



Can tortoises facilitate the germination of plants with dry fruits? A simulation of the effects of *Chelonoidis carbonarius* (Testudines: Testudinidae) on the germination of *Leucaena leucocephala* (Fabaceae)

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Abstract

Ingestion of seeds by vertebrates may influence seed germination and dispersion. However the mechanisms responsible by these effects and the importance of turtles in these interactions are still poorly understood. This study aims to evaluate how *Chelonoidis carbonarius* influences the germination process of *Leucaena leucocephala* using an experiment conducted at the Federal University of Ceará, Fortaleza-CE, Brazil, that simulates the passage of the seeds of this plant through the digestive tract of *C. carbonarius*: stomach acidic condition and seeds sown in feces. We demonstrated that sowing the seeds in feces of *Chelonoidis carbonarius* has a negative effect on the germination of *Leucaena leucocephala*, decreasing the germination speed index of the seeds, while the acidic conditions have a neutral one. This study is a first attempt to understand how turtles and tortoises may influence the seed germination, and the implications of these findings in natural environments deserve further investigations.

Key words: acidic conditions, germination speed index, invasive species, simulation, turtles.

Resumo

A ingestão de sementes por vertebrados pode influenciar a germinação e a dispersão dessas sementes, porém pouco se sabe sobre os processos responsáveis por essa influência e sobre a importância dos quelônios nessas interações. Este estudo tem como objetivo avaliar como *Chelonoidis carbonarius* influencia o processo de germinação de *Leucaena leucocephala* usando um experimento conduzido na Universidade Federal do Ceará, Fortaleza-CE, Brasil, que simula a passagem das sementes dessa planta através do trato digestivo de *C. carbonarius*: condições ácidas do estômago do animal e a semeadura das sementes nas fezes. Foi demonstrado que a semeadura das sementes nas fezes de *Chelonoidis carbonarius* tem um efeito negativo sobre a germinação de *Leucaena leucocephala*, diminuindo o índice de velocidade de germinação das sementes, ao passo que as condições ácidas tem um efeito neutro. Este estudo é uma primeira tentativa de entender como os jabutis podem influenciar a germinação das sementes e as implicações destes resultados em ambientes naturais merecem mais investigações.

Palavras-chave: condições ácidas, índice de velocidade de germinação, espécies invasoras, simulação, quelônios.

Introduction

Ingestion of seeds by vertebrates may affect seed germination, but the processes related to this influence is a topic still poorly understood (Traveset 1998). Turtles and tortoises are examples of animals that have fruits in their diets, and some studies found that these animals may influence the germination and dispersal of

plants that they eat (e.g., Liu *et al.* 2004; Strong & Fragoso 2006; Jerzolimski *et al.* 2009; Padgett *et al.* 2010; Blake *et al.* 2012). The ingestion of seeds by these animals may increase or decrease the number of germinated seeds and the germination speed, which vary according to the plant species (Liu *et al.* 2004). Tortoises can act in this germination course, accumulating seeds and

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placing them in a medium rich in nutrients (feces), removing germination inhibitors (Birkhead *et al.* 2005), or increasing seed coat permeability using the acidic composition of gastric secretions (Gardener *et al.* 1993).

Chelonoidis carbonarius (Spix 1824), popularly known as “Red-footed Tortoise”, is an herbivorous tortoise widely found in the Brazilian territory (Rueda-Almonacid *et al.* 2007). These animals mainly eat leafy vegetables, fleshy fruits (Wang *et al.* 2011), dry fruits of legumes (Birkhead *et al.* 2005), and they disperse some plants in the Amazon Forest (Strong & Fragoso 2006). In captive conditions, we observed that individuals of *C. carbonarius* also freely eat many parts of *Leucaena leucocephala* (Lam.) de Wit, a legume plant that is very common in the area where these captive tortoises forage. However, so far, no study has evaluated how this tortoise may influence the germination of plants with dry fruits, such as *L. leucocephala*.

Leucaena leucocephala, within the Fabaceae family, is an exotic legume plant, native from Mexico and geographically distributed across the tropical region (Souza *et al.* 2007). This species has been considered as invasive in many countries on all continents (except Antarctica) and is cited by IUCN (International Union for Conservation of Nature) as one of the 100 most aggressive invasive species on the planet (GISD - Global Invasive Species Database 2000). This species has natural seed dormancy caused by seed coat impermeability to water (Teles *et al.* 2000; Fonseca & Jacobi 2011; Tadros *et al.* 2011), common among tropical species and found in most of the legumes (Kigel & Galili 1995). Therefore, the exposure to acidic conditions, a common dormancy breaking mechanism, may accelerate the germination rate of this species.

The distribution of invasive species in the invaded areas is influenced by various processes, such as introduction effort, climate matching, species traits and global trade, and in plants, dispersion by vertebrates is a trait positively associated with the success of the invaders (*e.g.*, Lloret *et al.* 2005; Banks *et al.* 2015; Mahoney *et al.* 2015). This understanding is the first step to properly forecast the invasion spread and to be able to control it. Considering this conservation issue and the observations regarding the feeding ecology of *C. carbonarius* reported before, our study aimed to evaluate how this tortoise influences the germination process of *L.*

leucocephala using an experiment that simulates the passage of its seeds through the digestive tract of the tortoise (including exposition to acidic conditions and immersion in feces). Besides simulating the passage through the digestive tract, we also evaluated the effects of acidic conditions and immersion in feces separately. This approach is a novelty of our study, because we are trying to understand the processes related to the seed germination rather than simply evaluating the germination values of eaten seeds.

Material and Methods

Seeds of *Leucaena leucocephala* were equally harvested from six plants randomly chosen in January 2013 at the Federal University of Ceará, Fortaleza-CE, Brazil (3°45' S and 39°02' W, datum WGS84). The area has an average elevation of 16 m above sea level and a warm sub-humid tropical climate according to the Köppen classification (Reddy 1983) with average annual rainfall of 1,338 mm and average temperature of 26–28°C. The rainy season is concentrated from January to May (INMET 2014).

The weight of 1,000 seeds was 40.17 g with 15.3% moisture. The seeds were separated in four treatments: Treatment 1 (control): seeds kept in sand substrate for germination without dormancy breaking; Treatment 2: Seeds immersed in a solution of hydrochloric acid (HCl) and distilled water at pH 2.0 for 30 min (Brasil, 1992) and taken to germination in sand substrate; Treatment 3: seeds collected and sown in the feces of the animal for germination; Treatment 4: seeds collected and sown in the feces for germination after immersion in HCl solution for 30 min. Treatments with chemical scarification were selected in order to examine whether exposure to HCl at concentration = 0.01 mol/L or pH 2.0, a concentration similar to that found in the stomach of tortoises (Barboza 1995), could act as a mechanism for dormancy breaking. The feces used in the experiment were fresh and humid, and they were obtained from one individual of *C. carbonarius* kept in the Núcleo Regional de Ofiologia da Universidade Federal do Ceará (NUROF-UFC). We used the treatments with only HCl and only feces in order to evaluate which of these processes most influenced the germination of *L. leucocephala* seeds that are eaten by *C. carbonarius*, which would face both steps (equivalent to treatment 4).

Each treatment had four replicates with 25 seeds each (= 400 seeds). Each replicate was

placed in moist chambers containing washed and autoclaved river sand at constant temperature of 25 °C and with 20-W fluorescent lamps, giving a radiant flux of about 50 mol m⁻² s⁻¹ (Rosa & Ferreira 1998) for 12 hours/day. The experiment was kept for 30 days with daily observations and followed the recommendations of the Rules for Seed Analysis (Brasil 1992). Seeds with root protrusion of 0.5 cm were considered as germinated, a cut-off commonly used in other germination studies (*e.g.*, Maguire 1962; Paulsen & Högstedt 2002; Souza *et al.* 2007).

We evaluated differences in cumulative germination (cumulative proportion of seeds germinated in each day) among the four treatments using Generalized Linear Models (GLM) with Binomial error family and logit link function, because the response variable range between 0 and 1 (Guillén *et al.* 2009; Benítez-Malvido *et al.* 2014). The model had cumulative germination as a response variable and time (the number of days since the seeds were sowed), time², treatment, time × treatment, time² × treatment as explanatory variables. This linear model provides three parameters for each treatment: 1) starting germination (the regression intercept), 2) initial germination rate, and 3) variation in germination rate; and we used Analysis of Deviance with chi-square tests to evaluate if these parameters varied among the treatments (Guillén *et al.* 2009; Benítez-Malvido *et al.* 2014). Differences among the treatments were evaluated using Z tests and a significance level of 5% ($P < 0.05$). These analyses were performed using the R software ver. 3.1.2 (R Core Team 2014).

We also calculated the Germination Speed Index (GSI), using daily counts of germinated seeds at 30 days after sowing, according to the equation proposed by Maguire (1962): $GSI = \sum (n_i/t_i)$, where: n_i = number of seeds that germinated in time 'i', and t_i = time after conducting the test. We used this evaluation to infer about seed vigor and germination speed after dormancy break.

Results

Cumulative germination percentages were low in all the treatments (Fig. 1). We observed that germination percentages were higher and similar in treatments 1 and 2 when compared to the other treatments, with maximum values of 32.5 and 37% respectively. The lowest value (14%) was found in treatment 4.

We observed that the interaction time² × Treatment was not significant, indicating that there is no difference in the variation of germination rate among the treatments (Tab. 1). Because of this, we ran a second model excluding this variable to evaluate the treatment effects. In general, we observed that treatments using feces had lower starting germination values than the others, and the treatment 3 had the highest initial germination rate (Fig. 1).

There was no difference between the treatment using HCl (treatment 2) and the control (treatment 1) in GSI. However, the use of feces (treatment 3) and its combination with HCl (treatment 4) provided a reduction in germination speed, which represents a delay in the germination process in these treatments (Fig. 2).

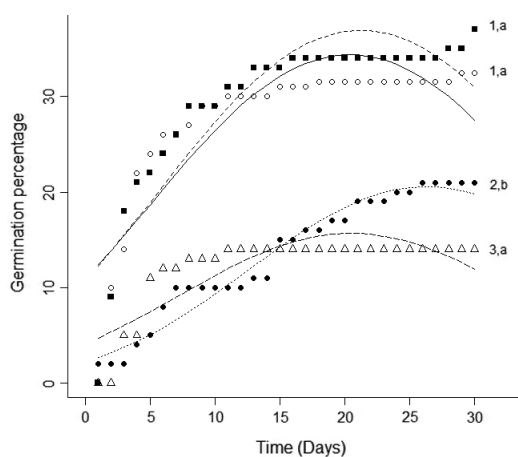


Figure 1 – Accumulated germination percentage of *Leucaena leucocephala* seeds throughout the experiment. Open circles = treatment 1: control; dark squares = treatment 2: immersion of seeds in a solution of hydrochloric acid (HCl) and distilled water at pH 2.0 for 30 min; dark circles = treatment 3: seeds collected and sown in *Chelonoidis carbonarius* feces for germination; and open triangles = treatment 4: seeds collected and sown in *C. carbonarius* feces for germination after immersion in HCl solution for 30 min. Different numbers represent differences in the intercept (starting germination) among the treatments; lower case letters represent differences in initial germination rate among the treatments. Continuous line = treatment 1; dashed line = treatment 2; dotted line = treatment 3; long dashed line = treatment 4.

Table 1 – Analysis of deviance performed to evaluate the significant predictors in our model using chi-square tests. The response variable was the accumulated germination percentage of *Leucaena leucocephala* seeds in each day of the experiment, and the explanatory variables were treatment, time and time².

Explanatory variable	Deviance	Df	P
Time	192.40	1	<0.001
Time ²	82.92	1	<0.001
Treatment	480.22	3	<0.001
Time × Treatment	25.04	3	<0.001
Time ² × Treatment	0.48	3	0.92
Model	781.07	11	
Residual	116.33	108	
Total	897.40	119	

Discussion

We expected that the HCl at pH 2.0, also present in the stomach of tortoises (Barboza 1995), would accelerate and facilitate the germination process due to an increase in seed permeability, but it was not confirmed by our results. We should emphasize, however, that germinative capacity was maintained even after exposing the seeds to HCl. This maintenance of germination is common in seeds eaten by turtles and tortoises (Jerozolinski *et al.* 2009; Kimmons & Moll 2010). The neutral effect of acidic conditions on seed germination could suggest that *C. carbonarius* could disperse seeds of *L. leucocephala*, because the animal could transport seeds of this plant for long distances after eat them, and they would still be able to germinate. This mechanism was already suggested by some authors for other species of turtles and tortoises (Jerozolinski *et al.* 2009; Kimmons & Moll 2010; Padget *et al.* 2010). Seeds ingested by chelonians usually have low damage percentage (Kimmons & Moll 2010; Padget *et al.* 2010), reinforcing the neutral effect on germination rates highlighted before.

It was expected that the animal's feces would increase the germination of *L. leucocephala* due to the supply of water and nutrients, acting as a fertilizer (Traveset & Verdú 2002). The highest initial germination rate observed for the treatment 3 (Fig. 1) may be related to an excess of nutrients or a progressive liberation of germination

inhibitors in feces (Paulsen & Högstedt 2002), which initially stimulates seedling emergence of the germinated seeds, but in this case did not ensure higher accumulated germination or higher germination speed over time (Figs. 1, 2). The lowest germination speed indexes observed for treatments that simulated ingestion of *L. leucocephala* seeds by *C. carbonarius* allow us to state that there was a delay in germination processes. A decrease in germination values was also observed in seeds ingested by other turtles and tortoises (Liu *et al.* 2004; Kimmons & Moll 2010). Fungal infection was observed in treatments using feces as substrate, which may have influenced their results. However, fungal growth in seed samples deposited in fecal material may be considered normal, because this substrate has several natural contaminants, and germination chamber has conditions of temperature, humidity, and photoperiod that favor such proliferation (Ferreira 1989).

Data obtained in the laboratory may not represent field conditions due to the complexity of factors that influence the biological processes of germination (Floriano 2004). Therefore, in the future, it would be important to replicate this experiment in a natural environment. It would be also interesting to perform a palatability analysis in these future studies in order to evaluate how often *C. carbonarius* prefer to eat dry fruits, such as those from *L. leucocephala*, instead of fleshy fruits in

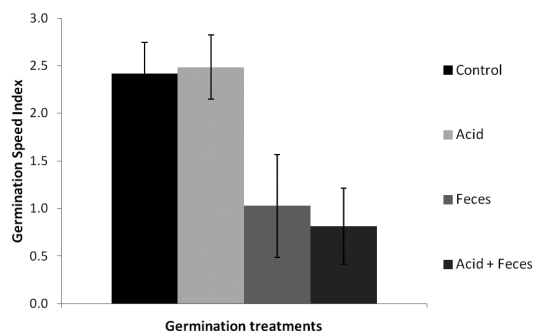


Figure 2 – Germination speed index (GSI) of *Leucaena leucocephala* seeds after 30 days. Control = treatment 1 (control), Acid = treatment 2 (immersion of seeds in a solution of hydrochloric acid (HCl) and distilled water at pH 2.0 for 30 min), Feces = treatment 3 (seeds collected and sown in *Chelonoidis carbonarius* feces for germination) and Acid + Feces = treatment 4 (seeds collected and sown in *C. carbonarius* feces for germination after immersion in HCl solution for 30 min). Error bars represent the standard errors of the means (n = 4).

natural conditions. This test was not performed in our study because we had access to only one individual of the Red-footed Tortoise, which could produce a biased result of food preference.

We demonstrated that sowing the seeds in feces of *Chelonoidis carbonarius* has a negative effect on the germination of *Leucaena leucocephala*, decreasing the germination speed index of the seeds, while the acidic conditions have a neutral one. This study is a first attempt to understand how turtles and tortoises may influence the seed germination, and the implications of these findings in natural environments deserve further investigations.

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