05) in each of the four soil types.

No earthworms aestivated in the sandiest soils (2.1 and 2.2) until the lowest moisture content (~12.39 wt%), whereas earthworms were found aestivating in the loam (2.4) and clay (6S) soils even at the highest moisture content (~19.7 wt%) (**Fig. 4.3**). All earthworms in the clay soil were aestivating at each of the three gravimetric sampling points.

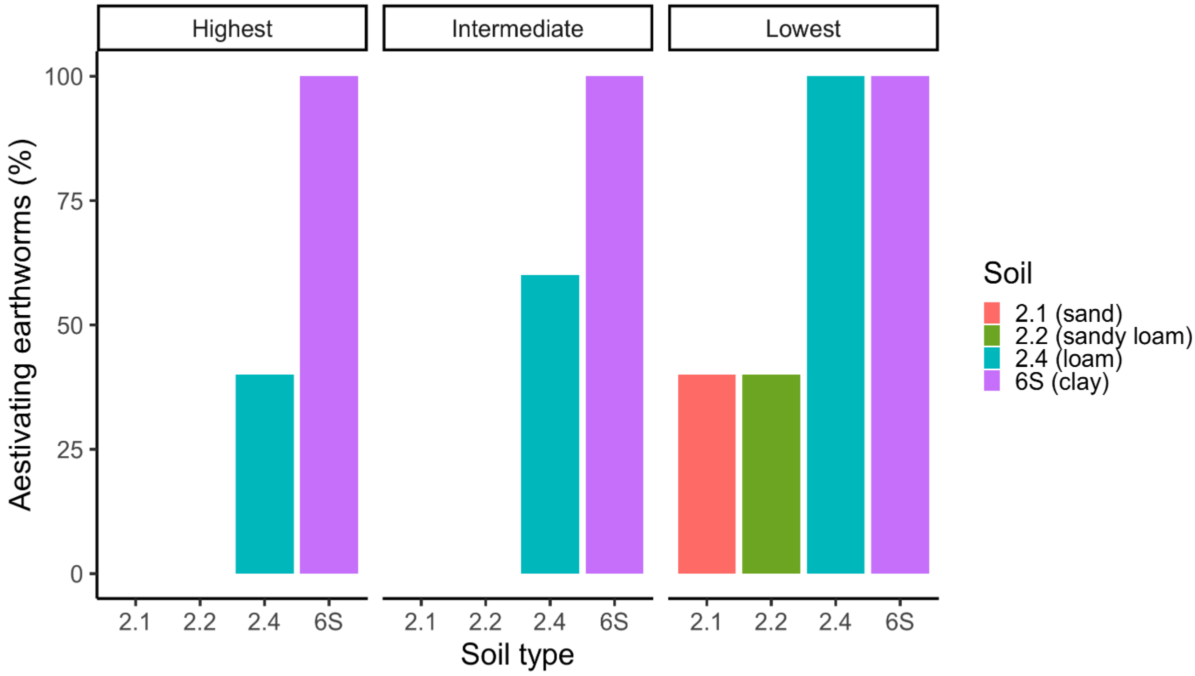

Figure 4.3. The percentage of *Al. chlorotica* (n = 5) found aestivating at each gravimetric moisture sampling point (highest = ~19.7 wt%, intermediate = ~15.55 wt% and lowest = ~12.39 wt%) in each of the four soil types.

4.4.2 Change in earthworm mass during hydration

Overall, there was a significant effect of the state of earthworms on their pre-hydration mass (ANOVA: $F = 205.194$, $df = 1, 187$, $p < 0.001$), but this was also dependent on the soil type (ANOVA: $F = 17.66$, $df = 2, 187$, $p < 0.001$). Prior to hydration, earthworms found aestivating were significantly lower in mass than those found active, except those in the sand (2.1) (Tukey, $p < 0.05$) (**Fig. 4.4**). After 24 hours hydration, there were no significant differences in the mass of earthworms found in either state when measured post-hydration (Tukey, $p > 0.05$) (**Fig. 4.5**). **Figure 4.6** shows the relationship between the mass of *Al. chlorotica* in the constant and drying treatments when weighed pre and post 24 hours hydration on wet filter paper. For *Al. chlorotica* in the constant control conditions, most points lie below the dashed line indicating that earthworms lost mass over the hydration period. In contrast, the direction of the change in mass of earthworms exposed to drying conditions depended on the state they were found in as those that aestivated tended to gain mass, while those that remained active lost mass during the hydration period (**Fig. 4.6**).

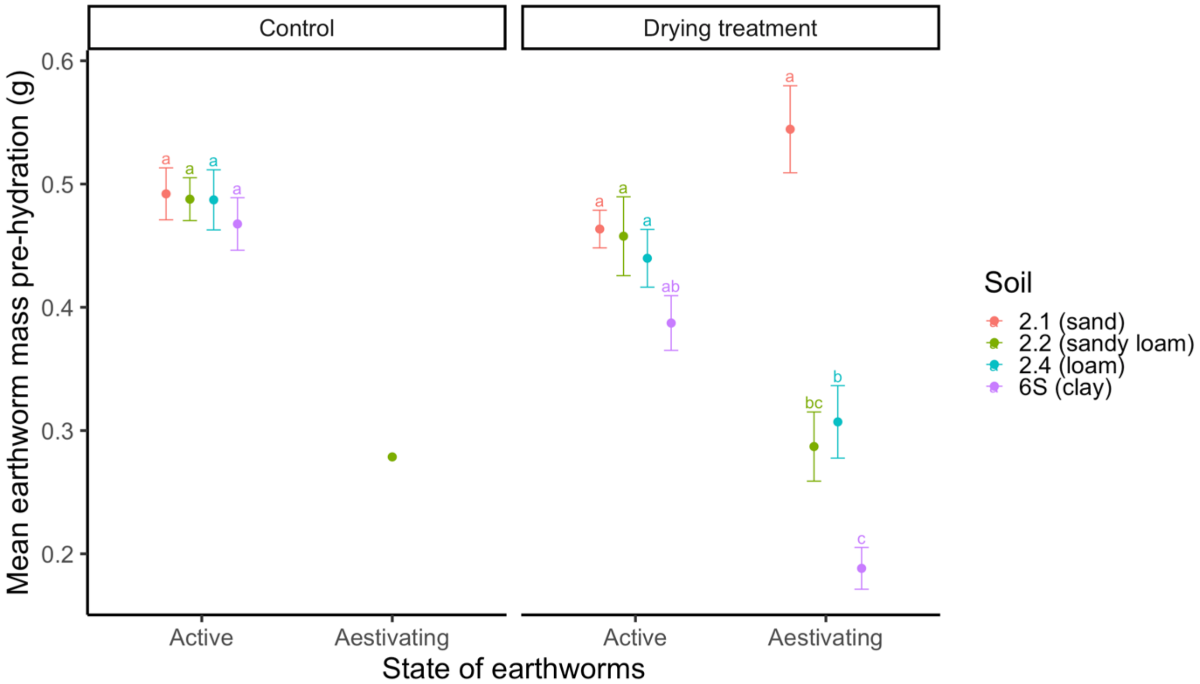

Figure 4.4. The mean mass of earthworms found active (both in the control and drying treatments) and aestivating in each soil type pre 24 hours hydration. Error bars represent standard error. Treatments with different letters differ to a statistically significant degree ($p < 0.05$).

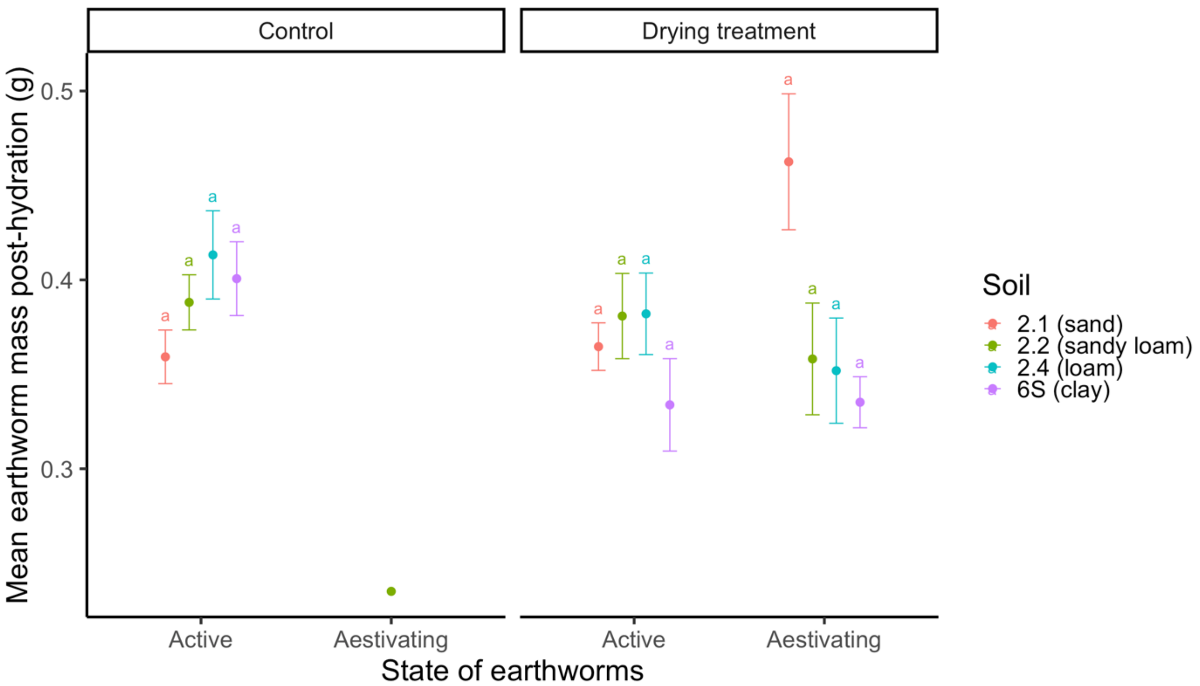

Figure 4.5. The mean mass of earthworms found active (both in the control and drying treatments) and aestivating in each soil type post 24 hours hydration. Error bars represent standard error. Treatments with the same letter do not differ to a statistically significant degree ($p > 0.05$).

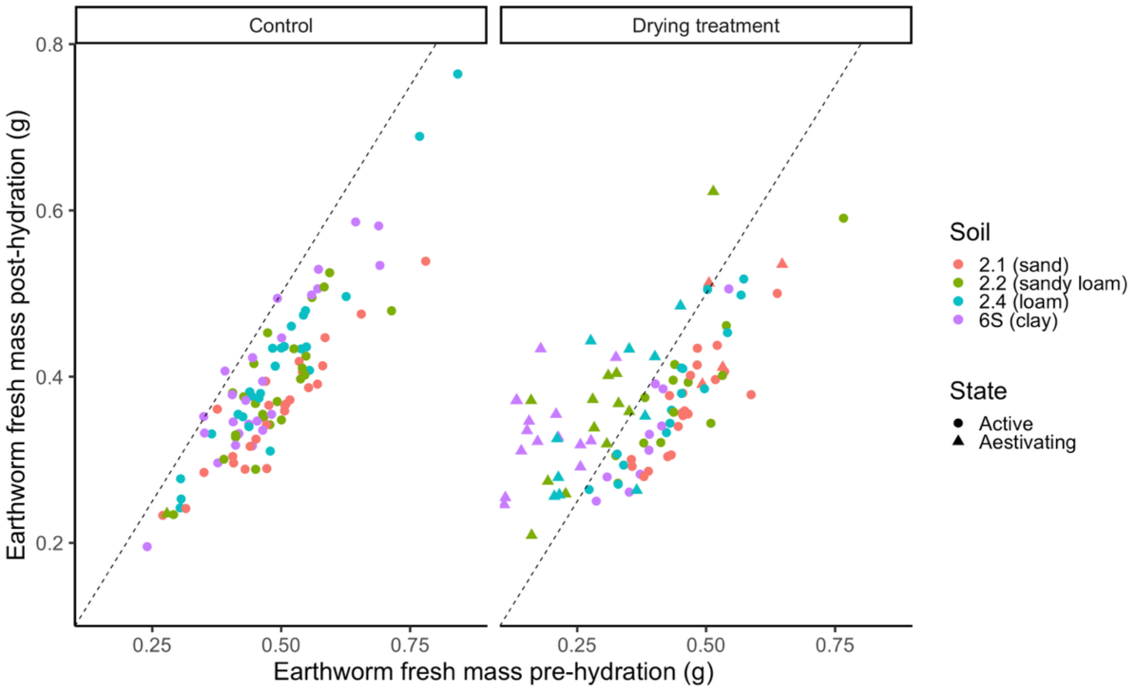

Figure 4.6. The mass of *Al. chlorotica* pre- and post-exposure to 24 hours hydration, grouped by treatment (constant control or drying conditions). Colours represent each soil type. Shapes represent the state of earthworms on destructive sampling (circles = active, triangles = aestivating). Dashed lines represent no change in mass.

4.4.3 Influence of water potential (pF) on earthworm mass

There were significant overall main effects of soil type (ANOVA: 12.204, df = 3, 48, p < 0.001) and water availability (pF) (ANOVA: F = 12.423, df = 3, 48, p < 0.001) on the mean change in *Al. chlorotica* mass when weighed on removal from drying soil moisture conditions (pre-hydration). The effect of water availability also varied significantly with soil type (ANOVA: F = 4.228, df = 6, 48, p < 0.01) (**Fig. 4.7**). At the most available water content (~pF 1.59) all earthworms had increased in mass (by between ~36-53 %) and there were no significant differences between soils (Tukey, p > 0.05). Similarly, at the intermediate water availability (~pF 2.92) most earthworms had increased in mass and by a similar magnitude (~54-55 %), except for those in soil 2.2 which were no different from their starting mass and thus differed significantly from the other soil types (Tukey, p > 0.05). At the least available water content (~pF 4.05) earthworms in soils 2.2, 2.4 and 6S had decreased in mass since the start of the experiment by between ~3 and 26 %. For those in soils 2.4 and 6S these changes in mass were significantly different to changes in the intermediate water availability (Tukey, p < 0.05). In contrast, those in soil 2.1 still showed an increase in mass by ~68 % in the least available water content. Earthworms in the control conditions had increased in mass by between ~32 and 120 % compared to their starting mass at every sampling point (**Fig. S4.4**). When earthworms subjected to drying conditions were compared to their corresponding control groups sampled at the same time (but coming from optimal soil water conditions), there was a significant interactive effect between sampling point, soil type and treatment group (ANOVA: $F = 2.266$, df = 6, 96, $p < 0.05$). At the most available water content, earthworms subjected to drying conditions had increased in mass by a similar amount to their corresponding control group (**Fig. S4.4**). At the intermediate availability those in soil 2.1 and 2.2 had increased by a significantly smaller proportion compared to their control groups and at the least available water potential earthworms in the controls had gained significantly more mass compared to those in the drying conditions in all soil types except for 2.1 (Tukey, $p < 0.05$).

After 24 hours hydration, all changes in mass were now positive and there were no longer any significant main effects of water availability (ANOVA: $F = 1.008$, df = 2, 48, $p = 0.372$) or soil type (ANOVA: $F = 1.823$, df = 3, 48, $p = 0.156$) or their interaction (ANOVA: $F = 0.847$, df = 6, 48, $p = 0.541$) on the change in *Al. chlorotica* mass for those in the drying conditions (**Fig. 4.8**). However, marked differences remained between earthworms from the constant optimal moisture control and drying treatments at the intermediate and least available water potentials (**Fig. S4.5**). In particular, earthworms in soil 2.2 exposed to the least available water potential had increased by significantly less mass than earthworms in the same soil sampled at the same time but from optimal water conditions (Tukey, $p < 0.05$).

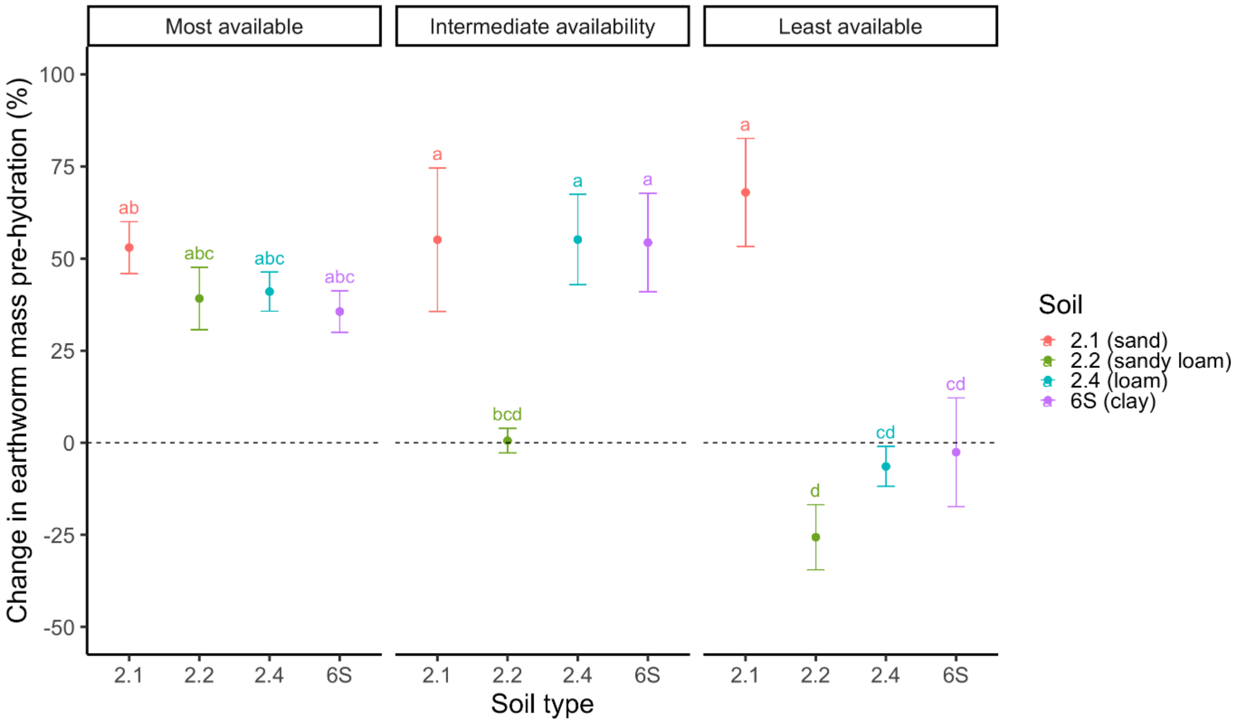

Figure 4.7. The mean change in earthworm mass (n = 5) relative to starting mass in each soil type at each of the three water potentials (of differing water availability) prior to hydration. Error bars show standard error. Treatments with different letters differ to a statistically significant degree ($p < 0.05$).

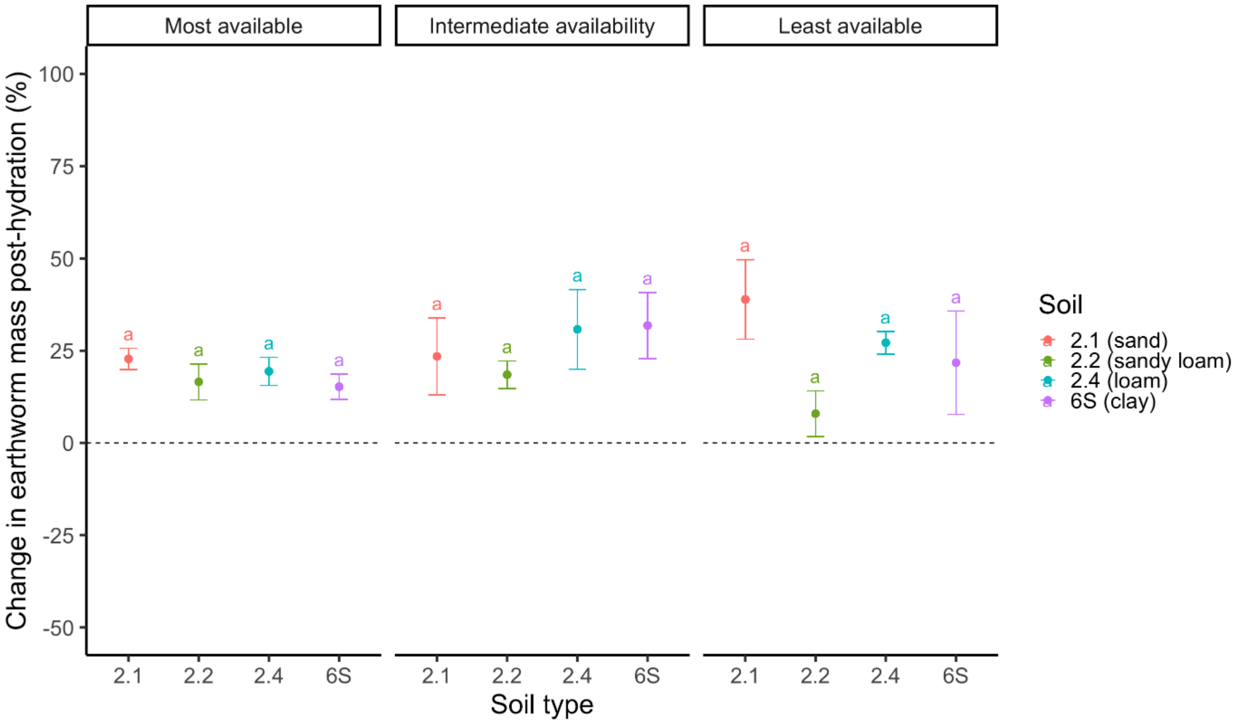

Figure 4.8. The mean change in earthworm mass (n = 5) relative to starting mass in each soil type at each of the three water potentials (of differing water availability) post-hydration. Error bars show standard error. Treatments with the same letter do not differ to a statistically significant degree (p > 0.05).

4.4.4 Influence of gravimetric moisture content (wt%) on earthworm mass

As with water potential, changes in mass measured pre-hydration relative to the start of the experiment were significantly influenced by main effects of soil type (ANOVA: F = 61.587, df = 3, 48, p < 0.001) and gravimetric moisture content (ANOVA: F = 6.581, df = 2, 48, p < 0.01). The effect of gravimetric soil moisture also significantly varied with soil type (ANOVA: F = 4.302, df = 6, 48, p < 0.01) (**Fig. 4.9**). At the highest moisture content (~19.7 wt%), all earthworms had increased in mass (by ~39-53 %) since the start of the experiment, except for those in the clay soil (6S) which had decreased by ~3 % and differed significantly from those in the sand soil (2.1) (Tukey, p < 0.05). At the intermediate gravimetric moisture content (~15.55 wt%) the changes in mass had become more negative for those in the clay soil (decreasing in mass by ~45 %) and this was significantly different from earthworms in all other soil types (Tukey, p < 0.05). Earthworms in the other soil types had increased in mass since the start of the experiment, but the increase for those in the loam soil was lower than in the highest moisture content (although not significantly, Tukey p > 0.05). At the lowest moisture content (~12.39) changes in mass were only positive for earthworms in the sand (2.1) and sandy loam (2.2). Earthworms in the clay soil (6S) had lost a similar amount as in the intermediate moisture content, while those in the loam had now decreased in mass (by ~6 %) since the start of the experiment and were significantly different from their change in mass in the highest moisture content (Tukey, p < 0.05). All earthworms in the control conditions had increased in mass since the start of the experiment by between ~46 % to ~99 % when weighed at every sampling point (**Fig. S4.6**). At the highest moisture content, earthworms in the drying conditions had gained a similar mass as those in the control conditions sampled at the same time, except for those in the drying clay soil (6S) which had gained significantly less mass (Tukey, $p < 0.05$). Earthworms in the drying clay conditions remained significantly different from their corresponding control in terms of changes in mass at every sampling point (Tukey, $p < 0.05$). The differences between control and drying groups increased as the drying groups lost more water and by the lowest gravimetric moisture content, those in the drying loam soil were also significantly different to their control (Tukey, $p < 0.05$).

After 24 hours hydration, all earthworms from the drying conditions had increased in mass (by between ~16-34 %) and there were no longer significant effects of soil type (ANOVA: F = 0.325, df = 3, 48, p = 0.807), gravimetric water content (ANOVA: F = 0.325, df = 2, 48, p = 0.725) or their interaction (ANOVA: F = 0.414, df = 6, 48, p = 0.866) (**Fig. 4.10**). Differences remained between earthworms from the controls with optimal moisture conditions and those from the drying treatments at each gravimetric sampling point (**Fig. S4.7**), mainly for those in the loam (2.4) and clay soils (6S), but these differences were not significant (Tukey, $p > 0.05$).

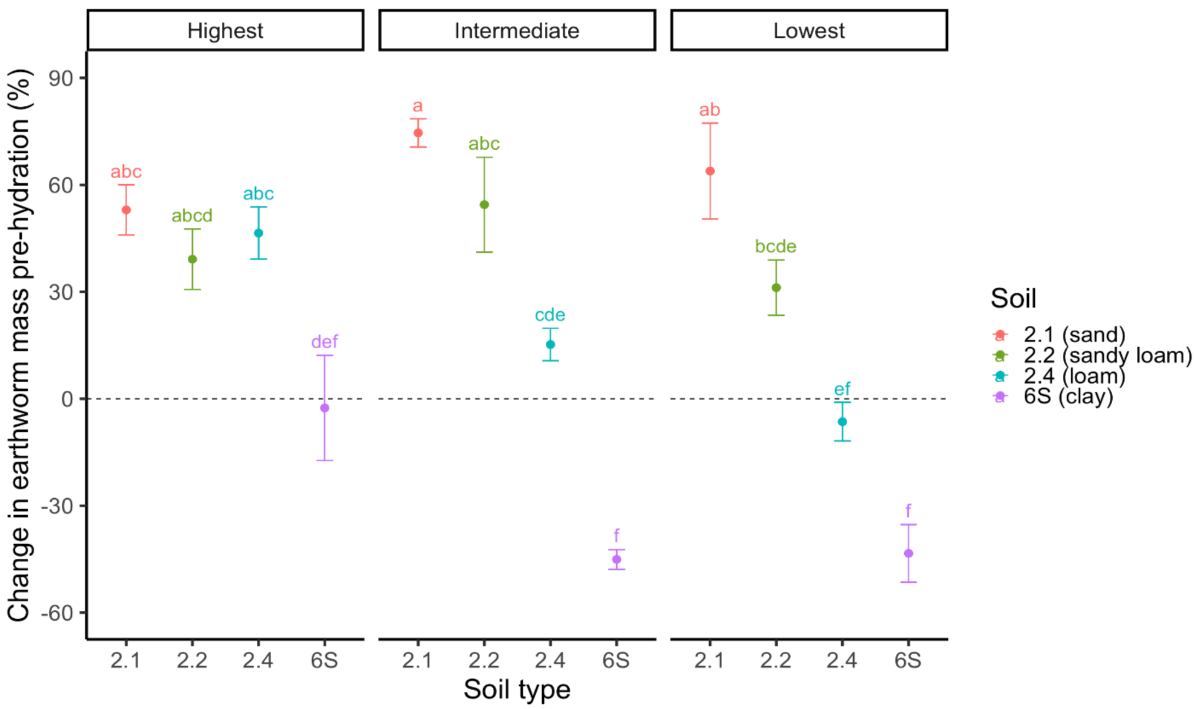

Figure 4.9. The mean change in earthworm mass (n = 5) relative to starting mass in each soil type at each of the three gravimetric moisture values (of differing water content) pre-hydration. Error bars show standard error. Treatments with different letters differ to a statistically significant degree ($p < 0.05$).

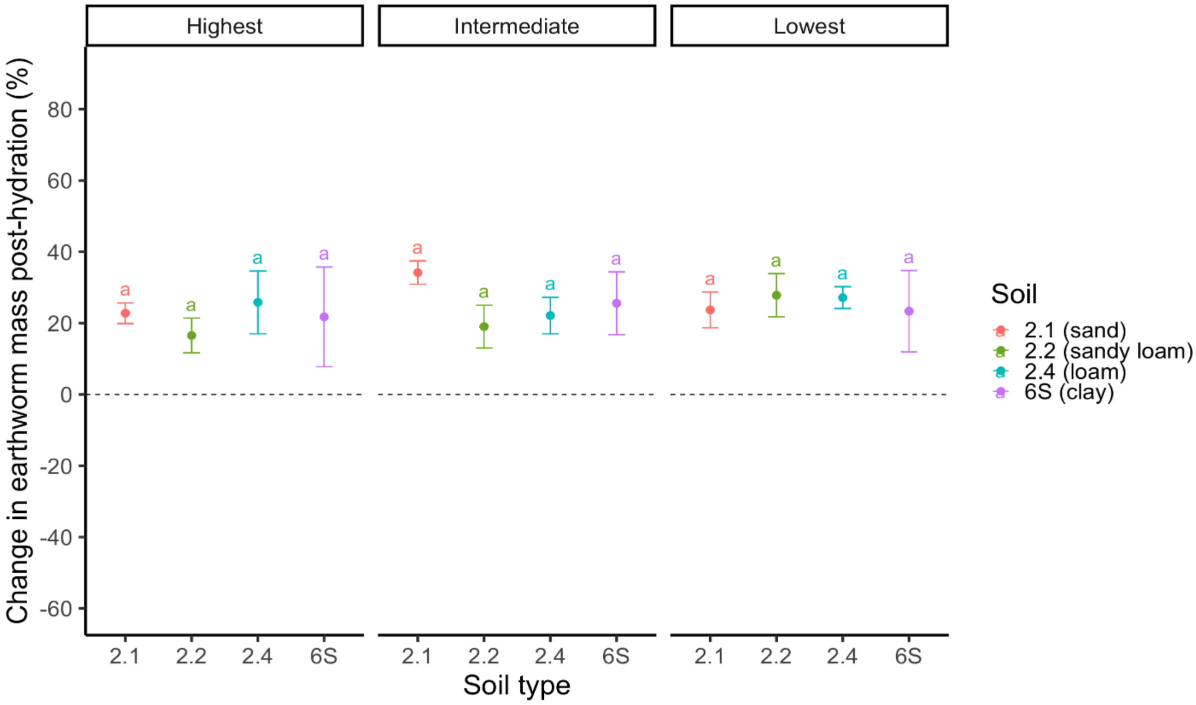

Figure 4.10. The mean change in earthworm mass (n = 5) relative to starting mass in each soil type at each of the three gravimetric moisture values (of differing water content) post-hydration. Error bars show standard error. Treatments with different letters differ to a statistically significant degree ($p < 0.05$).

4.4.5 Changes in Al. chlorotica mass pre- and post-hydration against continuums of water availability and water content

To better examine when *Al. chlorotica* in the drying conditions began to lose mass in each soil, changes in mass were also plotted against water potential (pF) and gravimetric moisture content (wt%) on a continuous scale (**Fig. 4.11**). Pre-hydration, the effect of water potential (Generalised additive model: adjusted $R^2 = 0.708$) and gravimetric water content (Generalised additive model: adjusted $R^2 = 0.712$) on earthworm mass change depended strongly on soil type. In the sand soil (2.1), there was no significant effect of water availability ($p = 0.661$) or gravimetric moisture ($p = 0.812$), and earthworms gained mass at all water potentials. In contrast, soil water potential ($p < 0.001$) and gravimetric moisture ($p < 0.001$) had significant nonlinear relationships with changes in earthworm masses in soils 2.2, 2.4 and 6S. At high water contents and availabilities, all earthworms had gained mass relative to their starting mass, but with increasing water loss the changes in mass became more negative. The threshold at which earthworms lost mass relative to their starting mass was dependent on soil type. Those in the sandy loam (2.2) were most sensitive to changes in water potential, with negative changes in mass observed at higher water availabilities (~pF 2.9) compared to those in the loam (2.4) and clay (6S) soils (in which negative changes were first observed above ~pF 4) (**Fig. 4.11)**. In terms of gravimetric moisture thresholds, earthworms in the clay soil were found below their starting mass at just below ~19 wt%, whereas those in the loam soil (2.4) did not show negative changes in mass until ~12 wt%

and those in the sandy loam remained at or above their initial masses until the soil moisture content declined below 10 wt% (**Fig. 4.11**).

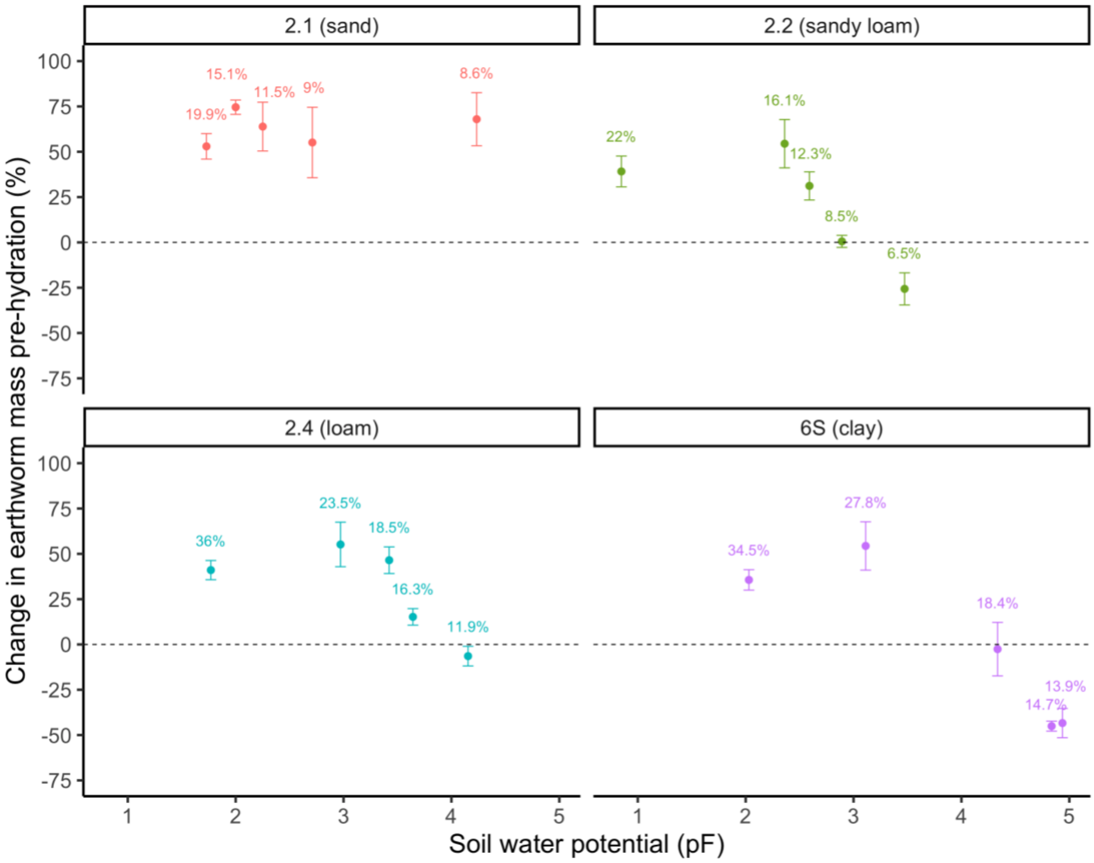

Fig 4.11. The mean (n = 5) change in *Al. chlorotica* mass relative to their starting mass measured pre-hydration relative to the soil water potential (pF) for each soil type. Error bars show standard error. Percentages above points represent the corresponding gravimetric moisture content for each pF value.

After 24 hours hydration, neither the water potential (Generalised additive model: adjusted R^2 = 0.028) nor the gravimetric moisture content (Generalised additive model: adjusted R^2 = 0.011) were significantly related to changes in earthworm mass for any soil type, with all earthworms greater in mass than the start of the experiment (**Fig. 4.12**).

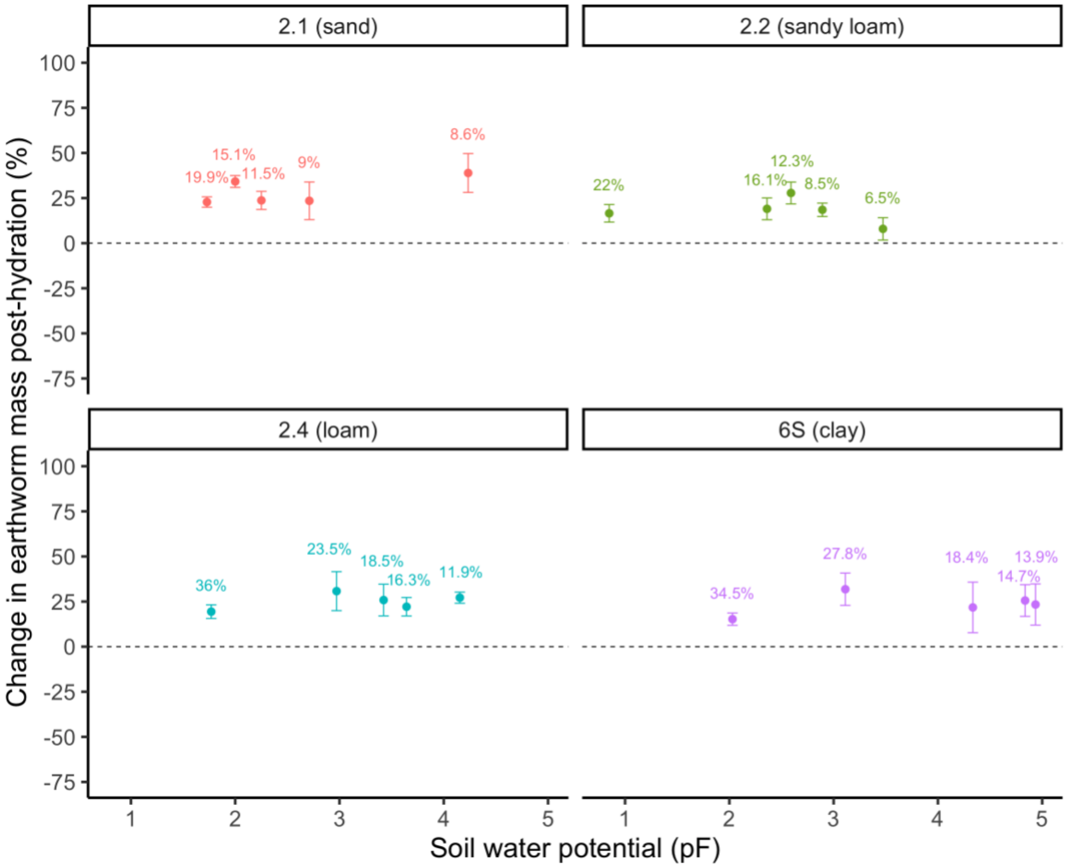

Fig 4.12. The mean (n = 5) change in *Al. chlorotica* mass relative to their starting mass measured post-hydration relative to the soil water potential (pF) for each soil type. Error bars show standard error. Percentages above points represent the corresponding gravimetric moisture content for each pF value.

4.5 Discussion

In the control conditions (~pF 0.8 to 1.6), almost all earthworms (99 %) remained active throughout the experiment, consistent with Friis *et al.* (2004), who found no *Aporrectodea caliginosa* (Savigny, 1826) formed aestivation cells in 'wet controls' kept at 18 % moisture (~pF 1-2). In contrast, in the drying treatment, there was a significant effect of water potential on the proportion of aestivating *Al. chlorotica*, which varied with soil type. As predicted, the incidence of aestivation increased with decreasing water availability. All *Al. chlorotica* were active at the highest availability (~pF 1.59) and all were aestivating in the lowest (~pF 4.05) except in the sand (2.1). In the sandier soils (2.1 and 2.2), some aestivation also occurred at the intermediate water availability (~pF 2.92). These findings agree with evidence from studies suggesting that lumbricids are generally active only in soils when water is more available than ~pF 3 to 3.3 (100-200 kPa) (Gerard, 1967; Kretzschmar and Bruchou, 1991; Nordström, 1975; Rundgren, 1975; Baker *et al.*, 1993). For instance, *Aporrectodea longa* (Ude, 1885) cast production and thus burrowing rates were severely restricted above pF 2.48 (30 kPa) and they induced aestivation at pF 3.22 (Kretzschmar and Bruchou, 1991). In *Ap. caliginosa*, Holmstrup (2001) found all juveniles remained active at water potentials of ~pF 2.08 (12 kPa) but entered aestivation above ~pF 2.3 (20 kPa), with a steep increase between pF 2.3 to 2.48 (20 and 30 kPa) and almost all aestivating at pF 2.6 (40 kPa). Later studies confirmed that adult *Ap. caliginosa* also form aestivation chambers in soils of 30 kPa (Bayley *et al.*, 2010), suggesting that these lower thresholds are not a result of earthworm maturity. Therefore, these different thresholds likely represent species-specific differences in drought tolerance which may reflect physiological differences.

Water availability, soil type, and their interaction significantly affected changes in *Al. chlorotica* mass on immediate removal from the soil. At the most available (~pF 1.59) and intermediate (~pF 2.92) water potentials, earthworms had increased in mass relative to their starting mass, though increases were negligible in the sandy loam and significantly lower than their corresponding control group. At the least available water potential (~pF 4.05), earthworms had lost up to ~26 % of their initial mass, except those in the sand. Similarly, Kretzschmar and Bruchou (1991) found *Ap. longa* lost as much as 60 % of their initial weight when exposed to pF 3.5 and 4.3. Water potentials below pF 2.78 had little effect on *Ap. longa* mass, although mass did still fluctuate, which they suggest is likely due to losses from casting and gains from ingestion (Kretzschmar and Bruchou, 1991). In contrast, *Ap. caliginosa* mass declined at water potentials greater than ~pF 2.08 (12 kPa) (Holmstrup, 2001). These findings reinforce that earthworm responses to drying are species-specific. Such differences may be in part explained by osmotic regulation as species differ in their osmotic pressures. It has been suggested that earthworms generally lose body water through osmosis when the soil water potential increases above ~pF 3.6 (~400 kPa) as the osmotic pressure of their body fluids becomes higher than the surrounding soil (Bayley *et al.*, 2010; McDaniel *et al.*, 2013a; Holmstrup *et al.*, 2016). If aestivation is a precautionary response to drying before harmful desiccation occurs (Holmstrup *et al.*, 2016) we may therefore expect aestivation to be induced at water potentials below that of their body fluid osmolality. Consistent with this, *Al. chlorotica* in the sand and sandy loam entered aestivation at ~pF 2.92 in the present study. However, earthworms tolerated lower water availabilities in the loam and clay and did not aestivate until ~pF 4.05. This supports Doube and Styan (1996) findings of a significant positive correlation between the percentage of clay and the water potential (pF value) at which the distribution and thus behaviour (avoidance) of *Aporrectodea trapezoides* (Dugès, 1828) did not diverge from the controls.

Earthworm activity and changes in mass were also significantly influenced by water content expressed in terms of gravimetric moisture, though variation was explained more strongly by soil type. In the sand and sandy loam, aestivation was absent until the lowest sampling point (~12.39 wt%) in which over half remained active. This supports findings from a previous experiment (chapter 2) where all *Al. chlorotica* remained active at 18 wt% but aestivation was observed at 13 wt%. Similarly, Díaz Cosín *et al.* (2006) found aestivation of *Hormogaster elisae* (Álvarez, 1977) earthworms was rare at 20 % moisture while the highest proportion were found aestivating at 10 % moisture particularly in spring and summer. Moreover, in the present study, earthworms in the sand and sandy loam remained greater than their initial mass even at the lowest of the three gravimetric moisture sampling points (~12.39 wt%). In contrast, *Al. chlorotica* were aestivating even at the highest of the three gravimetric sampling points (~19.9 wt%) in the loam and clay, with 100 % aestivation and a ~3 % loss of mass for those in the clay. This is consistent with the prediction that those in the more clay-rich soils would lose mass at a higher gravimetric moisture content. Changes in mass became more negative with decreasing moisture content for earthworms in the clay, loam and sandy loam and by ~15.55 wt%, those in the clay had lost ~45 % of their initial mass.

These differences in responses to equal gravimetric moisture contents likely reflect soil water retention properties resulting in different water availabilities at the same bulk water content. Both Díaz Cosín *et al.* (2006) and a previous experiment (chapter 2) involved soil with a similar composition to the sandy loam (2.2) used here which may explain the similar responses of earthworms to gravimetric moisture conditions. Whereas, at ~19.9 wt% the water availability was already quite reduced in the loam (>pF 3) and clay (>pF 4). Similarly, Doube and Styan (1996) found that the threshold at which *Ap. trapezoides* moved out of soil (avoided unfavourable conditions) was influenced by an interaction between water content and soil type ranging from 10 wt% (15 kpa, pF 2.2) in sandy loam, 12 wt% (25 kPa, pF 2.4) in loam and 20 wt% (300 kpa, pF 3.4) in clay. However, Doube and Styan (1996) found water potential was a more important factor than bulk content in determining the behaviour of *Aporrecotdea rosea* (Savigny, 1826) as they moved out of soil at pF 3.4 (300 kpa) irrespective of soil type or gravimetric moisture content which ranged from 7 - 20 wt%. Therefore, gravimetric thresholds are soil-dependent, and both water potential and soil texture are necessary to predict responses for some species.

Despite large mass losses of *Al. chlorotica* in the drying conditions, no mortality was observed. Similarly, Holmstrup (2001) found *Ap. caliginosa* survived exposure to 14-day drought periods with water potentials as low as pF 3.53 (Holmstrup, 2001). Moreover, there is evidence that *Al. chlorotica* can survive and recover from losses of up to 75 % of their body water (Roots, 1956). After just 24 hours of hydration, changes in *Al. chlorotica* mass became positive meaning they had gained mass since the start of the experiment (from 0.291 ± 0.074 g to 0.376 ± 0.087 g), irrespective of the soil type and degree of drying they had experienced. Consequently, although those found aestivating were significantly lower in mass than those found active (except in sand), they were able to gain water during hydration while active earthworms lost mass by depurating their gut contents resulting in no lasting significant differences. This is consistent with McDaniel *et al.* (2013a) who found that when *Ap. caliginosa* exposed to 1-, 2-, or 3-week cycles of drought stress were returned to higher moisture conditions (wet filter paper) they were able to rehydrate with no lasting negative effects on their biomass. Similarly, Díaz Cosín *et al.* (2006) found *H. elisae* were able to recover their initial body mass once placed into soil with a higher moisture content (20 wt%), although over a longer period of around one week (6.5 ± 3.6 days). Such rapid recoveries of mass support the suggestion that decreases in earthworm fresh mass are mostly due to water loss through urine production, in addition to the evacuation of gut contents and secretion of mucus in the construction of aestivation chambers (Holmstrup, 2001).

Although changes in mass were positive and less marked overall after the period of hydration, some differences in the extent of mass gained remained between earthworms exposed to control and drying treatments, particularly for those which had been subjected to the greatest degree of drying. Assuming *Al. chlorotica* were fully hydrated after 24 hours on wet filter paper, this suggests that some differences were a result of lower tissue mass. These differences may reflect time spent in aestivation when feeding is suspended

(Reinecke and Reinecke, 2007). In contrast, earthworms in the control conditions remained active, continued feeding and gained mass throughout (**Fig. S4.8, S4.9**). This suggests that food was not a limiting factor and indicates that differences in final biomass were instead a result of the limited water conditions, period of aestivation and associated suspended feeding of those in the drying conditions. In addition, differences in mass could be explained by reduced clitellum size which was observed for some individuals from the drying conditions (**Fig. S4.10**). Such regression of secondary sexual structures has been reported to be a key feature of aestivation in earthworms (Olive and Clark, 1978; Jiménez *et al.*, 2000). Some aestivating earthworms were also found with constricted body segments, particularly at their caudal end. These physical differences are likely a result of a severe lack of body water and thus prioritisation of more vital body segments for survival. Jiang *et al.* (2023) state that some tissue loss during aestivation may be a result of adipose tissue being broken down to produce adenosine triphosphate (ATP), while other organs such as those involved in digestion may lose mass through lack of use. In earthworms, some changes in mass due to tissue loss may not be problematic as undifferentiated stem cells allow for regeneration of localised body segments by developing blastema into new tissue (Christyraj *et al.*, 2025).

In the sand, *Al. chlorotica* gained mass irrespective of the degree of drying they were subjected to and were rarely observed in aestivation, even at the least available water potential (~pF 4.05). In contrast, aestivation was prevalent for those in the more clay-rich soils 2.4 and 6S which had considerably lower sand contents (33.1 % and 22.4 % respectively compared to 88.2 % for 2.1). One possible explanation for the apparent lack of aestivation and negative effect on earthworm mass in the sandiest soil (2.1) could be its lower density and texture which remained loose as it dried, allowing earthworms to remain active and keep feeding. In contrast, the loam and clay soils hardened as they dried, constraining their movement and activity as the energy required to burrow increases with increasing soil mechanical resistance (Arrazola-Vasquez *et al.*, 2022). This higher energetic cost combined with the reduction in soil water likely resulted in the onset of aestivation. Moreover, the sand reached the lowest available water content (pF 4.05) after only 25 days in the experiment compared to 45, 44 and 36 days for the other soils (2.2, 2.4 and 6S respectively) (**Table S4.1**). Therefore, earthworms in the sand were not exposed to drying experimental conditions for as long, reaching their final sampling point over 20 days before earthworms in the other soils (**Fig. S4.11**). Studies have found a correlation between increasing length of drought stress and proportion of *Ap. caliginosa* in aestivation (McDaniel *et al.*, 2013a). Similarly, Díaz Cosín *et al.* (2006) found that while some *H. elisae* maintained an active state after 2 weeks at 10 wt% moisture, 4 months at 10 % moisture resulted in 100 % aestivating. Therefore, it is possible that negative effects would occur with increasing duration of drought-exposure for earthworms in the sand. In this experiment, soils were gradually dried rather than kept at constant water levels, therefore future work is needed to investigate whether *Al. chlorotica* respond with aestivation at higher/more available water contents if their duration of exposure is increased.

Moreover, aestivation chamber quality also varied with soil type. When present in sandy soils, chambers were fragile and poorly sealed, whereas those in the clay-rich soils were more durable and remained intact during destructive sampling. Similarly, Bayley *et al.* (2010) found that a sandy loam soil (35 % coarse sand, 45 % fine sand, 9.4 % silt, 8.9 % clay) contained aestivation chambers which broke apart on handling, while those in a slightly less sandy soil (38.4 % coarse sand, 23.6 % fine sand, 22.3 % silt, 13 % clay) were more suitable for handling and extraction. This suggests that chamber construction is constrained by soil texture and may thus influence the viability of aestivation. In general, soils with high sand contents (> 80 %) are considered unsuitable for earthworms and constrain their distribution and activity, likely due to the abrasive nature of sand particles (Booth *et al.*, 2000; El-Duweini and Ghabbour, 1965). In contrast, some studies suggest coarse sand can support earthworms and increase digestive abilities by aiding in the grinding of plant residues in the gut (Eisenhauer *et al.*, 2009) although, the effect of sand content was negative for *Octolasion tyrtaeum* (Savigny, 1826), which increased in abundance and biomass with decreasing sand content, while there was no effect on *Ap. caliginosa* (Eisenhauer *et al.*, 2009). Thus, soil suitability for inhabitation depends on both species-specific traits and textural properties, with clay soils generally more conducive to aestivation chamber construction but sandier soils permitting continued activity under short-term drought.

4.6 Conclusion

Overall, these results demonstrate that thresholds inducing aestivation are dependent both on the extent of drying but also the soil type. In general, earthworm activity and changes in mass were more similar across soils at equal water availabilities. In contrast, when responses were compared at equal gravimetric moisture contents, more of the variation could be explained by soil type. This suggests that the availability of water is a more important driver of earthworm behaviour than bulk water content. Although, soil type modulates responses as aestivation and mass loss occurred at lower pF values in sandier soils. *Al. chlorotica* lost as much as 45 % of their initial mass when exposed to dry conditions, and those that aestivated tended to be significantly smaller than those that remained active. However, after 24 hours in high moisture conditions, all earthworms previously exposed to drying conditions rehydrated and so changes in mass became positive relative to the start of the experiment. This rapid recovery demonstrates the effectiveness of aestivation and suggests that mass losses are transient and mostly reflect body water loss which can be regained on return to more favourable conditions. Despite this, some differences in mass between those exposed to the drying and constant optimal (control) conditions persisted after hydration suggesting differences in tissue mass, likely a result of suspended feeding and reduced growth of aestivating earthworms. In addition, aestivating earthworms with regressed sexual characters were observed which may indicate potentially longer-lasting detrimental impacts of exposure to drying conditions, particularly for their reproductive output. Overall, these results suggest that *Al. chlorotica* are incredibly resilient to desiccation but that their responses to drought are shaped by complex interactions between soil texture and water availability. These interactions determine not only their physiological responses and changes in mass but also the restrictions imposed on their movement, and their ability to construct aestivation chambers, which is notably more successful in soils with a higher clay content. Further work investigating thresholds that induce aestivation in other lumbricid species in a range of soil types to determine potential tolerance ranges under different environmental contexts will be useful for predicting how their activity may be limited by droughts expected with climate change.

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4.8 Supplementary materials

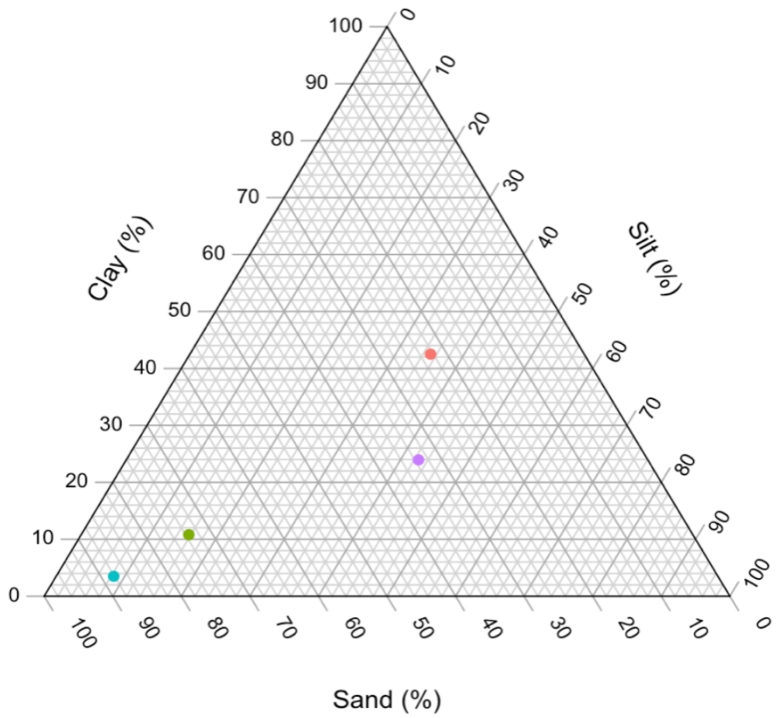

Figure S4.1. The relative proportion (%) of clay, silt and sand particles within the four soils: 2.1 (blue), 2.2 (green), 2.4 (purple) and 6S (red). Based on data of mean particle size distribution (The chemical and physical characteristics of standard soils, LUFA Speyer 2022).

Figure S4.2. Hyprop devices containing a saturated soil core placed on a balance, used to determine the water retention curves of each soil type.

HYPROP method:
Each soil core (250 ml) was packed with one of the four soil types, a nonwoven cloth was placed on one side along with a saturation plate before the core was gently placed into a tub filled with deionised water up to 5 mm below the sample rim and left overnight to fully saturate through capillary action until the surface was shiny. The tensio-shafts have a porous ceramic tip and a shaft which was filled with water which was degassed by vacuum to avoid air bubbles. The sensor unit was also filled with degassed water. Each tensio-shaft was gently screwed into the sensor unit and the pressure was monitored to ensure it was increasing but not exceeding 200 kPa to avoid damaging the pressure sensor. A silicone gasket was added to avoid soil entering the sensor unit and several function checks (speed of response, zero point etc.) were carried out to ensure the pressure was responding as intended. Two holes were made in each soil core using an auger and the sensor unit was inverted over the soil core so that the tensio-shafts could be carefully inserted into the holes making sure not to compress the soil. The soil core was upturned, saturation plate and cloth removed and then the soil sample was fixed to the sensor unit using clips and placed onto a balance. The balance was connected to a computer with the HYPROP software and measurements commenced. Recording was terminated when the soil sample had reached the air entry phase whereby the tension value drops abruptly to zero as air enters the ceramic tips. The soil core was removed from the sensor unit and oven dried at 105 °C for 24 hours to determine the dry weight of the soil sample.

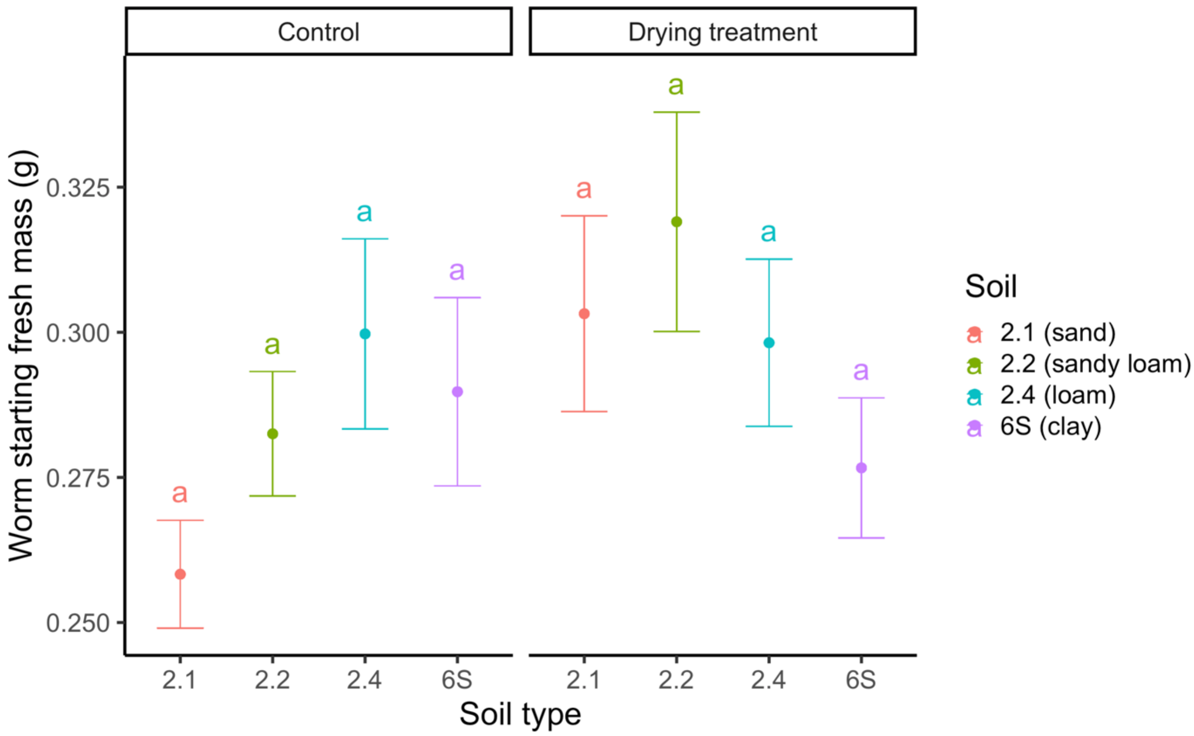

Figure S4.3. Mean initial fresh mass of *Al. chlorotica* (n = 25) when weighed prior to the start of the experiment for each of the four soil types, grouped according to treatment group for those that would be subjected to either constant control or drying conditions. Error bars show standard error. Treatments with the same letter do not differ to a statistically significant degree (p > 0.05).

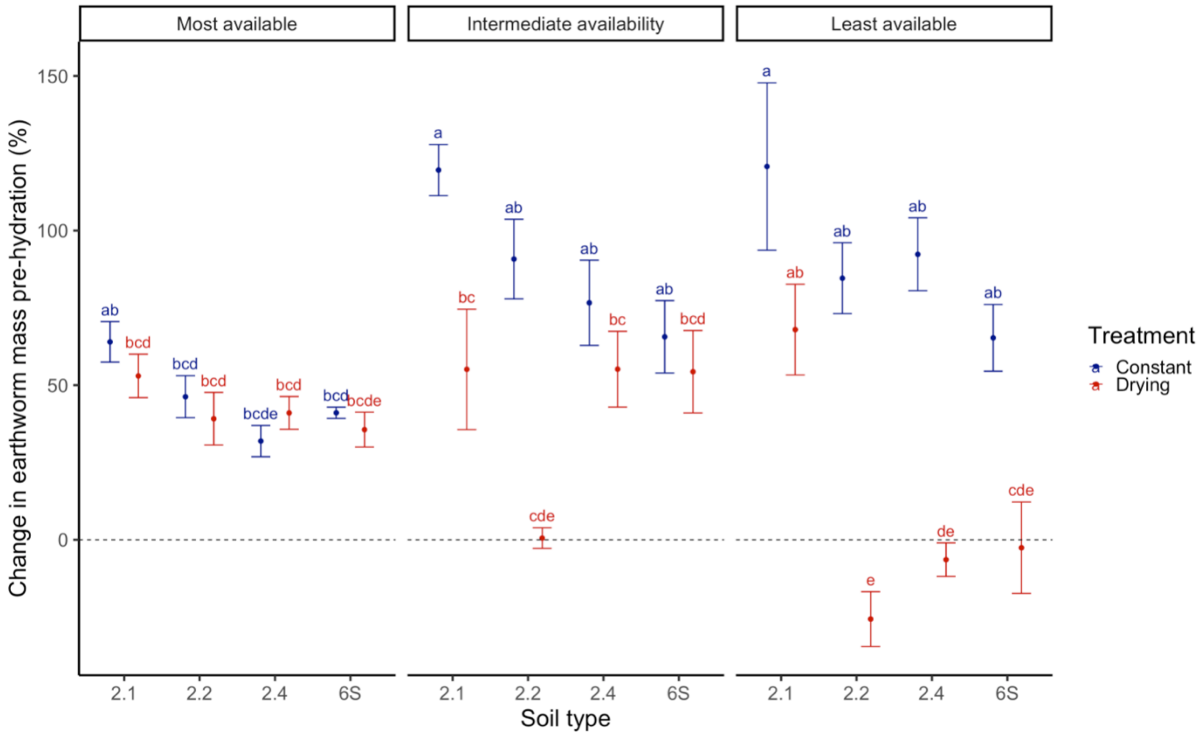

Figure S4.4. The mean change in earthworm mass (n = 5) relative to starting mass at each of the three water potentials (wet, medium and dry) for each soil type prior to hydration. Earthworms in the constant control conditions (blue) were sampled at the same time and those in the drying soils (red). Error bars show standard error. Treatments with different letters differ to a statistically significant degree ($p < 0.05$).

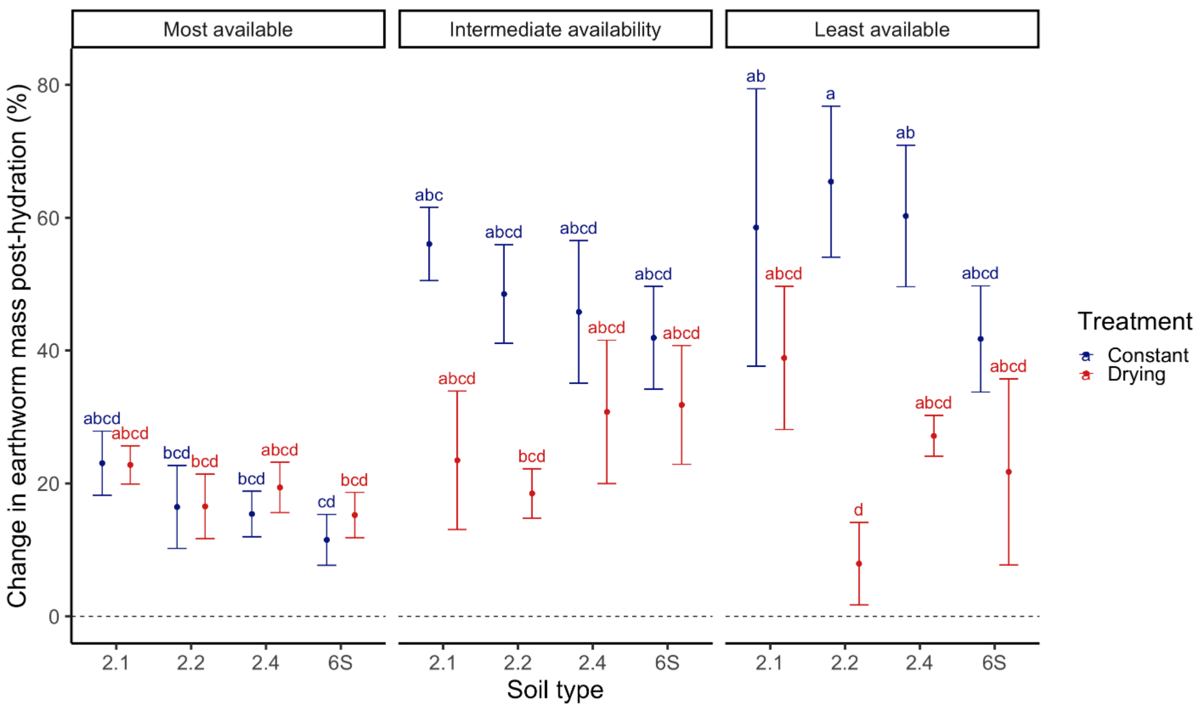

Figure S4.5. The mean change in earthworm mass (n = 5) relative to starting mass at each of the three water potentials (wet, medium and dry) for each soil type after 24 hours hydration. Blue = constant control conditions, red = drying soils. Error bars show standard error. Treatments with different letters differ to a statistically significant degree ($p < 0.05$).

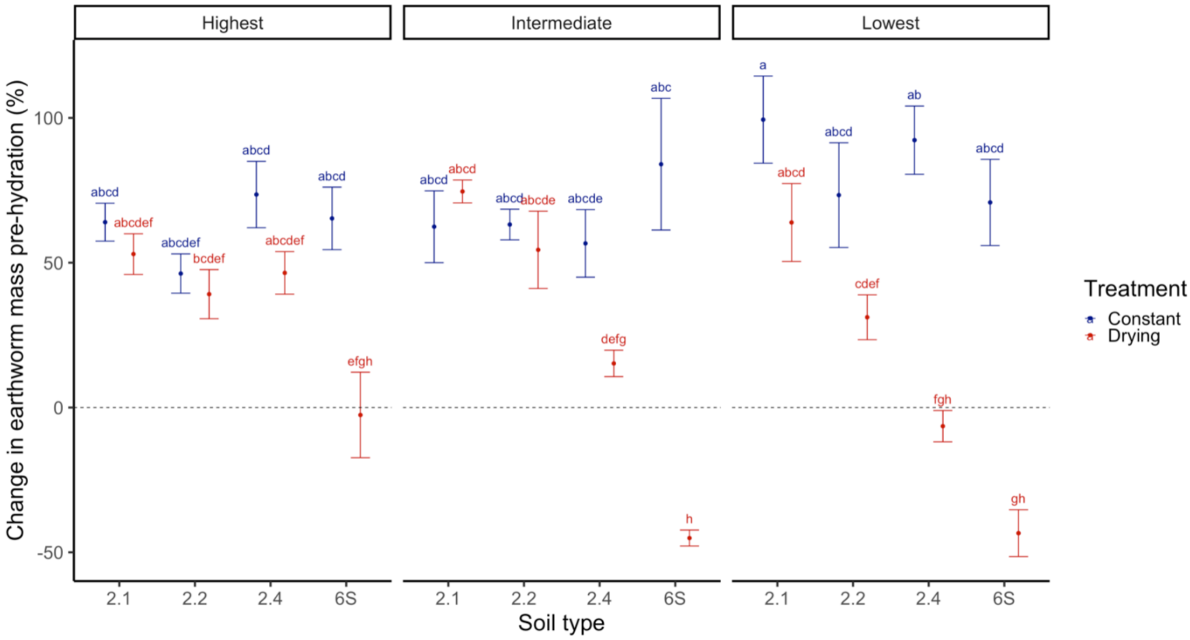

Figure S4.6. The mean change in earthworm mass (n = 5) relative to starting mass at each of the three gravimetric moisture sampling points (highest, intermediate and lowest water contents) for each soil type pre-hydration. Earthworms in the constant control conditions (blue) were sampled at the same time and those in the drying soils (red). Error bars show standard error. Treatments with different letters differ to a statistically significant degree ($p < 0.05$).

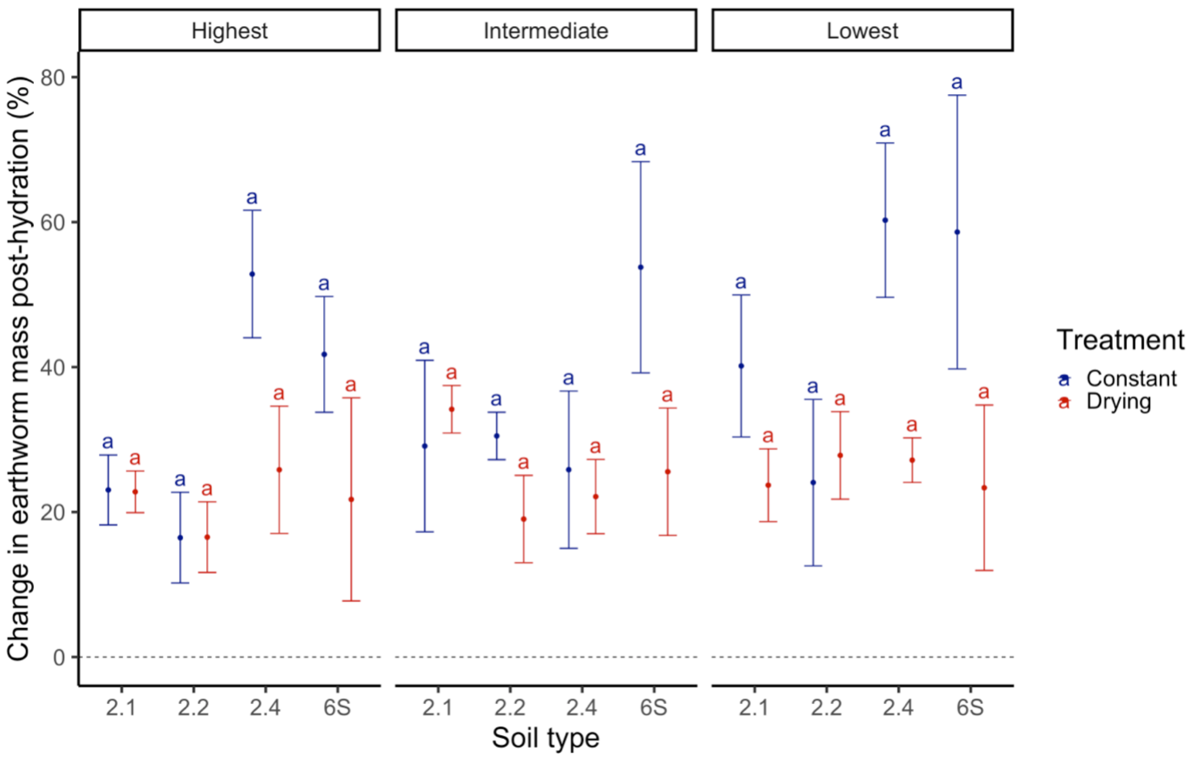

Figure S4.7. The mean change in earthworm mass (n = 5) relative to starting mass at each of the three gravimetric moisture sampling points (highest, intermediate and lowest water contents) for each soil type after 24 hours hydration. Earthworms in the constant control conditions (blue) were sampled at the same time and those in the drying soils (red). Error bars show standard error. Treatments with different letters differ to a statistically significant degree ($p < 0.05$).

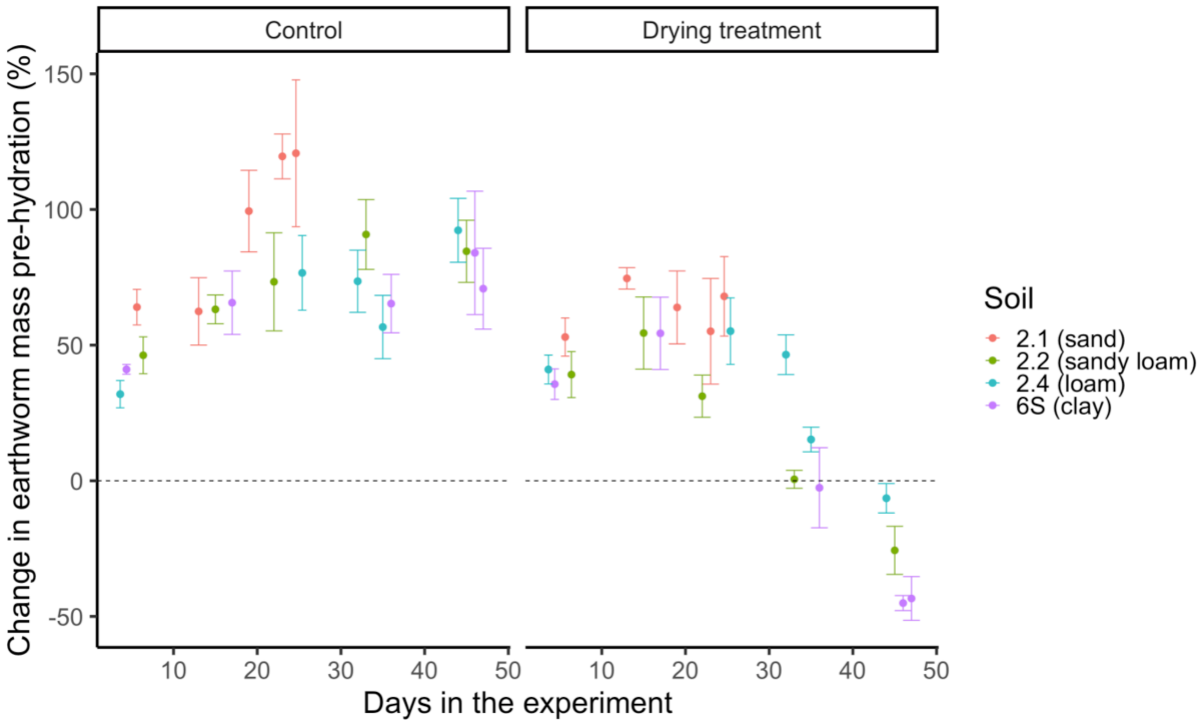

Figure S4.8. The mean change in earthworm mass (n = 5) from the start of the experiment to the point of destructive sampling (pre-hydration), for earthworms in the constant control (y = 0.735x + 55.836) and drying (y = -1.964x + 77.048) soils. Colours represent each soil type: red = 2.1 (sand), green = 2.2 (sandy loam), blue = 2.4 (loam) and purple = 6S (clay). Error bars show standard error. Dashed line represents no change in mass (y = 0 %).

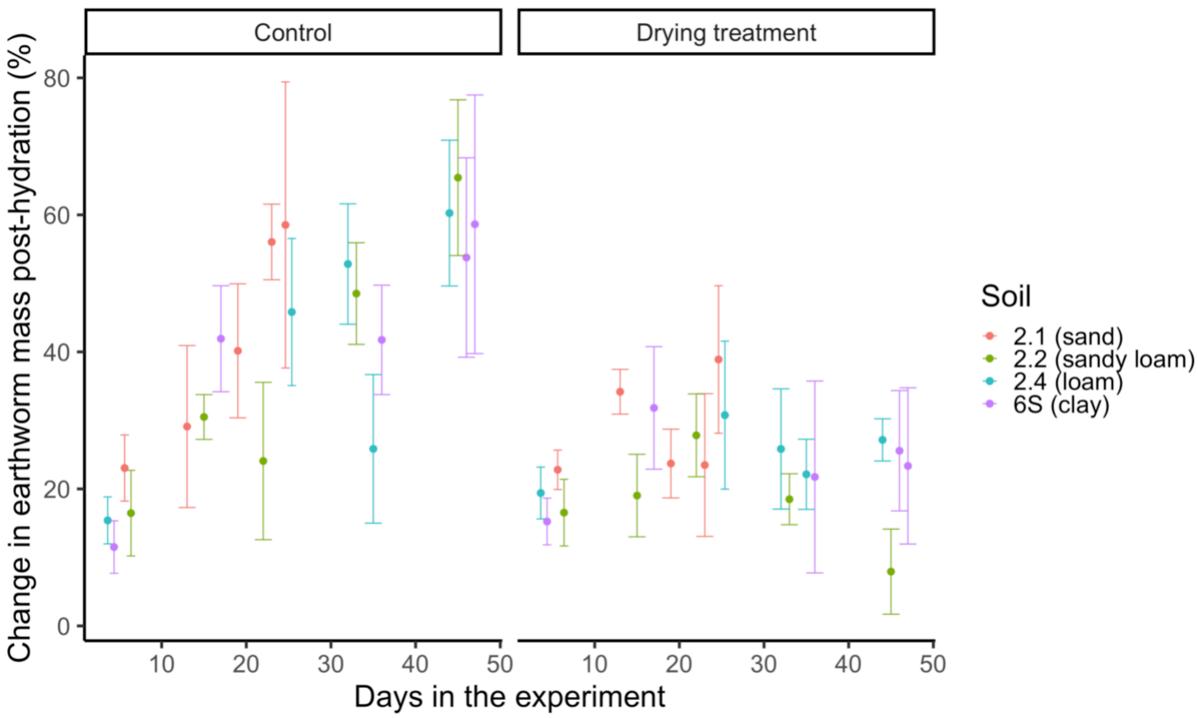

Figure S4.9. The mean change in earthworm mass (n = 5) from the start of the experiment to after 24 hours of hydration, for earthworms in the constant control (y = 0.939x + 16.647) and drying (y = -0.004x + 23.897) soils. Colours represent each soil type: red = 2.1 (sand), green = 2.2 (sandy loam), blue = 2.4 (loam) and purple = 6S (clay). Error bars show standard error.

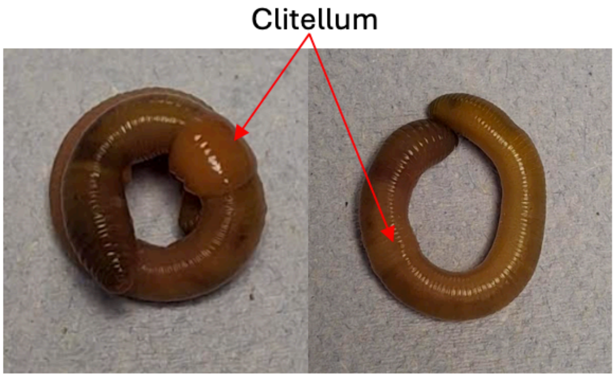

Figure S4.10. Adult *Al. chlorotica* with fully developed (left) and regressed clitellum (right).

Table S4.1. The day of the experiment soils reached each of the sampling points.

Soil	pF 1.59	pF 2.92	pF 4.05	19.7 wt%	15.55 wt%	12.39 wt%
2.1	6	23	25	6	13	19
2.2	6	33	45	6	15	22
2.4	4	25	44	32	35	44
6S	4	17	36	36	46	47

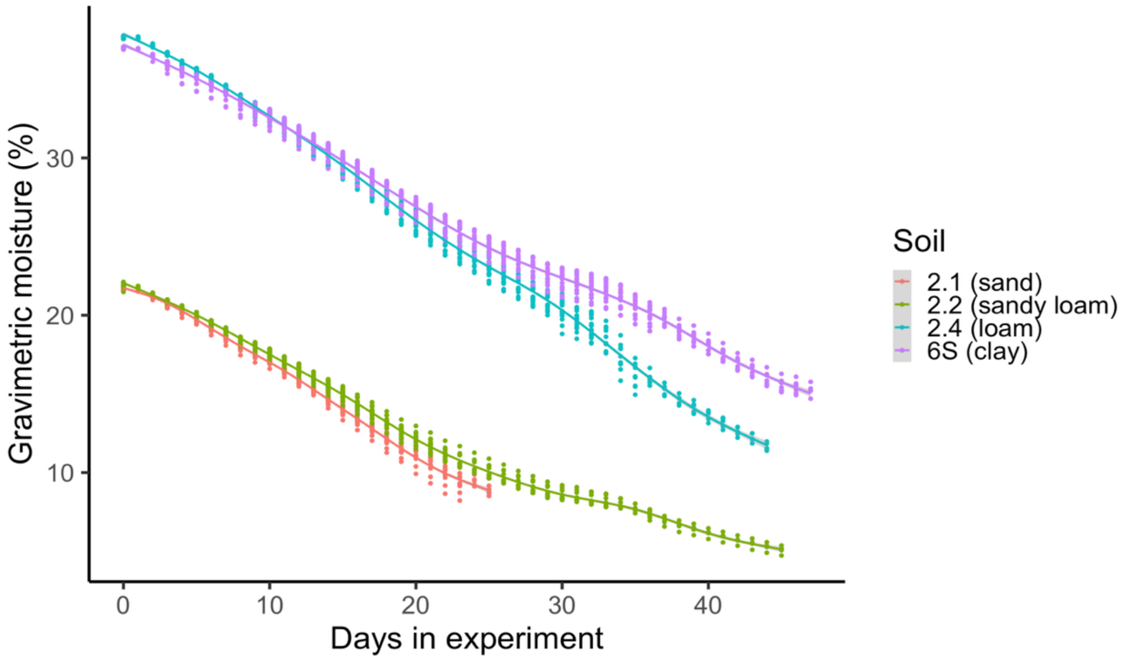

Figure S4.11. The change in the gravimetric moisture content (%) of each of the four soils over time in the drying conditions. Drying rates were as follows; y = -0.552x + 22.309 for 2.1, y = -0.422x + 21.646 for 2.2, y = -0.615x + 38.506 for 2.4 and y = -0.486x + 37.18 for 6S.

Chapter 5

Earthworm population dynamics and aestivation in response to changing soil abiotic conditions

5.1 Abstract

Earthworms are vital ecosystem engineers, contributing to the provision of key soil processes. Their diversity and activity are strongly influenced by soil moisture, making them vulnerable to the increased frequency and intensity of climatic extremes expected under climate change. Some species survive harsh conditions by entering aestivation, a state of low metabolic activity in which they remain until favourable conditions return. However, little is known about which species use this strategy and under what conditions it is induced. To investigate earthworm responses to changing soil abiotic factors, earthworm communities were sampled monthly for one year at two UK farms, recording their state as active or aestivating on collection. In addition, soil moisture measurements were taken and soil cores extracted to determine bulk density, pH and soil organic matter (SOM) content in the laboratory. The reproductive state of individual earthworms was recorded before their DNA was extracted from mucus by swabbing their epidermis. Earthworm populations at both farms showed similar trends with significant non-linear relationship between earthworm densities and soil moisture, peaking at around 40-50 vol% but declining at higher moistures. Aestivation was most common below moisture contents of 25-30 vol% but also occurred under saturated conditions (>45-50 vol%), suggesting that it is a general stress response to suboptimal conditions. During the driest months, communities were less diverse and dominated by four burrowing species (*Aporrectodea longa* (Ude, 1885), *Allolobophora chlorotica* (Savigny, 1826), *Aporrectodea rosea* (Savigny, 1826) *and Aporrectodea caliginosa* (Savigny, 1826)) that underwent aestivation, with litter-dwellers being mostly absent. Very few reproductively mature (clitellate) individuals were found, particularly in dry summer months, which could reflect adults regressing their sexual features during aestivation. SOM, pH, bulk density and temperature also influenced population dynamics, but these effects were often confounded by seasonal moisture stress. Overall, these results highlight soil moisture as the primary driver of earthworm aestivation and population structure. The rapid rebound in earthworm density and species richness observed following dry conditions, likely mostly due to migration from areas with more favourable conditions but also hatching of more drought-tolerant cocoons, indicates some resilience of earthworm populations. However, the extent to which populations can recover will likely depend on local environmental context and the frequency and severity of future climatic extremes.

5.2 Introduction

Earthworms are vital ecosystem engineers, capable of modifying the structure and function of the soil environment (Lavelle *et al.*, 1997; Cunha *et al.*, 2016; Schon *et al.*, 2017). Through their activities, earthworms provide essential ecosystem services including nutrient cycling, soil carbon storage and enhanced crop yields (Blouin *et al.,* 2013; Singh *et al.*, 2019). Traditionally, earthworms have been categorised into three ecological groups: epigeic (litter-dwellers), anecic (deep-burrowing, surface feeders) and endogeic (soil-dwelling, humus feeders), based on their morphology and life history (Bouché, 1972). Some of these traits have been linked to the services they provide (Marichal *et al.*, 2017). For instance, Van Groenigen *et al.* (2014) meta-analysis found the presence of earthworms enhanced crop yields, with increases of between 18 % for epigeic and 32 % for anecic species. Similarly, Huang *et al.* (2020) found anecic species had the greatest effect on litter decay, followed by epigeics, while endogeics had negligible effects. Moreover, they found that the presence of all three ecological groups was associated with greater litter decomposition than the presence of one or two groups alone (Huang *et al.*, 2020). Despite this, these three ecological groups were not intended to describe functional roles nor be discrete categories and many species exhibit traits associated with more than one category (Bottinelli *et al.*, 2020). More recently, Capowiez *et al.* (2024) examined the bioturbation behaviour of 50 earthworm taxa under standardised laboratory conditions and used this to develop 6 new functional groups (**Table 5.1**).

Table 5.1. Earthworm functional groups defined by Capowiez *et al.* (2024), with examples for some UK species.

Functional group	**Description**	**Species**
Intense tunneler	Very large and pigmented, surface feeding and casting, make extensive burrows, linked to anecics	*Aporrectodea nocturna* (Evans, 1946)
Burrower	Large and pigmented, surface feeding and casting, make a few true burrows, higher activity near surface, consume more litter than intense tunnelers, linked to epi-anecics	*Aporrectodea caliginosa* (Savigny, 1826), *Aporrectodea longa* (Ude, 1885), *Lumbricus terrestris* (Linnaeus, 1758)
Shallow bioturbator	Non-pigmented small earthworms, low surface activity, make shallow galleries in the soil, linked to epi-endogeics	*Allolobophora chlorotica* (Savigny, 1826)
Deep bioturbator	Non-pigmented large or average, low surface activity, make galleries deep in the soil, consume no litter, linked to hypo-endogeics	*Aporrectodea icterica* (Savigny, 1826), *Octolasion cyaneum* (Savigny, 1826)
Litter dweller	Pigmented small earthworms, very high surface activity, make few shallow galleries, linked to epigeics	*Eiseniella tetraedra* (Savigny, 1826), *Lumbricus castaneus* (Savigny, 1826), *Lumbricus rubellus* (Hoffmeister, 1845), *Satchellius mammalis* (Savigny, 1826)
Intermediate	Species without marked characteristics, both pigmented and non-pigmented	*Aporrectodea cupulifera* (Tetry, 1937), *Aporrectodea rosea* (Savigny, 1826)

Soil hydromechanical resistance, a product of compaction and moisture, strongly influences the burrowing ability and activity of earthworms (Kretzschmar, 1991; Ruiz *et al.*, 2021). Seasonal dynamics, particularly soil moisture, therefore, play a central role in shaping hydromechanical properties and act as the main constraint on earthworm occurrence and activity (Ruiz *et al.*, 2021). These constraints are expected to intensify under climate change, as the increased frequency and severity of extreme climatic events such as droughts (Seneviratne *et al.*, 2021) pose a threat to earthworm populations that depend on soil moisture for cutaneous respiration (Lee, 1985). Some earthworms have developed behavioural strategies to survive periods of drought, most notably aestivation (Lee, 1985).

During aestivation, earthworms coil into a knot within a soil chamber to reduce their exposed surface area (McDaniel *et al.*, 2013a; Bayley *et al.*, 2010), repress their metabolic activity and digestive processes (Tilikj *et al.*, 2023) and remain in this protective state to minimise water loss until more favourable conditions return (McDaniel *et al.*, 2013a; Friis *et al.*, 2004). However, the response of species to drying conditions is largely determined by the niche they occupy, with aestivation typically most observed in endogeic earthworms. Some deep-burrowing species may instead migrate vertically to deeper depths to avoid desiccation (Potvin and Lilleskov, 2017), while litter-dwelling species are more exposed at the soil surface and have limited ability to escape drought by burrowing (Eggleton *et al.*, 2009). There is evidence, although limited, to suggest some epigeic species are capable of some form of aestivation, though not necessarily within sealed chambers (Jiménez *et al.*, 2000). Such behavioural differences in response to moisture limitation and the physical resistance of soils will likely alter species presence and activity patterns, leading to shifts in earthworm community compositions under the harsher and more prolonged conditions expected with climate change. In turn, models predict that climate change-driven declines in earthworm functional richness will affect the provision of ecosystem services (Fourcade and Vercauteren, 2022). For instance, soil processes such as nitrogen mineralisation and carbon cycling will likely be influenced by changes in seasonal activity of earthworms (e.g. the onset of aestivation) and their interactions with other soil organisms (Potvin and Lillieskov, 2017; Walsh *et al.*, 2019).

To predict and mitigate the impacts of climate change on earthworm communities and their ecosystem services, it is crucial that we better understand the soil conditions that support or hinder earthworm activity. Accordingly, the need for more time series data assessing the response of earthworm populations to environmental conditions to establish wider trends has been highlighted (Eggleton *et al.*, 2009). As agricultural land covers ~72 % of total UK land area (FAOSTAT, 2023), it is important to study, particularly as it is also often degraded due to intensive management, which reduces its resilience to climate perturbations (Siebert *et al.*, 2019).

Traditional methods of earthworm identification involve using keys to identify morphological features. This can be problematic as some species have variable morphology and so are difficult to differentiate visually (Perez-Losada *et al.*, 2009), especially when preserved as they lose their pigmentation. In addition, identification using keys relies on the presence of sexual organs such as the clitellum to differentiate between earthworm species (Christyraj *et al.*, 2025; Richard *et al.*, 2010) and so juveniles cannot be identified. Consequently, many studies have neglected the juvenile component of earthworm communities, despite it often comprising the largest part and being key to future population replenishment (Torppa *et al.*, 2024; Eggleton *et al.*, 2009). Molecular techniques such as DNA barcoding (which involves the identification of species from DNA samples, often using high-throughput sequencing) largely resolve these problems due to their ability to detect juveniles and cryptic species, providing a more comprehensive understanding of earthworm community dynamics (Liu *et al.*, 2025). For instance, Torppa *et al.* (2024) found almost twice as many species through DNA barcoding compared to morphological identification.

This study investigates earthworm community responses to seasonal changes in soil properties (moisture, soil organic matter (SOM), bulk density, pH and temperature) over a one-year period in two UK agricultural sites. DNA metabarcoding was used to identify earthworm species, but rather than extracting DNA from tissue, the skin of individual earthworms was swabbed, enabling them to remain alive. Non-destructive skin swabbing has been effectively used for DNA extraction in amphibians such as great crested newts (*Triturus cristatus*) (Ward *et al.*, 2019), European tree frogs (*Hyla arborea*), alpine newts (*Ichthyosaura alpestris*) (Prunier *et al.*, 2012) and some small-bodied fish (*Danio rerio* and *Gasterosteus aculeatus)* (Tilley *et al.*, 2021) but is a novel approach for earthworm identification.

It was predicted that decreasing soil moisture would negatively affect earthworm density and species richness, and alter community composition, with drier months likely to comprise fewer litter-dwelling and deep burrowing species. Similarly, the percentage of earthworms in aestivation was predicted to increase with decreasing soil moisture. Additionally, the percentage of clitellate earthworms was expected to decrease with decreasing soil moisture, reflecting suboptimal conditions for growth and development. Moreover, adults and juveniles were predicted to differ in their propensity to aestivate, specifically, juveniles were expected to be more commonly found aestivating due to their smaller size and higher surface-area-to-volume ratio, making them more vulnerable to desiccation. Given that neutral soil pH and higher SOM are generally associated with higher earthworm abundance and biomass (Johnston, 2019; Hoeffner *et al.*, 2021), earthworm densities, species richness and the percentage of clitellate earthworms were expected to increase with increasing SOM, a key food source for earthworms and to be highest at more neutral pH values. In contrast, the density, species richness and percentages of adults and active earthworms were predicted to decrease with increasing soil bulk density and temperature due to the increased mechanical constraints imposed on movement in compact soil and the negative relationship expected between soil temperature and moisture respectively.

5.3 Methods

5.3.1 Study site characterisation, earthworm collection and soil analyses

Sampling took place every month from January to December 2023 in fields at two UK farms. Warren paddock at Spen farm, Tadcaster (53°52'25.9"N, 1°19'33.4"W) had been in pasture since 2012 before being converted back to arable cropping in August 2023 (**Fig. S5.1**). The soil is well-drained, loamy and calcareous (Holden *et al.*, 2019). The mean annual temperature range is 5.99 °C (min) to 13.97 °C (max), with mean annual precipitation of 619.63 mm (MET Office, Church Fenton weather station data for 1991-2020, <10 km from the site). Long jump field at Whirlow Hall farm, Sheffield (53°20'51.5"N, 1°31'59.8"W) is in permanent pasture and rotationally grazed by both cattle and sheep (**Fig. S5.1**). Hand-texturing (following method modified from Thien, 1979) revealed that the soil is also loamy, but has a higher stone content and less clay than Warren paddock. The mean annual temperature range is 6.92 °C (min) to 13.71 °C (max), with mean annual precipitation of 831.55 mm (MET office, Sheffield weather station data for 1991-2020, <5 km from the site).

In each field, 6 soil pits (~18 x 18 cm wide at the surface and ~20 cm deep) were dug in a W-pattern, approximately 15 m apart, in the same section of the field every month for a year. From August onwards sampling in Warren paddock was moved to the field margin (~1-

metre width) on request of the farmer to avoid disturbance as the field had been sown with oilseed rape. Consequently, sampling in a W-pattern was no longer possible so was instead conducted in the margins parallel with the field, maintaining 15 m between pits. Soil and surface plant matter were extracted from each pit, placed onto plastic trays and hand-sorted for approximately 30 minutes to remove all earthworms. Vermifuges were not used for extraction (e.g. mustard suspension) in order to observe the activity state of earthworms on extraction. Earthworms were separated into different 750 ml plastic containers with perforated lids ('Nationwide Paper' brand rectangular reusable plastic food containers with lids, purchased from Amazon) depending on the pit they were from and whether they were found active (outstretched and moving in the soil) or aestivating (curled into a knot, often within a soil chamber). Earthworms were transferred to a controlled temperature room and kept in field soil at 15 ± 1 °C and complete darkness until required for identification.

Four replicate volumetric soil moisture readings were taken both at the soil surface (0-5 cm) and base of each pit (20-25 cm depth) using a moisture probe (ML3 ThetaProbe Soil Moisture Sensor, Delta-T Devices). Three soil cores (100 cm^3) were taken at the soil surface (0-7 cm) (using a Royal Eijkelkamp corer) before being transferred to the laboratory where they were oven dried at 105 °C until their mass had stabilised. Dry masses were used to calculate soil bulk density on a g/cm^3 basis. Loss on ignition as a proxy for soil organic matter (SOM) was determined by placing soil into crucibles of known mass, burning at 400 °C in a muffle furnace for 4 hours and then calculating the percentage decrease in mass (as in Jensen *et al.*, 2018, but with reduced temperature to minimise structural water loss). Soil pH values were measured for three replicate soil samples per month on a suspension of 10 g air dried soil in 25 ml deionised distilled water (DIW), mixed in an end over end shaker working at 30 rpm for 15 minutes (The Analysis of Agricultural Materials – Ministry of Agriculture, Fisheries and Food) using a SciQuip 930 Precision pH/Ion meter calibrated with standards of pH 10, 7 and 4. Daily mean soil temperatures were extracted for each sampling day at Spen farm from Stanley *et al.* (2025) using measurements recorded by TDT sensors both at 5 cm and 20-25 cm depths. Equivalent data was not available for Whirlow Hall farm. To allow inference of water potential from the volumetric moisture content, HYPROP 2 instruments (UMS, 2015) which use tensiometers to measure changes in the mass and water potential of saturated soil samples were used to generate a water retention curve for soil from each site (**Fig. S5.2**, further details in supplementary material)

5.3.2 Identification of earthworm species through DNA metabarcoding

Individual earthworms were rinsed with DIW to remove adhering soil particles before being placed into individual containers. The ecological group of each earthworm was determined (as epigeic, endogeic or anecic) in addition to their species identity when evident morphologically (according to the Sherlock key, 2018), and they were classified as either clitellate (fully formed clitellum so sexually mature) or aclitellate (without a clitellum - sexually immature, including those both with and without development of a tubercula puberatis, the glandular thickening of the clitellum). Details of the full metabarcoding procedure are described in the supplementary materials. Briefly, the skin of each earthworm was swabbed with a sterile cotton swab. DNA extraction was carried out using ammonium acetate precipitation (as in Nicholls *et al.*, 2000; Richardson *et al.*, 2001, with some modifications described in the supplementary material). Swabs were placed into 1.5 ml Eppendorf tubes containing 300 µl Digsol with added sodium dodecylsulfate (20 % SDS) and 10 µl proteinase K, sealed and incubated in a rotating oven at 55 °C for one hour to lyse the cells. Swabs were then removed from tubes using forceps (cleaned with 10 % bleach between samples) and 300 µl of ammonium acetate was added to precipitate the DNA. Centrifugation was performed to separate the supernatant in addition to two washes and further centrifugation with ethanol. The ethanol was discarded and the tubes were left until any remaining ethanol had evaporated. To elute the DNA, 25 µl of LowTE buffer was added and the tubes were left overnight at 4 °C before being placed into a freezer at -20 °C for longer-term storage.

A primer pair, EwB (forward) and EwH2 (reverse) (**Table 5.2**), was designed to target highly conserved regions of the 16S region of mitochondrial DNA flanked by variable regions, producing an amplicon of ~226 bp. Primer EwB (Biernert *et al.*, 2012) was modified to generate 24 primers, each containing a unique 10 bp barcode (**Table. S5.1**), allowing DNA samples to be pooled into groups of 24. Primer EwH2, positioned between previously described primers EwE and EwF (Biernert *et al.*, 2012, **Fig. 5.1**), was designed based on a consensus sequence obtained from aligning the 16S sequences of UK earthworm species available in the NCBI database using the program Geneious Prime (version 2022.2.2). Primer pair specificity was verified in silico through the Primer-BLAST feature (NCBI, 2022), confirming that the primers amplify segmented worms, predominantly earthworms, but also enchytraeids and marine polychaetes. Initial PCRs (melting temperature (Tm) 52 °C, 40 cycles) using DNA from skin swabs of 10 earthworms of known species identity produced bands of the expected size, though some double bands were observed. PCR program optimisation involved running a PCR with a gradient of annealing temperatures which revealed that clear single bands were reliably obtained at 56 °C and above. Primer specificity was further tested by PCR using DNA from non-target species (insect, bird, amphibian and mammal including human), a positive control (earthworm) and negative control (water). Gel electrophoresis revealed clear amplification of earthworm DNA but also some degree of amplification of other invertebrate species: *Harmonia axyridis* (ladybird, Pallas, 1773), *Platycheirus albimanus* (hoverfly, Fabricius, 1781), butterfly, *Longitarsus rubiginosus* (flea beetle, Foudras, 1860), *Philaenus spumarius* (meadow froghopper, Linnaeus, 1758) and *Empis livida* (dance fly, Linnaeus, 1758). These species, however, are unlikely to be amplified from DNA extracted from earthworm skin swabs. Sanger sequencing of the 10 initial earthworm samples confirmed correct species identity, supporting the use of this primer pair for downstream metabarcoding.

For PCR1, a final volume of 20 µl was made by adding 4 µl of extracted DNA (or ddH_2O for negative controls) to each well in a 96 well plate along with 16 µl master mix (2 µl ddH_2O, 2 µl Ew_H2 primer, 2 µl EW_B primer and 10 µl myTaq). Plates were run in an Eppendorf PCR machine with the following program: 15 minutes at 95 °C then 35 cycles of 30 seconds at 94 °C, 90 seconds at 56 °C and 60 seconds at 72 °C, followed by 10 minutes at 72 °C.

Table 5.2. The 5' to 3' sequence and size (base pairs) of primers EwB (untagged) and EwH2.

Primer	5' to 3' sequence	Size (bp)
EwB (forward)	CAAGAAGACCCTATAGAGCTT	21
EwH2 (reverse)	CCCTAAGCCAACATCGAGGT	20

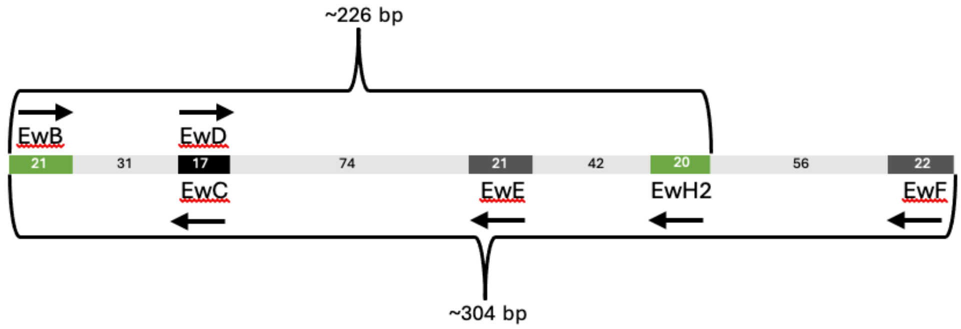

Figure 5.1. The amplicon produced by primers EwB to EwH2 and its position relative to the other 16S primers described in Biernert *et al.* (2012).

PCR1 products were visualised on a 1 % agarose gel and the presence of clear bands of expected fragment size indicated successful amplification. The concentration of DNA in each sample, evaluated using fluorometry, was used to determine the volume required to form each pool so they were present in a relatively equal concentration. Pooled DNA was purified through a 1.5x bead clean (AMPure XP beads) to remove unwanted PCR products (e.g. primer dimer) before LowTE was added to elute the DNA. PCR2 was performed with 10 µl myTaq, 8 µl DNA, 1 µl ddH_2O and 1 µl Illumina primers to add unique identifying indexes to each pool of DNA. The PCR program ran for 15 minutes at 95 °C then 12 cycles of 10 seconds at 98 °C, 30 seconds at 65 °C, 30 seconds at 72 °C, followed by 72 °C for 5 minutes. An increase in amplicon size indicated successful attachment of the indexes when pre- and post-PCR2 DNA pools were run on a TapeStation (using high sensitivity buffers and tapes). DNA concentrations of each pool (quantified using fluorometry) along with the final volume wanted were used to determine the specific volume of each pool required to be combined into a final pool. A second 1.5 x bead clean was carried out on this final pool to remove any leftover reagents or primer dimer from PCR2.

In preparation for sequencing, the pooled DNA was diluted in replicated triplicates down to 1/100, 1/1,000 and 1/10,000. To a skirted plate, 2 µl of each of the 1/1,000 and 1/10,000 dilutions was added to each well along with 2 µl of the 6 standards from a KAPA kit in triplicate replicates as well as 3 no template controls (NTCs of sample dilution buffer). Eight µl of SYBR fast mix (6 µl SYBR fast with added primers and 2 µl of ddH_2O) was added to each of the sample and standard wells. The plate was run in a QuantStudio 12k flex machine and the values were used to determine the concentration of DNA in the pool. The required concentration (nM) was divided by the actual concentration of the DNA (nM) (reported by the qPCR) machine and multiplied by the volume wanted (µl) to determine the volume needed to make the next dilution. The remainder of the dilution was made up with a volume of LowTE determined by subtracting the volume of DNA needed from the total volume wanted to give the new dilution. The qPCR process was repeated until the concentration reached ~4nM at which point it was prepared for sequencing by the genomics laboratory team at the NERC Environmental Omics Facility and loaded into an Illumina iSeq 100 following the system guidelines.

5.3.3 Bioinformatics and data analysis

The Illumina iSEQ produced paired end reads (2x150-bp) which were passed through the DADA2 pipeline (DADA2, 2022) using Bessemer HPC for quality control to: merge reads, remove unknown bases (Ns), filter and trim sequences, and remove chimeras. The National Center for Biotechnology Information taxonomic nucleotide BLAST database (NCBI, 2024) was used to assign amplicon sequence variants (ASVs) to species and data were filtered through Taxonomizr (2024) to include only those with sequence matches above 90 % similarity. MEGAN 6 (Hudson *et al.*, 2016) and R (version 4.4.1, R Core Team, 2024) were used to generate an ASV table from the BLAST output. As some samples contained multiple ASVs, a random forest (RF) machine learning approach was carried out in R using the "randomForest" package to assign final species to samples. The RF model was trained with 500 decision trees using the ASV counts and a subset of samples for which the species was 'known' (based on morphological identification, **Fig. S5.3**). Rare species with few occurrences were included in the training data if 100 % of their ASVs were consistent. The trained model was then used to predict the identity of the remaining 'unknown' samples, while out-of-bag (OOB) predictions (made by trees that did not see the samples during training) were used to predict the 'known' training samples to avoid re-fitting the model. The overall out-of-bag estimate of error rate was 5.43 % and ~92.9 % of the samples had a RF prediction consistent with the BLAST output from the ASV sequences. Some species with lower representation in the model had higher error rates and so their RF predictions were not always consistent with the BLAST output based on the ASV sequences. Therefore, a final filtering step was run so that in samples when the RF prediction was inconsistent with the BLAST output, they were assigned the species consistent with the majority of their ASV counts. This enabled identification of an additional 2.9 % of the samples resulting in identification of 95.8 % of the total samples. The remaining 4.2 % of the samples were either not successfully sequenced (very low/no reads) or had no dominant species in their ASVs (too contaminated), and these were classified as 'Unidentified'. Earthworm ecological and functional groups were determined based on Bouché (1972) and Capowiez *et al.* (2024) classifications respectively. As Capowiez *et al.* (2024) did not specify the functional group of *L. festivus*, here it is estimated to be a litter dweller given its higher association with the epigeic category (66.1 % epigeic, 33.9 % anecic) based on morphological and ecological traits reported in Bottinelli *et al.* (2020). Of the 'Unidentified' samples, based on their morphology, ~76 % were anecic, ~18 % epigeic and ~6 % endogeic, reflecting disparities in the quantity of DNA extracted from earthworm species. For instance, endogeic species were observed to release coelomic fluid more frequently when under stress and so higher concentrations of DNA were extracted from these earthworms compared to anecic and epigeic species. However, this also likely meant that there was a higher chance of endogeic DNA being transferred to the skin of other earthworms when they were housed together. Despite rinsing earthworms with DIW prior to swabbing, this may not always have been sufficient to remove contamination, resulting in some samples with ASVs from multiple species.

All statistical analyses were carried out using R (version 4.4.1, R Core Team, 2024). For measurements of soil properties including soil moisture (at 0-5 and 20-25 cm depths), SOM, pH, bulk density and temperature (at Spen farm only as equivalent data were unavailable for Whirlow Hall farm) t-tests were used to compare overall means at each farm. Linear models were also fitted separately for each farm with month as a fixed effect to assess temporal variation in soil properties within the year. At Whirlow Hall farm, February is missing data for soil moisture, SOM, pH and bulk density due to equipment unavailability. Both active and aestivating earthworms were included in calculations of earthworm abundance, richness and diversity. Earthworm abundances were given as densities, expressed as numbers of individuals per metre squared and means of 6 pits were calculated to get monthly averages. Both Shannon and Simpson's diversity indexes were calculated as monthly averages (of earthworms in the 6 pits, excluding the 'unidentified' samples) to give the most comprehensive measure of both the diversity and evenness of the earthworm communities. Statistical tests were performed after checking assumptions of normal distribution (Shapiro-Wilk test), linearity and constant variance through diagnostic plots. Where assumptions were not met and could not be resolved with data transformations, non-parametric tests were performed. T-tests were conducted to determine significant differences between farms in earthworm densities, species richness, species diversity (Shannnon's and Simpson's diversity), functional richness, functional diversity (Shannon and Simpson's diversity) and percentage of clitellate earthworms. Data for Shannon's and Simpson's species diversity at Spen farm did not meet the assumptions of ANOVA and so non-parametric Kruskal-Wallis tests were conducted instead. Post-hoc multiple comparison tests (Tukey for ANOVAs and Dunn's for Kruskal-Wallis) were performed to determine significant differences between treatments. The activity state of earthworms was assessed by calculating the percentage of earthworms found in aestivation in each soil pit. After adding a small scaling adjustment ((proportion aestivating*(n-1) +0.5)/n) to avoid zeros (Smithson and Verkuilen, 2006), beta regressions were carried out to look for statistical differences in the proportion of earthworms aestivating each month. A binomial GLM was conducted to assess the likelihood of aclitellate versus clitellate earthworms aestivating. PERMANOVAs were conducted on Bray-Curtis dissimilarity values calculated using the "vegan" package and NMDS were performed to assess differences in the composition of earthworm species and functional groups in each farm. Generalised additive mixed models (GAMMs, using the 'mgcv' package) with a negative binomial family were used to determine significant relationships between abiotic soil parameters (soil moisture at 0-5 cm, pH, SOM, bulk density, with the addition of temperature (5 cm) for Spen farm) and earthworm densities, species richness, percent of clitellate earthworms and densities of clitellate and aclitellate earthworms. The same models were applied but with a beta regression family (logit link) to assess significant relationships between abiotic soil parameters and the proportion of and aestivating (adjusted to account for zero values) earthworms. Due to the strong positive correlation between soil moisture measured at 0-5 and 20-25 cm (**Fig. S5.4**) at both Spen farm (Linear regression: $F = 128.8$, $df = 1,70$, $p < 0.001$, $R^2 = 0.64$) and Whirlow Hall farm (Linear regression: $F = 89.757$, $df = 1, 64$, $p < 0.001$, $R^2 = 0.58$), only the 0-5 readings were included in the GAMMs. Overall, there was no significant difference between the soil temperature at 5 cm depth compared to 20-25 cm depth (ANOVA: F = 0.919, df = 1, 48, p = 0.345, **Fig. S5.5**), so only 5 cm data were included in the GAMMs.

5.4 Results

5.4.1 Soil properties

Overall, Spen farm had a higher mean soil moisture content than Whirlow Hall farm at both 0-5 cm and 20-25 cm depths, although these differences were not statistically significant (**Table 5.3**). Spen farm had a significantly lower mean organic matter content, a significantly higher mean pH and a significantly higher mean soil bulk density compared to Whirlow Hall farm (**Table 5.3**).

Table 5.3. Mean and standard error values of soil properties averaged over the year for both Spen and Whirlow Hall farm. T-tests were conducted to determine significant differences between farms (indicated by asterisks, ns = not significant), with degrees of freedom, t-values and p-values given for each comparison.

Soil properties	Spen	Whirlow Hall	df	t-value	p-value
Moisture (vol%, 0-5)	38.8 ± 3.5	38.5 ± 3.8	10	0.225	0.826 (ns)
Moisture (vol%, 20-25)	28.3 ± 2.3	26.5 ± 1.9	10	2.118	0.06 (ns)
Organic matter (%)	8.2 ± 0.1	9.5 ± 0.1	10	-7.521	p < 0.001 (***)
pH	6.9 ± 0.04	5.6 ±0.03	10	26.948	p < 0.001 (***)
Bulk density (g/cm^3)	1.1 ± 0.03	0.9 ± 0.02	10	9.787	p < 0.001 (***)

Soil moisture showed similar significant variation across months at both Spen farm and Whirlow Hall farm (**Table 5.4, Fig. 5.2**). At Spen farm, soil moisture in the top 5 cm was significantly higher in March and October-December compared with all other months, while May and June were significantly drier (Tukey, $p < 0.05$). At 20-25 cm, soil moisture was significantly higher in October-December compared to all months and lower in May and June compared to all months except February (Tukey, $p < 0.05$). Similarly, at Whirlow Hall farm, soil moisture at 0-5 cm was significantly higher in March-April and October-December than all other months and significantly lower in May (Tukey, $p < 0.05$). At 20-25 cm depth, May again had the lowest values, significantly lower than March-April and September-December (Tukey, $p < 0.05$). In contrast, SOM did not vary significantly across months at either Spen farm or Whirlow Hall farm (**Table 5.4, Fig. S5.6**). Soil pH varied significantly with month at Spen farm, being significantly lower in August than February-March and June-July (**Table 5.4, Fig. S5.7**). At Whirlow Hall farm, pH was also lowest in August but did not vary significantly across months (**Table 5.4, Fig. S5.7**). Soil bulk density showed significant monthly variation at both Spen farm and Whirlow Hall farm (**Table 5.4**, **Fig. S5.8**). November had the highest bulk density, significantly higher than April at Spen farm and May at Whirlow Hall farm (Tukey, $p < 0.05$). At Spen farm, soil temperature varied significantly with month (**Table 5.4, Fig. S5.5)**. As expected, the lowest soil temperatures (<~8 °C) occurred in late autumn and winter, while the highest soil temperatures (>~15 °C) were recorded in late spring and summer. Correlations between soil parameters are given in **Table S5.2.**

Table 5.4. ANOVA results of linear models assessing differences in soil properties across months at Spen and Whirlow Hall farm. Linear models were fitted separately for each farm with month as the main effect. Degrees of freedom (df), F-statistics and p-values are reported for each soil property and farm. Significant differences across months are indicated by asterisks, ns indicates not significant. NA indicates that the model could not be fitted due to insufficient data.

Soil properties	Spen			Whirlow Hall		
	df	F	p-value	df	F	p-value
Moisture (0-5 cm)	11, 60	66.417	p < 0.001 (***)	10, 55	59.638	p < 0.001 (***)
Moisture (20-25 cm)	11, 60	31.982	p < 0.001 (***)	10, 55	10.557	p < 0.001 (***)
Organic matter	11, 24	1.89	0.093 (ns)	10, 22	1.349	0.266 (ns)
pH	11, 24	3.77	p < 0.01 (**)	10, 22	1.949	0.092 (ns)
Bulk density (g/cm^3)	11, 24	4.619	p < 0.001 (***)	10, 22	2.457	p < 0.05 (*)
Temperature (°C)	11, 48	1565.451	p < 0.001 (***)	NA	NA	NA

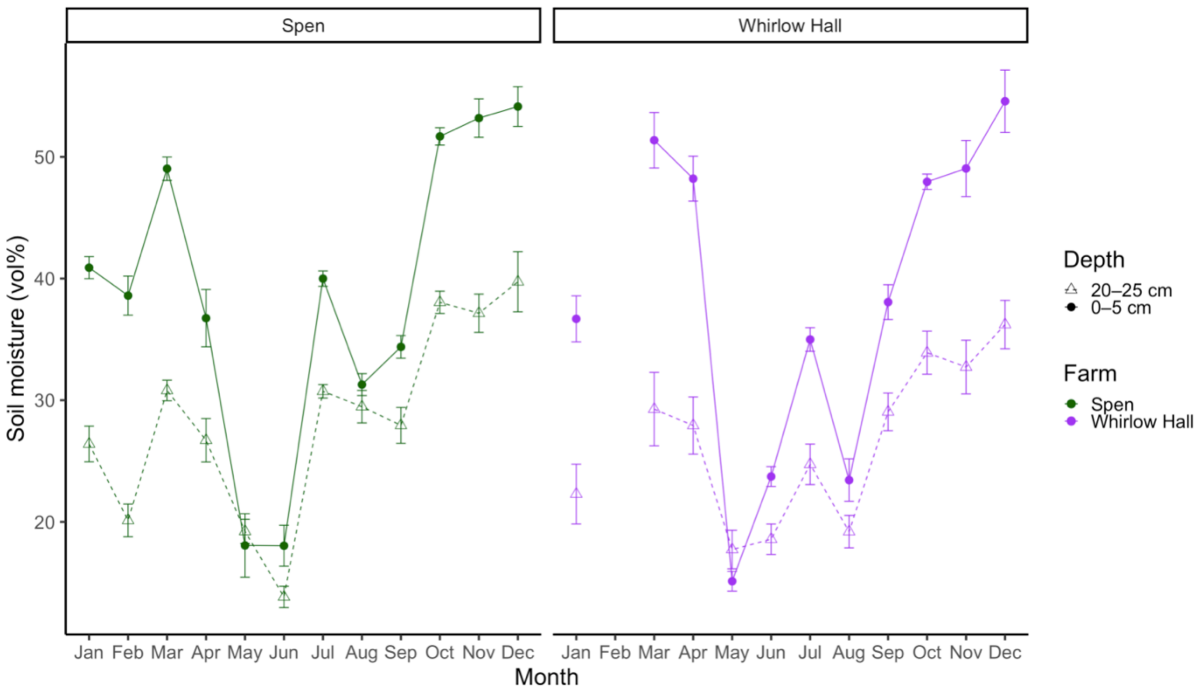

Figure 5.2. Mean soil moisture (vol%, n = 6) each month in both farms (green = Spen, purple = Whirlow Hall), measured at depths of 0-5 cm (filled circle, solid line) and 20-25 cm (hollow triangle, dashed line). Error bars show standard error.

5.4.2 Earthworm communities between farms and months

Over the whole year, Whirlow Hall farm had ~10 % more earthworms than Spen farm, with mean densities of 601 (± 67) and 545 (± 47) earthworms per m^2 respectively. However, this difference was not statistically significant (**Table 5.5**). Earthworm densities varied significantly with month, showing similar seasonal patterns at both Spen farm and Whirlow Hall farm (**Table 5.6, Fig. 5.3**). Earthworm densities were highest in March and April (>800/m^2) and lowest in June (<300/m^2) (**Fig. 5.3**). At Spen farm, the density of earthworms was significantly lower in June and November than March and April (Tukey, $p < 0.05$). At Whirlow Hall farm, May and June had a significantly lower earthworm density than February, March, April and October (Tukey, $p < 0.05$). March and April also had a significantly higher density of earthworms than August and December at Whirlow Hall farm ($p < 0.05$).

Table 5.5. Mean and standard error values of earthworm parameters averaged over the year for both Spen and Whirlow Hall farm. T-tests were conducted to determine significant differences between farms (indicated by asterisks, ns = not significant), with degrees of freedom, t-values and p-values given for each comparison.

Earthworm parameters	Spen	Whirlow Hall	df	t-value	p-value
Earthworm density (earthworms per m^2)	545 ± 47	601 ± 67	11	-1.503	0.161 (ns)
Species richness	4.43 ± 0.24	4.08 ± 0.16	11	1.59	0.14 (ns)
Shannon diversity (H)	1.24 ± 0.06	1.13 ± 0.04	11	1.628	0.132 (ns)
Simpson's diversity (1-D)	0.65 ± 0.02	0.61 ± 0.02	11	1.298	0.221 (ns)
Functional group richness	3.3 ± 0.1	2.8 ± 0.1	11	4.093	p < 0.01 (**)
Functional diversity (H)	1.01 ± 0.04	0.76 ± 0.03	11	5.382	p < 0.001 (***)
Functional diversity (1-D)	0.59 ± 0.02	0.46 ± 0.02	11	5.12	p < 0.001 (***)
Clitellate earthworms (%)	44.3 ± 2.8	22.8 ± 2.5	11	6.23	p < 0.001 (***)

Table 5.6. Differences in earthworm parameters across months at Spen and Whirlow Hall farm. Linear models (or Kruskal Wallis tests for Spen farm species diversity) were fitted separately for each farm with month as the main effect. Degrees of freedom (df), F-statistics and p-values are reported for each parameter and farm. Significant differences across months are indicated by asterisks, ns indicates not significant.

Earthworm parameters	Spen			Whirlow Hall		
	df	F	p-value	df	F	p-value
Earthworm density (earthworms per m^2)	11, 60	3.956	p < 0.001 (***)	11, 60	7.716	p < 0.001 (***)
Species richness	11, 60	4.265	p < 0.001 (***)	11, 60	1.795	0.075 (ns)
Shannon diversity (H)	11	37.611	p < 0.001 (***)	11, 60	1.636	0.112 (ns)
Simpsons diversity (1-D)	11	37.361	p < 0.001 (***)	11, 60	1.845	0.066 (ns)
Functional richness	11, 60	3.759	p < 0.001 (***)	11, 60	1.287	0.254 (ns)
Functional diversity (H)	11, 60	5.223	p < 0.001 (***)	11, 60	1.494	0.158 (ns)
Functional diversity (1-D)	11, 60	3.775	p < 0.001 (***)	11, 60	1.609	0.12 (ns)
Clitellate earthworms (%)	11, 60	3.29	p < 0.01 (**)	11, 60	2.674	p < 0.01 (**)

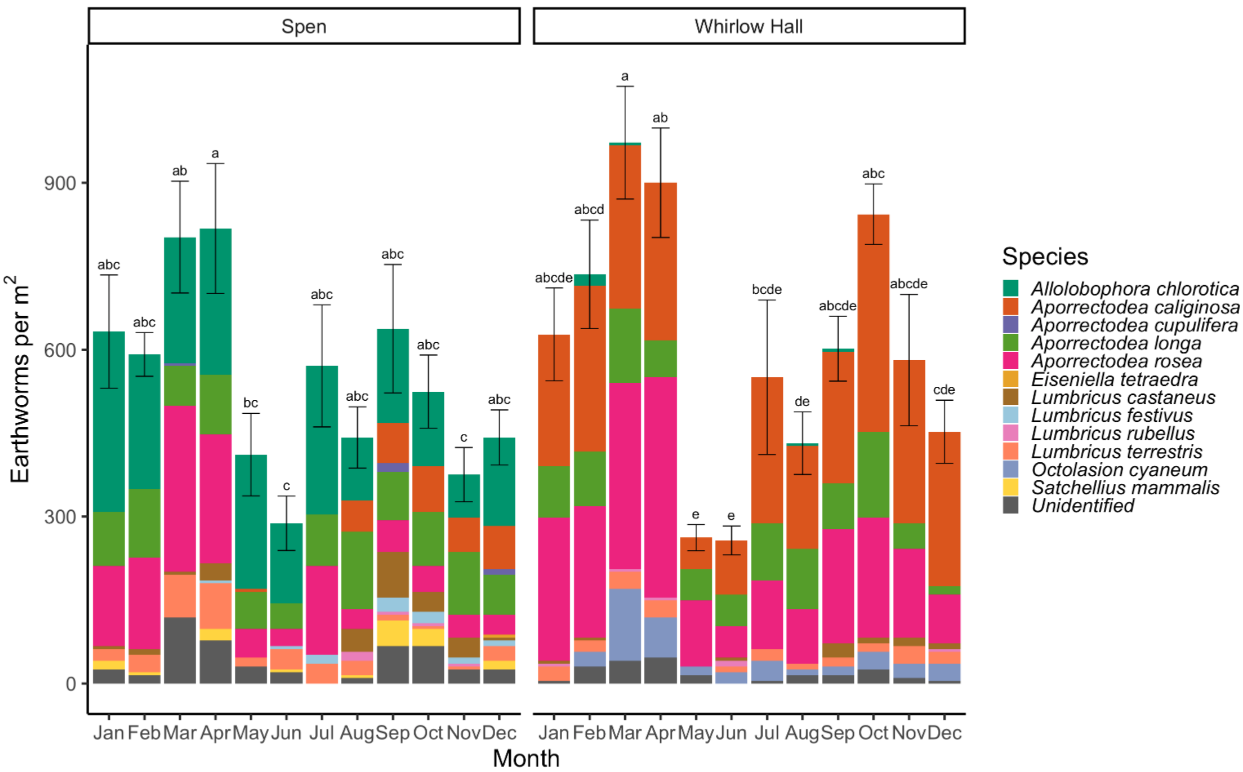

Figure 5.3. Mean density of earthworms (per m^2, n = 6) each month per farm. Colours represent different earthworm species. Error bars show standard error around total mean density. Bars with different letters represent farm-specific statistically significant months ($p < 0.05$).

As shown by the clear separation in the NMDS plot (**Fig. 5.4**), species composition differed significantly between Spen farm and Whirlow Hall farm (PERMANOVA: F = 77.989, df = 1, 143, R^2 = 0.355, $p < 0.001$). In total, eleven species were recorded at Spen farm, four of which were unique to that site (*Aporrectodea cupulifera*, *Eiseniella tetraedra*, *Lumbricus festivus*, and *Satchellius mammalis*). Eight species were recorded at Whirlow Hall farm, with one species (*Octolasion cyanenum*) unique to that site. The dominant species at Spen farm was *Al. chlorotica*, while *Ap. caliginosa* dominated at Whirlow Hall, each contributing over one third of the earthworms collected (**Fig. S5.9**). The second and third most abundant species at both sites were *Ap. rosea* and *Ap. longa* respectively. Seasonal variation in community composition was also evident (**Fig. 5.5**). At both farms, May and June had the most distinct species composition compared with all other months. At Spen farm, communities from January to April and July were similar to each other but differed from those recorded from August through to December (**Fig. 5.5**).

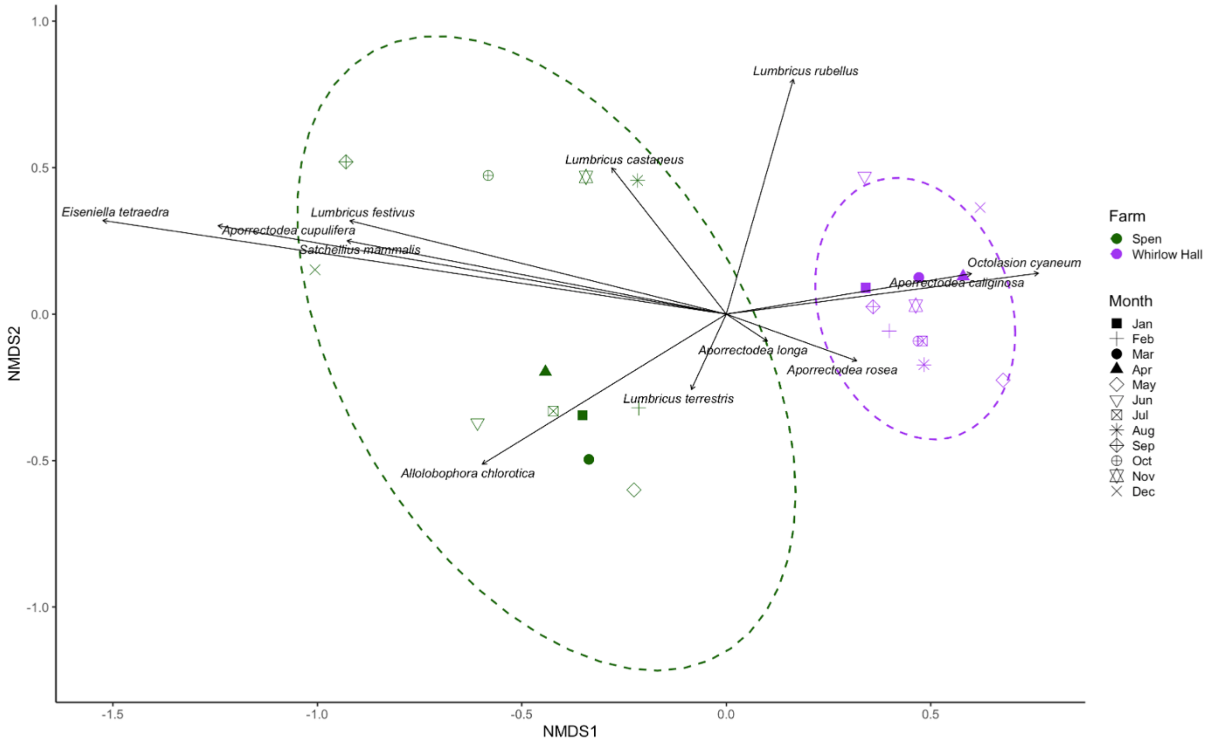

Figure 5.4. NMDS ordination of mean earthworm species composition for Spen (green) and Whirlow Hall (purple) farms. Shapes represent each month. Closer points are more similar in species compositions according to the Bray-Curtis dissimilarity. Arrow directions represent the gradient of increasing abundance for that species and arrow length is the strength of the relationship. Months near the origin/centre of the ordination have an average community composition. Ellipses around each farm are based on the covariance of points.

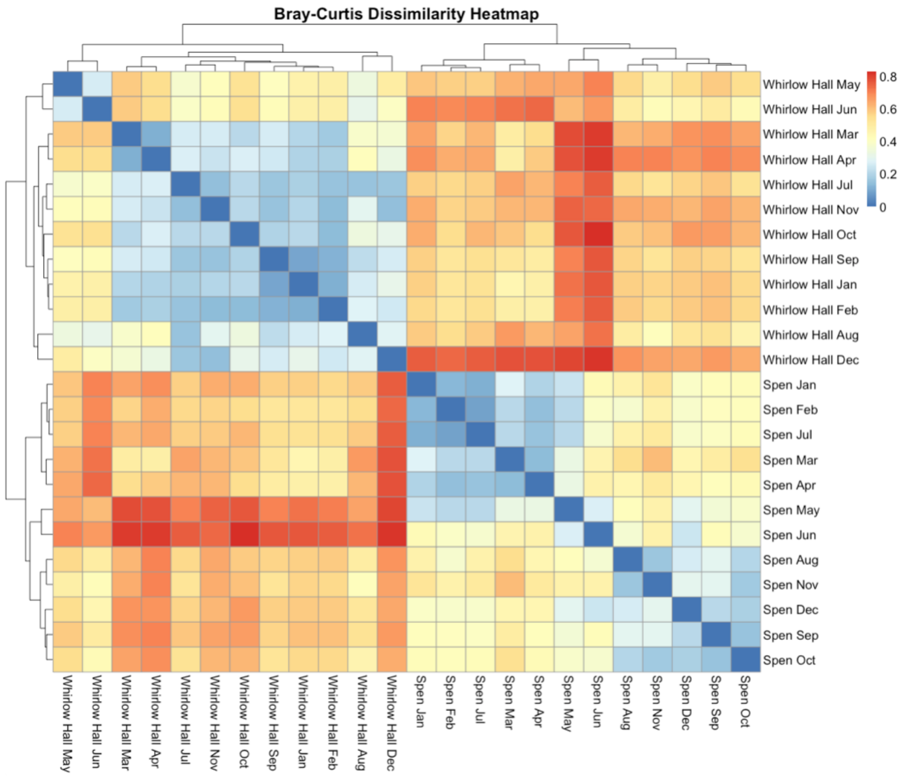

Figure 5.5. Heatmap showing pairwise Bray-Curtis dissimilarity values in the community composition of earthworms found each month at the two farms. A value of 0 (dark blue) indicates the months are identical while 1 (dark red) indicates they are completely unique. Dendrogram lines show how months cluster based on the similarity of their earthworm species composition.

5.4.3 Species richness and diversity

Compared with Whirlow Hall farm, Spen farm had a slightly higher overall mean species richness, Shannon diversity and Simpson's diversity, however t-tests revealed these differences were not significant (**Table 5.5**). At Spen farm, species richness and diversity varied significantly with month (**Table 5.6, Fig. 5.6**). In particular, species richness and diversity were significantly lower in May compared with September and October ($p < 0.05$). At Whirlow Hall farm, species richness and diversity were also lowest in May, but monthly differences were not statistically significant (**Table 5.6, Fig. 5.6**).

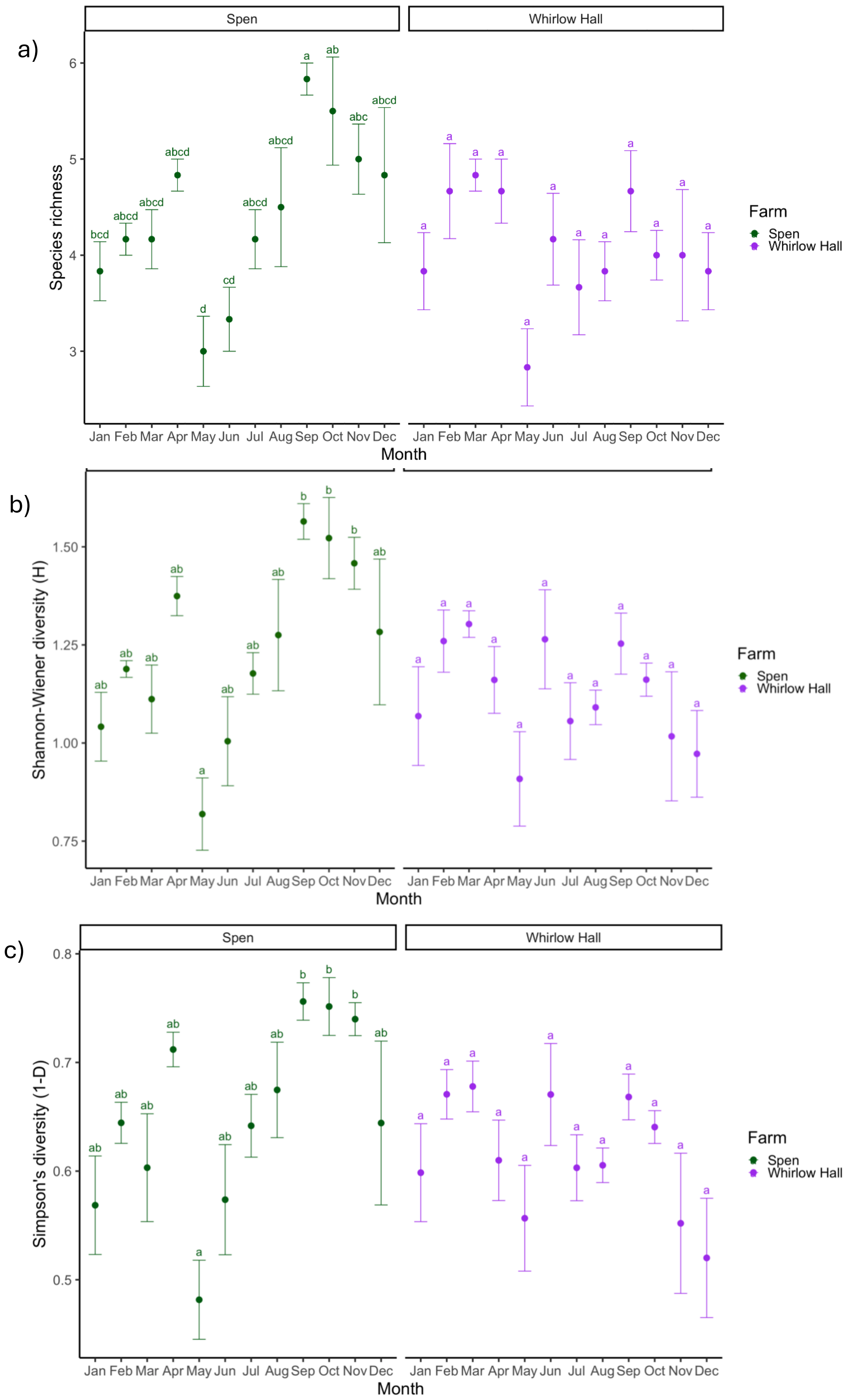

Figure 5.6. The mean (n = 6) a) species richness, b) Shannon-Wiener diversity index, and c) Simpson's diversity index values of earthworms every month at each farm. Error bars show standard error. Points with different letters represent farm-specific statistically significant months ($p < 0.05$).

5.4.4 Earthworm functional groups

Across the year, five functional groups were recorded at Whirlow Hall farm, while four groups were found at Spen farm, though these occurred more consistently throughout the year (**Fig. 5.7**). Earthworm functional group composition differed significantly between farms (PERMANOVA: F = 75.491, R^2 = 0.347, df = 1, 142, p < 0.001), mainly due to the absence of deep bioturbators at Spen farm and the lower abundance of shallow bioturbators and litter dwellers at Whirlow Hall farm (**Fig. 5.8**). Overall, Spen farm had a significantly higher mean functional group richness and functional group diversity compared to Whirlow Hall farm (**Table 5.5**).

Seasonal variation in functional group composition was also evident. At Spen farm, earthworm functional group richness varied significantly across months (**Table 5.6**, **Fig. 5.9**). May and June had a significantly lower functional group richness and diversity compared with April (Tukey, p < 0.05). In contrast, at Whirlow Hall farm, neither functional group richness nor diversity showed significant variation across months (**Table 5.6, Fig. 5.9**). In May, no litter dwellers were found at either farm (**Fig. 5.7**). At both farms, May and June were the most distinct in their functional group composition compared to the other months (**Fig. 5.10**). At Spen farm, communities from January to April and July were similar to each other, whereas August to December formed a distinct group.

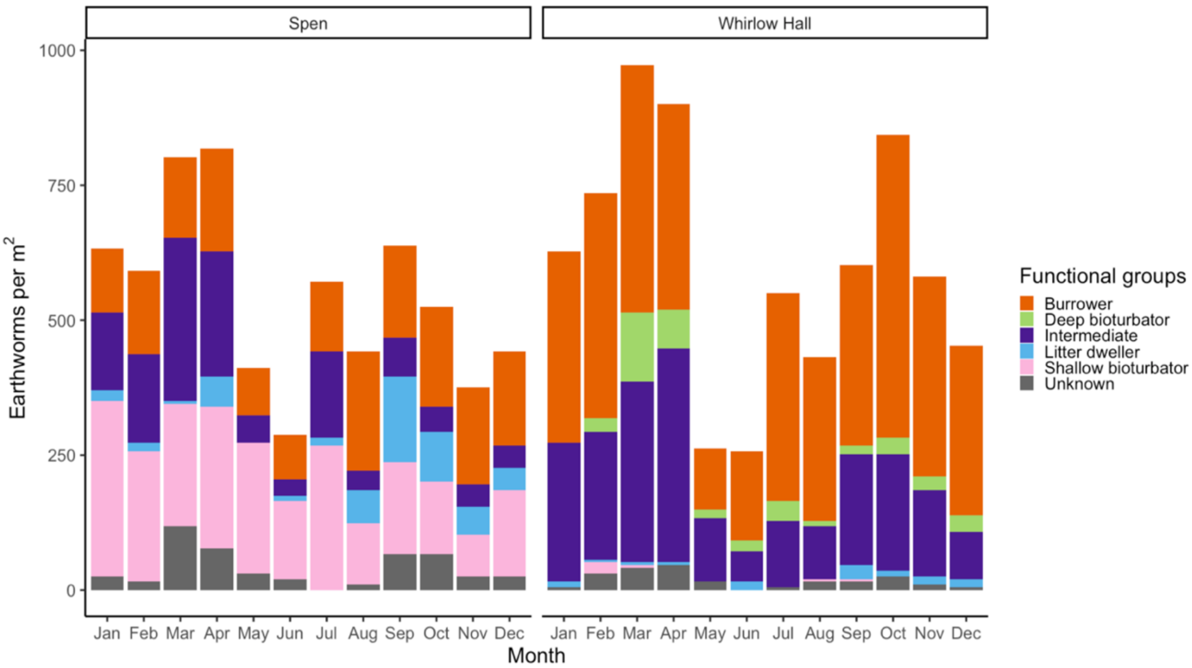

Figure 5.7. Mean density of earthworms (per m^2, n = 6) every month per farm, coloured depending on the functional group they belong to (according to Capowiez *et al.*, 2024). Burrower = *Ap. longa*, *L. terrestris*, *Ap. caliginosa*; shallow bioturbator = *Al. chlorotica*; deep bioturbator = *O. cyaneum*; intermediate = *Ap. rosea*, *Ap. cupulifera*; litter dweller = *E. tetraedra*, *L. castaneus*, *L. rubellus*, *S. mammalis*, *L. festivus*.

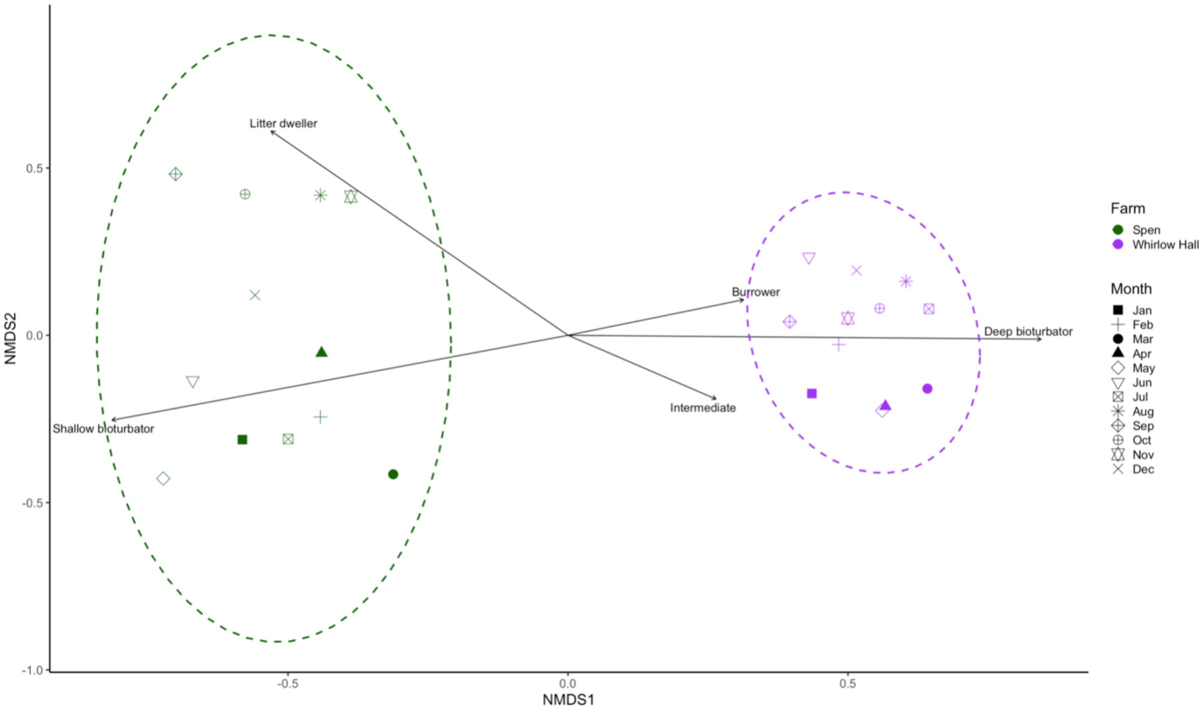

Figure 5.8. NMDS ordination of mean earthworm functional group composition for Spen (green) and Whirlow Hall (purple) farms. Shapes represent each month. Closer points are more similar in functional group composition according to the Bray-Curtis dissimilarity. Arrow directions represent the gradient of increasing abundance for that functional group and arrow length is the strength of the relationship. Months near the origin/centre of the ordination have an average functional group composition. Ellipses around each farm are based on the covariance of points.

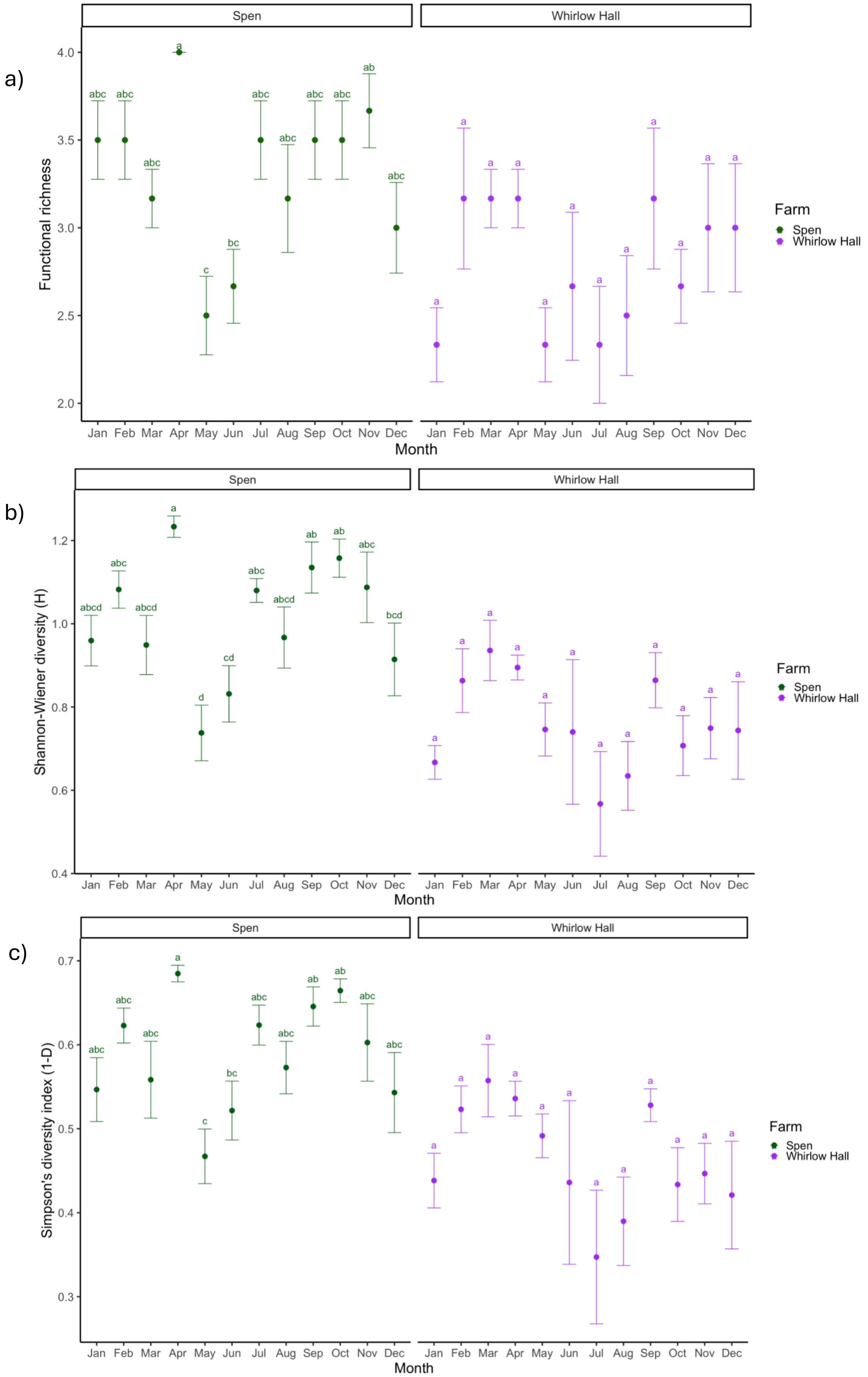

Figure 5.9. The mean (n = 6) a) richness, b) Shannon-Wiener diversity index, and c) Simpson's diversity index values of earthworm functional groups every month at each farm. Error bars show standard error. Points with different letters represent farm-specific statistically significant months ($p < 0.05$).

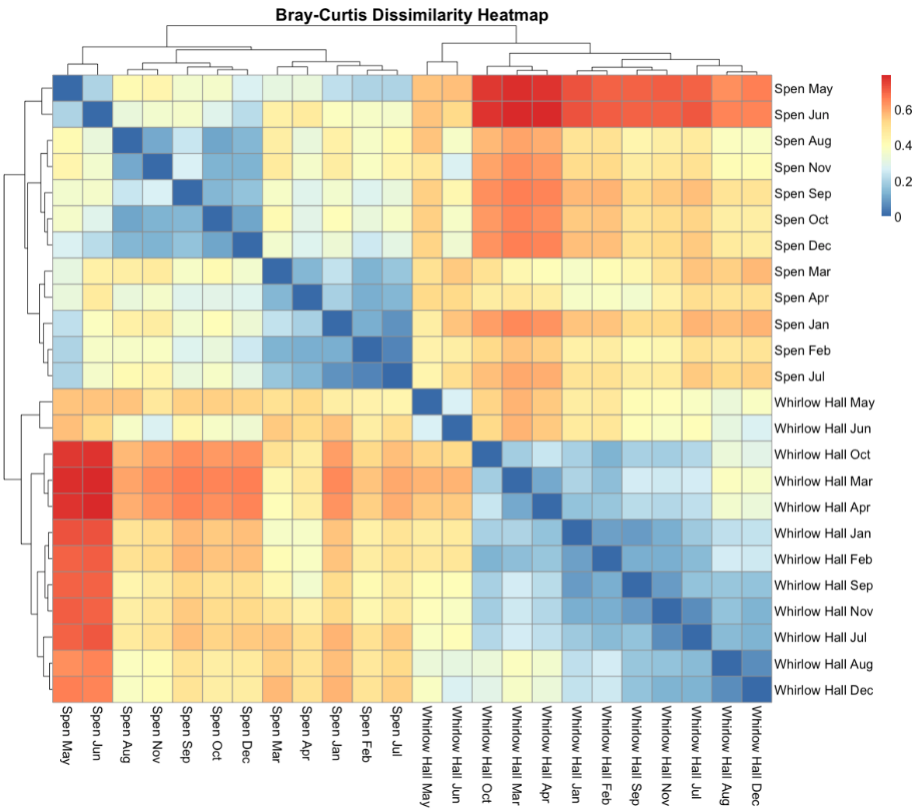

Figure 5.10. Heatmap showing pairwise Bray-Curtis dissimilarity values in the composition of earthworm functional groups found each month at the two farms. A value of 0 (dark blue) indicates the months are identical while 1 (dark red) indicates they are completely unique. Dendrogram lines show how months cluster based on the similarity of their functional groups.

5.4.5 Abiotic drivers of earthworm communities

There was a non-linear relationship between soil moisture and earthworm density at both farms, which was significant at Whirlow Hall farm (GAMM: $x^2 = 14.919$, $p < 0.01$) but not at Spen farm (GAMM: $x^2 = 6.352$, $p = 0.077$). In general, earthworm densities increased with soil moisture until reaching a maximum at ~40 vol% at Spen farm and ~50 vol% at Whirlow Hall farm, after which densities declined (**Fig. 5.11**).

The density of individual species also varied with moisture content at both farms (**Fig. 5.12**). At Spen farm, *Al. chlorotica*, *Ap. longa* and *L. terrestris* were found at the greatest range of moisture contents of ~10-59 vol% at 0-5 cm (~11-46 vol% at 20-25 cm), followed by *Ap. rosea* found between ~14-59 % at 0-5 cm (~12-46 vol% at 20-25 cm). *L. rubellus* was observed over the narrowest range of moisture values at 0-5 cm (~29-50 vol%), while at 20-25 cm *Ap. cupulifera* had the most restricted range (~29-46 vol%). A single *E. tetraedra* individual was recorded at the highest moisture content (~59 vol% at 0-5 cm, 46 vol% at 20-25 cm). No *Ap. cupulifera* individuals were found below 34 vol% at 0-5 cm (~29 vol% at 20-25 cm). At Whirlow Hall farm, *Ap. longa*, *Ap. rosea*, *Ap. caliginosa* and *O. cyaneum* were found over the widest range of moisture contents (~14-63 vol% at 0-5 cm depth). *Ap. longa* was found over the broadest range of moisture values at 20-25 cm (~14-41 vol%). In contrast, *L. castaneus* and *Al. chlorotica* had the smallest ranges at Whirlow Hall farm of ~26-52 vol% and ~22-50 vol% respectively at 0-5 cm (~18-33 vol% and ~19-31 vol% respectively at 20-25 cm). Despite these species-level occurrence patterns, soil moisture had no significant effect on species richness at either Spen farm (GAMM: z = 1.85, p = 0.064) or Whirlow Hall farm (GAMM: z = 0.272, p = 0.785) (**Fig. S5.10**). No other soil parameters significantly affected earthworm species richness.

At Spen farm, soil temperature explained more of the variation in earthworm densities (GAMM: x^2 = 12.08, p < 0.05) than soil moisture. Densities peaked at ~10 °C but began to decline at higher temperatures (**Fig. 5.13**). At Whirlow Hall farm, bulk density had a significant effect on earthworm density (GAMM: x^2 = 16.687, p < 0.05), with low bulk density generally associated with low earthworm density. No such effect was observed at Spen farm (GAMM: x^2 = 2.09, p = 0.149) (**Fig. S5.11**).

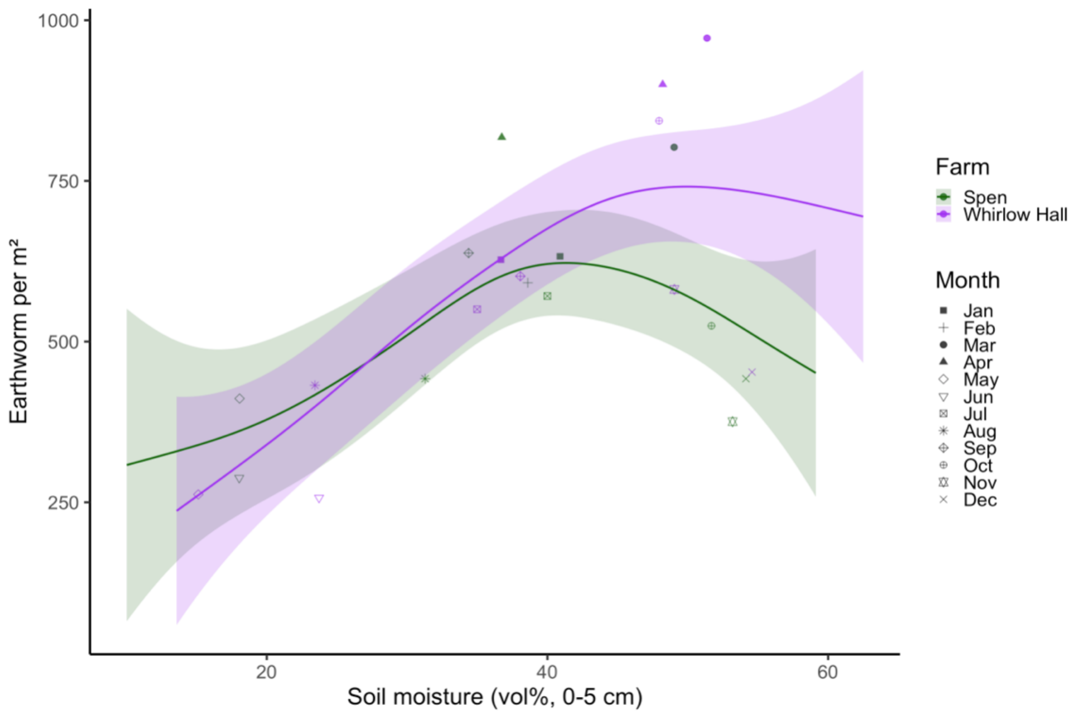

Figure 5.11. The relationship between mean soil moisture content (vol%, 0-5 cm, n = 6) and earthworm density (individuals per m^2, n = 6) in both farms (green = Spen, purple = Whirlow Hall). Shapes represent each month. Curves are estimated using generalised additive models (GAMs) and shaded ribbons represent standard error.

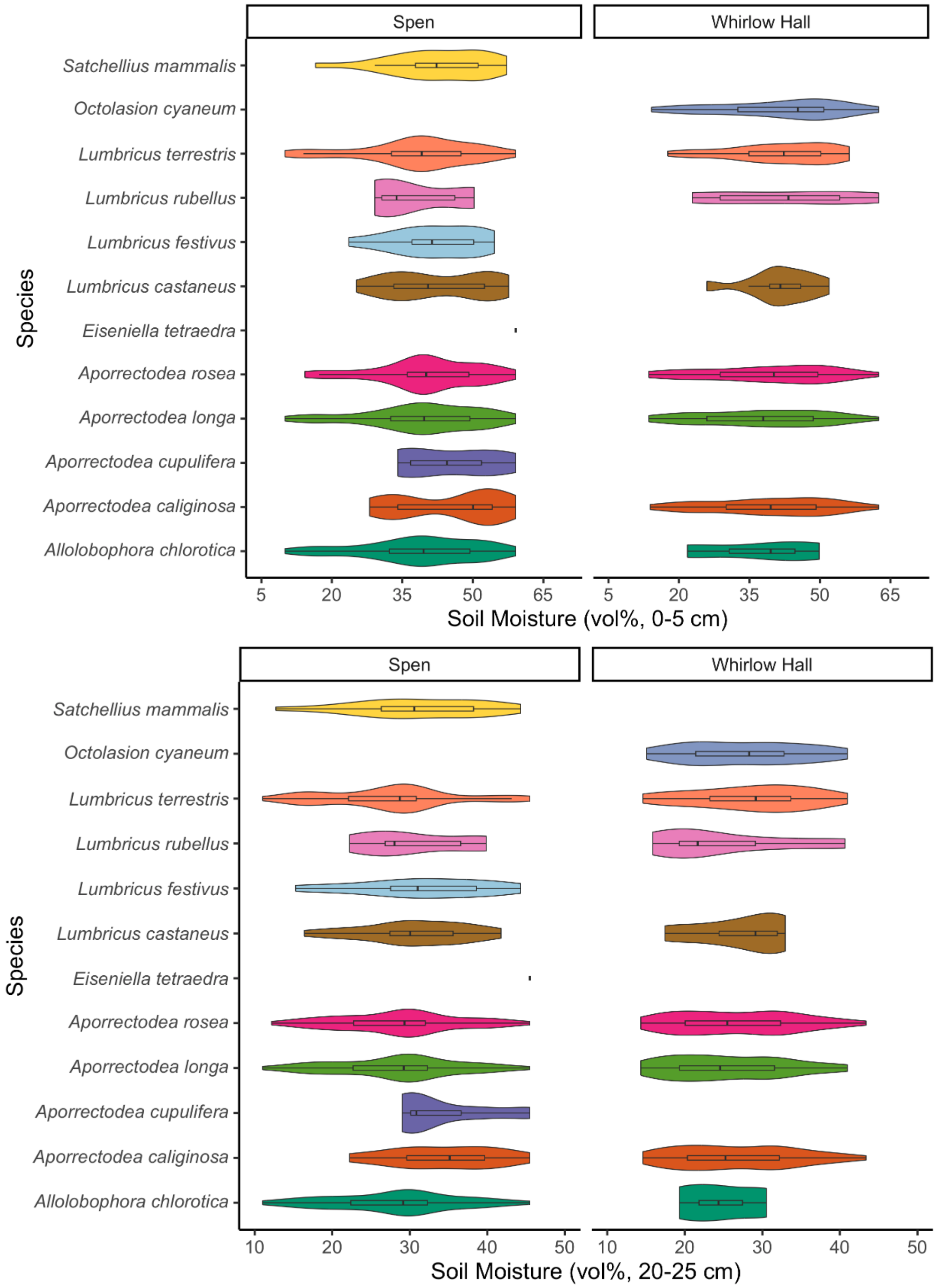

Figure 5.12. Species occurrences with soil moisture content at (a) 0-5 cm and (b) 20-25 cm depth, split by farm. The violin height represents observation density (number of individuals) and tails represent the observation range. Boxplots show values of the median, minimum, maximum and interquartile range.

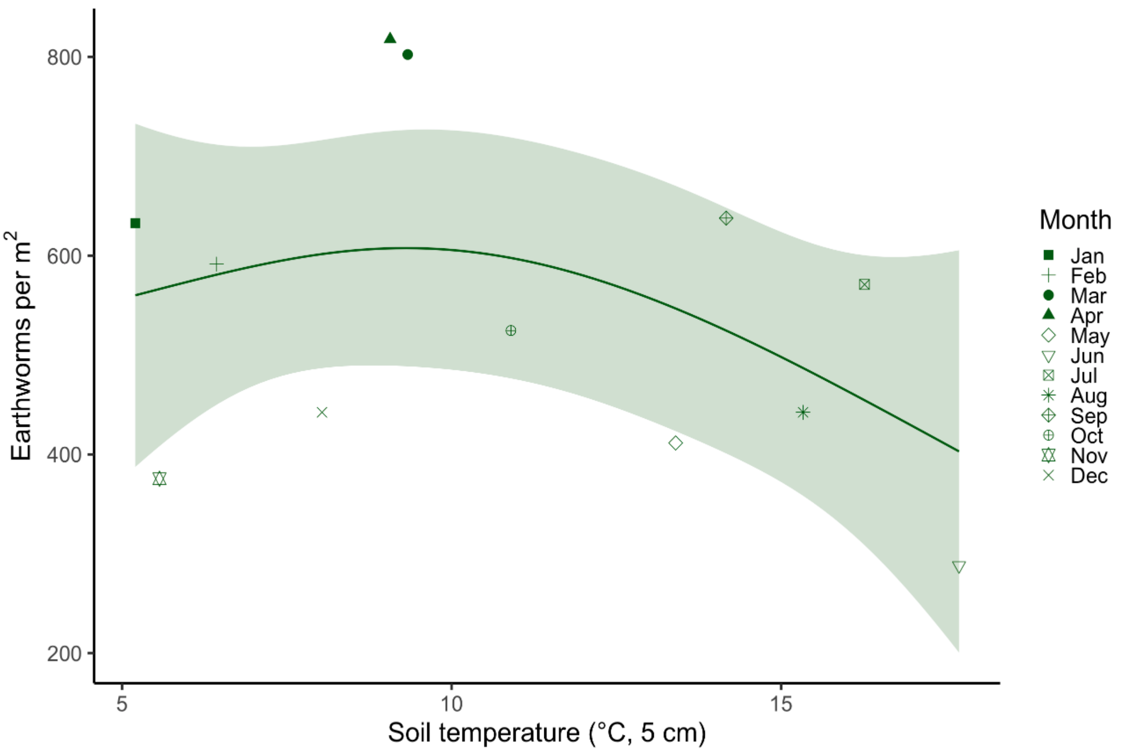

Figure 5.13. The relationship between mean soil temperature (°C, 5 cm, n = 3) and earthworm density (individuals per m^2, n = 6) at Spen farm. Shapes represent each month. The curve is estimated using a generalised additive model (GAM) and the shaded ribbon represents standard error.

5.4.6 Earthworm age structure

Overall, 33.96 % of the 2674 earthworms collected were clitellate and 66.04 % were aclitellate. Spen farm had a significantly higher mean percentage of clitellate earthworms than Whirlow Hall farm (**Table 5.5**). The proportion of clitellate earthworms also varied significantly within a year at both Spen farm and Whirlow Hall farm (**Table. 5.6, Fig. 5.14**). At Spen farm, June had the lowest percentage (~20 %), significantly smaller than January, August, September, November and December (all >46 %). At Whirlow Hall farm, June and December had the smallest percentage of clitellate earthworms (<12 %), significantly smaller than March (>37 %). At both farms, the clitellate earthworm density (per m^2) was also lowest in June (**Fig. 5.15**). Densities of clitellate worms peaked in spring at both farms, while aclitellate densities were highest in March and April at Spen farm, and April and October at Whirlow Hall farm (**Fig. 5.15**). The species most commonly found aclitellate were of the burrowing (e.g. *Ap. longa* and *L. terrestris*) and litter dwelling (e.g. *L. rubellus* and *L. festivus*) functional groups (**Fig. 5.16**). At both farms 100 % of *Ap. longa* were aclitellate in June, and this was also the case in December at Whirlow Hall farm (**Fig. 5.16**).

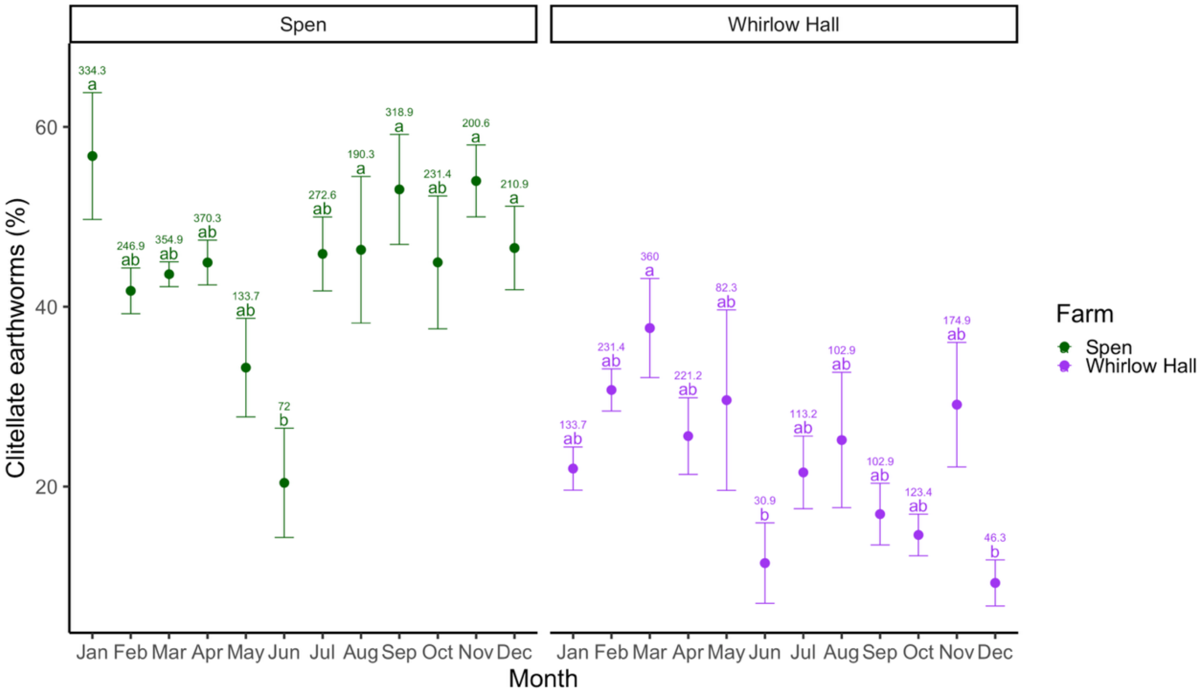

Figure 5.14. The mean percentage (%, n = 6) of clitellate earthworms every month at each farm. Error bars show standard error. Points with different letters represent farm-specific statistically significant months ($p < 0.05$). Numbers above bars represent the mean density of clitellate earthworms (per m^2) each month.

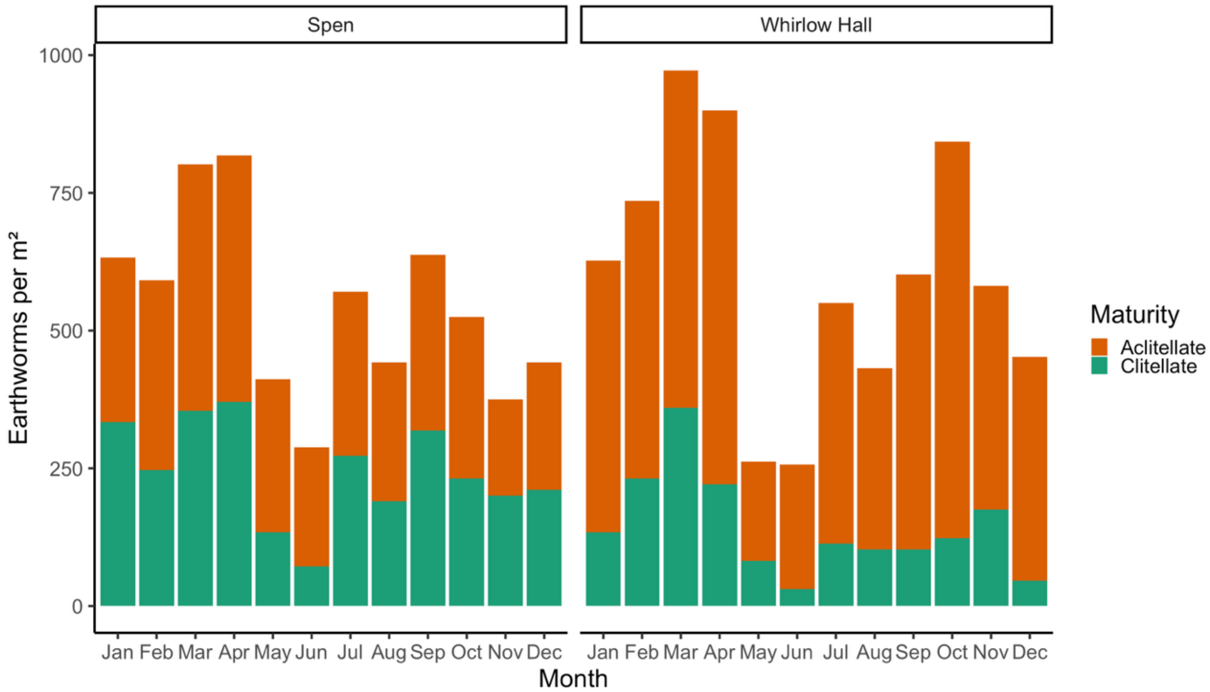

Figure 5.15. The mean density of clitellate (green) and aclitellate (orange) earthworms (per m^2, n = 6) every month at each farm.

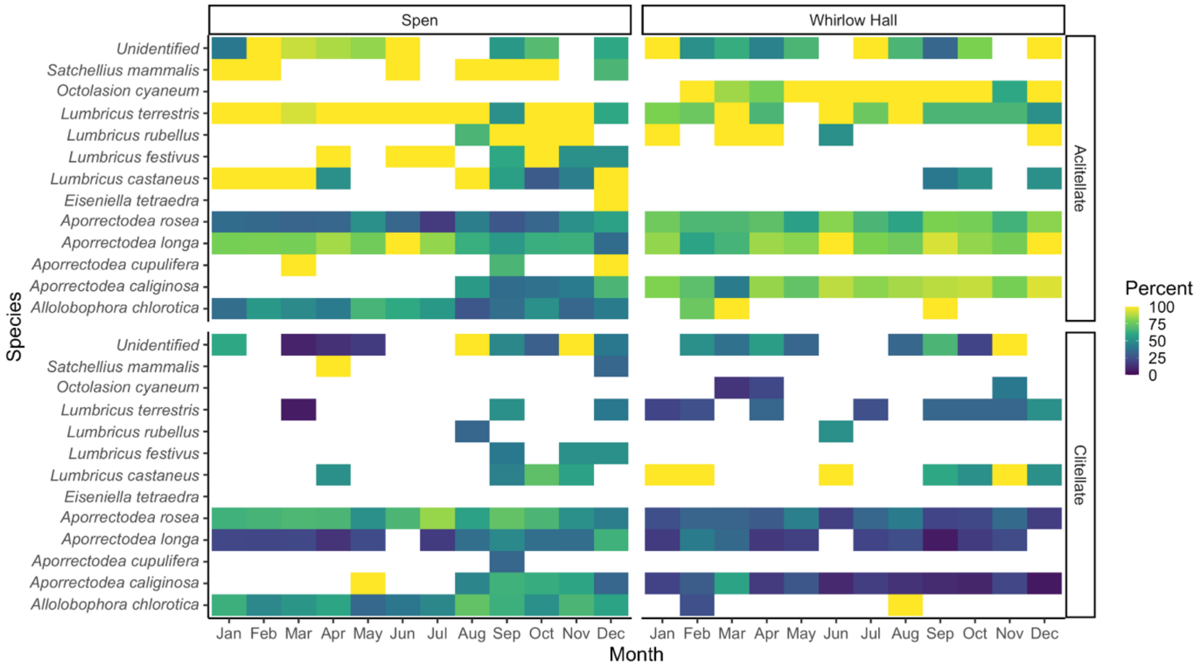

Figure 5.16. The percentage (%) of clitellate and aclitellate earthworms of each species every month at each farm.

Soil moisture significantly influenced the percentage of clitellate earthworms at Spen farm (GAMM: x^2 = 16.275, p < 0.01), but not at Whirlow Hall farm (GAMM: x^2 = 3.439, p = 0.409). At Spen farm, the proportion of clitellate individuals increased with moisture until around half the individuals were clitellate at ~30 vol%, after which further increases in moisture had little effect (**Fig. 5.17**). Moreover, the percentage of clitellate earthworms was significantly negatively correlated with soil pH at both Spen farm (GAMM: z = -2.234, p < 0.05) and Whirlow Hall farm (GAMM: z = -2.075, p < 0.05) (**Fig. 5.18**).

Clitellate earthworm density (per m^2) was significantly associated with soil moisture at both Spen farm (GAMM: x^2 = 12.046, p < 0.01) and Whirlow Hall farm (GAMM: x^2 = 10.982, p < 0.05) (**Fig. 5.19**). Soil moisture also significantly influenced the density of aclitellate earthworms at Whirlow Hall farm (GAMM: x^2 = 11.169, p < 0.01) but had no effect at Spen farm (GAMM: x^2 = 1.395, p = 0.524) (**Fig. 5.20**). At Spen farm, soil temperature significantly affected both clitellate (GAMM: x^2 = 9.993, p < 0.05) and aclitellate (GAMM: x^2 = 9.821, p < 0.05) densities (**Fig. S5.12, Fig. S5.13**). Soil pH was marginally negatively associated with the density of clitellate earthworms at Whirlow Hall farm (GAMM: z = -1.893, p = 0.058) and positively, though not significantly, at Spen farm (GAMM: z = 1.107, p = 0.268, **Fig. S5.14**). There was no significant relationship between soil pH and aclitellate earthworm density at either Spen farm (GAMM: z = 1.263, p = 0.207) or Whirlow Hall farm (GAMM: z = 0.199, p = 0.842) (**Fig. S5.15**).

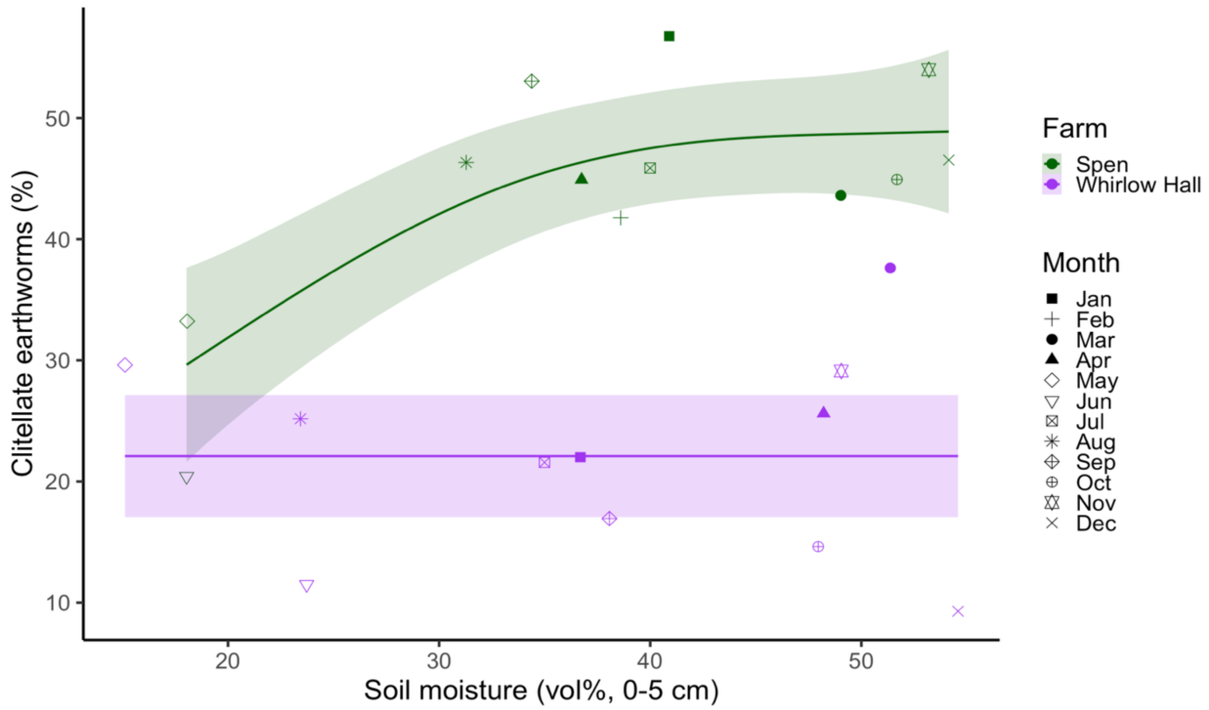

Figure 5.17. The relationship between mean soil moisture content (vol%, 0-5 cm, n = 6) and the percentage of clitellate earthworms (%, n = 6) in both farms (green = Spen, purple = Whirlow Hall). Shapes represent each month. Curve for Spen was estimated using a generalised additive model (GAM). Shaded ribbons show standard error.

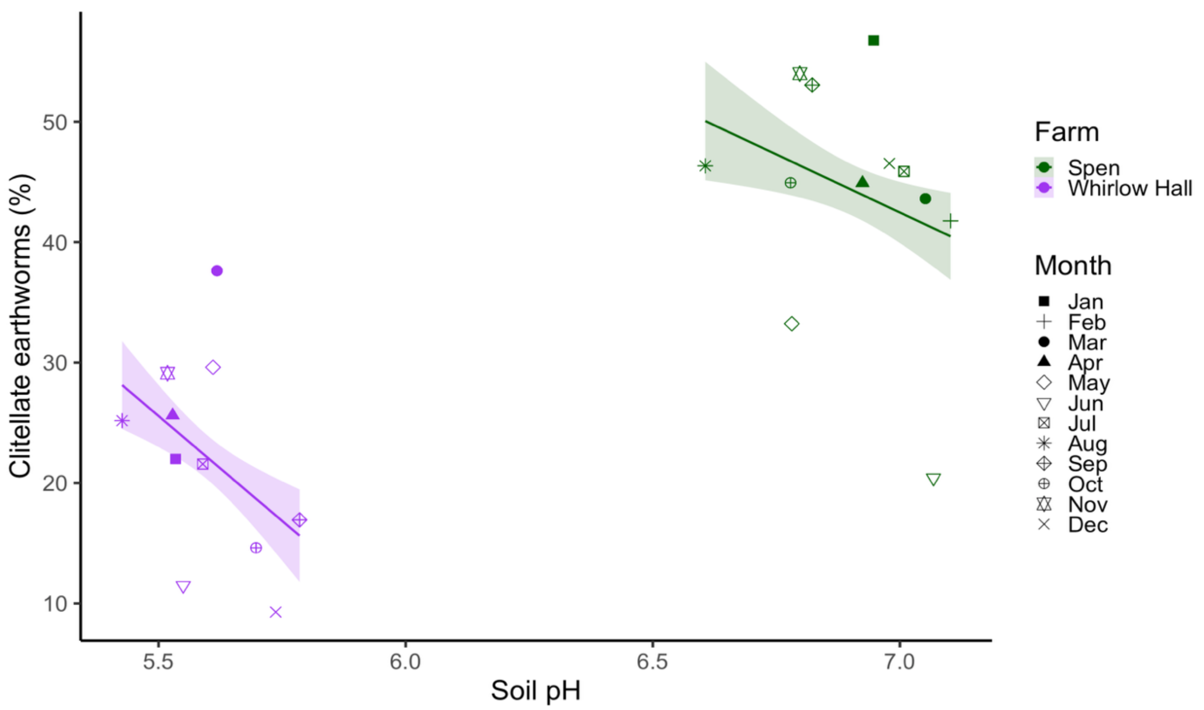

Figure 5.18. The relationship between mean soil pH (n =3) and the percentage of clitellate earthworms (n = 6) in both farms (green = Spen, purple = Whirlow Hall). Shapes represent each month. Lines fitted using linear regression. Shaded ribbons show standard error.

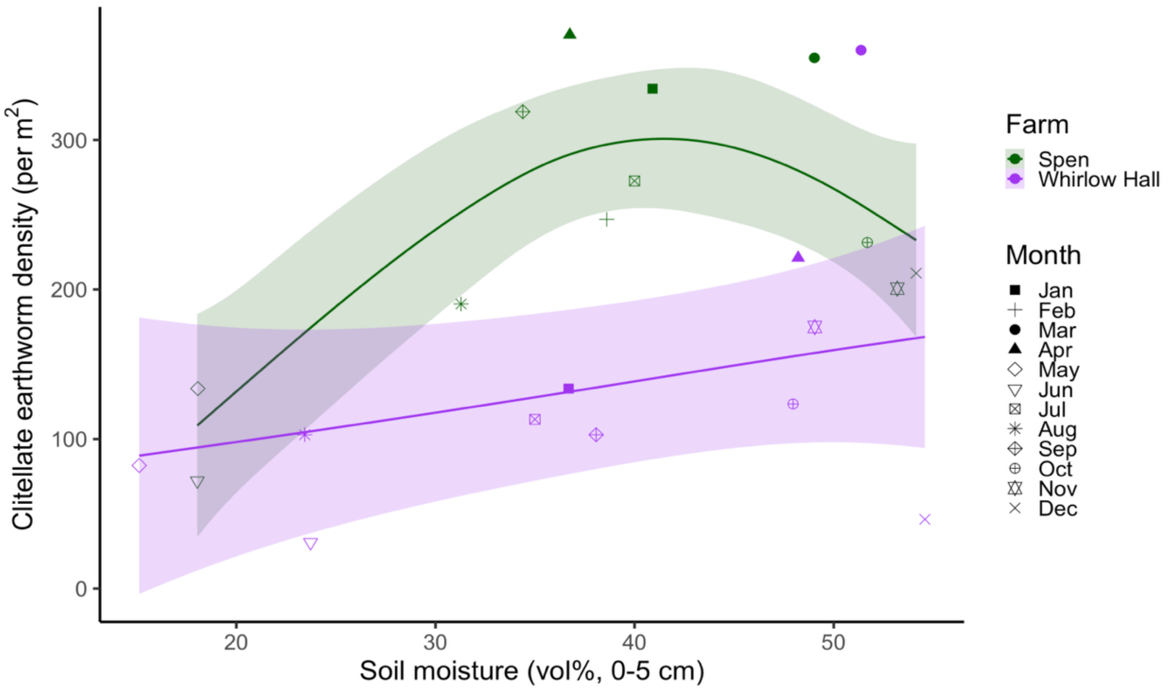

Figure 5.19. The relationship between mean soil moisture content (vol%, 0-5 cm, n = 6) and the density of clitellate earthworms (per m^2, n = 6) in both farms (green = Spen, purple = Whirlow Hall). Shapes represent each month. Curves estimated using a generalised additive model (GAM). Shaded ribbons show standard error.

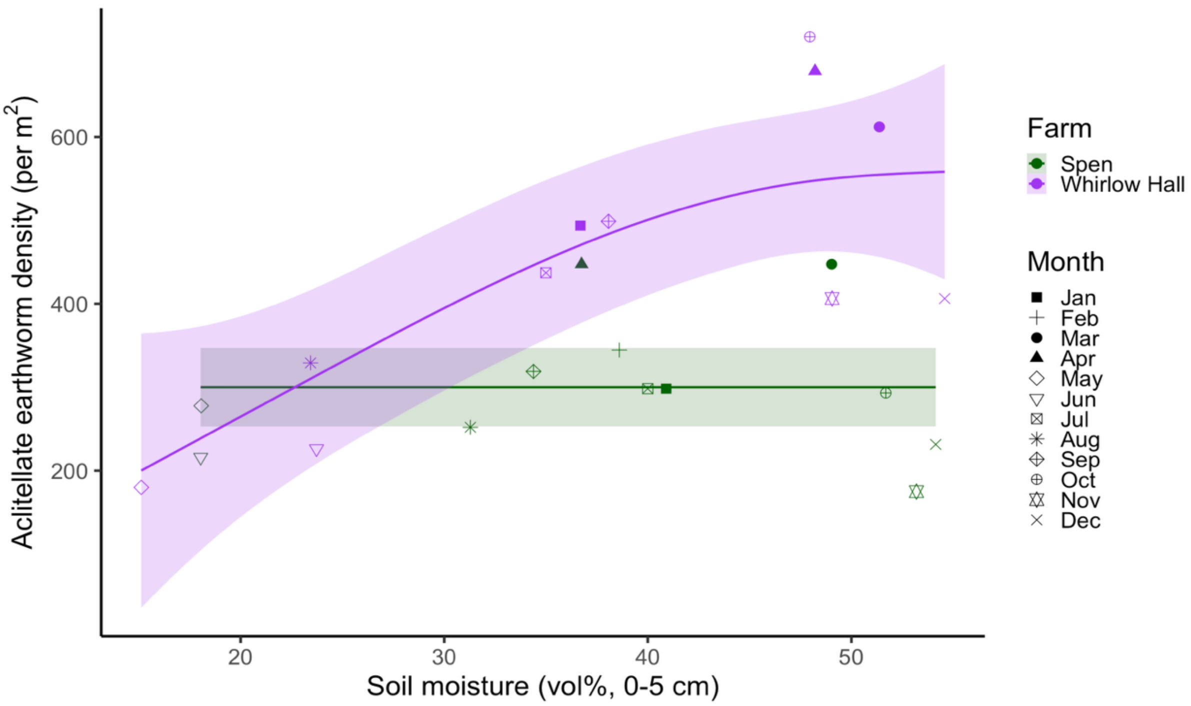

Figure 5.20. The relationship between mean soil moisture content (vol%, 0-5 cm, n = 6) and the density of aclitellate earthworms (per m^2, n = 6) in both farms (green = Spen, purple = Whirlow Hall). Shapes represent each month. Curves estimated using a generalised additive model (GAM). Shaded ribbons show standard error.

5.4.7 Earthworm aestivation

The percentage of earthworms in aestivation varied significantly with month at both Spen farm (Beta regression: Z = 4.826, $p < 0.001$) and Whirlow Hall farm (Beta regression: Z = 4.778, $p < 0.001$) (**Fig. 5.21**). At Spen farm, over 70 % of the earthworms were found aestivating in May and June, significantly more than in all other months (Tukey, $p < 0.05$). In contrast, only ~1 % of the population was observed aestivating in February, March and April at Spen farm. At Whirlow Hall farm, ~47 % of earthworms were aestivating in June, significantly greater than in all other months (Tukey, $p < 0.05$). Smaller peaks were also observed in May (~18 %) and August (~12 %). In December, aestivation occurred at both farms with ~14 % of the population at Spen farm and ~5 % at Whirlow Hall farm. There was no significant difference between aclitellate and clitellate earthworms in the likelihood of aestivation, as both groups were represented in similar proportions (binomial GLM: Z = 0.167, p = 0.867, **Fig. S5.16).** There was a strong species-specific response in the behaviour of earthworms. At both farms, the same four species were found aestivating: *Ap. rosea*, *Ap. longa*, *Ap. caliginosa* and *Al. chlorotica.* In June at Spen farm, 100 % of the *Al. chlorotica* and *Ap. longa* were aestivating, along with the majority of *Ap. rosea,* although a small percentage of the latter remained active (**Fig. 5.22**).

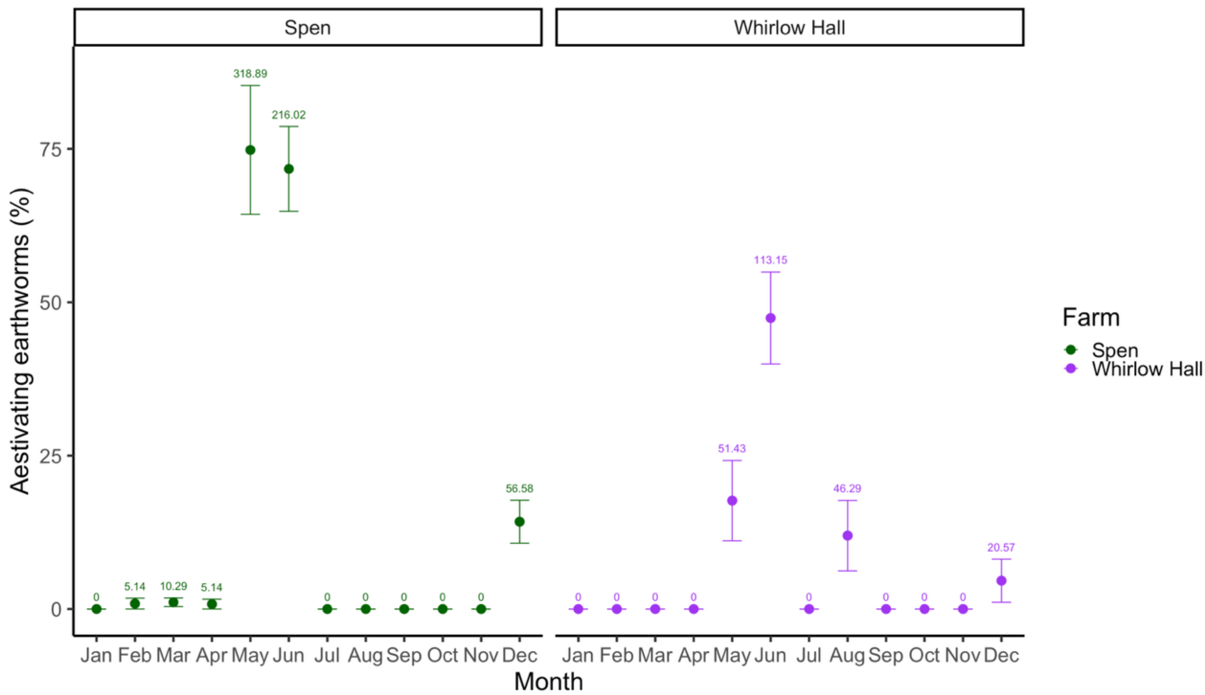

Figure 5.21. Mean percentage of aestivating earthworms (%, n = 6) each month in both farms (green = Spen, purple = Whirlow Hall). Error bars show standard error. Numbers above bars represent mean density of aestivating earthworms (per m^2) each month.

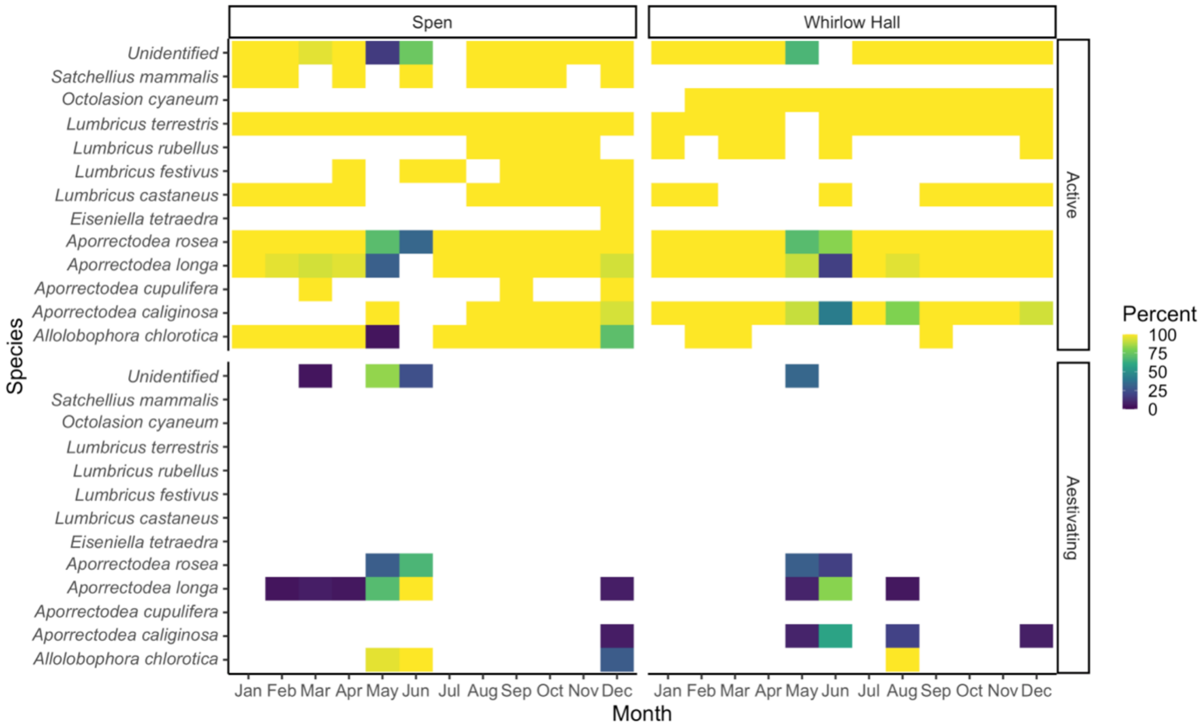

Figure 5.22. The percentage of each earthworm species found active (top) and aestivating (bottom) every month at each farm.

Soil moisture had a non-linear effect on the percentage of aestivating earthworms, which was highly significant at both Spen farm (GAMM: x^2 = 17.74, $p < 0.01$) and Whirlow Hall farm (GAMM: x^2 = 31.95, $p < 0.001$) (**Fig. 5.23**). At Spen farm, moisture contents below ~25-30 vol% led to a sharp increase in the percentage of earthworms found aestivating. Between 30-45 vol%, aestivation was rare but increased again in soils above ~45-50 vol% moisture. A similar pattern occurred at Whirlow Hall farm, although the maximum percentage of earthworms found aestivating in a month was lower at ~50 % of the population compared to ~75 % at Spen farm. Earthworm species responded differently to soil moisture. *Ap. rosea* were never found aestivating at moisture contents above 30 vol%, whereas some individuals of *Ap. longa*, *Ap. caliginosa* and *Al. chlorotica* were (**Fig. 5.24**). Soil bulk density also significantly affected aestivation at both Spen farm (GAMM: x^2 = 26.909, $p < 0.001$) and Whirlow Hall farm (GAMM: x^2 = 53.8, $p < 0.001$). The highest percentages of aestivating earthworms generally occurred in soils with lower bulk densities, but some aestivation was also observed in the densest soils at Spen farm (**Fig. 5.25**).

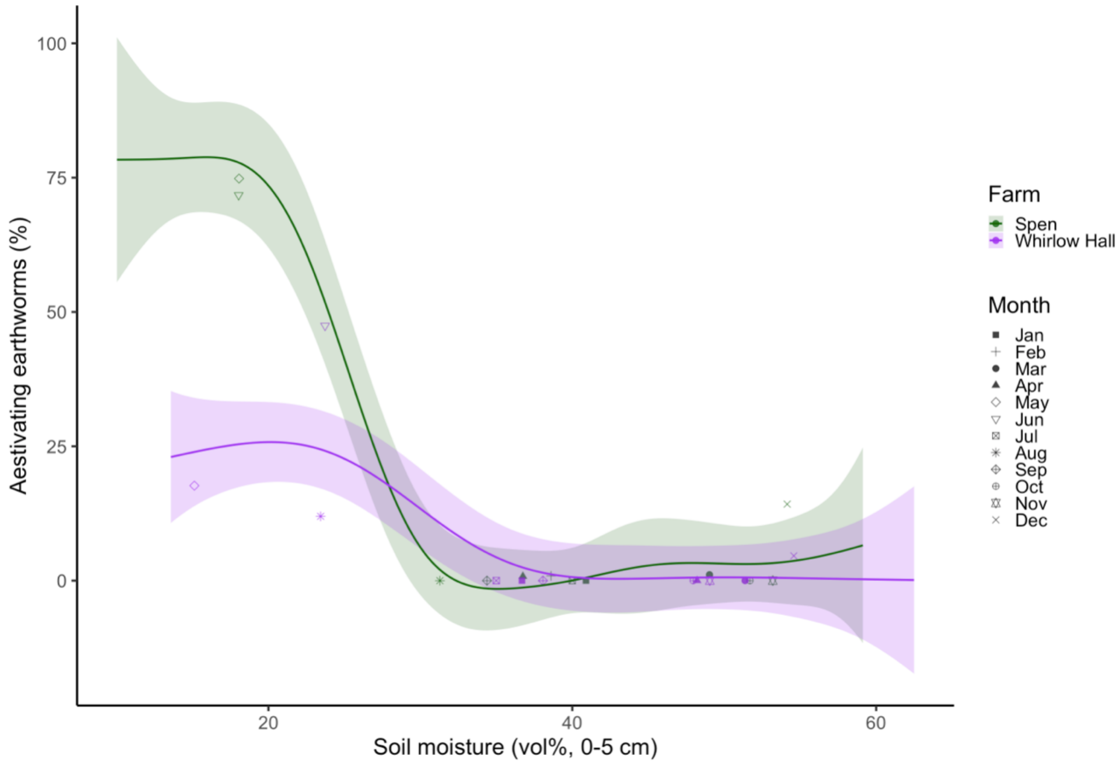

Figure 5.23. The relationship between mean soil moisture content (vol%, 0-5 cm, n = 6) and aestivating earthworms (%, n = 6) in both farms (green = Spen, purple = Whirlow Hall). Shapes represent each month. Curves are estimated using generalised additive models (GAMs). Shaded ribbons show 95 % confidence intervals around the fitted curves.

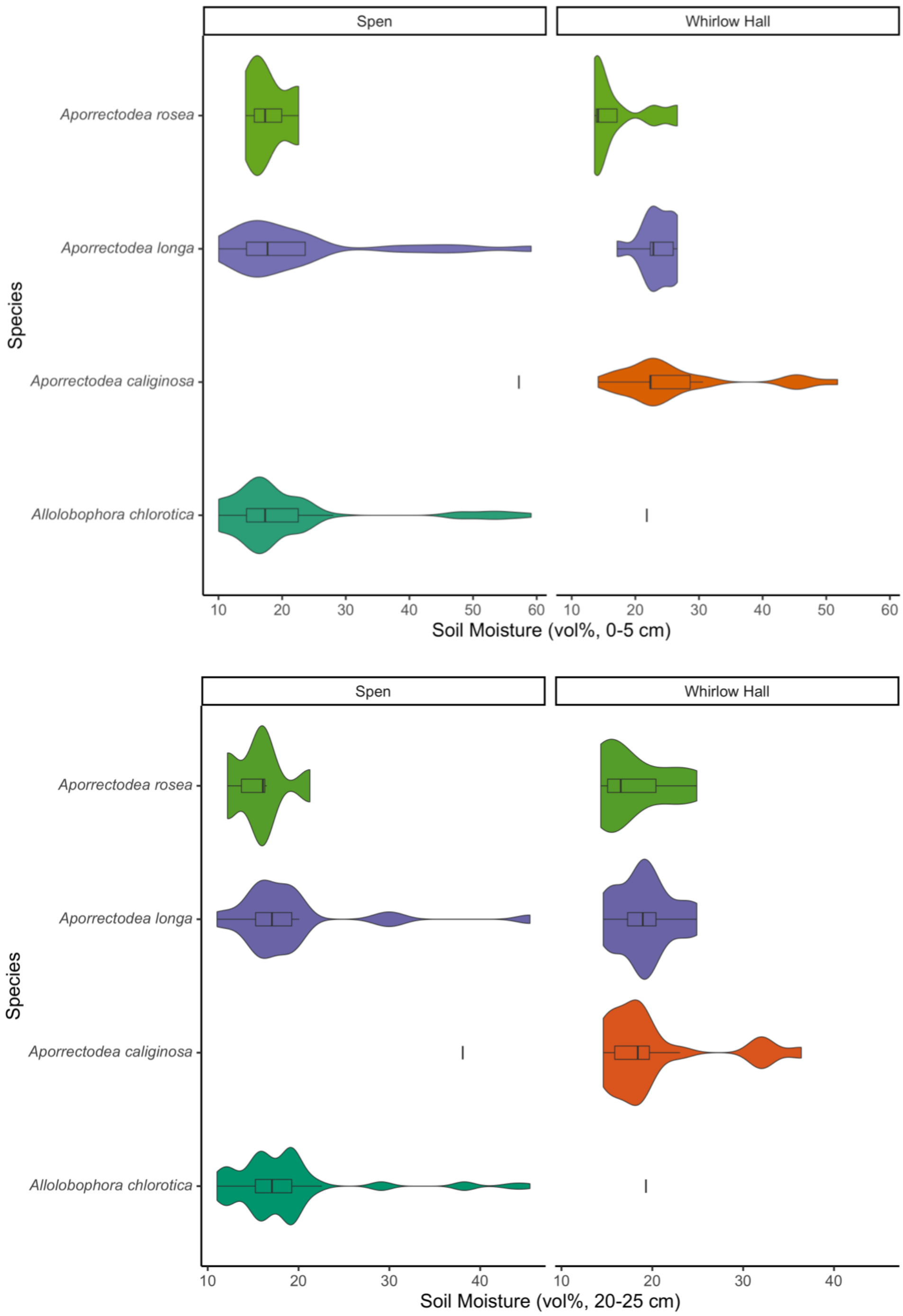

Figure 5.24. The distribution of occurrences of each species found aestivating with soil moisture content at (a) 0-5 cm and (b) 20-25 cm depth, split by farm. The height of the violin represents the density of observations (number of individuals), and the tails represent the range of values in which the species occurred in aestivation. Boxplots show values of the median, minimum, maximum and interquartile range.

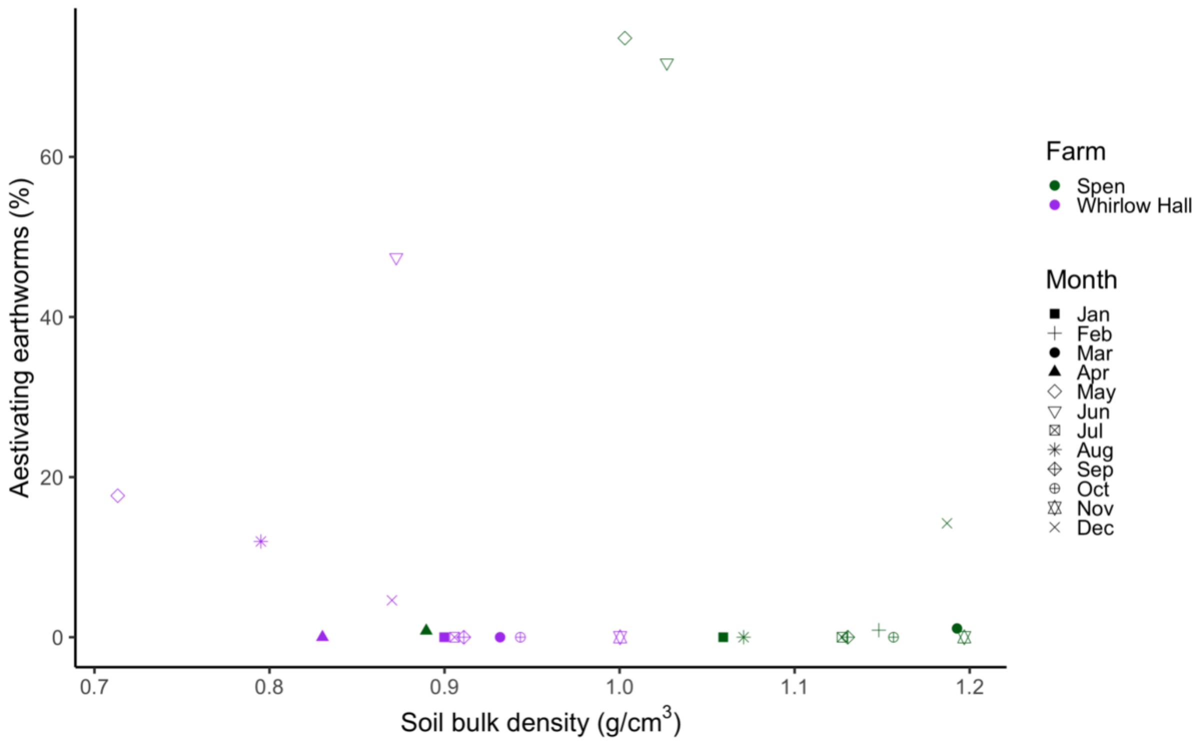

Figure 5.25. The relationship between mean soil bulk density (g/cm^3, n = 3) and the proportion of aestivating earthworms (%, n = 6) in both farms (green = Spen, purple = Whirlow Hall). Shapes represent each month.

5.5. Discussion

5.5.1 Earthworm densities

Overall, earthworm densities were approximately 10 % higher at Whirlow Hall farm than at Spen farm with 601 ± 67 and 545 ± 47 earthworms per m^2 respectively. These values fall within ranges reported in other studies with Hoeffner *et al.* (2021) recording mean densities of 517 ± 57 earthworms per m^2 across 24 grasslands, while Rutgers *et al.* (2016) found between 600 – 800 per m^2 in a nationwide soil monitoring study across Europe. Contrasting soil properties help explain overall differences in earthworm populations. Whirlow Hall farm soils were characterised by a higher mean soil organic matter content (9.5 ± 0.1 vs 8.2 ± 0.1%), but a lower mean pH (5.6 ±0.03 vs 6.9 ± 0.04), and mean bulk density (0.9 ± 0.02 vs 1.1 ± 0.03 g/cm^3) than Spen farm. The elevated SOM at Whirlow Hall farm is likely linked to manure inputs from grazing livestock, which can both enhance organic matter availability as a food source for earthworms (Lee, 1985), and soil aggregation, thereby influencing both soil porosity and the reciprocal bulk density (Guhra *et al.*, 2022; Robinson *et al.*, 2022). Consistent with this, SOM and bulk density were significantly negatively correlated at both farms. At Spen farm, shifting sampling to the field margins was not associated with distinct changes in soil parameters such as SOM, pH, and bulk density, suggesting that vegetation structure and litter inputs were comparable to the main field when in permanent pasture. Therefore, the shift in sampling location alone did not likely influence the density of earthworms. This is supported by previous work at Spen farm finding pasture fields and margins did not differ significantly in their soil bulk density, organic carbon or earthworm densities (757.5 ± 426.2 vs 673.6 ± 326.9 per m^2 for pasture and margins respectively) (Holden *et al.*, 2019).

The strongest seasonal fluctuations occurred in soil moisture, which showed similar temporal patterns at both farms and as expected, earthworm densities were strongly influenced by soil moisture. Many studies report positive effects of soil moisture and precipitation, on earthworm abundance and biomass (Liu *et al.,* 2025; Tondoh, 2006; Fournier *et al.*, 2012, Ahmed *et al.*, 2025; Singh *et al.*, 2016; Garnsey, 1994; Martay and Pearce-Higgins, 2018; Rutgers *et al.*, 2016; Walsh *et al.,* 2019; Torppa *et al.*, 2024). However, the relationship in the present study was non-linear. Generally, earthworm densities increased with moisture up to ~40 vol% at Spen and ~50 vol% at Whirlow Hall farm, then declined under more saturated conditions. Accordingly, the highest earthworm densities (>800/m^2) were recorded in spring (March, April) and autumn (September, October), when intermediate soil moisture (~35-50 vol%) provided favourable conditions. In contrast, the lowest earthworm densities (<300/m^2) were recorded in late spring/early summer (May, June) when topsoil moisture fell below 25 vol% and again in early winter (December) when moisture exceeded ~50 vol%. This pattern is consistent with previous observations of higher earthworm abundance and biomass in early spring and autumn corresponding with higher soil moisture than in summer (Singh *et al.*, 2020; Martey and Pearce-Higgins, 2018; Barnes *et al.*, 2023; Baker *et al.*, 1993; Garnsey, 1994). Seasonal declines in earthworm densities likely reflect a combination of migration to deeper soil layers and mortality (Baker *et al.*, 1993) due to a lack of free water (critical for facilitating oxygen uptake for respiration, Holmstrup, 2001) during drier conditions, or anoxic conditions during wetter conditions. For example, Plum and Filser (2005) observed a complete absence of earthworms after a summer flood, attributing this to the low oxygen availability associated with saturated soils. Consistent with this, Kiss *et al.* (2021) found submerged earthworms died when water became oxygen deficient whereas survival rates were high when the water was aerated with ambient air oxygen.

Soil temperatures at Spen farm also showed strong seasonality and, as expected, were significantly negatively correlated with soil moisture in the top 5 cm. Consequently, there was a significant non-linear effect of soil temperature on earthworm densities which peaked at ~10 °C in March and April and declined at both higher and lower temperatures, with the lowest density in June when the highest temperatures (~18 °C) and lowest moistures were recorded. Similar diminishing earthworm densities have been recorded following spring as soils became warmer and drier (Eggleton *et al.*, 2009; Walsh *et al.*, 2019). SOM content remained at a relatively stable level throughout the year at both farms and in contrast to the prediction, had no significant effect on the density of earthworms, suggesting it was within favourable ranges. This is consistent with findings by Torppa *et al.* (2024) of no significant association between earthworm density and SOM, which they attributed to SOM levels being sufficiently high to no longer be a limiting factor.

5.5.2 Composition of earthworm species and functional groups

Spen farm supported a greater overall species richness than Whirlow Hall farm (11 vs 8), although one fewer functional group was present (4 vs 5). The most abundant species were *Al. chlorotica* (shallow bioturbator) at Spen and *Ap. caliginosa* (burrower) at Whirlow Hall farm, followed by *Ap. rosea* (intermediate) and *Ap. longa* (burrower) at both farms. These findings align with Ashwood *et al.* (2024), who identified these species among the five most frequently recorded across the British Isles. In contrast, *L. rubellus,* the fourth most common species nationally, contributed only ~0.5 % of the total earthworm populations at both Spen and Whirlow Hall farm, likely reflecting habitat preferences, since 28 % of *L. rubellus* records in Ashwood *et al.* (2024) were from woodlands. Previous sampling at Spen farm also report that *Al. chlorotica* was the most dominant species (Holden *et al.*, 2019; Prendergast-Miller *et al.*, 2021). High densities of *Ap. caliginosa* and *Ap. rosea* at Whirlow Hall farm may reflect the influence of grazing, as Torppa *et al.* (2024) also found these species were more common in grasslands under high grazing intensities. Given their burrowing abilities, these species may benefit from manure inputs without the risk of trampling that typically negatively affects earthworms that live or feed near the soil surface (Chan and Barchia, 2007). Overall, endogeics dominated both farm populations, consistent with evidence that managed ecosystems favour these species while supporting fewer anecic and epigeic species compared with natural ecosystems (Johnston, 2019). Endogeics are generally more resilient to disturbance (e.g. due to tillage) than anecic and epigeic species, which require more stable conditions and are sensitive to surface disruption (Johnston, 2019; Briones and Schmidt, 2017). The shallow litter layer typical of grasslands and croplands may further restrict epigeic/litter dwelling species abundances (Liu *et al.*, 2025). Indeed, Whirlow Hall farm showed a lower density and diversity of litter dwellers and a lower density of shallow bioturbators, likely reflecting reduced vegetation cover and greater disturbance due to grazing (Chan and Barchia, 2007), whereas Spen farm retained intact cover until ploughing in August.

Seasonal dynamics in community composition were consistent across both farms and mirrored patterns in earthworm density and soil moisture. In general, species and functional richness peaked in early spring (March and April) and autumn (September), and declined in May. This agrees with Eisenhauer *et al.* (2009), who also found significant seasonal effects on earthworm communities. At Spen farm, species richness tended to increase with soil moisture, though this effect was not significant. The most distinct earthworm community compositions occurred during the driest summer months (May and June) at both farms. In May and June, *Ap. rosea* and *Ap. longa* dominated at both farms, alongside *Al. chlorotica* at Spen and *Ap. caliginosa* at Whirlow Hall farm. These species were also recorded across the widest soil moisture ranges at each farm, suggesting greater tolerance to hydrological extremes. Briones *et al.* (2009) reported similar results in grassland mesocosms, where artificial soil warming significantly reduced earthworm communities to just *Ap. caliginosa*, *Al. chlorotica* and *Ap. longa*, suggesting these species may have higher thermal tolerance and adaptive strategies to cope with the associated water loss. However, differences emerged between farms as for instance, *Al. chlorotica* was observed over a broad range of moisture contents at Spen farm but was restricted to narrower conditions at Whirlow Hall farm. This highlights the importance of local soil environments in mediating species responses (Eggleton *et al.*, 2009).

Species' vulnerability to climate stress may also depend on their ecological niche and depth of soil occupancy. Endogeic and anecic species, which burrow more deeply, are buffered from surface-extremes, whereas epigeics are restricted to the litter layer and are thus more exposed (Fourcade and Vercauteren, 2022). In the present study, litter-dwelling species (such as *L. rubellus* and *L. castaneus*) occurred in the narrowest range of moisture conditions and were completely absent from both farms in May, consistent with their expected inability to avoid drought by burrowing (Eggleton *et al.*, 2009). Their decline is also consistent with experimental warming studies, where even moderate temperature rises (3.5 °C) eliminated epigeic species from a soil system (Briones *et al.*, 2009). Conversely, the low species diversity recorded in December at Whirlow Hall farm likely reflected flooding stress, as species responses to saturated conditions depends on traits such as body size and ecological strategies (Kiss *et al.*, 2021). The lower species and functional richness and diversity observed here is consistent with the functional homogenisation and loss of rare earthworm species predicted to occur with climate change which will likely be detrimental to ecosystem processes (Fourcade and Vercauteren, 2022).

Two of the rarest species were detected only at Spen farm: *Ap. cupulifera* (6 individuals) and *E. tetraedra* (1 individual). *Ap. cupulifera* is considered very rare and, until recently, was only collected in the British Isles from Ireland (Natural England, 2014). While the sequences generated most closely matched *Ap. cupulifera*, they were not exact matches to the single 16S sequence that is currently present in the reference database for this species and were also close matches to sequences for *Ap. georgii* and *Ap. jassyensis* (although these species are not believed to occur in the British Isles). This highlights the need for greater sequencing of earthworm species to build up the reference database and improve the ability to identify species through molecular methods alone. All individuals identified as *Ap. cupulifera* were very small (~25-35 mm), even when clitellate (**Fig. S5.17**), and thus it is probable that individuals of this species may be often misidentified as juvenile and thus overlooked or discarded in morphology-based surveys where keys are used for identification (which requires adult specimens). In addition, *Ap. cupulifera* were only recorded in soils above 29 vol% while *E. tetraedra* was found at ~60 vol%, consistent with reports that it is adapted to aquatic environments and thus inhabits wetlands and waterlogged soils (Capowiez *et al.*, 2024; Ashwood *et al.*, 2024). The ephemeral nature of the conditions under which this species occurs likely accounts for the lack of individuals. Identification of such species which may go undetected by conventional sampling methods might be improved using eDNA, a low disturbance approach which involves extracting and sequencing DNA from soil samples to determine species presence (Bienert *et al.*, 2012).

5.5.3 Earthworm age structure

Across both farms, aclitellate earthworms dominated, with only one third of the individuals reproductively mature. This is consistent with previous studies showing that juveniles generally comprise the majority of earthworm populations in managed soils. For instance, Liu *et al.* (2025) reported a mean of 37.8 % adults in croplands, Torppa *et al.* (2024) found 33 % adults among 2419 earthworms, and Domínguez and Bedano (2016) found juveniles comprised ~70 % of the earthworm population. At Whirlow Hall farm, the percentage of aclitellates was significantly higher than at Spen farm (76.1 % vs. 54.9 % respectively). This higher percentage mirrors Singh *et al.* (2021) finding of ~76 % juveniles from their earthworm extractions and Liu *et al.* (2025) finding of 77 % juveniles in grasslands. Llanos *et al.* (2025) documented an even higher percentage (82.8 %) of the total earthworms as juveniles. These comparisons highlight that while juveniles typically comprise the majority of earthworms in agricultural soils, site-specific conditions such as soil type, management and disturbance regimes shape the relative proportions.

The percentage of clitellate earthworms also varied seasonally, with aclitellate earthworms most dominant in summer, particularly in June when all *Ap. longa* were aclitellate at both farms. This agrees with Eggleton *et al.* (2009) who reported an absence of mature earthworms from more than half of their soil pits in the drier months, and Walsh *et al.* (2019) who observed that adults accounted for ~50 % of populations between April and June before declining to very low percentages in summer. Eisenhauer *et al.* (2009) similarly found that juveniles dominated populations in both spring (~79 %) and autumn (~75 %), but their relative abundance and biomass differed between seasons. Juveniles were relatively more abundant and had a greater biomass in spring, while adults were more abundant and had a higher biomass in autumn (Eisenhauer *et al.*, 2009). However, no such patterns of density were evident at farms in the present study. At Spen farm, the percentage of clitellate earthworms increased with soil moisture until stabilising at a ~50:50 split at ~30 vol% moisture. This is consistent with Wever *et al.* (2001), who found that juvenile *Ap. tuberculata* failed to become clitellate below 25 wt% moisture. Conversely, there was no significant effect of soil moisture on the percentage of clitellate earthworms at Whirlow Hall farm, and the lowest percentage was recorded in December when the soil was saturated, suggesting that while low moisture limits development, excessive waterlogging can also suppress maturation. Moreover, the density of clitellate earthworms was significantly associated with soil moisture at both farms and decreased above ~40 vol% at Spen farm. In contrast, the density of aclitellate earthworms was only significantly affected by soil moisture at Whirlow Hall farm, with aclitellate densities remaining relatively stable irrespective of soil moisture at Spen farm but varying with soil temperature and reflective of total earthworm densities, peaking at ~9 °C. Together, these findings reinforce soil moisture as a primary driver of earthworm development and reproductive investment, with both lower and upper thresholds constraining maturity.

Importantly, aclitellate dominance may not solely reflect juvenile abundance. It was observed in previous laboratory experiments, and has been documented in the literature, that some species reabsorb their clitellum and gonads during aestivation and in response to extreme climatic conditions (Jiménez *et al.*, 2000; Edwards and Bohlen, 1996), making them harder to distinguish from juveniles when identified solely on secondary sexual characters. For instance, Baker *et al.* (1993) found that 60 % of the *Ap. rosea* identified as subadults between April and June were in fact adults with regressed clitella. This suggests that moisture stress, rather than recruitment of juveniles alone, may partially explain seasonal shifts in apparent age structure. Where possible, future studies should aim to differentiate true juveniles from adults with regressed clitella, as this distinction has major implications for interpreting earthworm population dynamics and reproductive potential. Key questions remain including how quickly earthworms regain reproductive function once favourable conditions return. In addition, it is not yet known whether all earthworm species are capable of this regression.

Soil pH also significantly influenced age structure, with the percentage of clitellate earthworms declining as pH became closer to neutral at both farms. The density of clitellate earthworms also decreased with increasing pH at Whirlow Hall farm, whereas this relationship was positive at Spen farm. Neutral soil pH is generally associated with higher earthworm abundance and biomass (Johnston, 2019; Hoeffner *et al.*, 2021) and so more clitellate earthworms were expected to be found in the higher pH soils. However, it could be that the lower percentage of clitellate earthworms reflects more suitable conditions for cocoon hatching, increasing the fraction of juveniles. Alternatively, the patterns observed may instead reflect covariation with soil moisture as some of the higher soil pH values (e.g. June at Spen farm and December at Whirlow Hall farm) coincide with months where soils were under moisture stress when clitellum regression might also be most prevalent.

Species-specific differences in maturity were also apparent. Burrowers such as *Ap. longa* and *L. terrestris* and litter-dwellers such as *L. rubellus* and *L. festivus* were more frequently found to be aclitellate than clitellate throughout the year, suggesting possible differences in vertical distribution or maturation dynamics. For instance, Grant (1955) proposed that adults are more capable of migrating to deeper depths to avoid harsh conditions as they produce more coelomic fluid during desiccation which may keep them mobile. They may also be better able to penetrate the soil than smaller immature individuals. However, this cannot explain the higher frequency of aclitellate litter dwellers, which live at the soil surface and do not tend to retreat deep into the soil. Alternatively, differences in developmental timing of species may lead to adults of some species appearing sooner (Walsh *et al.*, 2019) and thus being observed more frequently. For instance, faster-maturating species or those with broader moisture tolerance ranges may produce detectable adults over a wider temporal window, whereas slower-maturing species and those with narrower tolerance ranges remain aclitellate for longer as the opportunity for maturation will be more restricted. Species-specific maturation rates have been reported by Lowe and Butt (2002) who found *Ap. longa* took twice as long to become clitellate than *Al. chlorotica* at 15 °C (24 versus 12 weeks respectively). Moreover, Lowe and Butt (2007) demonstrated that maturation rates in *Al. chlorotica* (which has both a green and pink morph) varied both by morph and soil moisture. After 24 weeks, 87 % of the green morph were clitellate under moist conditions (29 wt% moisture), compared to only 18 % in drier conditions (21 wt% moisture). In contrast, 38 % of the pink morph reach maturity regardless of soil moisture conditions (Lowe and Butt, 2007). Accordingly, Plum and Filser (2005) suggest high juvenile to adult ratios are a result of repeated hydrological stress (floods and droughts).

5.5.4 Aestivation

Patterns of aestivation were strongly seasonal, with pronounced peaks in May and June and a smaller rise in December. At both farms, the same four species were observed in aestivation: *Al. chlorotica* (shallow bioturbator), *Ap. rosea* (intermediate), *Ap. longa* (burrower), and *Ap. caliginosa* (burrower). All of these species have been reported to aestivate previously. For example, *Ap. longa* was observed entering aestivation in May and re-emerging between August and September (Morgan and Winters, 1991). Although endogeic species are generally considered to be more likely to adjust their activity in response to shifts in moisture and temperature driven by climate change than anecic species (Potvin and Lilleskov, 2017), the presence of *Ap. longa* in aestivation suggests that this strategy is not strictly tied to functional group.

The maximum monthly percentage of aestivating earthworms reached ~75 % at Spen farm (May) and 50 % at Whirlow Hall farm (June). In most months, both active and aestivating individuals of the same species co-occurred, although in June at Spen farm, *Al. chlorotica* and *Ap. longa* were exclusively found in aestivation. This inter-individual difference in activity state cannot be explained by maturity as, contrary to the prediction, there was no significant difference in the likelihood of aestivation between aclitellate and clitellate earthworms. This is consistent with Díaz Cosín *et al.* (2006), who also reported no maturity-related differences in *Hormogaster elisae* (Álvarez, 1977) aestivation. Similarly, Garnsey (1994) found both juveniles and adults in aestivation, although smaller individuals were found nearer the soil surface. However, it is also important to consider that some of these aclitellate individuals may in fact be reproductively mature individuals that had regressed their clitellum, further complicating classification. That some individuals remained active even under conditions that induced aestivation in conspecifics suggests both species- and individual- level variation in stress responses.

As expected, aestivation was strongly driven by soil moisture. The relationship was non-linear, with most aestivation occurring below ~25-30 vol%, negligible rates at intermediate values and an increase when soils exceeded ~45-50 vol%. Walsh *et al.* (2019) also observed the onset of aestivation generally occurred at average soil moisture contents below 25 vol% (14.4 ± 5.5 vol% – 22.9 ± 3.9 vol%). Accordingly, the highest rates of aestivation occurred in May and June under the lowest soil moisture conditions at the farms in the present study. This aligns with observations of maximum activity during wet winter months and early spring and widespread aestivation during summer coinciding with low moisture and high temperatures (Gerard, 1967; Garnsey, 1994; Baker *et al.*, 1993; Potvin and Lilleskov, 2017). Further support comes from Díaz Cosín *et al.* (2006) laboratory experiments which found the highest percentage of aestivating *H. elisae* in summer (40.7 %), followed by spring (29.6 %), with lower rates in winter (18.5 %) and autumn (11.1 %) under constant temperature (18 °C). Importantly, aestivation rarely occurred when the soil moisture was 20 wt%, even in summer (Díaz Cosín *et al.*, 2006). Notably, no aestivation was recorded in July, even though it had one of the warmest soil temperatures at Spen farm, confirming Walsh *et al.* (2019) finding that providing soil moisture conditions are sufficient, earthworms can tolerate high temperatures. In addition, aestivation was observed in December at both farms when soils were saturated (~55-60 vol%), implying that waterlogging and associated oxygen limitation can also trigger aestivation. Species differed in their tolerance ranges as while *Ap. longa*, *Ap. caliginosa* and *Al. chlorotica* were found aestivating under both dry and saturated conditions, *Ap. rosea* were only found aestivating in dry soils. Cold temperatures may also contribute to overwinter aestivation, as reported by Potvin and Lilleskov (2017) who found that 30 % of the *Ap. caliginosa* aestivated during the driest month (August) while 50 % aestivated during the coldest month (February). However, this did not explain the December observations, since colder soils in January and February showed little aestivation. These results indicate that aestivation is not a fixed, obligatory seasonal diapause, but rather an environmentally induced survival strategy driven by both desiccation and anoxia.

Water availability to earthworms is ultimately determined not only by water content but by water potential, which varies with depth and soil texture (Kretzschmar and Bruchou, 1991). In the present study, aestivation was most frequent at pF values >3.5 (**Fig. S5.2**), when free water is typically no longer readily available in soil (Friis *et al.*, 2004). This is also consistent with findings that earthworm activity is limited above pF 3-3.3 (Gerard, 1967; Kretzschmar and Bruchou, 1991; Nordström, 1975; Rundgren, 1975; Baker *et al.*, 1993). Upper soil layers lose water at a faster rate than deeper layers (Nepstad *et al.*, 2002), which are more buffered and consequently moisture fluctuations were more pronounced in surface soils (0-5 cm) than deeper in the profile (20-25 cm). Rather than aestivate, some individuals may therefore avoid desiccation by moving to deeper depths where water potential remains within viable thresholds (Kretzschmar and Bruchou, 1991). For example, anecic earthworms such as *L. terrestris* are known to exploit this strategy and can maintain activity under harsh soil conditions by retreating deeper into their vertical burrows (Potvin and Lilleskov, 2017). Similarly, Baker *et al.* (1993) observed that most earthworms were concentrated in the top 10 cm of soil during wet periods but retreated deeper in drier months. Observations suggest some species are forced below 20-30 cm in arid conditions, beyond typical sampling depths (Ahmed *et al.*, 2025; Potvin and Lilleskov, 2017). Subsequently, hand-sorting may underestimate the presence of anecic earthworms relative to shallower burrowing species (Chan, 2004; Callaham and Hendrix, 1997). Aiding detection of such species, eDNA-based approaches offer a more comprehensive method for assessing community composition (Llanos *et al.*, 2025), though they have limited capacity to reliably determine abundances and cannot provide information on earthworm maturity and activity states. In the present study, hand-sorting was chosen as the most suitable method as chemical extraction methods (e.g. mustard suspension) may prematurely awaken dormant earthworms, confounding estimates of aestivation. Although Garnsey (1994) found most earthworms aestivated between 15-20 cm irrespective of species, soil texture, or moisture content, any inactive earthworms below this depth would have been missed here.

Soil bulk density also appeared to influence the percentage of earthworms in aestivation. Surprisingly, most aestivating individuals were found in lower-density soils, contrary to the prediction that denser soils would constrain movement and promote aestivation. This may indicate that aestivation chamber formation was easier in less dense soils, however, the highest earthworm densities were also found in denser soils. These apparent contradictions are likely in part explained by the strong correlation between soil moisture and bulk density. In general, wetter soils were denser and some soil slumping was observed, particularly at Spen farm, likely due to reduced friction between soil particles weakening the aggregate structure and decreasing porosity. Whereas the lower density of drier soils may be a result of soil particles, mainly clay, shrinking and forming cracks (Gimbel *et al*., 2016), thus reducing the mass of soil per volume. Therefore, the effects of bulk density were largely secondary to soil moisture given its influence on the soil texture. Moreover, while higher bulk densities can impair the movement of soil organisms, the effects of compaction are likely more severe on larger organisms (Nawaz *et al.*, 2013) and there is evidence to suggest that bulk density only becomes a limiting factor for soil organisms and their functioning when it reaches 1.7g/cm^3 (Beylich *et al.*, 2010), well above the densities recorded here (0.9-1.1 g/cm^3). Observations of aestivation were restricted to only a few months in the present study and so clearer associations with soil properties may be expected to emerge with greater incidences of aestivation.

5.5.5 Community recovery

Despite sharp declines in earthworm densities during drought, populations recovered rapidly when soil moisture returned. For example, earthworm densities almost doubled from June to July as surface soil moisture rose from ~16 vol% to ~40 vol%. This was mostly driven by clitellate individuals whose densities at both farms more than doubled (increased by >265 %) from June to July, whereas the density of aclitellate earthworms increased by

~38 % at Spen farm and ~93 % at Whirlow Hall farm. Therefore, this rebound likely reflects upward migration of clitellate individuals from deeper layers where conditions remained viable or recolonisation from surrounding areas acting as refuges during the harsh conditions. Emergence of hatchlings on return to favourable conditions likely also played a role, as some species produce drought-resistant cocoons capable of withstanding extreme desiccation (Petersen *et al.*, 2008). For instance, *Dendrobena octaedra* cocoons can survive up to ~95 % water loss under realistic drought conditions and when desiccation occurred gradually (over 10-days), compared to acutely, they lost water at a slower rate and had more time to accumulate osmolytes (particularly sorbitol and alanine) (Petersen *et al.,* 2008). Such adaptations enable earthworm populations, especially litter dwellers, which are exposed to the harshest conditions with limited ability to escape, to recover quickly following drought (Eggleton *et al.*, 2009; Holmstrup and Loeschcke, 2003). Although cocoons were rarely encountered in the present study, this is likely due to hand-sorting being an inefficient method for estimating cocoon numbers (Baker *et al.*, 1993). Future studies should therefore quantify cocoons alongside juvenile and adults to better resolve population dynamics under changing soil conditions. Further work investigating the extent to which cocoons can remain viable in desiccating conditions and earthworms can endure in aestivation is necessary to understand the recovery potential of earthworm populations.

Recovery also depends on the environmental context. At Spen farm, in addition to earthworm densities, species richness and diversity also increased following the driest months. From August onwards, litter-dwelling species became more common, coinciding with improved soil moisture conditions and the shift in sampling to the field margins. Field margins are typically less disturbed, enriched by hedgerow litter inputs and often support a greater abundance and diversity of earthworm species compared to conventional arable soils (Prendergast-Miller *et al.*, 2021). Torppa *et al.* (2024) reported greater abundance of epigeic species in soils with higher SOM and moisture, and Hoeffner *et al.* (2021) reported observed higher species richness in grasslands adjacent to hedgerows, especially for epigeic and anecic groups. However, Holden *et al.* (2019) found that field margins and pasture fields at Spen farm had similar soil properties and did not differ significantly in terms of earthworm community composition. Similarly, no distinct differences in soil properties were found between the margins and the main field before August in the present study. Therefore, the greater occurrence of litter dwellers observed from August onwards may reflect migration of displaced individuals from the main field following ploughing and conversion to arable cropping. Overall, these findings highlight the importance of habitat heterogeneity in enhancing resilience, by providing local refuges that may buffer against impacts of climatic extremes and disruptive land management practices.

The ability of earthworm populations to rebound will likely depend on the frequency, duration and intensity of drought. The minimum moisture content recorded in the present study was ~14 vol% (20-25 cm depth at Spen farm). Moreover, even in the driest months, earthworm densities remained relatively high >250/m^2, comparable to mean densities of 320 earthworms per m^2 recorded in Swedish grasslands (Torppa *et al.*, 2024). This suggests that the conditions experienced in the present study were within the tolerance range for recovery, but repeated, prolonged droughts are likely to have more severe effects. Epigeic species, in particular, may be vulnerable to local extinctions under recurrent desiccation in long, dry summers (Eggleton *et al.*, 2009). Moreover, greater negative responses of earthworm populations might be expected in more degraded agroecosystems as they are often more vulnerable to the impacts of climate change (Siebert *et al.*, 2019). Evidence from other systems supports this as, for instance, Liu *et al.* (2025) found earthworm communities in grasslands recovered in favourable conditions following drought conditions, whereas those in croplands did not. Consistent with this, Johnston (2019) found that the relationship between climate and earthworm communities was largely underpinned by plants and their functional traits. In contrast to monoculture-dominated croplands, grasslands and pastures typically support greater plant species richness, which has been positively correlated with earthworm abundances (Zaller and Arnone, 1999; Barnes *et al.*, 2023). This suggests that management practices maintaining plant diversity and soil structure may be as critical as climatic conditions in shaping recovery potential. It would be valuable to explore earthworm dynamics across soils under different management practices to better understand the factors that promote and restrict their activity. This could help predict the impacts of climate change on earthworms and broader ecosystem functioning, useful in guiding strategies to mitigate climate-related consequences and enhance resilience (both ecological and economic) in agricultural ecosystems.

Although care was taken to determine activity states, it is possible that the number of aestivating individuals were underestimated in the present study. Soil excavation and hand-sorting may damage some aestivation chambers, causing earthworms to uncoil. However, as noted by Walsh *et al.* (2019) earthworms extracted from aestivation chambers often remain relatively unresponsive and so are usually distinguishable from active individuals. Moreover, it is also important to acknowledge that sampling reflected single monthly timepoints, capturing conditions at the moment of collection, but not shorter-term fluctuations. Therefore, it was not possible to determine whether an individual had been aestivating in the days preceding sampling nor whether it would have entered aestivation in the following days. Some individuals showed morphological features that may indicate they had recently been in aestivation e.g. constricted and yellow caudal segments (observed mainly in those aestivating, but also some found active) (**Fig. S5.18**). This is possibly linked to metabolic shifts during aestivation and a build-up of excretory products due to a period of inactivity and subsequent concentration of the coelomic fluid associated with desiccation. For instance, Tilikj and Novo (2022) investigated transcriptional changes associated with aestivation and found genes coding for proteins involved in energy-consumptive processes such as digestion are switched off while those leading to nitrogenous waste accumulation are upregulated, possibly as a mechanism for mitigating stress.

5.6 Conclusion

Here a non-destructive DNA extraction method and metabarcoding in combination with random forest modelling was successfully applied to identify individual earthworms to species level, presenting a novel method of earthworm identification. Overall, earthworm communities at each farm were distinct with 11 species present at Spen farm and 8 at Whirlow Hall farm. Although largely dominated by the same few species, seasonal shifts in community composition were pronounced, with richness and diversity generally lowest during late spring/early summer droughts and highest in autumn. Across both farms, most individuals were aclitellate, consistent with existing findings for agricultural contexts. This likely reflects both differences in timing of maturation and development and environmental constraints such as those imposed by soil moisture, pH and SOM content. Regression of the clitellum under stressful conditions, particularly during aestivation, likely also contributes to the percentage of aclitellate individuals, however the consequences of this for subsequent reproduction are unknown. Climate change-driven increases in drought and flooding might be expected to exacerbate hydrological stress and thus reduce the duration of conditions suitable for reproduction and maturation.

Aestivation was a key survival strategy, occurring primarily under low soil moisture (below ~30-25 vol%) but also under saturated conditions (~55-60 vol%), suggesting that both desiccation and hypoxia may trigger this behavioural response. Local conditions such as soil texture and bulk density also modulate responses governed by soil moisture. Only four species, *Al. chlorotica*, *Ap. rosea*, *Ap. longa* and *Ap. caliginosa* were observed aestivating, with active and dormant individuals co-occurring, demonstrating species and individual level variation in stress responses. However, this could not be explained by differences in maturity as aclitellate and clitellate earthworms showed similar likelihoods of aestivation. These four species were also the most abundant earthworms over the whole year, likely owing to their broader moisture tolerance ranges and ability to aestivate. In contrast, rare species were confined to specific moisture niches, emphasising the role of microhabitat heterogeneity in sustaining biodiversity. With an increased frequency and severity of droughts a shift in earthworm community compositions may therefore be expected, favouring species with broader tolerance ranges and adaptive survival strategies, with subsequent changes to the delivery of ecosystem services.

Rapid population recovery following increases in soil moisture, likely primarily facilitated by a combination of migration from deeper depths and neighbouring soils but also hatching of drought-resistant cocoons, highlights the resilience of earthworm communities. However, the capacity of earthworm populations to recover and return to normal functioning is likely to be context-dependent with intensely managed landscapes potentially less resilient due to greater soil degradation and lower biodiversity.

Overall, these findings reinforce the central role of soil moisture as the primary driver of earthworm abundance, richness, and functional composition, but also emphasise the importance of species-specific strategies and landscape context in mediating resilience. This illustrates the combined influence of environmental conditions and anthropogenic pressures on soil ecosystem structure and resilience. Greater temporally and spatially intensive sampling is required to develop a more comprehensive understanding of seasonal earthworm activity and community composition and gain further insights into the potential impacts of climate change on earthworm populations. This could include deeper sampling, continuous soil moisture and temperature monitoring and a combination of hand-sorting and eDNA techniques to avoid disturbing dormant individuals whilst capturing diversity in the most efficient and reliable way.

5.7 Acknowledgements

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5.9 Supplementary materials

Figure S5.1. Warren paddock at Spen farm prior to conversion to arable cropping (left), and Long jump field at Whirlow Hall farm (right).

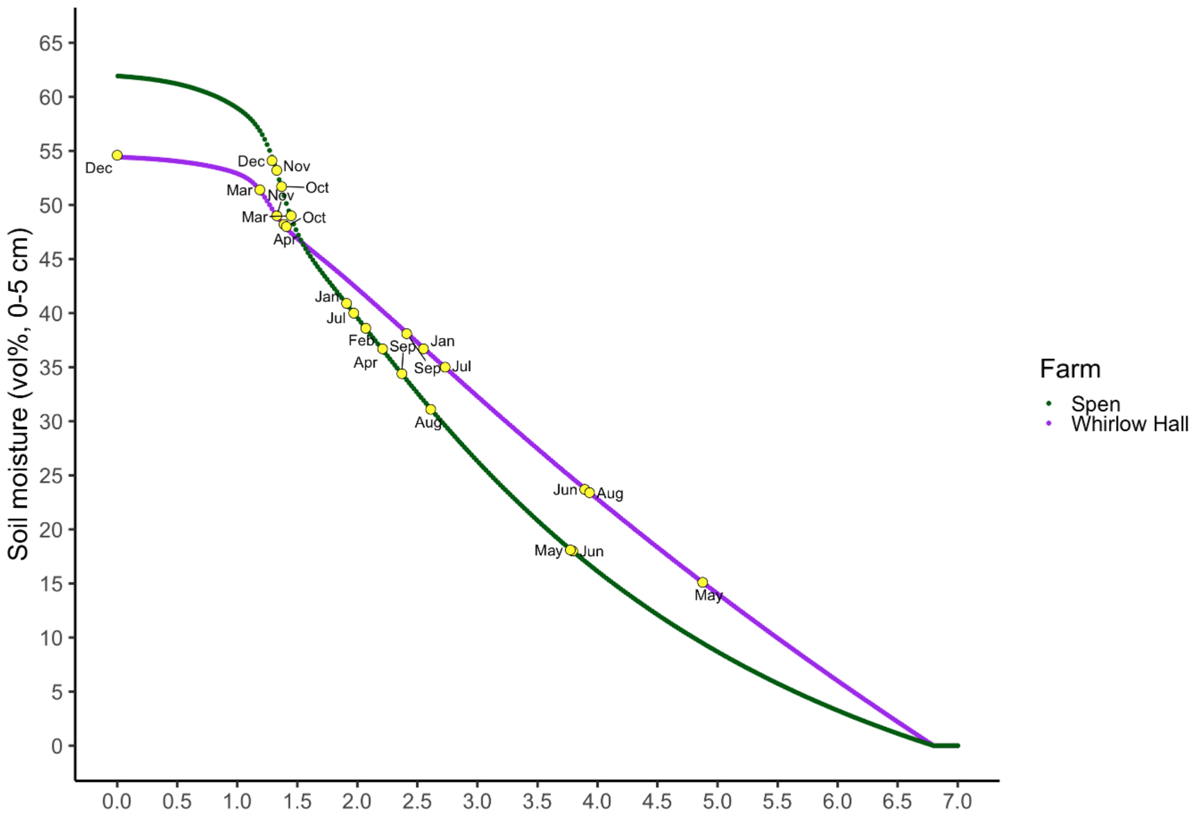

Figure S5.2. Water retention curves for soils from Spen and Whirlow Hall farm (densities of 0.9 and 1.1 g/cm^3 respectively). Data are generated from HYPROP values with curves fitted based on the PDI-variant of the binomial constrained van Genuchten model (m = 1 -1 /n).

Hyprop method:
For each farm, a core (250 ml) was packed with soil. A nonwoven cloth was placed on one side along with a saturation plate before the core was gently placed into a tub filled with deionised water up to 5 mm below the sample rim and left overnight to fully saturate through capillary action until the surface was shiny. The tensio shafts have a porous ceramic tip and a shaft which was filled with water which was degassed by vacuum to avoid air bubbles. The sensor unit was also filled with degassed water. Each tensio-shaft was gently screwed into the sensor unit, and the pressure was monitored to ensure it was increasing but not exceeding 200 kPa to avoid damaging the pressure sensor. A silicone gasket was added to avoid soil entering the sensor unit and several function checks (speed of response, zero point etc.) were carried out to ensure the pressure was responding as intended. Two holes were made in each soil core using an auger and the sensor unit was inverted over the soil core so that the tensio shafts could be carefully inserted into the holes making sure not to compress the soil. The soil core was upturned, saturation plate and cloth removed and then the soil sample was fixed to the sensor unit using clips and placed onto a balance. The balance was connected to a computer with the HYPROP software and measurements commenced. Recording was terminated when the soil sample had reached the air entry phase whereby the tension value drops abruptly to zero as air enters the ceramic tips. The soil core was removed from the sensor unit and oven dried at 105 °C for 24 hours to determine the dry weight of the soil sample.

Ammonium acetate precipitation (modified from Nicholls *et al.*, 2000; Richardson *et al.*, 2001)**:**

1. For each sample, add 10 µl Proteinase K (10 mg/ml) and 300 µl Digsol (to make 500 ml: 20 ml 0.5M EDTA, 3.425 g NaCl, 25 ml 1M Tris-HCl and 430 ml ddH_2O, then add 25 ml 20 % SDS after autoclaving) to a 1.5 ml flip-top Eppendorf tube.
2. Swab the earthworm using a sterile cotton swab and add to the tube, snapping wood so the lid will close.
3. Vortex and then place into rotating oven at 55 °C for 1 hour to digest.
4. Once digested remove swab using forceps (clean forceps with 10 % bleach between to avoid contamination) and add 300 µl 4M ammonium acetate to each sample
5. Vortex several times over period of at least 15 mins at room temperature to precipitate the proteins i.e. min 5, 10, 15
6. Centrifuge for 10 mins at 13,000 rpm.
7. Pipette the supernatant (clear liquid containing DNA) into a clean labelled Eppendorf tube, discard the protein pellets which may be at the bottom or floating on the top
8. Add 1 ml (1000 µl) of 100 % ethanol
9. Invert the tubes gently several times to precipitate the DNA
10. Centrifuge for 10 mins at 13,000 rpm
11. Pour off ethanol
12. Add 500 µl 70 % ethanol and invert several times to rinse pellet (may not be visible)
13. Centrifuge at 15,000 rpm for 5 mins in case the pellet has dislodged from the bottom of the tube

14. Pour out the ethanol in a smooth movement. Stand tubes upside down on clean tissue for 30-60 mins to dry
15. Once fully dry add approximately 25 µl Low TE (to make 500 ml: 5 ml 1M Tris-HCl, 100 µl 0.5M EDTA, 495 ml ddH_2O and autoclave)
16. Flick sample to dislodge pellet
17. Place tubes in fridge at 4°C overnight to dissolve.
18. Store at -20°C long term in the freezer

PCR1 preparation using primers and extracted DNA:
Dilute primers from stock to 5M (5 ml primer, 95 ml LowTE).
A PCR master mix was made for each of the 24 tagged primers:

- 10 µl of myTaq
- 2 µl of forward primer (one of the 24 EW_B primers)
- 2 µl of reverse primer (EW_H2)
- 2 µl of sterile double distilled water

Into each well of a PCR plate add:

- 4 µl of DNA (or ddH_2O for negative control)
- 16 µl of one of the 24 master mixes

Seal the plate with a clear adhesive film onto the well plate, spin to remove air bubbles and place into an Eppendorf PCR machine with the following program:

- 15 minutes at 95 °C

Then 35 cycles of:

- 30 seconds at 94 °C,
- 90 seconds at 56 °C
- 60 seconds at 72 °C

Followed by 10 minutes at 72 °C.

Making and running the PCR products in a gel:
To make a 1% gel for 100 ml gel tray, in a conical flask add:

- 1 g agarose powder
- 100 ml TBE buffer

Microwave this mixture for 3 mins, swirling the flask at regular intervals (every min) to prevent the gel settling at the bottom.
Once removed from the microwave, ensure there are no crystals visible in the liquid, if there are still crystals then microwave for a bit longer.
Rinse the outside of the flask under cold water to cool the mixture (do not allow to cool too much as the gel will begin to set).
In a fume cupboard add 0.5 µl of Ethidium bromide per every 10 ml TBE buffer.
Tape the edges of the gel tray, insert combs into the tray to form the wells.
Pour the gel and leave to set (~ 20 mins).

For the DNA mixture, in each well add:

- 4 µl distilled water,
- 2 µl of 6x orange g
- 4 µl of the DNA (PCR products)

Try to minimise time between making this mixture and pipetting it into the gel to avoid evaporation.
Remove the comb/s and tape.
Pipette DNA into wells, making sure there is no air in the end of the pipette. Only put the tip of the pipette into the well being careful not to damage the well walls. Pipette a ladder in the first and last well of each comb.
Attach the power cords, set to 110 volts and leave to run for 40-45 minutes.
Remove the gel from the tray and visualise under UV to see the bands of DNA.

Fluorometry:
Make a quantifluor mixture with:
- 19 ml ddH_2O,
- 1 ml TE
- 50 ml dsDNA dye

Seven standards were made to 100, 50, 25, 12.5, 6.25, 3.125 and 0.
To each well of a black plate, add 2 ml DNA (or standard) along with 200 ml of the quantifluor mix before being placed into the fluorometer.

Purification with Promega Pronex beads:
1. Allow an aliquot of beads to reach room temperature (~30 mins), and vortex to resuspend them.
2. Add resuspended Pronex beads (1.5x the volume of each pool – mix well by pipetting up and down 10 times and incubate at room temperature for 5 minutes. *Using 1.5x bead concentration will remove any reagents left over from the first PCR which could affect the following PCR.*
3. Place on a magnetic rack to separate beads from the solution. When the liquid is completely clear, aspirate **the supernatant and discard**. Do not disturb the pellet of separated magnetic beads and do not remove the samples from the magnetic plate.
4. With the samples still on the magnetic place, add 200 µl Pronex wash buffer. Incubate at room temperature for 30 seconds, then carefully aspirate out and discard.
5. Repeat step 4.
6. Allow the beads to dry at room temperature for a few minutes, while on the magnetic stand. *Drying will allow traces of ethanol to evaporate, but over-drying the beads (if the pellet cracks) can significantly decrease elution efficiency*.
7. Remove samples from the magnetic plate and elute with 15 low TE (the same volume as the initial pool)- mix well by pipetting up and down 10 times (ensure that the beads are fully immersed and mixed with the low TE).
8. Place on a magnetic rack to separate beads from the solution (~ 1 minute).
9. Remove solution containing your DNA to a fresh 1.5 ml Eppendorf tube without carrying over any beads.
10. Spin the tubes containing the beads in the centrifuge and replace onto the magnetic rack so that any remaining liquid can be aspirated and added to the new tubes. Discard the beads.

PCR2 to add unique identifying sequences to each sample:
PCR2 was carried out with:

- 10 ml myTaq
- 8 ml DNA
- 1 ml ddH_2O
- 1 ml Illumina primers

The PCR program ran for:

- 15 minutes at 95 °C

Then 12 cycles of:

- 10 seconds at 98 °C
- 30 seconds at 65 °C
- 30 seconds at 72 °C

Followed by 72 °C for 5 minutes.

Quantification of amplicons (PCR products) using Tapestation:
First remove the High sensitivity screen tape and high sensitivity reagents from the fridge for approximately 30 minutes to reach room temperature.
To a Tapestation tube add:

· 2 µl of the high sensitivity buffer
· 1 µl of DNA (PCR products)
· 1 ml ddH_2O

Add the cap then spin on the mini spinner, vortex for 1 minute at 2000 rpm, then spin again. Add the tape to the Tapestation, put samples into the machine and leave to run.

qPCR (QuantStudio 12k flex machine)

1. Make a serial dilution of each library: 100, 1000 and 10000 -fold.
    1. For 100-fold dilution, Combine 99 µl of dilution buffer with 1 µl of the library. Vortex.
    2. For 1000-fold dilution, take out 1 µl from 1st dilution (100-fold) and add 9 µl of dilution buffer. Vortex.
    3. For 10000–fold, take out 1 µl from 2nd dilution (100-fold) and add 9 µl of dilution buffer. Vortex.
2. Repeat step 2 two times to produce three independent dilutions of the library.
3. Prepare SYBR master mix as follows:

· 6 µl SYBR Fast mix + primers
· 2 µl ddH2O

5. Add 2 µl of the kit standards, diluted sample libraries (only 1/1000 and 1/10000), or dilution buffer (no template control) to appropriate wells in the 96-well plate.
6. Dispense 8 µl of the SYBR master mix to the appropriate wells in the 96-well plate.
7. Spin plate and ensure that there are no bubbles at the bottom of wells or debris on the bottom of the plate. Cover plate with a clear adhesive film.
8. Set up reaction on ABI's Quantstudio software. Define the wells used for the standards (task = standard, set appropriate values), no template control (task = NTC), and samples (task = unknown).
9. Set the reaction volume to 10 µl and the following qPCR profile:

5 min at 95°C
35 cycles of the following:
30 sec at 95°C
45 sec at 60°C

10. Determine the concentration of DNA in the pool using the qPCR values.
11. Divide the required concentration (nM) by the actual concentration of the DNA (nM) given by the qPCR machine and then multiply by the volume wanted (ml) to determine the volume of pooled DNA needed to make the next dilution. Make the remainder of the dilution up with a volume of LowTE determined by subtracting the volume of DNA needed from the total volume wanted.
12. Repeat qPCR on diluted DNA until a final concentration of 4 nM is reached.
13. The final pool is now ready for Illumina sequencing.

Table S5.1. The sequence of each of the 24 forward EwB primers with unique 10bp tags (red).

Primer	5' to 3' sequence
EwB_F_BC1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAACAAACACCAAGAAGACCCTATAGAGCTT
EwB_F_BC2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATTAGCTGGGCAAGAAGACCCTATAGAGCTT
EwB_F_BC3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCACGAGTTAACAAGAAGACCCTATAGAGCTT
EwB_F_BC4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTACAATCATCAAGAAGACCCTATAGAGCTT
EwB_F_BC5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCAGGATACCAAGAAGACCCTATAGAGCTT
EwB_F_BC6	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTTTAGGCCTCAAGAAGACCCTATAGAGCTT
EwB_F_BC7	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAACTAGCAACAAGAAGACCCTATAGAGCTT
EwB_F_BC8	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGGGCTCACCAAGAAGACCCTATAGAGCTT
EwB_F_BC9	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGCCACGTTCAAGAAGACCCTATAGAGCTT
EwB_F_BC10	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGAAATTCGTCAAGAAGACCCTATAGAGCTT
EwB_F_BC11	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACCTTAGATCAAGAAGACCCTATAGAGCTT
EwB_F_BC12	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTACTGCCGACAAGAAGACCCTATAGAGCTT
EwB_F_BC13	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAACCACCAGCAAGAAGACCCTATAGAGCTT
EwB_F_BC14	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGTTGAGGACAAGAAGACCCTATAGAGCTT
EwB_F_BC15	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTATACCCGGGCAAGAAGACCCTATAGAGCTT
EwB_F_BC16	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTCTCGCGCCAAGAAGACCCTATAGAGCTT
EwB_F_BC17	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACCACCTAATCAAGAAGACCCTATAGAGCTT
EwB_F_BC18	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGATTACTCACAAGAAGACCCTATAGAGCTT
EwB_F_BC19	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGGGCCGATTCAAGAAGACCCTATAGAGCTT
EwB_F_BC20	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAGGGATGGTCAAGAAGACCCTATAGAGCTT
EwB_F_BC21	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGAATGCACACAAGAAGACCCTATAGAGCTT
EwB_F_BC22	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTTGTCTTAGCAAGAAGACCCTATAGAGCTT
EwB_F_BC23	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGCGGTTGGTCAAGAAGACCCTATAGAGCTT
EwB_F_BC24	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTAATCTAGCAAGAAGACCCTATAGAGCTT

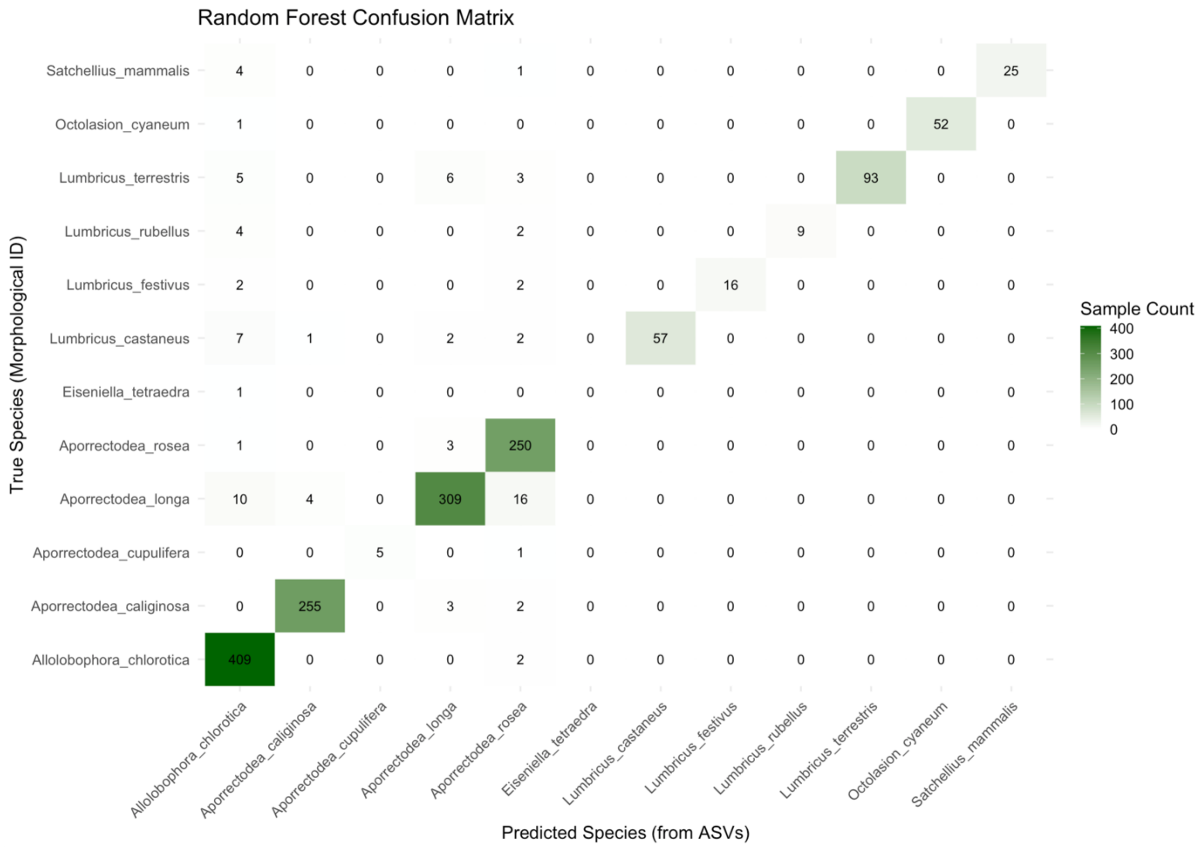

Figure S5.3. The confusion matrix of predicted species (from ASV counts) against the true identification produced by the random forest model, trained using data from the known samples identified morphologically.

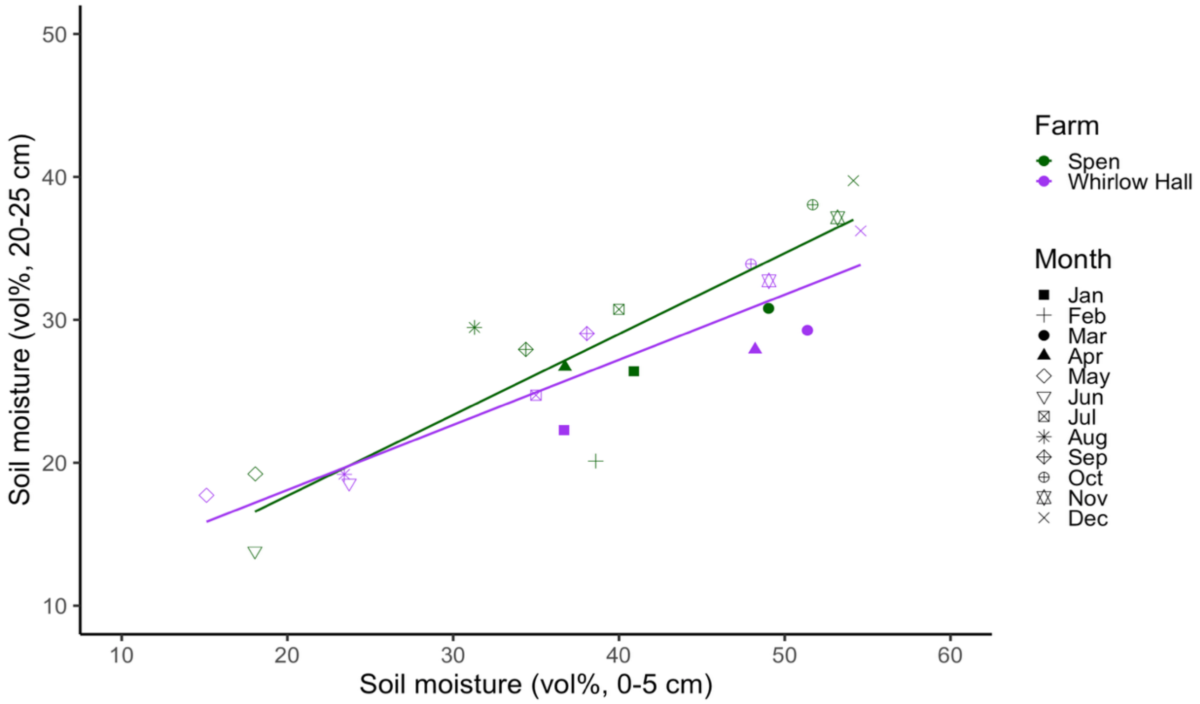

Figure S5.4. The relationship between mean soil moisture content (vol%, n = 6) at 0-5 cm and 20-25 cm depth in both farms (green = Spen, purple = Whirlow Hall). Shapes represent each month. Lines are fitted using linear regression: Spen y =0.537x + 7.496, Whirlow Hall y = 0.444x + 9.435.

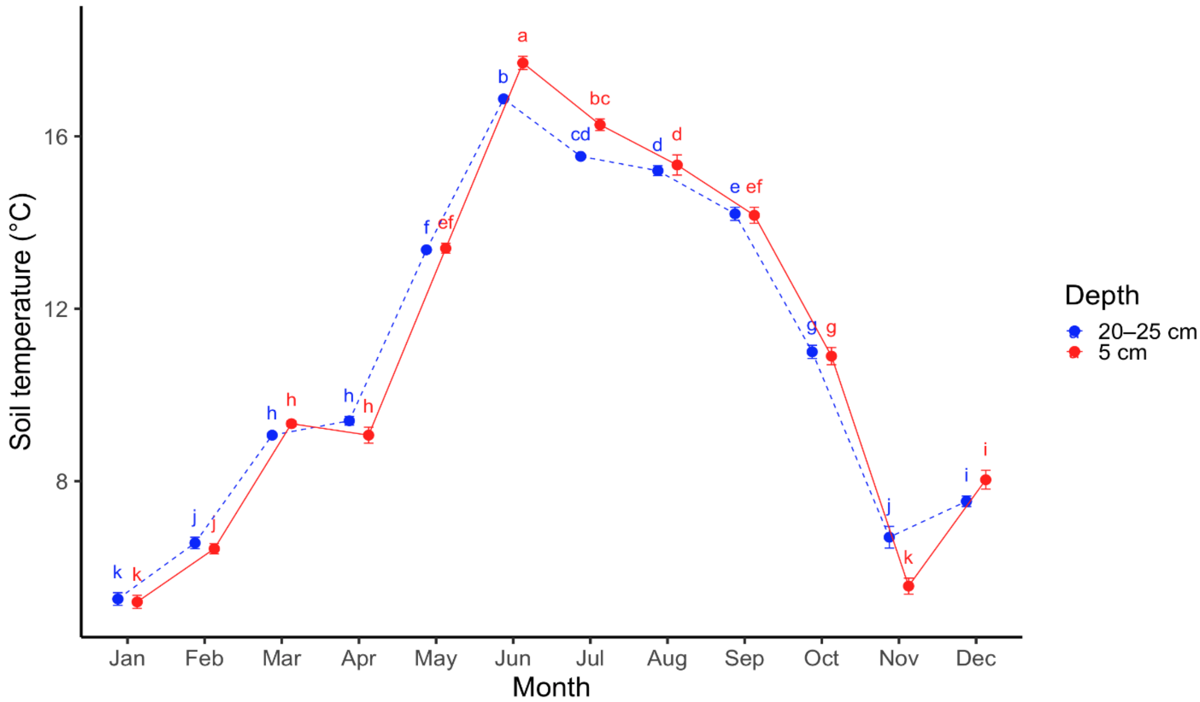

Figure S5.5. Mean soil temperature (°C, n = 3) each month at Spen farm, measured at depths of 5 cm (red solid line) and 20-25 cm (blue dashed line). Error bars show standard error. Points are offset for ease of legibility. Points with different letters differ to a statistically significant degree ($p < 0.05$).

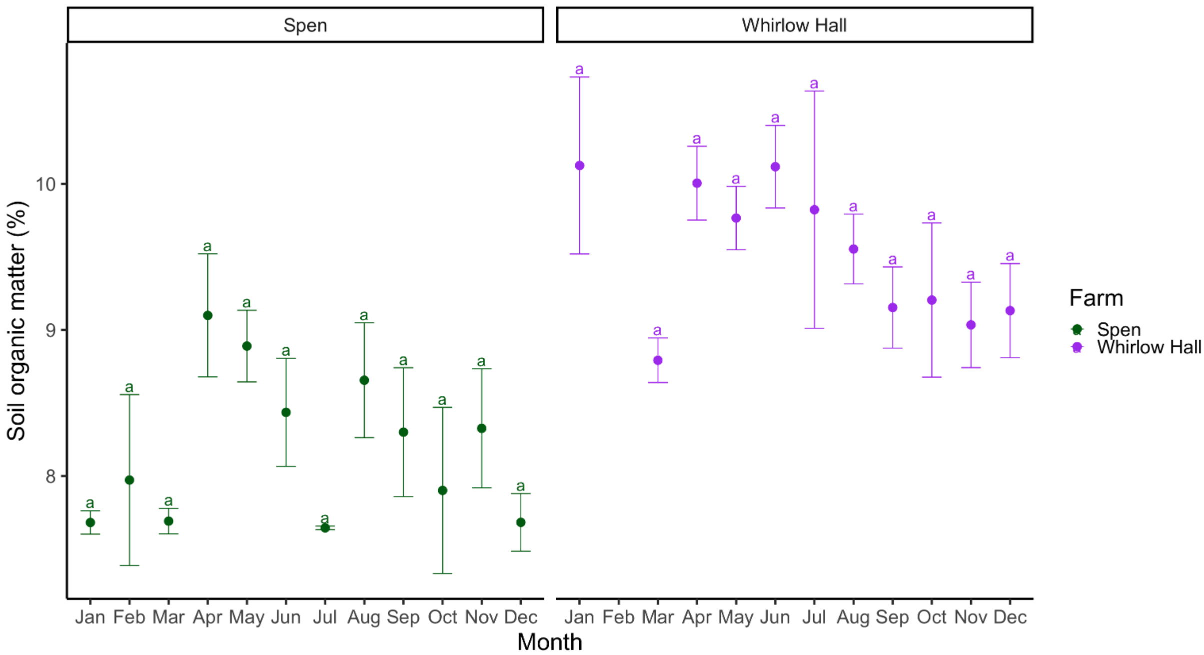

Figure S5.6. Mean soil organic matter (%, n = 3) each month for each farm. Error bars show standard error. For each farm, points with the same letters do not differ to a statistically significant degree ($p > 0.05$).

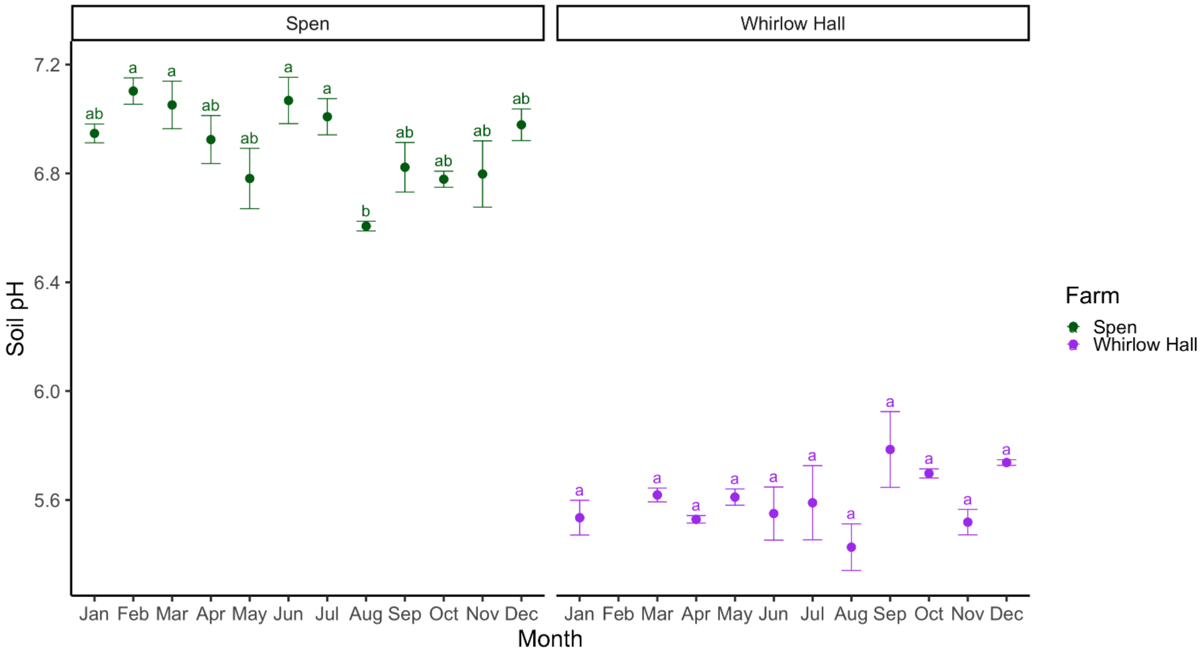

Figure S5.7. Mean soil pH (n = 3) each month for each farm. Error bars show standard error. Points with different letters represent farm-specific statistically significant months ($p < 0.05$).

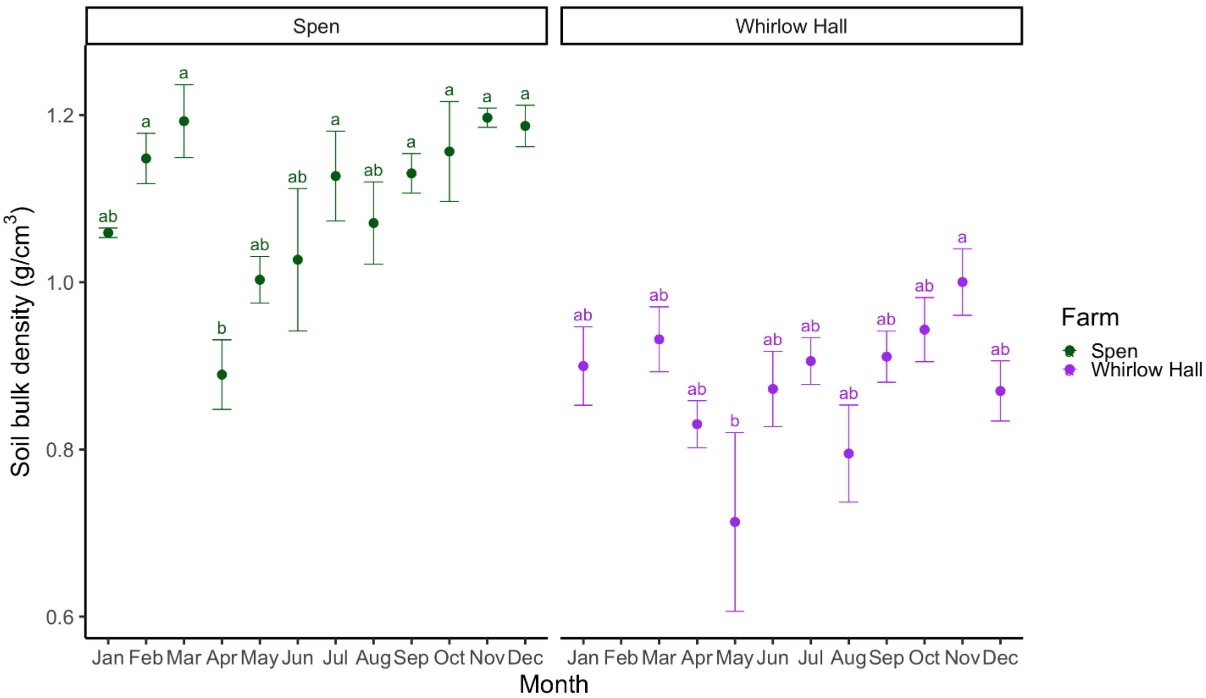

Figure S5.8. Mean soil bulk density (g/cm^3, n = 3) each month for each farm. Error bars show standard error. Points with different letters represent farm-specific statistically significant months ($p < 0.05$).

Table S5.2. The Spearman's rank correlation coefficient values (rho) for relationships between soil parameters at both Spen farm (S) and Whirlow Hall farm (WH). Significant relationships are indicated as (*) = p < 0.05, (**) = p < 0.01, (***) = p < 0.001.

	Soil moisture (vol%, 0-5 cm)	**Soil organic matter (%)**	**Soil pH**	**Soil bulk density (g/cm³)**	**Soil temperature (°C, 5cm)**
Soil moisture (vol%, 0-5 cm)		S = -0.554 (***); WH = -0.551 (***)	S = 0.017; WH = 0.242	S = 0.77 (***); WH = 0.534 (***)	S = -0.567 (***)
Soil organic matter (%)	S = -0.554 (***); WH = -0.551 (***)		S = -0.378 (*); WH = -0.519 (**)	S = -0.511 (**); WH = -0.537 (**)	S = 0.21
Soil pH	S = 0.017; WH = 0.242	S = -0.378 (*); WH = -0.519 (**)		S = 0.052; WH = 0.191	S = -0.07
Soil bulk density (g/cm³)	S = 0.77 (***); WH = 0.534 (***)	S = -0.511 (**); WH = -0.537 (**)	S = 0.052; WH = 0.191		S = -0.35 (**)
Soil temperature (°C, 5 cm)	S = -0.567 (***)	S = 0.21	S = -0.07	S = -0.35 (**)	

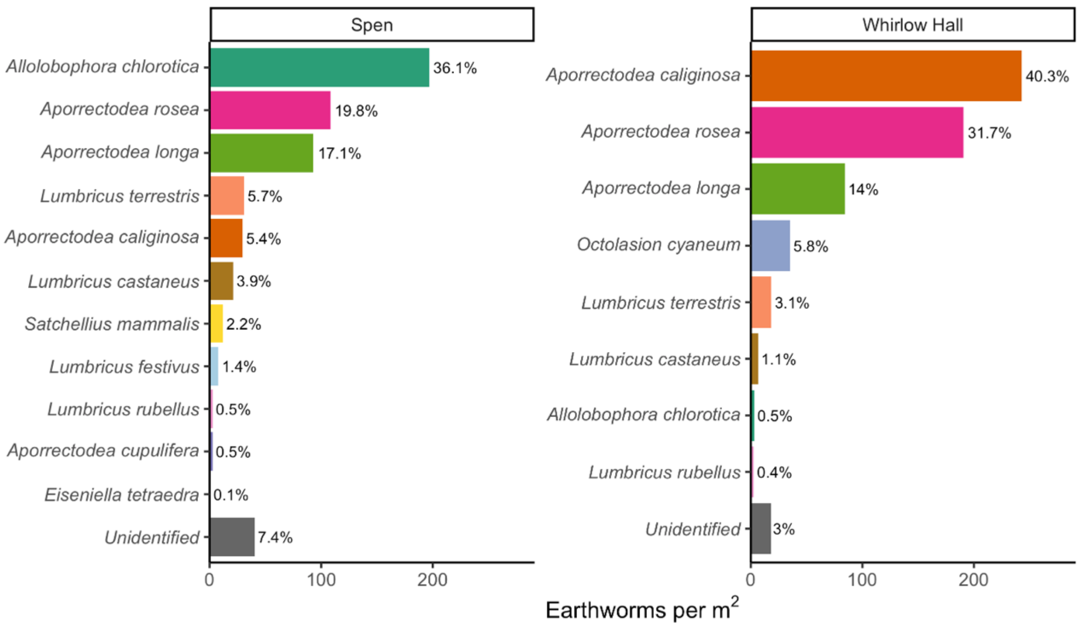

Figure S5.9. The overall density of each earthworm species and their relative abundance (% of the community composition) at Spen and Whirlow Hall farm.

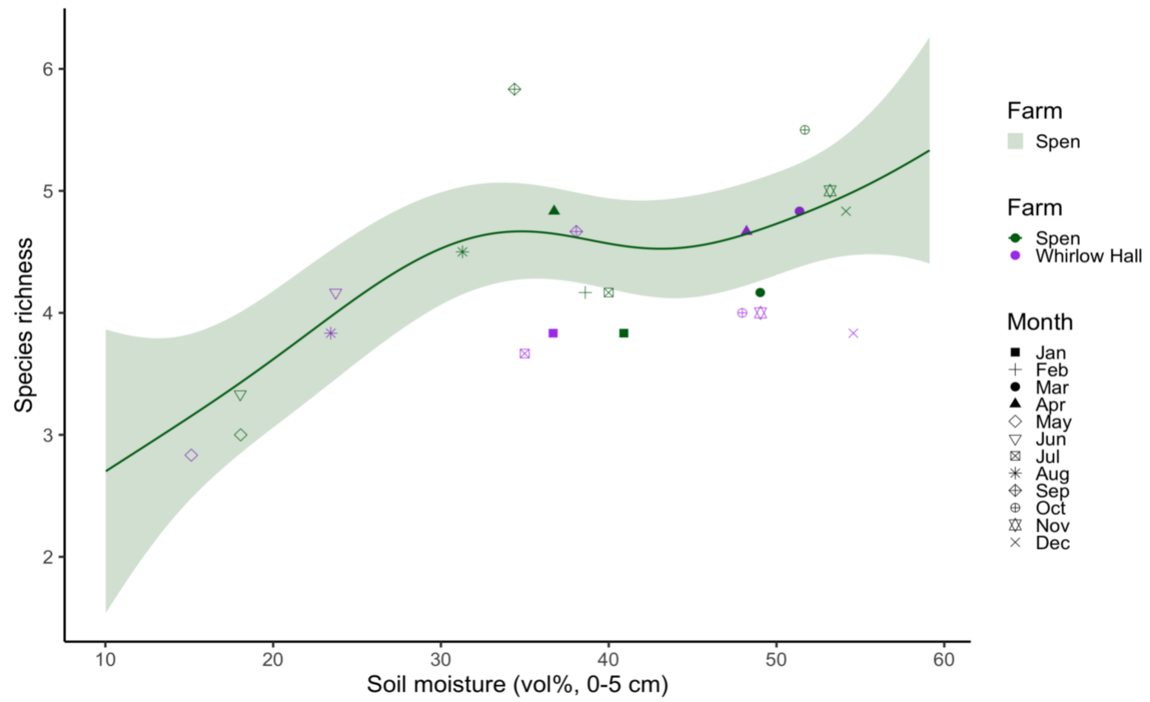

Figure S5.10. The relationship between mean soil moisture content (vol%, 0-5 cm) and earthworm species richness in both farms (green = Spen, purple = Whirlow Hall). Shapes represent each month. Curve estimated using generalised additive model (GAM). Shaded ribbon shows 95 % confidence intervals around the fitted curve.

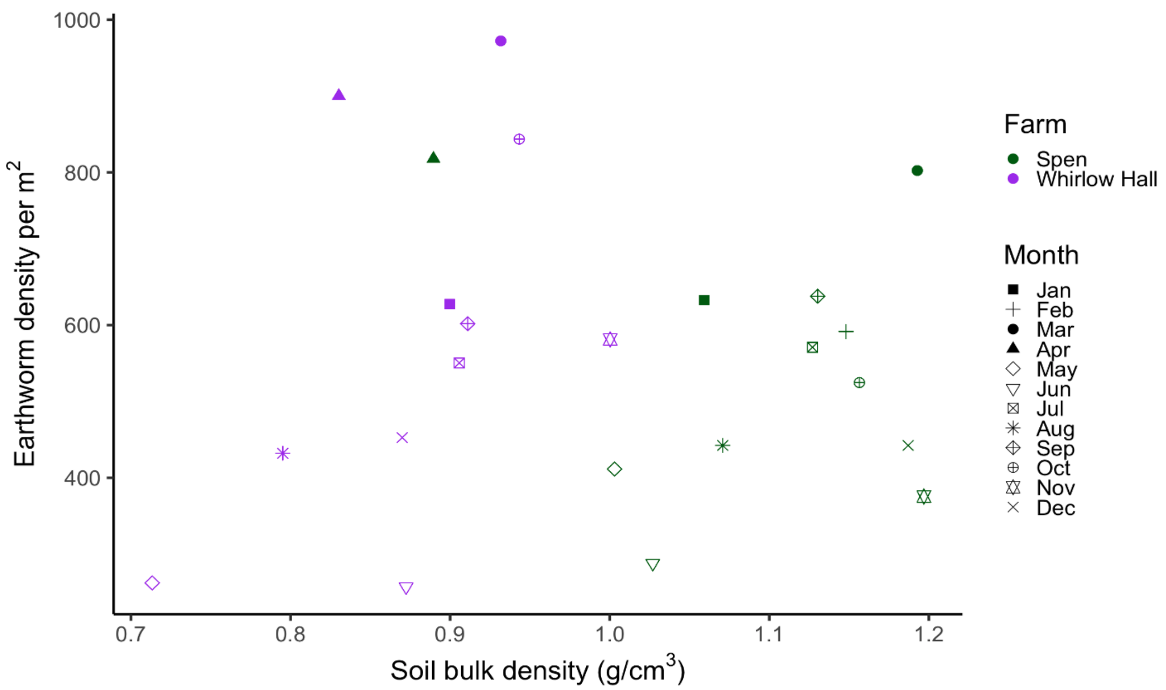

Figure S5.11. The relationship between mean soil bulk density (g/cm^3, n = 3) and earthworm density (individuals per m^2, n = 6) at Spen farm (green) and Whirlow Hall farm (purple). Shapes represent each month.

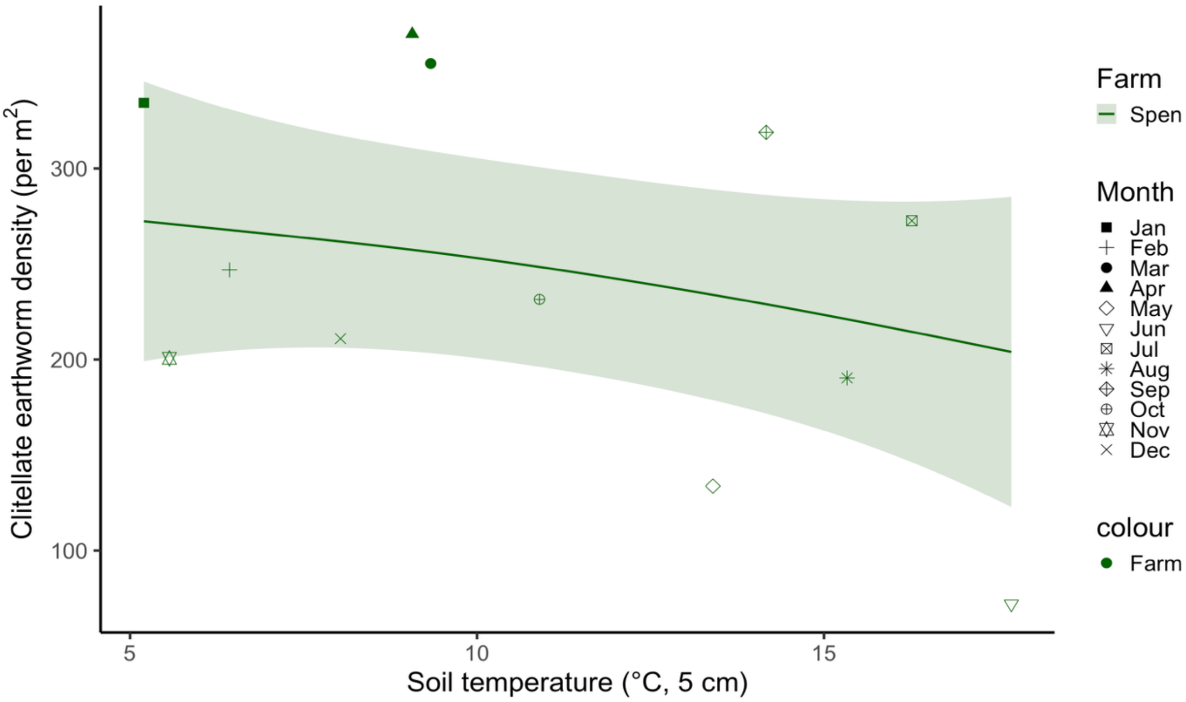

Figure S5.12. The relationship between mean soil temperature (°C, 5 cm, n = 3) and the density of clitellate earthworms (per m^2, n = 6) at Spen farm. Shapes represent each month. The curve is estimated using a generalised additive model (GAM) and the shaded ribbon represents standard error.

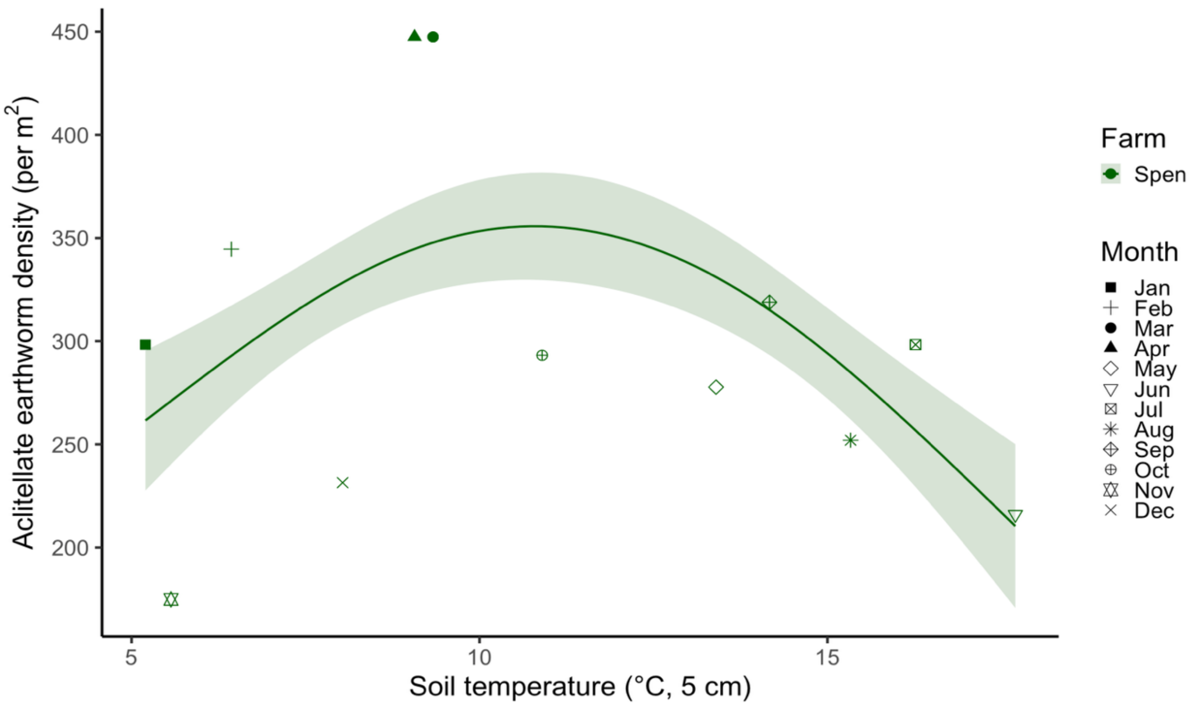

Figure S5.13. The relationship between mean soil temperature (°C, 5 cm, n = 3) and the density of aclitellate earthworms (per m^2, n = 6) at Spen farm. Shapes represent each month. The curve is estimated using a generalised additive model (GAM) and the shaded ribbon represents standard error.

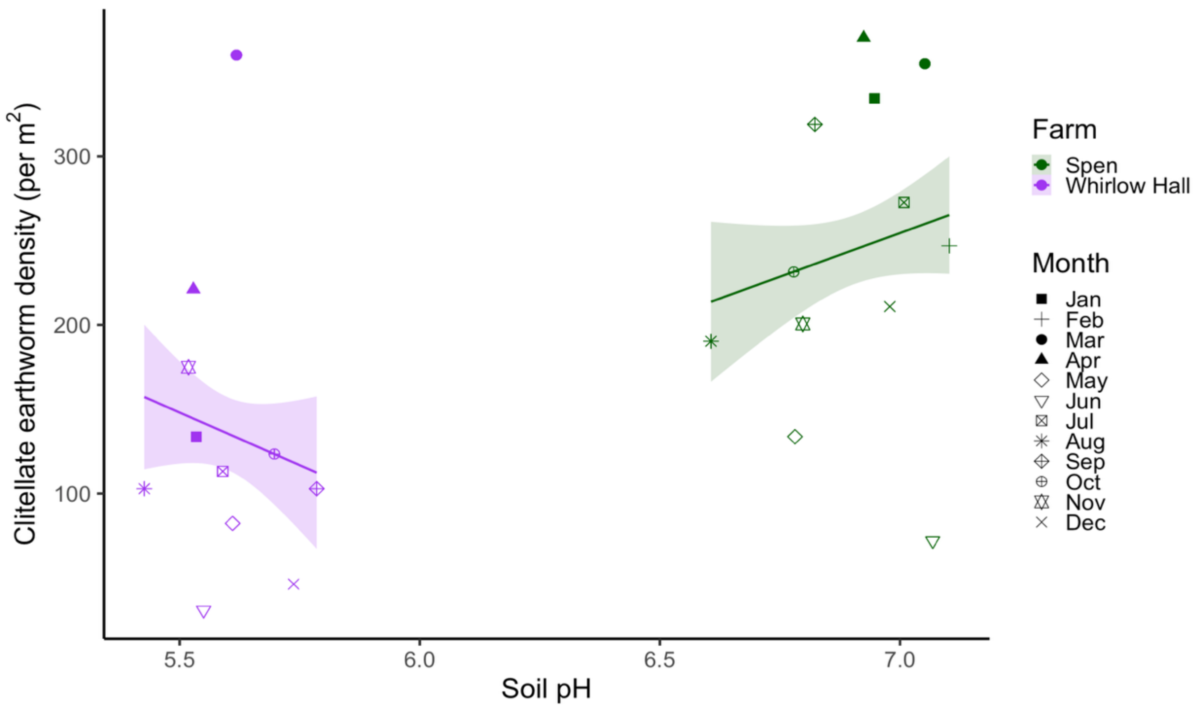

Figure S5.14. The relationship between mean soil pH (n =3) and the density of clitellate earthworms (per m^2, n = 6) in both farms (green = Spen, purple = Whirlow Hall). Shapes represent each month. Lines fitted using linear regression. Shaded ribbons show standard error.

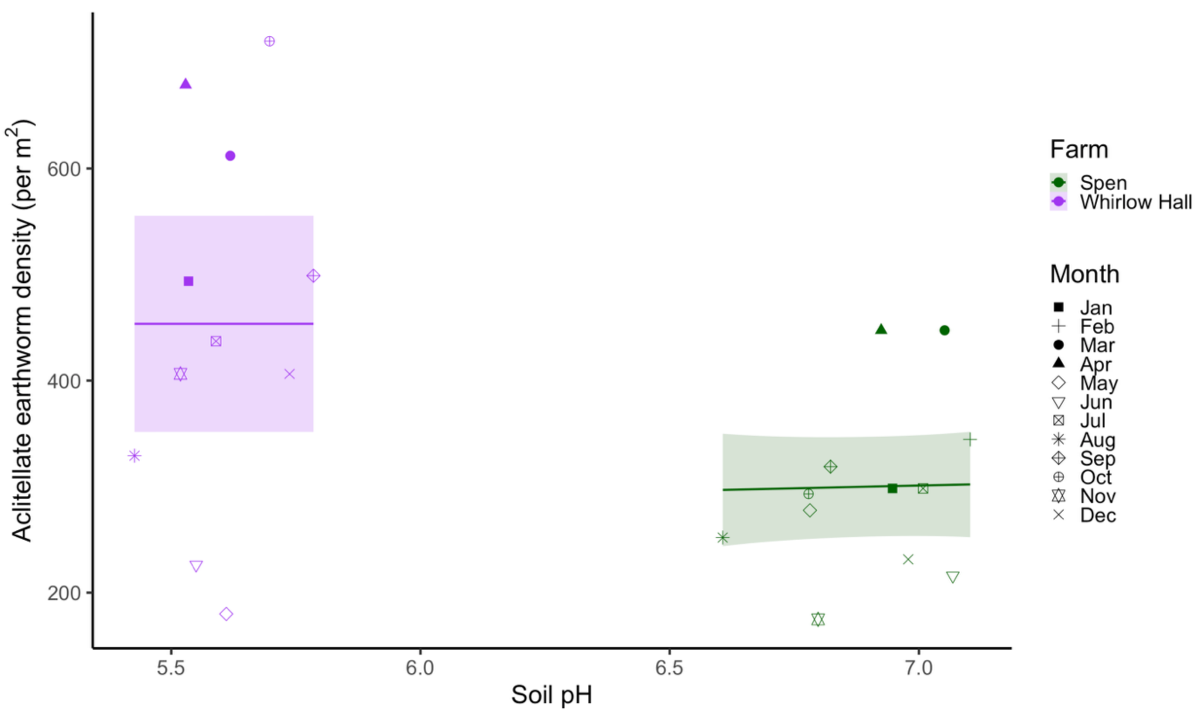

Figure S5.15. The relationship between mean soil pH (n =3) and the density of aclitellate earthworms (per m^2, n = 6) in both farms (green = Spen, purple = Whirlow Hall). Shapes represent each month. Lines fitted using linear regression. Shaded ribbons show standard error.

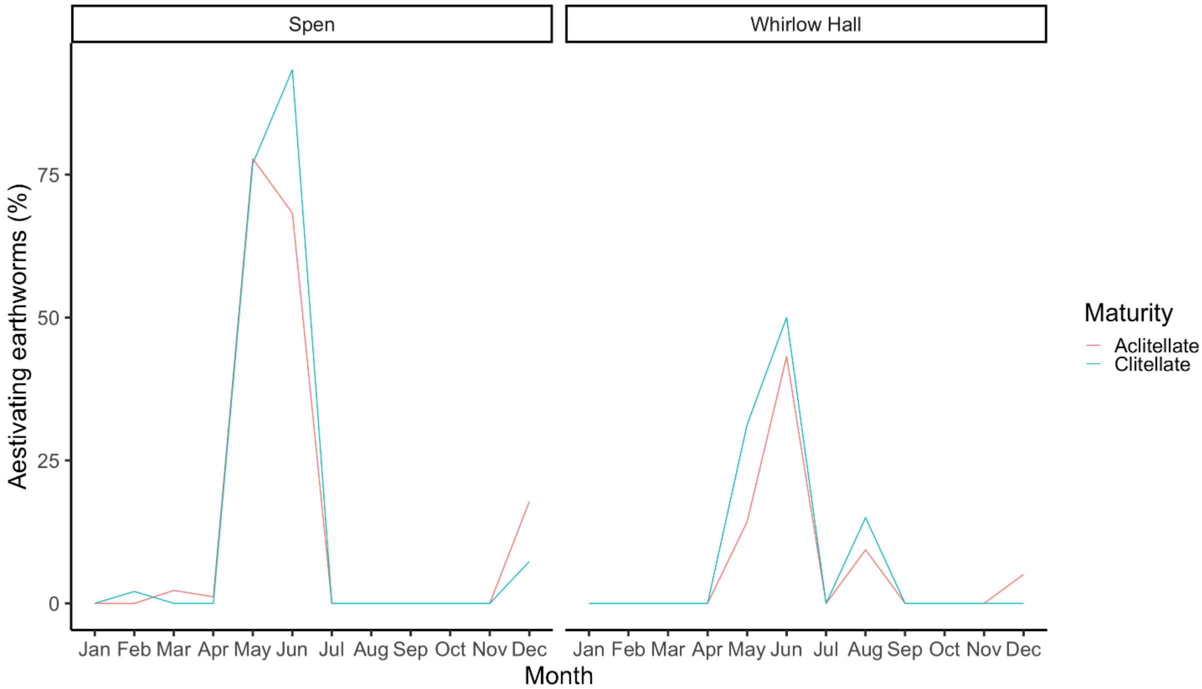

Figure S5.16. The percentage of earthworms found in aestivation each month at each farm, depending on whether they were clitellate (blue) or aclitellate (red).

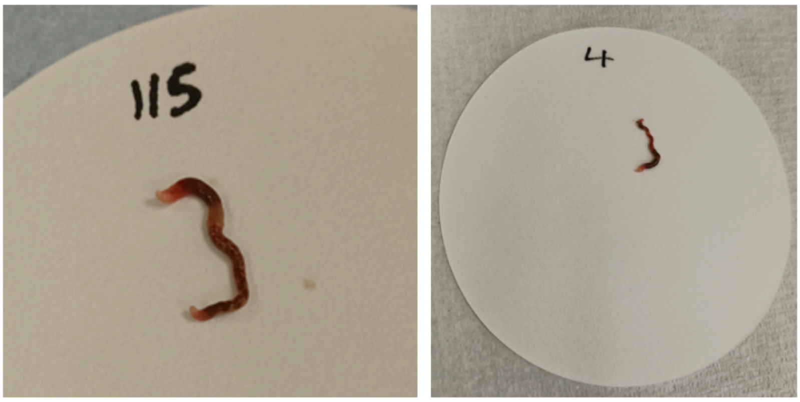

Figure S5.17. Clitellate (left) and aclitellate (right) *Aporrectodea cupulifera* earthworms on filter paper discs (70 mm in diameter).

Figure S5.18. Constricted, yellow caudal segments of earthworms collected from the field.

Chapter 6

Overall conclusions

This thesis demonstrates that soil water is the primary driver of earthworm activity, survival, reproduction and community dynamics, with aestivation acting as a key behavioural strategy enabling survival in periods of stress. However, the effectiveness of aestivation is shaped by the soil type, drought severity, species-specific traits, and landscape context, with important consequences for population recovery and community composition under climate change.

Drivers of aestivation

Aestivation in *Allolobophora chlorotica* (Savigny, 1826) and other earthworm species was shown to be an environmentally induced strategy rather than an obligatory diapause as individuals remained active in summer when moisture conditions were high. Specific moisture thresholds that determine transitions between activity, aestivation and mortality were clearly identifiable. In controlled experiments (chapters 2 and 3), aestivation was triggered between 18-11 wt% (in Kettering loam soil) but mortality was induced below this, indicating a lower limit of tolerance. Importantly, soil texture modulated these thresholds and earthworm mass loss and aestivation were induced at lower moisture contents in sandy soils (~12 wt%) compared to more clay-rich soils (~20 wt%), where water availability and structural properties constrained activity (chapter 4). These findings align with water potential measurements, where aestivation became more common above ~pF 3 (chapter 4). Field studies confirmed that most aestivation occurred in drier summer months at ~15-24 vol% moisture, generally above ~pF 3.6 (chapter 5). However, aestivation was also observed under waterlogged conditions in winter (>40-50 vol%, chapter 5), likely reflecting stress from associated anoxic conditions. Together this indicates that aestivation is likely a flexible response to multiple stressors, including desiccation, hypoxia and suboptimal soil pore space, rather than being driven by drought alone.

Community-level responses

At the farm scale, DNA metabarcoding revealed distinct species assemblages with earthworm communities strongly shaped by seasonal hydrology. Diversity was lowest in dry summer months, with populations dominated by four aestivating species (*Al. chlorotica* (Savigny, 1826), *Aporrectodea caliginosa* (Savigny, 1826), *Aporrectodea rosea* (Savigny, 1826) and *Aporrectodea longa* (Ude, 1885)). These species, which live within the soil, persisted across broad moisture ranges, whereas litter-dwelling species had more restricted ranges and largely disappeared under drought. The co-occurrence of active and aestivating individuals of the same species further suggests intraspecific variation in stress responses, which here could not be explained by differences in maturity. These findings highlight the role of both behavioural traits and microhabitat heterogeneity in maintaining biodiversity under variable conditions.

Costs of aestivation and harsh conditions

While aestivation is an effective short-term survival mechanism for earthworms, it imposes costs when drought is severe or repeated due to their limited ability to prevent water loss. *Al. chlorotica* exhibited significant mass losses which became greater with increasing soil desiccation (~3 % mass loss at ~20 wt% and ~45 % mass loss at ~16 wt% in clay soils, chapter 4). Mortality was induced from ~13 wt% moisture, with 100 % mortality observed at and below ~10 wt% in an initial experiment using a loam soil (chapter 2). Repeated drying exposures amplified effects on earthworm mass, resulting in lighter individuals and reduced reproductive output (chapter 3). Cocoon production was diminished following multiple bouts of aestivation, with early cocoons lighter, of lower viability and slower to hatch. Regression of the clitellum in stressed individuals indicates prioritisation of resource allocation to body segments more vital for survival and further suggests longer-term reproductive constraints. These findings emphasise that reproductive success is strongly mass-dependent, meaning that any factor reducing body size, whether through water loss, suspended feeding, or repeated stress, ultimately compromises fecundity.

Resilience and recovery

Despite these costs, earthworms displayed remarkable resilience. Irrespective of the degree of drying or soil type, individuals rapidly regained mass after rehydration, often exceeding their initial body weight. This indicates that most losses are transient and reflect water and gut contents rather than differences in tissue mass, highlighting the ecological importance of aestivation in enabling rapid recovery when favourable conditions return. At the population level, recovery was also evident in the field, with species richness and densities rebounding following drought. In particular, the density of clitellate individuals increased by over 260 % from June to July. The rebound in earthworm numbers likely primarily reflects vertical migration and recolonisation from surrounding areas but also the hatching of drought-resistant cocoons. Reserves of tolerant cocoons may allow populations to re-establish even if mortality occurs in adults and is likely of particular benefit to litter-dwelling species which have limited means of escaping drought. However, the resilience of populations depends on the duration of periods with more favourable conditions and if climatic extremes become more frequent, populations may not have sufficient time to rebuild before the next stress event. This may result in smaller, less diverse earthworm communities, with local extinctions, particularly of species with more restricted tolerance ranges.

Future work

This research highlights a number of important avenues for future study. Understanding the thresholds and mechanisms underlying earthworm responses is essential for predicting soil ecosystem resilience under future climate change scenarios. In particular, more work is needed on the long-term impacts of aestivation on reproductive output, including how long negative consequences last, to help determine the duration of favourable conditions required for populations to recover. A key area of interest lies in the regression of secondary sexual characters, most notably the clitellum, during periods of stress. The conditions which induce this and the extent to which clitellum regression affects reproductive capacity, cocoon viability, and recovery time remains largely unknown but will strongly influence the capacity of populations to rebuild following harsh conditions. One approach would be to expose clitellate earthworms to gradual soil drying with sampling at set intervals to assess clitellum regression, before transferring groups with different levels of regression to favourable conditions and monitoring reproductive output and cocoon quality (mass, viability and incubation time) over time. Such an experiment would provide insight into the recovery of clitellum function and the longevity of negative impacts on fecundity.

The influence of body size, life stage and species identity on the onset of aestivation also warrants further investigation, as does the comparison of aestivating versus non-aestivating individuals to assess adaptive benefits under varying climatic scenarios. Experiments could involve exposing multiple species and maturity stages to controlled drought stress and recording changes in their mass and activity and at set thresholds of soil moisture. Comparisons of reproductive output during recovery would help to determine the costs and potential adaptive benefits of aestivation across functional groups. The responses of epigeic species and those that do not employ aestivation as a survival strategy in suboptimal conditions remain poorly understood and represent a significant knowledge gap. Future work should also explore thresholds that induce aestivation across different environmental contexts and land management practices, expanding research beyond temperate regions to improve global predictions.

Questions remain regarding the maximum duration of aestivation, which is likely dependent on the severity of water loss. This could be tested by inducing aestivation under simulated droughts of varying intensities and assessing survival at regular time points to determine whether earthworms can survive longer in aestivation in milder conditions. In addition, most studies involve abrupt termination of aestivation by manually extracting earthworms from their chambers, leaving the dynamics of recovery poorly understood including time taken to re-activate under natural moisture regimes, and how this varies with soil depth or disturbance. Experiments using mesocosms subjected to realistic rewetting regimes with periodic destructive sampling could track reactivation under natural moisture gradients. Understanding these processes is important for predicting recovery windows and population resilience in environments subject to increasingly frequent and irregular rainfall events.

Cocoon dynamics also deserve greater attention, as they may be critical to population recovery after drought. Outstanding questions include how long cocoons can remain viable under stressful conditions, their tolerance thresholds, and the effects of moisture content on development and hatching. Cocoons of known age could be exposed to combinations of low moisture and high temperature for varying durations and then incubated under favourable conditions to measure hatching time, success and juvenile growth. Exploring cocoon drought-resistance may also have applied value as, for instance, they could be used in reintroductions for soil restoration in combination with adults to increase chances of population persistence given their potential greater tolerance to climatic extremes.

Future studies should also consider interactive and indirect effects of climate change. The complexity of ecosystems means that soil moisture rarely changes in isolation, and temperature, soil texture, and aboveground vegetation interact to shape earthworm responses. Experimental approaches combining multiple stressors will be necessary to disentangle these effects, particularly in field settings where indirect pathways through vegetation loss, soil microbiota shifts, or altered nutrient cycling may amplify impacts. Greater research into ecological interactions between earthworms and other soil fauna, including enchytraeids and microbes, would further improve our understanding of how whole soil communities may respond to drought.

Finally, the application of skin swabbing to extract DNA from earthworms presents a novel, non-destructive method for earthworm identification. However, this approach produced some unexpected challenges, primarily due to species-specific differences in the release of coelomic fluid, which led to variation in DNA yield and more prominent transfer of endogeic species DNA between individuals when together in soils. Contamination issues were mitigated using a machine learning approach with a random forest model trained on a subset of samples of known species identity. Future applications of skin swabbing for earthworm identification should therefore consider including cross-validation of a subset of samples through alternative methods (e.g. morphological identification using a key or DNA extraction from earthworm tissue). These reference datasets can be used both to confirm species identities and to provide training material for machine learning approaches in cases where contamination occurs.

Final conclusions and implications

Collectively, this thesis highlights both the resilience and vulnerability of earthworms to changing climatic conditions. Species such as *Al. chlorotica* can endure short-term desiccation by entering aestivation, enabling survival under drought and rapid recovery once favourable conditions return. However, this strategy has costs, with negative effects on mass and reproduction increasing with repeated aestivation. Moreover, cumulative and prolonged periods of environmental stress will reduce the time available for growth and reproduction, constraining population dynamics and ultimately reducing biodiversity. Therefore, more frequent and severe climatic extremes will likely reshape earthworm communities, favouring more tolerant, generalist species at the expense of specialists, with subsequent consequences for soil functioning and the provision of crucial ecosystem services. The capacity of earthworm populations to persist will not only depend on species-specific traits, but also on soil quality, texture, and the broader landscape context. Thus, interactions between climatic stressors and anthropogenic pressures will be critical in determining ecosystem resilience. Protecting soil health, structure and biodiversity through more regenerative management practices which seek to reduce soil degradation may help to buffer against climatic extremes and sustain earthworm populations. Finally, more spatially and temporally extensive research into earthworm behaviour and ecology will not only aid functional group classification but will also be essential to anticipate community responses under climate change. Such knowledge will be critical for informing strategies that preserve soil ecosystems and the services they provide.