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Earthworm invasion in North America: food resource competition affects native millipede survival and invasive earthworm reproduction

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22   **Abstract**

23   The invasive non-native earthworm *Amynthas agrestis* (Goto and Hatai 1899) has

24   recently been documented invading forests of the Appalachian Mountains in the

25   southeastern United States. This epigeic earthworm decreases the depth of organic soil

26   horizons, and this may play a role in the decrease of millipede richness and abundance

27   associated with *A. agrestis* invasion.   To investigate the mechanisms behind these

28   effects, *A. agrestis* and the millipede *Sigmoria ainsliei* (Xystodesmidae) were placed into

29   microcosms with soil and either L horizon, F and H horizon, or a combination L/FH

30   treatment. Microcosms were destructively sampled and reconstructed with the same

31   treatments every four weeks to assess faunal fresh weight change and survival. Soils

32   from earthworm treatments were wet-sieved for cocoons to assess treatment effects on

33   reproduction. On average, millipede mortality occurred 88 days sooner in treatments that

34   did not have FH horizon material, and within all litter treatments millipedes tended to

35   survive longer when *A. agrestis* was absent. Earthworms maintained higher fresh weight

36   in L/FH than FH or L treatments. With a single exception, no *A. agrestis* cocoons were

37   recovered from microcosms that also contained *S. ainsliei*. The results suggest that *A.*

38   *agrestis* and *S. ainsliei* may compete for food resources, particularly the smaller particle

39   material in the FH horizons of the forest floor. Millipedes may exert some biotic

40   resistance to *A. agrestis* invasion, as diminished earthworm fecundity was observed in

41   experimental units containing both species.

42

43   Keywords: Millipede; earthworm; *Sigmoria; Amynthas;* competition; food preference;

44   invasive species

45

1 Introduction

Non-native earthworm invasion is a truly global phenomenon in which invasive earthworm species are invading every continent, except Antarctica (Hendrix et al., 2008). These earthworm species also have origins on every continent, except Antarctica. In North America, earthworms of Asian origin (the genera *Amynthas*, *Metaphire*, *Pheretima*, and *Pithemera*) have recently been documented in the northeastern (Steinberg et al., 1997; Burtelow et al., 1998; Bohlen et al., 2004a,b), central (Snyder, unpublished results), and southeastern (Callaham et al., 2003; Snyder et al., 2011) regions of the United States, although these earthworms have been known in North America since the early 20th century (Garman, 1888; Gates, 1937).

Earthworm invasion can significantly alter forest ecosystems. Physical changes to the forest floor through consumption of organic horizons, mixing of organic and mineral horizons, and burrowing and casting activities can impact biogeochemical cycling (Bohlen et al., 2004a,b,c). Earthworm invasion can also impact soil fauna communities through competition and through the significant alteration of soil profile and structure (Bohlen et al., 2004b,c; Frelich et al., 2006). Although much is known about the interactions of invasive earthworms with soil micro- and mesofauna, less is known about interactions with detritivorous macrofauna, such as millipedes (Migge-Kleian et al., 2006). Bonkowski et al. (1998) found that earthworms benefited from consuming millipede (*Glomeris marginata*) fecal pellets in a European Beech forest. However, in a microcosm experiment, millipedes were negatively affected by earthworms (*A. corticis*), but earthworms may have similarly consumed millipede fecal material (Snyder et al.,

3

68 2009). Snyder et al. (2009) found that although the millipede *Pseudopolydesmus erasus*

69 was epigeic and *A. corticis* was endogeic, *P. erasus* acquired less C during the four-week

70 course of the experiment in the presence of *A. corticis*, and it is likely that over longer

71 time scales, this C deficit could affect growth, weight maintenance, survival, and/or

72 reproductive output.

73   *Amynthas gracilis* invasion in forests of New York, USA was found to reduce O

74 horizon organic matter (Steinberg et al., 1997; Burtelow et al., 1998). Similarly, invasion

75 of *A. agrestis* in the Great Smoky Mountains, USA, reduced the depth of the FH horizon

76 (a combination of the F and H horizons) (Snyder et al., 2011). Millipedes reside in and

77 consume FH horizon, and Snyder et al. (2011) found that millipedes were negatively

78 affected by this *A. agrestis* invasion, both in terms of abundance and species richness.

79 The field observations of Snyder et al. (2011) motivated us to explore the mechanisms

80 behind this interaction, and a microcosm experiment was performed to test whether these

81 two taxa competed for food resources in the L or FH horizons, and whether earthworms

82 and millipedes benefited from the presence of these resources. This microcosm

83 experiment was novel in its approach towards creating a longer-term study (i.e., Months

84 instead of weeks). Earthworms, and to a lesser extent millipedes, burrow in the soil and

85 this prevents regular monitoring of faunal survival and fresh weight without causing

86 disturbance. In order to facilitate data collection, all microcosms were destructively

87 sampled every four weeks and fauna were put into newly constructed microcosms of the

88 same treatment.

89

90 **2 Methods**

91   Millipedes and earthworms were collected by manually searching through leaf

92   litter at the Great Smoky Mountains Institute at Tremont (Blount Co., Tennessee, USA;

93   35°38'22" N, 83°41'17" W), within the Great Smoky Mountains National Park (GSMNP)

94   in early June 2007. Earthworms and millipedes were kept separate during transport to the

95   laboratory. The two taxa were also stored separately until the beginning of the

96   experiment in containers with soil and litter from the collection site.

97   Microcosms consisted of 1 l transparent plastic containers with perforated snap-on

98   lids. Each microcosm received 500 ± 5 g of air dried soil that was then mixed with $70 \pm$

99   5 mL tap water. Soil was a commercially acquired ultisol (USDA soil taxonomy) from

100   the top 25 cm of a recently cleared forested site in Clarke Co., GA, USA. Soil was

101   screened through a 4.75 mm sieve to remove large aggregates and rocks. Litter was

102   previously collected from GSMNP and defaunated via Berlese extraction for 72 hours,

103   followed by air-drying. Dominant tree species at the litter collection site were *Acer* spp.,

104   *Quercus* spp., *Liquidambar styraciflua*, *Liriodendron tulipifera*, and *Pinus strobus*

105   (Snyder et al., 2011).

106   Litter treatments were defined by particle size: litter was 4.75 mm sieved to

107   separate unfragmented leaves (L horizon) from fragmented and partially decomposed

108   organic matter (FH, combined F and H horizons). Large rocks, twigs, seeds and nuts

109   were discarded. Organic layer treatments were L (15 ± 0.1 g of L horizon), L/FH ($7.5 \pm$

110   0.1 g each of L and FH horizon), or FH (15 ± 0.1 g of FH horizon). Litter was misted

111   with a standard quantity (~7 mL) of tap water when microcosms were constructed. Three

112   fauna treatments were established: two *Amynthas agrestis* individuals (mean fresh weight

113   0.86 ± 0.036 g each); one adult male *Sigmoria ainsliei* (mean fresh weight 2.26 ± 0.038

114 g); and two *A. agrestis* and one *S. ainsliei* together. *Amynthas agrestis* were all clitellate

115 or pre-clitellate. All individuals were approximately the same size and due to the annual

116 nature of their life cycle (Reynolds, 1978; Callaham et al., 2003; Snyder et al., 2011) all

117 individuals were similar in age. Individuals were randomly assigned to treatments with 6-

118 7 replicates for a total of 76 microcosms. However, at the end of the experiment four

119 experimental units were found to contain *Amynthas corticis* rather than *A. agrestis*; these

120 were excluded from subsequent analyses.

121   All microcosms were kept in the dark at 20˚C (± 2ºC). Each microcosm was

122 misted with tap water weekly, except early in the incubation when microcosms were

123 misted every 3 d. Incubation began in June 2007 and continued until all fauna died

124 (except *A. corticis* mentioned above).

125   Microcosms were destructively sampled every four weeks. After destructive

126 sampling, new microcosms were constructed and the surviving fauna were weighed and

127 placed into the new microcosms. Earthworms were rinsed in tap water to remove soil

128 and gently dried on a paper towel prior to weighing. If any fauna (earthworm or

129 millipede) from the original treatment were alive, then a new microcosm was constructed,

130 if all fauna in a particular microcosm had died, then that microcosm was terminated. In

131 this way, longevity of every individual could be assessed. Soils from treatments that

132 included earthworms were wet-sieved through a 2 mm sieve to assess cocoon production.

133 After the first cocoons collected were found to be only slightly larger than 2 mm in

134 diameter, a 1.4 mm sieve was employed to ensure cocoon capture.

135   Millipede and earthworm survival and fresh weight data were analyzed using a

136 general linear model (GLM), with the LSMEANS option for post-hoc tests. Data used in

137 the GLM analysis for earthworm survival were the calculated average days of survival

138 for the two worms in each microcosm. Fresh weight changes through 12 weeks

139 (millipedes) and 16 weeks (earthworms) were analyzed using a repeated measures

140 analysis; beyond this point there were insufficient replicates for robust analyses. Cocoon

141 production was assessed with a t-test comparing between Months 1-3 and 4-7, and GLM

142 comparing between Months 4, 5, 6, and 7. All statistical analyses were completed in SAS

143 (Version 9.2).

144

145 **3 Results**

146 *3.1 Survival and Growth*

147   Millipedes lived a mean time of 136.8 ± 10.6 d ($n = 36$) from the beginning of the

148 experiment (Fig. 1). The overall model testing fauna and litter effects was significant (P

149 $= 0.0002$). Millipede survival was significantly affected by litter ($P < 0.0001$), with

150 survival time significantly decreased in L relative to FH ($P < 0.0001$) and L/FH ($P =$

151 0.0012). However, differences in millipede survival times between L/FH and FH

152 treatments were not statistically significant ($P = 0.0567$). There was a trend for

153 earthworm presence to decrease millipede survival time, but this was not statistically

154 significant ($P = 0.0750$). Specifically, when *A. agrestis* was absent, millipedes survived

155 an average of 26 days longer in L/FH and 54 days longer in FH (Fig. 1). Overall,

156 millipedes survived 47.4% (69 days) longer in the L/FH treatment and 58.1% (106 days)

157 longer in the FH treatment relative to the L treatment. There was no interaction between

158 litter and fauna ($P = 0.4655$).

159   Mean time to *A. agrestis* mortality was 117.9 ± 4.1 d (*n* = 36 experimental units)

160   from the initiation of the experiment. The first and second *A. agrestis* mortality within

161   each experimental unit were 31.8 ± 5.6 d apart (*n* = 36). There was no evidence for

162   earthworm survival being affected by millipede presence or litter type (*P* = 0.2771, Fig.

163   2). In microcosms with both earthworms and millipedes, at least one earthworm survived

164   longer than the millipede in every replicate.

165   Millipede fresh weight (Fig. 3A) did not differ between treatments at the

166   beginning of the experiment (*P* = 0.5294) or at the last measurement before mortality (*P*

167   = 0.9010). There were significant differences between litter treatments but earthworms

168   did not impact millipede fresh weight (Fig. 3A, Table 1, analyzed through week 12).

169   Millipede fresh weight increased significantly more in FH relative to L treatments (*P* =

170   0.0100), but neither were significantly different from L/FH treatments (FH vs. L/FH *P* =

171   0.1351; L vs. L/FH *P* = 0.1412). Within-subjects tests for effects over time and

172   interactions with time were all non-significant (data not shown).

173   Earthworm fresh weight (Fig. 3B) did not differ between treatments at the

174   beginning of the experiment (*P* = 0.6190). A significant impact of litter (*P* = 0.0180), but

175   not of millipede treatments (*P* = 0.9531, Table 1), was observed in fresh weight changes

176   through week 16: *A. agrestis* maintained a higher fresh weight in L/FH than in FH and L

177   treatments. Earthworm fresh weight decreased over time (Table 2, Fig. 3B, *P* < 0.0001),

178   anda time by litter interaction was also significant (*P* = 0.0211).

179   *3.2 Earthworm cocoon production*

180   Cocoons were detected beginning in the fourth month and in every subsequent

181   month of incubation (Fig. 4). In Months 4-7, microcosms in which cocoons were

8

182   recovered contained 2.06 ± 0.44 cocoons; this was a significant increase over the zero

183   cocoons recovered during Months 1-3 (t-test, n = 17, P = 0.0003). Numbers of cocoons

184   recovered in Months 4-7 were not significantly different from one another (GLM, P =

185   0.8952). Cocoons were recovered from microcosms that began a month with either one or

186   two earthworms, but the number of cocoons per microcosm was not significantly

187   different due to this factor (P = 0.5381). There were a total of 28 cocoons recovered

188   during the experiment, and only one of these was recovered from a microcosm that also

189   contained a live millipede. Three cocoons were recovered in millipede treatments after

190   millipede mortality. Litter treatment did not influence the number of cocoons recovered

191   per microcosm (P = 0.7868).

192   **4 Discussion**

193   Based on inferences from field (Snyder et al., 2011) and microcosm (Snyder et

194   al., 2009) studies that Asian invasive earthworms may compete with native North

195   American millipedes, we designed a microcosm experiment to evaluate longer-term

196   interactions between two species focusing on the potential for food competition. 'Longer-

197   term' in this case is relative to most microcosm experiments and also to the putative life-

198   span of these taxa, i.e., the experiment continued for months rather than weeks. This

199   methodology had the advantage of allowing measurement of fresh weight and survival

200   while limiting the frequency of disturbance. Although the disturbance to the microcosms

201   may seem substantial - the entire microcosm was destroyed and replaced – in reality the

202   stress to the organisms was quite brief and limited as much as possible. In practice, each

203   organism was quickly located, weighed, and placed into a new microcosm in a matter of

204   seconds. The alternative of searching for surviving individuals, weighing, and returning

205 to the same container would have the potential of differentially disturbing experimental

206 units depending on how quickly and easily individual organisms were discovered,

207 collected, and measured.

208   Presence of FH material was important for *Sigmoria ainsliei*: millipede survival

209 time decreased greatly without FH material and biomass increased the most in the FH

210 treatment. However, earthworm fresh weight, but not survival, was highest in the

211 treatment with both particle sizes (L/FH). In FH and L/FH, we observed that *A. agrestis*

212 consumed nearly all FH material within each four-week time period. This finding is

213 consistent with field observations and data showing that a decrease in FH horizon

214 correlates with *A. agrestis* invasion (Snyder et al., 2011), and supports the hypothesis that

215 *A. agrestis* directly causes this decrease through consumption.

216   In L/FH and FH treatments, there was also a trend that *S. ainsliei* survived a

217 shorter amount of time when *A. agrestis* was present, but this was not statistically

218 significant. However, we propose that from biological standpoint, this may indeed be a

219 relationship worthy of further exploration. Interestingly, when both species were present,

220 millipedes almost always died first, and this suggests that when the two are in close

221 proximity, the invasive earthworms may outcompete millipedes and eventually exclude

222 them. In these same litter treatments (FH and L/FH) there was also a very weak trend that

223 *A. agrestis* survived longer in treatments without millipedes. However, in L treatments,

224 *A. agrestis* tended to survive longer in the presence of millipedes, suggesting that

225 earthworms may benefit from millipede presence in L treatments, probably through

226 consumption of litter that had been processed by millipedes, as has been observed in

227 other studies (Bonkowski et al., 1998; Snyder et al., 2009). Earthworms were also

228   observed to burrow into mineral soil during the incubations, and may have been able to

229   exploit organic matter in the mineral soil in addition to resources supplied on the soil

230   surface (Zhang et al., 2008; Callaham et al., unpublished results). This behavior is

231   consistent with the findings of Zhang et al. (2008) who showed that *Amynthas* had

232   substantial dietary flexibility, and this possible additional source of organic matter may

233   help explain why earthworm survival was not affected by litter treatment, as well as why

234   the effect of millipede presence was not statistically significant. When availability of

235   aboveground resources was limited, *A. agrestis* may have burrowed and consumed soil

236   organic matter, while adult *S. ainsliei* were restricted to feeding on surface organic

237   horizons. Millipedes appear to have inhibited reproductive potential, possibly through

238   this same mechanism. In the presence of millipedes, earthworms may have spent more

239   energy burrowing to access lower quality food resources. This combination may have led

240   to less energy being available to devote to cocoon production.

241   *4.1 Conclusions and future perspectives*

242   Overall, the data from the present study are consistent with, and help to elucidate,

243   observations from field studies (Snyder et al., 2011), and other lab experiments with

244   invasive earthworms and millipedes (Snyder et al., 2009). *Amynthas agrestis* invasions

245   being associated with decreased F/H horizon depth and decreased millipede abundance

246   (Snyder et al., 2011) served as a starting point to ask questions about what the potential

247   mechanisms behind these relationships might be. From our microcosms, we now have

248   evidence that two of the organisms involved in the field study will consume the same

249   food sources, and that when they are kept in proximity to one another, these organisms

250   affect one another's longevity and reproductive output. Although microcosms are, of

251 necessity, quite simple relative to the natural systems they are meant to simulate, they can

252 nevertheless offer important insights particularly into mechanistic relationships (Drake

253 and Kramer, 2012; Cadotte et al., 2005). We suggest that our study has uncovered just

254 such a mechanistic relationship between *A. agrestis* and *S. ainsliei*, but we also

255 recommend that much more detailed work should be undertaken to examine the trophic

256 ecology and resource use of these organisms in their native habitats. Such work will be

257 crucial if we are to have fuller understanding of effects of earthworm invasion, and

258 imperative to the future development of successful management approaches to control

259 earthworm invasions in the Southern Appalachian Mountains.

260   Our data lends support to the hypothesis that earthworms and millipedes compete

261 for partially decomposed leaf material, but many questions remain. Greater cocoon

262 production in the absence of millipedes supports the competition hypothesis and suggests

263 that millipedes may provide some biotic resistance to invasion. Future studies could

264 offset the natural variability in earthworm and millipede mortality, and improve their

265 statistical power, by including more replicates. Additionally, initiating treatment

266 conditions on younger individuals may produce stronger responses. Maintenance of

267 laboratory cultures (Lowe and Butt, 2005) will be a critical step in our ability to perform

268 more of these experiments.

269

270 **Acknowledgements**

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348

349 Figure Captions

350 Fig. 1. Mean survival (± SE) of *Sigmoria ainsliei* from initiation of the incubation with

351 (M+W) and without (M) earthworms. Litter treatments were litter (L), litter and FH

352 material (L/FH), and FH only (FH).

353

354 Fig. 2. Mean survival (± SE) of *Amynthas agrestis* from initiation of the incubation with

355 (W+M) and without (W) millipedes. Litter treatments were litter (L), litter and FH

356 material (L/FH), and FH only (FH).

357

358 Fig. 3. Mean fresh weight change of surviving fauna (± SE) since the beginning of the

359 experiment, expressed as percent of initial mass: millipedes (A) and earthworms (B) in

360 different litter treatments through 12 weeks (millipedes) or 16 weeks (earthworms) of the

361 incubation. Different letters indicates significant *P*-values at α=0.05 within one sampling

362 time.

363

364 Fig. 4. Cocoon production by *Amynthas agrestis* over the duration of the incubation.

365 Mean (± SE) number of cocoons recovered per microcosm (A) and number of

366 earthworm-containing microcosms from which cocoons were (shaded) and were not

367 (open) recovered (B). Cocoon recovery in Months 4-7 was greater than 0 (Months 1-3, *P*

368 = 0.0003; n = 17).

369

370

371

372   Table 1. Results of repeated measures GLM analyses tests of hypotheses for between

373   subjects effects on fresh weight change from initiation of the incubation. Fresh weight

374   was measured every four weeks. Millipede data were analyzed until week 12 and

375   earthworm data until week 16. Significant *P*-values at α=0.05 indicated by an asterisk.

376

Source	df	SS	MS	*F*	*P*
Millipede Fresh Weight					
Fauna treatment	1	3.72×10^{-6}	3.72×10^{-6}	0.00	0.9912
Litter treatment	2	0.21553	0.10777	3.59	0.0438*
Fauna * Litter	2	0.03781	0.01891	0.63	0.5411
Error	23	0.68954	0.02997		
Earthworm Fresh Weight					
Fauna treatment	1	0.00274	0.00274	0.00	0.9531
Litter treatment	2	7.73328	3.8666	5.00	0.0180*
Fauna * Litter	2	1.19285	0.59642	0.77	0.4761
Error	23	14.6819	0.77273		

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380 Table 2. Results of repeated measures GLM analyses tests of hypotheses for within

381 subjects effects on earthworm fresh weight change from initiation of the incubation.

382 Fresh weight was measured every four weeks and analyzed until week 16. Significant *P*-

383 values at α=0.05 indicated by an asterisk.

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Source	df	SS	MS	*F*	*P*
Time	3	19.2486	6.41619	86.54	< 0.0001*
Time * Fauna	3	0.18038	0.06013	0.81	0.4930
Time * Litter	6	1.21529	0.20255	2.73	0.0211*
Time * Fauna * Litter	6	0.07883	0.01314	0.18	0.9820
Error (Time)	57	4.22590	0.07414		

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