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Measurement of Fecal Corticosterone Metabolites as a Predictor of the Habituation of Rhesus Macaques (*Macaca mulatta*) to Jacketing

Amy E Field,[1,*] Cynthia L Jones,[1] Richard Kelly 3rd,[1] Shannon T Marko,[1] Steven J Kern,[2] Pedro J Rico[1]

Jacket use in NHP is a common practice and is often considered a form of refinement during experiments necessitating extended periods of catheterization. An important consideration when using jackets is the physiologic effects that jacketing has on NHP and its potential to confound research. Several studies have evaluated the stress response and habituation of NHP to various forms of restraint, but none have looked directly at the timeframe necessary for the habituation of rhesus macaques (*Macaca mulatta*) to jackets. We set out to determine whether 3 d was a sufficient timeframe for this species to become habituated to a jacket, with or without an undershirt, by evaluating 2 major physiologic parameters. After jacket placement, we measured food consumption and collected fecal samples to measure fecal corticosterone metabolites (FCM) daily for 2 wk. FCM measurements for NHP without undershirts were significantly increased for days 2 and 3 after jacketing before returning to baseline levels. FCM measurements for NHP with undershirts were significantly increased for only 1 d after jacketing, suggesting that the undershirt has a positive effect on jacket habituation. There were no measurable differences in food consumption during the jacket habituation period. Furthermore, no significant differences were noted between sexes. These findings suggest that FCM levels return to baseline 3 d after jacketing and could be a useful predictor of jacket habituation in rhesus macaques.

Abbreviation: FCM, fecal corticosterone metabolites.

The 1985 amendments to the Animal Welfare Act through the 2011 edition of the *Guide for the Care and Use of Laboratory Animals* demonstrate expanding emphasis on promoting the behavioral wellbeing of NHP in laboratory research.[1,21] However, there is still much to be learned on the topic of procedural habituation and adaptation of NHP to routine husbandry and experimental procedures, such as jacketing and tethering. The *Guide*, which carries regulatory weight in the United States, stresses the necessity of habituation but remains unclear on how this can be accomplished. The *Guide* states that habituation should be encouraged whenever possible but that the type and duration of habituation necessary will be determined by the procedure's complexity.[21] The European Commission's Directorate on the welfare of nonhuman primates also emphasizes the beneficial effects of adapting animals to experimental routines but, like the *Guide*, remains vague on the methods of adaptation.[35] Although jacketing is a relatively common practice in the laboratory setting, few published data outlining optimal practices regarding jacket habituation in rhesus macaques are available. Often, individual investigators in consultation with the veterinarian and IACUC develop their own guidelines and standard operating procedures on habituation timeframes. These timelines typically are based on existing empirical literature, other facilities current practices', and the investigators' personal experiences.[34]

Given the general paucity of empirical literature on the subject of jacket habituation, the Association of Primate Veterinarians recently released a set of guidelines for jacket use in NHP. The guidelines state that animals should be habituated to wearing a jacket and tether at least 1 to 2 wk prior to study initiation and that, in most cases, whether an animal will tolerate a jacket (that is, no signs of maladaptive behavior) is apparent within the first 24 to 48 h.[2] Although these guidelines do not carry the regulatory weight of the *Guide*, they can be very useful in developing NHP jacketing procedures and appropriate habituation timeframes within a facility. Even given these guidelines, selecting an appropriate timeframe after which the majority of NHP can be considered confidently to be habituated to jacketing is still extremely challenging, in light of differences in species, sex, and experimental variables.

At our institution, jacket placement prior to indwelling intravenous catheterization in NHP has become increasingly more common as a routine procedural practice. As characterization of appropriate animal models continues to progress and improve, many animal studies require frequent blood sampling for continual evaluation of host immune responses as well as identification of biomarkers of infection. Frequent blood sampling often necessitates the use of an indwelling catheter in an effort to avoid the confounding variables associated with repeated restraint and sedation. The placement of an indwelling catheter in an NHP requires placing the animal into a nonrestraining jacket, which is designed to protect the catheter from interference from both the cage environment and the animal itself.[16] The catheter then is routed through a tether system which is attached to the cage wall. This practice allows flexibility in animal movement within the cage and enables the investigator to perform repeated blood collections without having to restrain or anesthetize the NHP.[22,32]

The catheter–jacket–tether system is used as a method of refinement to reduce the pain and distress of NHP. The system

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[1]Veterinary Medicine Division (VMD), and [2]Statistician, United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, Maryland.
[*]Corresponding author. Email: amy.e.field.mil@mail.mil

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is intended to eliminate the effects that anesthetic use and capture-related stress have on physiology, activity, and appetite, which are key clinical assessments in many infectious disease models.[16] However, it is important not to discount the physiologic stress exhibited by the animal, due to jacket placement, with regard to altering these parameters. The National Research Council defines stress as the effect of external and internal factors (stressors) that can cause changes in biologic equilibrium.[20,31] Such stress induces an adaptive response in an effort to return to baseline physiologic parameters or a previous mental state. This adaptive response is manifested by changes in physiology, psychologic state, and behavior.[31] For many species, efforts to systematically habituate or desensitize subjects to procedures during the pre-experimental acclimation period actually may increase the variability in experimental data.[34] Stress responses and habituation of NHP to various forms of restraint have been studied extensively.[4,12,31-33,40] However, no study has looked directly at the physiologic parameters of fecal corticosterone metabolites (FCM) and food consumption with regard to determining the timeframe after which rhesus macaques can be considered habituated to jacketing. Habituation to jacketing must be established before data collection can begin, thus eliminating a key confounding variable related to physiologic stress.

Chronic stress can be defined as a constant and long-term exposure to a stressor. Many components of the physiologic system can be measured in an effort to evaluate the stress response, including the hypothalamic–pituitary–adrenal axis (that is glucocorticoids), cardiovascular mediators such as catecholamines, immune factors such as cytokines and lymphocytes, as well as behavioral and CNS changes. Unfortunately, many of these stress parameters are difficult to obtain without directly manipulating the animal (that is, restraint for sedation or evaluation, blood collection for immunologic parameters) or, at a minimum, physically interacting with the animal, which may directly affect the stress values. In the research setting, there are also a variety of ways to specifically evaluate glucocorticoid levels, many of which include directly measuring cortisol values through the collection of serum, urine, feces, saliva, or hair. Glucocorticoid values are one of the most frequently evaluated biomarkers of stress in vertebrates, and the change in these values over time can be indicative of an animal's response to prolonged stressors, such as changes in their social or physical environments.[5,9,18,19,26,41] Upon exposure to a stressor, the hypothalamic–pituitary–adrenal axis is activated, leading to the secretion of hormones such as glucocorticoids in amounts proportional to the magnitude of the perceived threat.[33] More importantly, chronically elevated glucocorticoid levels can markedly affect metabolism and even lead to suppression of the immune and reproductive systems.[14,27,29] Furthermore, long-term increases in glucocorticoid levels have been associated with hyperglycemia and neuronal cell death.[14]

Another important consideration in the type of collection method chosen is the timeframe that the researcher is trying to capture. For example, serum cortisol samples reflect circulating steroid levels integrated over seconds to hours.[5] In comparison, urine and feces reflect circulating steroid levels over several hours to days, whereas hair integrates steroids over the entire period of hair growth which can be months to years.[5] Fecal glucocorticoid assays are reliable in detecting endogenous changes in the adrenal activity of a variety of species.[18,19,41] Lee and colleagues evaluated fecal glucocorticoid changes in response to anesthetization in rhesus macaques; in that study, fecal glucocorticoid metabolite levels were first elevated at 26 h and peaked at 38 h, before returning to baseline at 48 h.[24] In another study comparing the metabolism and excretion of cortisol in 3 NHP species, including long-tailed macaques (*Macaca fascicularis*), peak levels of radioactivity in urine were recovered at 5.5 h, whereas peak levels in feces were recovered within 26 h.[2] In an effort to evaluate stress levels that most closely reflect changes over the course of a 24-h period and with the least amount of physical interaction with animals, we chose FCM as the primary means of evaluating physiologic stress in NHP after jacketing.

Historically, jacketing of NHP in our facility has been performed at least 3 d prior to indwelling catheter placement by using a standard double-layer, nylon-mesh primate jacket (Lomir Biomedical, Malone, NY) without an undershirt. The present study was undertaken to determine whether this 'habituation timeframe' of 3 d is sufficient for rhesus macaques to be considered habituated to a jacket in preparation for catheter surgery and entry into a study. To assess jacket habituation, we assessed 2 key physiologic parameters, FCM and food consumption. In addition, in conjunction with another study evaluating lesion incidence in NHP wearing primate undershirts (beneath jackets),[23] we evaluated whether the presence or absence of this undershirt had an effect on FCM values and food consumption. Determination of the optimal time course for jacket habituation would aid in stabilizing NHP prior to study initiation and increase confidence in the validity of the research data being collected.

Materials and Methods

Subjects. A total of 20 (10 female, 10 male) experimentally naïve, Chinese-origin rhesus macaques (*Macaca mulatta*; age, 3.5 to 7 y; weight, 4.8 to 7.8 kg) were used for this study. Each NHP received a baseline health assessment, including a CBC count and blood chemistry, and was determined to be clinically normal on physical examination. All animals were seronegative for measles virus, *Macacine herpesvirus* 1, SIV, and simian T-cell leukemia virus. All animals were negative for *Mycobacterium tuberculosis* by tuberculin skin test at least 6 mo prior to the study. To ensure the applicability of results to Animal Biosafety Level 3 and 4 environments, an exemption for partial- or full-contact housing was approved by the IACUC in light of the anticipated stress of permanent social separation from a cagemate, the nature of the diseases studied, and safety and sanitation concerns. All macaques arrived at our facility from Worldwide Primates (Miami, FL) at least 1 y prior to study initiation. Two weeks prior to the start of the study, all animals were transferred to the same room and singly housed in 4.5-ft^2 cages with 4 cages per rack (Allentown Caging Equipment, Allentown, NJ), with visual and auditory contact with conspecifics at all times. A single caretaker was trained to monitor food intake and was placed in charge of the room for the 2 wk prior to and throughout the duration of the study. Environmental conditions were maintained as recommended in the *Guide for the Care and Use of Laboratory Animals* (temperature, 68 to 72 °F; relative humidity, 30% to 70%; and 12:12-h light:dark cycle).[21] Animals were fed once daily (at 0800) with a commercial primate diet (2050 Teklad Global 20% Protein Primate Diet, Harlan Laboratories, Frederick, MD). Fresh water chlorinated and filtered at the municipal level was provided ad libitum via an automated watering system (Edstrom Industries, Waterford, WI). Due to the necessity of close monitoring of food intake, supplemental food enrichment was not provided. Each NHP was provided 2 toys at all times in its cage as a form of physical enrichment, according to an established schedule of toy enrichments (Challenge Ball, Kong, football, and Dental

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Star [Bio-Serv, Frenchtown, NJ]) as outlined by our institute's husbandry and care program.

Humane care guidelines. All research was conducted under an IACUC-approved protocol in compliance with the Animal Welfare Act, PHS policy, and other federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by AAALAC and adheres to the principles stated in the *Guide for the Care and Use of Laboratory Animals*.[21]

Health assessment and grouping. A qualified veterinarian or veterinary technician conducted baseline health assessments which included a physical exam, CBC count, and serum chemistry on all NHP one week prior to study initiation. Upon completion of the baseline evaluations, all animals were matched by sex and weight (± 0.3 kg) for statistical purposes with equal numbers of female and male macaques in each group. From each statistical match, one macaque was randomly assigned to be fitted with a jacket only (group A), whereas the other matched animal was assigned to be fitted with both a jacket and undershirt, which was designed to be placed beneath the jacket (group B; Figure 1). All NHP in both groups were fitted for jackets (Lomir Biomedical, Notre-Dame-de-l'Île-Perrot, Quebec, Canada) according to the recommended weight ranges for Rhesus macaques provided by the manufacturer. The jacket sizes ranged from medium to extra-large and were confirmed to be the correct fit by ensuring that a caregiver could place 2 fingers between the jacket and skin at the neck, armpit, and waist regions. This study was conducted in collaboration with another study evaluating jacket-associated lesions, wherein all NHP were sedated once each week and jackets were assessed regularly for appropriate fit, signs of damage, and cleanliness.[23]

Experimental design. Baseline measurements for daily food consumption and FCM sample measurements were obtained 4 times prior to jacketing, including once on each of the 3 d prior to jacketing and the morning of jacketing. On the morning of jacketing (day 0), food was withheld, and any food from the previous day was removed from the cage. Jackets were placed on the macaques in the afternoon, approximately 3 h after the fourth (and final) baseline observation. For jacketing, all NHP were anesthetized with ketamine (3 to 10 mg/kg) and dexmedetomidine (0.02 to 0.05 mg/kg) intramuscularly. During recovery, atipamazole (0.2 to 0.5 mg/kg) was given intramuscularly. All daily fecal collections were performed prior to any sedation event in an effort to avoid alterations in stress responses due to sedation of the NHP.

All animals were fed 12 biscuits plus one piece of fruit, placed directly into the cage, once daily. All fruit was consumed completely, thus daily food consumption was determined by counting the number of biscuits remaining in either the cage or pan under the cage every morning between 0700 and 0730, prior to biscuit and fruit removal and cage cleaning. All animals were scored for appetite: 1, at least 8 biscuits consumed; 2, a total of 4 to 7 biscuits eaten; 3, no to 3 biscuits eaten.

Daily fecal samples were collected from all animals at the same time each morning, approximately 1 h after the morning feeding and cage cleaning. Samples were collected for 14 d after jacketing, except day 6, when NHP were fasted and sedated for a thorough jacket-fit assessment. The technician was instructed to use a tongue depressor to collect a small amount of the freshest feces (approximately 2 to 7 g) that was not contaminated with urine, from directly within the cage or below the cage (in the pan). All individual samples were collected into a prelabeled plastic bag, placed into a freezer (−7 to −10 °C) for storage until the end of the study, and then submitted for FCM testing to the

Figure 1. Primate undershirt and jacket

Smithsonian Conservation Biology Institute, where samples were individually freeze-dried, crushed, and transferred to storage tubes. A 0.2-g aliquot of dried feces for extraction was placed in a 16 × 125 tube; 5 mL 90% ethanol and 100 µL corticosterone tracer (for recovery assessment) were added to each tube. Samples were capped with rubber stoppers and placed on the multipulse vortexer for 30 min. Samples were centrifuged at 1500 × *g* for 20 min, and supernatants were decanted into another set of labeled tubes. Each fecal pellet was combined with another 5 mL 90% ethanol and vortexed for 30 s. Samples were centrifuged for 15 min at 1500 × *g* and the supernatant added to that decanted previously. The combined supernatant was dried down and diluted 1:8 in dilution buffer (0.1 M $NaPO_4$, 0.149 M NaCl; pH 7.0) for corticoid analysis. Samples were analyzed by using a Corticosterone Enzyme Immunoassay kit (part no. K014-H5, lot no. 12CS001a, Arbor Assays). At the reference laboratory, the assay was validated analytically for rhesus macaques by demonstrating parallelism between dilutions of fecal extracts (1:2 to 1:128) and the standard curve and high recovery (>90%) of the corticosterone standard added to fecal extracts before analysis.

Throughout the course of this study, each NHP was observed individually daily by a single trained observer for identification of signs of maladaptation to the jacket, including signs of jacket manipulation, locomotor stereotypy, physical withdrawal, and self-injurious behavior. The observer was directed to perform only cageside assessments and to look for overt and significant signs of wounding, jacket disruption and tears, and stereotypies. These behavioral observations were performed 4 times prior to the jacketing event (as a baseline) and throughout the 14 d after jacketing.

Statistical analysis. A total of 3 linear mixed models were fitted to estimate the average change from baseline of FCM levels at each experimental day after jacketing. Estimates were made for each day for each group (A, B) and for the difference between the 2 groups by using a model consisting of a fixed day × group interaction. Estimates were made for each sex (female, male) and for the difference between the 2 sexes by using a model consisting of a fixed day × sex interaction. Additional estimates were made for each day after controlling for effects of group, sex, and animal pairing (by age, sex, and weight) by using a model consisting of fixed effects of day, group, sex, and animal pairing. Each model also included a repeated-day effect with a spatial power covariance structure to account for repeated measures. FCM values were log10-transformed to satisfy assumptions of residual normality and homoskedasticity. *P* values for each comparison were adjusted by simulation to account for multiple comparisons.

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Generalized estimating equations were used to estimate the odds ratios of decreased appetite scores between groups (jackets only compared with jackets and undershirts) or between female and male macaques. These estimates were made overall for the duration of the experiment as well as on each experimental day where estimable. Multinomial probabilities and simultaneous confidence intervals for appetite scores were estimated by using a previously described method.[37]

Comparisons of proportions of response scores on each day after jacketing were compared with the proportions of response scores before jacketing by modeling responses with generalized estimating equations to account for repeated measures. To account for complete separation (that is, all observations from a single day yield the same response value), those specific days were excluded from the analysis to produce an identifiable model. As such, experimental days representing a single value could not be compared with the other days in the experiment.

Comparisons of response proportions at each postjacketing day with the prejacketing period were evaluated as odds ratios. These odds ratios were the fraction of the odds of a higher appetite measurement on the given day divided by the odds of a higher appetite measurement in the prejacketing period. An increased odds ratio on a given day is interpreted as an increased likelihood of a change in appetite on the given day compared with the response observed before jacketing. *P* values for each comparison were adjusted by simulation to account for multiple comparisons. Statistical analyses were performed by using SAS version 9.3 (SAS Institute, Cary, NC) and R version 3.0.2 (www.r-project.org). All *P* values are considered significant at an α level of 0.05.

Results

All NHP were evaluated for the full 14-d observation period. Of the 20 NHP, 19 remained within their jackets throughout the entire course of the study. One NHP in the jacket-only group removed the jacket twice within the 14-d observation period; the jacket was replaced immediately on discovery. All 20 NHP were included in the data analysis to ensure statistical relevancy of the objective being measured (time to habituation to jacketing). In this study, the most common sign of maladaptation to the jacket was destruction of the jacket. On day 1 after jacketing, 13 of the 20 NHP were observed to be manipulating or causing jacket destruction. By day 3, only 2 NHP were noted to be actively manipulating or causing jacket destruction.

FCM measurements. FCM levels in macaques wearing jackets only (group A) were significantly increased on days 2 to 3 after jacketing compared with baseline values (day 2, 14.41-fold increase [95% CI, 4.41 to 47.10; $P < 0.0001$]; day 3, 3.57-fold increase [95% CI, 1.06 to 12.02; $P = 0.0396$]). In addition, FCM levels in macaques with jackets only showed a trend toward being increased on day 1 after jacketing, with a nontrivial effect size of 2.61-fold increased [95% CI, 0.87 to 7.88; $P = 0.0879$]. FCM levels in macaques wearing both jackets and undershirts (group B) were significantly increased compared with baseline on day 1 after jacketing (day 1, 4.48-fold increase [95% CI, 1.49 to 13.50; $P = 0.0078$]). By day 2, FCM levels in macaques wearing undershirts and jackets were no longer increased compared with baseline (day 2, 2.03-fold increase [95% CI, 0.64 to 6.39; $P = 0.2270$]; Figure 2). No significant difference was detected in the change in FCM levels from baseline between female and male NHP at any day after jacketing. FCM levels in macaques wearing jackets only were significantly below baseline on day 9 (day 9: 0.19 fold decrease [95% CI, 0.06 to 0.063; $P = 0.0065$]) and were borderline lower in macaques wearing undershirts and jackets on day 10 (day 10, 0.31-fold decrease [95% CI, 0.09 to 1.05; $P = 0.0570$]).

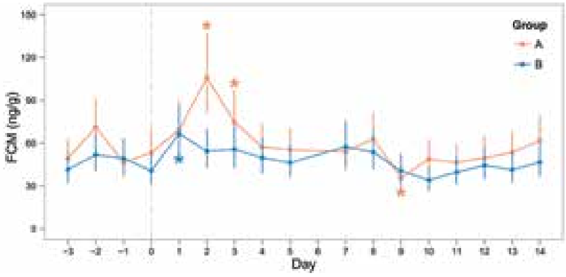

Figure 2. Mean FCM values (ng/g) and 95% confidence intervals according to jacketing group (A, jackets only; B, jackets and undershirts). Dashed line indicates the day of jacketing. Asterisks indicate significant changes from baseline. Fecal data on day 6 are omitted due to sedation of macaques for jacket assessments. FCM levels were significantly ($P < 0.04$) above baseline on days 2 and 3 in macaques that were jacketed only (group A) and on day 1 for those wearing undershirts and jackets. FCM levels were significantly ($P = 0.0065$) below baseline on day 9 in macaques wearing jackets only and showed a trend toward a significant decrease ($P = 0.0570$) on day 10 in animals wearing undershirts and jackets.

Food consumption. All NHP consumed 8 to 12 biscuits and all fruit daily both before and after jacketing. There were no significant differences in food consumption after jacketing when compared with baseline food consumption values. On addition, there were no significant differences in the overall odds of having a lower appetite score between groups as well as between sexes. None of the NHP had a significantly decreased appetite, according to our measures. Therefore, food consumption did not appear to be a significant factor in determining the jacket habituation timeframe and will not be discussed further.

Discussion

When performing a common laboratory practice such as jacketing of NHP, it is imperative to take into consideration the physiologic effects of the procedure and their significance as a possible confounder in research data. The current study set out to evaluate 2 key physiologic parameters, FCM and food consumption, to determine whether 3 d was sufficient time for rhesus macaques to be considered habituated to primate jackets prior to catheter implantation and entry into a study. Whereas the *Guide* remains rather vague on the expected duration of habituation, the jacket-use guidelines from the Association of Primate Veterinarians indicate that an NHP should be habituated to wearing the jacket for at least 1 to 2 wk prior to study initiation. Our study results suggest that this recommended period overestimates the time required for jacket habituation, especially for rhesus macaques. However, these guidelines consider jacket habituation to include all aspects of the restraint system (that is tether–swivel system), whereas in our study, we specifically evaluated the jackets themselves. Furthermore, as mentioned previously, these guidelines do not have regulatory authority and as such were developed as general guidelines for consideration when assessing the use of jackets. It remains necessary that professional judgment should be used in development of institutional policies and practices when evaluating jacket habituation timeframes. Our study was intended to provide information that would help researchers, animal caregivers, veterinarians, and IACUC to make better informed and guided decisions regarding the appropriate timeframe within which to deem this species habituated to a jacket, thereby decreasing the risk of the confounding variable of stress associated with the jacket.

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FCM measurements have been used extensively as a measure of physiologic stress in a wide variety of species, including macaques, and have proven to be a very useful, noninvasive method of assessing an animal's wellbeing and for complementing behavioral and physiologic studies.[3,8,17,24,36,38,41-43] In a study evaluating alopecia in rhesus macaques, levels of immunoreactive cortisol metabolites in feces were evaluated and negatively correlated with alopecia, suggesting a relationship between activity of the hypothalamic–pituitary–adrenal axis and hairloss.[38] Another study compared the effects of various anesthetic protocols on fecal glucocorticoid measurements as a measure of anesthesia-induced stress in both rhesus and cynomolgus macaques (*M. fascicularis*).[24] The study determined that none of the anesthetic protocols had significantly different effects on the fecal glucocorticoid measurements.[24] Furthermore, in a study using cynomolgus macaques, urinary cortisol values were evaluated in response to jacketing and tethering.[12] Urinary cortisol values were measured for approximately 1 wk to assess jacket and tether adaptation, and the authors determined that some aspects of tethering adaptation did produce urinary cortisol evidence of stress but that all animals showed adaptation to most procedures within 1 d.[12] One study indicated that NHP may take as little as a week to accept a jacket and tether system, after which 95% of the study population no longer attempted to remove or chew at the jacket or escape the tether; these animals therefore were considered to be conditioned satisfactorily and were placed into research studies.[28]

Our study showed that for rhesus macaques in the jacket-only group, FCM values remained significantly higher than baseline for 3 d after jacketing before they returned to baseline by day 4. Interestingly, when compared directly with the jackets-only animals, macaques wearing undershirts with jackets had significantly increased FCM values only on day 1 after jacketing. This finding may reflect the undershirt's ability to protect the NHP from irritation or rubbing from the jacket, thus decreasing overall stress and increasing comfort and habituation to the jacket. In the collaborative study by Kelly and colleagues regarding lesion incidence and severity performed within our institution, macaques wearing the undershirt and jacket had fewer back and neck lesions than did animals that wore the jacket only.[23] One therefore might conclude that the undershirt especially protected the back and neck regions, thus achieving quicker habituation to the jacket. These results support our hypothesis that 3 d is sufficient time to allow rhesus macaques to habituate physiologically (that is, through measurement of FCM) to jacketing in preparation for catheter surgery or for placement into a study.[23] In addition, the results of the present study act as a form of biologic validation of the sensitivity of the EIA assay in detecting the 3-d elevation from baseline in FCM levels that correlated with the stressful jacketing event.

The FCM values for the jackets-only group were lower than baseline values on day 9 and were borderline lower on day 10 in macaques wearing undershirts and jackets. This result may be due to the fact that these days occurred on the weekend, when the facility is much quieter and there is much less human interaction throughout the day than on a typical weekday. This same effect might have played a role on weekend days 2 and 3, when the FCM values might have been even higher if they had been measured on a weekday. Future investigations to evaluate the effects of weekend compared with weekday jacket habituation are warranted.

The sedation event on day 0 might have caused an increase in the FCM values 24 to 72 h after jacketing. Although our study did not include an unjacketed control group to assess the effects of sedation on FCM values, there were no significant increases in FCM values on days 7 and 8 (after sedation on day 6). This pattern suggests that the initial significant increase of FCM (after day 0) was not solely a function of the sedation event. Therefore, although the sedation event may have had a mild effect on FCM levels, the significant increase of FCM levels on days 0 through 3 after jacketing were more likely a result of the stress due to the jacketing event. In a future study, we recommend the inclusion of an unjacketed control group, to enable closer evaluation of the effects of sedation on FCM values.

Our study only used NHP that were naïve to the jacketing procedure. Increased experience with the jacket could reduce the novelty effect and alter the timeframe needed for habituation. Additional investigation is recommended to assess the effect of NHP with previous jacketing experience on jacket habituation timeframe. One study of urinary excretion of cortisol from rhesus macaques habituated to chair restraint found that habituation to the chair led to no increases in cortisol values.[40] The findings suggested that monkeys were well trained to the restraint and that an acclimation period of restraint, prior to starting experiments, was not necessary for previously habituated monkeys.[40] Another potential area of research is to evaluate any differences in habituation timeframes associated with jacketing alone compared with jacketing and tethering at the same time.

Our study addressed FCM changes and food consumption, 2 of the many physiologic parameters of stress in evaluating jacket habituation in rhesus macaques. However, another important physiologic component of stress in NHP is the presence of atypical or maladaptive behavior. Although many of the factors involved in the etiology and maintenance of atypical behaviors are not completely understood, these behaviors are thought to reduce tension and anxiety as a method of coping with stress.[15,25,30,39] Habituation to a defined stressor has been shown to cause reductions in behavioral agitation.[33]

The guidelines regarding jacket use in NHP from the Association of Primate Veterinarians discuss the need to identify signs of maladaptation, which can include destruction of the jacket, poor appetite, depressed or aggressive attitude, decreased activity, self-injury, and stereotypic behavior.[2] At our facility, the 4 most common atypical behaviors noted by our staff as common indicators of maladaptation to the jacket include: jacket manipulation, locomotor stereotypy, physical withdrawal, and self-injurious behavior. During the course of our study, each NHP was observed individually daily by a single trained observer for identification of any of these signs of maladaptation. Because our study was not a behavioral study, the observer was directed to perform only cageside assessments, looking for overt and significant signs of wounding, jacket disruption and tears, and stereotypies. These behavioral observations were performed prior to jacketing and throughout the 14 d after jacketing. As might be expected, the most common sign of maladaptation was destruction of the jacket. On day 1 after jacketing, 13 of the 20 NHP were observed to be manipulating or causing jacket destruction. By day 3, only 2 NHP were noted to be actively manipulating or causing jacket destruction. This drop in jacket manipulation percentages from 65% on day 1 to 10% by day 3 could stand as an additional indicator of habituation to the jacket. Furthermore, whereas there was no evidence of physical withdrawal or self-injurious behavior in any NHP prior to jacketing, there were several NHP that showed evidence of physical withdrawal (3 of 20) and self-injurious behavior (4 of 20) after jacketing. With the exception of one animal, all other NHP appeared to return to normal baseline behaviors (that is

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lack of observed atypical behavior) within 3 d after jacketing, a finding that is suggestive of behavioral habituation to the jacket. However, additional behavioral analysis with 24-h observation is warranted before objective evidence of jacket habituation can be determined.

As stated previously, other physiologic parameters of stress include cardiovascular, immune, and CNS changes. Unfortunately, many of the methods within which to collect these parameters can themselves directly alter the stress levels of the NHP. In our study, FCM was chosen as the method of evaluating stress given that appropriate samples could be collected noninvasively and enabled evaluation of stress levels that reflect changes over the course of a 24-h period. Additional studies are necessary to determine whether these other physiologic parameters mirror the FCM response to jacketing, with habituation within 3 d.

Another important consideration is the role that species plays in defining the length of time required for habituation to the jacket and tether. Although similarities in the behavioral ecology of macaque species are present, subtle differences may affect the success of specific management strategies to include procedural practices such as jacketing.[11] Rhesus macaques that scored high on the personality trait of 'sociability' had higher responses immunologically to immunization and social relocation, when compared with less sociable macaques[6,7] In addition, some temperament-associated constructs such as behavioral inhibition in certain animals or species are associated with increased stress sensitivity, which would play an important role in assessing the success of habituation in a given species.[10] One study that assessed the behavior, appetite, and urinary cortisol responses of pigtailed macaques (*M. nemestrina*) and long-tailed macaques (*M. fasicularis*) to cage or room changes determined that these 2 macaque species have behavioral, ecological, and possible temperament differences, such that their responses to the laboratory environment might therefore be quite different.[13] Our study focused primarily on habituation of rhesus macaques to jacketing. However future studies are warranted to evaluate whether jacket habituation timeframes differ among the various species of macaques.

In conclusion, our FCM results support our hypothesis that 3 d is a sufficient timeframe after which rhesus macaques can be deemed habituated to jacketing, with or without an undershirt, in preparation for catheter surgery and entry into a study. There were no measurable differences in food consumption during the jacket habituation period and no significant differences noted between sexes. Finally, the presence of an undershirt beneath the jacket may have had a positive effect by decreasing the habituation timeframe of rhesus macaques to jacketing.

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