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Effects of Pair Housing on Behavior, Cortisol, and Clinical Outcomes during Quarantine-like Procedures for Rhesus Macaques (*Macaca mulatta*)

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Abstract

Background: Compatible pair housing of macaques in research settings increases species-typical behaviors and facilitates beneficial social buffering. It is not yet established whether these benefits are maintained after intrafacility transfer and domestic quarantine, which are two stressors that can lead to behavioral and clinical abnormalities.

Methods: We evaluated 40 adolescent male rhesus macaques who were single- or pair-housed immediately following an intrafacility transfer. We measured behavior, fecal cortisol, body weight, and diarrhea occurrence. Body weight and diarrhea occurrence were also retrospectively analyzed in an additional 120 adolescent rhesus who underwent a similar transfer.

Results and Conclusions: Pair-housed macaques exhibited less of some undesirable behaviors (e.g., self-clasping) and experienced less diarrhea than single-housed subjects; however, no significant differences in cortisol levels or alopecia measures were found. The demonstrated beneficial effects of pair housing for rhesus macaques following intrafacility transfer and adjustment suggest pairing upon arrival at a new facility will bolster animal welfare.

Keywords

quarantine; transfer; social housing; fecal metabolites; stress; diarrhea; welfare; abnormal behavior

Introduction

Quarantine is an established practice in both human and veterinary medicine, in which a restriction of movement of people, animals, and goods is put in place to prevent the spread of disease or pests [1].

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Ethics Statement and Conflict of Interest Statement
The authors confirm that the ethical policies of this journal, as noted on the Journal of Medical Primatology author guidelines page, have been adhered to. This research was conducted under an Emory University IACUC approved protocol and in accordance with USDA Animal Welfare Regulations and the US National Research Council's Guide for the Care and Use of Laboratory Animals.

Nonhuman primates (NHPs) undergo routine quarantine procedures upon arrival at a new facility to monitor health status and prevent disease spread in established colonies. While domestic quarantine denotes animal isolation upon transfer between institutions within the United States [2], internal facility transfers from differing locations within one institution may warrant quarantine-like procedures. When rhesus macaques (*Macaca mulatta*) are received from external domestic facilities, Emory National Primate Research Center (ENPRC) quarantine entails a 90-day period which includes fecal evaluations, physical exams, blood sampling, intrapalpebral tuberculin (TB) testing carried out every two weeks, and chest radiographs, prior to release of the animals into the general population. However, when animals are transported from the ENPRC Field Station breeding facility to the main research campus, a post-intrafacility transfer adjustment period occurs over four weeks, in which physical examinations are performed and three consecutive negative TB tests are required prior to release to the general population. Although the length of the stay prior to release into the general colony differs between the two types of facility transfers, stressors associated with transport, housing changes, and novel environments are similarly in effect for these new arrivals in both domestic quarantine and in post-intrafacility transfer adjustment periods [2]. Due to these similarities, we anticipate that the effects of pair housing during this 4-week adjustment period will also generalize to domestic quarantine periods.

Within quarantine and adjustment periods, although groups of NHPs are often placed by shipment into separate rooms, there is no explicit regulatory guidance on how to house new arrivals when they enter a research facility [3,4]. While single housing of primates during quarantine procedures is used to reduce potential disease transmission between individuals and may simplify management practices, in recent years there has been a transition to pair housing NHPs from the same shipment, aiming to promote the benefits of social housing [5,6].

It is widely accepted that shipment and subsequent housing changes are stressors experienced by NHPs that can result in stress-induced behavioral, immune, and clinical pathologic changes [7–9]. The monkeys experience social changes (removal from kin or peers), environmental changes (movement from outdoor to indoor space, and into much smaller enclosures), nutritional changes (foods provided vary), human interaction changes (different people working with them, at much closer distances), husbandry changes (indoor cage washing procedures), and macro environmental changes (artificial light cycle instead of natural lighting) [9]. Reports in both rhesus [10] and cynomolgus macaques[11] have shown persistent perturbations in peripheral blood mononuclear cell, T and B cell levels and functions after transport, which can alter animals' vulnerability to infection or lead to reactivation of already latent diseases. Effects on hematocrit values, total bilirubin, and serum cortisol elevations have also been documented, and persist beyond quarantine procedures [12,13]. Prolonged alterations in these and other physiologic parameters can affect clinical and experimental outcomes, thus more investigation in this area, and ways to decrease or even prevent these changes, are warranted to reduce impact on research and health [14].

There are many documented benefits of pair housing macaques in the research setting [15]. Pair housing increases affiliative interactions, exploration, and other species-specific behaviors, and decreases the incidence of abnormal activities and self-injurious behaviors in the midst of active research [3]. Socially-housed rhesus macaques spend more time feeding and exploring, and less time performing self-directed behaviors such as excessive grooming [16]. Pair-housed individuals also experience decreased self-injurious, abnormal, and stereotyped behaviors when compared to those in single housing [17,18]. Guidelines regard social housing as the industry standard to help promote the psychological well-being and social needs of these animals. For example, the National Research Council's 2011 *Guide for the Care and Use of Laboratory Animals* specifies social housing (i.e., living in a compatible group or pair) as the default for NHPs, as a way to promote these benefits. It suggests that single housing social species should be the exception when applicable for experimental needs or veterinary medical concerns. The United States Department of Agriculture (USDA) Animal Welfare Regulations also requires that the environmental enhancement plans of institutions include specific provisions for the social needs of NHPs, which can often be met through appropriate social housing in healthy animals. There are several other international and national groups that provide guidance on pair and other forms of social housing to promote species-typical behaviors [19–21].

Compatible pair housing induces the effects of social buffering, a characteristic of social mammals whereby living among conspecifics improves recovery from stress. With social buffering, individuals experience less overall stress or recover more rapidly after a stressful event when housed with conspecifics compared to when they are not [22]. Previous work has shown positive effects of social buffering of macaques in various research settings and during long-distance transport [23]. Juvenile rhesus relocated from large and moderately-sized social settings to form a new, smaller group show fewer behavioral abnormalities if moved with at least one familiar companion compared to those moved to the smaller group alone [24]. Rhesus macaques visually exposed to the restraint and anesthesia of others in their housing room show fewer signs of anxiety when paired, compared to the reactions of those housed alone [25]. However, the impact of social buffering varies amongst species; its benefits can depend on the relationship of the conspecifics involved and the degree of stress imposed on an individual animal [22,26].

Here, we define stress as an event that threatens and can change normal physiologic and behavioral homeostasis. Inciting stressors are often stimuli that are uncontrollable, unpredictable, or novel to the subject experiencing them [27–29]. A commonly studied physiologic system that stress can alter is the hypothalamic-pituitary-adrenocortical system [30]. Changes in this system are quantified and monitored through the elevated release of cortisol. To quantify this perceived stress in rhesus, various methods of measuring cortisol can be utilized, including via sampling of plasma, saliva, urine, hair, and fecal analysis [17]. Each of these sampling methods differs in the point in time the analyte measures cortisol, invasiveness of the procedure to obtain a sample, and the accessibility of the sample while animals are pair housed. Many of the ways to assess cortisol are difficult to obtain without manipulating an animal, which may alter subsequent results [17,31–34]. We thus employed fecal cortisol measures for the evaluation of stress, which provides the least manipulation of the animal, eliminates the need for anesthesia, and reflects cortisol levels over a longer time course (24 hours) compared to other methods of collection, making it the most appropriate evaluation method for this study [31,34].

Although studies have explored the advantages of pair housing and social buffering, it is not known whether these clinical and behavioral benefits are noted and maintained after transfer, and during domestic quarantine and post-intrafacility transfer adjustments. The general clinical presentations of NHPs during this period are not well described, although immune parameters and blood work abnormalities were previously reported [10–13]. It is also unclear whether stress-related clinical abnormalities are altered through social housing, and what effects full contact pair housing has on stress while animals acclimate to new environments. To better understand the extent of social buffering and how it may effectively reduce stress, we evaluated the effects of pair housing on behavior, cortisol, and clinical outcomes after intrafacility transfer and during quarantine-like adjustment in rhesus macaques.

We hypothesized the incidence of abnormal and anxiety-related behaviors, alopecia, and fecal cortisol concentrations would be reduced among pair-housed rhesus macaques as compared to single-housed macaques. The lower incidence and a smaller diversity of abnormal behaviors displayed (Table 1), comparatively fuller hair coats, and decreased cortisol levels would demonstrate the beneficial effects of pair housing following intrafacility transfer. We hypothesized the incidence of clinical abnormalities, specifically diarrhea and weight loss, would be reduced in individuals living in pairs when compared to single-housed macaques as prior research has found that pair-housed macaques require less veterinary intervention for diarrhea than do their single-housed counterparts [35] and compatible pairings are not associated with weight loss [15,35,36]. With fewer stress-associated clinical abnormalities, we posited that clinical intervention by veterinary staff would also decrease when caring for pair-housed macaques when compared to those individually housed. We used both prospective and retrospective methods to test these hypotheses; only clinical measures were assessed in the retrospective study.

Materials and Methods

Animals

For the prospective study, subjects were male Indian-origin rhesus macaques (*Macaca mulatta),* aged 2–3 years, born at the Emory National Primate Research Center (ENPRC) Field Station (FS) (Lawrenceville, GA) undergoing intrafacility transfer to the ENPRC Main Center (MC) (Atlanta, GA) from winter 2018 through fall 2019. These individuals were mother-reared, and group housed in outdoor runs or compounds (with attached indoor enclosures) comprised of 10 to 180 macaques prior to study start. The retrospective study included male and female mother-reared rhesus macaques, matched for age and early rearing to the prospective study subjects, but who underwent similar transfer from 2014 – 2018. “Chronic diarrhea” cases, defined as any potential subjects with a previous history of more than three recorded incidents of diarrhea within a year, were excluded during subject enrollment to minimize impact on clinical assessments. Final subject counts were 40 males for the prospective study; 60 male and 60 females for the retrospective study. For the entire study, subjects totaled 160 (100 male, 60 female). Of the 40 subjects enrolled into the prospective study, 20 were single housed, and 20 were pair housed (all male). Of the 120 subjects in the retrospective study, those enrolled included 60 pair housed (30 females, 30 males), and 60 that were single housed (30 females, 30 males) during a post-intrafacility transfer adjustment period.

Intrafacility transport at the ENPRC involved relocation of animals via ground transportation in an IACUC approved climate-controlled van. Macaques were individually housed within secure transport boxes during the hour-long transport and conditions were compliant with legal requirements of local and state governments, as well as with the requirements of the USDA Animal Welfare Regulations. Transferred macaques then began the post-intrafacility transfer adjustment period and were housed for four weeks in indoor-only caged housing specifically reserved for newly transferred FS animals. Each rhesus received intradermal tuberculin skin tests every two weeks for three consecutive negative tests before release from the adjustment housing area and moved for research assignment. These procedures at ENPRC are similar to commonly accepted industry standard quarantine procedures [37].

Humane care guidelines.

All animals enrolled in this study had continuous access to fresh drinking water and had unrestricted access to commercial chow (LabDiet 5037, Purina Mills International, St Louis, MO) twice daily. Routine enrichment provided to all animals included fresh produce, foraging devices, and other manipulanda. All animals on the prospective study were research-naïve and free of simian immunodeficiency virus, simian T-lymphotropic virus, simian type D retroviruses, and herpes simian B virus. The facilities and Division of Animal Resources at ENPRC are fully AAALAC-accredited. Procedures involving all study animals were approved by the Emory University IACUC and were conducted in accordance with USDA Animal Welfare Regulations [3] and the *Guide for the Care and Use of Laboratory Animals* [1].

Pair Compatibility Assessments (Prospective)

For the prospective study, both single- and pair-housed subjects who met the inclusion criteria described above were selected from animals already destined for permanent transfer to the ENPRC MC based on other ENPRC project assignments. Housing type, including partners, were specified by FS Colony Management personnel who were not involved in the study. Housing assignments were affected by the number of animals being permanently transferred to the MC at a single time point (i.e. if five animals fitting the study criteria were transferred, only 2 pairs could be made). Pair-housed subjects were introduced via a previously described transport pairing method [38], whereby animals are moved directly from their individual transport boxes to adjoining caging, forming full contact pairings, upon arrival at MC. Due to the random matching of pairs at the time of transfer, their social compatibility was evaluated via pair intake assessments, which included 50 minutes of focal observations over the five weeks following pair formation by a trained behavioral management technician. Observations evaluated affiliative, agonistic, and neutral behaviors shared between paired macaques. Other observation (thrice-weekly checks by behavioral management staff, daily observation by veterinary technicians and animal care staff) for compatibility assessment was also conducted. Incompatible pairs were to be removed from study to ensure we were evaluating the impact of compatible pairing on the measures of interest. Of the 40 subjects enrolled into the prospective study, 20 were single housed, and

20 were pair housed; no pairs were found to be incompatible and thus none were removed from the study. Upon completion of the adjustment period and study, single-housed animals were pair housed per ENPRC Behavior Management Unit (BMU) protocols.

Behavioral Data Collection (Prospective)

Behavioral assessments were performed using a behavioral observation check sheet extrapolated from a set of operational definitions (Table 1) which emphasized abnormal and other undesirable behaviors (e.g., fear, anxiety, depression). Chow tossing was a targeted behavior to evaluate if excessive removal of normal ration by the monkey correlated with weight loss. Pan fishing was recorded to evaluate if incidence of diarrhea correlated with increased intake of items from the cage pan. Behaviors were recorded via a 1/0 sampling method with a thirty-minute interval. Each animal received a total of 16 observation time points, thus the maximum possible measured occurrence of a behavior for one subject was 16. Observation began within 24 hours of arrival and continued for 28 days, the end of the adjustment period after transport. The thirty-minute observations were performed four times per week, balanced between the morning and evening, totaling 8 hours of observation per animal, and thus 320 hours of observations total across the study. A 2-minute habituation period preceded the start of each observation period. Caging location for observations was based off availability within quarantine rooms. When possible, regardless of single or paired housing, animals on study were housed within the same rack caging system, (i.e., two over two or quadrant rack caging), thus observations could be performed on a maximum of four animals simultaneously through a single thirty-minute time interval. Interobserver reliability testing was performed for all observers prior to and periodically throughout the project. Reliability between observers was maintained at greater than 85% agreement across all 18 behaviors recorded. Any animals observed to have self-injurious or other debilitating behavioral abnormalities would be reported to BMU and veterinary staff for further intervention.

Fecal Sample Identification and Collection (Prospective)

Feces were collected from the cage pan at 7-day intervals for four weeks during the adjustment period, totaling four fecal samples per individual and 160 samples for the entire study. Collection was performed in the morning between 7:30 – 10:00 am after cages were cleaned and animals fed. A tongue depressor was used to collect a small amount of fresh feces (2–3g) uncontaminated by urine, placed in a 2ml cryovial and frozen in −80C freezer for storage until the end of the study. Lake Pigments (MakeYourOwn Chemistry Connection Co., Conway AR) were used as a method to differentiate feces between paired animals. Blue (FD&C Blue No 1 Lake 41%) and yellow (FD&C Yellow No 5 Lake 38%) pigments were given in half inch-long pieces of banana (about 150mg of pigment) 12–24 hours before fecal collection. Feces containing blue pigment was readily differentiated cage side via visual inspection. All animals in pairs received either a blue or a yellow pigmented banana. Individually housed animals also received pigmented bananas 12–24 hours prior to fecal collection to maintain uniformity in human interaction. At the time of administration, uneaten pigmented bananas were removed to prevent ingestion by cage mate and confounding fecal coloration.

Cortisol Extraction (Prospective)

Purification of fecal samples was performed in the Assay Services unit at the Wisconsin National Primate Research Center using a 2-part extraction method. First 0.1g of solid feces was suspended in 5mL of 50% ethanol by vortex mixing for 8 minutes. The suspension was then spun at 3000XRPM for 10 minutes and 4mL of liquid supernatant was collected with a 1mL aliquot undergoing solid phase extraction (SPE) and the rest being stored in a refrigerator. C18 reversed phase SPE 100mg columns were first washed with 1mL of 100% methanol followed by 1mL of ultra-filtered water on a positive pressure manifold. The 1mL aliquot of liquid supernatant was then loaded onto the column and washed using 1mL of 20% methanol. Cortisol was collected using (2) 1mL aliquots of 100% methanol (2mL total) and then the samples were dried in a water bath and reconstituted in 1mL of 100% ethanol for storage in a refrigerator until assay.

Cortisol analysis was performed at Assay Services with an in-house competitive enzyme-immunoassay using cortisol conjugated to horseradish peroxidase (F:HRP). 200uL aliquots of unknowns were dried down and resuspended in assay buffer and F:HRP then pipetted into a clear 96-well microtiter plate coated with a commercially available anti-cortisol antibody along with reference standards and quality control (QC) pools. Each sample well contained the equivalent of 1.333mg of feces. After a 2-hour competitive binding period at room temperature the plate was washed 5X in an automated plate washer and substrate solution containing hydrogen peroxide was pipetted to all wells. This produced a green color reaction that was stopped with an acid stop solution after 45 minutes and read on a Molecular Devices plate reader. The SoftMax Pro 7 data acquisition software was then used to construct a calibration curve using the reference standards and calculate the nanograms of cortisol per gram of feces for the unknowns and 2 different QC pools. Intra- and inter-assay coefficients of variation were found to be 2.04% and 1.13% and 7.99% and 9.84% respectively using the QC pools made from rhesus macaque feces and extracted in the same way as the unknowns.

Alopecia Scoring (Prospective)

To evaluate possible changes in hair coat, alopecia scoring was performed on the prospective subjects within 48 hours of arrival to the MC and within 48 hours of being released following the adjustment period. Alopecia scoring guidelines established by ENPRC BMU were followed using a 4-point system, for scoring hair coverage to determine if individuals needed further intervention for clinical evaluation or behavioral needs. Under the 4-point system, 1 = 0 to 25% hair cover, 2 = 26 to 50% hair cover, 3 = 51 to 75% hair cover and 4 = 76 to 100% hair cover. Interobserver reliability on the 4-point scale was maintained at over 85% agreement.

Prospective and Retrospective Clinical Assessments

For both the prospective and retrospective studies, clinical assessment included collecting the recorded incidence of diarrhea, trauma, and any other abnormal clinical presentation that occurred during the four-week period following intrafacility transfer. During this adjustment period, routine twice daily observations were performed by ENPRC veterinary staff and abnormalities were recorded in clinical observation room logs. Diarrhea was recorded as any incidence of abnormal, loose stool. For this study, diarrhea that occurred for more than one day was reported as a single incidence from day of onset until resolution. If diarrhea appeared to resolve, but recurred within three days, this was recorded as a separate incidence. Any injury inflicted on a subject by another monkey, and other abnormalities, including a subjective assessment of anorexia, where more than half of offered chow remained in the cage, was collected from the clinical observation room logs. Treatment, diagnostics, and necessary sedations for any clinical abnormalities were under the purview and discretion of the overseeing veterinarian.

Per routine veterinary care, ENPRC veterinary staff record daily observations in area specific clinical observation logs. This information was evaluated for the aforementioned abnormalities via weekly evaluations of logs (prospective subjects) or the computerized records system (retrospective subjects). Weights were recorded preceding transfer, at access for tuberculosis testing during the adjustment period (three measures), and after release from the post-transport adjustment. Succeeding weights were collected at the subject's next regular health surveillance testing. For abnormal clinical presentations, the date of initiation of medical treatment and monitoring performed by ENPRC veterinary staff was also reported.

Statistical Analyses

A nonparametric Wilcoxon rank sum test was utilized to determine if there were differences in the mean measured occurrence of each behavior based on housing type (single, paired). Each observed behavior was recorded as a 1 (present) or 0 (absent), thus for statistical analysis, the total number of observation sessions in which the behavior was recorded was analyzed. The maximum possible measured occurrence of a behavior for one subject was 16, the number of observations per subject. Due to the large number of tests conducted, an adjusted alpha level of 0.05 was used to determine statistical significance.

Cortisol values were evaluated via mixed model analysis with fixed effects of housing type, collection week, and interaction of housing type and collection week, along with random effects for individual animal and cage. One animal was excluded from the cortisol analysis, owing to errors in sample collections, thus 154 fecal samples were assessed. Three subjects had only three of the four samples collected but were still included in the assessment. To further evaluate relationships between behavior and cortisol, the association between individual average cortisol levels and a combined incidence of all abnormal behaviors for each subject was measured by a Spearman correlation, overall and in the subset of monkeys who displayed whole body stereotypies. In addition, cortisol levels were compared between subjects who displayed whole body stereotypies versus those who did not by a nonparametric Wilcoxon Rank-Sum test (with alpha level of 0.05).

Weights were evaluated against a weight nomogram [39] to compare normal growth rates to study subjects' weights. To assess if the subjects' weights differed over time by housing type, a single mixed effect descriptive analysis model (with alpha level of 0.05) was performed for both the retrospectively and prospectively collected weights.

A chi-square test (with alpha level of 0.05) was performed to evaluate the occurrence of diarrhea and associated medical interventions (e.g., administration of medication, fluids, probiotics) across housing type during the period of study for all subjects.

Results

Pair Compatibility Assessments

After five weeks of pair intake assessments, all pairs were deemed compatible. No pairs were removed from study due to social incompatibility.

Behavioral Measures (Prospective)

Of the 18 behaviors recorded, two (digit sucking and self-biting) were not displayed by any subject so were not analyzed, and six behaviors showed significant differences, across the two housing conditions (see Table 2). Single-housed subjects showed significantly higher incidence than pair-housed subjects of fecal-directed behaviors p=0.025, hair plucking p=0.013, hunched posture p=0.0005, other stereotyped behaviors p=0.006, self-clasping p=0.001, and whole-body stereotypies p=0.042. At alpha=0.05, floating limb and fearful behaviors did not show a significant difference between housing type but did trend higher in single-housed subjects. The behaviors that varied by social housing condition were quite prevalent in our subjects: of the single-housed subjects, 25% showed fecal-directed behaviors, 30% whole body stereotypies, 45% hair plucking, 50% self-clasping, 55% hunched postures, and 55% other stereotyped behaviors. All other behaviors were unaffected by social housing condition.

Fecal Cortisol Measures (Prospective)

Mean fecal cortisol concentrations among all subjects were 13.06 ng/g at week one, 8.07ng/g at week two, 7.82ng/g at week three, and 7.20 ng/g at week four, showing a significant decline across time (p=0.0014). There was no significant difference in mean cortisol levels between single and paired subjects, throughout the collection period (Table 3). There was no correlation detected between cortisol level and the total occurrence of abnormal behaviors (Spearman r = −0.04, p=0.80). In the six monkeys who displayed whole body stereotypies, the median (Q1, Q3) cortisol level was 8.43 (4.43, 10.55) was not significantly different then in the 33 monkeys who did not display whole body stereotypies whose median (Q1, Q3) cortisol levels were 8.14 (4.43, 10.55); Wilcoxon Rank-Sum test p=0.68.

Clinical Measures

Prospective Study Subjects: Descriptive Information—All subjects were within normal limits of the weight nomogram [39], which was between 3.355 kg (SD = 0.51) and 7.52 kg (SD = 1.295) for male subjects. All subjects had an alopecia score of “4,” indicating 76–100% of hair coverage, upon entry into and again at the end of the period of study, so no changes were detected, and no statistical analysis was conducted. There were no reports for anorexia, thus anorexia was excluded from analysis. Subsequently, the behavioral observation of “chow tossing” was removed from further assessment because our intention was to look for a relationship with anorexia. One single-housed animal incurred a minor injury from an adjacently caged animal (not in this study) who was able to reach the subject. Routine veterinary care was provided for this subject and a single dose of meloxicam (0.2mg/kg) was provided as therapy. No other wounds were reported for any other subject so wounding data were not statistically analyzed. Five of 20 (25%) single-housed monkeys had an incidence of diarrhea, all of which resolved with no medical intervention (Table 4). Three of 20 (15%) pair-housed subjects had an incidence of diarrhea, and all received medical intervention via antibiotic therapy. No subject had more than one incidence of diarrhea during the study period.

Retrospective Study Subjects: Descriptive Information—All subjects were within normal limits of the weight nomogram, which was between 3.355kg (SD 0.51) and 7.52 (SD = 1.295) for the male subjects and 3.295 kg (SD = 0.42) and 5.95kg (SD = 0.685) for female subjects. Data on alopecia was not evaluated in the retrospective study. No subjects were reported to have anorexia or trauma. Of single-housed subjects 12 of the 60 (20%) had an incidence of diarrhea; of these 12 subjects, 10 (83.3%) had medical intervention via antibiotic therapy. Regarding the pair-housed subjects, two of the 60 (3.3%) subjects had an incidence of diarrhea; one was treated with antibiotics while the other's diarrhea resolved without intervention (Table 4).

Combined Prospective and Retrospective Subjects: Statistical Evaluation— When data were combined, retrospective and prospective subjects' weights revealed a significant difference between single- and pair-housed subjects (p=0.04), with paired subjects experiencing significantly more weight gain than single-housed subjects across the study period (Table 5). When data were combined, the records of diarrhea cases revealed that 21.25% of single-housed subjects had an incidence of diarrhea compared to 6.25% of pair-housed subjects, constituting a statistically significant difference between housing types (p=0.006), depicted in Table 6. There was no significant difference across housing type for subjects requiring medical intervention (p=0.093) although there was a trend toward more intervention among the pair-housed subjects.

Discussion

In general, our findings supported hypotheses that abnormal behaviors would be reduced and that there would be improved health measures in pair-housed rhesus macaques subjects when compared to those single-housed following an intrafacility transfer. There was an increased occurrence of some abnormal behaviors in the single-housed, juvenile rhesus macaques compared to pair-housed counterparts during this 28-day adjustment period. Behaviors indicative of anxiety did not vary across the social housing condition. However, a hunched posture behavior, shown to be associated with depression [7,40] was expressed in more single-housed subjects than paired, and there was a trend toward more fear-related behaviors among the single-housed subjects. No significant difference was found in fecal cortisol concentration between subjects in the two housing types, or in extent of hair loss. At the end of the four-week adjustment period, pair-housed monkeys gained more weight and had experienced significantly fewer occurrences of diarrhea compared to their single-housed counterparts, which is in support of our hypotheses. Although our results did not support the supposition that cortisol levels would be decreased in paired animals, results did show a significant decline in cortisol levels from the start to end of the adjustment period.

Behavior

Behaviors recorded significantly less often among paired subjects included feces-directed behaviors, hair plucking, hunched posture, stereotypies (both whole body stereotypies and other types), and self-clasping (Table 2).

Feces-directed behaviors, such as fecal smearing, in captive NHPs are often attributed to lack of environmental stimulation and this can be perpetuated in animals that are single housed who have reduced opportunities for social interaction and engagement [41]. The higher incidence of fecal behaviors in our single-housed animals may reflect this decreased opportunity for stimulation. Although monkeys enrolled in this study had access to enrichment devices (e.g., KONG® toys, foraging devices) within standard ENPRC enrichment practices, compatible pair housing provides additional opportunities for activity and engagement that cannot be replaced by inanimate objects, underscoring the value of social housing in quarantine settings.

Our assessment showed a significant difference in the display of a hunched posture by monkeys between housing types. This presentation, where the macaque is inactive, seated or slumped in a fetal-like position (unrelated to sickness), has previously been determined to be a physical manifestation of depression [7,40,42]. In humans, depression can have a myriad of effects on biological systems, including and not limited to inflammatory, neuroendocrine, and metabolic pathways [43]. Reports of weight loss, anhedonia, hypercortisolemia, and positive response to anti-depressant therapy in rhesus displaying a hunched posture further exemplify physiologic and clinical changes that can occur with depression [44,45]. Beyond the welfare implications [7,40,46], the associated physiologic changes create an unstable animal model that may compromise data collection for research studies, especially if these changes occur during quarantine and adjustment periods prior to study start.

In this study, hunched posture onset presented on average of 6.7 days into the adjustment period and had a prevalence rate of 55% in single-housed animals compared to 0% in those paired. Comparatively, previous studies have shown rates of 18.9% in single-housed rhesus (61% of which were adult males) [40] and between 38–45% in adult female rhesus macaques for the subordinate individuals within pairs exposed to chronic low-social status [47]. The higher prevalence in single-housed animals in the current study, in addition to absence of hunched postures in pairs, suggests the effects of social buffering [25] may be more active and beneficial in quarantine settings or after housing changes, and may indicate a need for additional social support during these times for younger juvenile macaques like our subjects.

In this study, whole body stereotypies (e.g., flipping, pacing, circling), other stereotypies (i.e., skin pulling and tail grasping), and self-clasping had a significantly higher occurrence in single-housed animals. These behaviors are often associated with “isolation syndrome” (a syndrome resulting from infant rearing in complete isolation leading to poor development of normal facial expression, motor responses, heightened emotional responses, and inability to build positive social relationships [48]), younger ages, and nursery rearing [18,48,49]. Both whole body stereotypies and self-directed abnormal behaviors are postulated to be coping mechanisms, in that the completion of the behavior has anxiolytic effects for better handling stressful events [48,50]. The occurrence of these behaviors during the adjustment period exemplifies the stress associated with post-transfer procedures and environmental alterations. Our findings also corroborate previous reports of these behaviors occurring less in pair-housed animals compared to those singly housed when undergoing stressful scenarios [16,18,22,23,25].

Single-housed animals also had significantly increased incidence of hair plucking. Hair plucking and associated behaviors in single-housed animals such as ingestion of pulled hair or hair pulling without removal are reported to be a result of poor environmental stimulation, similar to fecal behaviors, and can be reduced or ameliorated with increased opportunities for exercise, foraging, and social interaction [51,52]. While hair plucking is an abnormal behavior that can lead to self-induced alopecia [53], we saw no difference in hair scores in any animal during the study period. Although no alopecia difference was noted, previous reports show social housing can have positive effects on animal hair coat quality [54], suggesting that our findings of hair plucking in single-housed subjects may lead to coat abnormalities in the future.

For alopecia scoring, all subjects received a baseline and post-adjustment hair score of “4,” which indicated 75–100% hair coverage. Although no change in hair score was observed, the general sensitivity of this measure is not high, as animals could potentially lose 24% of their hair coverage while still retaining the same score. Although there is a close relationship between alopecia and hair plucking, our findings add to reports that have found varying correlations between hair loss and hair plucking behavior. For example, one study found hair pullers are more likely to have alopecia, however hair plucking did not explain the alopecia found in a significant portion of their study group (57–69% of animals with low hair coverage did not hair pull) [54]. This low level of alopecia associated with hair plucking also correlates with low percentages (4–7%) of hair plucking associated with alopecia in laboratory housed rhesus in another report [52]. The relationship between stress and alopecia still warrants further investigation, as both positive and no correlation between stress indicators and alopecia have been reported [53]. Although there are several other factors that come into play with hair loss, hair plucking, and stress [34,51–53], increased incidence of hair plucking among single-housed subjects may still lead to notable hair loss over time and warrants further evaluation if apparent within quarantined animals.

Although no significant difference was found between housing types, the floating limb behavior trended higher in our single-housed animals. Previous reports have found floating limb behavior in rhesus correlates with an increased occurrence of self-injurious behaviors [18,55–57]. This progression may be associated with an animal’s reaction to the elevated limb as the behavior is performed: whether they ignore the limb or react to it (likely by attacking or biting). In one retrospective analysis consisting of a 3-year data set, researchers found 87% of monkeys who displayed floating limb also displayed self-biting behaviors, and regardless of immediate reactivity to the raised limb, a substantial proportion of monkeys displaying floating limb behaviors eventually did perform self-biting [55]. No self-injurious or self-biting behaviors were recorded in our study. Because of the close relationship between floating limb and self-injurious behaviors, closer behavioral monitoring should be performed on animals displaying floating limb behaviors during quarantine procedures and as those monkeys age.

Self-clasping, a self-directed behavior in which the subject clutches their own body with their hands or feet, was also found to show a significant increase in occurrence in single-housed subjects. This behavior is often associated with juveniles in isolation rearing as well as those who have undergone various nursery rearing strategies [58]. Including this behavior, there are a host of behavioral changes and abnormalities that are often associated with nursery rearing [59]. Although all our targeted behaviors did not show significant differences based on housing type, it is worth noting that we also recorded bizarre posturing and eye behaviors (poking, pressing, saluting). These behaviors, including self-clasping, are generally associated with nursery rearing and long periods of single housing and social isolation [18,58,60]. The display of these abnormal behaviors in this subject pool is somewhat unexpected, as the rhesus macaques selected were mother-reared until at least two-years-old and experienced minimal single housing (besides singular clinical interventions) prior to this study. These behaviors presenting during the adjustment period (including self-clasping displayed in half of the single-housed subjects) indicates there are other factors contributing to the onset of their occurrence, such as being secondary to transfer stressors or short-term, dramatic social (i.e., removal from group housing) and environmental (e.g., indoor housing, increased human interaction) changes. Although long-term effects of social restriction on NHP abnormal behavior have been evaluated [18,49], further research on the onset of presentation for bizarre posturing, eye behaviors, floating limb, self-clasping, and hunched posture are needed to better understand how quickly these abnormal behaviors can develop following a short-term psychosocial stressor.

At least once during the adjustment period, all animals showed anxiety behaviors (i.e., scratching, yawning, teeth grinding, body shaking) while 67.5% showed fearful behavior (e.g., crouching, freezing, fear grimace). These categories of behavior may indicate distress in rhesus macaques [5], thus these findings support the notion that the transfer and changes occurring during the adjustment period are stress-inducing. Across the two housing conditions, there were no significant differences in the display of anxiety or fear behaviors, but fear trended higher in single-housed animals (p=0.0989), suggesting possible social buffering in coping with fear among pair-housed animals.

One limitation in this study's behavioral assessment includes the nature of the behavioral data collection method itself. Observations were performed with a 1/0 sampling method on a maximum of four animals simultaneously through a single thirty-minute time interval. Although one-zero sampling methods do not provide information on how often or for how long a behavior occurs during the interval selected, they do enable simpler recordings for behaviors of interest and improved interobserver reliability when multiple individuals are conducting observations[61]. In this study, observations occurring on more than one animal were performed. This removes the potential assumption of independence for these observations. However, in this study the data was treated as statistically independent for each individual animal. This data collection method was developed for the routine behavioral management program to detect and distinguish animals of concern. While not the best practice for data analysis, this method does provide information to evaluate the contrasts in behavior between the two housing conditions assessed.

Cortisol

There was no significant difference in fecal cortisol levels between single-housed and pair-housed subjects which did not support our hypothesis. These findings echo that from a previous report, in which plasma cortisol was used to assess effects of housing condition in juvenile (about 2–3 years old) rhesus macaques after periods of single housing and pair housing. A dexamethasone suppression test was used to evaluate plasma cortisol levels, and no difference was found between housing conditions [62]. Although the sampling methods differ, the outcomes are consistent across the two studies. However, other studies have found that fecal cortisol levels were reduced in pair-housed compared to single-housed monkeys. One study reported significantly lowered levels in settled pairs of socially-experienced, biotelemetry-implanted adult males who were introduced to one another compared to when they were living individually [63]. Another report showed significant decreases in plasma cortisol levels for a within-subjects study of juvenile female rhesus that were initially in a free-ranging setting, single-housed for a year, and then brought to stable social group after single housing. During individual housing, females showed downregulation of cortisol, but increases in other hormonal measures such as prolactin [64]. The variation in findings may be a result of how cortisol is measured [17,63], or age (juvenile vs adult), sex (female vs male), and prior housing condition (animals indoor cage-housed in a research setting vs wild caught vs, as in our case, captive mother-reared social groups) differences across studies, all of which could influence stress perception [17,65]. Baseline cortisol assessment was not performed in the current study and a comparison of such may be warranted to further evaluate changes in cortisol in our study population after intrafacility transfer. It is also possible that an underlying confound of available space may have affected our results. Previous reports have noted that increased exercise and movement, leading to hypothalamic activation and increased corticotrophin-releasing hormone excretion and the subsequent release of cortisol [63–65], can contribute to increase in fecal cortisol levels. In socially-housed rhesus, generally there is more activity, play, and movement compared to those in individual housing [15,66], and in this case, they also had additional cage space, which can contribute to more movement opportunities [1]. In this study, all animals were housed in caging in accordance with both federal regulations and AAALAC recommendations, but those in pairs had access to double the floor space of single-housed animals due to the combined floor space of two cages. The possibility that exercise-induced increases in cortisol levels occurred in paired-housed, while stress-related increases occurred in single-housed subjects, could contribute to our finding of no difference between the groups. In future assessments, equal floor space should be provided to remove this confound.

Here, our cortisol findings showed no association with our behavioral findings; there was a significantly higher occurrence of abnormal behaviors, as well as higher prevalence of fear, in single-housed animals whilst no significant difference in cortisol levels between housing type was found. We found no relationship between individuals' cortisol levels and their total occurrence of abnormal behavior. Reduced cortisol values among those showing stereotypy would be predicted by the hypothesis that stereotyped behavior in a sub-optimal environment may serve a coping function through one of several routes, as proposed by Mason and colleagues [66,67]. However, our measure of stereotyped behavior was fairly simplistic (without true frequencies or durations of the behavior, and over a short period of time), so this result should be interpreted cautiously.

In this study, the significant decline in fecal cortisol levels across both housing types throughout the adjustment period further supports other recommendations for periods of acclimation and conditioning to a new environment after major changes and prior to research manipulation [1,9,14,21].

Clinical Assessments

To the authors' knowledge, this is the first report assessing the clinical outcomes across two social housing strategies during a post-intrafacility transfer period in rhesus macaques. Clinical results partially supported our hypotheses as pair-housed animals were less likely to have a bout of diarrhea compared to single-housed animals, but there was no statistically significant difference in veterinary intervention (e.g., administration of medication, fluids, probiotics, or other treatment modalities) between housing types indicating that the single-housed subjects were not ill enough to require more care. This may be attributed to more prevalent self-limiting bouts of diarrhea in our single-housed animals, compared to longer standing (> 4 days) diarrhea cases in some subjects who were paired. Although all animals with diarrhea did not have fecal examination or culture performed, most with longer standing diarrhea were found to have enteric pathogens requiring treatment. Further assessments evaluating microbial composition of diarrhea cases was beyond the scope of this report but could be evaluated in differentiating pathogenic versus stress associated alterations leading to diarrhea in recent transfer situations in future studies. Diarrhea is a useful marker of comprehensive health as it is one of the leading causes of morbidity in captive NHPs [68–72]. Outside of infectious entities, reports of antemortem idiopathic etiologies of diarrhea in association with stress and anxiety have been reported [68,73] and in stressful situations, such as housing changes and quarantine, are hypothesized to increase in occurrence. With social housing often improving behavioral outcomes [74], here we show that positive effects of clinical outcomes are also possible, with only 6% of pair-housed subjects having diarrhea, compared to 21% of those individually housed.

We assessed "pan fishing" behavior (exploring and foraging in the waste pan under the monkey's cage), to determine if increased intake of items from the cage pan (which may be fecal contaminated) would be associated with an increased occurrence of diarrhea. Between both housing groups, all animals except one displayed pan fishing; there was no significant difference across housing type and no association between occurrence of pan fishing and diarrhea. However, with a small sample size within the prospective subject group who had an episode of diarrhea and pan fishing observed (n = 8), additional targeted assessment of autoinfection and diarrhea in association with this behavior may warrant further investigation in captive social settings.

Our results indicated that pair-housed animals' weight gain was significantly higher than those in single housing. Due to our subject groups' ages, weight gain is expected [39], so in this study we were interested in evaluating if maintaining expected weight gain would be more likely in those who were paired rather than single housed due to the reduction in stress and associated diarrhea hypothesized to occur with pairing [68,69,71]. Although psychosocial stress has been linked to obesity and emotional feeding in adult rhesus [75], weight fluctuations in association with stress and laboratory housing in juvenile macaques warrants further investigation. Here, although animals in both housing types were within the institutional nomogram of weight, with greater weight gain, individuals in pairs are more robust for research assignment and manipulation. Various research protocols and procedures can result in weight loss, thus animals starting studies with higher weights that are still within a normal body condition provides for a more clinically stable animal model.

In summary, the present study shows that rhesus macaques between 2–3 years old have better clinical and behavioral outcomes when undergoing post-transport adjustment periods in pairs rather than being housed alone. The presence of better behavioral outcomes illustrates active social buffering in the face of quarantine-like adjustment periods. Increased behavioral abnormalities associated with depression, fear, and social restriction increased in single-housed animals presents both a welfare concern and study variant that should be addressed through social housing modifications [41,48,62,74], especially during acclimation procedures such as quarantine. The decreased clinical abnormalities and increased weight gain in individuals who were paired presents a healthier study model for active research. Although studying the effects of social housing on longer domestic and international quarantine periods is warranted, with our current findings we propose that social housing during quarantine should be more routine in support of both the psychologic and physiologic welfare of rhesus macaques.

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Data Availability Statement

Research data are not shared.

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Table 1:

Behavioral Ethogram

Behavior	**Definition**
Anxiety	Behaviors associated with a state of stress or uneasiness (unlike fear, may not be in response to an identifiable trigger); Include scratching, yawning, teeth grinding, body shaking
Fear	Behaviors associated with escape or avoidance. Fear behaviors are associated with a specific triggering stimulus (e.g., a threat from another primate); Include fear grimace/bared-teeth grin, crouching, freezing, cringing, bracing against cage walls
Abnormal Behaviors	**Definition**
Bizarre posturing *	Holding seemingly uncomfortable or unnatural positions; ex. hind leg extensions
Digit sucking	Oral behavior marked by lips being sealed around a digit (finger or toe). Cheeks may be seen moving during sucking.
Other self-injurious behavior	Any self-injurious behavior not otherwise specified by this list (self-slapping, head banging, etc.)
Other stereotypy *	Ritualistic behaviors not involving locomotion that are not included in another abnormal behavior category; include but not limited to skin pulling, tail grasping
Eye behaviors	Movement directed at an animal's own eye and/or eyelid, including saluting, eye covering, and eye poking
Feces-directed behavior	Smearing or rubbing fecal material onto a surface; coprophagy
Floating limb	Subject raises arm or leg in the air, with the appearance of no control of the arm or leg
Hair pluck	Pulling out/removing one's own hair, may or may not be followed by ingestion of the plucked hair
Hunched posture	Animal is inactive and seated in a slumped or fetal-like position with head at or lower than shoulder level, back rounded, shoulders forward, and limbs drawn toward center of the body. This behavior is only recorded if the animal's eyes are open or if you are unable to determine whether the eyes are open. If the eyes are closed, score this as inactive (which includes sleeping). Self-clasping (clutching of one's own body with hands or feet) is scored separately.
Regurgitation & re-ingestion	Animal regurgitates/vomits food or liquid and ingests vomited material
Self-bite	Rapidly biting oneself with force; may or may not break the skin
Self-clasp	Clutching of one's own body with hands or feet; This is scored separately from hunched posture;
Urine drink	Urophagia; licking or sucking of urine either directly from the penis or pooled on a surface; urine must be visible to be scored (i.e., penis sucking is scored as a self-directed stereotypy)
Whole body stereotypy *	Repetitive behavior patterns involving whole body movements that do not serve an obvious function; this behavior is recorded after 3 repetitions (flipping, pacing, circling, rocking, swaying)
Miscellaneous Behaviors	**Definition**
Pan fishing	Manual (hand) manipulation or investigation of items inside pan followed immediately with hand, fingers, or item retrieved from the pan touching the mouth or tongue (fecal content involved scored as "feces" behavior)
Chow tossing	Manual (hand) depositing of chow outside of the cage

* Behavior recorded only after occurring for 3 seconds or more

Table 2:

Comparison of behavior occurrence by housing type

Behavior	Single Housed (N=20)	Pair Housed (N=20)	p-value
Anxiety	15 (14.5,16)	15 (13,16)	0.646
Fearful	1 (0.5,4)	1 (0,1)	0.0989
Abnormal Behaviors			
Bizarre posturing	0 (0,0)	0 (0,0)	0.2933
Digit suck	\	\	\
Eye behaviors	0 (0,1)	0 (0,1)	0.912
Feces-directed	0 (0,0.5)	0 (0,0)	**0.025**
Floating limb	0 (0,0)	0 (0,0)	0.088
Hair pluck	0 (0,2)	0 (0,0)	**0.013**
Hunched posture	1 (0,2)	0 (0,0)	**0.0005**
Other self-injurious behavior	0 (0,0)	0 (0,0)	0.089
Other stereotype	0 (1,1)	0 (0,0)	**0.006**
Regurgitation & re-ingestion	0 (0,0)	0 (0,0)	0.349
Self-bite	\	\	\
Self-clasp	0.5 (0,1)	0 (0,0)	**0.001**
Urine drink	0 (0,0)	0 (0,0)	0.931
Whole body stereotypy	0 (0,1)	0 (0,0)	**0.042**
Miscellaneous Behaviors			
Chow tossing	0 (0,1.5)	0 (0,0)	0.179
Pan fishing	7 (3.5,10.5)	3.5 (2,8)	0.085

Median (Q1 Lower Quartile, Q3 Upper Quartile) and p values are reported for Wilcoxon rank sum test

\ Denotes absence of behavior (none displayed in either housing setting)

Table 3:

Average cortisol levels (ng/g) at each collection week compared by housing type

Week	Single Housed (*N=19) Mean (SE)	Pair Housed (N=20) Mean (SE)	Mean difference (95% CI)	p-value
1	13.45 (1.71)	12.72 (1.70)	0.73 (−4.05, 5.51)	0.7610
2	7.99 (1.71)	8.18 (1.70)	−0.20 (−4.97, 4.57)	0.9350
3	6.49 (1.71)	9.12 (1.70)	−2.63 (−7.40, 2.14)	0.2774
4	6.94 (1.85)	7.47 (1.79)	−0.52 (−5.63, 4.59)	0.8401

From mixed model with two random effects (animal identification and cage) and three fixed effects (housing, week, week with visit), mean difference (SE) and p-value for single-housed versus pair-housed subjects are reported.

*The observation of one animal is excluded due to sample collection error.

Table 4:

Raw data for diarrhea occurrence separated by subject group

Prospective Subjects with:	**Single Housed (n=20)**	**Pair Housed (n=20)**
No diarrhea	15	17
Diarrhea	5	3
Medical intervention	0	3
Retrospective Subjects with:	**Single Housed (n=60)**	**Pair Housed (n=60)**
No diarrhea	48	58
Diarrhea	12	2
Medical intervention	10	1

Table 5:

Weight change compared by housing type

Collection Period (i.e., week 1 to week 2)	Single Housed (N=80) Mean Weight Change in kgs (SE)	Pair Housed (N=80) Mean Weight Change in kgs (SE)	Mean Difference Between Single and Paired Weights (SE)	p-value
1–2	0.28 (0.16)	0.42 (0.16)	−0.13 (−0.26, −0.01)	0.06
2–3	0.21 (0.16)	0.22 (0.16)	−0.02 (−0.15, 0.12)	0.82
3–4	0.17 (0.15)	0.20 (0.16)	−0.03 (−0.17, 0.11)	0.68
4–5	0.39 (0.16)	0.50 (0.16)	−0.12 (−0.25, 0.02)	0.10
Overall	0.26 (0.08)	0.34 (0.08)	−0.07 (−0.14, −0.00)	**0.04**

From mixed model with two random effects (animal identification and cage), mean difference (SE) and p-value for single-housed versus pair-housed subjects are reported.

Table 6:

Evaluation of diarrhea and medical intervention among all subjects (prospective and retrospective combined)

Covariate	Level	Single Housed (N=80)	Pair Housed (N=80)	p-value
Incidence of diarrhea	No	63 (78.75)	75 (93.75)	**0.006**
	Yes	17 (21.25)	5 (6.25)	
Medical intervention	No	70 (87.5)	76 (95)	0.093
	Yes	10 (12.5)	4 (5)	

The parametric p-value is calculated by chi-square test.