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Evaluating Outcomes of Diazepam Administration in Gradual Steps Introductions of Adult Male and Female Rhesus Macaques (*Macaca mulatta*)

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Social housing remains one of the best forms of environmental enhancement for nonhuman primates (NHPs). The gradual steps (GS) method, a 2-step plan involving an initial phase of limited physical contact (protected contact [PC]) prior to full contact (FC), is widely used for introducing macaque pairs. Recent evidence has suggested that administration of diazepam prior to FC introduction, without a PC phase, improves the success rate of pairings among adult male rhesus macaques (*Macaca mulatta*). Nevertheless, given the popularity of the standard GS method, there is considerable interest in using diazepam along with this technique. We hypothesized that administering a single dose of diazepam prior to the PC phase would improve the success rates of isosexual pairings of unfamiliar adult macaques. Twelve males and 12 females were studied in each of 3 groups, with 2 different doses of oral diazepam (2.5 or 3.2 mg/kg) and controls introduced without diazepam. Pairs were deemed successful after 14 consecutive days of compatible FC housing. Among males, success rates for the low diazepam, high diazepam, and control groups were 67%, 50%, and 67%, respectively. Among females, the corresponding values were 50%, 33%, and 17%. There were no significant differences in overall introduction success rates for either sex. However, among females, the success rates during the initial PC phase were significantly higher in introductions involving the lower dose of diazepam (83%) than among controls (33%). Descriptively, in both sexes, less severe wounding patterns were observed with the lower dose compared with either the high dose or control groups. Our results suggest that diazepam administration prior to the PC phase of the GS method does not improve pairing outcomes for either sex in rhesus macaques. However, diazepam may have some utility in moderating wounding during unsuccessful introductions.

Abbreviations and Acronyms: C, control; FC, full contact; GS, gradual steps; HD, high diazepam; LD, low diazepam; PC, protected contact; TNPRC, Tulane National Primate Research Center

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Introduction

It is well documented that nonhuman primates (NHPs) are social animals that benefit from social housing.[2,3,7–9,13,17,23,25] Current regulations specify that social housing is the default unless an animal is deemed overly aggressive, debilitated, suspected of having a contagious disease, or is unable to be housed compatibly with available social partners; veterinary and research exemptions are permitted as well.[26] However, forming successful adult pairs of rhesus macaques (*Macaca mulatta*) can be challenging, thus prompting behavioral scientists, veterinarians, and/or animal care staff to identify and implement new strategies to improve safety and outcomes.

Several methods have been developed with varying levels of success and not without limitations.[25] These are generally classified into two categories based on the overarching strategies employed: full contact (FC), where animals move directly from single housing to FC, or protected contact (PC), which involves an initial PC phase allowing either visual-only or visual/physical contact between potential pair members through barriers (consisting of mesh or dividers with bars or openings of various sizes) prior to moving to FC. FC strategies include transport and anesthetization. PC strategies include rapid steps, cage-run-cage, and gradual steps (GS).

FC strategies generally have the advantages of being relatively quick to implement and potentially limiting initial wounding. Monkeys paired with the transport method, which entails moving the animals in transport cages within or between facilities, may spend more energy coping with environmental changes rather than directing energy toward the new partner.[15,25] Meanwhile, postanesthetic sedation of monkeys paired with the anesthetization method, which entails anesthetizing the animals and allowing them to recover in FC, may limit anxiety-driven behaviors during early interaction and allow the introduction to unfold in calmer settings. As for the disadvantages of FC strategies, the initial inhibition of wounding may lead to a false sense that animals are compatible, and more staff time may be required to assess compatibility after the pair is established. Moreover, the transport method may limit staff's ability to separate partners engaged in fights and may not be feasible due to facility housing configurations and space limitations, while anesthetization may lead to the monkeys recovering at different

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rates with associated ramifications (for example, conscious animals potentially wounding still-anesthetized partners).[25]

PC strategies generally have the advantages of minimizing injury risk in the early phases, incremental compatibility assessments, and promotion of confidence in some monkeys as they have the choice to approach their prospective partners. They are also flexible in terms of how they can be applied according to available caging. The rapid steps method simply includes a 24-h visual contact phase before progressing to FC and has the additional benefits of requiring less staff time and providing the ability to see a full range of dominant-submissive behaviors earlier in the pairing process. Cage-run-cage initially involves a PC phase within the home environment (that is, cage). This is followed by the first FC phase, which occurs in a larger enclosure away from the home environment (that is, run), which provides the advantages of additional flight space and preventing influence from other familiar animals housed in the home environment. The final FC phase occurs back in the home environment (that is, cage) after initial compatibility assessments. The GS method is similar to rapid steps, but typically includes a longer PC phase that allows for limited physical contact before progressing to FC, which, in addition to providing some physical contact between prospective partners, has the advantage of longer initial compatibility assessments. The GS method has been successfully applied to varying ages of both sexes and is often used for introductions of high-risk animals. However, both cage-run-cage and GS require more staff time and can also create a false sense of security, as contact aggression may not occur until animals are paired in FC. Lastly, these strategies may create frustration among the animals due to the delay before FC.

All of the above illustrate the nuances of developing and implementing pairing techniques for macaques. Numerous studies have reported on their application with wide variability in the techniques employed and their relative success rates. Thus, tailoring a socialization plan to any population of macaques is always a challenging task involving the consideration of many factors, including animal demographics, housing configurations, space, and staff availability. There is no one-size-fits-all approach, often necessitating the use of a mixture of these methods to maintain successful social housing programs for NHPs.

Consequently, these limitations and nuances have prompted behavioral scientists, veterinarians, and/or animal care staff to investigate novel strategies, especially those involving the use of pharmacological interventions. Diazepam was recently selected to assess its utility in FC introductions of unfamiliar adult male rhesus macaques.[15] That study reported that pairing animals dosed with diazepam and eliminating the PC phase was highly successful (94%) in comparison to standard GS pairings (45%).[15] These results, in concert with the plethora of research on diazepam's anxiolytic and sedative properties via its modulatory effects at GABA receptors[5,20,22] and anticonflict/positive behavioral effects in rhesus macaques,[6,10,16,21] suggest that the administration of diazepam is a useful strategy to improve success rates of pair introductions. Furthermore, diazepam administration in this context may address the aforementioned drawbacks of other introduction methods, even considering the known potential paradoxical effects of diazepam in a variety of species.[11,12,14,19]

However, concerns persist regarding the implementation of FC introductions involving pharmacological interventions, and the GS method is still typically employed by most institutions housing NHPs.[1] Due to the hesitance to forego the GS method, there is considerable interest in building on these findings[15] by examining the role of diazepam in addition to, rather than in place of, PC when pairing adult rhesus macaques.

The aim of this study was to examine outcomes using this diazepam dosing strategy for forming isosexual pairs of both male and female rhesus macaques. In addition, we wanted to determine the utility of a lower dose of diazepam to reduce difficulties in gaining animals' cooperation with consuming the medication and to limit the potential for adverse effects associated with benzodiazepines. We implemented 2.5 mg/kg as the lower dose, as this was previously reported to have increased social grooming, approach, and contact and decreased aggression among socially housed rhesus macaques without adverse effects.[16] We hypothesized that employing diazepam in GS pairings of adult rhesus macaques would be more successful than the common practice of implementing GS pairing without medication and produce comparable results to FC pairings using diazepam. Furthermore, we hypothesized that introductions using a lower dose of diazepam (2.5 mg/kg PO) would have similar success rates to those using a higher dose (3.2 mg/kg PO), thus refining the dosing strategy.

Materials and Methods

Animals. Eighty potential subjects were screened for inclusion and were chosen based on physical examination findings. Individuals were anesthetized with ketamine (10 mg/kg IM; Zoetis, Lincoln, NE) to perform physical examinations and blood sampling for CBC and serum chemistry screenings. The principal investigator performed all physical examinations and reviewed all laboratory results. Any potential subjects found to have clinical conditions requiring treatment or suggestive of underlying pathology were promptly removed from enrollment and received appropriate veterinary care. Based on these assessments, 8 animals were removed from the study and replaced before group allocation occurred. The remaining subjects included 72 Indian ancestry rhesus macaques, 36 adult males and 36 adult females, born at the Tulane National Primate Research Center (TNPRC) in Covington, LA.

At the time of enrollment, male subjects ranged in age from 4.86 to 16.77 y (mean of 10.34 y) and weighed between 7.4 and 20.8 kg (mean of 11.70 kg). Female subjects ranged in age from 4.20 and 23.66 y (mean of 12.28 y) and weighed between 5.50 and 12.80 kg (mean of 7.85 kg). All subjects were mother-reared in social groups housed in outdoor field cages. Macaques were free of *Macacine alphaherpesvirus* 1, SIV, simian betaretrovirus, and simian T cell lymphotropic virus. Twelve males and 12 females were assigned to each of 3 groups: 1) low diazepam (LD; 2.5 mg/kg PO), 2) high diazepam (HD; 3.2 mg/kg PO), and 3) controls (C; no diazepam).

At the start of the study, animals were individually housed in interconnecting stainless steel NHP enclosures exceeding the minimum standards set forth by the USDA and *The Guide for the Care and Use of Laboratory Animals.*[18,26] Male subjects' lifetime tenure of single housing ranged from 120 to 1679 d (mean of 660 d) and time since last social housing ranged from 68 to 415 d (mean of 164 d). Female subjects' lifetime tenure of single housing ranged from 76 to 919 d (mean of 334 d) and time since last social housing ranged from 30 to 560 d (mean of 143 d). Animals weighing less than 10 kg were housed in a single enclosure with 0.4 m^2 of floor space and 0.9 m of vertical space. Animals weighing 10 to 20 kg had access to 2 side-by-side enclosures, providing 0.8 m^2 of floor space and 0.9 m of vertical space. Lastly, animals weighing more than 20 kg had access to 3 side-by-side enclosures, providing 1.2 m^2 of floor space and 0.9 m of vertical space. Caging configurations were composed of two tiers. Each was outfitted with foraging devices, perches, and various manipulanda both installed on and loose in the enclosure.

Study subjects' caging areas were maintained in sheltered outdoor housing, which typically serve as acclimation areas for animals moving from indoor housing to the outdoor breeding colony enclosures. The buildings are constructed with chain-link fence walls and are equipped with temperature-moderating elements, including retractable plastic panels and metal shutters, heaters, and industrial-sized fans that are used as necessary, such as when outdoor temperatures drop below 10 °C or rise above 23.9 °C. Due to the design and purpose of these buildings, the animals were exposed to a wider range of heat and humidity than for indoor-housed animals. Rooms were maintained with a temperature range of 12.2 to 36.1 °C and a relative humidity range of 27% to 100%. Lighting was maintained with a 12-h light/12-h dark cycle, although the animals were to some degree also exposed to natural daylight. Unlike animals housed in typical indoor housing, animals housed in sheltered outdoor housing are able to visualize and hear relatively loud and varying levels of weather events and human activity associated with vehicles and equipment used for husbandry and property maintenance.

Subjects were fed a standard commercial diet (LabDiet fiber-plus monkey diet, LabDiet, St. Louis, MO) twice daily with ad libitum access to water via an on-demand water valve. Subjects received forage, fresh fruit and/or vegetables, and other food items at least 5 times per week in accordance with the TNPRC Environmental Enhancement Program.

All experiments were ethically reviewed and approved by the IACUC at the TNPRC. All NHPs were treated in accordance with the *Guide for the Care and Use of Laboratory Animals* and PHS policy in a facility that is fully accredited by AAALAC.

Pair selection and group assignment. Pairs were selected based on disparate weights when possible, according to standard TNPRC policy, and for research purposes, no subjects had prior familiarity. Pairs were then randomly assigned to 1 of 3 introduction groups and the end observer was blinded to group allocation. Introductions were conducted using 1 of 2 strategies: 1) with diazepam (LD or HD), in which pairs were gradually introduced to one another following administration of either LD or HD prior to the initial PC phase before moving into FC; or 2) no diazepam (C), in which pairs were gradually introduced to one another (also beginning with a PC phase before moving into FC) while not under the influence of any pharmaceutical manipulations. Groups consisted of 1) male LD and female LD (2.5 mg/kg PO; n = 12 males; n = 12 females); 2) male HD and female HD (3.2 mg/kg PO; n = 12 males; n = 12 females); and 3) male C and female C (n = 12 males; n = 12 females).

Pairing procedures. All introductions were conducted in accordance with TNPRC standard practices for social housing and as previously described.[15] All subjects were moved into adjacent enclosures separated by solid panels at least 3 d before the commencement of introductions. During the initial phase, individuals were placed in PC by replacing the solid panel with a PC panel that permits limited physical contact. PC panels consisted either of a set of vertical bars spaced 3 cm apart (Allentown, Allentown, NJ) or a stainless-steel partition with multiple 5.08- to 7.62-cm openings (Lab Products, Seaford, DE).

Video recordings were initiated just prior to implementation of PC and continued for a maximum of 2 h. The camera (model HDR-CX405 HD 9.2 MP Handycam camcorder, Sony Electronics) was positioned so that both animals could be visualized at all times regardless of their locations within the enclosures. Pairs were also observed in person for a minimum of 10 min by the principal investigator and behavioral technician to ensure initial pair compatibility in each phase. Based on prior experience and criteria established by the TNPRC Unit of Behavioral Management, animals were separated if wounding, persistent or escalating aggression, or fear was observed. Pairs separated during PC were not reattempted as a pair or with other study subjects. The duration of PC ranged from 0 to 13 d (mean of 7.6 d) for all male groups and 0 to 15 d (mean of 3.9 d) for all female groups.

The decision to move forward with FC phases for each pair was based on the prevalence and patterns of behaviors exhibited by the animals, such as aggression, affiliation, proximity, or distress. Pairs not displaying affiliation or proximity were allowed to proceed if no problematic behaviors were observed. If subjects showed behavior that did not merit immediate separation but was deemed concerning, additional video footage was collected and assessed for aggressive, rank-related, or fearful behavior. If potential pairs were successful in PC for a minimum of 2 d, they were introduced into FC by removal of the PC panel. The same observational and behavioral assessments used for the PC phases were also employed for the FC phases. After the initial introduction, animals were monitored according to TNPRC standard FC pairing procedures, including behavioral monitoring by direct observation and video recording executed in the same manner as for the PC phases. Pairs were considered successful after 14 d of FC housing without the need for separation.[7,25] In pairs that successfully graduated from PC, the duration of FC ranged from 0 to 14 d (mean of 12 d) for all male groups and 0 to 14 d (mean of 9 d) for all female groups.

Diazepam preparation. Diazepam tablets (2, 5, or 10 mg/tablet; Covetrus, Dublin, OH) were either inserted into a high-value food item or crushed and ground using a pill cutter and subsequently mixed into high-value food items.

Diazepam pairing procedures. Subjects assigned to the diazepam treatment groups were introduced in the same manner as described above.

Animals in the LD groups were administered 2.5 mg/kg diazepam PO in high-value food items 30 to 45 min prior to initiation of the PC phase. Those assigned to the HD groups were administered 3.2 mg/kg diazepam PO in the same manner. The time between diazepam administration and initiation of the PC phase was determined by the observation of behavioral effects (for example, anxiolysis, sedation) in each animal while individually housed. Subjects were monitored for potential signs of sedation including somnolence, decreased interaction with partner and/or attentiveness to the external environment, and motor deficits. Animals did not exhibit overt signs of sedation or any obvious evidence of adverse effects or motor deficits after diazepam administration.

Data collection. All introductions were video recorded during the PC and FC phases for up to 2 h each. These videos were subsequently coded by a single behavioral technician blinded to subject group allocation. A single 10-min session was coded from the initiation of each pair's PC and FC phases using one-zero sampling with 30-s intersample intervals. Data were coded using an established social introduction ethogram originally developed by the TNPRC Unit of Behavioral Management.

Across all subjects and phases, a total of 59 coded video sessions were coded. All male pairs had at least one video recorded session for a total of 34 sessions (n = 18 PC; n = 16 FC). All female pairs had at least one video recorded session for a total of 27 sessions (n = 17 PC; n = 10 FC). The camera malfunctioned during the PC introduction of one female pair from the HD group; therefore, a recording was not obtained or subsequently coded for that pair's PC phase. Only 25 (n = 16 PC; n = 9 FC) of the 27 female recorded sessions were coded and subsequently analyzed due to early pair separation. One pair from the LD group had to be separated at 6 min into the PC phase, while another pair from the same group had to be separated at 2 min into the FC phase. Data from pairs that had to be separated prior to the 10-min mark of any GS phase were excluded. The maximum number of coded sessions per pair was 2 (one each for the PC phase and FC phase) depending on the outcome of the PC phases. Additional behavioral monitorings of varying durations were conducted to assess pair compatibility for management purposes but were not implemented to generate study data.

C PC phase video recordings began immediately after the pairs were introduced, with the first 10 min of these recordings being used for behavioral coding. In the PC phase of the diazepam groups' introductions, video recordings began immediately after administration of diazepam (to confirm the drug's consumption and document its effects) but prior to introducing the animals. The 10-min video recordings that were subsequently used for behavioral coding of diazepam PC phases began immediately after replacement of the solid panel with the PC panel. All groups' FC phase video recordings began immediately after the pairs were placed in FC, with the first 10 min being used for behavioral coding.

Statistical analysis. Data for males and females were analyzed separately using IBM Statistical Package for the Social Sciences (SPSS) for Windows, version 27.0. Results were considered significant with $\alpha \leq 0.05$. Introduction success rates between the LD, HD, and C groups of each sex were compared using Fisher exact tests.

Behavior analyses were performed using the frequencies of each behavior summed across all of an individual's observations. Behaviors were grouped into categories for analysis as indicated in Table 1. The abnormal behavior category as well as submissive behavior were dropped from the analyses because they were observed at extremely low levels and among only a few subjects. Therefore, analysis included aggressive, affiliative, and anxiety-related behaviors. Kruskal-Wallis tests were used to compare groups.

Similar to previously described methods,[15] we examined our data with an eye toward identifying confounding factors that may have predisposed the LD, HD, or C groups of each sex toward successful pairings, and ANOVA tests were used to evaluate differences between study participants in 6 individual characteristics: lifetime tenure in single housing, days since most recent housing in a social setting, age, age difference between cage mates, weight, and weight difference between cage mates. Data are presented as the mean and SEM.

Backward stepwise binary logistic regression analyses were employed to identify potential individual characteristics or behaviors that could have predicted pair success in each group.

Results

Male pairing outcomes. Four of 6 (66.7%) LD, three of 6 (50.0%) HD, and four of 6 (66.7%) C introductions were successful (Figure 1A). All but two of the male pairs, both from the HD group, successfully completed the PC phase and graduated to the FC phase (Figure 1B).

Fisher exact tests indicated that there were no significant differences in success between LD and HD pairings ($P = 0.680$), nor

Table 1. Ethogram of behaviors used during observations

Aggressive behaviors	
Aggressive contact/biting	Physical contact involving biting or biting attempts
Aggressive contact/no biting	Physical contact without involvement of the mouth (for example, pushing, pulling, grabbing, and scratching)
Displaying	Vigorous shaking, slamming, or bouncing off of the cage
Threatening	At least one of the following partner-directed gestures: ears flattened against the head, brow retracted, open-mouth stare, head bobbing, slap surface or slap at the partner without making contact, and lunging (high-speed aggressive intention movement toward another animal)
Affiliative behaviors	
Cothreatening/solicit cothreat	Alternating threats and glancing at the partner, who may or may not join in the threatening
Grooming	Manipulating, brushing, or licking of fur (or eyes, wounds) of another animal with the mouth and/or both hands. Includes both groomer and animal receiving grooming
Lip-smacking	Bringing the lips together rapidly, resulting in a smacking sound; teeth are covered. Directed at potential partner
Mounting	Common usage, with or without pelvic thrusting and penetration and with or without foot clasp. Includes both mounter and animal being mounted
Playing/play soliciting	Nonaggressive, lively actions performed with another individual with or without direct physical contact (for example, chasing), without piloerection, but with relaxed facial expressions
Rump presenting	A posture involving a stance on all fours with the hind quarters elevated and the tail raised. In some animals the tail may be lifted to the side rather than raised. In some instances, animals may place their heads between their legs. Rump presents may be accompanied by brief tail flicks. Directed at potential partner
Anxiety-related behavior	Body shuddering, scratching, yawning, or teeth grinding
Abnormal behavior	Animal performs species atypical behaviors (for example, hair plucking, self-directed, or locomotor stereotypies)
Submissive behavior	
Fear grimacing	Grin-like facial expression involving retraction of the lips, exposing teeth

Behaviors were grouped into 5 categories for analysis and are indicated in bold. Note that abnormal behaviors and fear grimacing are presented but were observed too infrequently to include in analysis.

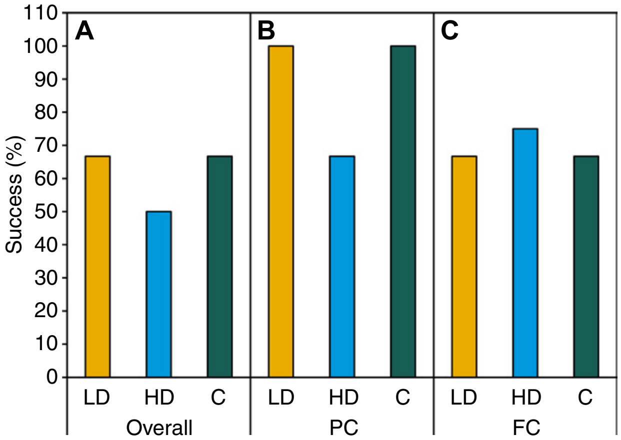

Figure 1. Male groups' introduction success rates. Fisher exact tests indicated that there were no significant differences between any groups overall (A) or during either the PC phase (B) or the FC phase (C). Data are plotted as percentages of pairs that successfully completed the full GS method (A), that successfully completed the PC phase and graduated to the FC phase (B), and that successfully completed the PC phase then remained in the FC phase for 14 d (C).

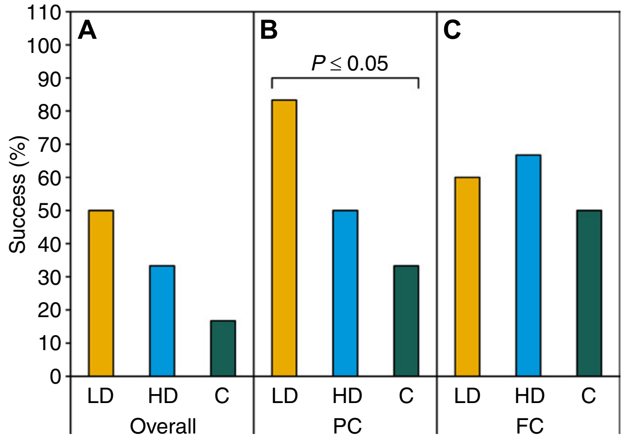

Figure 2. Female groups' introduction success rates. Fisher exact tests indicated that there were no significant differences between any groups overall (A) and during the FC phase (C). Success differed significantly between only the LD and C groups during the PC phase (B). Data are plotted as percentages of pairs that successfully completed the full GS method (A), that successfully completed the PC phase and graduated to the FC phase (B), and that successfully completed the PC phase then remained in the FC phase for 14 d (C).

were there significant differences in success between C pairings and either LD ($P = 1.000$) or HD ($P = 0.680$) pairings (Figure 1A). Despite 100% PC phase success in the LD and C groups, these were not significantly higher than that of the HD group (66.7%; $P = 0.093$) (Figure 1B), as revealed by Fisher exact tests. Furthermore, among male pairs that successfully completed the PC phase, there was no difference in their successes during the FC phase ($P = 1.000$) (Figure 1C).

One individual in one of the HD pairs that failed during the PC phase was wounded by his partner, requiring separation and veterinary care. The other pair was deemed incompatible during observations on the first day of PC due to escalating aggression and was subsequently separated. Both HD and C FC phase failures were the result of wounding that required separation for veterinary care. Both of the LD pairs that failed during the FC phase were deemed incompatible during observations, including wounding in one individual that did not require veterinary care.

Female pairing outcomes. Three of 6 (50.0%) LD, two of 6 (33.3%) HD, and one of 6 (16.7%) C pairings were successful (Figure 2A). One, three, and four of the LD, HD, and C pairs, respectively, failed during the PC phase (Figure 2B). Two, one, and one of the LD, HD, and C pairs, respectively, failed during the FC phase (Figure 2C).

Fisher exact tests indicated that there were no significant differences in success between LD and HD pairings ($P = 0.680$), nor were there significant differences in success between C pairings and either LD ($P = 0.193$) or HD ($P = 0.640$) pairings (Figure 2A). The success rate of the PC phase in the LD group (83.3%) was significantly higher than that of the C group (33.3%; $P = 0.036$) but not the HD group (50.0%; $P = 0.193$) (Figure 2B), and there was no difference in PC phase success between the HD and C groups ($P = 0.680$) (Figure 2B). However, the significance of the LD PC phase introductions was not predictive of overall pairing success, as there was no difference in success between female pairs of any group at the FC phase that successfully completed the PC phase ($P = 1.000$) (Figure 2C).

Three of the C PC phase failures involved wounding, two of which did not require veterinary care, while the third did require care for one individual. The remainder of the PC phase failures in the LD, HD, and C groups were the result of incompatibility due to escalating aggression as assessed during observations. The HD FC phase failure was the result of wounding that required veterinary care. The LD and C pairs that failed during the FC phase were deemed incompatible during observations.

Comparisons of male behaviors. Kruskal-Wallis tests revealed no differences in aggressive, affiliative, or anxious behaviors between the male groups during either introduction phase (Table 2).

Comparisons of female behaviors. Kruskal-Wallis tests detected no differences in aggressive or affiliative behaviors between the female groups during either introduction phase (Table 3). However, these tests did indicate differences in anxious behaviors during the PC phase ($P = 0.013$) (Figure 3). The C pairs exhibited anxious behaviors during the PC phase more frequently than did both the LD ($P = 0.006$) and HD pairs ($P = 0.031$), which were not statistically different from each other ($P = 0.568$).

Comparisons of male characteristics. ANOVA tests indicated that age difference from social partner was significantly different between the male groups ($P = 0.005$) (Table 4), with post hoc analyses revealing that only HD and C pairs differed in this measure ($P = 0.004$).

Comparisons of female characteristics. ANOVA tests indicated that days since last socially housed and weight difference from social partner were significantly different between the female groups ($P = 0.010$ and $P = 0.023$, respectively) (Table 5), with post hoc tests revealing that only LD and C pairs differed in days since last socially housed ($P = 0.009$) and only LD and HD pairs differed in weight difference from social partner ($P = 0.028$).

Identifying predictors of pairing outcomes. We attempted backward stepwise binary logistic regression analyses to identify individual characteristics or behaviors that could have predicted pair success in each group. However, these could not be performed due to small group sizes or the overall outcomes of the introductions. These analyses were feasible, however, when collapsing the diazepam groups into one group for each sex (that is, LD+HD). Here, greater age difference between social partners was predictive of introduction success when using diazepam in the male ($P = 0.005$) pairing groups (Table 6).

In contrast, greater age difference was predictive of introduction failure when using diazepam in the female ($P = 0.027$)

Table 2. Kruskal-Wallis tests revealed that were no significant differences in affiliative, aggressive, or anxious behaviors between the male groups during either introduction phase

	Kruskal-Wallis	*P*	LD (mean ± SE)	HD (mean ± SE)	C (mean ± SE)
PC					
Aggression	1.276	0.528	0.079 ± 0.019	0.217 ± 0.087	0.096 ± 0.044
Affiliation	5.820	0.054	0.179 ± 0.051	0.042 ± 0.016	0.150 ± 0.057
Anxiety	3.193	0.203	0.329 ± 0.078	0.238 ± 0.078	0.388 ± 0.068
FC					
Aggression	0.358	0.836	0.004 ± 0.004	0.012 ± 0.013	0.008 ± 0.006
Affiliation	4.010	0.135	0.246 ± 0.123	0.019 ± 0.013	0.104 ± 0.056
Anxiety	0.695	0.706	0.138 ± 0.044	0.219 ± 0.083	0.138 ± 0.030

Table 3. Kruskal-Wallis tests revealed that there were significant differences in anxious behaviors between the female groups during the PC phase

	Kruskal-Wallis	*P*	LD (mean ± SE)	HD (mean ± SE)	C (mean ± SE)
PC					
Aggression	0.004	0.998	0.175 ± 0.087	0.110 ± 0.041	0.092 ± 0.026
Affiliation	2.381	0.304	0.015 ± 0.007	0.105 ± 0.053	0.021 ± 0.011
Anxiety	8.612	0.013	0.085 ± 0.046	0.090 ± 0.030	0.325 ± 0.086
FC					
Aggression	0.332	0.847	0.056 ± 0.050	0.025 ± 0.017	0.025 ± 0.014
Affiliation	3.854	0.146	0.113 ± 0.085	0.083 ± 0.040	Not observed
Anxiety	0.359	0.835	0.200 ± 0.095	0.200 ± 0.066	0.163 ± 0.094

There were no significant differences in affiliative or aggressive behaviors between the female groups during either phase.

pairing groups (Table 7). Lastly, greater frequency of aggressive behavior during the PC phase was predictive of introduction failure when using diazepam in the female pairing groups ($P = 0.041$) (Table 8).

Discussion

While research has clearly shown benefits of pair housing, successful pairing of unfamiliar adult rhesus macaques remains a challenging proposition replete with nuance. As such, current social housing research focuses on strategies to improve safety and outcomes. In light of recent evidence proving the utility of diazepam administration in direct FC introductions of adult male rhesus macaques,[15] the present study sought to test a diazepam strategy within the framework of the GS pairing method for both male and female rhesus macaques. The purpose was to see if comparable results could be achieved, namely that the use of diazepam would improve pairing success rates compared with the standard GS method and be equally successful as FC introductions using diazepam, while also attempting to refine the diazepam dosage. Lastly, we attempted to identify factors that could have confounded our findings by assessing whether individual group differences in behavior frequencies and specific subject characteristics predicted introduction outcomes.

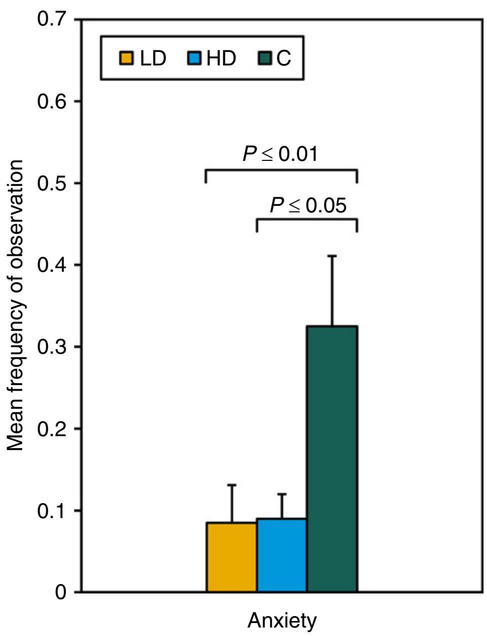

Figure 3. Female groups' frequency of anxious behaviors. Kruskal-Wallis tests indicated that C pairs exhibited significantly higher levels of anxious behaviors than both the LD and HD pairs. Data are plotted as the mean ± SE frequency of observations.

In this study, we introduced adult male and female rhesus macaques in GS after either LD or HD administration prior to the PC phase and compared outcomes to GS pairing attempts for C animals introduced without diazepam. We found that diazepam administration did not improve overall introduction success in either sex at either dose tested in this study. Although LD administration was associated with increased success in the PC phase in females, it did not influence final introduction outcomes. However, because there may be instances where animals can only be introduced into PC, this result may indicate that diazepam is a useful tool for said purposes.

Because diazepam was effective in direct FC introductions of adult male rhesus macaques,[15] it is possible that the addition of a PC phase may have been an inhibiting factor, despite the use of diazepam. The idea has been postulated that perhaps the PC phase of the GS method instills frustration in macaques that can lead to downstream aggression and unsuccessful pairing outcomes. Diazepam may reduce anxiety during initial interactions but cannot moderate potential frustration that may develop over time, particularly because pairs remained in PC for a minimum of 2 d and up to 13 and 15 d in at least one male and female pair, respectively. It could also be that diazepam

Table 4. ANOVA tests revealed that age difference from social partner was significantly different between the male HD and C groups while duration of single housing, days since last socially housed, age, weight, and weight difference from social partner were not significantly different between any male groups

	Sum of squares	*df*	*P*	LD (mean ± SE)	HD (mean ± SE)	C (mean ± SE)
Duration single housing (d)	5,992,653.6	35	0.979	660.9 ± 96.0	677.6 ± 133.5	642.1 ± 135.2
Days since last socially housed	186,097.6	35	0.194	194.5 ± 33.8	156.3 ± 8.8	142.1 ± 7.7
Age (y)	506.5	35	0.713	9.8 ± 1.0	11.1 ± 1.0	10.1 ± 1.3
Age difference (y)	307.4	35	0.005	5.3 ± 0.9	4.0 ± 0.7	7.7 ± 0.5
Weight (kg)	357.4	35	0.837	11.8 ± 1.1	12.1 ± 0.8	11.3 ± 0.8
Weight difference (kg)	173.8	35	0.967	5.3 ± 1.0	5.3 ± 0.3	5.1 ± 0.5

Table 5. ANOVA tests revealed that days since last socially housed and weight difference from social partner were significantly different between the female LD and C groups while duration of single housing, age, age difference, and weight were not significantly different between any female groups

	Sum of squares	*df*	*P*	LD (mean ± SE)	HD (mean ± SE)	C (mean ± SE)
Duration single housing (d)	1,773,220.8	35	0.207	257.7 ± 60.0	322.9 ± 58.7	420.7 ± 71.8
Days since last socially housed	461,127.0	35	0.010	86.0 ± 20.7	123.8 ± 25.5	219.3 ± 39.5
Age (y)	959.5	35	0.613	11.8 ± 1.6	11.5 ± 1.6	13.5 ± 1.5
Age difference (y)	793.8	35	0.753	6.4 ± 1.8	5.2 ± 1.3	6.6 ± 1.0
Weight (kg)	86.7	35	0.795	7.9 ± 0.6	8.1 ± 0.4	7.6 ± 0.3
Weight difference (kg)	87.2	35	0.023	2.7 ± 0.6	1.1 ± 0.3	1.4 ± 0.3

Table 6. Greater age difference between social partners was predictive of male pair introduction success when utilizing diazepam while single housing duration, time since last socially housed, age, weight, weight difference, and frequencies of anxious, aggressive, and affiliative behaviors were not predictive of pair outcome

	B	SE	Wald χ^2	*df*	*P*	Odds ratio
Age difference (y)	0.598	0.215	4.672	1	0.005	1.818

Table 7. Greater age difference between social partners was predictive of female GS introduction failure when utilizing diazepam while single housing duration, time since last socially housed, age, weight, weight difference, and frequencies of anxious, aggressive, and affiliative behaviors were not predictive of pair outcome

	B	SE	Wald χ^2	*df*	*P*	Odds ratio
Age difference (y)	−0.409	0.185	4.878	1	0.027	0.664

effectively muted the subjects' behaviors to such a degree that individuals were unable to engage in the initial exchange of interactions necessary for developing social bonds with their partners (which was only compounded by the addition of a PC phase where direct interaction of pairs could not readily occur). However, because the pairs that were administered diazepam did not exhibit overt signs of sedation or any obvious evidence of adverse effects, and pairing outcomes were similar across all groups, this lends more support to the notion that PC alone may have impeded success when comparing these results to the success rates of diazepam FC introductions of adult male rhesus macaques.[15] It is also possible that the length of PC in this study have may have impeded success, as shorter durations (24 h or less) were not assessed and longer durations (greater than 2 d) were imposed either due to the nature of the animals' interactions, or because of external factors, such as the schedule of research procedures or the day of the week. We do not advance introductions during the weekend, nor for some pairs in the latter part of the work week, due to reduced staff presence during the weekend.

Table 8. Greater frequency of aggressive behaviors during the PC phase was predictive of female PC failure when utilizing diazepam while single housing duration, time since last socially housed, age, age difference, weight, weight difference, and frequencies of anxious and affiliative behaviors were not predictive of PC outcome

	B	SE	Wald χ^2	*df*	*P*	Odds ratio
PC						
Aggression	−3.677	0.18	4.172	1	0.041	0.025

Another aspect that may codify success or at least a refinement in this study was the wounding characterized in each of the groups. Because wounding requiring veterinary care only occurred in the HD and C groups for each sex, this may indicate that the lower diazepam dose elicits safer introductions than at least standard GS pairings. A previous study reported that diazepam doses of 0.1 and 10 mg/kg decreased aggressive acts (including attacks) by 50% and 80%, respectively, by the dominant monkey in paired rhesus macaques. Dominance hierarchy was also unaltered regardless of whether the drug was administered to the dominant or submissive partner.[6] Despite not replicating a similar pattern in terms of increased dose corresponding with less severe wounding, it nonetheless supports the notion that diazepam diminishes attacks in paired rhesus. Descriptively, a larger proportion of female LD PC phase pairings were successful and, at least, no worse in male LD PC phase pairings, suggesting that the use of LD merits continued experimentation to test whether these wounding patterns and outcomes are seen with larger sample sizes.

Diazepam is widely used in human and veterinary medicine as a potent sedative in combination anesthetic protocols but also, imperatively, as an antiepileptic and anxiolytic therapy. It is also well documented that it can produce paradoxical effects (that is, excitation and anxiety) in a variety of species.[11,12,14,19,24] However, these effects are not widely documented in NHP species and were not expected outcomes of its use in this study, nor do our results suggest that these paradoxical effects were observed. Although only statistically significant in the female diazepam groups, lower frequencies of anxious behaviors during PC (the only phase when the drug would have been directly effective) were associated with diazepam administration in both sexes, with the HD groups exhibiting lower frequencies than the LD groups, evincing the expected and desired anxiolytic effect of the drug. However, descriptively, aggression was seen more frequently while affiliation was exhibited less frequently in the male HD group during the PC phase compared with the male LD and C groups, which were essentially the same in both of these measures. This potentially lends more credibility to the notion that PC instills frustration and can lead to escalating aggression, while diazepam may have a negative compounding effect. Females were significantly less anxious during PC when given diazepam, which makes the significant difference in PC outcomes less surprising. Diazepam administration in females otherwise had slightly different associations with behaviors in the PC phase than was observed in males. Descriptively, aggression was exhibited more frequently in the female LD group, while affiliation was exhibited more frequently in the female HD group. These associations are somewhat perplexing, particularly in female LD pairs, as they had greater success and less severe wounding at the PC phase despite more frequent displays of aggression. Conversely, the male behaviors seemed to be more congruent with each group's respective outcomes and wounding patterns. It is probable that the limitations of this study make it difficult to fully interpret the variety of behavioral associations observed here.

We found that most subject characteristics were uniformly distributed across individuals in each treatment group of both sexes. This homogeneity of subject characteristics essentially eliminated potential confounds in our data. While desirable from a study design perspective, this also made it more challenging/impossible to identify characteristics that could have predicted pair successes/failures in light of the lack of variability of introduction success rates across groups. As such, we were unable to perform most analyses on the individual groups to identify predictors of outcomes. However, by collapsing the diazepam groups into one for each sex, we were able to determine that age difference between social partners was predictive of male pair success, such that the greater the difference in age, the more likely the pairs were to remain successfully paired. This aligns with previous studies[4,8] where social partners of disparate ages (which is also typically associated with disparate weights) were found to be more consistently successful than social partners less disparate in these measures. Conversely, in females, greater difference in age between social partners was predictive of pair failure. The reason for this difference in females is not immediately clear, nor is it congruent with other study findings, but it may be due to inherent differences between males and females in terms of social interactions. Lastly, another predictive factor identified in females after collapsing the diazepam groups was the frequency of aggressive behaviors exhibited during the PC phase of introductions. As expected, the greater the frequency of aggressive behaviors, the lower the odds of pair success. Persistent aggression or those resulting in wounds are one of the main factors used to determine whether to terminate a pairing attempt.

This study has several limitations that need to be addressed. As previously mentioned, sample sizes and the frequency of introduction successes significantly limited our analyses. These issues could be mitigated by larger sample sizes. In addition, the social introduction ethogram used to code behavioral observations was designed with an eye toward both the feasibility of data collection while closely monitoring social introductions, and maintaining consistency between live and videotaped observation. This was achieved by selecting a subset of behaviors derived from a standardized and inclusive rhesus macaque ethogram. As such, this limited ethogram does not cover the full breadth of behaviors subjects may have engaged in or that were representative of each behavioral category (for example, submissive behaviors limited to only fear grimacing). Use of an expanded ethogram may elucidate additional predictors of social introduction outcomes in future studies. Also, the influence of external parameters (for example, movements of animals in and out of adjacent enclosures, weather, levels of human activity), considering the subjects' unique housing environment, could not be directly measured. Furthermore, adhering to a strict 2-d minimum PC phase duration likely significantly impacted the efficacy of the diazepam during the FC phase of the introductions. While this design was necessary to approximate GS rather than rapid steps[25] and provide a discrete contrast between other methods employed,[15] it would be beneficial to repeat the study by allowing pairs to graduate to FC more quickly as their behaviors permit. We also attempted to refine the diazepam dosage in an effort to achieve the same result while significantly reducing the amount of drug needed to do so. Despite the refinement, both of these oral doses can be challenging to administer for several reasons: the amount of drug given is quite large, making it extremely difficult to ensure it is evenly distributed and the animal receives the full dose, and the difficulty of administering unpalatable oral medication to any NHP, especially if repeated doses are required, (that is, attempting diazepam administration on different days or with different animals). These issues are further compounded by the drug scheduling of diazepam, which adds regulatory burden to the proceedings (for example, DEA licensing, enhanced documentation, and scrutiny of medical records). Lastly, as this study was limited to only rhesus macaques, these results cannot be extrapolated to other macaque or NHP species.

In conclusion, diazepam administration did not improve the overall outcomes of GS introductions of unfamiliar adult male and female rhesus macaques. However, LD may be a useful tool for PC-only introductions and may elicit safer (that is, less severe wounding) interactions during social housing attempts, suggesting that further experimentation with LD using larger sample sizes is warranted. Furthermore, because it has proven utility in FC introductions, this may indicate either that diazepam simply does not work in the broader context of the GS method, or that GS introductions may need to be reevaluated as an effective method for socially housing rhesus macaques.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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