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Effects of temporary grassland introduction into annual crop rotations and nitrogen fertilisation on earthworm communities and forage production

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Abstract

Earthworms contribute to a wide range of ecosystem services in agriculture. Most studies focusing on impacts of annual crop management on earthworm communities are related to soil tillage, fertilisation or pesticides, while the effect of introducing temporary grassland into an annual crop rotation remains largely unknown. In this context, the aims of the present study were to evaluate effects of (i) introducing grassland into a crop rotation, (ii) grassland duration and (iii) grassland fertilisation on earthworm communities and grassland forage production in a long-term experimental station in western France. Three years of highly fertilised (230 kg ha^{-1} yr^{-1} of mineral nitrogen) grassland preceded by three years of annual crop rotation was compared to an annual crop rotation without grassland and to six years of grassland either lightly (30 kg ha^{-1} yr^{-1} of mineral nitrogen) or highly (230 kg ha^{-1} yr^{-1} of mineral nitrogen) fertilised. Grassland introduction into a crop rotation significantly increased earthworm abundance, biomass, and diversity, especially for anecic species, and improved the functional structure of the earthworm community. Grassland duration and fertilisation increased earthworm abundance and biomass, especially for anecics, without affecting endogeic species and earthworm diversity. Grassland duration did not increase forage production, but the increase in fertilisation did. Three years of highly fertilised grassland significantly increased earthworm abundance and biomass, improved the functional structure of the earthworm community and produced as much forage per year as six years of highly fertilised grassland. Overall, the introduction of grassland into annual crop rotations appears an effective way to rapidly increase earthworm community parameters and, given the important role of earthworms in soils, enhance soil functioning.

Keywords: Grassland management; grassland duration; mineral fertilisation; soil biodiversity; ecological category

1. Introduction

In response to global changes, and because farmers are facing crucial issues, annual cropping systems are changing; focussing not only on fertility performance (yield), but also seeking to promote other ecosystem services supported by soils, such as climate regulation through carbon sequestration, water flux and water quality regulation, maintenance of soil structure to limit soil erosion, and biodiversity conservation (Albizua et al., 2015; Keesstra et al., 2016; Chabbi et al., 2017). Therefore, even if the economical aspect is a crucial pillar for farmers, environmental and social sustainability are becoming more important leading to increasingly complex rotations that include temporary grassland (Albizua et al., 2015; Lemaire et al., 2015; Tsiafouli et al., 2015). Introducing grassland into an annual crop rotation has several demonstrated benefits, including improving soil structure (Loaiza Puerta et al., 2018), soil organic matter storage (Crème et al., 2018; Panettieri et al., 2017), yield of subsequent crops (Franzluebbers et al., 2014) and soil organism community parameters (van Eekeren et al., 2008; Postma-Blaauw et al., 2010; Tsiafouli et al., 2015).

Earthworms are known to play an important role in the provision of soil ecosystem services (Blouin et al., 2013) including contributing to element regulation (Marinissen and de Ruiter, 1993), plant production (van Groenigen et al., 2014), soil structure maintenance (Blanchart et al., 2009; Pérès et al., 2010) and water regulation (Bastardie et al., 2003), which are particularly valued in agricultural environments (Bertrand et al., 2015). There is considerable evidence that the implementation of an annual crop after a grassland leads to a rapid decrease in earthworm abundance and diversity, however, the effects of introducing grassland after annual crops on earthworm communities remain poorly studied (van Eekeren et al., 2008; Postma-Blaauw et al., 2010; Crotty et al., 2016). The available literature tends to be biased towards experimental sites with limited (3-5 species) earthworm diversity (van Eekeren et al., 2008;

Postma-Blaauw et al., 2010). However, it is widely reported that earthworm diversity may be much higher in agricultural contexts ranging from six to eleven species per field (Cluzeau et al., 2012; Richard et al., 2012). Therefore, effects of introducing grassland after annual crops could modify the development earthworm communities (Bouché, 1977; Satchell, 1980; Butt, 1993) and their contribution to ecosystem services.

Since earthworms are influenced by the presence and quality of food and soil properties (Curry, 2004; Rutgers et al., 2016; Hoeffner et al., 2021), grassland management factors such as stocking rate, cutting frequency and fertilisation modify earthworm communities (Cluzeau et al., 1992; Muldowney et al., 2003; Bueno and Jiménez, 2014). For example, in grassland Curry et al., (2008) observed positive relationships between nitrogen (N) application and earthworm abundance and biomass. Mineral fertilization, by increasing primary production, increases the food supply for earthworms and increases protection against predators and climatic hazards (Curry et al., 2008; Mariotte et al., 2016; Stroud, 2020). However, to our knowledge, no previous study focused on the impact of N application on the restoration of grassland earthworm communities.

The first objective of the present study was to evaluate both the effects of introducing grassland into an annual crop rotation and grassland duration on the composition of the earthworm community and grassland forage production. We hypothesised that introducing grassland into a crop rotation would have a positive effect on earthworm community parameters that in turn would increase with grassland duration. The second objective was to evaluate the effects of grassland mineral fertilisation on the composition of earthworm communities and grassland forage production. We hypothesised that fertilising grassland would have a positive effect on earthworm community parameters and forage production. A treatment of three years of fertilised grassland preceded by three years of annual crop rotation (grain maize, wheat and lastly

85 barley) was compared to treatments of six years of an annual crop rotation without grassland
86 (grain maize, wheat, barley, grain maize, wheat and lastly barley) and six years of fertilised
87 grassland (fertilised either highly or lightly).
88
89 **2. Materials and methods**
90 **2.1 Site description and experimental design**
91 The experimental site is part of the Agro-ecosystems, Biogeochemical Cycles and
92 Biodiversity long-term experimental stations on environmental research (SOERE ACBB),
93 managed by the French National Institute of Agricultural Research and Environment (INRAE),
94 located in Lusignan (46°25′12.91″ N; 0°07′29.35″ E), France. The climate is oceanic, with a
95 mean annual precipitation of 796 mm and mean annual temperature of 11.4 °C, a monthly
96 minimum of -1.9 °C and a monthly maximum of 30.6 °C (data from Agroclim, 2006-2011). In
97 2011, soil cores (0-30 cm depth) were collected at 16 locations to characterise bulk density, pH
98 and carbon (C) and nitrogen (N) content for the site. Soil cores were sent to the analytical
99 laboratory of INRAE (Arras, France) for pH, C and N measurements following the NF ISO
100 10390, 10694 and 13878 respectively. Bulk density was measured with calibrated cylinders. The
101 soil is a Plinthic Cambisol (Moni et al., 2010) with a silt loam texture, the 0-30 cm soil layer is
102 characterised by a pH-H_20 of 6.67, C content of 10.67 mg g^{-1}, N content of 1.27 mg g^{-1} and bulk
103 density of 1.48 g cm^{-3} (Supplementary Table S1, data from Arras, France, soil analysis
104 laboratory).
105 Experimental treatments were established in 2005 and management of the crops and
106 grasslands was ensured according to agricultural practices to achieve yields and forage
107 production close to the potential determined in the region by soil and climate. Before the start of
108 the experiment, the site was homogenously cultivated as a succession of winter triticale (2004-

2005), winter barley (2003-2004) and rapeseed, winter wheat or winter barley (2000-2002). Previously, the site was cultivated under annual crops and mown grassland rotations for at least 70 years. During the period 1990-2000, the field was covered with 2/3 annual crops and 1/3 temporary grasslands.

Four treatments were compared (Table 1): (i) six years of fertilised annual crop rotation (grain maize, wheat, barley, grain maize, wheat and lastly barley) (AC); (ii) three years of highly fertilised grassland preceded by three years of annual crop rotation (grain maize, wheat, barley) (G3N+); (iii) six years of highly fertilised grassland (G6N+) and (iv) six years of lightly fertilised grassland (G6N-). Each treatment was replicated four times randomly (i.e. four blocks). For more details about the experimental design, see Kunrath et al. (2015). Crops and grass were sown after ploughing to a depth of 25 cm. Maize was sown in April and harvested in October; winter wheat was sown in October and harvested in July; barley was sown in November and harvested in July; after barley harvest, soil was left fallow till sown with maize. Grain maize residue was crushed and left on the soil surface, while the straw of wheat and barley was removed. After barley harvest, soil remained bare until maize was sown in March. Grasslands were sown as a mixture of perennial ryegrass (*Lolium perenne* cv. Milca), tall fescue (*Festuca arundinacea* cv. Soni) and cocksfoot (*Dactylis glomerata* cv. Ludac). Grasslands were cut to 5-7 cm and harvested three or four times per year (depending on seasonal climate and grassland productivity of the year). Nitrogen (ammonium nitrate) was the only mineral element added through mineral fertilizer application. For annual crops, N application rates and timing were adjusted each year using the PC-AZOTE program (for more details, see Kunrath et al. 2015). On average over the experimental period, N application on annual crops was 106 kg N ha^{-1}. For grasslands, regular estimations of the Nitrogen Nutrition Index (NNI) were made to maintain a NNI of between 0.9 and 1.0, i.e. close to a non-limiting N nutrition allowing for potential herbage production (for

more details, see Kunrath et al. 2015). On average over the experimental period, annual N application was 230 kg ha^{-1} on highly fertilised grassland (divided among applications performed after cutting) and 30 kg ha^{-1} on lightly fertilised grassland.

2.2 Earthworm sampling and laboratory analyses

Earthworm sampling for the present study was performed in March 2004 before the establishment of the experimental site, and in March 2011 (after the harvest of the barley for AC and in grassland for G3N+, G6N+ and G6N-). In 2004, earthworms were sampled at nine locations homogeneously distributed to cover the entire site, which provided a baseline value of the earthworm community before experimental establishment. In 2011, earthworms were sampled in each plot, at three locations spaced at least 10 m apart and at least 10 m from the plot edge, before sowing the maize in AC and during the grassland phase of G3N+, G6N+ and G6N-. Earthworms were sampled according to the ISO 23611-1 protocol modified according to Pérès et al., (2010), which combines chemical and physical extraction. Briefly, each earthworm-sampling event consisted of applying three 10 L applications of water with an increasing concentration of formaldehyde (0.25%, 0.25% and 0.40%) on one square meter. To recover earthworms unable to reach the surface, a block of soil (25 × 25 × 20 cm), corresponding to an area of 1/16 square meter, was hand-sorted inside the sample square (for more details, see Pérès et al., 2010).

In the laboratory, earthworms were counted, individually weighed (± 10 mg), assigned to a stage of development (juvenile, sub-adult and adult), identified to species level using Bouché's (1972) key and assigned to an ecological category: epigeic, epi-anecic (*Lumbricus* sp.), strict-anecic (*Aporrectodea* sp.) or endogeic (Bouché, 1977; Jégou et al., 1998; Hoeffner et al., 2019). Juvenile earthworms that could not be determined at the species level (29% of the dataset) were allocated to species according to the proportion of adult and sub-adult identified within a square

meter and within the same functional group. The earthworm community was analysed by total abundance, total biomass, abundance and biomass of ecological categories, species richness, and species evenness (i.e. similar distributions of species).

2.3 Forage production and composition

In 2010, grass was cut three times during the year (April, June, and July). Above-ground biomass was estimated by cutting an area of 1.5 × 5.0 m with an experimental harvester (Haldrup, Germany). Grass was dried in an oven at 70 °C and weighed to determine dry matter content. It was then ground for chemical analysis. Total C and N concentrations were determined by the Dumas method using an elemental analyser (Carlo Erba EA 1108).

2.4 Statistical analyses

Statistical analyses were performed using R software 3.5.2 (R. Core Team, 2020). All differences were considered significant at $P < 0.05$. The conditions of normality and homoscedasticity were tested.

We firstly used linear mixed-effects models ("nlme" package), followed by Tukey HSD tests for post hoc pairwise comparisons, to assess differences between 2004 (i.e. before the establishment of the experimental site) and 2011 with the four treatments (annual crop, highly fertilised 3- and 6-year-old grassland and lightly fertilised 6-year-old grassland) on total earthworm abundance, biomass and species richness. To account for pseudo-replication the random part of the model specified that earthworm-sampling events were nested within a block. Secondly, we used linear mixed-effects models ("nlme" package), followed by Tukey HSD tests for post hoc pairwise comparisons, to test in 2011 effects of the four treatments (annual crop, highly fertilised 3- and 6-year-old grassland and lightly fertilised 6-year-old grassland) on each

181 earthworm community parameter. To account for pseudo-replication, the random part of the
182 model specified that earthworm-sampling events were nested within a block. We then used
183 separate one-way ANOVAs, followed by Tukey HSD tests for post hoc pairwise comparisons, to
184 assess differences in 2010 for forage production and composition (C and N) between the
185 treatments. Next, we calculated Pearson correlations between grassland forage production and
186 composition (C and N) and earthworm community parameters.
187
188 **3. Results**
189 Compared to the 2004 baseline, total earthworm abundance and biomass in 2011
190 decreased significantly by 3.5 and 5.5 times in AC respectively and increased by 1.9 and 2.3
191 times in G6N+ respectively (ANOVA, $P < 0.001$, Fig. 1a and b), while no significant difference
192 was observed for G3N+ and G6N-. In addition, earthworm species richness decreased by 1.4
193 times in AC (ANOVA, $P < 0.001$, data not shown) whereas no difference was observed between
194 2004 and 2011 for the grassland treatments (G3N+, G6N- and G6N+).
195 In 2011, on the whole experimental site, 13 earthworm species were observed (Table 2):
196 one epigeic (*Lumbricus castaneus*), four epi-anecic (dominated by *Lumbricus centralis*), three
197 strict-anecic (dominated by *Aporrectodea longa longa*) and five endogeic (dominated by
198 *Allolobophora chlorotica chlorotica* and *Aporrectodea caliginosa caliginosa*, Table 2). Epigeic
199 earthworms were only present in highly fertilised grasslands (G3N+ and G6N+) and absent in the
200 other treatments (AC and G6N-, Table 2).
201 In 2011, total earthworm abundance and biomass were significantly higher in G3N+ than
202 in AC (4.6 and 8.3 times, respectively; ANOVA, $P < 0.001$, Fig. 1a and b, Supplementary Table
203 S2). Except for epigeic earthworms, abundance and biomass of ecological categories were
204 significantly higher in G3N+ than in AC (ANOVA, $P < 0.008$, Fig. 1a and b). These differences

were greater for epi-anecic (153.3 and 130.0 times, respectively) than for strict-anecic (15.3 and 16.5 times, respectively) or endogeic earthworms (2.8 and 3.9 times, respectively, Fig. 1a and b). Interestingly, there was an unequal proportion between strict-anecic and epi-anecic in G3N+, as strict-anecic abundance and biomass were 3.2 and 2.1 times higher (Fig. 1a and b). Earthworm species richness was 1.6 times higher in G3N+ than AC (ANOVA, $P < 0.001$, Supplementary Table S2), due to the absence in AC of *L. castaneus* (epigeic), *Lumbricus terrestris* (epi-anecic), *Aporrectodea giardi* (strict-anecic), *Ethnodrilus zajonci* (endogeic) and *Octolasion cyaneum* (endogeic). Species evenness did not differ significantly between G3N+ and AC (ANOVA, $P > 0.05$, Supplementary Table S2).

In 2011, regarding the effects of grassland duration (G3N+ vs G6N+), total abundance and biomass were 1.5 times higher in G6N+ than G3N+ (ANOVA, $P < 0.001$, Fig. 1a and b), due to the presence of strict-anecic earthworms (ANOVA, $P < 0.003$, Fig. 1a and b). Epigeic, epi-anecic and endogeic abundance and biomass did not differ significantly between G3N+ and G6N+ (Fig. 1a and b). Moreover, earthworm species richness, species evenness, and forage production and composition (C and N) did not differ significantly between G3N+ and G6N+ (Table 3 and Supplementary Table S2).

In 2011, regarding the effects of mineral fertilisation (G6N+ vs G6N-), total earthworm abundance and biomass were 1.6 and 1.4 times higher, respectively, in G6N+ than G6N-, due to the presence of strict-anecic earthworms (ANOVA, $P < 0.001$, Fig. 1a and b). Once again, epigeic, epi-anecic and endogeic abundance and biomass, as well as earthworm species richness and species evenness, did not differ significantly between G6N- and G6N+ (Supplementary Table S2). Interestingly, there was also an unequal distribution of strict-anecic and epi-anecic as strict-anecic abundance was 3.1 and 3.5 times higher than epi-anecic abundance in G6N+ and G6N- respectively (Fig. 1a). Nevertheless, forage production was 3.3 times as high in G6N+ as in G6N-

229 (ANOVA, $P < 0.001$, Table 3), whereas forage composition did not differ between them with a
230 mean C content of 432.81 ± 0.83 (SE) mg g^{-1} and a mean N content of 24.91 ± 1.17 (SE) mg g^{-1}
231 (ANOVA, $P > 0.448$, Table 3). Finally, neither forage production nor composition (C and N)
232 correlated significantly with any earthworm community parameter ($R^2 < 0.56$, $P > 0.05$, data not
233 shown).
234
235 **4. Discussion**
236 In the present study, between 2004 and 2011, total earthworm abundance and species
237 richness in the annual crop treatment declined sharply (71 and 49%, respectively), which
238 highlights the strong negative effect of annual crop practices applied in our study on earthworm
239 communities. In 2011 earthworm communities were characterized by an average of 92.0 ind. m^{-2}
240 and 4.5 species m^{-2}. Although these 2011 values are consistent with results obtained from annual
241 crops in other experimental fields (van Eekeren et al., 2008; Postma-Blaauw et al., 2010; Roarty
242 et al., 2017), they are noticeably lower than values recorded in non-experimental fields (Decaëns
243 et al., 2008; Cluzeau et al., 2012; Frazão et al., 2017). Among 52 crops in western France,
244 Cluzeau et al. (2012) observed total earthworm abundance of 215 ind. m^{-2} and 7.4 species m^{-2},
245 and Frazão et al. (2017), among 13 crops with semi-natural field edges in south-western
246 Netherlands, observed total earthworm abundance of 414.1 ind. m^{-2} and 4.9 species m^{-2}. The low
247 values in the present study could have been related to the absence of organic fertilisation, which
248 is known to be beneficial for soil fauna (Marhan and Scheu, 2005; Bünemann et al., 2006;
249 Cluzeau et al., 2012) or changes in the use of pesticides that are known to be detrimental to soil
250 fauna (Pelosi et al., 2013). Results could also be explained by the basic and short annual crop
251 rotation (maize, wheat, barley) without cover or inter-crops which left the soil bare for five
252 months in winter, which can be detrimental for earthworms (Schmidt et al., 2001; Edwards,

253 2004). Conversely, between 2004 and 2011, total earthworm abundance and species richness in
254 grassland treatments stabilized or slightly increased to reach an average of 479.9 ind. m^{-2} and 7.3
255 species m^{-2}, respectively, which is of similar magnitude to those found in previous studies. For
256 example, Didden (2001) observed a total earthworm abundance of 384 ind. m^{-2} and 2.1 species
257 m^{-2} in 20 Dutch grasslands while Cluzeau et al. (2012) observed a total abundance of 350.0 ind.
258 m^{-2} and 9.6 species m^{-2} in 47 French grasslands. In the present study, we observed that grassland
259 treatments (G3N+, G6N+ and G6N-) had higher earthworm abundance, biomass and species
260 richness than those observed in the annual crop treatment. This result reinforces previous studies
261 which showed that annual crops benefit earthworms less than grasslands (Decaëns et al., 2008;
262 Rutgers et al., 2009; Tsiafouli et al., 2015). The lower values under crops could be explained by
263 negative effects of soil tillage (Chan, 2001; Pelosi et al., 2016; Briones and Schmidt, 2017), less
264 food resource for earthworms (Schmidt et al., 2001; Edwards, 2004) and negative effects of
265 pesticide application (Pelosi et al., 2013). Interestingly, this result was obtained in all grassland
266 treatments, including the young grassland, i.e. three years of grassland preceded by three years of
267 crops (G3N+), which demonstrates that the earthworm community can recover rapidly, even in a
268 grassland preceded by three years of annual crops.

269 The increase in earthworm species richness in grassland could be explained by their
270 ability to colonise soil at a rate of 1.5-14 m $year^{-1}$ (Eijsackers, 2011) from surrounding edges or
271 by residual surviving populations in the field (Lagerlöf et al., 2002; Roarty and Schmidt, 2013;
272 Hoeffner et al., 2021). Nevertheless, even though species richness differed between crop and
273 grassland treatments, the four treatments had similar species evenness. All ecological categories
274 of earthworms had higher abundance and biomass in grassland treatments (G3N+, G6N- and
275 G6N+) than in the annual crop treatment (except epigeic earthworms, whose density was too low
276 to be discussed). However, abundances of epi-anecic and strict-anecic earthworms increased

277 more than that of endogeics, because the former are more sensitive to soil tillage due to their
278 permanent or semi-permanent vertical burrows (Chan, 2001; Peigné et al., 2009; Briones and
279 Schmidt, 2017). Moreover, anecic earthworms may be more sensitive to pesticide application,
280 especially fungicides and insecticides (Pelosi et al., 2013), and to bare-soil conditions (Lowe and
281 Butt, 2002; Marhan and Scheu, 2005; Frazão et al., 2019). An increase in epi-anecic and strict-
282 anecic earthworms in grassland would alter soil functioning substantially, transferring more plant
283 litter into the soil (Jégou et al., 1998; Hoeffner et al., 2019), increasing tubular soil porosity,
284 which may increase water infiltration (Jégou et al., 1997; Bastardie et al., 2003), and increasing
285 soil surface roughness by producing surface casts, which may decrease soil erosion (Le Bayon et
286 al., 2002). Regarding the ratio between anecic sub-categories, strict-anecic abundance was on
287 average 3.2 times as high as epi-anecic abundance in grassland treatments. This relative
288 difference has been recorded in the literature: in western France, Decaëns et al. (2008) observed a
289 ratio of 2.5 among 22 grasslands, and Richard et al. (2012) observed one of 4.9 and 3.7 for a 5-
290 and 42-year-old grassland, respectively. Regarding effects of endogeics, despite the smaller
291 increase in their abundance and biomass in grassland treatments compared to those in annual crop
292 rotation, the ecosystem functions they provide, such as soil structure maintenance (Le Couteulx et
293 al., 2015), may also have been improved.

294 While the present study demonstrated that introducing three years of grassland into a crop
295 rotation rapidly benefits earthworm communities, it also showed that total earthworm abundance
296 and biomass do not peak after three years of grassland, as they were still lower than those after
297 six years of grassland. Considering the six years of grassland treatments (G6N- and G6N+) as
298 permanent grassland, our results contrast with those of van Eekeren et al., (2008), who observed
299 that earthworm communities can be restored more rapidly, as total earthworm abundance and
300 species richness in two- and three-year-old grasslands were similar to those in a permanent

301 grassland. Furthermore, Postma-Blaauw et al., (2010) observed that total earthworm abundance
302 and biomass in three-year-old grasslands were 1.5 times higher than in permanent grassland.
303 These differences between the rates of response of the earthworm community to grassland
304 introduction could be explained by differences in cutting frequency (depending on the growth
305 rate of the grass), annual crop rotations preceding grassland introduction and soil properties such
306 as soil texture (loamy *vs* sandy-loam texture), soil structure and organic matter content. In
307 addition, the studies of van Eekeren et al. (2008) and Postma-Blaauw et al., (2010) observed total
308 earthworm abundances that were generally much lower than in the present study (i.e. lower
309 baseline) with different and fewer earthworm species (five including three endogeics and three
310 including two endogeics, respectively) than the present study (13 species including four epi-
311 anecic, three strict-anecic and five endogeic). Thus, the lower total earthworm abundance and
312 demographic strategies of different species could lead to different responses of the earthworm
313 community to grassland introduction (Bouché, 1977; Satchell, 1980; Butt, 1993). In the present
314 study, higher total earthworm abundance and biomass after six compared to three years of
315 grassland was due mainly to a large increase in anecic species abundance (1.8 and 1.7 times
316 higher for epi-anecic and strict-anecic earthworms, respectively). Thus, grassland duration could
317 have significant consequences for soil functioning, especially soil porosity and associated
318 ecosystem services such as soil water regulation, due to the burrow network of anecic species
319 (Jégou et al., 1997, 2001; Bastardie et al., 2003). Contrasting results were obtained for the
320 provisioning ecosystem service (i.e. forage production). Indeed, while the present study
321 demonstrated greater effects of six than three years of grassland in a crop rotation on the
322 earthworm community, no effect on forage production was observed. This result contrasts with
323 the meta-analysis of van Groenigen et al., (2014) that showed a positive relation between total
324 earthworm abundance and plant growth, reaching +30% of plant growth in pasture without

legumes. This contrasted finding could be explained by several mechanisms not measured in the present study, such as climate parameters (Fay et al., 2003) or deficiency in macronutrients (e.g. P, K, S, Ca, Mg) in the soil (Maathuis, 2009) which could have limited grass growth and explains the apparent low levels of N and C recovery (Whitehead, 1995; Graux et al., 2020). Indeed, Graux et al., (2020) predicted a grassland dry matter yield of 7.7 t ha^{-1} yr^{-1} over the 1984-2013 period in the same district of our study site, which is noticeably higher than those observed in the current grassland treatments (5.4, 5.5 and 1.7 t ha^{-1} yr^{-1} for the G3N+, G6N+ and G6N- respectively).

The present study demonstrated that grassland N fertilisation did not modify earthworm diversity but did increase total earthworm abundance and biomass by 1.4 and 1.6 times, respectively, for the highly fertilised grassland (230 kg N ha^{-1} yr^{-1}). This positive effect of N fertilisation in grassland has been attributed to an increase in primary production, resulting in a greater supply of plant-derived material and thus a greater supply of food and refuge for earthworm communities (Muldowney et al., 2003; Curry et al., 2008). This assumption was confirmed in the present study by annual forage production being 3.3 times higher in highly fertilised than lightly fertilised grassland. This increase in the food resource was beneficial for anecic species, especially strict-anecics, whose abundance and biomass increased by 1.7 and 2.0 times, respectively. Therefore, to decrease use of mineral N fertilisers, future studies could examine effects of organic grassland fertilisation or the addition of legumes to the grassland during sowing on earthworm communities and the possible consequences on soil functioning, which are still rarely studied (Haas et al., 2019).

5. Conclusion

The present study clearly illustrated positive effects of introducing grassland into an annual crop rotation on earthworm abundance, biomass, species richness and functional structure. Further, this study highlights that earthworm communities can react quickly, at least within three years, to the introduction of grassland into an annual crop rotation. Grassland duration (3 years *vs* 6 years) and fertilisation (N- *vs* N+) increased earthworm total abundance and biomass and were beneficial for anecics (strict-anecics), without affecting endogeics, which means that ecosystem services provided by earthworms may become more effective as grassland duration and fertilisation increase. Nevertheless, increased grassland duration did not increase forage production, but increased grassland fertilisation (30 *vs* 230 kg N ha^{-1} yr^{-1}) did. In order to reach both forage performance and earthworm community restoration, the introduction of three years of grassland in the rotation, combined with nitrogen fertilisation, appears (in our context) as the most suitable management technique. Ideally, maintaining the grassland for more than three years could allow a better earthworm community restoration.

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576 **TABLES**

577 **Table 1.** Experimental design of the study site with the four treatments. Six years of fertilised
578 annual crop rotation (grain maize, wheat, winter barley, grain maize, wheat and lastly winter
579 barley) (AC), three years of highly fertilised grassland preceded by three years of annual crop
580 rotation (grain maize, wheat, barley) (G3N+), six years of highly fertilised grassland (G6N+) and
581 six years of lightly fertilised grassland (G6N-). The vertical bold lines indicate the date of
582 sampling (March 2004 and 2011).

	2003	2004	2005	2006	2007	2008	2009	2010	2011
AC	Barley	Triticale	Maize	Wheat	Barley	Maize	Wheat	Barley	Maize
G3N+	Barley	Triticale	Maize	Wheat	Barley	Grassland			Maize
G6N+	Barley	Triticale	Grassland						Maize
G6N-	Barley	Triticale	Grassland						Maize

583

584 **Table 2.** Names, associated ecological category and mean of abundance (ind. m^{-2}) of the 13 earthworm species observed per
585 experimental treatment in 2011 (n = 12). Values are means ± SE: six years of fertilised annual crop rotation (grain maize, wheat,
586 winter barley, grain maize, wheat and lastly winter barley) (AC), three years of highly fertilised grassland preceded by three years of
587 annual crop rotation (grain maize, wheat, barley) (G3N+), six years of highly fertilised grassland (G6N+) and six years of lightly
588 fertilised grassland (G6N-).

Earthworm species or sub-species	Ecological category	AC	G3N+	G6N+	G6N-
Lumbricus castaneus (Savigny, 1826)	Epigeic	0	0.4 ± 0.2	4.1 ± 3.1	0
Lumbricus rubellus rubellus (Hoffmeister, 1843)	Epi-anecic	0	0	0	1 ± 1
Lumbricus terrestris (Linné, 1758)	Epi-anecic	0	0.1 ± 0.1	0	0
Lumbricus centralis (Bouché, 1972)	Epi-anecic	0.3 ± 0.1	40.7 ± 9	74.6 ± 14	35.6 ± 7
Lumbricus festivus (Savigny, 1826)	Epi-anecic	0	0	0.3 ± 0.3	0
Aporrectodea longa longa (Ude, 1985)	Strict-anecic	8.2 ± 3	112.9 ± 16	171.8 ± 34	106.8 ± 21
Aporrectodea giardi (Savigny, 1826)	Strict-anecic	0	2.7 ± 3	1.0 ± 0.4	1.6 ± 1
Aporrectodea caliginosa meridionalis (Bouché, 1972)	Strict-anecic	0.1 ± 0.1	6.0 ± 1	15.3 ± 5	8.0 ± 4
Aporrectodea caliginosa caliginosa (Savigny, 1826)	Endogeic	29.3 ± 7	91.5 ± 19	108.4 ± 25	91.8 ± 23
Allolobophora chlorotica chlorotica (Savigny, 1826)	Endogeic	31.4 ± 8	100.1 ± 21	92.4 ± 23	93.3 ± 16
Allolobophora rosea rosea (Savigny, 1826)	Endogeic	2.7 ± 2	5.8 ± 4	13.0 ± 6	7.0 ± 3
Octalasium cyaneum (Savigny, 1826)	Endogeic	0	0.1 ± 0.1	0.3 ± 0.2	0.5 ± 0.3
Ethnodrilus zajonci (Bouché, 1972)	Endogeic	0	1.3 ± 1	4.1 ± 4	1.3 ± 1

589 **Table 3.** Mean annual above-ground forage production (dry matter, t ha^{-1}) and composition
590 (carbon and nitrogen concentrations, mg g^{-1}) per experimental grassland (n = 4) in 2010. Values
591 are means ± SE: three years of highly fertilised grassland preceded by three years of annual crop
592 rotation (grain maize, wheat, barley) (G3N+), six years of highly fertilised grassland (G6N+) and
593 six years of lightly fertilised grassland (G6N-). Forage production was summed over multiple
594 cuts. Different letters denote significant differences among treatments with a > b (Tukey HSD
595 test). *F*-values and associated *P*-values are indicated.

Characteristic	G3N+	G6N+	G6N-	*F*	*P*
Forage production (t ha^{-1})	$5.37^{a} \pm 0.21$	$5.50^{a} \pm 0.35$	$1.69^{b} \pm 0.07$	80.01	< 0.001
Carbon (mg g^{-1})	$434.5^{a} \pm 0.7$	$432.9^{a} \pm 1.3$	$431.0^{a} \pm 1.8$	0.78	0.489
Nitrogen (mg g^{-1})	$27.1^{a} \pm 1.7$	$25.3^{a} \pm 2.2$	$22.4^{a} \pm 1.6$	0.88	0.448

596
597

598 **FIGURE**

599 Fig. 1 Mean earthworm abundance (a) and biomass (b) by experimental treatments in March
600 2011: six years of fertilised annual crop rotation (grain maize, wheat, winter barley, grain maize,
601 wheat and lastly winter barley) (AC), three years of highly fertilised grassland preceded by three
602 years of annual crop rotation (grain maize, wheat, barley) (G3N+), six years of highly fertilised
603 grassland (G6N+) and six years of lightly fertilised grassland (G6N-). Bars represent standard
604 deviations of the means (n = 12). Different letters (uppercase for the total abundance and
605 lowercase for each ecological category abundance) denote significant difference between the
606 treatments based on post-hoc Tukey's test. The dashed line represents the mean value observed at
607 the experimental site in March 2004.

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