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A Fluorescent Marking and Re-count Technique Using the Invasive Earthworm, *Pontoscolex corethrurus* (Annelida: Oligochaeta)

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ABSTRACT.—We used a fluorescence technique to mark and re-count the invasive earthworm, *Pontoscolex corethrurus* from PVC tubes established in a forest and a recently abandoned pasture in Puerto Rico to test the effects of the labeling treatment on earthworm population survival over time. A fluorescent marker was injected into the earthworms in the middle third section of the body, between inside and outside walls. Five labeled juveniles and two unlabeled adults of *P. corethrurus* were incubated in the PVC tubes under field conditions and retrieved after 2, 4, 8, and 16 weeks (labeled population; four PVC tubes harvested per date and site for a total of 32 tubes). Concurrently, an additional set of 8 PVC tubes contained five unlabeled juveniles and two unlabeled adults each of *P. corethrurus* (unlabeled population) was established in each site (forest and pasture; 16 tubes total) to compare the percent remaining of worms in tubes containing labeled and unlabeled populations after 2 and 16 weeks (4 replicates per date). We found that (1) the fluorescent marker was still present in the earthworm tissue after four months, (2) the percent remaining of the total earthworm population was not significantly different between the forest and pasture sites, and (3) thirty percent of the labeled worms were lost after 2 weeks, but the population remained stable between week two and four of the experiment. Our results suggest that fluorescent markers can be a useful tool for carrying out short term mark and re-count studies of earthworms.

KEYWORDS.—*Pontoscolex corethrurus*, earthworms, exotic, invasion, tropics, Puerto Rico, fluorescent, mark and recapture, re-count

INTRODUCTION

The ability to individually identify organisms is an important research component for effective conservation and management programs. Methods of marking or tagging individual organisms are especially important in genetics and selective breeding studies, where individual identification is essential (Arce et al. 2003). External identification tags have been found inappropriate for a variety of organisms as may affect their behavior, welfare or survival (Beuckers et al. 1995; Willis and Babcock 1998; Doupé et al. 2003). Internal identification often causes few biological effects but visual identification is difficult (Guy et al. 1996; Arce et al. 2003). Injections of visible fluorescent markers have been successfully applied to aquatic fauna like fish (e.g., Doupé et al. 2003) and shrimp (e.g., Welker et al. 1975); but are not as often used in the study of soil invertebrates.

Earthworms are among the most important macro-decomposers in tropical moist to rain forests (Anderson and Swift 1983). Over the last twenty years, an increasing number of researchers have emphasized the importance of earthworms in affecting soil processes (e.g., Lee 1985; Edwards and Bohlen 1996). They can have a critical influence on accelerating the decomposition of soil organic matter and improving soil physical conditions for plant growth and nutrient uptake (Blair et al. 1995; González and Seastedt 2001). Earthworms can also be

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important in determining the availability and cycling of phosphorus (López-Hernández et al. 1993). They are able to feed on soils poor in organic matter (Lavelle 1983), and can stimulate the micro-flora to degrade soil organic matter by producing assimilable carbon (Barois 1992).

The abundance and community structure of earthworms and the establishment of invasive earthworms in areas previously inhabited by native worms can be determined by the land use history (González et al. 2006). In Puerto Rico, deforestation occurred extensively during the late 1800's and the beginning of the 20th century. Much of the deforested area in the montane slopes was converted to cattle pastures. This conversion triggered an increase in earthworm abundance from 50 worms per m^2 in relatively mature forests to 850 worms per m^2 in pastures at 25 cm depth (Zou and González 1997). *Pontoscolex corethrurus* (Annelida: Oligochaeta: Glossoscolecidae), an invasive earthworm, is the dominant species in the pastures and is commonly found in the mature forests of Puerto Rico. *Pontoscolex corethrurus* is a poly-humic endogeic species with a pantropical distribution (Spain et al. 1990). The individuals of this species are small, with large populations, normally geophagous and cast within the soil (Lavelle et al. 1987). Given its expansive distribution, *P. corethrurus* can be a model organism for studying the comparative effects of invasive and native earthworm species on ecosystem processes in the tropics. The use of a marking and re-count technique for earthworms would enhance such comparative studies and could help us understand their biology, demography, selective feeding strategies, and resource competition dynamics. Thus, this research is designed to 1) study a fluorescence technique to mark and re-count *P. corethrurus* in enclosures containing forest and pasture soils under field conditions, and 2) determine the effects of the labeling treatment on the percent remaining of labeled individuals over time at each site. To our knowledge, this is the first study describing a mark and re-count technique using a fluorescent marker on earthworms.

Materials and Methods

Study area

This study was conducted in a subtropical wet forest (Ewel and Whitmore 1973) near El Verde Field Station (18°20′N, 65°49′W) and a pasture near the town of Sabana (18°18′N, 65°50′W) in the Luquillo Experimental Forest in northeastern Puerto Rico. The elevation at the sites is about 420 m above sea level. Annual air temperature in Luquillo is 22.3°C (Brown et al. 1983), and mean annual precipitation is 3524 mm (García-Martinó et al. 1996), with rainfall distributed more or less evenly throughout the year. The dominant soils are moderately well drained Oxisols belonging to the Zarzal series with high clay content and of volcanic and sedimentary origin (Soil Survey Staff 1995). The vegetation in the forest is dominated by tabonuco trees (*Dacryodes excelsa* Vahl) (Zimmerman et al. 1995); while, the vegetation in the pasture is mostly composed of a grass-vine-fern community (Aide et al. 1995).

Sampling

Within each of the sites, a 1-m^2 plot was established. At each plot, the soil was removed to a depth of 12 cm and hand-sorted over a sieve to collect worms of *Pontoscolex corethrurus*. Sixteen enclosures were established using PVC tubes (15 cm wide × 30 cm long) filled with soil to a depth of 25 cm within the plot at each site. All the PVC tubes were secured with nylon cloth at both ends to prevent predation and/or migration of earthworms. Five labeled juveniles and two unlabeled adults of *P. corethrurus* (labeled population) were incubated in the PVC tubes and retrieved after 2, 4, 8, and 16 weeks (4 replicates per date). The juveniles were labeled with a fluorescent marker provided by the Elastomer Starter Kit of the Northwest Marine Technology Company, Inc. (Olympia, WA). The fluorescent marker was injected (0.02 cc), with a syringe, into the earthworms in the middle third section of their body between the inside and outside walls. After injection, the earthworms were left to rest for 48 hrs in trays supplied with soil and litter. Surviving earthworms were removed from the trays and then placed inside of the PVC tubes, covered with a thin layer of soil, and left for incubation in the field. Concurrently, an additional set of 8 PVC tubes each containing five unlabeled juveniles and two unlabeled adults of *P. corethrurus* (unlabeled population) was established in each site (forest and pasture; 16 tubes total) to compare the percent remaining of worms in tubes containing labeled and unlabeled populations after 2 and 16 weeks (4 replicates per date). Two adult worms were included into the PVC tubes containing labeled and unlabeled juveniles to determine demographic parameters. We will not discuss here the data on the demographic parameters. To determine whether we were successful at extracting all earthworms and cocoons from the soil, we used another set of 8 PVC tubes containing no earthworms as controls at each site. These PVC tubes (controls) were harvested after 2 and 16 weeks in the field. Each retrieved PVC tube was hand-sorted for earthworms. No cocoons or worms were found in the control PVC tubes. Earthworms were separated into juveniles and adults as recovered from the PVC tubes containing the labeled and unlabeled populations. Each worm was examined for fluorescent marker under a fluorescent flashlight.

Statistical analyses

To look at the effects of forest and time on the percent remaining and/or number of earthworms, MANOVA analyses were performed by using the 1) percent remaining of juvenile, adult and total worms, and 2) the number of labeled and unlabeled juvenile and adults as dependent variables and site (forest vs. pasture) and time (0, 2, 4, 8, 16 wks) as independent variables (SPSS 11.5 for Win). SNK (Student-Neuman-Keuls) tests ($\alpha = 0.05$) were used to determine a significant effect of time within an earthworm category. One way ANOVAs and SNK ($\alpha = 0.05$) were employed to look at the effect of time on the percent remaining of *P. corethrurus* in PVC tubes containing labeled and unlabeled populations within each worm category and site. Likewise, ANOVAs and SNK were used to determine significant treatment effect of the labeling technique within an earthworm category and time.

RESULTS

The fluorescent marker lasted for 16 weeks inside the earthworm body, although most of the labeling moved to the posterior section. Over time, the percent remaining of *P. corethrurus* declined (Fig. 1). Overall, the percent remaining of juveniles and total worms of *P. corethrurus* did not significantly vary during the first eight weeks of the experiment while the percent

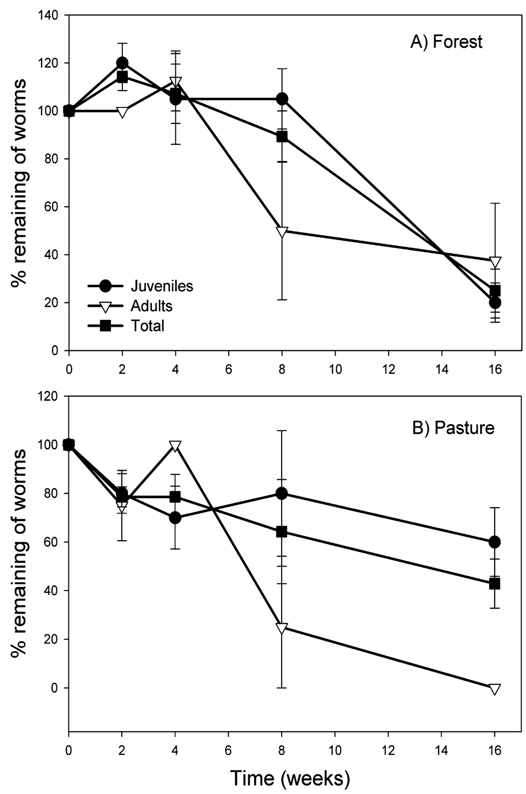

FIG. 1. Percent (± SE) of juvenile, adult and total earthworms (labeled plus unlabeled) of *Pontoscolex corethrurus* remaining in PVC tubes established in a forest and a pasture site in the Luquillo Mountains, Puerto Rico. Data from both sites combined. Common letters within an earthworm category indicate no significant effect of time using SNK (Student-Neuman-Keuls) tests; $\alpha = 0.05$.

remaining of adult worms significantly decreased after four weeks (Fig. 1). In the forest and pasture, the number of labeled juveniles and unlabeled adults decreased (Fig. 2A, B), whereas the number of labeled adults increased at times (although not significantly; P = 0.06, Power = 0.66) during the 4-month incubation period (Table 1). There was a significant forest and time interaction on the number of unlabeled juveniles of *P. corethrurus* (Table 1); as in the forest, the number of unlabeled juveniles increased during the initial 8 weeks and decreased in the later 8 weeks (Fig. 2A) but in

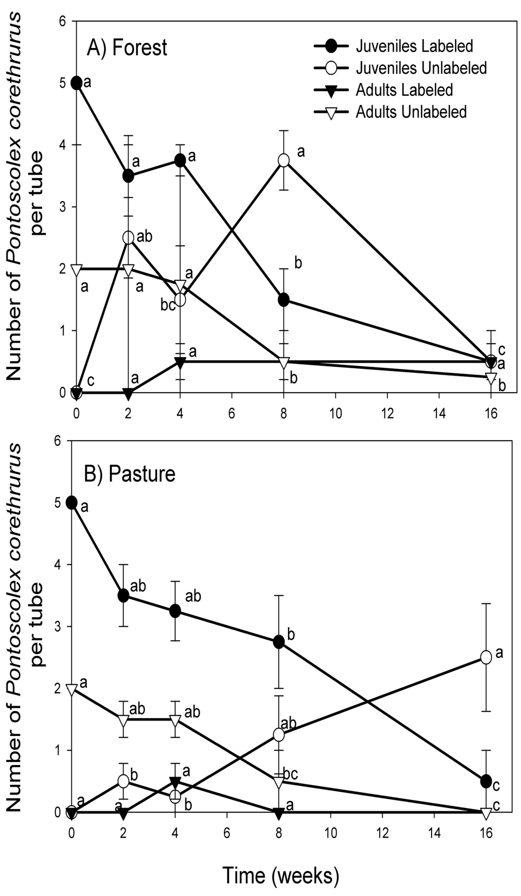

FIG. 2. Mean number (± SE) of labeled and unlabeled juveniles, and labeled and unlabeled adults of *Pontoscolex corethrurus* per tube after 2, 4, 8, and 16 weeks of incubation in A) a forest and, B) in a pasture site in the Luquillo Mountains, Puerto Rico. Common letters within an earthworm category for each forest type indicate no significant effect of time using SNK tests; α = 0.05.

the pasture, the number of unlabeled juveniles steadily increased to the highest number by the end of the experiment (Fig. 2B). When all data are combined, there is no effect of site (forest vs. pasture) on the percent remaining of labeled juveniles (ANOVA, F = 0.25, P = 0.62). The population of labeled juveniles declined 30% after two weeks but maintained at this level between weeks two and four (Fig. 3). After week four, the percent remaining of labeled juveniles significantly decreased and reached the lowest level by the end of the experiment (Fig. 3). Meanwhile, the total labeled worms declined after two weeks, slightly increased by week four and thereafter declined until the end of the experiment (Fig. 3). When we combine the data for both sites, and compare the mean percent of remaining worms of *P. corethrurus* (labeled plus unlabeled worms) in the PVC tubes of labeled and the unlabeled populations, we found no significant differences of site (MANOVA Between Subject Factors; P = 0.44, Power = 0.18) and treatment (P = 0.71, Power = 0.10) on the percent remaining of worms. However, if we split the data by site, some interesting patterns emerge; as there was a significant forest and treatment interaction on the percent of remaining worms in the PVC tubes of labeled and unlabeled populations (P = 0.006, Power = 0.84). In the forest and in the pasture, the percent of remaining worms (juveniles, adults and total) in the labeled population remained the same within the first two weeks (see Fig. 4; with the exception of the declined on total worms in the pasture after 2 weeks) and significantly declined by the end of the experiment with the exception of the remaining juveniles in the pasture. The remaining juveniles in the unlabeled population of the pasture remained about the same during the four month period. At this site, the juvenile worms in the labeled population maintained a loss of 20% between weeks 2 and 16 (Fig. 4D). In the forest, the percent of remaining adults and total worms in the unlabeled population decreased after two weeks and moreover, at two weeks of incubation the percent of adult and total worms lost was significantly higher in the unlabeled than in the

TABLE 1. Effect of forest (F; forest vs. pasture), time (T; 0, 2, 4, 8, 16 wks after the worm labeling) and the forest and time interaction on the number of earthworms of juveniles labeled (JL), juveniles unlabeled (JU), adults labeled (AL), adults unlabeled (AU). Degrees of freedom (df), F and P values, Power, and R^2 for the corrected model for a two-way MANOVA are presented.

Source	Dependent variable	df	F	*P*	Power
Forest (F)	Juveniles Labeled (JL)	1	0.25	0.62	0.08
	Juveniles Unlabeled (JU)	1	5.07	0.06	0.59
	Adults Labeled (AL)	1	3.00	0.09	0.39
	Adults Unlabeled (AU)	1	1.60	0.22	0.23
Time (T)	JL	4	25.29	**<0.001**	1.00
	JU	4	6.11	**0.001**	0.97
	AL	4	2.62	0.06	0.66
	AU	4	22.15	**<0.001**	1.00
F × T	JL	4	0.94	0.46	0.26
	JU	4	5.86	**0.001**	0.96
	AL	4	1.12	0.36	0.31
	AU	4	0.35	0.84	0.12
Corrected model		R^2			
	JL	0.78			
	JU	0.64			
	AL	0.37			
	AU	0.75			

labeled population (Fig. 4 B and C). In contrast, the percent of total remaining juvenile and total worms were significantly higher in the unlabeled and in the labeled population after week two (Fig. 4 D and F). Adult worms were not significantly different between the labeled and unlabeled population at week two (Figure 4E) in the pasture.

DISCUSSION

Traditionally, mass-marking techniques have been primarily used in birds (by banding) and arthropods—particularly for insects. Paints, dyes and stains have been widely used in studies of insect population dynamics (e.g., Coppedge et al. 1979; Haagsma et al. 1993). However, fluorescent markers have several advantages over liquid paints, and they are becoming the best technique for color-marking insects and aquatic fauna. The technique allows for rapid and inexpensive marking of large numbers of individuals (Turchin 1998). It is also environmentally safe, cost-effective and easy to use. The injections of visible fluorescent markers have been successfully applied to aquatic fauna like fish (e.g., Doupé et al. 2003) and shrimp (e.g., Welker et al. 1975) but to our knowledge injections of fluorescent markers have not been previously described in earthworms.

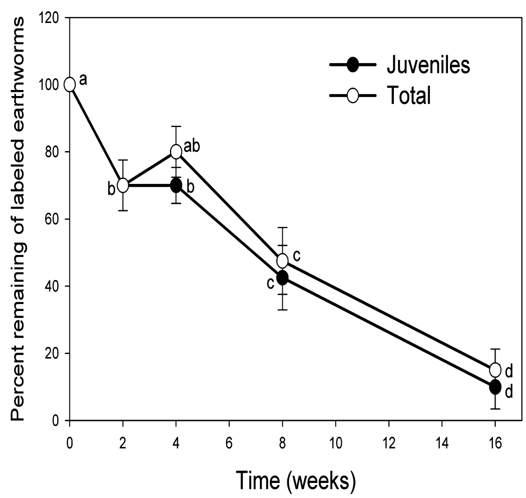

FIG. 3. Percent remaining (± SE) of labeled juvenile and total earthworms of *Pontoscolex corethrurus* in PVC tubes established in a forest and a pasture site in the Luquillo Mountains, Puerto Rico. Data for both sites are combined. Common letters indicate no significant effect of time within a worm category using SNK tests; one-way ANOVA; α = 0.05.

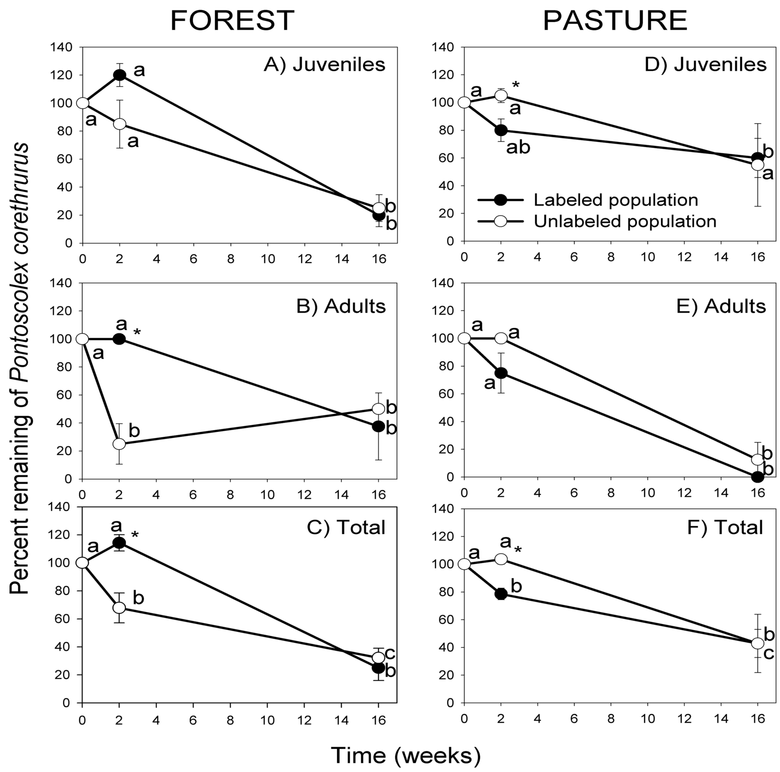

FIG. 4. Percent remaining (± SE) of juvenile, adult and total earthworms of *Pontoscolex corethrurus* after 2 and 16 weeks of incubation in labeled and unlabeled populations established in A-C) a forest and, D-F) a pasture site in the Luquillo Mountains, Puerto Rico. The labeled population consisted of five labeled juveniles and two unlabeled adult worms per tube. The unlabeled population consisted of five unlabeled juvenile and two unlabeled adult worms per tube. Common letters indicate no significant effect of time within a population using SNK tests; one-way ANOVA; $\alpha = 0.05$. Asterisks indicate a significant treatment effect of the labeling technique within an earthworm category and week (one-way ANOVA; $\alpha = 0.05$).

Since the fluorescence was still present in the earthworm tissue after four months we propose that the injection of a fluorescent marker is an effective technique to mark and re-count earthworms from enclosures. Overall 30% of the labeled individuals of earthworms died within the first two weeks of the injection (Fig. 3), but on average that decrease in the survival of the labeled population was not significantly different than the unlabeled population ($P = 0.71$). Moreover, the percent remaining of juvenile, adult and total worms was significantly higher in the labeled than in the unlabeled population in the forest site. Interestingly, we found mixed effects of the labeling treatment on the number of juvenile earthworms in the forest and pasture sites. The number of labeled and unlabeled juvenile earthworms in the pasture differed after 2 weeks of incubation, suggesting a direct effect of labeling on the survival rate of the labeled earthworms. In the pasture, all 5 unlabeled juveniles survived in the unlabeled population (no fluorescent treatment) 2 weeks after the incubation, whereas only 4 labeled juveniles survived during the same period. Thus, injecting the fluorescent marker into earthworms caused a 20% of mortality 2 weeks after the treatment in the pasture (See Fig. 4D). After a significantly negative declined of the juveniles in the labeled population in the pasture, we observed that the juvenile worms in the labeled population maintained at a loss of 20% between weeks 2 and 16. As with any marking technique, it is important to retain for a sufficient period of time the organisms of interest to ensure that the marker will not affect them. From our study, a 2-wk retention time of earthworms marked via fluorescent injections seems appropriate.

It has also been stated that an "ideal maker" should persist without inhibiting the "normal behavior" of the targeted population (Hagler and Jackson 2001). We found that there seems to be no deleterious effect of the fluorescent treatment on the reproductive behavior of the labeled population of earthworms as there was recruitment of unlabeled juvenile earthworms in the forest (highest number of unlabeled juveniles after eight weeks), and in the pasture (steadily increase until the end of experiment) sites (see Fig. 1). In addition, the percent remaining of adult worms was significantly higher in the labeled than in the unlabeled population in the forest site (see Fig. 4B). Although not significantly, there was an increased in the number of labeled adults at times throughout the four month period, suggesting the recruitment of labeled juveniles into the category of labeled adult worms (Fig. 2 and also see the difference between percent remaining labeled juveniles and total earthworms, Fig. 3). Potentially, we see no disruptive effect of the fluorescent treatment on the growth of the labeled individuals as well; given an initially greater sample size of labeled juveniles of earthworms in this experiment.

Pontoscolex corethrurus, is one of the most widespread invasive species around the world. Because of its adaptations, *P. corethrurus* has established everywhere in the moist tropical regions, including over 56 tropical countries (Fragoso et al. 1999). As it is tolerant to dry microclimate conditions of disturbed grassland (e.g., González et al. 1996; Zou and González 1997; Fragoso et al. 1999; Lapied and Lavelle 2003; Sánchez-De León and Zou 2003; Decaëns et al. 2004), this earthworm can reach an abundance of 1000 individuals per square meter (25 cm deep) in disturbed agricultural pastures (Zou and González 1997). It was shown that *P. corethrurus* inhabits soils that are highly compacted, have low pH, high temperature and low moisture regimes and poor organic inputs (e.g., Henrot and Brussaard 1997; Römbke et al. 1999; García and Fragoso 2002; Decaëns et al. 2004; among many others). In this study, we observed a significant forest and treatment effect on the number of unlabeled juveniles (see Table 1); at week two, the survival of unlabeled juveniles was higher in the pasture than in the forest site. Also, unlabeled adults survived better in the pasture than in the forest site (Fig. 4B and E). Thus, results from this study confirm previous literature on the resistance ability of *P. corethrurus* to pasture conditions.

Pontoscolex corethrurus and other tropical invasive earthworms (e.g., *Perionex excavatus*, *Dichogaster modigliani* and *Polypheretima elongata*) are often considered continuous breeders with high fecundity (Bhattacharjee and Chaudhuri 2002). High fecundity and short incubation period with high hatching success are adaptative strategies that enable them to survive drastic environmental changes—like heat, drought and predation in the soil (Pianka 1970; Bhattacharjee and Chaudhuri 2002). Thus, in this study, the survival of the juvenile population in the pasture could be explained as the result of this response to selection pressure. Also in this study, we observed that in the both the forest and pasture, the number of labeled juveniles and unlabeled adults decreased (Fig. 2A, B). Therefore, the study might have been influenced by the change from wet to dry season during the time period; which may explain the sharp decline in the worm population near the end of the incubation period.

In the end, the injection of fluorescence markers can be useful to mark and recount earthworms because 1) it allows for marking juveniles as small as 0.2 grams of fresh weight and adults as large as 30 grams of fresh weight, 2) it has long mark detection time, and 3) it has high level of readability for different colors. When using the technique, we recommend evaluation of the sample size of the earthworms to be marked (as initial death rates can be significant) and retaining the labeled earthworms for a sufficient period of time so to ensure that the marker will not affect them (about 2 wks). Our results suggest that fluorescent markers can be a useful tool for carrying out short term mark and re-count studies of earthworms and it can help us understand their basic biology, demography, and ethology.

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