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Interspecies variation in survival of soil fauna in flooded soil

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Abstract

While many studies have examined the effects of flooding on earthworm population distributions, few studies have investigated physiological and behavioural responses of earthworms to the low oxygen conditions caused by flooding. An earthworm's skin is its oxygen exchange organ, allowing earthworms to survive in flooded environments provided that the water contains sufficient dissolved oxygen. Individuals of three species of earthworm, the anecic *Lumbricus terrestris* (Linneaus, 1758), the green morph of the endogeic *Allolobophora. chlorotica* (Savigny, 1826) and the epigeic *Lumbricus castaneus* (Savigny, 1826) were placed in reconstituted groundwater that was either kept aerated or kept in a sealed container so that dissolved oxygen was gradually consumed as the earthworm respired. Oxygen saturation of the water was measured over time in sacrificial triplicate replicates from each treatment at discrete time points, with earthworm death recorded. Before treatments, oxygen levels in all treatment tubes were 9.53 (± 0.64) mg O_2 L^{-1}. *L. terrestris*, a large species which emerges at night to forage at the soil surface died when oxygen levels reached 0.82 (± 0.46) mg O_2 L^{-1} after approximately 36 hours. *L. castaneus*, a smaller species which lives on the soil surface, died when oxygen levels reached 3.60 (± 2.01) mg O_2 L^{-1} after approximately 168 hours. *A. chlorotica,* which is similar in size to *L. castaneus*, lives in the upper 20 cm of soil and is known to aestivate during the summer, did not die, even when oxygen levels reached 1.49 (± 0.40) mg O_2 L^{-1} after 280 hours. The results suggest that earthworm respiration is closely linked to both body size and to behavioural ecotype. These findings suggest that if flooding increases in frequency resulting in episodic reductions in soil oxygen levels, the species composition of earthworm communities may change, with an increased presence of endogeic earthworms which show a responsive plasticity to flooding events.

Keywords: earthworms; flooding; oxygen requirements; ecotypes; aestivation; traits

1. Introduction

The soil environment is one in which both biotic and abiotic factors can be highly heterogeneous over scales ranging from hectares to millimetres (Ettema and Wardle, 2002). The spatial heterogeneity of factors such as soil aggregates, microorganisms, moisture and organic matter lead to a highly spatially diverse distribution of the soil oxygen concentration both within pore space and aggregates (Sexstone et al., 1985; Parkin, 1993; Stoyan et al., 2000;). As earthworms burrow through the soil they can therefore encounter a range of different physico-chemical environments within a short distance. The respiratory system of the earthworm allows for passive diffusion of oxygen across the cuticle and epidermal tissues, as long as there is sufficient moisture to facilitate gas exchange and sufficient oxygen for respiration (Edwards and Lofty, 1977). The use of the skin as the organ of gas exchange also allows earthworms to survive for some time in oxygenated water. An experiment performed by Roots (1956), found that earthworm survival in aerated water without food varied between species, with individuals of *Allolobophora chlorotica* (Savigny, 1826) and *Lumbricus terrestris* (Linneaus, 1758) each surviving a mean average of 137 days and *Lumbricus rubellus* (Hoffmeister, 1843) surviving 78 days (Roots, 1956).

The differing lengths of survival in oxygenated water first noted by Roots (1956) may be linked to the different ecological niches that the earthworm species exploit. Earthworm species are broadly divided into three categories: anecic earthworms, which live in deep, vertical burrows and emerge at night to forage on the soil surface, endogeic earthworms, which live in and feed on the upper 20 cm of soil, and, epigeic earthworms, which live within and forage in leaf litter on the soil surface (Bouché, 1977). These three distinct habitats may be subject to different levels of oxygenation, which may in turn mean that earthworms of different ecotypes are adapted to differing levels of oxygen availability. As soil moisture and organic matter disperses with increasing soil depth (Stoyan et al., 2000), transitions between oxic and anoxic zones become smoother, meaning there are fewer distinctly anoxic and distinctly oxic zones, and more regions existing at partial oxygenation. This, along with the formation of deep burrows which may conduct oxygen down to deeper layers of soil (Lavelle, 1988) may mean that anecic earthworm species are less likely to be subject to the heterogeneity of soil oxygenation potentially encountered by the endogeic species. Epigeic earthworms live in and consume litter on the soil surface. In the soil surface litter environment, microbial activity is highly dependent on moisture and temperature, which could lead to highly variable oxygen concentrations; however, the fact that the litter is on the soil surface, where oxygen can easily be replenished from the atmosphere, may mean that epigeic earthworms do not need to display any long term adaptations to cope with low oxygen conditions.

While earthworms of all ecotypes are likely adapted to some degree of oxygen stress, with *L. terrestris* and *L. rubellus* having been found to produce lactic acid (Davis and Slater, 1928) and other metabolites associated with anaerobic respiration (Gruner and Zebe, 1978), their ability to tolerate anoxic conditions is still unknown. A number of field studies have found that flooding causes a decrease in earthworm abundance and biomass, but also reduces the overall diversity of earthworm species (Plum, 2005; Plum and Filser, 2005; Kiss et al., 2021). As flooded soil can reach anoxic levels within as little as 24 hours (Ponnamperuma, 1984; Kiss, 2019), understanding how earthworms of different ecotypes respond to decreasing oxygen concentrations may inform understanding of earthworm population dynamics in regularly flooded regions. This study could also aid understanding of how previously undisturbed populations may shift with the increased frequency and intensity of flooding predicted to occur in many regions with global climate change (Hirabayashi and Kanae, 2009; Kundzewicz et al., 2014).

In this study, three common European earthworm species (*L. terrestris, A. chlorotica,* and *Lumbricus castaneus* (Savigny, 1826)) representing anecic, endogeic and epigeic ecotypes respectively, were maintained in sealed treatment tubes or in aerated control tubes filled with a reconstituted groundwater solution. These tubes were destructively sampled at set time points to determine how the dissolved oxygen concentration within the solution changed over time. The hypothesis for the study was that the dissolved oxygen concentration at which individuals die will differ between species, depending on characteristics such as the size of the individual or species behavioural patterns. This study aims to quantify differences in oxygen requirements between the three earthworm species, to suggest mechanisms for why these differences may occur, and to suggest how these differences may affect earthworm populations both at present and with predicted future climate change.

2. Materials and Methods

2.1. Earthworm collection

Adult, clitellate *L. terrestris* were purchased from Wiggly Wrigglers Ltd (Blakemere, UK); adult, clitellate *A. chlorotica* and *L. castaneus* were collected from pasture fields at Spen Farm, near Leeds (SE 44300 41700). The same experimental methodology was used for each species, but changes were made to sampling times based on scoping studies (not reported). *L. terrestris* was selected as the anecic study species due to its prevalence in UK and Western European soils (Rutgers et al., 2016). *A. chlorotica* was selected as the endogeic study species as it was the most common species in arable and pasture field sites (Natural England, 2014). *L. castaneus* was selected as the epigeic species as it was similar in size to *A. chlorotica*, and could be collected in sufficient numbers from the Spen Farm site.

2.2. Experimental design

Earthworms were depurated for forty-eight hours at 10°C on damp blue roll to empty their gut contents (Arnold and Hodson, 2007). Blue roll was changed approximately every 12 hours to prevent re-ingestion of soil matter. A greater number of earthworms than required were depurated to allow for any individuals that appeared to be in poor condition post depuration to be discarded. Following depuration, forty-eight individuals of each species were selected and their weight, length and diameter recorded. Earthworms were weighed on a four place Ohaus Adventurer Pro balance. To determine length and diameter earthworms were photographed against 1 mm^2 graph paper and dimensions were measured manually with a rule using the grid for scale.

Individual earthworms were added to 50 ml centrifuge tubes that were filled to the brim with reconstituted groundwater (Arnold et al., 2007) which had been pre-cooled to 10°C in a controlled temperature cabinet. Twenty-four of the tubes were sealed with centrifuge tube lids. The remaining twenty-four tubes were capped with lids that had been modified by drilling seven holes of approximately 2.5 mm width in the lid. A length of flexible plastic tubing of approximately 2.5 mm internal diameter was inserted through one of these holes. After the earthworms were placed in the tubes, the treatment tubes were returned to the 10°C controlled temperature cabinet. The control tubes were connected to a peristaltic pump set to rotate at 90 RPM to aerate the reconstituted groundwater solution. The size and shape of the pumps meant that they could not fit in the 10°C controlled temperature chamber, so the control experiment was conducted in a 15°C controlled temperature room. Despite the fact that the treatment and control tubes were maintained at different temperatures, this was considered justified as the purpose of the controls was to demonstrate that the earthworms could survive in the solutions if the solutions were kept aerated so that oxygen levels did not deplete. While temperatures of 15°C are optimal for high rates of earthworm cocoon production and growth under laboratory culture conditions (Lowe and Butt, 2005), earthworms are active in the soil at 10°C and this is a more realistic temperature for earthworm activity, so it was deemed more appropriate to run the main experiment at that temperature (Edwards and Lofty, 1977). As metabolic rates in earthworms are highly influenced by external temperature (Meehan, 2006), when seeking to understand how earthworm oxygen requirements may vary between species in flooded soils it was important to maintain the treatment tubes in temperatures that more accurately represent flooded UK soils than the 15°C control tubes.

2.3. Measurements

Measurement intervals were determined in preliminary studies using the different species, when prior methodologies were being tested (data not reported here). Preliminary tests used just the earthworm-bearing sealed tubes to determine the length of time for which the earthworms of different species were likely to survive. By taking measurements regularly over the course of these preliminary tests, we were able to determine appropriate sampling time points in the main experiment in order to obtain interpretable response curves for each species whilst taking into account the different time scales over which the different earthworms responded. The time points at which measurements were taken for *L. terrestris* were 0, 3, 6, 9, 24, 33, 48, and 72 hours following immersion. For *A. chlorotica*, the time points were 0, 9, 24, 48, 96, 144, 216, and 288 hours following immersion. For *L. castaneus*, the time points were 0, 9, 24, 48, 72, 120, 168, and 216 hours following immersion. At each interval, three tubes from both the treatment and control sets were selected at random using a random number generator and opened – these destructively sampled replicates are referred to as ‘sacrificial replicates’. By employing this methodology, rather than repeated measurements of the same tube, each measurement was independent, thus avoiding pseudoreplication in our experimental design. Immediately after opening each tube, the percent oxygen saturation, the concentration of oxygen in solution (mg O_2 L^{-1}), and the solution temperature were measured using a Thermo Scientific Orion Star A223 and Star A23 Portable Dissolved Oxygen Meter. Immediately following oxygen measurements, the pH of the solution was measured using a Thermo Orion 420A plus pH/ISE Meter, calibrated with pH 4, pH 7 and pH 10 buffers. The earthworm from the tube was removed, blotted on blue roll, and its weight, length, and width recorded. Weight was measured using a four decimal place Ohaus Adventurer Pro balance and length and width measured manually with a rule from photographs of earthworms against 1 mm^2 graph paper. The earthworms were tested to see if they were alive using a response test, in which they were prodded near the sensitive mouth parts using a sharp needle (OECD, 1984). If the earthworm did not respond to the prodding and, in the case of *A. chlorotica,* which appeared to show a behavioural response to submersion, did not show any signs of movement after two minutes on the bench surface, during which it was weighed and measured, it was recorded as dead. If earthworms were alive, they were removed to damp soil for later release.

2.4. Data analyses and statistics

Data were analysed using RStudio (R Core Team, 2019). The oxygen concentration in solution for each replicate at each time point was normalised per gram biomass of earthworm using the initial earthworm fresh biomass, and per unit surface area of earthworm. Initial biomass was used for calculations to account for variation in time since earthworm death at sampling, which may have led to a change in mass, but at an unknown rate, due to loss of earthworm active control of osmoregulation (Carley et al., 1983). Earthworm surface area was calculated using the initial length and width of the earthworms and by assuming that the earthworms were perfect cylinders. pH values were converted to proton concentrations prior to statistical analysis. Datasets were statistically tested for normality using a Shapiro-Wilk test and visual examination of the data distribution, and non-parametric equivalents of statistical tests used where necessary. To determine how the oxygen concentration changed in the control and treatment tubes over time for each species, a generalised linear model (GLM) was performed as a non-parametric equivalent to a two-way analysis of variance (ANOVA) comparing the effects of treatment and time point on the oxygen concentration for each earthworm species. This was performed for the absolute oxygen concentration (mg O_2 L^{-1}) in solution, the concentration per gram fresh initial earthworm biomass (mg O_2 L^{-1} g^{-1}) and the concentration per mm^2 fresh initial surface area (mg O_2 L^{-1} mm^{-2}) for each individual. Tukey *post hoc* tests were used to determine where differences between combinations of timepoint and treatment lay. To determine whether the oxygen concentration at which individuals of *L. terrestris* and *L. castaneus* died differed, a two-way t-test for the absolute concentration of oxygen, and a Wilcoxon signed ranks test for the concentration of oxygen normalised by both gram fresh biomass of the earthworm individual and by the initial surface area of the earthworm was used. A further hypothesis, that the oxygen concentration at which individuals of *L. terrestris* and *L. castaneus* died differed significantly from the plateaued oxygen concentration observed in the *A. chlorotica* experiments, was tested using a Kruskal-Wallis test, and pairwise Wilcox *post hoc* testing. To determine if the mass gained by earthworms in solution differed between control and treatment, between live and dead earthworms, and the effect of the time spent immersed in solution, a three-way GLM was performed separately for both *L. terrestris* and *L. castaneus*. As no individuals of *A. chlorotica* died during the experiment, a GLM was performed comparing the mass gained between individuals in the treatment and control tubes and the time submerged. Tukey *post hoc* testing was used to determine how the mass gain differed between control species, live individuals, and dead individuals. To determine whether the pH of the solution changed with time and between control or treatment tubes, GLMs acting as a non-parametric two way ANOVA were performed with the pH of the solution as the dependent variable and measurement time point and treatment or control as the factors. Tukey *post hoc* tests were used to determine where differences between combinations of timepoint and treatment lay.

3. Results

3.1. Solution oxygen concentration and earthworm response

There was a significant effect of the sampling time point ($P < 0.01$), the treatment ($P < 0.01$), and the interaction term between the two ($P < 0.01$) on the oxygen concentration in solution for *L. terrestris* (Fig. 1), *A. chlorotica* (Fig. 2), and *L. castaneus* (Fig. 3).

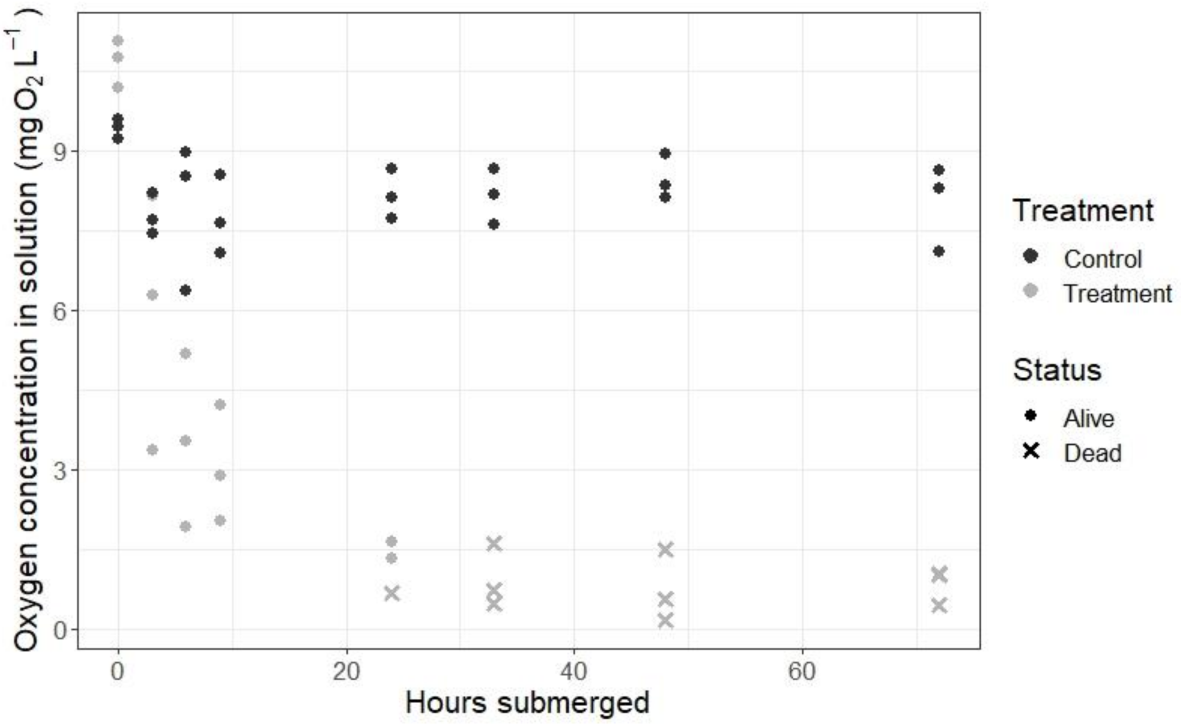

Fig 1. The changes in oxygen concentration in control and treatment tubes containing individuals of *Lumbricus terrestris* over time.

No individuals of *L. terrestris* died in the control tubes over 72 hours. In the treatment tubes, 100% earthworm mortality was reached by 36 hours submerged. Tukey *post hoc* testing showed that, for individuals of *L. terrestris,* the oxygen concentration in the control and treatment tubes did not significantly differ from hours 0 to 6. From hours 9 to 72, the oxygen concentration was significantly lower in the treatment tubes than in the control tubes ($P < 0.05$). Across all sampling time points, there was no significant difference in the oxygen concentration in the control tubes. From hours 9 to 72, the oxygen concentrations in the treatment tubes did not differ significantly.

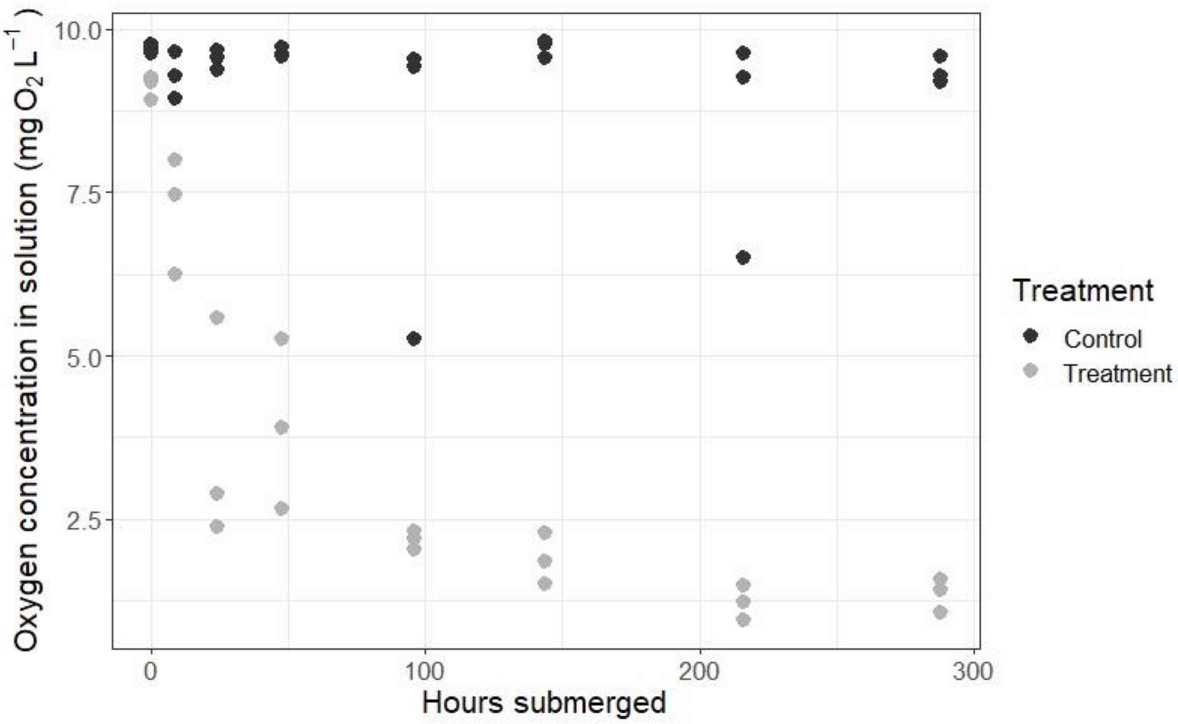

Fig. 2. The changes in oxygen concentration in control and treatment tubes containing individuals of *Allolobophora chlorotica* over time.

No individuals of *A. chlorotica* died over the 288 hour sampling period. Tukey *post hoc* testing showed that, for individuals of *A. chlorotica*, the oxygen concentration in the control and treatment tubes did not significantly differ from hours 0 to 9. From hours 24 to 288, the oxygen concentration in the treatment tubes was significantly lower than in the control tubes ($P < 0.05$). Across all sampling time points, there was no significant difference in the oxygen concentration of the control tubes. Between hours 24 to 288, the oxygen concentrations within the treatment tubes did not differ significantly.

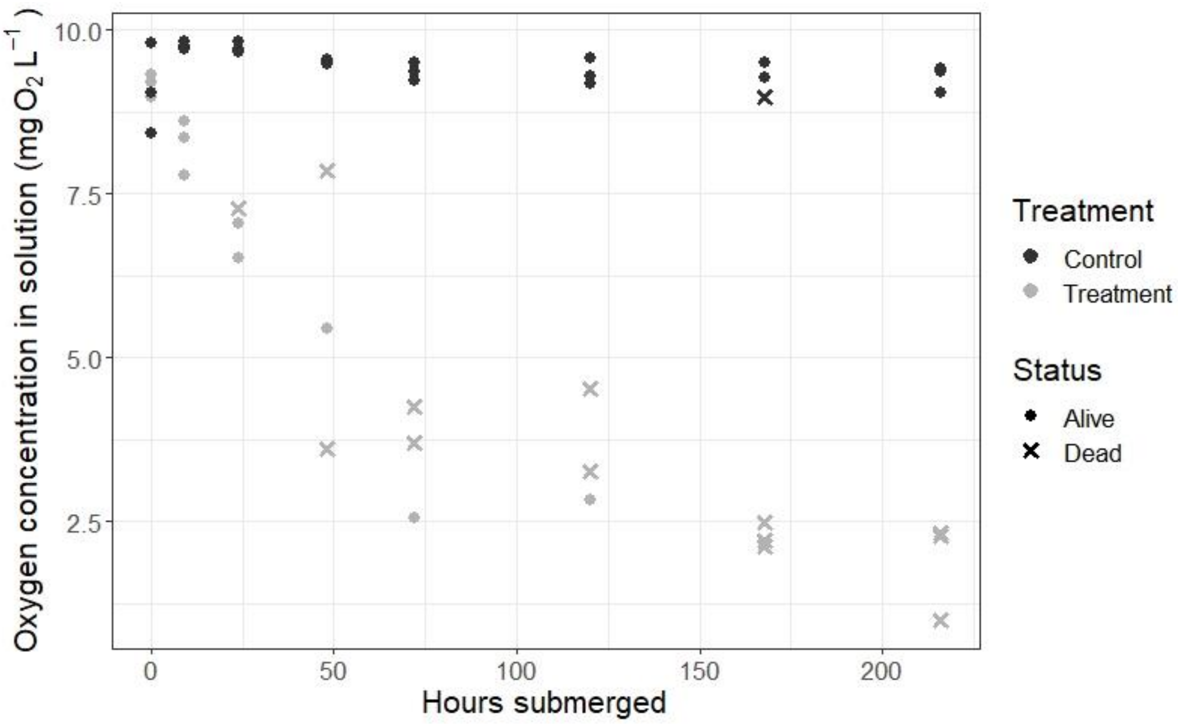

Fig. 3. The changes in oxygen concentration in control and treatment tubes containing individuals of *Lumbricus castaneus* over time.

One individual of *L. castaneus* was found dead in the control tubes, at hour 168. In the treatment tubes, 100% mortality was reached by 168 hours submerged, with 66% mortality reached at 28 hours. Tukey *post hoc* testing showed that, for individuals of *L. castaneus*, the oxygen concentration in the control and treatment tubes did not significantly differ from hours 0 to 9. From hours 24 to 216, the oxygen concentration in the treatments tubes was significantly lower than the oxygen concentration in the control tubes ($P < 0.05$). Across all sampling time points, there was no significant difference in the oxygen concentration of the control tubes. Within the treatment tubes, the oxygen concentration was significantly higher at hours 24 and 48 than the sampling time points between hours 72 and 216 ($P < 0.05$).

The mean values of the normalised oxygen concentration per gram biomass of individuals of each earthworm species for each time point are presented in Table 1. There was a significant effect of time point ($P < 0.01$) treatment ($P < 0.01$) and the interaction term between the two ($P < 0.01$) for all three earthworm species.

Tukey *post hoc* testing showed that for all three earthworm species, there was no significant difference in the oxygen concentration normalised per gram at each time point for the control tubes ($P < 0.05$). For individuals of *L. terrestris*, the oxygen concentration per gram biomass in the treatment tubes began to be significantly lower than the control tubes from hour 9 ($P < 0.05$), with no significant difference in the oxygen concentration per gram biomass in the treatment tubes from hour 6 ($P < 0.05$). For individuals of *A. chlorotica*, the oxygen concentration per gram biomass in the treatment tubes began to be significantly lower than the control tubes from hour 24 ($P < 0.05$), with no significant difference in the oxygen concentration per gram biomass in the treatment tubes from hour 24 ($P < 0.05$). For individuals of *L. castaneus*, the oxygen concentration per gram biomass in the treatment tubes began to be significantly lower than the control tubes from hour 72 ($P < 0.05$). From hour 24, there was no significant difference in the oxygen concentration per gram biomass in the treatment tubes ($\mathrm{P} < 0.05$).

Table 1. The mean and standard deviation oxygen concentration normalised by the gram biomass of the individual (mg O_2 L^{-1} g^{-1}) in control and treatment tubes containing individuals of *Lumbricus terrestris* (n = 48), *Allolobophora. chlorotica* (n = 48), and *Lumbricus castaneus* (n = 48) at each time point (n = 3 for each species time point). For each species, cells marked with the same letter within control and treatment columns are not significantly different (*P* > 0.05; Tukey *post hoc* testing).

L. terrestris			***A. chlorotica***			***L. castaneus***		
Hours submerged	**Oxygen concentration normalised per unit biomass (mg O_2 L^{-1} g^{-1})**		**Hours submerged**	**Oxygen concentration normalised per unit biomass (mg O_2 L^{-1} g^{-1})**		**Hours submerged**	**Oxygen concentration normalised per unit biomass (mg O_2 L^{-1} g^{-1})**	
	Control	**Treatment**		**Control**	**Treatment**		**Control**	**Treatment**
0	2.58 (± 0.34) **ef**	3.16 (± 0.67) **f**	**0**	57.15 (± 12.15) **d**	59.54 (± 2.99) **d**	**0**	79.64 (± 13.06) **bc**	61.33 (± 7.03) **bc**
3	1.96 (± 0.20) **de**	1.42 (± 0.78) **bcde**	**9**	54.08 (± 19.31) **d**	47.75 (± 16.15) **bcd**	**9**	78.11 (± 24.06) **c**	77.88 (± 8.29) **c**
6	1.99 (± 0.50) **def**	0.84 (± 0.43) **abcd**	**24**	54.97 (± 4.35) **d**	23.62 (± 13.84) **abc**	**24**	76.26 (± 11.60) **c**	47.15 (± 1.95) **abc**
9	1.48 (± 0.44) **de**	0.74 (± 0.42) **abc**	**48**	61.59 (± 7.79) **d**	22.15 (± 5.94) **ab**	**48**	61.67 (± 24.08) **c**	49.26 (± 27.09) **abc**
24	1.98 (± 0.38) **ef**	0.28 (± 0.13) **ab**	**96**	53.89 (± 21.64) **d**	13.78 (± 1.47) **a**	**72**	75.17 (± 16.39) **bc**	25.93 (± 8.47) **ab**
33	2.21 (± 0.20) **cde**	0.25 (± 0.19) **ab**	**144**	54.33 (± 6.80) **d**	10.93 (± 3.75) **a**	**120**	80.64 (± 14.46) **c**	28.83 (± 10.34) **ab**
48	1.76 (± 0.12) **ef**	0.19 (± 0.19) **a**	**216**	52.70 (± 12.93) **cd**	8.88 (± 1.40) **a**	**168**	81.64 (± 13.06) **c**	14.52 (± 1.67) **a**
72	2.38 (± 0.72) **ef**	0.21 (± 0.08) **a**	**288**	63.69 (± 4.18) **cd**	7.64 (± 0.68) **a**	**216**	79.64 (± 25.56) **c**	12.37 (± 2.88) **a**

The mean values of the oxygen concentration normalised per unit surface area (mm^{-2}) for each time point are presented in Table 2. There was a significant effect of time point ($P < 0.01$) and treatment ($P < 0.01$) for all earthworm species, with a significant interaction term ($P < 0.01$) present for *L. terrestris* and *A. chlorotica*.

Tukey *post hoc* testing showed that for all earthworm species, there was no significant difference in the oxygen concentration normalised per mm^2 surface area at each time point for the control tubes ($P < 0.05$). For individuals of *L. terrestris*, the oxygen concentration per mm^2 surface area in the treatment tubes began to be significantly lower than the control tubes from hour 24 ($P < 0.05$), with no significant difference in the oxygen concentration per gram biomass in the treatment tubes from hour 3 ($P < 0.05$). For individuals of *A. chlorotica*, the oxygen concentration per mm^2 surface area in the treatment tubes became significantly lower than the oxygen concentration per mm^2 at 9 hours in both the treatment and control tubes from 48 hours onwards ($P < 0.05$). There was no significant difference in the oxygen concentration per mm^2 in the treatment tubes from 24 hours ($P < 0.05$). For individuals of *L. castaneus*¸ oxygen concentration per mm^2 surface area for individuals in the treatment tubes was significantly lower than the oxygen concentration in the control tubes ($P < 0.05$). Tukey *post hoc* testing of the interaction terms showed that the only statistically significant difference occurred between the treatment tubes at hours 168 and 216, which were significantly lower than the control tubes at hour 9 ($P < 0.05$).

Table 2. The mean and standard deviation oxygen concentration normalised by the mm^2 surface area of the individual ($\mu g\ O_2\ L^{-1}\ mm^{-2}$) in control and treatment tubes containing individuals of *Lumbricus terrestris* (n = 48), *Allolobophora chlorotica* (n = 48), and *Lumbricus castaneus* (n = 48) at each time point (n = 3 for each species time point). For each species, cells marked with the same letter within control and treatment columns are not significantly different ($P > 0.05$; Tukey *post hoc* testing).

L. terrestris			***A. chlorotica***			***L. castaneus***		
Hours submerged	**Oxygen concentration normalised per unit surface area ($\mu g\ O_2\ L^{-1}\ mm^{-2}$)**		**Hours submerged**	**Oxygen concentration normalised per unit surface area ($\mu g\ O_2\ L^{-1}\ mm^{-2}$)**		**Hours submerged**	**Oxygen concentration normalised per unit surface area ($\mu g\ O_2\ L^{-1}\ mm^{-2}$)**	
	Control	**Treatment**		**Control**	**Treatment**		**Control**	**Treatment**
0	4.26 (± 1.63) **d**	4.38 (± 0.54) **d**	**0**	44.89 (± 11.65) **e**	46.53 (±3.99) **e**	**0**	63.14 (± 22.58) **ab**	63.69 (± 19.24) **ab**
3	2.88 (± 0.53) **cd**	2.53 (± 1.42) **abcd**	**9**	37.78 (± 11.27) **cde**	36.36 (± 11.02) **bcde**	**9**	84.49 (± 12.05) **b**	76.18 (± 27.95) **ab**
6	3.17 (± 0.84) **cd**	1.27 (± 0.42) **abc**	**24**	48.97 (± 1.78) **e**	18.82 (± 10.45) **abcd**	**24**	64.84 (± 32.57) **ab**	47.34 (± 15.56) **ab**
9	3.24 (± 0.67) **cd**	1.44 (± 0.98) **abc**	**48**	57.20 (± 12.67) **e**	15.89 (± 2.77) **abc**	**48**	81.19 (± 38.19) **ab**	55.04 (± 33.70) **ab**
24	3.75 (± 1.05) **d**	0.48 (± 0.21) **ab**	**96**	43.50 (± 16.53) **de**	12.24 (± 0.56) **ab**	**72**	60.02 (± 28.60) **ab**	21.69 (± 14.63) **ab**
33	2.71 (± 0.22) **bcd**	0.36 (± 0.28) **a**	**144**	33.51 (± 9.26) **cde**	8.05 (± 3.18) **a**	**120**	59.44 (± 10.36) **ab**	21.07 (± 5.47) **ab**
48	2.86 (± 0.11) **cd**	0.28 (± 0.28) **a**	**216**	47.66 (±7.89) **de**	6.00 (± 1.48) **a**	**168**	63.48 (± 13.18) **ab**	13.81 (± 4.05) **a**
72	3.16 (± 1.19) **cd**	0.36 (± 0.27) **a**	**288**	42.50 (± 3.97) **de**	5.65 (± 0.88) **a**	**216**	74.46 (± 50.81) **ab**	13.44 (± 3.97) **a**

3.2. Differences in oxygen concentration at which earthworms died

As no individuals of *A. chlorotica* died, they were not included in this portion of the statistical testing. However, the average oxygen concentration at the timepoints at which oxygen concentrations ceased to reduce (hours 144 to 288) was included for comparison to that at which individuals of *L. terrestris* and *L. castaneus* died. The absolute, and biomass- and surface area-normalised oxygen concentrations recorded for each dead individual of *L. terrestris* and *L. castaneus* were compared (Table 3). Individuals of *L. terrestris* died at a significantly lower oxygen concentration than individuals of *L. castaneus* when considering the absolute oxygen concentration ($P = 0.003$), concentration normalised to biomass ($P < 0.0001$) and concentration normalised to surface area ($P < 0.0001$). There was no significant difference between the statistically constant oxygen concentrations across hours 144 to 288 in treatment tubes containing living *A. chlorotica* and the concentrations at which *L. castaneus* died. However, *L. terrestris* died at significantly lower oxygen concentrations ($P < 0.0001$).

Table 3. The mean and standard deviations of the absolute, biomass-normalised and surface area-normalised oxygen concentrations recorded for each dead individual of *Lumbricus terrestris* (n = 10) and *Lumbricus castaneus (*n = 13), and the oxygen concentrations in treatment tubes between hours 144 and 288 for *Allolobophora chlorotica* (n = 9). Within the same row, cells marked with the same letter are not significantly different ($P > 0.05$; pairwise Wilcox *post hoc* testing).

	L. terrestris	***L. castaneus***	***A. chlorotica***
Absolute oxygen concentration (mg O_2 L^{-1})	0.82 (± 0.46) **a**	3.60 (± 2.01) **b**	1.49 (± 0.40) **b**
Oxygen concentration normalised by biomass (mg O_2 L^{-1} g^{-1})	0.21 (± 0.14) **a**	26.34 (± 19.13) **b**	9.15 (± 2.90) **b**
Oxygen concentration normalised by surface area (µg O_2 L^{-1} mm^{-2})	3.28 (± 2.11) **a**	2.43 (± 2.13) **b**	6.57 (± 2.13) **b**

3.3. Earthworm mass gain

Individuals of each earthworm species increased in mass over the duration of the experiment (Table 4). There was no significant difference in the mass gained (g) by individuals of *L. terrestris* between the control and treatment tubes and live and dead individuals, but there was a significant effect of the time spent submerged ($P < 0.001$), with individuals from 6 hours submerged gaining significantly more mass than the individuals retrieved at 0 hours submerged ($P < 0.05$).

Individuals of *A chlorotica* in the treatment tubes gained significantly more mass ($P = 0.028$) than individuals in the control tubes, and showed a significant interaction between the treatment and the time submerged ($P = 0.01$). Treatment individuals submerged for 96 and 216 hours gained significantly more mass than control individuals submerged for 0, 9, 48, 216 and 288 hours, and than treatment individuals submerged for 0, 9, and 24 hours ($P < 0.05$).

There was no significant difference in the mass gained between the control and treatment tubes for individuals of *L. castaneus*, and no significant effect of the hours submerged. There was a significant difference between live and dead individuals of *L. castaneus* ($P < 0.0001$) and a significant effect of the interaction term between treatment and control tubes and living and dead earthworms ($P = 0.002$). Tukey *post hoc* testing showed that dead treatment individuals gained significantly more mass than alive treatment, alive control, or dead control earthworms ($P < 0.05$).

Table 4. The mean and standard deviations of the mass (g) before and after the experiment and the mass change (g) observed in control and treatment individuals of *Allolobophora chlorotica*, and the mass gain in control and live and dead treatment individuals of *Lumbricus castaneus* and *Lumbricus terrestris*. Within the mass change column, cells of the same species marked with the same letter are not significantly different (P > 0.05; Tukey *post hoc* testing). Cells marked with N/A indicates no earthworms within that category.

			Control tubes			
	Before		**After**		**Mass change**	
A. chlorotica	0.17 (± 0.03)		0.22 (± 0.06)		0.05 (± 0.04) **a**	
			Treatment tubes			
A. chlorotica	0.16 (± 0.03)		0.25 (± 0.07)		0.09 (± 0.07) **b**	
	Before		**After**		**Mass change**	
	Live earthworms	**Dead earthworms**	**Live earthworms**	**Dead earthworms**	**Live earthworms**	**Dead earthworms**
			Control tubes			
L. castaneus	0.14 (± 0.03)	**N/A**	0.15 (± 0.03)	**N/A**	0.01 (± 0.01) **a**	**N/A**
L. terrestris	3.93 (± 0.60)	**N/A**	4.75 (± 0.90)	**N/A**	0.83 (± 0.49) **a**	**N/A**
			Treatment tubes			
L. castaneus	0.13 (± 0.02)	0.15 (± 0.03)	0.19 (± 0.08)	0.22 (± 0.07)	0.02 (± 0.02) **a**	0.11 (± 0.05) **b**
L. terrestris	4.19 (± 0.78)	4.17 (± 0.45)	5.27 (± 1.36)	5.54 (± 0.72)	1.08 (± 0.93) **a**	1.37 (± 0.42) **a**

3.4. Changes in solution pH

The pH of the solutions fluctuated between 6.20 and 7.79 throughout the experiment. There was a significant effect of time ($P < 0.0001$), treatment ($P < 0.0001$) and the interaction term between the two ($P < 0.0001$) on the solution pH for *L. terrestris*, *A. chlorotica*, and *L. castaneus* (Table 5).

Tukey *post hoc* testing of the pH values over time points and treatments for individuals of *L. terrestris* found that the pH of the solution in the treatment tubes at time points 48 and 72 hours were significantly lower than the pH of control tubes at 3 and 72 hours ($P < 0.05$). The general trends showed that pH values were significantly lower in the treatment tubes than in the control tubes ($P < 0.05$), and that pH values were significantly lower in hours 28 and 72 than in hours 0 to 24 ($P < 0.05$).

Tukey *post hoc* testing for *A chlorotica* found that the pH of the solution in the treatment tubes was significantly lower than the control tubes at time point 288 hours ($P < 0.05$). The pH values from 48 to 288 hours were significantly lower than 0 to 9 hours, with no significant difference in the pH between hours 48, 96, 144, and 288. The general trend shows decreasing pH with time in both the treatment and control tubes, but with the exception of time point 288 there is no significant difference between the pH in the control and treatment tubes at each time point.

Tukey *post hoc* testing for *L. castaneus* found that the pH of the solution in the treatment tubes was significantly lower than the control tubes ($P < 0.05$), with the solution pH decreasing over time.

Table 5. The mean and standard deviation solution pH in control and treatment tubes containing individuals of *Lumbricus terrestris* (n = 48), *Allolobophora chlorotica* (n = 48) and *Lumbricus castaneus* (n = 48) at each time point (n = 3 for each species time point). For each species, cells marked with the same letter within control and treatment columns are not significantly different (*P* > 0.05; Tukey *post hoc* testing).

L. terrestris			***A. chlorotica***			***L. castaneus***		
Hours submerged	**Solution pH**		**Hours submerged**	**Solution pH**		**Hours submerged**	**Solution pH**	
	Control	**Treatment**		**Control**	**Treatment**		**Control**	**Treatment**
0	7.35 (± 0.09) **abcd**	6.91 (± 0.13) **abc**	**0**	7.61 (± 0.08) **f**	7.41 (± 0.04) **e**	**0**	7.53 (± 0.06) **ef**	7.61 (± 0.05) **f**
3	7.71 (± 0.08) **e**	7.37 (± 0.06) **abcd**	**9**	7.30 (± 0.07) **cde**	7.22 (± 0.01) **bcde**	**9**	7.61 (± 0.09) **f**	7.54 (± 0.04) **ef**
6	7.62 (± 0.06) **de**	7.02 (± 0.06) **abc**	**24**	7.39 (± 0.11) **de**	7.19 (± 0.09) **abcd**	**24**	7.47 (± 0.11) **def**	7.24 (± 0.03) **abcd**
9	7.31 (± 0.19) **abcd**	6.86 (± 0.01) **ab**	**48**	7.21 (± 0.14) **bcde**	6.99 (± 0.03) **ab**	**48**	7.43 (± 0.10) **cdef**	7.22 (± 0.09) **abcd**
24	7.40 (± 0.02) **bcd**	7.07 (± 0.56) **abcd**	**96**	7.08 (± 0.10) **abc**	6.93 (± 0.04) **ab**	**72**	7.31 (± 0.14) **bcde**	7.13 (± 0.07) **abc**
33	7.25 (± 0.02) **abc**	6.65 (± 0.15) **abc**	**144**	1.09 (± 0.14) **abc**	6.91 (± 0.04) **ab**	**120**	7.13 (± 0.16) **abc**	6.94 (± 0.15) **ab**
48	7.19 (± 0.06) **abc**	6.47 (± 0.03) **ab**	**216**	6.89 (± 0.12) **ab**	6.79 (± 0.08) **a**	**168**	7.16 (± 0.26) **abcd**	6.65 (± 0.07) **a**
72	7.47 (± 0.15) **cde**	6.25 (± 0.06) **a**	**288**	7.36 (± 0.10) **de**	6.82 (± 0.05) **a**	**216**	7.21 (± 0.12) **abcd**	6.60 (± 0.06) **a**

3.5. Observed behavioural responses

Although not quantified, it was observed that individuals of *A. chlorotica* exhibited a behavioural response similar to aestivation after being submerged for some time. Before treatment individuals were relaxed and moved normally whilst being weighed and kept on the laboratory workbench. However, when replicates were sampled after 24 hours and onwards individuals were curled into a tight ball, and it was only after a period of up to two minutes on the workbench in ambient air that they uncurled and began moving again and responding to stimulation (Fig 4).

4. Discussion

4.1. Control vs treatment deaths

The control and treatment individuals were maintained at different temperatures, with control individuals maintained at 15°C and treatment individuals at 10°C. However, the deaths of treatment individuals are unlikely to be due to the temperature at which they were maintained. The 10°C at which treatment individuals were maintained is within the temperature range at which individuals are still found active in the field (Edwards and Lofty, 1977), and *L. terrestris* and *A. chlorotica* both exhibit normal behaviours such as reproduction when maintained at 10°C (Butt, 1991; Butt, 1997). In other studies (not reported), we have maintained earthworms at 5°C in soil for several months with no mortality occurring, further suggesting that the difference in mortality in this experiment is not due to the 10°C solution temperature.

Only one control earthworm death occurred: an individual of *L. castaneus* at hour 168. This indicates that the earthworm death is likely not linked to starvation or being maintained in the tubes. If it was, then there would likely have been more than one death out of the 24 control replicates of *L. castaneus*, and 72 total control replicates across all earthworm species. Roots (1956) found that, in aerated water and without a supply of food, *L. terrestris* and *A. chlorotica* were able to survive an average of 137 days when submerged, while *Lumbricus rubellus*, an epigeic species, was able to survive an average of 78 days. The fact that the duration of the experiment was well within these limits together with the survival of earthworms at temperatures of 10°C and below for extensive periods of time, the soil activity of earthworms at temperatures below 10°C, and the death of only one individual in the control tubes, show that earthworm deaths in the 10 °C tubes were not due to them being kept in solution, starvation, or temperature conditions but were instead due to other factors such as the depletion of oxygen or changes in pH.

For all three of the earthworm species, the pH of the treatment tubes was significantly lower than that of the control tubes. In both the treatment and control tubes there is a general trend of decreasing pH across all time points. This acidification of the reconstituted groundwater is likely due to the production of CO_2 during earthworm respiration. CO_2 is highly soluble in water, where it dissolves to form carbonic acid ($CO_{2(aq)} + H_2O \leftrightarrow H_2CO_{3(aq)}$) at a saturated concentration of 1.97 g L^{-1} at 15°C (Dean, 1972).

The higher pH in the control tubes compared to the treatment tubes may be attributed to the control mechanism of continuous aeration with ambient air. Aeration with oxygen is a mechanism frequently used in aquaculture to strip carbon dioxide from solution (Summerfelt et al., 2000), which in turn leads to an increased pH. The pH in the treatment solutions was lower in the tubes containing individuals of *L. terrestris* and *L. castaneus* at the end of the experiment than the tubes containing *A. chlorotica*. This may be a result of reduced respiration rates in *A. chlorotica*, which could be a survival tactic used by the species in response to reduced oxygen or high stress conditions (see below).

In culture, earthworms are able to tolerate a pH range of 4.5 to 7 (Lowe and Butt, 2005). In this study, pH values ranged from 6.2 to 7.8. While the highest pH is slightly above that which Lowe and Butt reported as preferable, other studies have found that the aversion to soil pH values of above 7.0 is slight, and weaker than the aversion earthworms show for very acidic soils, although the authors did not provide a reason for this observation (Baker and Whitby, 2003). In this study, therefore, although changes in pH were observed over time, and differences between control and treatment tubes were observed, these are unlikely to have contributed to the earthworm deaths.

4.2. Earthworm mass gain

Individuals of *L. castaneus* showed a significantly higher mass gain in the dead treatment earthworms relative to the single dead control earthworm which did not gain any mass, with no significant difference in the mass gain between the control and treatment live earthworms. The difference in mass gain between the dead treatment earthworms and the live earthworms in both treatment and control tubes is likely because whilst 100% earthworm death in the randomly selected tubes occurred by 168 hours, 66% of earthworm death was reached by 48 hours. As tubes were randomly selected, it may be the case that many of the earthworms had been dead for some time when their tube was randomly selected between the 48- and 168-hour sampling times. Earthworms actively control their osmoregulation

(Carley et al., 1983); in the period between death and sampling, the individuals of *L. castaneus* may have gained significant quantities of water, and thus mass, via osmosis.

Individuals of *A. chlorotica* gained significantly more mass in the treatment tubes than in the control tubes, with the mass gained by treatment individuals in comparison to control individuals increasing as the time submerged increased. *A. chlorotica* is able to aestivate (Edwards and Lofty, 1977), meaning that the species is able to enter a period of dormancy in response to high temperatures and dry conditions. Studies performed on another endogeic aestivator, *Apporectodea caliginosa*, found that, when aestivating in soil, the earthworm water content increased in the early stages of aestivation. The earthworms increased their osmolarity, which resulted in the passive update of water from the soil; this strategy allowed increased chances of survival in hot and dry conditions (Bayley et al., 2010). We suggest that *A. chlorotica* is exhibiting a similar strategy to that which they exhibit when soil conditions become too hot and dry, and increased their osmolarity as they entered a dormant state, resulting in the passive uptake of water. As the earthworms were submerged in solution it seems likely that the mass gain was higher than may be observed in soil due to either greater differences in osmolarity between the earthworm and the surrounding fluid and/or the greater fluid:earthworm ratio in the solutions leading to a larger supply of available water.

There was no effect of treatment or status on the mass gain of *L. terrestris*. While there was an effect of the time spent submerged, the significant difference lay between individuals removed at 0 hours submerged gaining significantly less mass than individuals submerged for greater than 6 hours, and no significant difference observed between individuals submerged for more than 6 hours. It may be the case that mass changes due to osmosis did occur, but, as a larger bodied organism, the mass changes represented a smaller percent increase of the total mass of individuals, meaning that statistically any mass changes were masked within variance in the dataset.

4.3. Absolute oxygen concentration

One of the major findings of this study was that none of the individuals of *A. chlorotica* died during the experiment, despite being immersed in water for nearly 300 hours (12 days). However, the consumption of oxygen showed a similar pattern to that observed in both *L. terrestris* and *L. castaneus*, with the oxygen concentration reducing rapidly in the early stages of the experiment before plateauing. From the period of 144 hours to 288 hours, the mean oxygen concentration in the tubes containing *A. chlorotica* (1.49 ± 0.40 mg O_2 L^{-1}) was significantly higher than the mean oxygen concentration at which individuals of *L. terrestris* died (0.82 ± 0.46 mg O_2 L^{-1}), but did not significantly differ from the mean oxygen concentration at which individuals of *L. castaneus*, a similarly sized earthworm, died (3.60 ± 2.01 mg O_2 L^{-1}). *L. terrestris* is a larger earthworm than both *L. castaneus* and *A. chlorotica*, and thus has less surface area of body wall per unit mass to exchange oxygen across which might suggest that it would die at higher oxygen concentrations but this is not observed. Similarly, *L. terrestris* survived at lower oxygen concentrations when these were normalised by biomass and by surface area. This suggests that the difference in oxygen requirements between species may be a result of adaptation to the different lifestyles exhibited by the different earthworm ecotypes.

4.4. Earthworm traits

The key driver in the differences in the oxygen requirements between *L. terrestris* and *L. castaneus* is likely a result of differences between the characteristics of the two ecotypes, related to the organism's lifestyle and strategy rather than characteristics of the preferred soil habitat. As an anecic species, *L. terrestris* leads what could be described as a mostly sedentary lifestyle, living in deep vertical burrows in the soil. Laboratory experiments have shown that the oxygen consumption of other anecic species is greatest during night time periods compared to during the day (Chuang and Chen, 2008) when the earthworms typically emerge to forage on the soil surface. This suggests that the anecic earthworms may experience more extremes in their physical activity rates, and therefore oxygen consumption, than epigeic or endogeic species. The lifestyle of epigeic species, on the other hand, is dramatically different. Living in, and consuming, the soil-litter layer, the ecotype displays many of the characteristics of rapid colonisers (Eijsackers, 2011), rapidly coming to dominate in regularly disturbed areas (Pižl, 2001; Klok et al., 2006). Satchell (1980) first suggested that epigeic earthworm species are *r* strategy organisms, and that their life strategy differs to that of anecic earthworms. *r* strategy organisms favour large productivity (MacArthur and Wilson, 1967), and typically have a shorter lifespan, greater reproductive output, and a smaller size of individuals, while *K* species are characterised by a longer lifespan, reduced reproductive output, and a larger individual size. Butt and Lowe (2011) summarised studies regarding a number of characteristics between earthworm species, including the number of days required to grow to maturity, the number of days required to incubate cocoons, and the number of hatchlings per cocoon. Their data suggest that *L. terrestris* has more *K*-like strategies than epigeic earthworms such as *L. rubellus* and *Eisinea fetida* (Savigny, 1826) (Butt and Lowe, 2011). Other studies on the lifespan of earthworms (Lakhani and Satchell, 1970; Mulder et al, 2007), the growth to maturity (Edwards, 1988) and the number of hatchlings produced per cocoon (Butt, 1997) also support this observation. At the time of writing, we are not aware of any laboratory studies performed to determine the potential lifespan of *L. castaneus* individuals, and it must be remembered that the practical lifespan of the organism in the field is likely much shorter than under laboratory conditions, due to factors such as predation and food availability.

The lifestyle of an *r* species organism, prioritising reproduction and growth to sexual maturity at the cost of lifespan of the individual, likely comes at a higher metabolic cost than that of a *K* species. Traits such as the smaller body mass typically associated with *r* strategists are associated with higher metabolic rates (Brown et al., 2004), while *K* strategist organisms typically show reduced energy wastage in comparison to *r* strategists due to their maintenance of population equilibrium (Southwood et al., 1974). Applying these *r* and *K* strategies to *L. castaneus* and *L. terrestris* respectively would explain why *L. castaneus* individuals died at a much higher oxygen concentration than *L. terrestris*. For an *r* species, the survival of the individual in a flooding event is less important, as the high reproductive output of cocoons, each with a high number of individuals per cocoon, such as *E. fetida* producing 3.3 hatchlings per cocoon (Edwards, 1988), means that an *r* species is likely to rapidly recolonise after a disturbance event, despite lower survival rates of individuals in low oxygen, flooded conditions.

It is not just the mass gain observed in *A. chlorotica* which indicates that the species may be aestivating in the treatment tubes. The aestivation process involves a number of behavioural characteristics, where the individuals excavate a chamber which is lined by mucus and then roll themselves into a tight knot, with the head and tail tucked into the centre; the mucus lining is thought to help minimise water loss from the body (Edwards and Lofty, 1977). While it is not possible for the earthworms to excavate a chamber when kept in solutions, and any mucus excreted would have dissolved into solution, the behavioural response of aestivation was still observed (Fig. 4).

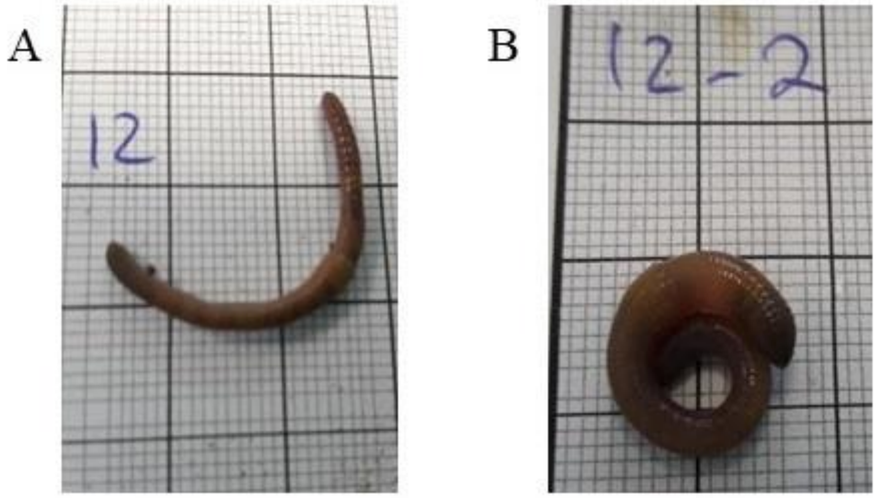

Fig. 4. The same individual of species *Allolobophora chlorotica* before (A) and after (B) submergence in a treatment tube for 144 hours. (To see the photograph in colour, the reader is referred to the web version of this article.)

A. chlorotica comprises two colour morphs; one pink, the other green. The pink morph prefers drier conditions than the green morph (Satchell, 1967) to the extent that lower soil moisture significantly restricts the growth of green morph juveniles (Lowe and Butt, 2007), with some studies arguing for their classification as two separate species on the basis of breeding experiments (Lowe and Butt, 2008) and genetic analysis (King et al., 2008). The precise reasons for the strong preference of high soil moisture content is still not known, but there is evidence that the colouration differences are due to a differing haem pigment between the two morphs (Kalmus et al., 1955). In this study, the green morph of *A. chlorotica* was used, although this does not show up well in Fig. 4 due to poor lighting. It may be the case that the different haem pigments present in the two morphs have different oxygen affinities thereby making oxygen available for respiration to the green morph at lower concentrations than for the pink morph. Research into earthworm haemoglobin has focussed heavily on *L. terrestris* (e.g. Reichert and Brown, 1908; Chen et al, 2015) and so lack of data means that this hypothesis remains unproven.

However, previous studies have demonstrated differences in the oxygen affinities of the haemoglobin of *L. terrestris* and *Apporectodea longa* (Ude, 1885) (Haughton et al., 1958) and, between the haemoglobin of these two species and the giant earthworm *Glossoscolex giganteus* (Leuckart, 1835) (Johansen and Martin, 1966) suggesting that significant differences may exist between the haem pigments of the two colour morphs. In the field, the higher soil moisture conditions which the green morph of *A. chlorotica* prefers are more likely to be associated with low soil oxygen availability (Ponnamperuma, 1984; Kiss, 2019), suggesting that the species is perhaps able to exploit a niche that other earthworm species are unable to.

With its small body size, and relatively fast growth to maturity and cocoon incubation period, *A. chlorotica* is more similar in lifestyle to the *r* strategy, epigeic species than to the *K* strategy, anecic species. However, this study suggests that for this species, the aestivation response overrides any expected oxygen requirements associated with either the *r* or *K* strategy.

4.5. Field site context

This study does not represent field conditions. While this study has focused on the absolute oxygen concentrations required for survival, some experiments have found that earthworms are able to survive for 120 days in flooded soil samples (Ausden et al., 2001). This suggests that earthworm behavioural responses in flooded soil may be equally as influential for a species surviving a flooding event as their absolute oxygen requirement. For example, while developing the methods for this experiment, we initially used open beakers but individuals of *L. terrestris* were observed exhibiting a ‘snorkelling’ behaviour, where a segment of the body was maintained out of the water, allowing the earthworms to respire. Our final experimental design with sealed tubes purposefully prevented this behaviour as we wished to assess fatal oxygen concentrations. Another consequence of our initial open beaker design was that oxygen was able to diffuse across the air-water interface; in these experiments earthworm mortality was low. In the field, provided flood waters are not excessively deep, earthworms that emerge on to the soil surface would have access to oxygenated water just below the air-water interface, though this may make them vulnerable to predation. Despite these departures from ecological realism our study remains ecologically informative; it supports the idea that earthworms are observed in significant numbers on the soil surface following intense periods of rainfall in order to avoid suffocation. Soils are rapidly depleted in oxygen when water logged (Ponnamperuma, 1984), with oxygen concentrations reaching levels below which earthworms have been demonstrated to die in this study within 4 to 8 hours, depending on the soil's organic matter content (Kiss, 2019).

This study also helps to explain some of the patterns of earthworm distribution observed in previous studies. While *A. chlorotica* is one of the most common species of earthworm in the UK, representing 34% of all UK earthworms (Natural England, 2014), and is found in high abundances throughout Northern and Central Europe (Dinter et al., 2013), their presence in regularly flooded sites has been recorded in a number of studies (Plum, 2005; Plum and Filser, 2005; Kiss et al., 2021), with little variance in their abundance regardless of recent flooding events (Zorn et al., 2005). The persistence of *A. chlorotica* populations in soils may in part be due to the ability of the species to resist extremes in soil environmental conditions, such as both drought and flooding conditions.

This study highlights the differences in oxygen requirements between earthworm species with different life strategies, which may impact their survival in flooding events. As climate change is predicted to lead to increased flooding across a number of regions globally (Hirabayashi and Kanae, 2009; Kundzewicz et al., 2014), this may have wider consequences on earthworm diversity and distributions of earthworm species in fields likely to flood. Because of this, and given our results, further experiments which are more ecologically realistic are warranted to more fully investigate how changes in oxygen levels in flooded soils impact earthworms. For example, the depth of standing water on flooded soils above which rates of oxygen diffusion result in anoxic soils could be determined together with experiments in which a gradient of oxygen concentrations in flooded soils is established to determine at which level earthworms would actively move out of the soil volume and whether lateral or vertical movement is preferred. Our research also highlights the need for additional research into aestivation and its triggers, as the response may be more wide ranging than currently understood.

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