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Endozoochory and Germination of Selected Ingested Seeds by Malayan Box Turtles (*Cuora amboinensis*) from Laguna Province, Philippines

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Authors' contributions

This work was carried out in collaboration between both authors. Author KEGI was responsible for the concept and design, experimentation and data acquisition, data and statistical analyses, and manuscript preparation. Author LVP was responsible for the concept and design, supervision of the experiment and data acquisition, checking and correcting data and statistical analyses, manuscript review, and preparation for publication. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Malayan box turtles' (*Cuora amboinensis*) ecological niche are essential in an ecosystem but are often overlooked. This study investigated the germination of selected seeds that passed through the gut of Malayan box turtles to determine its role in promoting seed dispersal and aiding seed germination.
Study Design: Experimental approach.
Place and Duration of Study: Pamantasan ng Lungsod ng Maynila (University of the City of Manila) and Dasmarinas, Cavite between June 2016 to March 2017.
Methodology: The seeds that passed through the turtle's gut (Gut Passed Seeds) and seeds that did not pass through its gut (Mechanically Extracted Seeds) underwent comparative germination test. The Germination Rate (GR) and Percent Germination (%GR) of each group were determined

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in the study. Seed shadowing was also conducted to evaluate the turtle's seed dispersal capacity (endozoochory).
Results: Results showed that after gut passage, seed GR and %GR were enhanced on *Lycopersicon esculentum, Carica papaya, Psidium guajava,* and *Muntingia calabura*. However, Germination Rate and Percent Germination of *Passiflora quadrangularis* decreased after gut passage. Statistical analyses revealed that there is a significant difference in the GR and %G of *M. calabura* and %G of *L. esculentum*, and *P. quadrangularis*. Thread trailing method showed that *C. amboinensis* can disperse seeds at a distance of 24.8 to 52.8 meters.
Conclusion: This study demonstrates the important role of *C. amboinensis* in the ecosystem through its contribution to plant seed germination and dispersal. It showed that Malayan box turtles are not only seed dispersal agents but are also important in the germination of seeds that they have ingested and defecated.

Keywords: Cuora amboinensis; malayan box turtle; seed germination; endozoochory; seed dispersal.

1. INTRODUCTION

Malayan box turtle (*Cuora amboinensis*) is known for being well distributed in different regions of the Philippines as well as in other countries of South East Asia [1]. *C. amboinensis* is a hard-shelled turtle commonly found in semi aquatic environment such as rice fields, rivers, ponds, marshes and swamps [2]. The turtle is abundant in Palawan and has been sighted in different areas of Laguna province of the Philippines [3].

C. amboinensis' occurrence and distribution is extensive in the past decades, but in recent years its number declined because of the following factors: habitat loss, introduction of alien predators and competitors, and illegal trading [4]. It is also widely popular among pet shops and trade market as they are used as a delicacy and for traditional medicine [5,2]. *C. amboinensis* is considered a Vulnerable Species according to the IUCN list in 2016.

This turtle is an omnivore but mostly subsist on plant diet. In water, it feeds on water plants, mollusks and crustaceans, while on land it feeds on invertebrates, vegetation and fruits [1]. The fecal examination conducted by Ives et al. [5] revealed seeds and other vegetative matter, which implies the species' inability to masticate and totally digest the ingested seeds.

In spite of the fact that this turtle species is abundant in the Philippines, still, there is scanty information and very few studies about its ecological importance [2]. This study centers on the important ecological role played by *Cuora amboinensis* in terms of its ability in conserving, dispersing, and germinating the seeds of selected fruit bearing plants and the possible symbiotic relationship it plays in the ecosystem with other plant biota. Furthermore, there is limited information investigating the fate and viability of the seeds defecated by *C. amboinensis*, and if gut passage significantly affect seed germinability. The study also covered the evaluation of the potential role of the said turtle in the ecosystem as a seed dispersal agent.

2. METHODOLOGY

2.1 Collection of the Test Organism

Cuora amboinensis were collected from the province of Laguna, Philippines. The collection site is comprised of different habitat and environment such as rivers, creeks, swamps, wood piles and rocky areas. Sampling was also done in plantation areas and rice fields where turtles are frequently seen according to an interview done among the locals. Notes on the description of the area where each turtle was collected were recorded. Juvenile turtles were not used as experimental subjects, and the sex ratio of the collected turtles was disregarded.

2.2 Acclimation and Fecal Analysis

Collected turtles were acclimated for seven days in the experimental area. The turtles were placed in 46-cm. diameter basin surrounded by 3ft x 6.5 ft plastic chicken wires (13 mm holed). Each basin contained 5 cm. tap water to stimulate defecation [6]. For every stool observed from the container, fecal seed recovery was conducted. Recovered seeds were collected, counted and identified up to the species level. For the entire 7 days, the turtles were not given any food to allow elimination of eaten food for seed identification and quantification [7]. The basins each contain 1-

cm water depth for the whole span of the research.

2.3 Preference Test

A preference test was conducted to assess the food preference of *Cuora amboinensis* in the wild. A total of six types of food were offered to each turtle. These were the natural foods of *C. amboinensis* in the wild reported by Ives et al. [5] and Schoppe et al. [1]. These include water spinach, fresh water fish, mushroom, insects, amphibians, and fruits. Pointing system was used to evaluate the food preferability of *C. amboinensis* [7].

2.4 Fruit Feeding

Collected fruits were grouped into two: experimental and control. Both were composed of same fruit species. The experimental group was subjected to gut passage by feeding it to the turtle. The control group was not fed to turtles; instead, it was reserved for direct seed germination test. Set-up A was fed with fruit not found in the feces. Set-up B to E were fed with different fruit species found in initial fecal analysis. Feeding was conducted every night time when turtles were active. The time of feeding and initial seed number were also noted every night. Water on each set-up was changed every 2 days. The retention time inside the turtle's gut was also recorded.

2.5 Seed Microscopic and Weight Analysis

Defecated seeds were recovered by separating the seeds from other fecal matter using a sieve (1 to 2 mm mesh). Representative Gut Passed Seeds (GPS) and Mechanically Extracted Seeds (MES) were observed under the scanner of a compound light microscope to determine the morphological effects of gut passage on the seeds. The species of the plants in which the seeds are classified into were also identified in this study. In addition, the weight in grams of GPS and MES were measured using analytical balance to determine the effects of gut passage on the weight of the seeds.

2.6 Germination Test

The GPS and MES groups were planted at same time. Nursery poly bags were used as the germination container where a total of 5 cm thick organic soil was placed. Each polybag contains 10 seeds that were watered daily to moisten the soil [8]. The germination rate was calculated by noting how many seeds germinated each day, from the time of planting until all or none of the seeds germinated. The emergence of any part of the seedlings (i.e. radicle) was considered germinated seed [8,9]. The collective number of germinated seeds present from the time of planting was recorded daily. For non-germinating seeds, an allowance of 1 week was allotted. After the allowable time, Percent Germination (%G) was determined from the number of seeds that germinated and the number of seeds that did not germinate.

2.7 Thread Trailing Method (Seed Shadowing)

The Malayan box turtles were subjected to seed shadowing to determine the dispersal capacity of the species. A 100-meter sewing thread (0.10 mm diameter) rolled in a bobbin cylinder were attached on the turtle's carapace using a 1 cm wide rubber. Turtles were released on different areas of the sampling site. The minimum retention time for each plant species was designated on each turtle. The displacement covered by the turtle after the given retention time was considered as the dispersal distance [10,11]. After seed shadowing, the turtles were released individually on the respective areas where each was initially captured.

2.8 Statistical Analysis

One-way ANOVA and Post Hoc test were used to analyze the significant differences in percent germination and germination rate of selected plant species. T-test was used to determine the significant differences between percent germination and germination rate of Gut Passed Seeds and Mechanically Extracted Seeds. Differences were considered significant at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Initial Fecal Analysis

The fecal analysis suggested that *C. amboinensis* are also frugivores because of the seeds found from their feces. Similar observations have been obtained from the work done by Ives et al. [5] when they found seeds in the feces of *C. amboinensis* in Sulawesi, Indonesia.

There were 15 seeds found and collected from the feces of 7 wild *C.* amboinensis (Table 1). These seeds were *Polyscias florosa* (Araliaceae), *Psidium guajava* (Myrtaceae), *Carica papaya* (Caricaceae), and *Passiflora quadrangularis* (Passifloraceae). In addition, 2 remnant calyx of *Muntingia calabura* (Muntingiaceae) were also found from the fecal material (Fig. 1).

3.2 Preference Test and Fruit Feeding

The preference test revealed that fruits were highly preferred by Malayan box turtles, while other food types were either fairly or poorly preferred by *C. amboinensis* (Table 2).

The ripe fruit with seeds intact of *Lycopersicon esculentum, Carica papaya, Passiflora quadrangularis, Psidium guajava* and *M. calabura* were fed to *C. amboinensis. L. esculentum* was used to replace *Polyscias florosa* because the fruiting phenology of the latter was not in season. *L. esculentum* was chosen to represent other fleshy fruit species and is a known part of the diet by other turtle species [12]. The other fruits were the plant species identified from the fecal analysis.

Table 1. Frequency of the seeds and other fruit parts found from *C. amboinensis* fecal samples

Turtle code	Family	Species	Frequency
1	*Araliaceae*	*Polyscias florosa*	2
4	*Myrtaceae*	*Psidium guajava*	4
6	*Muntingiaceae*	*Muntingia calabur* (Calyx)	3
9	*Caricaceae*	*Carica papaya*	1
13	*Caricaceae*	*Carica papaya*	2
14	*Passifloraceae*	*Passiflora quadrangularis*	1
15	*Passifloraceae*	*Passiflora quadrangularis*	2

Fig. 1. Seeds and fruit part found from feces of C. amboinensis : a) *Polyscias florosa*, b) *Psidium guajava*, c) *Carica papaya*, d) *Passiflora quadrangularis*, e) *Muntingia calabura* (Calyx)

Table 2. Preference test for *C. amboinensis*

Foods	Points	Preferability
Water spinach	1.2 ± 1.1	Fair
Fresh water fish	1.4 ± 1.1	Fair
Mushroom	1 ± 0.84	Fair
Insect	0.07 ± 0.26	Poor
Amphibian	0.2 ± 0.4	Poor
Fruit	2.9 ± 0.25	Very good
Ratio	**Points**	**Preferability**
0/3	0	Poor
1/3	1	Fair
2/3	2	Good
3/3	3	Very good

3.3 Seed Microscopic and Weight Analysis

Microscopic seed analysis was conducted and showed noticeable physical changes (Fig. 2) such as seed color [13]. The sarcotesta of *L. esculentum, C. papaya*, *P. guajava* and *M. calabura* were removed by digestion inside the gut [6].

The seed weight of *L. esculentum, C. papaya*, *P. guajava* and *M. calabura* were reduced due to the removal of sarcotesta on the seeds [14]. On the contrary, the weight of *P. quadrangularis* seeds was increased after gut passage due to liquid absorption while inside the gut, and it was also attributed for softer seeds [15]. In addition, the seed weight loss after gut passage was due to seed coat scarification [16].

3.4 Germination Test

The ability of the seed to germinate after passing through the animal's gut is a least concern in many studies; however this aspect is important in order to understand the evolutionary relationship of plant and animal interaction [17]. Gut Passed Seeds (GPS) and Mechanically Extracted Seeds (MES) were subjected to germination test in order to assess and investigate seed viability, germinability, Germination Rate (GR), and Percent Germination (%G). Of the 5 test plants, 4 showed an enhanced (GPS > MES) germination rate (Fig. 3) and percent germination (Fig. 4) after gut passage. These plants were *L. esculentum* (0.6190 seed/day, 96.67%), *C. papaya* (0.445 seed/day, 86.67%), *P. guajava* (0.468 seed/day, 93.33%) and *M. calabura* (0.753 seed/day, 21.22%). However, in the case of *P. quadrangularis*, (0.579 seed/day, 76.67%) germination rate and percent germination were decreased after gut passage.

The germination characteristics of seeds were modified after gut transit. This modification was responsible for variation in germination responses [16]. Table 3 enumerates the effects of *Cuora amboinensis'* gut passage on seed germination of 5 different plant species. There were factors that contribute in germination activity after gut passage. First, seed coat scarification after gut passage positively contributes to germination. When seeds are scarified or abraded, the water, light and air are more permeable, thus faster germination and improved the germination rate [13,14]. Even though turtles have short gut, the retention time or exposure of the seeds inside the gut made the seeds abraded by the mechanical and chemical processes of the digestive tract. Furthermore, [17] reported the presence of symbiotic microorganisms that inhabit the turtle's caecum are responsible for seed coat polymer fermentation. It was also attributed to weight loss by [9]. *L. esculentum*, *C. papaya, P. guajava* and *M. calabura* seeds slightly to significantly lost weight as they have enhanced germination rate. On the contrary, *P. quadrangularis* was negatively affected by scarification because seed abrasion immediately absorbs water and other digestive substances while in the gut. It was said so because after gut passage, the *P. quadrangularis* seeds gain weight and became softer. As a result, the germination rate of the said plant was reduced.

The second factor was deinhibition. A strong chemical inhibitor that occurs in sarcotesta blocks the biochemical pathway of germination [16,8], thus affected the germinability rate and percentage. The active agent in this case is an aromatic glycoside [16]. The result of germination test on the five plant species above mostly favoured Gut Passed Seed (GPS) since sarcotesta were removed after gut passage. On the other hand, the Mechanically Extracted Seeds (MES) were not observed to have been sarcotesta-free. MES were directly exposed to germination inhibitors. For this reason, MES produced a lower rate of germination than GPS. However, opposite results were observed for *P. quadrangularis* because GPS were greatly affected by the first factor, and its MES were affected by the second factor since some are considered sarcotesta-free seeds.

The last factor was seed cleaning or the removal of sarcotesta. Liu et al. [6] noted that pulp removal in the digestion process could be the reason of enhanced germination percentage. The sarcotesta decreased germination activity by altering the seeds microenvironment such as osmotic pressure caused by the high sugar content of ripe fruits, and by light-blocking that prevents enough light reaching the seedlings to stimulate germination [16]. Seed cleaning is a key process because it reduces the inhibition effect of high osmotic pressure, light-blocking and secondary metabolites in seed germination. Seed cleaning is necessary to remove germination inhibitors present in the fruit pulp [18]. In addition, the removal of sarcotesta permits water to be absorbed by the seeds.

Fig. 2. Seed microscopic analysis showing Gut Passed Seeds and Mechanically Extracted Seeds of a) *Lycopersicon esculentum*, b) *Carica papaya*, c) *Psidium guajava*, d) *Passiflora quadrangularis*, e) *Muntingia calabura*

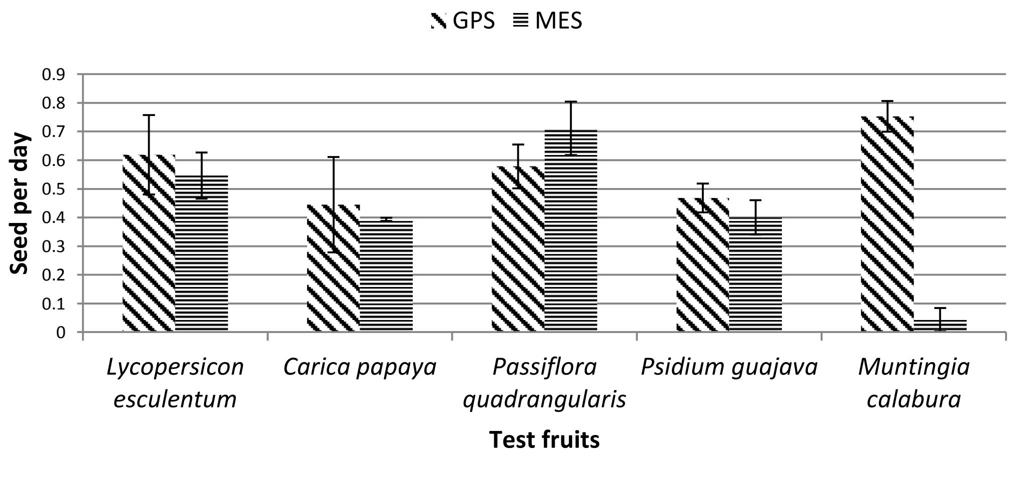

Fig. 3. Germination rate (GR) of gut passed seeds (GPS) and mechanically extracted seeds (MES) of 5 test fruits

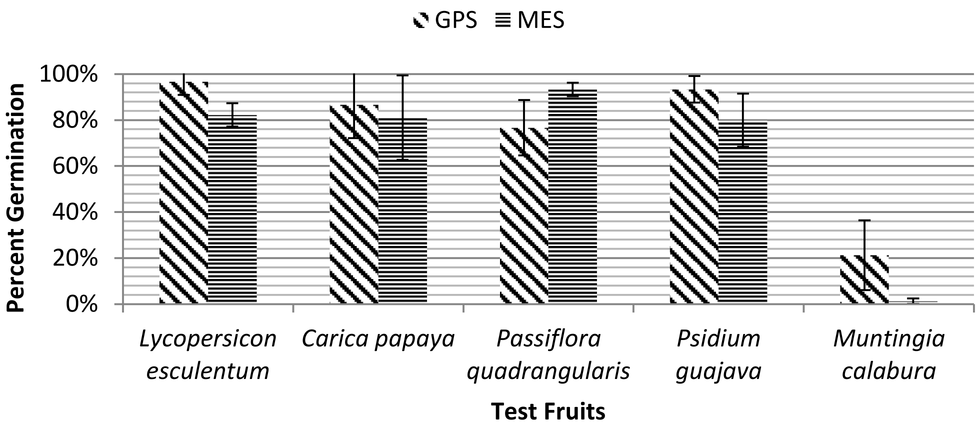

Fig. 4. Percent germination (%G) of gut passed seeds (GPS) and mechanically extracted seeds (MES) of 5 test fruits

Table 3. Effects of gut passage on seed germination

Plant species (seeds)	Germination Rate	Percent Germination
Lycopersicon esculentum	Enhanced	Enhanced
Carica papaya	Enhanced	Enhanced
Passiflora quadrangularis	Reduced	Reduced
Psidium guajava	Enhanced	Enhanced
Muntingia calabura	Enhanced	Enhanced

Among the five test plant species, post-hoc analyses revealed that there is no significant difference in percent germination among the four plant species: *L. esculentum, C. papaya, P. quadrangularis* and *P. guajava* since their percent germination are close to one another (76.67 to 96.67%). However, *M. calabura* is significantly lower as compared to the other plant species (21.22%). This is possibly because *M. calabura* has the smallest seed size (0.3 mm) among the test plant species. Liu et al. [6] reported that small seeds are more likely to be affected negatively than larger seeds.

Only *M. calabura* has a significant difference in germination rate between GPS and MES. Braun and Brooks [7] reported the enhanced germination rate for small seeds after animal's gut passage. Smaller seeds are most likely to have thinner seed coat than larger seeds. Seed coat scarification enhances the rate of germination. On the other hand, *L. esculentum, P. quadrangularis* and *M. calabura* have significant differences between the percent germination of GPS and MES. This can be attributed to the effects on weight after gut passage. *P. quadrangularis* significantly gain weight after gut passage. Seeds that gain weight absorbed digestive substances while inside the gut that made the seeds less viable [15]. The other plant species lost weight. *L. eculentum* and *M. calabura* slightly lost weight after gut passage. Seeds with slight reduction in weight are more likely to be more viable for germination than seeds with significantly reduced weight after gut passage [19].

3.5 Seed Shadowing

The results of this study indicate the prospective role of *Cuora amboinensis* in the ecosystem as a seed dispersal agent because of the seeds found from its feces. To assess the extent of the capability *C. amboinensis* in dispersing the seeds it ingest, seed shadowing was conducted along with the measurement of the dispersal distance by the turtle before defecation or while seeds are inside its gut. Thread trailing method was used to determine the dispersal capacity of the turtle as prescribed by previous researchers in evaluating spatial movements and pathway structure of turtles [20].

Given the minimum retention time of 30.97 hrs to 72.99 hrs, *C. amboinensis* were able to disperse seeds of five plant species at a distance of 24.84

meters to 52.83 meters. Diesmos [17] and Traverset [3] reported the advantage of long retention time. The longer the retention time of the seeds inside the turtles' gut would most likely result in seeds being dispersed at a farther distance as observed in the case for *C. papaya* and *M. calabura*. The first has the shortest retention time (30.97 hrs), thus dispersed at the shortest distance (24.84 m), whereas the second has longer retention time (71.7 hrs), hence were dispersed at a farther distance (52.83 m) than the first. However, it is not always the general rule like as in the case of *P. quadrangularis* and *P. guajava*. *P. guajava* has longer retention time than the first but it was dispersed at a shorter distance. Several factors affect the dispersal capacity of the Malayan box turtle. These factors observed include 1) the behavior of each turtle since there were individuals that were very active, and some were less active; 2) Differences in movement capacity because some turtles are fast moving, while others are slow moving; and lastly 3) the topographical features of the area trailed by the turtle for seed shadowing.

The seed dispersed away from parent trees is crucial for plant fitness and survival [21]. The seed dispersal potential of a turtle is measured by its capability of depositing seeds at a considerable distance [10]. This considerable distance is the dispersal distance or the displacement of the place of seed ingestion to the place of seed defecation. Frugivores such as turtles contribute in population dynamic of different plant species and are important for evolution of plant-frugivore interaction. However, if there is an adverse condition, and seeds are unfavorable for germination, this turtle may contribute effectively by increasing the seed rain, a vital process to maintaining the soil seed bank since seeds are still viable [9].

4. CONCLUSION

Cuora amboinensis has significant roles in an ecosystem. It contributes to plant seed germination and dispersal. The study revealed that passage of seeds through *C. amboinensis* gut enhances germination rate and percent germination for *Lycopersicon esculentum, Carica papaya, Psiudium guajava* and *Muntingia calabura*; while reduces GR and %G of *Passiflora quadrangularis*. Furthermore, *C. amboinensis* enables seeds to be dispersed at a distance, thus allowing plants to be distributed over a large geographical area in the ecosystem.

ETHICAL APPROVAL

In this study, the turtles were handled following protocols of the ethical treatment of experimental animals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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