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Titi monkey father-daughter bond-related behaviors explain stress response variability

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Abstract

Social interactions regulate our behavior and physiology, and strong social bonds can buffer us from stress. Coppery titi monkeys (*Plecturocebus cupreus*) are socially monogamous South American monkeys that display strong social bonds. Infants form selective bonds with their fathers, making them ideal for studying father-daughter bonds. We established a method for quantifying variability in expression of bond-related behaviors in females (n =12), and the present study is the second to use this method for explaining titi monkey responses to behavioral tests. We also investigated how manipulations of oxytocin (OT) and vasopressin (AVP) influenced juvenile behavior and physiology. Subjects received acute intranasal treatments of saline, low/medium/high OT, low/high AVP, or OT receptor antagonist (OTA) prior to an acute social separation. General linear mixed-effects model results revealed fathers were significant behavioral and physiological stress buffers for their daughters, as evidenced by fewer distress vocalizations (p < 0.001), less locomotion (p < 0.001), and lower plasma cortisol (p < 0.001) in a social separation paradigm.

Females vocalized less if they exhibited greater expression of bond-related behaviors with their fathers as infants (p = 0.01), and this stress-buffering effect remained even when the daughter was separated from the father (p = 0.001). While treatments did not alter behaviors, OTA treatment caused the largest rise in plasma cortisol (p < 0.001), suggesting blockade of OT receptors can inhibit fathers' stress-buffering effects. Remarkably, females with greater expression of father-daughter bond-related behaviors exhibited an overall reduced physiological separation distress response (p = 0.04). Findings from the present study advance current knowledge of the neurobiological mechanisms foundational to female bonds and help inform how social disruptions may differently impact individuals based on expression of bond-related behaviors.

Keywords

Stress buffering; separation distress; oxytocin; arginine-vasopressin; cortisol; social bond

1. Introduction

Social interactions can regulate behavioral, psychological, and biological processes, and strong social support may buffer individuals from stress-induced behavioral and physiological changes (Uchino, 2006). Poor or absent social relationships have been associated with adverse health outcomes and increased risk of mortality in humans (Holt-Lunstad et al., 2010). Psychosocial factors may underly gender differences in rates and repercussions of affective disorders like major depressive disorder (Almeida and Fletcher, 2022), and social risk factors may contribute to women's increased likelihood of developing affective disorders. It is critical to study how social bonds and stress buffering may affect health outcomes, particularly in women.

Several neuropeptides and steroids are important for social bonds and stress buffering. Studies of monogamy in prairie voles (*Microtus ochrogaster*) and titi monkeys (*Plecturocebus spp.*) have demonstrated the importance of oxytocin (OT) and arginine-vasopressin (AVP) for pair bonding (Bales et al., 2017). OT and AVP interact with the hypothalamic-pituitary-adrenal (HPA) axis, with OT activity inhibiting and AVP enhancing HPA-axis activity, and these interactions may help explain the stress buffering effects of social bonds (Gobrogge and Wang, 2015). These interactions are also important for modulating an individual's sexually differentiated roles in prairie vole pair bonding. In this species, females require low circulating CORT for pair bond formation, and exhibit high CORT following pair bonding if exposed to a novel male, whereas males exhibit the opposite trends (Young et al., 2010). Understanding interactions between OT, AVP, and HPA-axis activity may help disentangle observed sex differences in the neurobiology of social bonds and stress buffering.

Social environments can influence neuroendocrine systems (Kelly and Vitousek, 2017), and relationship quality may be one such factor that may help explain variability in stress responses. In humans, adults in high-quality romantic relationships exhibit higher endogenous levels of plasma and salivary OT (Holt-Lunstad et al., 2015) and plasma AVP (Gouin et al., 2012). Daughters in supportive father-daughter relationships had lower

salivary CORT responses to a stress task compared to daughters in coercive relationships (Byrd-Craven et al., 2012). While these studies begin to elucidate links between relationship quality and the neuroendocrine system, it is still unknown *how* variability in social bonds, OT, AVP, and CORT interact and impact stress buffering.

To that end, non-human primate (NHP) research is critical for understanding interactions between the neuroendocrine system and social bonds in a controlled environment. Titi monkeys are socially monogamous South American monkeys that live in the Amazon basin. In their natural environment, titi monkeys live in small family groups, exhibit bi-parental care of offspring, and display classic attachment behaviors, including maintaining proximity with attachment figures as well as distress upon separation from and stress buffering by their attachment figures (Bales et al., 2021). Because titi monkeys exhibit attachments and family structures similar to humans and share the conserved forms of OT and AVP with humans (Bales et al., 2017), they are excellent for research topics with human health applications.

The primary attachment figure for titi monkey infants and juveniles is the father. Laboratory tests have shown that, when given a choice between interacting with their mothers or fathers, infants will choose their fathers (Mendoza and Mason, 1986). Titi monkey infants also show behavioral and physiological signs of distress if they are separated from their fathers, but virtually no response when separated from their mothers if their father is present (Mendoza and Mason, 1986). Infants likely form a strong attachment to their fathers because fathers spend more time carrying their infants than mothers (Karaskiewicz et al., 2021). While titi monkeys consistently exhibit a selective attachment to their fathers, there is variability in the degree to which they display bond-related behaviors, such as proximity maintenance (Carp et al., 2016; Witczak et al., 2022). Neurobiological mechanisms important for social bonding and stress buffering are likely impacted by this variability in expression of bonding behaviors.

One way to investigate the role of neuropeptides in attachment-related behaviors is to study the effects of exogenous manipulations of OT and AVP during a social challenge. Intranasal treatments have shown that these hormones exert central effects, altering neuronal activity and cognition in both humans (Bakermans-Kranenburg & Van Ijzendoorn, 2013) and non-human species (including prairie voles and titi monkeys; Freeman & Young, 2016). Changes in brain activity and behavior may be due to a combination of central and peripheral effects of intranasal treatments (Yao et al., 2023). Our laboratory has successfully used intranasal OT and AVP in a number of previous studies, and our results have shown that these treatments result in dose-dependent alterations in a variety of titi monkey social behaviors (Arias-del Razo et al., 2022b, 2020; Jarcho et al., 2011). An oxytocin receptor (OTR) antagonist (OTA) that is highly selective for OTR in humans and NHPs (Pettibone et al., 1995) has also been used successfully for behavioral pharmacology experiments in marmosets (*Callithrix jacchus;* Smith et al., 2010) and rhesus macaques (*Macaca mulatta*; Boccia et al., 2007). No study has investigated how variation in expression of father-daughter bond-related behaviors impacts behavioral and physiological responses to exogenous manipulations of the OT and AVP system. The main objectives for the present study were to understand how variability in expression of father-daughter bond-related behaviors and manipulations of the OT and AVP systems impact the behavioral and

physiological correlates of separation distress and stress buffering in juvenile female titi monkeys. We tested several predictions:

1. Higher expression of father-daughter bond-related behaviors, as evidenced by more distress upon separation and greater proximity maintenance as infants and juveniles, would enhance separation distress and stress buffering in a social isolation paradigm.

2. OT treatments would reduce separation distress and enhance stress buffering, but with possible dose-dependent effects.

3. OTA and AVP treatments would dose-dependently enhance separation distress and diminish stress buffering.

2. Methods

2.1 Subjects & Housing

Subjects were 12 juvenile female titi monkeys (ages 14–18 months) and their fathers (N = 12 fathers). Subjects were housed at the California National Primate Research Center and lived in their natal groups with their parents and any older siblings. This housing condition approximates typical titi monkey social groups in the natural environment. Families were housed in a 1.2 m × 1.2 m × 2.1 m or 1.2 m × 1.2 m × 1.8 m stainless steel cage with four horizontal perches, a food bowl, and two water dispensers. They were fed twice daily with Purina New World monkey chow, rice cereal, carrots, apples, and bananas. They were kept on a 12-hour light, 12-hour dark cycle, with lights coming on at 0600 hours and turning off at 1800 hours. The room temperature was maintained at approximately 21°C. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Davis, and complied with legal requirements of the United States, Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, and National Institutes of Health guide for the care and use of Laboratory animals.

2.2 Pharmacological Treatments

We weighed subjects every two weeks to ensure each subject received treatments based on their current weight. For the OT treatments, we dissolved OT acetate salt (Santa Cruz Biotechnology, Dallas, Texas, USA), in saline at three concentrations: 0.8 IU/kg, 8.0 IU/kg, and 80.0 IU/kg. Given that previous studies from our laboratory have shown titi monkeys exhibit variable responses to chronic intranasal OT treatments of 0.8 IU/kg OT (Arias-del Razo et al., 2020), we chose 0.8 IU/kg OT as our lowest dose and chose two higher doses (8.0 IU/kg and 80.0 IU/kg OT) to investigate a potential dose-response curve. These higher doses have been used in rodents without adverse effects (Bales et al., 2013). For the AVP treatment, we dissolved AVP acetate salt (Sigma Aldrich, Burlington, Massachusetts, USA), in saline at two different concentrations: 40 IU/kg and 80 IU/kg. We selected these dosages for AVP treatments because those were previously shown to affect social behavior in male titi monkeys (Jarcho et al., 2011). For the OTA treatment, we dissolved L-368,899 (Bio-Techne, Minneapolis, Minnesota, USA), in saline at 10 mg/kg. Previous studies in marmosets observed behavioral responses to lower (3 mg/kg; Kotani et al., 2017) and higher dosages (20 mg/kg; Smith et al., 2010), so we picked a dosage that was in between these

values to ensure effectiveness of the treatment while minimizing the risk of adverse side effects. We aliquoted and stored treatments at −80°C until use. Before each use, we thawed individual doses but kept them cold prior to administration. To administer the treatment, trained personnel entered the subject's home cage, captured the subject in a small transport box (60 cm × 30 cm × 30 cm), wrapped the subject using a towel, and administered a total of 180 μl of treatment using a manual single-channel pipette. To minimize the risk of treatment loss, we administered 45 μl of treatment in one nostril and then gently covered that nostril with a finger for a few seconds until the compound was absorbed into the nasal mucosa (the other nostril was left uncovered). We then repeated this process three additional times, alternating nostrils, to administer a total volume of 180 μl of treatment. If any treatment was expelled during administration, we re-administered the approximate amount that was lost. We then returned the subject home.

2.2 Social Separation Data Collection

For the juvenile testing, female subjects began testing at 14 months of age and completed testing approximately 15 weeks later. Subjects were habituated to capture, handling, and treatment procedures prior to the start of testing. At the start of testing, all subjects went through one baseline blood draw. We collected an awake 0.5 ml femoral blood sample at 1130 hours using 1.0 mL syringes pretreated with heparin. Subjects were captured in a transport cage, hand captured, and manually restrained for sample collection. All blood samples were collected within 5 minutes of entry into the subject's home-cage. Following blood collection, samples were immediately placed on ice, centrifuged at $1,610 \times g$ at 4°C, and the plasma was extracted and stored at −80°C until assay.

One week later, subjects underwent 14 separation tests: seven in which mothers were removed, leaving the females with their fathers, and seven in which both parents were removed. At 1030 hours, subjects received an intranasal dose of one of seven treatments (either saline, 0.8 IU/kg OT, 8.0 IU/kg OT, 80.0 IU/kg OT, 40 IU/kg AVP, 80 IU/kg AVP, or 10 mg/kg OTA). Separation conditions and treatment order were counter-balanced across all subjects. Tests were spaced one week apart to ensure drug washout between tests. This period was likely more than adequate to ensure washout, as all treatments were expected to be completely out of the subject's system after 48-hours (Jarcho et al., 2011; Smith et al., 2010); however, it should be noted oxytocin can have long-lasting effects (Alaerts et al., 2021). This one-week period was also beneficial for keeping the developmental period relatively consistent between and within subjects while minimizing stress on the subjects and their families.

After dosing, females were returned to their families for a 30-minute uptake period. At approximately 1100 hours, we removed either the mother (stress buffered condition) or both the mother and father (separation distress condition) and temporarily relocated them to a separate building where they did not have visual, auditory, or olfactory access to the subject during the testing period. For the one subject that had an older sibling in the family group, that sibling was also removed after the uptake period in both conditions. During the separation period, we live scored vocalizations using Behavior Tracker (www.behaviortracker.com) and recorded vocalizations using a solid-state digital

flash recorder (Marantz PMD660) and a directional condenser microphone (RODE NTG2). We used previously defined classifications of titi monkey vocalizations (Clink et al., 2019) to define all vocalizations we identified by ear. The actual scoring of the vocalizations was done live by the first author (Witczak). All tests were video recorded and later scored using Behavior Tracker (www.behaviortracker.com) for locomotion. Locomotion was defined as moving one body length or more and being in motion for at least one second.

At approximately 1130 hours, we collected an awake 0.5 ml femoral blood sample using the methods described above. Families were reunited in the home cage and we video-recorded parent-offspring interactions for 15 minutes following the reunion.

Urine samples were collected three times per week to track female subjects' reproductive status. It was not expected that females would be cycling at this age while still in their natal group (Conley et al., 2022).

2.3. Plasma Cortisol Assay

We used an enzyme immunoassay validated for titi monkeys to estimate plasma CORT concentrations from blood samples (for detailed assay methods, including chemical and biological validation, see Witczak et al., 2021). A total of six plates were assayed, with intra-assay CVs of 4.5%, 13.2%, 10.0%, 9.0%, 0.1%, and 8.1%, with an inter-assay CV of 19.2%.

2.4. Quantification of Expression of Bond-related Behaviors

Two important behavioral indicators of a strong, selective attachment are distress upon separation from and preference for maintaining proximity to the attachment figure (Bales et al., 2021). For the present study, we quantified four indicators of separation distress and five indicators of proximity maintenance for 12 subjects (Table 1; Supplementary Methods S1.1). These measures were used to quantify the expression of bond-related behaviors for titi monkeys.

We quantified separation distress in two ways at two developmental time points, using archival data from an infant open field (IOF) test that was conducted when subjects were four months of age and from the saline condition of present study when subjects are 14–18 months of age. For IOF testing, subjects were placed in the center of a one meter by one meter testing arena with a six-by-six square floor grid. One wall included a wire mesh grate that allowed auditory, visual, and olfactory access to a stimulus animal that was placed in a transport box on the other side of the grate. During this testing, subjects experienced four five-minute trials, each with a different stimulus animal on the other side of the grate (their father, their mother, their oldest sibling [if they had any], and an empty box; for more details regarding our IOF paradigm, see Larke and colleagues [2017] and Savidge and Bales [2020]). Our four measures of separation distress were number of line crosses during IOF testing (*IOF Locomotion*), number of vocalizations during IOF testing (*IOF Vocalization*), duration of time spent locomoting during juvenile testing (*Juvenile Locomotion*), and number of separation distress vocalizations during juvenile testing (*Juvenile Vocalization*). For each measure of separation distress, we calculated the percent change in behavior from

the father condition to the alone condition ([{behavior when alone – behavior with father} / behavior with father] * 100).

To quantify proximity maintenance, we used data collected from the present study, IOF testing, and infant carry scan sample data collected when infants were 0–9 months of age (for a description of methods, see Karaskiewicz et al., 2021). To calculate *Juvenile Proximity*, we quantified the duration of time subjects spent in proximity, contact, or tail-twining with their father during the 15-minute reunion period after experiencing the separation distress condition and receiving saline for the present study. *IOF Proximity* and *IOF Grate* were quantified during the father condition of IOF testing and measured as time spent in proximity to the father and time spent touching the grate separating the subject and the father. For these three predictors, we calculated the percentage of time that females were in social proximity with their fathers as the total time in social proximity out of the associated testing period ([duration of time in social proximity / test condition duration] * 100). We also calculated the percentage of time infants were carried by their fathers during the first nine months of life (*Infant Proximity*) by quantifying the percentage of time females were carried by their father out of all scan samples collected for that subject.

In human studies, parents' relationship quality has been found to be a predictor of children's future romantic relationship quality (Gager et al., 2016); therefore, we also included a measure of parents' time spent in social proximity (*Parent Affiliation*) as a measure of expression of bond related-behaviors. For the present study, we quantified the percentage of time parents were in proximity, contact, or tail-twining out of all scan samples collected on the parents during the first 14 months of our subjects' lives (for data collection methods, see Witczak et al., 2022).

For data analysis, all measures of bond-related behaviors were centered about the mean value for our 12 subjects. This allowed us to determine how variation in expression of bonding behaviors influences outcomes in our testing paradigm. Prior to analyses we assessed correlations between bond-related variables (Supplementary Table 1).

2.5. Data Analysis

All analyses were conducted in R Statistical Software (version 4.0.3, R Core Development Team, 2020). For all outcome measures, we performed a Shapiro Wilk test of normality and transformed non-normally distributed variables as necessary. All tests were two-tailed, and the significance threshold was set at .05. Prior to model interpretation, we performed a sensitivity analysis using G*Power 3 (Faul et al., 2007) to determine the minimum effect size (Cohen's f^2) that we could reliably determine based an α of .05, a desired Power $(1 - β)$ of 0.80, and our total sample size of $N = 168$ (12 subjects × 14 conditions).

We first identified which of the nine bond-related behaviors (Table 1) best explained variance in our three outcome variables (distress vocalizations, locomotion, plasma CORT). To identify best-fitting bond-related predictor variables, we ran stepwise regression using the *train()* function in the *leaps* (Lumley, 2020) package. This method iteratively adds and removes variables in the predictive model to identify which subset of variables results in the model with the lowest prediction error (Gareth et al., 2014). To simplify the stepwise

regression models, we first ran separate stepwise regression models for separation distress (*IOF Locomotion, IOF Vocalization, Juvenile Locomotion, Juvenile Vocalization*) and proximity maintenance (*Juvenile Proximity, IOF Proximity, IOF Grate, Infant Proximity, Parent Affiliation*) variables. Once we identified the top separation distress and proximity maintenance variables, we ran a final stepwise regression model with those top variables. The combination of bond-related variables that was identified as producing a model with the lowest prediction error was then used in our mixed-effects models.

We next ran general linear mixed-effects models (LMM) using the *lmer()* function in the *lmerTest* package (Kuznetsova et al., 2017), with animal identity as a random effect to account for repeated measures. Our models were specified as: $y_{ij} = \beta_0 + \beta_1 x^{(1)}_{ij} + \cdots + \beta_m x^{(m)}_{ij} + \eta_{i0} + e_{ij}$. In this model, y_{ij} denotes the *j*th observation taken of individual *i* for outcome variable *y*. Individual covariates included in the model are represented by *x* (covariates 1–m), which have fixed effects common to all individuals in the population (β). This model also includes a random effect of ID that is specific to each individual (η_{i0}). Residual errors are represented by e_{ij}. In our full model, fixed effects included *Treatment*, *Test Number*, *Condition* (separation, buffering), bond-related variables (identified by stepwise regression analyses), and interaction effects between *Condition* and each bond-related variable. To determine the best-fitting model, we used backwards selection to remove any non-significant fixed effects (Bentler and Mooijaart, 1989). We used a log likelihood ratio test to compare model fit to determine whether removing any fixed effects resulted in a better fitting model (Vuong, 1989; Supplementary Table 1). We then performed a log likelihood ratio test to compare the fit of our best model to that of the null model where we removed all fixed effects. The resulting final model, determined using a model comparison based on the likelihood of the model to the data, is the only one in which we evaluated the significance of parameters. Therefore, for each outcome variable, we had one model where we interpreted significance level. That model represented the most likely hypothesized relationship between parameters given the data. Because we only evaluated parameter significance in one model per outcome, post hoc corrections were not necessary (Vuong, 1989). For all significant predictors we calculated Cohen's f^2 as a measure of effect size (Selya et al., 2012). Based on Cohen's guidelines, $f^2 \geq 0.02$, $f^2 \geq 0.15$, and $f^2 \geq 0.35$ represent small, medium, and large effect sizes, respectively. We had strong *a priori* hypotheses regarding how bond-related variables and treatments would impact our outcome variables. Data for this study are available via Open Access ([dataset] Witczak et al., 2023).

3. Results

3.1. Separation Distress Vocalizations

Because the residuals for separation distress vocalizations were not normally distributed, we log-transformed the data using base e ($\log_e$ transformation). The model that included the main effects of *Condition, Infant Proximity, Juvenile Locomotion, IOF Proximity*, plus the interaction between *Condition* and *Juvenile Locomotion* and *Condition* and *IOF Proximity* best predicted separation distress vocalizations (Supplementary Table 2a; Supplementary Table 3a). The minimum effect size that could be significantly detected in this model is $f^2 = 0.084$.

The R^2 for this model was 0.6753. In general, females vocalize less with their fathers are present (Mean = 88.01, Range: 0 – 1561, SE = 19.88) than when they are tested alone (Mean = 462.27, Range: 27 – 1371, SE = 35.30; β= −2.184, SE = 0.014, t = −15.958, p < 0.001, f^2 = 1.361; Figure 1a; Supplementary Figure 1).

We found evidence to suggest that females also vocalize less if they spent more time being carried by their fathers as infants (β = −0.061, SE = 0.023, t = −2.709, p = 0.027, f^2 = 0.159; Figure 1b). There was a significant interaction effect between *Condition* and *Juvenile Locomotion* (β = 0.002, SE = 0.0004, t = 3.729, p < 0.001, f^2 = 0.066), suggesting females vocalize more when they are with their father and less when tested alone if they exhibit a greater expression of this bond-related behavior. We found a significant interaction between *Condition* and *IOF Proximity* (β = 0.024, SE = 0.005, t = 4.386, p < 0.001, f^2 = 0.092) which suggested that females that spent more time in proximity to their fathers during IOF testing vocalize more when the father is present but less when tested alone.

3.2. Locomotion Duration

The residuals for locomotion duration were normally distributed following a square-root transformation. The model that included the main effects of *Test Number, Condition, Infant Proximity, IOF Locomotion*, plus the interaction between *Condition* and *Infant Proximity*, best predicted locomotion duration (Supplementary Table 2b; Supplementary Table 3b). Our minimum effect size that could be significantly detected was f^2 = 0. 079.

The R^2 for this model was 0.3502. In general, females locomoted less when tested with their fathers (Mean = 194.06 seconds, Range: 14 – 513, SE = 12.90) compared to alone (Mean = 299.86 seconds, Range: 47 – 727, SE = 17.19; β = −3.307, SE = 0.614, t = −5.390, p < 0.001, f^2 = 0.480; Figure 2a; Supplementary Figure 2).

Females locomoted more as test number increases from one to 14, regardless of treatment or separation condition (β = 0.359, SE = 0.076, t = 4.701, p < 0.001, f^2 = 0.129). Females also locomote more during testing if they showed a greater increase in line crossing behavior from the alone to father condition during infant open field testing (β = 0.007, SE = 0.002, t = 3.295, p = 0.009, f^2 = 0.082). There was a significant interaction between *Condition* and *Infant Proximity* (β = −0.405, SE = 0.094, t = −4.288, p < 0.001, f^2 = 0.108; Figure 2b) such that females in the alone condition locomote more if they spent a lot of time being carried by their fathers as infants; however, in the father condition, females locomote less if they are carried more as infants.

3.3. Plasma Cortisol

The residuals for plasma CORT concentration were normally distributed following a log_e-transformation. The model that included the main effects of *Treatment, Condition, Juvenile Locomotion, IOF Grate,* and *Juvenile Proximity* best predicted CORT concentration (Supplementary Table 2c; Supplementary Table 3c). The minimum effect size we could determine based on this model was f^2 = 0.079.

The R^2 for this model was 0.5894. Based on the results of this best-fitting model, we have evidence to suggest that females exhibit a smaller rise in plasma cortisol when tested with

their fathers present (Mean = 444.44 ng cortisol/mL plasma, Range: 167.20 – 1041.8, SE = 21.57) compared to the alone condition (Mean = 593.03 ng cortisol/mL plasma, Range: 206.60 – 1113.00, SE = 21.63; β = –0.32, SE = 0.044, t = –7.264, p < 0.001, f^2 = 0.215; Figure 3a; Supplementary Figure 3).

Females treated with OTA exhibited the largest rise in plasma cortisol (Mean = 657.24 ng CORT/ml plasma, Range: 206.6 – 1113.0, SE = 49.61) compared to the saline condition (482.63 ng CORT/ml plasma, Range: 271.2 – 916.4, SE = 34.98). Females treated with OTA exhibited the largest rise in plasma cortisol, regardless of whether females were with their fathers or alone (β = 0.286, SE = 0.082, t = 3.472, p < 0.001, f^2 = 0.079). Females also exhibited a higher rise in cortisol if they spent more time in proximity to their father during IOF testing (β = 0.008, SE = 0.003, t = 2.598, p = 0.036, f^2 = 0.224), but a lower rise in cortisol if they spent more time touching the grate during IOF testing (β = –0.010, SE = 0.004, t = –2.478, p = 0.042, f^2 = 0.203; Figure 3b).

4.   Discussion

We found support for our prediction that greater expression of father-daughter bond-related behaviors would be associated with enhanced stress buffering. We had also predicted that OT treatments would enhance behavioral and physiological indicators of stress buffering while AVP and OTA treatments would enhance separation distress. When treated with OTA, females exhibited an elevated CORT response, even when they were tested with their father in the stress buffering condition. These findings suggest that activation of the OT system, and specifically of OTR, may be one important mechanism underlying fathers' ability to buffer their daughters from stress.

Females vocalized less when their fathers were present compared to when they were tested alone. If they were carried more as infants, females vocalized even less, suggesting fathers have a buffering effect with regards to distress vocalizations. Interestingly, females that exhibit more distress upon separation as infants and juveniles vocalize more when with their father but less when alone. When females are tested with their fathers, their fathers are experiencing a separation from their partner, and tend to vocalize frequently. Females in this condition that were themselves more distressed upon separation from their father (as infants) appear to somewhat mirror their fathers' behavior and vocalize frequently themselves. This behavior is similar to the synchronization of behaviors observed between titi monkey pair-mates during an acute social intruder challenge (Mercier et al., 2020). When these same females with greater expression of bond-related behaviors (more proximity maintenance and separation distress as infants) are tested alone, they vocalize less, suggesting the fathers may have some lingering buffering effects on these females. These findings are similar to those found in humans that have demonstrated that strong attachment relationships are able to buffer children and adults from stressors (Ditzen & Heinrichs, 2014). The mechanisms driving social buffering in titi monkeys may therefore be similar to those in humans.

Females also locomoted less when tested with their fathers compared to when alone; however, locomotion increased as test number increased, suggesting an effect of time and/or testing experience on locomotion behavior. Females that exhibited a larger increase

in line crossing during IOF testing also spent more time locomoting during both testing conditions. Given that infant locomotion predicted juvenile locomotion, this finding suggests locomotion may measure overall tendencies for females to exhibit "anxiety-like" behaviors during testing that may be consistent within an individual. Indeed, locomotion behavior is used in other NHP studies as a measure of temperament (Gottlieb et al., 2018). A previous titi monkey study also found that infant locomotion during IOF testing predicted anxiety-like behaviors during a novel response task in adulthood (Savidge and Bales, 2020). Our measure of locomotion may therefore reflect stable traits such as general activity level or anxious behavior.

Interestingly, for locomotion duration, we found a significant interaction effect between *Infant Carry* and *Condition* that was the opposite of our findings for separation distress vocalizations. Specifically, females that were carried more by their fathers as infants locomoted less in the father condition but more in the alone condition. It is possible locomotion and vocalization may have different social saliences for this species. In the wild, if a titi monkey lost visual access to a social other, displaying a visual signal of separation distress may not be as effective for eliciting reunion with that social other as an auditory cue. Locomotion may therefore serve a different purpose from distress vocalizations, instead reflecting some measure of overall activity in titi monkeys.

We had predicted that agonism of the OT system would decrease separation distress behaviors while agonism of the AVP system would increase separation distress; however, none of our treatments significantly explained vocalizations or locomotion. Titi monkeys share the conserved form of OT and AVP with humans (Bales et al., 2017; Lee et al., 2011) and our selected treatments have affected titi monkey behaviors in previous paradigms assessing social preference (Arias-del Razo et al., 2022a; Jarcho et al., 2011). It is possible that other neuropeptide systems are more relevant for mediating certain distress behaviors. For example, the kappa opioid system may be particularly important for mediating responses to short and long term separation from a pair bonded partner (Bales and Rogers, 2022).

Responses to exogenous manipulations of the OT and AVP systems may depend on age and sex as well as type of behavior. Adult male titi monkeys experiencing social separation do exhibit an endogenous rise in OT and a drop in AVP in response to separation from their pair-bonded partner (Hinde et al., 2016), suggesting these systems may be involved in separation distress responses in adult males. Adult titi monkeys that were chronically treated with intranasal OT or saline from 12–18 months of age exhibited treatment- and sex-dependent responses to a social separation challenge with acute manipulations of the OT system (Arias-del Razo et al., 2022b). The observed sex differences suggest females may respond differently to manipulations of the OT system, and those manipulations early in life can have lasting impacts that alter adult behaviors like vocalizations. One mechanism which may explain differing responses to acute exogenous treatments may be changes in OTR expression following chronic intranasal OT treatments (Huang et al., 2014). Interestingly, variability in locomotion was not significantly explained by chronic or acute intranasal treatments of OT and saline in either study (Arias-del Razo et al., 2022b; Huang et al., 2014). These findings in conjunction with the findings from the present study further

suggest other neuropeptide systems may explain locomotion behavior in response to social challenges.

We did find effects of treatment and expression of bond-related behaviors on physiological responses to social separations. Females exhibiting higher grate touching as infants were physiologically buffered from the stress of separation, but we saw the opposite effect if females spent more time in proximity to their father during IOF testing. This finding suggests these two behaviors (grate touching vs proximity) may be capturing different aspects of females' attachments to their fathers. Touching the grate takes more conscious effort than being in proximity to the father during IOF testing and therefore may be a better indicator of a female's attachment to her father. Indeed, a study examining adult titi monkey behavior during a preference testing paradigm found that females spent more time touching their father's grate than that of a stranger, despite not showing a significant preference for remaining in the father's proximity zone (Carp et al., 2016). Alternatively, grate touching may be an indicator of overall anxiety. Therefore, it is important to consider the different functional roles of various expressions of bond-related behaviors.

Our most striking finding was that females treated with OTA exhibited the largest rise in plasma cortisol compared to the saline condition, regardless of whether they were tested with their father or alone. In general, South American monkeys, including titi monkeys, have higher circulating levels of plasma cortisol compared to humans and Old-World monkeys (Klosterman et al., 1986); however, the values observed in the present study were within the expected range for titi monkeys based on prior laboratory experiments (Hennessy et al., 1995; Witczak et al., 2021). While blockade of OTR disrupted the stress buffering effect of fathers overall, females that spent more time touching the grate during IOF testing exhibited a lower rise in plasma cortisol during juvenile testing. These findings suggest that OTA can block the stress-buffering effects the father may have on his daughter, as we had predicted; however, if females exhibited a greater expression of father-daughter bond-related behaviors, as evidenced by spending more time touching the grate during IOF testing, they may have a reduced physiological response to a social stress paradigm. Therefore, while manipulations of the OT system may not significantly impact behavioral expressions of anxiety, blockade of OT receptors can enhance physiological separation distress. Previous research also found discrepancies between behavioral and physiological responses to social challenges (Hennessy et al., 1994), highlighting the importance of measuring both when investigating stress response and stress buffering. Our findings support the established connections between OT and the HPA-axis (Gobrogge and Wang, 2015). While we did not find support for our prediction that AVP receptor agonism would also exert anxiolytic effects, this was the first study to conduct AVP manipulations in juvenile female titi monkeys.

It is possible that females that exhibit more father-directed bond-related behaviors have alterations in their OT and AVP systems, for instance, in the density of receptors available. Several studies have illustrated alterations in neural circuitry associated with variability in early life experience and social affiliation in monogamous rodents and NHPs (López-Gutiérrez et al., 2022). For example, studies on OT and AVP receptor binding in the hippocampal formation found differences in receptor density based on parental status and

affiliation between titi monkey pair-mates (Baxter et al., 2023, 2020). It is possible that the degree to which females exhibit bond-related behaviors directed towards their fathers may also impact receptor density.

Eight of our nine bond-related predictors significantly explained variability in responses to a social separation challenge, suggesting it is important to incorporate measures of bonding behaviors in future studies. A previous study in titi monkeys similarly found early measures of attachment predicted adult anxiety-like behaviors and partner affiliation (Savidge and Bales, 2020), and a study conducted in humans also found that measures of infant attachment predict measures of anxiety later in development (Bar-haim et al., 2007). *Parent Affiliation* was the only measure that did not significantly explain variability in our outcome measures. It is possible that the relationship between titi monkey parents does not significantly impact the daughter's attachment to the father. In fact, it is likely that differences in the relationship between the father and daughter may have a stronger impact on the relationship between the parents, as evidenced by a recent study in titi monkeys examining changes in parent affiliation following the birth of an infant (Karaskiewicz et al., 2021).

The limitations of this study pose promising future directions for investigations of the neurobiology of social bonds. For titi monkeys, vocalizations can be coordinated between conspecifics (Clink et al., 2019); however, we only scored vocal behaviors of our test subjects. While we did not score fathers' vocalizations during testing, it would be interesting to quantify the degree of synchronization in vocalizations between fathers and daughters in future studies. Additionally, we only focused on father-daughter bonds for the present study; however, it would be valuable to examine the effects of pharmacological treatments and expression of father-son bond-related behaviors in male offspring. Given our lack of evidence for OT and AVP treatments to affect separation distress behaviors, it is likely other neuropeptides, like opioids, underly these behaviors. Future studies should repeat these social separation paradigms using exogenous opioid manipulations to determine whether titi monkeys exhibit stress buffering effects similar to those observed in other pair bonding animals (Bales and Rogers, 2022). While the AVP treatments we used in the present study have been used successfully in adult male titi monkeys (Jarcho et al., 2011), it is possible that our AVP treatments were not sufficient to activate HPA-axis activity in juvenile females. Conversely, our treatments may not have produced any effects if they were large enough to result in AVP binding to both AVP and OT receptors, given the ability of AVP and OT to bind to each other's receptors (Song and Albers, 2018). Our present study is unable to disentangle these two possibilities; therefore, future studies with different dosages of AVP may be useful. The present study is one of the first to link variability in expression of bond-related behaviors and receptor manipulations; however, we are unable to assess differences in OT and AVP system functioning in our sample. Future studies should directly investigate how the OT and AVP systems are altered by variation in expression of bonding behaviors in species that form strong, selective attachments. Finally, while our measures of expression of father-daughter bond-related behaviors explained variability in behavior and physiology, our measure of females' observations of parent bond-related behaviors (*Parent Affiliation*), did not explain separation distress and stress buffering. It would be helpful to conduct follow-up

experiments using a different behavioral paradigm measuring social preference to determine whether this predictor explains variability in female proximity maintenance behaviors.

Conclusions.

The present study is the first to examine how expression of female bonding behaviors throughout development and manipulations of the OT and AVP systems impact behavioral and physiological correlates of separation distress and stress buffering. Greater expression of father-directed bonding behaviors both as an infant and a juvenile predicted more behavioral distress upon separation and enhanced stress buffering physiology. Blockade of OT receptors led to increased CORT, a physiological indicator of separation distress. Interestingly, our treatments did not impact behavioral indicators of separation anxiety, suggesting other neuropeptide systems, like the opioid system, may underlie separation distress behaviors. Findings from the present studies advance current knowledge of the neurobiological mechanisms foundational to female titi monkey attachment relationships and help to inform how social disruptions may differently impact individuals based on the quality of their social bonds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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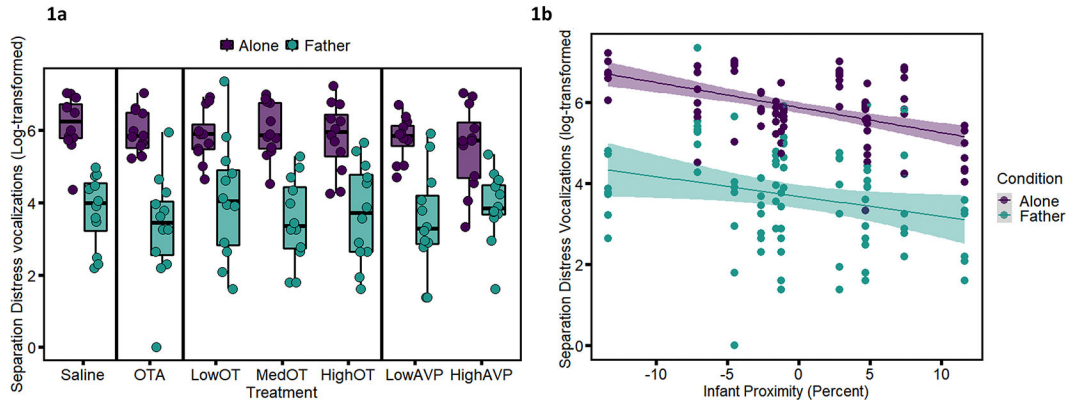
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Figure 1.

Juvenile separation distress vocalization frequency: a) main effect of pharmacological *Treatment* separated by *Condition* (alone or father) and b) interaction effect between *Condition* and *Infant Proximity.* During this separation paradigm, females generally vocalize less when tested with their fathers (a) and vocalize even less if they spend more time in proximity to their fathers as infants (b). OT = oxytocin; AVP = arginine-vasopressin; OTA = oxytocin antagonist; Saline = saline; LowOT = 0.8 IU/kg OT; OTA = 10 mg/kg OTA; MedOT = 8.0 IU/kg OT; HighOT = 80.0 IU/kg OT; LowAVP = 40 IU/kg AVP; HighAVP = 80 IU/kg AVP
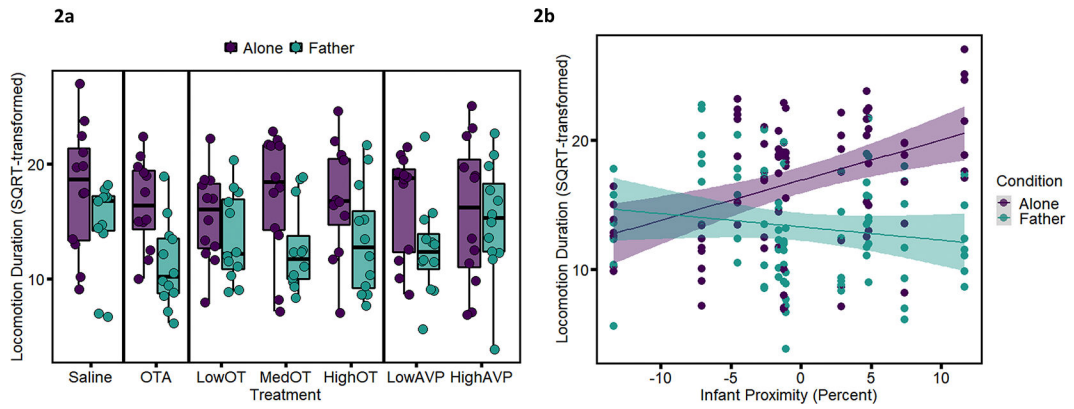
Figure 2.

Locomotion duration: a) main effect of pharmacological *Treatment* separated by *Condition* (alone or father) and b) interaction effect between *Condition* and *Infant Proximity*. During this separation test, females generally locomote less when tested with their fathers (a), and subjects that spend more time in proximity to fathers as infants locomote more when tested alone and less when tested with their fathers (b). OT = oxytocin; AVP = arginine-vasopressin; OTA = oxytocin antagonist; Saline = saline; OTA = 10 mg/kg OTA; LowOT = 0.8 IU/kg OT; MedOT = 8.0 IU/kg OT; HighOT = 80.0 IU/kg OT; LowAVP = 40 IU/kg AVP; HighAVP = 80 IU/kg AVP
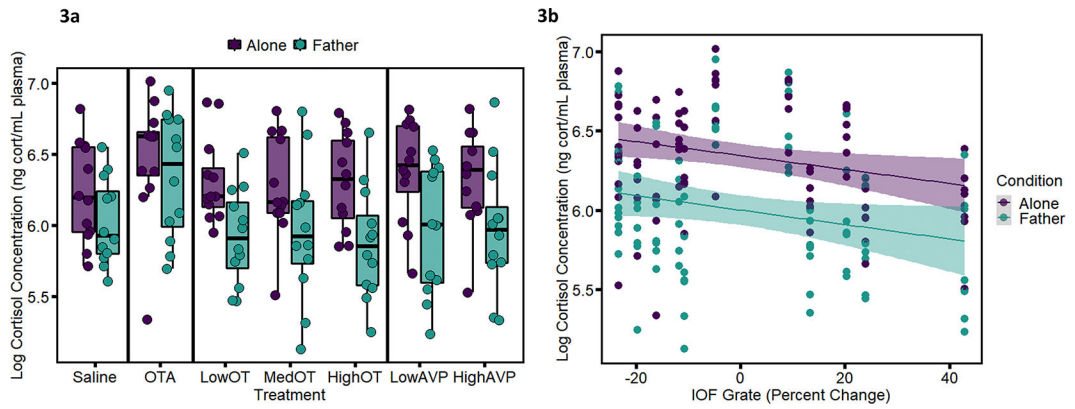
Figure 3.
Plasma cortisol concentration: a) main effect of pharmacological *Treatment* separated by *Condition* (alone or father) and b) interaction effect between *Condition* and *IOF Grate*. During separation tests, cortisol is lower when females are tested with their fathers (a) and females that spend more time touching the grate during infant testing exhibit lower cortisol concentrations, regardless of test condition (b). OT = oxytocin; AVP = arginine-vasopressin; OTA = oxytocin antagonist; Saline = saline; OTA = 10 mg/kg OTA; LowOT = 0.8 IU/kg OT; MedOT = 8.0 IU/kg OT; HighOT = 80.0 IU/kg OT; LowAVP = 40 IU/kg AVP; HighAVP = 80 IU/kg AVP

Table 1.

Ethogram for expression of bond-related behavior.

Behavior	Definition
Separation Distress Behaviors	
Greater percent change in behavior interpreted as greater expression of separation distress	
IOF Locomotion	Percent change in line crossing from the father to the empty box condition of infant open field (IOF) testing[a]
IOF Vocalization	Percent change in vocalizations emitted from the father to the empty box condition of IOF testing[a]
Juvenile Locomotion	Percent change in locomotion behavior from the stress buffered to the separation distress condition of juvenile separation testing when subjects are treated with saline[b]
Juvenile Vocalization	Percent change in total vocalizations emitted from the stress buffered to the separation distress condition of juvenile separation testing when subjects are treated with saline[b]
Proximity Maintenance Behaviors	
Greater percentage of time in behavior interpreted as greater expression of proximity maintenance	
Juvenile Proximity	Percentage of time subject spends in proximity, contact, or tail-twining with the father during a 15-minute reunion period immediately following juvenile social separation testing when subjects are treated with saline[b]
IOF Proximity	Percentage of time subject spends in proximity to the father during the father condition of IOF testing[a]
IOF Grate	Percentage of time subject spends touching the grate in the IOF arena during the father condition of IOF testing[a]
Infant Proximity	Percentage of time subject was carried by the father during the first nine months of life as measured during daily scan samples[c]
Parent Affiliation	Percentage of time parents of subjects spent in proximity, contact, or tail-twining with each other during the first 14 months of the subjec s life as measured during daily scan samples[d]

[a] see methods from (Larke et al., 2017; Savidge and Bales, 2020)

[b] see methods from present study (section 2.2)

[c] see methods from Karaskiewicz et al., 2021

[d] see methods from Witczak et al., 2022