Maternal separation produces lasting changes in cortisol and behavior in rhesus monkeys

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Maternal separation (MS), which can lead to hypothalamic pituitary adrenal axis dysfunction and behavioral abnormalities in rhesus monkeys, is frequently used to model early adversity. Whether this deleterious effect on monkeys is reversible by later experience is unknown. In this study, we assessed the basal hair cortisol in rhesus monkeys after 1.5 and 3 y of normal social life following an early separation. These results showed that peer-reared monkeys had significantly lower basal hair cortisol levels than the mother-reared monkeys at both years examined. The plasma cortisol was assessed in the monkeys after 1.5 y of normal social life, and the results indicated that the peak in the peer-reared cortisol response to acute stressors was substantially delayed. After 3 y of normal social life, abnormal behavioral patterns were identified in the peer-reared monkeys. They showed decreases in locomotion and initiated sitting together, as well as increases in stereotypical behaviors compared with the mother-reared monkeys. These results demonstrate that the deleterious effects of MS on rhesus monkeys cannot be compensated by a later normal social life, suggesting that the effects of MS are long-lasting and that the maternal-separated rhesus monkeys are a good animal model to study early adversity and to investigate the development of psychiatric disorders induced by exposure to early adversity.

In humans, early adversity can have long-term effects on the brain and behavior of animals, which increases vulnerability to the development of psychopathology. For example, studies have demonstrated abnormal behavior in adults who were mistreated as children (1, 2). In monkeys, maternal separation (MS) is frequently used to mimic early adversity. In a study using 6-d MS Tonkean macaques, self-injurious behavior was observed and social play decreased during the 9 wk following separation (3). Early separation often resulted in abnormally aggressive and reproductive activity (4). Another study that examined the maternal demonstrated decreased motor activity and body contact in MS monkeys compared with controls (5).

On the other hand, growing evidence also suggests that early adversity has significant long-term effects on the hypothalamic pituitary adrenal (HPA) axis, a major system that mediates neuroendocrine responses to stress (6). In this system, glucocorticoids (cortisol in primates, corticosterone in rodents) are released from the adrenal cortex during stressful conditions (7, 8). Normally, the temporary elevation of glucocorticoid hormone concentrations mobilizes energy stores and helps the organism survive an acute stressor. However, sustained adrenocortical secretion caused by chronic adversity can lead to severe impairment of brain regions, such as the hippocampus (9–11). Furthermore, several studies have shown that human HPA axis hyperactivity, which occurs in the early stages of psychiatric disorders, such as posttraumatic stress disorder (PTSD), may be blunted over time (12, 11). In this vein, the initial phase of hypercortisolism is followed by a latter phase of hypocortisolism. This switch in the pattern of cortisol release has been reported in human juveniles and adolescents that have experienced early adversity (13, 9, 12). Based on these endocrinological studies, an increased emphasis on the regulation of glucocorticoid release in animal models has been demonstrated to aid in determining the etiology of psychiatric disorders triggered by early adversity exposure.

In animal models, previous studies have shown that MS can lead to the dysfunction of the HPA axis, especially by modifying glucocorticoid release (12, 14). To date, most studies have examined the effects of MS in rats, with a higher corticosterone (CORT) level in the MS rats than in control rats following exposure to stress (15). In addition, the MS rats exhibited a different time profile for CORT release compared with control rats. Specifically, in the control group, CORT returned to basal levels, whereas it remained significantly elevated in the MS group (16, 17). In a study by Ladd et al. (18), plasma CORT levels peaked at 10 and 45 min following exposure to stressors in the control and MS groups, respectively. In general, these effects are long-lasting, but other studies have shown that they can be reversed in some conditions, such as environmental enrichment (19, 20).

Even though the results of the rat studies are promising, we should be cautious in applying this knowledge to humans. Aside from the quantitative and qualitative differences in the gene composition between these two species, primates demonstrate a different course of development than rodents. First, the primate brain is more mature at birth than the rodent brain (21). In addition, rodents generally exhibit a stress hyperresponsive period (22–25), which is absent in primates (21, 12). Therefore, nonhuman primates, the closest relatives to humans, are a more suitable model to examine early adversity in humans.

One line of the previous studies on nonhuman primates focuses on the basal cortisol levels. Most studies tend to support that mother-reared (MR) rhesus monkeys possess higher basal cortisol levels in comparison with surrogate peer-reared (S-P-R), peer-reared (PR), or nursery-reared (NR) individuals. Shannon et al. (26) demonstrated that 2-mo-old MR rhesus monkeys exhibited higher basal levels of plasma cortisol than either the S-P-R or PR animals, but the latter two groups did not differ from...
each other. Furthermore, according to Meyer et al. (27), 6-mo-old MR monkeys displayed significantly higher basal plasma cortisol levels than the S-P-R monkeys. In addition, some studies failed to discern a difference in cortisol baselines. For example, Clarke reported no difference in basal cortisol values between the MR and PR groups during the first 6 mo of life (28). There are many possible explanations for this inconsistency. We believe that one of the reasons might be in the methods used to measure the basal cortisol levels. When blood or fecal sampling was used, the sampling time points (29–32) and the stress caused by the capture and possible environmental disturbances shortly before the collection might have affected the cortisol levels (33–35).

One way to overcome this problem would be to analyze hair cortisol. The hair sampling technique has been available in rhesus macaques (36, 34, 37) and is a unique tool for assessing long-term changes in HPA system activity (36). In fact, hair cortisol represents the cumulative secretion of cortisol over months and is not affected by circadian rhythms or other factors. Thus, hair analysis provides a more reliable approach to assess the chronic effects of MS on baseline cortisol levels than blood or fecal sampling analysis. In addition, cortisol present in the hair reflects the biologically active, unbound fraction of this steroid in circulation (34). For these reasons, hair cortisol was measured in this study.

The other series of previous studies concentrate on the stress-induced increase in cortisol levels. These results revealed that cortisol levels were lower in the MS groups (PR, S-P-R, or NR) than in the control group (MR) when separation, isolation, or relocation was used as an acute stressor. In a study by Shannon et al. (26), S-P-R monkeys showed lower stress-induced cortisol levels than either the MR or PR group when tested at 3, 4, and 5 mo of age following 30 min of isolation (stressor). In another study, Capitanio et al. (38) demonstrated that 3- or 4-mo-old NR monkeys (similar to PR monkeys) showed significantly lower plasma cortisol levels 1 h following separation/relocation (stressor) than the age-matched MR controls. Similarly, Kinnally et al. (39) demonstrated that 3- or 4-mo-old MR infants had significantly higher cortisol levels than the NR group 2.5 h following separation/relocation (stressor).

In the studies demonstrating that PR monkeys had abnormal basal cortisol levels, the data were obtained immediately after the termination of separation. Thus, we questioned whether these deleterious effects of MS were reversible by later normal living experiences. In humans, individuals who developed psychiatric disorders related to early adversity usually had lived a normal social life before the occurrence of the diseases. The fact that in rodents the effects of MS on the HPA axis can be reversed by later experience (27, 28) makes this an important issue. To answer this question, we performed a long-term study to identify whether the effects that MS imposed on rhesus infants can be reversed after living a long and normal social life following the separation.

Results

Hair Cortisol Levels. Rearing condition significantly affected cortisol levels. The hair cortisol levels decreased significantly in the PR monkeys compared with the MR monkeys at 2 y of age \(F(1, 33) = 12.85, P = 0.001\) (Fig. 1A) or 3.5 y of age \(F(1, 29) = 5.25, P = 0.029\) (Fig. 1B).

Behavior Differences Between the PR and MR Monkeys. Compared with the MR monkeys, the PR monkeys demonstrated a significantly decreased duration \(F(1, 29) = 43.50\) (Fig. 2A and Table 1) and frequency \(F(1, 29) = 42.03\) (Fig. 2B and Table 1) of locomotion, decreased duration \(F(1, 29) = 6.58\) (Fig. 2E and Table 1) and frequency \(F(1, 29) = 4.54\) (Fig. 2F and Table 1) in the sitting together behavior, and an increased duration \(F(1, 29) = 23.00\) (Fig. 2C and Table 1) and frequency \(F(1, 29) = 39.54\) (Fig. 2D and Table 1) in stereotypical behaviors.

Cortisol Levels in Blood Samples. The data revealed significant differences in the time course of the cortisol response to stress between the MR and PR juveniles, with the peak cortisol response substantially delayed in the PR compared with the MR monkeys (Fig. 3). Specifically, MR individuals showed a significant increase in cortisol concentrations between the first two samples (time window 1 vs. time window 2) \(\mu(13) = -2.409, P < 0.05\), but this was not true for the PR monkeys \(\mu(10) = -0.386, P > 0.05\). In contrast, the PR monkeys demonstrated a significant elevation in cortisol levels from time window 2 to time window 3 \(\mu(4) = -6.129, P < 0.01\), which was absent in the MR juveniles \(\mu(4) = -0.985, P > 0.05\).

Correlation Between Hair Cortisol Levels and Plasma Cortisol Levels. There was no significant correlation between the cortisol values measured in the hair and in the plasma at baseline \(r = 0.293, P > 0.05\).

Discussion

In the present study, the effects of MS on the HPA axis function were evident in two separate tests following a long period of normal social life following the separation (1.5 and 3 y, respectively), indicating that it is a stable and long-lasting phenomenon. The PR monkeys had significantly lower basal levels of cortisol compared with the MR monkeys, and the peak in the PR monkey cortisol response to stress was substantially delayed. This study is unique in demonstrating that the deleterious effects of MS on the HPA axis of monkeys cannot be compensated for by later normal social life. In addition, this study is unique in using hair analysis to demonstrate that MS can reduce the level of the biologically active, unbound fraction of cortisol and is unique in having examined the time profile for the cortisol response to stressors in rhesus monkeys that have undergone MS. Importantly, this study identified the long-lasting abnormal behavioral patterns induced by MS following up to 3 y of normal social life. These results suggest that the MS rhesus monkeys are a good animal model to study early adversity in humans and could be used to investigate the development of psychiatric disorders induced by early adversity exposure.

The results obtained from examining the hair cortisol changes at 2 and 3.5 y of age in MS monkeys was consistent with a previous study on the long-term effects of early adversity in humans, which showed that adult women who had suffered from childhood mistreatment had low plasma cortisol levels (11). Consistent with these results, our study in monkeys was similar, whereby early adversity exerts an enduring impact on individuals, and the impacts could not be compensated for by later experience.
One explanation for the long-lasting hypocortisolism in the PR subjects could be a consequence of the down-regulation of HPA components induced by sustained early exposure to adversity after birth. During the development of certain psychiatric disorders (e.g., PTSD), a hypocortisolism phase evolves from an initial phase of hypercortisolism caused by sustained exposure to stress. It is possible that the juvenile PR subjects in the present study had already transitioned and were in the later phase of hypocortisolism at the time of sampling.

In addition to the difference in the basal hair cortisol levels, there were also significant differences between the MR and PR subjects in the time course of the cortisol response to stressor (capture/restraint) when blood sampling was obtained from the monkeys at the age of 2. Immediately after the presentation of stressor, the MR monkeys showed a significant elevation in blood cortisol levels. This is an adaptive strategy to help the animal cope with stressors in which the release of cortisol prepares the animal to escape from an unfavorable situation, and the cortisol-mediated negative feedback preserves the HPA axis from over-activation. Because of the effective feedback mechanism, there was a rapid poststress return to basal cortisol levels in the MR subjects. In contrast, the peak cortisol response was significantly delayed in the PR subjects. Because the blood samples were collected within only three time windows, with the last time window (20–30 min) being insufficient to detect the descent of the time profile in the PR subjects, it was difficult to determine whether cortisol reached peak levels within the third time window. Nevertheless, it was still clear that the PR monkeys responded to the stressor at a much slower pace than the MR monkeys, which reflects the existence of HPA axis dysfunction. Our results were similar to a study in rats (18), where plasma CORT levels peaked at 10 and 45 min after the stressor presentation in the control and the MS groups, respectively.

For blood analysis, the first sample collected within 8 min of the technician’s entry (time window 1) should reflect basal cortisol levels because it has been reported that 5 to 10 min is insufficient for stressors to change cortisol levels (40). The cortisol concentration measured in the first blood sample (time window 1) should, therefore, be comparable to that measured in the hair sample. Similar to a study by Clarke (28), the difference between the MR and PR monkeys in the cortisol baseline was absent in the blood samples (see the cortisol values within 8 min in Fig. 3). There are at least two possibilities for this inconsistency between the hair and blood samples. First, plasma cortisol levels are sensitive to even minor environmental disturbances. Even though the elapsed time (within 8 min) was short, it was difficult

![Fig. 2. Comparison of the three kinds of behaviors (mean ± SEM) between the PR (n = 11) and MR monkeys (n = 20) at 3.5 y of age. The behavioral measurements included the locomotion duration (A), locomotion frequency (B), stereotypical behavior duration (C), stereotypical behavior frequency (D), sitting together duration (E) and sitting together frequency (F).](image-url)
to ensure that the value measured was the true basal value. Second, hair and plasma cortisol do not reflect the same portion of the steroid present in circulation. It is reported that in plasma ~80% of cortisol is bound to cortisol-binding globulin, 10% to albumin, and the rest is in a free, unbound form (41). As stated in a previous study (34), hair cortisol was significantly correlated with the free fraction in the plasma, which accounts for less than 10% of the total cortisol measured in the blood analysis. Therefore, it was not surprising that no significant correlation between the hair and blood samples was detected.

In addition to the long-lasting change in the basal and stress-induced cortisol levels, long-term behavioral changes also occurred in the PR monkeys. In the social affiliative behavior, the PR initiated significantly less physical close sitting to their mates in the colony. Compared with the MR monkeys, the locomotion time and frequency decreased significantly in the PR monkeys. This finding was consistent with a previous study showing reduced mobility in MS monkeys in response to an acute stressor (5). In addition, the time and frequency of stereotypical behaviors increased significantly in our PR monkeys. Indeed, the long durations for all subsets of the stereotypical behaviors were observed in the PR monkeys mainly in pacing, digit sucking, and self-grasping, whereas the MR monkeys rarely exhibited such behaviors. The long-lasting stereotypical behaviors in our PR monkeys might reflect a state of high anxiety because one of the functions of stereotypical behavior is to enable individuals to dissipate anxiety by reducing arousal (42, 43).

The dysfunction of the stress systems is usually associated with the disruption of psychological processes, which is detected by measuring behavior. These abnormal behaviors, especially the stereotypical behaviors, observed in the MS monkeys might be a result, in part, of the abnormal function of the HPA axis. In fact, the disturbance of the HPA axis can influence the function of neurotransmitter systems (44), such as the serotonergic system, which is closely related to anxiety (45) that manifests in stereotypical behaviors.

Both the cortisol and behavioral differences between the PR and MR monkeys might be related to cognitive differences. Specifically, cognitive differences in stress appraisal rather than HPA dysregulation might account for the cortisol differences. Similarly, the possible cognitive differences might contribute to the behavioral differences. However, this theory does not negate our main finding that early adversity has a long-lasting impact on brain development. Furthermore, because the PR infants in our study were separated artificially by technicians to improve their survival, it is a possibility that the PR group was not randomly selected, even though around 80% were separated for reasons that were not related to genetic background, such as bad weather or an inexperienced mother. The remaining 20% were separated because the mother lacked the ability to produce enough milk or the baby was born too weak to survive, which might have some indirect relationship to genetics. All together, these factors might complicate our conclusions, but because MS is an intense manipulation of infant monkeys, we believe that our data provide valuable insight for the understanding of the early adversity on nonhuman primate brain development.

Conclusion

We have demonstrated that MS exerts long-lasting deleterious effects on the HPA axis function and the behavior patterns in rhesus monkeys. When the subjects were tested after 1.5 and 3 y of normal social life, consistent changes in basal cortisol levels were found in the MS monkeys. In addition, the peak cortisol response to stressor (capture/restraint) was significantly delayed in the PR subjects even after experiencing 1.5 y of normal social life. Compared with the MR subjects, the PR subjects spent significantly less time in locomotion and initiated less sitting together behavior, and their stereotypical behaviors were increased following 3 y of normal social life. These enduring impacts of MS on rhesus monkeys indicate that MS might be a good model to study early adversity.

Materials and Methods

Subjects. All animal care was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee of Kunming Institute of Zoology. The rhesus macaques (Macaca mulatta) were selected from two rearing conditions: mother-reared (22 monkeys) and peer-reared (13 monkeys). The MR infants lived with their biological mothers belonging to different small reproductive groups. Each group consisted of one adult male and four to five adult females with their offspring, and each group lived in a connected indoor (2.61 × 2.46 × 2.58 m)-outdoor (2.67 × 2.56 × 2.57 m) colony. Commercial monkey biscuits were provided twice a day with tap water ad libitum. In addition, the animals were fed with fruits and vegetables once daily. This rearing condition was maintained for the first 4 mo of life; afterward each infant was weaned and relocated to an indoor steel cage (0.74 × 0.71 × 0.74 m) with another age-matched MR peer-mate. Illumination was maintained on a 14/10 light/dark cycle (lights on 0700 hours; lights off 2100 hours), and the temperature was set at 22 °C.

The PR infants were separated from their mothers at birth under the following circumstances: (i) around 60% of the PR infants were the first born and separated by the caretakers because of their mothers’ lack of experience. The inexperienced mothers sometimes handled their first offspring incorrectly, which could lead to serious injuries or even death. Thus, under such conditions, the caretakers of the primate center had to take the babies away and raise them in the incubators. In fact, when the second infant was born, the former inexperienced mother would be able to raise the baby properly. (ii) Around 20% of the PR infants were separated from their mothers because the mothers had little breast milk to nourish them or the baby was too weak to survive. (iii) The final 20% were born when the weather was rainy and cold. A wet newborn could lead to sickness or death. As a result, when a newborn got wet, the caretaker would take the newborn away from the mother and raise him or her in an incubator until the weather improved before returning the baby monkey to the mother. If the mother refused to take the baby back, the little monkey would be raised in the incubator.

The PR monkey was reared alone for the first month of his or her life in an incubator, which was maintained at 32 °C with towels for the baby to cling to. During this period, the baby monkey was hand-fed with milk roughly nine times per day. After the first month, each infant was paired with another age-matched PR peer-mate in a steel cage (0.74 × 0.71 × 0.74 m). At approximately 7 mo of age, both the MR and PR monkeys were relocated to the connected indoor (2.61 × 2.46 × 2.58 m)-outdoor (2.67 × 2.56 × 2.57 m) colonies and reared together in mixed sex social groups without adults.

Experimental Design. To measure the effect of MS on HPA axis function, the hair samples were collected twice when the monkeys were 2 and 3.5 y of age. The behavior was recorded beginning on the second day following the second hair sampling (at 3.5 y of age) to investigate the behavioral changes following MS. Blood samples were collected to evaluate stress-induced cortisol secretion when the monkeys were 2 y of age. However, when we tried to collect the blood samples again at the age of 3.5 y, the primate center had changed the protocols for capturing monkeys in the colony to reduce the possibility of injuring the monkeys. Fast blood sampling was greatly limited under the new protocols, with the aim of not causing panic in the monkeys.
during the capture. We had tried to capture the monkeys within 8 min for the baseline plasma cortisol sampling, but all attempts failed. Thus, this study lacks the plasma cortisol data from the monkeys at the age of 3.5 y.

**Blood and Hair Sample Collection.** When the subjects were 2–3 y old, blood and hair samples were collected between 1330 hours and 1500 hours. Beginning at 1330 hours, each subject was captured by an experienced technician using a net and taken out of the colony for sampling. The monkey was manually restrained, 1.5 mL of blood was drawn from the femoral vein into a heparin lithium-treated vacuum collection tube. Next, hair from the back of the monkey’s neck was clipped using a pair of scissors and placed into a small pouch of aluminum foil for protection during storage. One hair sample and three blood samples were obtained from each monkey. The blood samples were collected within 8 min (baseline, time window 1), 10 to 15 min (time window 2), and 20 to 30 min (time window 3) from the technician’s entrance. The last two blood samples reflected the stress-induced cortisol levels, with the capture/restraint being the stressor. However, the stress of the capture, restraint, and blood collection, as well as the limitation on the volume of blood that could be safely drawn from each juvenile monkey, precluded the possibility of repeated sampling of the same monkey on the same day. As a result, the three blood samples were obtained 6 d apart, with the second and third sample collected 6 and 12 d after the first sample, respectively. Nevertheless, all samples were collected during the same period of the day. In addition, the order of samplings within the three time windows was counterbalanced in every colony.

When the monkeys were 3.5 y old, the hair samples were collected following the same procedures described above.

### Measurement of Hair Cortisol.

The hair cortisol extraction procedure was based on a protocol (26). Hair samples were initially washed twice in 5 mL isopropanol (3 min each) to remove surface contaminants, dried at 35 °C for 8 h, and then pulverized using a Retsch ball mill (Retsch M400) at 26 Hz for 2.5 min. Approximately 200 mg of the powdered hair was weighed and incubated in 4 mL of methanol at room temperature for 24 h with a slow rotation to extract cortisol. The samples were then centrifuged at 11,000 × g for 5 min, and 2 mL of the supernatant was pipetted into a centrifuge tube and dried under a stream of nitrogen gas. Finally, the sample was reconstituted with 0.25 mL of PBS and stored at −20 °C until assayed. The cortisol RIA was performed at the radioimmuno-laboratory of the second affiliated hospital of Kunming Medical College using commercially available kits. The CORTISOL RIA DSL-2000 and CORTISOL RIA KIT REF IM1841 were used in the first and second hair cortisol assays, respectively. A double-blind procedure was used in the cortisol extraction and analyses.

### Measurement of Plasma Cortisol.

The blood samples were first centrifuged at 8,000 × g for 10 min. The plasma fraction was then transferred to a 0.5 mL centrifuge tube and stored at −20 °C until assayed. The cortisol RIA was performed following the same procedures as applied in the hair analysis.

### Behavioral Data Collection and Analysis.

A digital camera fixed on a tripod was set up in front of the colony to record one of the monkeys in the cage. To avoid disturbing the animals, the observers stayed away from the cage (at least 5 m) during the tape recording. Three 30-min recordings were collected for each monkey per week, and a total of 4 h from each monkey (eight videos) was obtained. All of the behavioral analyses were conducted on the computer.

Although several monkeys may have appeared in a 30-min recorded video, we concentrated on only one monkey and analyzed his or her behaviors. The specific behaviors of the monkeys were defined as follows. Using the locomotor behavior of Monkey A (the focused monkey) as an example, when Monkey A started its locomotion in the video, the video was paused manually, and the precise time point displayed on the screen was recorded. Next, the video was resumed until the locomotion stopped, the precise time point was also recorded. The period (in seconds) between the two time points was considered as one episode of locomotion. Many episodes of locomotion might occur in a 30-min video. The duration of locomotion was calculated by adding all of the episodes, and the number of the episodes was counted at the frequency of locomotion. Afterward, the duration and frequency of locomotion were calculated from the eight recorded videos of the monkey were summed and then divided by the total time (in minutes, 8 × 30 = 240 min in total) of the behavioral recordings of the monkey, which yielded two values in terms of seconds per minute (duration) and times per minute (frequency) for later analyses. The behaviors of the studied monkey were analyzed similarly.

### Behavioral Categories and Definitions.

In previous studies, decreased activity, anxiety-related stereotypical behavior, and decreased affiliation behavior (e.g., sitting close) have been reported in MS monkeys (5, 45). Therefore, the locomotion, various stereotypical behaviors, and sitting together initiated by the monkey studied were analyzed in this study. These behaviors are defined in the **SI Text**.

The behavior mentioned above was quantified by its duration and frequency. During the evaluation of these two parameters, the videos of the recorded monkeys were rated by the technicans, who were blinded to the manipulation of the monkeys (whether they were PR or MR).

### Data Analysis.

A total of 35 (13 from PR monkeys and 22 from MR monkeys) and 31 (11 from PR monkeys and 20 from MR monkeys) hair samples were analyzed when the monkeys were 2 and 3.5 y old. The behavioral recordings from the same 31 monkeys were obtained when they were 3.5 y of age. The difference in the number of monkeys between the first and second sampling was because four monkeys were sold during the time period.

Because all subjects lived in social groups at the time of sample collection, it was impossible to accurately control for the time elapsed from the entry of the technician to the point of blood sampling. Consequently, two of the three sampling behavioral events fell into the same time window (within 8 min, 10–15 min, or 20–30 min) for several monkeys. In this situation, the cortisol values measured in the two samples were averaged to reflect the steroid level at a single sampling point. Therefore, the data for the three time windows were not available for all monkeys. The number of samples for each time window was described as follows: 17 MR and 13 PR samples were available in time window 1; 15 MR and 11 PR samples were in time window 2; 8 MR and 7 PR samples were in time window 3.

Data analysis was conducted using SPSS software. One-way ANOVA was used to assess the effects of the rearing conditions on the hair cortisol levels and behavioral measurements (the durations and frequencies of the rated behaviors). For the blood samples, paired-sample t tests were performed. For all analyses, the significance level was set at P < 0.05.

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