



## RESEARCH ARTICLE - ANTS

## Seed removal by ants in Brazilian savanna: optimizing fieldwork

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### Abstract

There has been an increase in the number of studies using seed removal by ants to evaluate ecosystem functioning; however, these studies encompassed varying time periods and used different types of seeds. Therefore, our aim was to evaluate differences in the proportion of seeds removed by ants in impacted and non-impacted sites in Brazilian savanna. Furthermore, we evaluated seed removal (1) during the morning and after a 24h period of seed exposure and (2) using natural and artificial seeds (manipulated resource to resemble natural seeds). The proportion of seeds removed was higher after the 24h exposure period (artificial seeds) regardless of site status, and more artificial seeds were removed than natural seeds. Our recommendations regarding sampling period depend on whether evaluating impacted or non-impacted sites. Although seed removal was greater after 24h in both impacted and non-impacted sites, we suggest that research evaluating the proportion of seeds removed in non-impacted sites should be performed only in the morning period to optimize the sampling time (removal of 60% during this period). When the aim is to compare non-impacted and impacted sites, we suggest evaluating after 24h of exposure, since the impacted sites experienced a higher proportion of seed removal during the afternoon and/or night time periods. Furthermore, we recommend the use of artificial seeds because they are easier to obtain and manipulate, and allow us to do comparisons between studies at different regions. We consider these findings an important first step towards standardizing future research on seed removal in Brazilian savannas by facilitating fieldwork and allowing comparisons to be made among different studies.

### Introduction

Ecological functions, such as seed removal, are extremely important for preservation of ecosystem stability (Naeem et al., 1999), due to regulatory processes of ecosystem functioning and the influence of several organisms in the ecosystem dynamics. However, such functions may be transformed or even lost due to landscape modifications caused by human interventions, which may even result in the complete loss of habitats, species and resources (Armbrecht et al., 2006; Ribas et al., 2012b; Edwards et al., 2014). Aiming to observe and evaluate such environmental modifications and their effects on ecological functions, many studies have widely used bioindicator organisms (e.g.: Braga et al., 2013; Costa et al., 2016; Koivula, 2011; Siddiget al., 2016).

Ants are frequently used as bioindicator organisms for evaluating different types of impacts, such as: agricultural practices (Armbrecht & Perfecto, 2003; Philpott et al., 2008), habitat fragmentation (Vasconcelos et al., 2006; Leal et al., 2012), fire (Parr et al., 2004; Andersen et al., 2006; Santos et al., 2008; Anjos et al., 2017) and mining (Dominguez-Haydar & Armbrecht, 2011; Rabello et al., 2015). Furthermore, ants are abundant in practically all environments, sensitive to environmental modifications and respond quickly to human disturbances (Ribas et al., 2012a; Schmidt et al., 2013). In addition, ants carry out important ecological functions and services (Folgarait, 1998; Philpott et al., 2010), such as ecosystem engineers (Frouz & Jilková, 2008), predation, biological control of agricultural pests (Armbrecht & Gallego, 2007; De La Mora et al., 2015; Morris et al., 2015) and seed



removal (Lima et al., 2013; Gallegos et al., 2014; Griffiths et al., 2017).

Seed removal by ants has been used by some studies in tropical regions to evaluate different types of environmental impacts (e.g.: Zelikova & Breed, 2008; Bieber et al., 2014; Gallegos et al., 2014; Leal et al., 2014); however, there is no standardized sampling method for such purposes. For example, the period of time evaluated and the seed type used to evaluate the effect of environmental impact on seed removal by ants vary widely (Christianini et al., 2012; Bieber et al., 2014; Leal et al., 2014; Rabello et al., 2015). Sometimes, researchers may have similar aims and use different methodologies for evaluating this, so it would be interesting to standardize methodology when the aims are similar. This lack of a standardized methodology hampers the comparison of responses of seed removal by ants in different vegetation types, habitats and biomes and prevents further advances towards a broader understanding of patterns of ecosystem functioning in response to environmental impacts.

Some researchers have determined the proportion of seeds removed by ants only after a period of 22 or 24 hours (Christianini et al., 2007; Bieber et al., 2014) or 48 hours (Rocha-Ortega et al., 2017) or 96 hours of seed exposure (Ferreira et al., 2011). Other studies followed this process during the morning and/or afternoon, with different periods of time of observation (Parr et al., 2007; Christianini et al., 2012; Gallegos et al., 2014; Leal et al., 2014). In addition to varying time periods, seed removal experiments have also differed in the types of seeds used, with some using natural seeds (Christianini et al., 2007; Parr et al., 2007; Christianini et al., 2012; Leal et al., 2014), and others using artificial seeds (manipulated resource to resemble seeds or fruits), both with varying compositions (Uehara-Prado, 2005; Bieber et al., 2014; Rabello et al., 2015). Thus, this is the first step towards a standardized methodology for the evaluation of seed removal by ants, facilitating the comparison of results among different ecological studies.

The aim of this present study is to evaluate the proportion of seeds removed by ants at different sites of Brazilian savanna, comparing the use of artificial or natural seeds and two different sampling periods. As a first step towards optimizing methods for evaluating seed removal by ants in savannas, we compared the proportion of seeds removed by ants between: (1) the morning (first hours of the day) and after a 24h period of exposure; and (2) between natural and artificial seeds. We predicted that the proportion of seeds removed by ants will be (1) higher after 24h of seed exposure since the seeds would be available on the ground for a longer period of time. We believe that seed removal occurs during different periods of the day, thus, after 24h of seed exposure may generate an additive effect of seed removal, probably because different ant species have foraging throughout all day. We also predicted that (2) there will be greater removal of artificial than natural seeds, since artificial

seeds present a standard size and are composed of a greater variety of nutrients attractive to ants, thus enhancing seed removal by different ant species.

## Material and Methods

### Study sites

We performed this study in Brazilian savannas within southern and northern metropolitan regions in the state of Minas Gerais, Brazil. These regions are classified as tropical with dry winters (April to September) and rainy summers (October to March). Sampling was carried out during January and March, period that corresponds to the rainy season, between the years 2012 and 2016. Our data come from the combination of other studies to consider different savanna vegetation types, regions, and types of impact, enhancing the broadness of the study. We sampled ants in 37 sites with native vegetation, hereafter referred to as non-impacted sites, including the following vegetation types: cerrado, cerrado *sensu stricto*, campo limpo, campo rupestre and dry forest. We also sampled 47 sites with different types of environmental impact, hereafter referred to as impacted sites, including: eucalyptus (*Eucalyptus* sp.) monoculture; post-mining rehabilitation with exotic grass (*Braquiaria decumbens* and *Melinis minutiflora*) and pigeon pea (*Cajanus cajan*); and camping sites (characterized by sandy soil with scarce or no herbaceous or shrubby vegetation).

### Evaluating the proportion of seed removal

We established a single transect in each site containing at least five sampling points, with a minimum distance of 20 m between each of them. We provided at least ten seeds (natural or artificial) per sampling point, and excluded them from vertebrates using a metal mesh cage (Henao-Gallego et al., 2012; Rabello et al., 2015). The variation in the number of seeds and sampling points is due to the use of different sampling design in different experiments that compose this manuscript.

The natural seeds were of *Croton floribundus* (Euphorbiaceae), a non-myrmecochorous species, with an average length of 4.7 and width of 4.4 mm (Paoli et al., 1995). The artificial seeds were 1.8 mm plastic beads weighing 0.03g and attached in an artificial paste attractive to ants (similar to Raimundo et al., 2004 and Rabello et al., 2015). Although the natural seeds were larger than the artificial seeds, both were classified as of small size according to the classification proposed by Pizo and Oliveira (2001).

To evaluate the influence of period of exposure (during the morning and for 24h) on seed removal proportion, we only used data from the studies with artificial seeds in order to standardize the type of seed. We counted the seeds removed after 4 h of exposition (seed removal during the morning period from 8 a.m. to 12 a.m.) and after 24 h of seed exposure in 24 sites: 11 impacted sites and 13 non-impacted sites.

To compare the seed removal proportion between natural and artificial seeds, we used data from 42 sites using natural seeds (27 impacted sites and 15 non-impacted sites) and 24 sites using artificial seeds (11 impacted sites and 13 non-impacted sites); the sites with artificial seeds were not the same as those with natural seeds. In this case we considered only data for the period of 24h seed exposure.

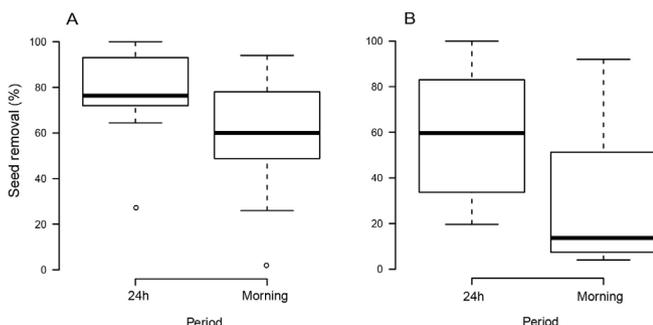
### Statistical analysis

In order to evaluate differences in seed removal proportion between the two seed exposure periods, we performed generalized linear mixed models (GLMM) with binomial distribution using the package *lme4* in the software R v.3.2.3 (R Development Core Team 2015). Seed removal proportion was the response variable, the exposure period (morning or 24 h) was the explanatory variable and the site (non-impacted or impacted) was the random variable. We used GLMM because we had pseudo replication of sampling points (due to sampling the same site during the morning and after a 24h period).

We used generalized linear model (GLM) for the seed type evaluation because there was no pseudo replication. For the GLM, seed removal proportion was the response variable and the type of seeds (natural or artificial) was the explanatory variable and we carry out the analyses separately for impacted and non-impacted sites. For both evaluations we used the average proportion of seeds removed per transect performing the analyses using the software R v.3.2.3 (R Development Core Team 2015).

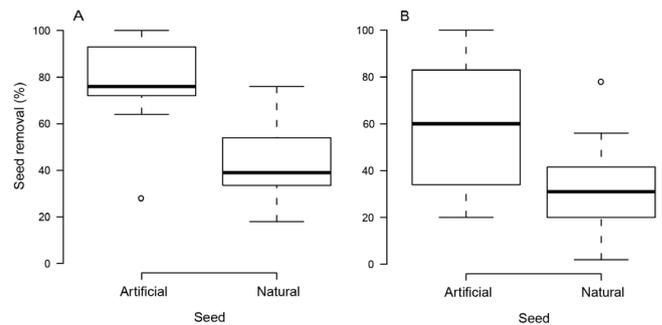
## Results

The proportion of seeds removed by ants was greater after seed exposure for 24 h than during the morning period in both non-impacted sites ( $\chi^2 = 318.68$ ;  $p < 0.0001$ ;  $df = 23$ ) (Fig 1A) and impacted sites ( $\chi^2 = 649.1$ ;  $p < 0.0001$ ;  $df = 19$ ) (Fig 1B). In non-impacted sites, the seed removal proportion during the morning period was 60%, which increased to 80% percent after the 24 h exposure period. In impacted sites, the seed removal proportion during the morning period was less than 20%, with an increase to 60% after the 24 h exposure period.



**Fig 1.** Proportion of artificial seeds removed by ants during the morning period and after 24 h of seed exposure in non-impacted (A) and impacted (B) sites.

Seed removal proportion for artificial seeds was greater than for natural seeds, both in non-impacted sites ( $F = 21.83$ ;  $p < 0.0001$ ;  $df = 26$ ) (Fig 2A) and impacted sites ( $F = 13.81$ ;  $p = 0.0007$ ;  $df = 36$ ) (Fig 2B).



**Fig 2.** Proportion of artificial and natural seeds removed by ants after 24 h of exposure in non-impacted (A) and impacted (B) sites.

## Discussion

The present study evaluated the proportion of seeds removed by ants at different sites of Brazilian savanna, comparing the use of artificial and natural seeds and two different sampling periods. We observed that the proportion of seeds removed by ants is influenced by the exposure period of the seeds, with it always being greater after a 24 h exposure period, regardless of the type of area (non-impacted or impacted). In addition, artificial seeds removal by ants was higher than natural seeds.

The proportion of removed seeds was lower in the morning period than after 24 h of exposure in both impacted and non-impacted sites. This is expected because seeds have a shorter period of exposure. Extending the seed exposure period to 24 h may have enabled ants with different diel foraging periods to exploit the resource, and an additive effect on seed removal by diurnal and nocturnal ants, may explain the observed higher seed removal proportion after the 24 h exposure period.

However, in non-impacted sites, the highest proportion of seed removal was observed during the morning period (60% out of 80% in 24 h), while in impacted sites few seeds were removed during the morning period (20% out of 60% in 24h). Thus, there seemed to be greater ant activity during the evening and/or night time period in these impacted sites, probably due to change in the composition of the ant community (Bieber et al., 2014). The lower ant activity in the morning could be a result of our impacted sites possibly having lost some functional groups of ant species which forage during the morning period. Thus, the higher seed removal proportion, in our study, during the afternoon would be due to different ant species composition between non-impacted sites which harbor ant species with different diel foraging (Aranda-Rickert & Fracchia, 2012). Furthermore, the greater complexity of vegetation in the non-impacted sites may support better microclimatic conditions for ants, including a decrease in soil exposure, which lessens the exposure of ants to dehydration

and predation, since they are sensitive to temperature and moisture (Traniello, 1989; Lima & Antonialli-Junior, 2013), thus, contributing to seed removal during the morning periods. Indeed, according to our personal observations, the ground of impacted sites had greater exposure to solar incidence and a lack of herbaceous vegetation, which may also have contributed to the differential ant species composition and activity (Williams et al., 2012).

Based on our results in savanna sites, we suggest that when the main aim of a study is to determine the seed removal proportion in non-impacted sites, sampling could only be performed during the morning period. We make this recommendation because the cost-benefit ratio is better in these sites, with 60% of seed removal occurring during the morning representing a significant sampling, and only 20% being removed during the remaining period. Additionally, when the aim of the study is to determine only seed removal in non-impacted sites, a faster sampling (4 hours during the morning) is more feasible and less costly than a sampling performed over a period of 24h, which contributes only 20% to total seed removal. It is also possible to collect the seed-removing ant species during this period. However, if the aim of the study is to compare seed removal proportion within impacted sites, samplings at least after 24 h of seed exposure are recommended, since seed removal in these sites may occur intensively during the night time. However, we think a future research would be interesting to evaluate and compare other periods of seed removal exposure, in order to verify if higher seed removal exposition (i.e. 48 h and 96 h as done by Rocha-Ortega et al., 2017; Ferreira et al., 2011 respectively) increases percentage of seed removal increasing the robustness of the study. Besides, we also consider important to evaluate other parameters of seed removal process such as seed-removing ant composition in diurnal and nocturnal periods, as well as, comparisons of these parameters between non-impacted and impacted sites. Species composition is important because it allows observing the presence of harvester (Pirk & Lopez-de-Casave, 2006; Belchior et al., 2012) and seed-disperser ants (Leal et al., 2014) which can provide a different destination for the seed. We observed that in the afternoon period, seed-removing ants re-start their foraging activity between 15h and 16h as was previously documented by Aranda-Rickert and Fracchia (2012). The period of exposure and type of seed may depend on the aim of the research, however, we consider really important to standardize the sampling methodology in ecological studies that evaluate different environmental disturbances and impacts on ecological function.

The seed removal proportion was greater when using artificial than when using natural seeds. This difference may be the result of the composition of the artificial seeds (being near 80% lipids), since the lipid-content have been shown to be major determinants of seed removal by ants (Pizo & Oliveira, 2001). Pizo and Oliveira (2001) showed that ants interact for longer periods of time and more frequently with

diaspores containing aril or pulp rich in lipids. It is likely that our natural seeds possessed a lower amount of lipid than artificial seeds since most seeds of plants of the genus *Croton* have 34 to 40% lipids (Adeyinka et al., 2013; Bello et al., 2014).

Artificial fruits have been used to evaluate ant-diaspore interactions (Henao-Gallego et al., 2012; Bieber et al., 2014; Rabello et al., 2015), and are considered an alternative to using natural diaspores, due to their convenience and reliability. Such artificial fruits can reduce problems with data collection due to shortage of natural fruits during experimental periods, the use of fruits that are less attractive to ants, and variation in chemical and morphological characteristics of fruits, which may differ geographically (Raimundo et al., 2004; Bieber et al., 2014). The use of artificial fruits may also help to standardize the methodology of determining the proportion of seeds removed by ants and facilitate comparisons among different studies from different regions (i.e., different biomes), which would not be possible using natural seeds. Moreover, attractive artificial fruits can be used in studies that evaluate the rehabilitation of degraded sites, which, in many cases, are initially devoid of natural seeds (Henao-Gallego et al., 2012; Rabello et al., 2015). In this way, artificial seed removal proportion and the presence of seed-removing ant species could determine, in the absence of natural seeds, whether the ecological function of seed removal is being recovered.

Finally, we consider artificial seeds to be an effective alternative to natural seeds for studies aiming to evaluate and monitor this ecosystem function under different types of impacts. The attractiveness of artificial seeds is an important factor to consider, especially when the sampling period does not coincide with the fruiting season or when there is no prior access to the site or knowledge about the fruiting plant species present there. In order to standardize the attractiveness of artificial seeds, we suggest using the same composition used in the present study (see also Rabello et al., 2015). This artificial seed composition was the same used by Raimundo et al. (2004), only with a modification in the type of lipid resource used in our study, and who also reported it as efficiently attractive to seed-removing ants. Our study suggests that standardizing the type of seed used and the period of sampling could maximize the acquisition of information regarding this important function of seed removal by ants. Lastly, it is important to emphasize that our study was conducted in Brazilian savanna, and so we recommend that future researchers carry out more comprehensive studies involving different phytophysiognomies to see how general these findings are.

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