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Behavioral Interactions and Mate Compatibility Influence the Reproductive Success of New England Cottontails (*Sylvilagus transitionalis*) in a Conservation Breeding Program

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Abstract: Wild populations of New England cottontails (NECs, *Sylvilagus transitionalis*) are declining and occupy a small proportion of their historic range. To conserve this species, wild-caught cottontails participate in a reintroduction breeding program. To increase the program's productivity, this study described breeding behavior in pairings with (n = 3 pairings) and without (n = 9 pairings) reproductive success. Females were paired with two males consecutively and behaviors were recorded using 24 h continuous video footage. Activity peaked between 19:00 and 06:00. Copulation was only observed in successful pairings, and the gestation period was 31 days. Successful pairings had a significantly higher rate of follows ($p < 0.001$) and a lower rate of dashes ($p < 0.0001$) compared to unsuccessful pairings. Females had significantly higher rates of charge ($p < 0.0005$), chase ($p < 0.0003$), and dash ($p < 0.0016$) than males. Males had higher rates of follow ($p < 0.005$) than females. Males and females altered their behavior in the presence of different potential mates, suggesting that mate compatibility may influence breeding program productivity. This study significantly expands the understanding of NEC breeding behavior and is applicable to the management of this imperiled species living at low densities in fragmented landscapes.

Keywords: lagomorph reproduction; social interactions; reintroduction

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1. Introduction

New England cottontails (*Sylvilagus transitionalis*, hereafter NECs) are the only native rabbit species in New England, USA, and are an important conservation concern. Rabbits are a staple prey source for many midsize predators such as foxes and birds of prey. Often, the conservation of these predators relies on lagomorph populations [1]. Current NEC populations are considered vulnerable, and wild populations are declining, with an estimated 13,000 individuals remaining [2,3]. Habitat destruction and the fragmentation of early successional forests have resulted in a reduction in the range of NECs to only 14% of their historic range [4]. Due to the current predicament of NECs, the species is at risk for further decline without human intervention. Therefore, Roger Williams Park Zoo (RWPZ), in collaboration with other agencies, created a reintroduction breeding program [5]. This important conservation strategy aims to re-establish or augment wild populations through the release of captive-bred individuals. The effective management of these programs requires an understanding of the species' reproductive biology, but the breeding behavior of NECs has not been described. Addressing the resulting gaps in knowledge has the potential to greatly increase the success of the breeding program.

NECs are a good candidate for a managed conservation breeding program because of their potential to be relatively prolific breeders. Does in the *Sylvilagus* genus are seasonally polyestrous and induced ovulators [6] and reproduce as early as 91 days old [7]. NECs are reported to have an average gestation period of 28 days, and kits are weaned 2 weeks after parturition [5]. NECs can have 3–4 litters per season (March–August), with an average of 3.5 kits per litter [8]. Thus, one female could theoretically produce 10.5–14.0 kits in a breeding season. The RWPZ conservation breeding program has an average of 10 female NECs participating every year, so the expected maximum productivity should be 105–140 kits born per year. Actual productivity is typically much lower [5], suggesting that management changes could improve the productivity of the program.

In other conservation breeding programs, an enhanced knowledge of breeding behavior has increased productivity. Animal managers use this knowledge to inform the number of individuals and the sex ratio placed together for breeding, when to pair individuals, and when breeding is imminent. For example, investigations of the behavior of captive female platypuses (*Ornithorhynchus anatinus*) and Ozark hellbender salamanders (*Cryptobranchus alleganiensis bishopi*) both allowed for better predictions of when breeding would occur, so that management could be adjusted accordingly [9,10]. Behavioral studies can also help determine which individuals are best suited for the breeding program, or specific potential mates. In cheetahs (*Acinonyx jubatus*), individuals who did not reproduce exhibited more fearful behaviors compared to those who were reproductively successful [11]. Behavioral variation among individuals can then inform the selection of more productive pairings, as in giant pandas (*Ailuropoda melanoleuca*) [12]. Understanding changes in individual behavior in the presence of different mates can help animal managers anticipate the success of a given pairing. Therefore, research on reproductive behavior not only provides a guide for proper care but is a valuable tool for increasing the productivity of the breeding program.

Investigating the breeding behavior of NECs would be particularly valuable because of the relatively low success rate for pairings. In 2018, the breeding success for pairings in the breeding program was only 44% [13], but due to a lack of observations, it is impossible to determine if the failed pairings resulted from a lack of copulation or infertile matings. Infertility would require veterinary intervention or removal from the program, while failure to copulate is a behavioral barrier that could be ameliorated by modifying breeding or husbandry practices. Non-productive pairings significantly impair the productivity of the program because each female only has three opportunities to mate in a given breeding season. In the interest of minimizing the handling of potentially pregnant females, pregnancy tests are not performed, because a non-invasive method does not yet exist for this species. Thus, females are removed from the program for at least 45 days after pairing to either give birth or confirm a lack of pregnancy, delaying the introduction of non-pregnant females to a new male. Improving our understanding of this species' behavior is a non-invasive method that could help determine which individuals are likely to breed, identify potential explanations for a lack of success in a given pairing, and improve the overall efficiency of the program.

To the best of our knowledge, there are no formal descriptions of breeding behavior in wild or professionally managed NECs beyond anecdotal observations. Their cryptic nature, both in choice of habitat and crepuscular activity pattern [14], largely precludes direct observations in the wild. However, the breeding behavior of other members of the genus has been studied. Before the taxonomic split between the Appalachian cottontail (*Sylvilagus obscurus*) and *S. transitionalis* [15], Tefft and Champman [16] published their observations of Appalachian cottontail behavior, despite referring to subjects as NECs. This study provided important descriptions of the behavioral repertoire of Appalachian cottontails and found behavioral variation between this species and other cottontails. For instance, Appalachian cottontails did not perform the displays of territorial defense or aggressive behaviors that were seen in the swamp rabbit (*Sylvilagus aquaticus*). Physical contact during reproductive dislodgments was rare in other species of cottontail, but females frequently made physical contact with males during these interactions in *S. obscurus* [16]. Although a detailed ethogram was developed, the small sample size (n = 3) and the nature of the naked-eye observations, conducted under unnatural field lighting for only part of the night, limits the applicability of this study to the ongoing NEC breeding program. To address these gaps, 24 h video observations of a larger sample size under natural lighting conditions are needed.

The lack of knowledge about NECs leaves questions about the mating strategies and reproductive behavior of this species unanswered. Yet, this information is crucial for breeding management and identifying barriers to reproductive success. Therefore, this study aimed to apply continuous and unobtrusive video monitoring of male-female pairings to (1) more completely describe the social behavior of male-female pairs of NECs and (2) to determine if copulation is failing to occur or if females are not carrying to term in non-productive pairings. We hypothesize that individuals will exhibit different behaviors depending on the potential mate in their presence and that males and females will behave differently from one another. Most importantly, we expect significant behavioral differences between successful and unsuccessful pairings.

2. Materials and Methods

2.1. Subjects and Housing

Four male and six female wild-caught NECs participating in the conservation breeding program at Roger Williams Park Zoo in Providence, Rhode Island, USA (41°49′30.8136″ N and 71°25′7.9824″ W) were the subjects of this study. Except for 2 females, the subjects were transferred to RWPZ 3 to 5 months before observations to acclimate to captivity. Females 1 and 2 participated in previous breeding seasons and had been at RWPZ since 2017 and 2014, respectively. Females were tagged in their left ear and males in their right. To ensure visibility and easy identification at night, red reflective tape was placed on the male's ear tag. Due to incorrect tagging, sex could not be determined from the video for pairings with Male 3.

The NECs were kept in laboratory cages until the start of the breeding season, when breeding pairs were placed in one of four enclosed outdoor pens. All pens are 2.13 m by 2.74 m and enclosed with a wood wall surrounded by fencing. All four pens contained hiding structures and two infrared security cameras mounted to opposite walls. These cameras provided continuous visibility of the entire pen. NECs were given a diet consisting of commercial rabbit feed, greens, and vegetation for browsing. Water was available ad libitum. Pens were serviced daily by zoo personnel to remove feces, refresh food and water, and perform any other husbandry tasks.

2.2. NEC Observations

Observations occurred between 21 March 2019 and 4 June 2019. RWPZ determined which individuals were paired together to maximize genetic diversity. Males were assigned to one of the four outdoor pens for the duration of the study. One female was placed in a pen with a male for approximately 48 h. Once this time period concluded, females were immediately moved into a different pen with a different male for 48 h. Each female was paired with two males during the study for a total of 12 pairings (Table S1). After the second pairings, females were removed from the outdoor pens and returned to their lab cages, where they remained until either the anticipated due date had passed after 45 days or after the female gave birth and weaned kits. All females were paired an additional two times throughout the 2019 breeding season, but no video was collected for analysis.

An ethogram of the most common social behaviors was developed based on previous research on Appalachian cottontails [16] and preliminary observations (Table 1, Video S1). The video was analyzed in two stages using BORIS (v. 7.13.8), an event-logging software [17]. In stage 1, 12 observers used continuous behavior sampling [18] to record all occurrences of social interactions. A social interaction was defined as the occurrence of any social behavior in the ethogram. In the second stage of analysis, five observers examined each social interaction to continuously record the specific behavioral events that comprised each interaction.

Table 1. Ethogram used in this study.

Behavior	Definition
Approach	One cottontail moves toward a recipient cottontail to a distance within three body lengths or less.
Dash	One cottontail moves towards a recipient. The trajectory of the approaching individual leads them past the approached individual within a distance of three body lengths or less from the recipient.
Charge	Following an approach, one of the individuals rapidly moves toward the other.
Follow	Results when an individual is approached, moves away, and is followed at slow speed by the approaching individual.
Chase	Results when an individual is approached, moves away rapidly, and is followed at high speed by the approaching individual.
Jump-Circle	After an approach, an individual rapidly exchanges places one or more times with the recipient; this may include low jumping over the other individual or continuous place exchange.
Attempted Mount	An individual climbs onto the back of the other while stationary or while moving; the individual may or may not hold on to the recipient's sides with forelimbs. Pelvic thrusting is absent.
Mount (presumed copulation)	An individual climbs onto the recipient's back while stationary or while moving and holds the recipient's sides with forelimbs; pelvic thrusting occurs.
Ejaculatory Fall-Off	During a mount, the mounting cottontail's hindlegs are thrown forward and the mounter falls backward off the recipient [19].

2.3. Interobserver Reliability

Interobserver agreement tests were performed for observers at both stages of analysis to ensure consistency in data collection. Using 4 h and 15 min of training video containing all behaviors listed in the ethogram except for follow, stage 1 observers were tasked with marking all social interactions. Percent agreement with a reference observer (HP) was calculated (189 total interactions). Stage 1 observers had a 93% or higher percent agreement with the reference observer. Stage 2 observers used the same videos but were required to correctly identify the type of behavior and the sex of the acting NEC. Pairwise comparisons between each observer and the reference observer (HP) were made using the kappa statistic for each behavior separately [20], and percent agreement was calculated for the correct identification of the actor's sex. Stage 2 observers had $k > 0.74$ agreement with the reference observer for each behavior ($k > 0.75$ is considered an "excellent" agreement) and an average of 90% agreement for sex identification. The total number of hours each observer coded and their scores are listed in Table S2.

2.4. Data Analysis

In total, 668 h of video and 12 NEC pairings were analyzed. The frequency of point events, as well as the date, time, and pen in which these events occurred, was recorded. When copulation was observed for a female, the gestation length was determined. The day copulation was first observed was considered day 1 of gestation, and the day of parturition was considered the last day of gestation.

Due to the inability to distinguish between Male 3 and the paired females, behavioral data are presented by reproductive outcome groupings. The reproductive outcome groupings were divided into pairings that produced offspring (successful pairings; SPs) and those that did not (unsuccessful pairings; UPs). Using R [21], descriptive statistics for all behaviors were generated and compared between SPs and UPs. Averages and standard deviations for each behavior were calculated for each grouping per hour and are reported as (mean $\pm$ SD). The analysis of individual behavior only focused on individuals from pairings in which both individuals could be definitively identified (Females 1, 2, and 6 and Males 1 and 4). These individuals were used to assess variations between sexes and potential mates. Because females that were paired with Male 2 were also paired with Male 3, the variation in their behavior cannot be ascertained. Rates for individually initiated behaviors were calculated in a similar manner as described above. In addition, the proportion of behaviors initiated by each sex is reported but only utilizes data from 6 total pairings comprised of Females 1, 2, and 6 and Males 1 and 4.

To determine if the mean hourly rates of each behavior were significantly different between SPs and UPs and between males and females, a Monte Carlo permutation *t*-test was conducted. This non-parametric method is suitable given the small sample size and the inability of the data to fit a known distribution. The null hypothesis posits that there is no significant difference between the two groups. Using the MKinfer package in R, 9999 permutations were performed by randomly reassigning labels to the observed data points [22]. The difference in mean between the two groups was calculated for each permutation. ***p***-values were computed as the proportion of permuted test statistics that were as extreme as, or more extreme than, the observed test statistic. A significance level of $\alpha = 0.05$ was used to determine statistical significance, and *p*-values for each behavior are reported.

3. Results

Three of the 12 pairings produced offspring; 14 kits were born, of which 5 survived to weaning (Table 2). The number of offspring produced per litter (4.67 ± 0.57 kits) and gestation length (31.67 ± 0.57 days) did not vary between females (Table 2). However, offspring survival was not consistent across all three females (1.67 ± 1.53). No pairings had all kits survive to release. Female 3 produced kits with the first male she was paired with, while Females 4 and 6 produced kits with the second male. Copulation was only observed in these three pairings. Two out of three pairings copulated after 24 h, and one pairing copulated both the first and second night. Copulation first occurred between 23:48–03:46 and occurred 494–2236 min after being paired (Table 2). Unlike offspring production, mounting and attempted mounts varied considerably between Male 3 and Male 4. Male 3 copulated with the females less than two times per female (Table 2). While Male 4 only mounted one female, he attempted to mount her 171 times and successfully copulated with the same female 19 times (Table 2). Out of the 22 mounts observed, only 4 concluded with the male rabbit falling off the female. There were three instances of ejaculatory fall-off in pairing M4F6, one in pairing M3F4, and none observed in pairing M3F3.

Table 2. List of copulation, gestation, and offspring data for SPs (n = 3).

Pair ID	Date of First Copulation	Time of First Copulation	Time till First Copulation (min)	Mount	Attempted Mounts	Gestation (Days)	Kits Born	Kits Released
M3F3	24 March 2019	03:46	2236	1	1	32	5	2
M3F4	30 March 2019	03:23	2149	2	0	31	4	0
M4F6	31 March 2019	23:48	494	19	171	32	5	3
		Avg. Sd.				31.67 ±0.57	4.67 ±0.57	1.67 ±1.53

3.1. Behavioral Differences between SPs and UPs

In total, 10,841 social interactions were observed during this study. A social interaction, as defined in this study, required the animals to be within three body lengths of each other. Therefore, all interactions had to be preceded by either an approach or a dash, meaning the occurrence of these behaviors can be used to evaluate the diel pattern of social activity. Social interactions occurred 27.5 times more often between 19:00 to 6:00 (7.578 ± 10.777) than between 8:00 to 16:00 (0.276 ± 1.209) (Figure 1).

All behaviors listed in the ethogram were observed in both pairing types except for attempted mounts and mounts, which were only performed by SPs. The behaviors dash and follow were performed at different rates by SPs and UPs (Figure 2). UPs have significantly fewer follows per hour (0.271 ± 0.543) than SPs (3.314 ± 7.099; $p < 0.001$). SPs had a lower average hourly rate of dashes (1.267 ± 1.872) than UPs (3.781 ± 5.907; $p < 0.00001$). While not significantly different, UPs performed 1.5× more charges on average (0.454 ± 0.751) than SPs (0.307 ± 0.8342).

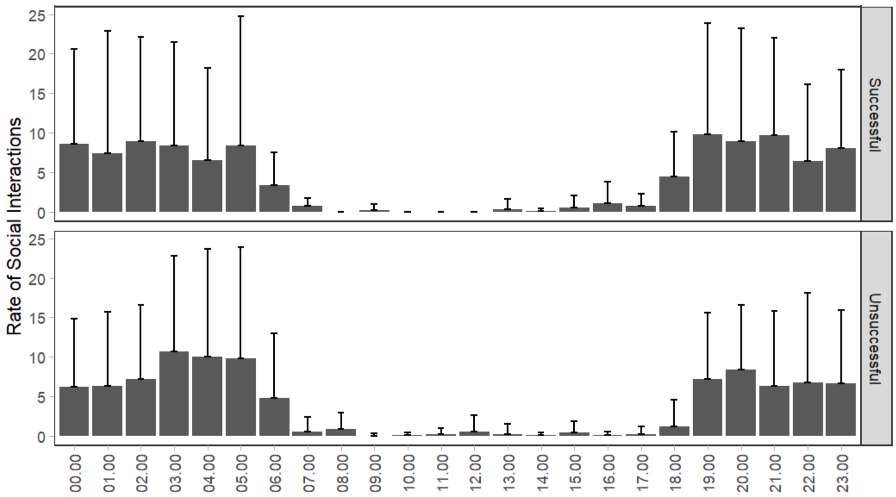

Figure 1. Hourly frequency (mean ± SD) of social interactions for male–female pairings that successfully reproduced (**top** panel) or did not reproduce (**bottom** panel).

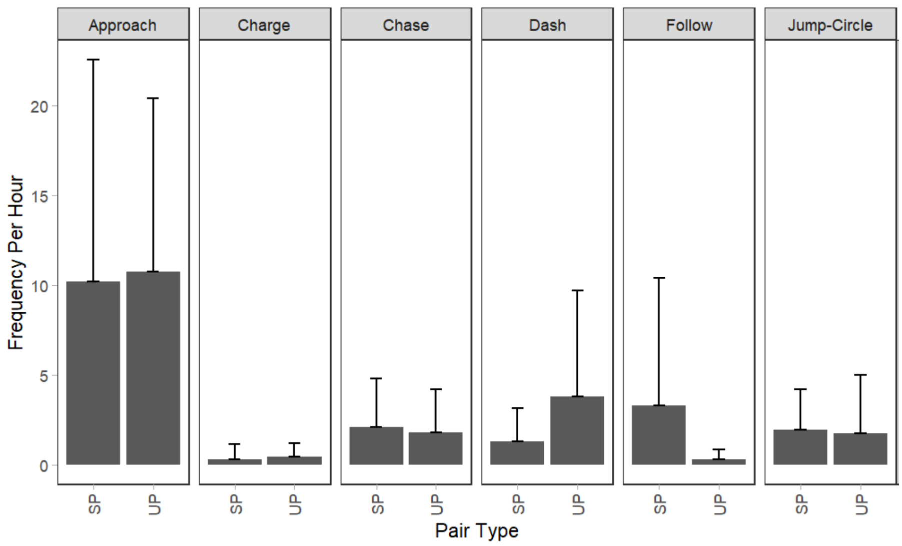

Figure 2. Hourly behavioral frequency between successful (SPs, n = 3) and unsuccessful pairings (UPs, n = 9).

3.2. Variation in Behavioral Frequency by Sex and Individuals

Both sexes performed all behaviors in the ethogram except for mounting and attempted mounts, which were exclusively performed by males. However, charge ($p < 0.0005$), follow ($p < 0.005$), chase ($p < 0.0003$), and dash ($p < 0.0016$) were performed at significantly different rates between the sexes according to the permutation tests. Females initiated a higher proportion of charges (0.686), chases (0.638), and dashes (0.651) while males initiated a higher proportion of follows (0.776) (Figure 3).

In general, individual NECs exhibited different behaviors within different pairings, as represented in Figures 4 and 5. For example, F1 performed dashes 7.5× more frequently with M1 (5.646 ± 9.407 dashes per hour) than with M4 (0.753 ± 1.519 dashes per hour). The two other females (F2 and F6) that were paired with these males also performed more dashes with M1 compared to M4 (Figure 4). Within pair F1M4, an unsuccessful pairing, F1 did not perform any jump-circles, and this pairing had the lowest rate of jump-circles (0.042 ± 0.222). However, that same female (F1) did initiate jump-circles at a rate of 0.655 ± 1.642 per hour with M1. When M4 was paired with F6, there was an average jump-circle rate of 3.261 ± 2.709 with both individuals initiating the behavior. F6 performed less jump-circles with M1 (0.291 ± 0.651) compared to M4 (2.310 ± 3.374).

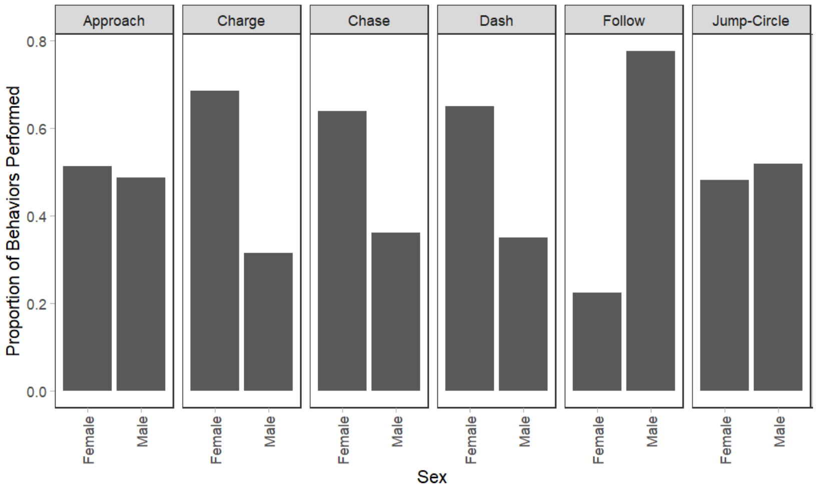

Figure 3. Proportion of occurrence of each behavior initiated by each sex.

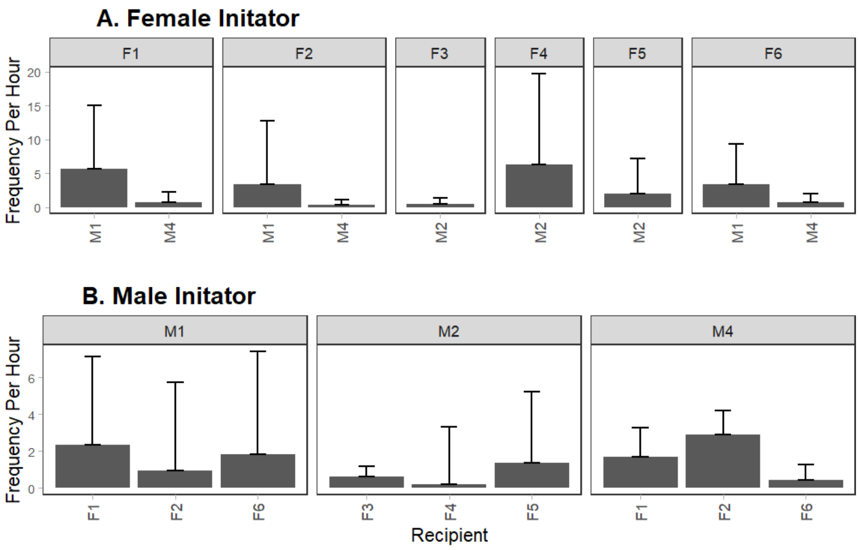

Figure 4. Average dash frequency per hour initiated by females (**A**) or males (**B**). The recipient is represented on the *X*-axis.

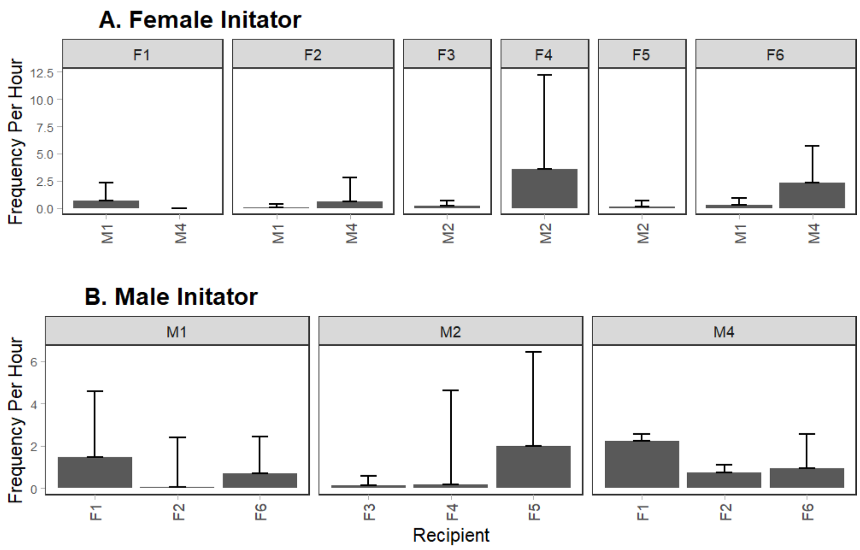

Figure 5. Average jump-circle frequency per hour initiated by females (**A**) or males (**B**). The recipient is represented on the *X*-axis.

4. Discussion

This study was the first to quantify breeding behavior in the imperiled New England cottontail. Using non-invasive observation methods, social behavior was found to peak during crepuscular hours but was performed throughout the night. Copulatory behavior was only observed at night. Both females and males initiated social interactions, and the relative frequency of initiating social interactions varies by pairing. NECs in successful pairings had differing behavioral rates from individuals in unsuccessful pairings. Most importantly, this behavioral study has provided insights into the low pregnancy rates in the breeding program, despite our small sample size. Pairings that did not produce kits did not copulate, but all pairings that copulated did produce kits, reducing concerns of infertility. The findings of the study will aid the further development of the conservation breeding program in zoos, which is required to ensure that the number of offspring released will sustain wild populations.

4.1. Study Design and Diel Activity

While the study design enables a detailed analysis of NEC behavior, the effects of managed care on reproductive success remain unknown. However, five of the six females in this study reproduced at least once during the 2019 breeding season [13], and the conservation breeding program has been broadly successful [5]. Failure to breed captive European rabbits (*O. cuniculus*) in a laboratory setting has been observed and necessitated the use of artificial insemination (AI) [23]. While AI has been used in other *Sylvilagus* species, its use in NECs is untested. This procedure also requires the unnecessary handling of NECs, and additional research is required to implement this procedure [24]. The unnatural setting could impact maternal care or increase the potential for fetal reabsorption. However, litter sizes in this study were typical of the species [7], suggesting that fetal reabsorption was minimal or absent, and the aborting of pregnancies did not occur, as copulation was only observed in successful pairings. Thus, management in the breeding program did not appear to negatively impact gestation, but steps may be taken to encourage mating and maternal care behaviors.

The exact frequency of social interactions in the wild is unknown, thus the program design of pairing a male and female together for 48 h may artificially inflate the number of interactions and activities observed. Similarly, sequentially pairing females with two males may impact behavior, as presumably wild females could interact with more than one male concurrently. Analysis of NEC activity over a 24 h period revealed increased activity throughout the night. Field studies using wildlife cameras showed cottontail (*Sylvilagus* spp.) active during 47% of the day but were unable to distinguish between eastern cottontails (*Sylvilagus floridanus*) and NECs [25]. If the hours of low activity in this study are considered inactive times, then the experimental rabbits were slightly more active than wild cottontails (58% of the time). Perhaps NECs are active for longer proportions of the day compared to ECs, or our study design caused the higher activity level, or simply detected activity levels with greater accuracy through continuous observations. These ex situ observations complement wild observations of NECs by continuously recording interactions, which is nearly impossible to accomplish with in situ studies.

Lagomorphs are known to exhibit seasonal changes in their activity levels. For instance, both swamp rabbits and eastern cottontails delay the onset of activity in early spring to coincide with the later sunset times [26]. Diel activity studies have shown wild lagomorphs exhibiting significantly less activity in exurban areas throughout summer and winter, with more crepuscular behavior in exurban areas during the summer [14]. However, in the present study, NEC activity was present throughout the night, with pair activity peaking a few hours before sunrise (which occurred between 6:20 and 6:45) and after sunset (which occurred between 19:00 and 19:15; Figure 1). These results align more closely with observations of lagomorphs displaying more nocturnal patterns in rural areas [14]. The nocturnal activity may indicate seasonal plasticity within the species, which is not uncommon in lagomorphs. For example, European hares (*O. cuniculus*) increase nocturnal activity from December to March [27], and studies on wild cottontails in New England found significantly higher rates of activity during the winter and fall [25]. The activity levels observed in our study may reflect the increased activity associated with late winter, but observations are only available for late winter–early spring, precluding comparisons across seasons. Future research should investigate the temporal plasticity of activity within this species to better understand these patterns.

4.2. Gestation Length

This study is the first to determine the exact gestation period for NECs, because it is the first to apply continuous behavioral observations. The average gestation length of 31 days for the three females that produced offspring was slightly longer than the previously reported 28-day gestation period [28]. In Dalke's study [28], copulation and parturition were difficult to observe. Dalke therefore calculated the days between the removal of the male and the loss of a heavy abdominal appearance in the female to estimate the gestation period. The disparity may be attributed to the small sample size in this study, but it is more likely that the ability to pinpoint copulation dates has produced a more accurate determination. Other congeners have even longer gestation periods; for example, *S. palustris* has a reported gestation length of 38 days [7]. This information will improve the management of potentially pregnant females by increasing the number of days staff monitor for pregnancy before females are placed with a new male.

4.3. Behavioral Differences between SPs and UPs

Behavioral, rather than physiological, barriers to reproductive success in the NEC conservation breeding program are supported by the observation of behavioral differences in SPs and UPs and a lack of copulation in UPs. This stands in contrast to a conservation program with Columbia Basin pygmy rabbits (*Brachylagus idahoensis*), in which pairs may copulate but not conceive [29]. Other behavioral differences may relate to the expression of interest in a potential mate. The higher rate of follows in SPs may be connected to the olfactory exploration of potential mates. The initiator of this behavior often performed an anal investigation of the other rabbit simultaneously or directly after following. This behavioral sequence was also observed in eastern cottontails and was almost exclusively performed by males [30]. While male NECs initiated around 80% of the follows observed, females still initiated 20%, differing from their eastern counterparts. Future work should investigate the potential for olfactory signaling of receptivity or mate quality. Another NEC behavior that differs in usage from other cottontail species is dash. In eastern cottontails and Appalachian cottontails, dashes were considered a pre-copulatory behavior [16,30]; in NECs it was most often performed by UPs. NECs also differ from domestic rabbits (*Oryctolagus cuniculus domesticus*). In the domestic rabbit, fall-off behavior is a clear indication of ejaculation [19]. Our observations have shown that ejaculation in NECs may not be accompanied by this behavior, as seen in pair M3F3. The lack of behavioral consistency with other lagomorphs may be caused by the small sample size limiting our ability to find trends. However, examination of other behaviors offers a better explanation for this discrepancy between NECs and other species.

One such behavior that offers an explanation is charge. While charges were not significantly different between SPs and UPs, the increased occurrence of the behavior in UPs is worth emphasizing. These charges were often used to disrupt unwanted social interactions or potential indications of aggression. Charges were an important behavior in reproductive dislodgement for the Appalachian cottontail, in which females would often bite and strike the male [16]. Mate aggression has been observed in non-cottontail lagomorph species such as Columbian Basin pygmy rabbits. Observations have shown females acting aggressively toward males before and after copulation has occurred [31]. A similar observation has occurred in this study; nearly 70% of charges and chases were initiated by females, despite females initiating more dashes and both sexes being equally likely to approach each other. Females may use these behaviors to determine which mates are compatible or displace incompatible mates to avoid copulation.

Female avoidance of unwanted mates can be used as an effective mechanism for controlling the paternity of her offspring. In chimpanzees (*Pan troglodytes verus*), when females resisted approaching males, they were able to avoid copulation 69.2% of the time [32]. The ability of males to consistently reengage with the females despite being chased away may signal potential fitness benefits. Male NECs continuously avoiding female charges may indicate high levels of endurance or speed. These male phenotypes may be selected by females because they offer direct or indirect benefits. If these traits are indicators of direct benefits, females may experience higher fertility or fecundity [33]. Indirect benefits can improve the viability of her offspring [34]. For instance, the phenotypes under selection may be especially important for this prey species to avoid predation. In pikas (*Ochotona princeps*), the male call rate was significantly correlated with the presence of a suitable den in the male's activity area [35]. Female pikas tended to mate with males with suitable single-entrance dens, which are speculated to be easier to defend from predators [35]. Charges may also enable NECs to avoid mating with undesired individuals. For example, female aggression towards males to prevent coercion from unwanted males was often observed in bonobos (*Pan paniscus*) [36]. Female NECs may avoid mating with less fit males in a similar fashion. Mate avoidance in this study is further supported by the lack of copulation or attempted mounts observed in pairings that did not produce offspring. Only two out of the four males in this study successfully copulated. However, there were stark differences between these males, with one male only attempting to mount once and the other male attempting 171 times. Additionally, the mounting behavior varied between these two individuals. Most importantly, despite being paired with two males consecutively, females only copulated with the male they produced offspring with.

Given that female NECs are only seasonally receptive to breeding, the lack of copulation may result from mistiming the reproductive receptivity in NECs, but the results of this study suggest this to be unlikely. The breeding season for this species is speculated to coincide with that of the eastern cottontail. The eastern cottontail breeding season typically ranges from late February or early March to late August or early September [8]. This study occurred well within this timeframe, and the occurrence of three pregnancies highly suggests that the female NECs were in breeding condition. Further support for behavioral, rather than physiological, barriers to pregnancy comes from the fact that females who became pregnant did so with either the first or second male they were paired with during the study, as opposed to consistently with the first or second male. Furthermore, five of the females reproduced in 2019. Females 1 and 5 both produced with males that they were not exposed to in the first round. Additionally, all the females were paired with at least one of the males who were known sires during the study [13]. A full veterinary exam to assess receptivity may not always be possible because the program aims to minimize the handling of these animals. Thus, in future breeding seasons, studies should be conducted to discover signs of reproductive receptivity in does. For instance, in domestic rabbits, receptive does display restlessness, chin rubbing, lordosis, and swollen, reddened vulva [37]. Perhaps female NECs exhibit similar behaviors that would allow caretakers to know when to pair females with males.

Based on the evidence provided, behavioral limitations are impairing the potential productivity of the conservation breeding program and therefore should be further investigated. The lack of copulation in UPs, the observation of individuals adjusting their behavior between pairings, and the differences in the rate of dashes and follows in SPs highly suggest that a form of female mate choice is occurring [38]. If so, then variation in NEC behavior likely influences the mating success of individuals. An important next step is to discover which individuals and behavioral characteristics are selected. However, the number of times NECs perform a behavior does not predict success. Future research should increase the sample size and focus on the differences in behavioral sequences between SPs and UPs to further our understanding of breeding behavior.

4.4. Ecology and Mate Choice

Mate avoidance may indicate mate choice is an important reproductive strategy in NECs, which may be fostered by the ecology of this species. The overlap of male and female NEC home ranges increases from 25.5% during the winter to 45.8% during the breeding season [39], suggesting that individuals are likely exposed to multiple potential mates before and throughout the breeding season. Females may be employing a pre-copulatory mate choice among males with overlapping home ranges. While mate choice may be occurring naturally for NECs, it has not been implemented in the conservation breeding program. Mimicking the natural breeding ecology may enhance NEC proceptivity. Mate choice has proven effective in lagomorph conservation breeding programs, as shown in Columbia Basin pygmy rabbits where pairing females with preferred males increases the probability of kit production and litter sizes [40]. In this study, individuals varied their behavior between different mates, and behavioral differences between SPs and UPs occurred. Additionally, two females did not copulate with the first male they were paired with but did copulate with the second; perhaps female mate choice strongly influences which males can successfully breed. Pre-copulatory mate choice may be especially important for species like NECs with induced ovulation, as the first male to copulate is more likely to sire the offspring, weakening post-copulatory selection mechanisms [41,42]. Since the goal of the present study was to describe the behavioral differences between SPs and UPs, the design did not test female choice. Future studies should examine if female NECs engage in mate choice through preference testing. If observed, future research should assess the potential benefits of pre-copulatory mate choice in NECs. If this mating strategy is important for wild NECs, then it also has implications for conservation and management, as NECs have low occupancy across fragmented landscapes [3], which may greatly reduce the ability of individuals to find preferable mates. Thus, it is important to understand the role mate choice plays in the breeding success of this species.

5. Conclusions

To our knowledge, this is the first study to use 24 h continuous data collection to observe NECs and document interactions between males and females, resulting in a more complete description of NEC social behaviors. This design improved the current knowledge about the diel activity and the gestation period of NECs and gave insights into the potential barriers hindering the reproductive output of the conservation program. The observation of copulation only occurring in pairs that produce offspring highly suggests a behavioral limitation to the breeding program's success, which may result from a limited understanding of the NEC ecology.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jzbg5030034/s1, Table S1: Pairing schedule; Video S1: Video ethogram; Table S2: Second stage interobserver reliability scores.

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