**Experimental evaluation of the viability in the *Juniperus deppeana* forest seed dispersal by endozoochory and diploendozoochory after wild zoo mammals’ ingestion.**

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***Abstract***

Carnivores participate in forest disturbance recovery by dispersing the seeds that pass through their digestive systems. The dispersal, survival, and colonization of forest seeds by endozoochory and diploendozoochory is a complex process that depends on multiple factors associated with the characteristics of the plants, environmental conditions, and other members of each forest ecosystem. The objective of this study was to evaluate the capacity of mammals for Juniperus deppeana seed dispersal with an experimental evaluation of endozoochory and diploendozoochory, through indices of recovery, viability, changes in testas, and retention of seeds in the digestive tract. *Juniperus deppeana* fruits were collected in the Sierra Fría Natural Protected Area, Aguascalientes, Mexico, and were administered in the diet of gray fox (*Urocyon cinereoargenteus*), coati (*Nasua narica*) and domestic rabbits (*Oryctolagus cuniculus*) these three mammals represented the endozoochory. For the diploendozoochory, seeds excreted by rabbits were incorporated into the diets of bobcat (*Lynx rufus*) and cougar (*Puma concolor*) in a local zoo. The seeds present in the scats were collected, and recovery rates and retention times were estimated; viability was estimated by X-ray optical densitometry, and testa thicknesses and surfaces were checked by scanning electron microscopy. The results showed a recovery of seeds greater than 70% in all the animals; the retention time was < 24 h in the endozoochory, but the time was longer (24-96 h) in the diploendozoochory (*P* < 0.05). Seed viability ( ± *SD*) was decreased in rabbits (74.0 ± 11.5 %) compared to fruits obtained directly from the canopy (89.7 ± 2.0 %), while gray fox, coati, bobcat and puma did not affect viability (*P* < 0.05). An increase in the thickness of the testas was also observed in seeds excreted from all mammals (*P* < 0.05). Through evaluation, our results suggest that mammalian endozoochory and diploendozoochory contribute to dispersal of *J. deppeana* by maintaining viable seeds with adaptive characteristics in the testa to promote resilience and forest restoration. In particular, felines (predators) can provide an ecosystem service through scarification and seed dispersal.

**Keywords:** Bobcat; carnivores; coati; cougar; gray fox.

**INTRODUCTION**

The endozoochory is the system by which animals consume the fruits produced by the plant. The seeds contained in the fruits pass through the digestive tract to later be defecated or regurgitated and dispersed (Cypher & Cypher, 1999; Kleyheeg & van Leeuwen, 2015). For this reason, why frugivorous animals are essential dispersers of plants. (Montiel, 2000). However, most of the studies that have evaluated dispersal by endozoochory have focused on small flying vertebrates, such as birds or bats (Aziz *et al*., 2021; Silva *et al*., 2021).

Mammals consume large numbers of fruits and keep the seeds in the digestive tract for relatively long periods. In the case of large species of mammals with large range of movement, allow the seeds to be dispersed several kilometers from the parent plant (Cypher & Cypher; Cousens *et al*., 2010). These plant-disperser interactions are essential for the dynamics of plant communities (Levin *et al*., 2003; Levine & Murrell, 2003), especially in settings where environmental disturbance and changes have occurred (Lundberg & Moberg, 2003; Montoya *et al*., 2008). In this context, terrestrial mammals like rabbits (Malo & Suarez 1995; Lezama-Delgado *et al*., 2016), foxes (Escribano-Ávila *et al*., 2014), and even largest herbivores (Campos-Arceiz & Blake, 2011), act as dispersers by endozoochory of different fruit species.

The passage of the seeds through the animal's digestive system is a critical phase of endozoochory; complex interactions are established among the characteristics of the plants. In addition, the frugivores mammals, and the environment, making it challenging to understand the dispersal phenomenon, seed colonization, and forest restoration (Cosyns *et al*., 2005). Therefore, seed feeding experiments are essential to determine which plant or mammal traits critically influence seed survival after intestinal passage and thus to assess colonization and dispersal capabilities. However, seed feeding experiments have presented contrasting results, with greater germination success in some cases (Mancilla-Leyton *et al*., 2011; Grande *et al*., 2013) and less success in others (D'hondt *et al*., 2011; Grande *et al*., 2013).

The potential for endozoochorous dispersal in many plant species has been evaluated using seed feeding experiments with herbivores, such as domestic rabbits (Cosyns *et al*., 2005) or germination tests of collected scat samples. However, many processes that influence germination success (density-dependent germination, alternate environmental conditions) are difficult to control under field conditions (Milotić & Hoffmann, 2016). Existing experiments focus on either the study of a single animal species or target domestic ruminant species, such as sheep and cattle (Cosyns *et al*., 2005).

Endozoochory evaluations have also been designed using wild animals under controlled conditions to test their role in the ecosystem. For example, Murray *et al*. (1994) studied the effect of a soluble chemical substance on the fruits of *Witheringia solanacea* by passing them through the digestive tract of a bird (*Myadestes melanops*), Godínez-Álvarez *et al*. (2002) examined the effect of intestinal passage on the proportion of germinated seeds of the species *Neobuxbaumia tetetzo* dispersed by bats and birds. A study by Graae (2004) evaluated the dispersal of seeds by the arctic fox (*Vulpes lagopus*) through a feeding experiment with subsequent analysis of the scats.

Diploendozoochory is a complex process involving the participation of two or more dispersing agents in sequence (prey and predator), where the involvement of a carnivore in the second phase of the process can influence the plant in three ways: the transport of the seeds, altering their viability and changing the abundance of dispersed seeds (Hämäläinen *et al*., 2017). Derived from the above, existing studies have been focused only on revealing the species involved and the number of seeds dispersed. Investigations in this area, one carried out by Sarasola *et al*. (2016), involved the cougar (*Puma concolor*), which, over a long distance, dispersed large numbers of seeds of herbaceous species initially consumed by its prey, the dove (*Zenaida auriculata*).

Recently, Rubalcava-Castillo *et al*. (2021) documented diploendozoochory in *Juniperus deppeana* seeds dispersed by bobcats (*Lynx rufus*) through their prey, the rabbits (*Sylvilagus sp*.). The juniper or táscate (*Juniperus deppeana*) had a fruit adapted to dispersal by endozoochory and diploendozoochory (Rubalcava-Castillo *et al*., 2020), a pioneer species with a wide distribution that colonizes post-disturbance areas and is vital in the recovery of the forests of the Sierra Fría Natural Protected Area (SF-NPA), Aguascalientes, Mexico (Díaz-Núñez *et al*., 2016). One of the factors associated with the wide distribution of the juniper is its dispersal of seeds through terrestrial mammals (Rubalcava-Castillo *et al*., 2021).

Studies of diploendozoochory have shown that strictly carnivorous predators, such as felines, could have extensive ecological functions that have been overlooked. However, research related to the understanding of diploendozoochory in cats is scarce, and no information can explain the efficiency of diploendozoochory and endozoochory through a feeding experiment. Thus, seed feeding experiments are essential to determine the adaptive characteristics and the time that seeds remain in the digestive tracts of dispersers. The previous variables critically influence the survival of the seeds after the intestinal passage; by which, through its evaluation, it is possible to determine the dispersal capacities of the different plant species. The objective of this work consists of evaluating the capacity of *Juniperus deppeana* seeds to be dispersed by mammals under an endozoochory and diploendozoochory model, through indices of recovery, viability, changes in testas, and retention of seeds in the digestive tract. Specifically, we wish to demonstrate whether there is a difference in the influence of endozoochory by frugivores and diploendozoochory by hypercarnivores on seed dispersal efficiency, seed retention, and seed viability. In addition, to know if the thickness of the testa of dispersed seeds decreases and influences seed survival after intestinal transit. This experimental evaluation is focused on determining the beneficial effects and deficits suffered by the seed when dispersed by one or another dispersal system by examining the effects on different animal species. This system would make it possible to determine the dispersal capabilities of this plant species by means of mammal zoochory.

**MATERIAL AND METHODS**

The experimental evaluation consisted of two stages: I) Adaptation of mammals to the ingestion of Juniperus deppeana seeds and post adaptation II) Modeling of experimental evaluation of endozoochory and diploendozoochory. Prior to the two stages, the collection of seeded fruits of the plant component was carried out, the *J. deppeana* shrub that produces globules that generally enclose five seeds inside; the fruits are consumed and dispersed by birds and frugivorous mammals (Livingston, 1972; Schupp et al., 1997). The animal component was also selected according to the dispersal system: gray fox, coati (omnivores) and rabbit (herbivore/feline prey) for endozoochory and bobcat and puma (predators) for diploendozoochory; it is documented that all selected mammals disperse seeds by one system or the other. At the beginning of stage I, seeds were incorporated to the usual diet of each mammal; this stage lasted 10 days with the objective of adapting the animals to the ingestion of the seed. Once the animals were adapted, stage II began to start the modeling, in which, again, the animals were offered the seeds in their diets for a period of 21 days. During the second stage, the retention time in the tracts was estimated and the seeds were collected from the scats. Subsequently, the scats seeds and those collected from the canopy (control) were subjected to X-ray and SEM tests.

***Control group***

A control group was established to test the effects of endozoochory and diploendozoochory in seeds from mammals in evaluation versus seeds taken directly from the tree. During the spring of 2019, according to the calendar of the northern hemisphere meteorological stations, ripe fruits with seeds were collected from twelve random individual *J. deppeana* trees of basal diameter >5 cm and height >2 m. The trees selected according to these criteria corresponded to adult plants with a high probability of bearing ripe fruits (Rubalcava-Castillo et al., 2020). The fruits were collected when they presented a reddish-brown color in temperate forests of the SF-NPA. Fruit collection was carried out in an area of 527 ha, within a temperate forest with a temperate subhumid climate and presents summer rainfall (Rzedowski, 1978) with an average annual precipitation of 650 mm (SEDESO, 1995).

***Mammal selection***

The selection of mammals was based on animal species (the gray fox, coati and bobcat) where the dispersal of *J. deppeana* seeds by endozoochory and diploendozoochory had been verified in the forests of the SF-NPA (Rubalcava-Castillo et al., 2021). Furthermore, it has previously been verified that rabbits can disperse *Juniperus* seeds by endozoochory in forests (Lezama-Delgado *et al*., 2016), therefore, 17 domestic rabbits (*Oryctolagus cuniculus*; California breed, young adults, no detectable morphological or physiological alterations, five males and 13 females were chosen) were used to represent the primary disperser (prey) in the diploendozoochory process. The rabbits were kept in cages 1.60 m wide and 50 cm high; in the morning they were fed first with the fruits and later with their usual diet consisting of a special mixture of alfalfa and standard commercial food.

The mammals were selected from species in captivity at the Center for Environmental, Cultural and Recreational Education of the Rodolfo Landeros Park in Aguascalientes City, Mexico. The species included were female specimens of the gray fox (*Urocyon cinereoargenteus*), coati (*Nasua narica*), bobcat, and cougar (one specimen per species). In the case of felines, it has been previously proven that the rabbit forms part of the diet of the bobcat (Rubalcava-Castillo *et al*., 2020) and the cougar (de la Torre & de la Riva, 2009; Monroy-Vilchis *et al*., 2009) in the region.

The animals were kept in zoo accommodations specially designed to meet the standards of space and habitability, where they were fed their usual diet and incorporated seeds. The gray fox diet was made with fruits (papaya and melon) and chicken; the coati diet was made with fruits (banana, papaya, watermelon, and melon) and chicken. The bobcat diet contained only chicken, and the cougar diet included veal and chicken.

***Adaptation of mammals to seed intake***

As part of the experimental evaluation, all mammals, including rabbits, underwent a 10-day adaptation period for the ingestion of forest seeds. In the first part (only rabbits), 3 whole fruits (equivalent to 15 seeds) were offered in the morning to each of the rabbits once a day to familiarize them with the taste, appearance, smell, and intake of the fruits. The rabbits were monitored to observe behaviors and effects from the offered fruits.

In the second part, at the same time as the rabbits, the gray fox, coati, and felines underwent an adaptation period of eating the seeds. The seeds were placed within the usual diet of mammals. Small pieces of the fruits were broken for gray foxes and coatis, and pieces of chicken were provided for felines. Portions of fruit/chicken with seeds were swallowed in a single bite by mammals to guarantee the intake of all the seeds and avoid choking problems. Five seeds were placed on each small piece of fruit and chicken; three pieces per day were offered to each mammal, and the animals were monitored to ensure they swallowed the food completely to ensure the total ration.

***Experimental evaluation of endozoochory and diploendozoochory***

The evaluation consisted of two main parts: (1) endozoochory and (2) diploendozoochory. For endozoochory, the seeds were offered to the gray foxes, coatis, and rabbits. Rabbits, in addition to carrying out the endozoochory of *Juniperus* (Lezama-Delgado *et al*., 2016), also represent the primary disperser and prey of felines in diploendozoochory. Therefore, diploendozoochory was divided into two stages: rabbits and felines. Part of the seeds dispersed by rabbits were used to analyze their endozoochory, and the other part was offered to felines to complete the transit of the seeds through two digestive tracts (prey and predator).

After the adaptation period, the same procedure was repeated for the ingestion of seeds in all mammals for 21 days. When the seeds were offered, the exact time of ingestion was recorded; after ingestion, inspections were carried out in the rabbit cages and habitats of other mammals at 1-hour intervals to collect and sweep the scats present. All scats were reliably collected from each habitat of each animal species over the entire plausible retention period, due to an exhaustive search of the entire area of the habitats, which had bare soil making visible all the scats deposited in that period of time and to make sure that the proportion of recovered seeds is an accurate reflection of the seeds lost to digestion rather than to the habitat.

Upon finding scats with seeds, the time of discovery was recorded to estimate the retention period in the mammalian tract, and the scats were placed in paper sacks. Until verifying the discovery of seeds in scats for each species, the corresponding ration of seeds was offered to each one the following day. The scats were washed in a sieve system to retain the seeds, which were dried and counted to estimate the percentage of recovered seeds.

For the second stage with felines, the seeds from the pellets were incorporated into the felines’ usual diet. The exact time of ingestion and the time of excretion were recorded. In this way, it was possible to complete the diploendozoochory system by obtaining in the scats of the felines the seeds that had initially passed through the digestive tract of the rabbits and later through the felines. The seeds found in the scats of all mammals were placed in sacks to be used later in viability tests and SEM analysis.

***Viability test***

The viability test used the control seeds and those from the scats by means of optical densitometry analysis using X-ray equipment (Faxitron X-Ray Corporation, Texas, USA, at 10 s and 26 kv intensity), according to the technique proposed by De La Garza and Nepamuceno (1986). The technique consists of observing the radiograph and distinguishing viable seeds with intact testas and embryos from nonviable ones by the presence of underdeveloped or incomplete embryos and empty seeds or lack of embryos. Seeds were placed on sheets of acetate paper to determine the percentage of viable and nonviable seeds based on the density of the radiographs. A densitometric analysis was made in each individual seed of the control and those dispersed by each mammal, based on the technique proposed by Rubalcava-Castillo *et al*. (2021).

***Wear on the thickness of the testas***

The thickness of the seed testas was analyzed, and the measurements of the control group were compared with those from the scats to determine if wear and other changes in the surfaces of the testas occur in the same way or whether there are differences in endozoochory and diploendozoochory. Whole seeds were used and cut in a sagittal shape; they were covered with yellow gold (4 min) in a Denton Vacuum apparatus (JFC-1100®, JEOL LTD, Tokyo, Japan). Once prepared, the seeds were placed inside the SEM chamber (JSM-35C®, JEOL LTD, Tokyo, Japan) (Dykstra & Reuss, 2003).

To analyze the wear of the seed testas produced by the passage through the digestive tract of mammals, the technique proposed by Rubalcava-Castillo *et al*. (2021) was used.

The technique consists of observing the seed inside the chamber to take thickness measurements in three parts of the testa with a 40× magnification: (1) the micropyle part; (2) the central part; and (3) the opposite to the micropyle, where three measurements were taken for each part. In addition, the qualitative characteristics of the surface and interior of the testas were recorded, including the loss of superficial vegetal layers and the presence of holes and cracks in the external and internal parts of the testas.

***Statistical analysis***

The average percentages of viable seeds recovered from the scats of the entire set of species were compared between the two dispersal systems: endozoochory and diploendozoochory, using the t-Student test for independent observations, in addition, an analysis of variance (ANOVA) and Tukey's honest significant difference (Tukey's HSD) were performed to compare the averages of recovered seeds, retention time and testa thickness between each species of mammal; both tests, at a confidence level of 95%, where the means a-b with a different literal in each variable presented statistical differences. The analyzes were performed using statistical software (Statgraphics, V. 16.1, 2012) with the average values ( ± *SD*) expressed in the response variables as: % of seeds recovered / % viability / thickness of testa (μm ± *SD*) and the explanatory variables: dispersal systems (endozoochory and diploendozoochory) and each species of mammal (gray fox, coati, rabbits, bobcat and cougar). Additionally, for the averages of the percentages of viability and thickness by SEM, a Dunnett`s test was carried out at 95% confidence to determine statistically significant differences between the averages of the percentages of viability and thickness of the seeds from each mammal with respect to those of the canopy (control).

***Ethics regulations for the use of animals in research***

The management of all the animal species used in the evaluation was carried out in compliance with the provisions established in the Ethics Regulations for the Use of Animals in Teaching and Research at the Autonomous University of Aguascalientes (CEADI-UAA) Code: DI-PL -NO-37, since the behavior and habitual handling of the animals was not altered.

**RESULTS**

***Experimental evaluation of endozoochory and diploendozoochory***

The average seed recovery was statistically different between the two dispersal systems (*t* = 6.31, g. l. = 85, *P* < 0.01). In the diploendozoochory, almost 100 % of the seeds that were offered in the scats of bobcats and cougar were recovered (94.7 ± 9.0 %), as opposed to conventional endozoochory (66.7 ± 33.1 %) (Figure 1a). Regarding retention times, there were statistical differences between endozoochory and diploendozoochory (*t* = 3.64, g. l. = 17, *P* < 0.01), because the latter presented a longer average retention time of seeds in the feline digestive tract (44.6 ± 23.4 h) in contrast to endozoochory, where the times were reduced by half (20.92 ± 6.21 h) (Figure 1b).

Significant differences in the average recovery between mammals (*F*4, 84 = 13.19, *P* < 0.01) were found. The Tukey`s test (*P* < 0.05), showed that only in the rabbits there was a significant difference compared to the rest of the animals, resulting in a lower percentage of recovery (28.7 ± 16.3 %). Most of the ingested seeds were destroyed by chewing, and mostly only seed remains were found at the time of reviewing the pellets. The remaining dispersers presented recovery percentages without significant differences, regardless of the dispersal system, including the gray fox (76.2 ± 28.3 %) in endozoochory and the bobcat (92.8 ± 11.8 %) in diploendozoochory (Figure 1c).

Seed retention times among mammals showed significant differences (*F*4, 84 = 30.53, *P* < 0.01). The bobcat (30.4 ± 11.1 h) and cougar (58.7 ± 24.3 h) that carry out diploendozoochory showed longer retention times than the gray foxes (21.7 ± 9.2 h), coatis (18.8 ± 1.46 h), and rabbits (24 ± 0.01 h) that perform endozoochory (Figure 1d).

***Viability***

No significant differences were found (*P* > 0.05) according to Dunnett’s paired test when comparing the averages viability of the control against the treatments. Although the seeds of the dispersers did not present significantly higher percentages than those of the canopy (87.0 ± 5.0 %), the animals do not affect viability either, except for the seeds from the rabbits (74.0 ± 11.5 %), which percentage was the lowest throughout the analysis (Table 1).

***Wear on the thickness of the testas***

Statistically significant differences (*P* < 0.05) were observed according to Dunnett’s test between the average thickness in the testa of the seeds from mammals and the control, particularly, in the coati and bobcat as compared to the seeds of the canopy; the seeds dispersed by these two mammals presented greater thickness in the testas. In addition, the ANOVA test indicated that there are significant differences between the averages of each treatment (*F*5,81 = 5.46, *P* < 0.01), verifying that the effect of wear that occurs in the seed coat is different in each animal. Seeds testas from all mammals, except those excreted by rabbits (729 ± 98.0 μm), were on average thicker than those of the canopy (731 ± 238 μm), particularly in coati (1106 ± 442 μm) and bobcat seeds (1142 ± 355 μm) (Figure 2).

Dunnett’s test showed that the seeds dispersed by the coati (1106 ± 442 μm) and the bobcat (1317 ± 238 μm) with 24 h of retention had significant differences compared to those of the canopy. Also, those seeds with the longest retention time in the cougars at 72 h (1002.0 ± 166.7 μm) and 96 h (936.8 ± 117.1 μm) showed significant increase in the thickness of the testas compared to the control (Table 2).

Additionally, qualitative characteristics in the testas after passing through the digestive tract of mammals were recorded. It was observed that the seed of the canopy presented an external vegetal layer that protects it (Figure 3a); this vegetal layer is removed when it passes through the digestive tract of mammals as in seed dispersed by rabbits at 24 h (Figure 3b). Also, the wear of a second vegetal layer was observed, as in the seed dispersed by the gray fox at 24 h (Figure 3c), and it was even possible to appreciate the formation of holes in the testa of the seed from the coati at 24 h (Figure 3d).

In the diploendozoochory, it was observed in the testa from the bobcat at 24 h that both the outer layer and the superficial layer were removed (Figure 4a), while there were cracks in the inner part that could possibly allow the flow of water into the interior (Figure 4b). In the cougar, it was also possible to appreciate the removal of the external plant layer and the superficial layer of the testa at 48 h (Figure 4c). At 72 h, the change in the surface of the testa was more noticeable (removal of the testa surface), and because it had a longer retention time, a certain permeability began to be observed throughout the width of the testa (Figure 4d).

**DISCUSSION**

The evaluation carried out in the present study demonstrated the participation of local mammals: fox and coati (endozoochory) and bobcat and cougar (diploendozoochory) as important parts in the dispersal of *J. deppeana* seeds. This potential for seed dissemination was evidenced by a high percentage of recovered and viable seeds. The retention time was longer than previously observed for birds and fruit bats, suggesting that there is potential for seed dispersal over a great distance; therefore, our results suggest that mammals that carry out endozoochory and diploendozoochory can contribute to mitigating disturbances in forest areas.

Studies based on a seed dispersal evaluation allow us to generate an increasingly detailed knowledge of the endozoochorous dispersal system. However, most of the evaluations have been done on birds and herbivores because of their availability and ease of handling. In the case of birds, it has been shown that the retention time in the digestive tract is very fast, with the dispersion of seeds in less than an hour (Murray *et al*., 1994; Cosyns *et al*., 2005; Pérez-Villafaña & Valiente-Benuet, 2009).

Seed dispersal by endozoochory in carnivorous mammals has been the object of study in the past two decades, demonstrating its importance in the ecosystem (Zarco-Mendoza *et al*., 2018; Pedrosa *et al*., 2019). Because most of the research has focused on medium-sized mammals, such as the fox and the marten (Graae *et al*., 2004; Tsuji *et al*., 2020), we studied 3 mammals that carry out the endozoochory: gray fox, coati and rabbit. In addition, felines were incorporated into the evaluation to demonstrate the participation of these large mammals in seed dispersal through the diploendozoochory process (Tsuji *et al*., 2011; Sarasola *et al*., 2016; Rubalcava-Castillo *et al*., 2020).

Our results showed that the recovery of the seeds in the frugivorous gray fox and coati mammals was relatively high with more than 70 % recovery. Our results support the results in previous models with these types of animals, including Graae *et al*. (2004), who obtained a recovery of 75 % in most of the plant species dispersed by the arctic fox (*V. lagopus*), and Varela and Bucher (2006), who obtained more than 84 % of seeds recovered from most of the plant species dispersed by South American foxes (*Lycalopex gymnocercus* and *Cerdocyon thous*). Thus, it is suggested that the gray fox and the coati are efficient species in the dispersal because most of the seeds they ate were dispersed, and the seeds not expelled were possibly destroyed by mechanical aspects of chewing or could not be found in the soil of the habitats (the intake of all the seeds that were offered within the diet of the animals was ensured).

When starting the diploendozoochory process in the primary disperser or predator prey, rabbits, we found a considerable decrease in the recovery of seeds contained in the pellets because of destruction by intense chewing (Cosyns *et al*., 2005) of most of the seeds at the time of offering the fruits for ingestion. In this way, we found a decrease in the number of seeds eliminated through the primary disperser; therefore, the number of seeds that the secondary disperser could disseminate was limited by the primary disperser. That is, the dispersal of seeds by predators will depend on the number of seeds that remain alive and integrated in the digestive tracts of the prey. The second part of diploendozoochory involving bobcat and cougar predators showed good results by recovering more than 90 % of the seeds, which makes strictly carnivorous mammals efficient seed dispersers. Although there was a significant reduction of seeds during their passage in the first disperser, it can be ensured that at least 90% of the seeds that remain in the prey will be disseminated by the secondary disperser. Consequently, secondary dispersal by carnivores could have important implications for the functioning of disturbed ecosystems (Hämäläinen *et al*., 2017) because they effectively disperse most of the seeds contained in the prey.

The frugivorous mammals, such as the gray fox and the coati, had short retention times to disperse the seeds in a maximum time of 21 h, while in other studies with medium-sized mammals, retention times of no more than 10 h were obtained (Varela & Bucher, 2006; Zhou *et al*., 2008; Tsuji *et al*., 2011). In turn, Graae *et al*. (2004) determined that the arctic fox has an average seed retention of 44-48 h, a much longer time than our results suggest. In the same way, the seed retention time in rabbits was not greater than 24 h, similar from that indicated by Cosyns *et al*. (2005), where they observed a retention time of 12 h. It is possible that the variation in retention times in our study and in the previous studies is the result of the species of seed offered and the elements in the animals’ diets. However, it is essential to know the retention time of the seeds in the intestines of the animals because it is an important stage in dispersal and can influence the distance the seeds can travel through the landscape (Tsuji *et al*., 2011). Diploendozoochory by felines had longer retention times than the endozoochory, with averages between 30 h and almost 60 h. Although those are very long times for seed retention, Pedrosa *et al*. (2019) also reported long average times of 70 h in seeds dispersed by wild boar (*Sus scrofa*). Particularly, the cougar presented very long retention times that were up to 96 h, so they can promote the dispersal of seeds at considerable distances from the mother plant (Varela & Bucher, 2006; Pedrosa *et al*., 2019).

Although the results were not statistically significant, seeds retained in the tracts of most mammals managed to maintain a viability equal to or greater than those of the canopy; therefore, the passage of the seeds in the tracts did not affect viability, except in seeds of the rabbits because of damage caused by intense chewing. In their study of arctic foxes, Graae *et al*. (2004) suggested that the seeds must be defecated within 12 h to remain viable; however, our results showed that gray fox and coati with a retention greater than 18 h managed to maintain viability in the seeds. This is similar to Rubalcava-Castillo *et al*. (2020), who reported that the gray fox and ringtail (*Bassariscus astutus*‎, which is the same family as the coati, *Procyonidae*) also managed to maintain viability in seeds of the species *J. deppeana*, suggesting that this plant species is adapted to dispersal by endozoochory. The effect of diploendozoochory on the viability of seeds dispersed by bobcat and cougar showed the best percentages of viability, even better percentages than in endozoochory, a result similar to that reported by Rubalcava-Castillo *et al*. (2021), who suggested that the seeds remain viable despite their passage through the digestive tract of the bobcat. This further confirms the importance of these carnivores in the process of regeneration of ecosystem since the seeds remain viable during their passage in the digestive tracts to finally be dispersed. However, the diploendozoochory process can have different effects on viability depending on the animal and plant species involved, as indicated by Nogales *et al*. (2015), where dispersal by the lizard (*Gallotia*) and its predator, the wildcat (*Felis*), showed a decrease in the viability in the seeds of two plant species (*Plocama pendula* and *Rubia fruticose*).

The testas of the seed are external barriers that protect the embryos from the external environment; therefore, the thickness of the testa is essential in surviving the passage through the intestine in animals and breaking dormancy (Jaganathan *et al*., 2016). However, a clear relationship has not been found between the physical characteristics of the seeds, such as thickness, and their retention times during the dispersal process (Willson 1989; Graae *et al*., 2004; Varela & Bucher 2006). Our results through SEM showed that the thickness of the testas in *J. deppeana* seeds dispersed by most mammals did not cause wear, and there was no decrease in the thickness of the testas during passage through the digestive tracts of mammals. This differs from what has been established in several studies in which the authors concluded that the exposure of the seeds to this digestive effect can cause changes in the internal and external structure of the testa, generally decreasing its thickness (Traveset *et al*., 2001; Nogales *et al*., 2007; Nogales *et al*., 2015). Our results suggest the opposite of the previous observations, registering an increase in the thickness of the seed testas from all mammals, except the rabbits. In the coati with endozoochory, the seed increased until reaching 1105 µm, and in the bobcat with diploendozoochory, the testa increased to 1141 µm. These thickness measurements are much larger than those mentioned by Venier *et al*. (2012), who reported a decrease in the thickness of the seeds of *Acacia aroma* (491 µm) when simulating their passage through cattle. The anomaly in the increase of the thickness of the testas in our model coincides with that recently reported by Rubalcava-Castillo *et al*. (2021), who also recorded an increase in the thickness of the seed testas of *Juniperus sp*. coming from scats of different mammals found in temperate forests, and they agreed that the increase in thickness may be a result of the prolonged time of the seeds in the tracts as previously verified. The seeds, when in contact with liquids in the intestines of animals, could absorb those liquids and cause swelling, such as that reported by Varela and Bucher (2006), who pointed out that the seeds of the *A. aroma* plant species dispersed by foxes showed signs of hydration.

In response to the long periods of seed retention in the endozoochory and diploendozoochory by mammals, the testas presented cracks, holes, and removal of plant layers on the surfaces, which can be a benefit for the seed by acquiring permeability characteristics for the entry of essential elements (water, light, oxygen) for germination (Schaumann & Heinken, 2002; Costea *et al*., 2016).

Also, importantly, the effect of double digestion on seed attrition seen in this experimental evaluation is probably overestimated relative to typical diploendozoochory that occurs in wildlife because, although in our study seeds pass through the entire digestive tract of both herbivores and carnivores, this is unlikely to happen, unless carnivores eventually consume lagomorph faeces that include seeds, which has not been proven so far. However, the experiment was designed in this way to be conducted in an ethical manner to avoid the sacrifice of the rabbits. Despite this, our results show the real effects of endozoochory and diploendozoochory on retention times and seed wear (scarification) with emphasis on predators. Thus, to demonstrate that predators can provide an ecosystem service through scarification and seed dispersal in landscapes.

***Conclusions***

The evaluation carried out in this study highlights the participation of mammals: fox and coati (endozoochory) and bobcat and cougar (diploendozoochory) as an important part in seed dispersal because of the high percentage of dispersed (recovered) seeds and the variable retention times of seeds that directly affect long-distance dispersal. Thus, our results from an evaluation with animals in captivity suggest that these endozoochory and diploendozoochory mammalian species seem to contribute to the dispersal potential by keeping the *Juniperus deppeana* seeds viable and presenting adaptive characteristics in the testa to resist digestive scarification.

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Table 1 Average viability percentages (± *SD*) by X-ray optical densitometry of seeds from the scats of mammals in captivity (gray fox, coati, rabbits, bobcat, and cougar) and from the canopy of the species *Juniperus deppeana*.

|  |  |  |
| --- | --- | --- |
| **Disperser species** | **Seeds (*N*)** | **Viability (%)** |
| Directly taken from the tree (control) | 90 | 87.0 ± 5.0 |
| Gray fox | 90 | 88.2 ± 21.2 |
| Coati | 90 | 90.3 ± 11.6 |
| Rabbit | 90 | 74.0 ± 11.5 |
| Bobcat | 90 | 91.9 ± 4.7 |
| Cougar | 90 | 90.9 ± 16.2 |

\*There were no statistically significant differences according to Dunnett's test (*P* < 0.05).

Table 2 Average SEM measurements of the thickness ( ± *SD*) of the seed testas of *Juniperus deppeana* from each of the mammals (endozoochory and diploendozoochory) and the canopy and the retention time for each animal species.

|  |  |  |  |
| --- | --- | --- | --- |
| **Dispersion type** | **Species** | **Retention time (h)** | **Thickness (µm)** |
| Directly taken from the tree (Control) | *J. deppeana* | 0 | 731 ± 238 |
| Endozoochory | Gray fox | ≤ 24 | 932 ± 199 |
|  | Coati |  | 1101 ± 442\* |
|  | Rabbit |  | 729 ± 98.0 |
| Diploendozoochory | Bobcat | ≤ 24 | 1317 ± 238\* |
|  |  | 38 | 966 ± 376 |
|  | Cougar | ≤ 24 | 806 ± 232 |
|  |  | 48 | 685 ± 123 |
|  |  | 72 | 1002 ± 167\* |
|  |  | 96 | 937 ± 117\* |

\* Statistically significant differences according to Dunnett's test (*P* < 0.05).

Figure 1 (a) Average percentages ( ± *SD*) of táscate (*Juniperus deppeana*) seed recovery and (b) average retention times (h) of táscate seeds in the digestive tracts through the dispersal systems of endozoochory (gray fox, coati, and rabbits) and diploendozoochory (bobcat and cougar). c) Average percentages of seed recovery and (d) mean retention times (h) in the digestive tract by each mammal species. The lines above the bars in the four subfigures indicate the *SD*. a-b Averages with different literals present statistically significant differences to t-Student test (*P* < 0.05) for the dispersion systems: endozoochory and diploendozoochory and Tukey’s HSD test (*P* < 0.05) for the different species of mammals.

Figure 2 Measurement of the average thickness ( ± *SD*) of *Juniperus deppeana* seed testas from mammals dispersers of the endozoochory and diploendozoochory and from the canopy by means of SEM. a-c Averages with different literals present statistically significant differences to the Tukey’s HSD test (*P* < 0.05) or \* for Dunnett’s test (*P* < 0.05).

Figure 3 *J. deppeana* seeds from the canopy (control) and the scats of mammals (endozoochory). (a) Control seed, with the presence of an external fibrous layer that protects the seed. (b) Seed dispersed by rabbits at 24 h, showing the removal of the outer layer. (c) Seed dispersed by gray fox at 24 h, showing the detachment of a second layer on the surface of the testa. (d) Seed dispersed by the coati at 24 h with testa showing holes.

Figure 4 *J. deppeana* seeds from the canopy (control) and scats of mammals (diploendozoochory). (a) Seed dispersed by the bobcat at 24 h, showing the removal of the outer layer. (b) Seed dispersed by the bobcat at 24 h, showing cracks observed inside the testa. (c) Seed dispersed by cougar at 48 h, showing a detachment of the external plant layer and the superficial layer of the testa. (d) Seed dispersed by the cougar at 72 h, showing detachment of the outer and superficial layers of the testa and permeability throughout the width of the testa.

**DATA ACCESSIBILITY STATEMENT**

Data will available from the Dryad Digital Repository

**AUTHORS’CONTRIBUTIONS AND CONFLICT OF INTEREST**

F.A.R.C. led and conducted this experimental evaluation and coordinated all aspects, including design, collection of scats, laboratory work, writing and statistical analysis. A.G.V.F. contributed significantly to writing, graphics monitoring, statistical analysis, corrections and of the experimental evaluation. J.J.L.R. contributed to the methodological design, laboratory work and writing. L.I.I.D. contributed to the revision and theoretical foundations. V.M.M.C. contributed to the laboratory work, revision, and theoretical foundations. A.J.M.J. contributed to the revision and theoretical foundations. J.S.R. acquired the funds and contributed significantly throughout the project with writing and corrections. No authors disclose any conflict of interest.

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