Effects of Acute Stress on Parental Behavior in Reproductively Naïve Male California Mice

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ABSTRACT

In many biparental mammalian species, such as California mice (Peromyscus californicus), new fathers exhibit affiliative behavior toward unfamiliar infants, whereas reproductively naïve males show highly variable behavioral responses to infants. The sources of this variation are unknown. We investigated the effects of acute stress on pup-directed behavior in reproductively naïve male California mice. Each mouse underwent three 10-minute tests with an unfamiliar pup at 48-hour intervals. Males in the stressed group (N=22) were stressed using subcutaneous oil injections, a common experimental stressor used in rodents, immediately before each of the first two tests. The controls (N=22) were left undisturbed to avoid any experimentally induced stress. Compared to controls, stressed mice spent significantly less time performing paternal behavior in tests 1 and 2, while only marginal differences were seen in test 3. In tests 2 and 3, significantly fewer stressed mice interacted with the pup than controls. These findings suggest that acute stress experienced by reproductively naïve males might contribute to both short-term and long-term differences in pup-directed behavior.

Key Words: Stress; reproductively naïve; California mouse; biparental; paternal behavior; subcutaneous injections; behavioral tests; experimental pups.

FACULTY MENTOR - Dr. Wendy Saltzman, Department of Evolution, Ecology, and Organismal Biology

Dr. Saltzman is a Professor in the Department of Evolution, Ecology, and Organismal Biology. She has a B.Aa in Animal Physiology from UC San Diego and a Ph.D. in Animal Behavior from UC Davis, and did her postdoctoral work at the University of Wisconsin, Madison. She returned to Southern California and joined the faculty of UCR in 2001. Dr. Saltzman's research focuses on neural and physiological consequences of parenthood and neuroendocrine influences on parental behavior in biparental rodents.

Nabeel Shaikh

Nabeel Shaikh is a fourth-year Biology major. Starting Summer 2021, he has studied the biological mechanisms underlying mammalian parenting in Dr. Saltzman's lab. Nabeel presented his projects at numerous conferences, including at Harvard University and Stanford University. He is a UC Riverside Undergraduate Education Mini Grant recipient and STEM Success Scholarship recipient. Nabeel hopes to pursue a M.D.-Ph.D. and aspires to be a physician-scientist.

Parihan Asif

Parihan Asif is a fifth-year Biochemistry and Cell, Molecular, and Developmental Biology major. In Dr. Saltzman's behavioral neuroendocrinology lab, Parihan focused on how stress and empathy are displayed in biparental species. Parihan has presented her research at UC Riverside, Stanford University, and Loyola Marymount University. Parihan plans to pursue a career in Emergency Medicine.

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INTRODUCTION

Parental care encompasses behaviors that positively contribute to the evolutionary fitness of the offspring (Klug and Bonsall, 2014). Mothers in all mammalian species provide parental care. In contrast, only about 6% of mammalian species exhibit biparental care, in which both fathers and mothers provide care for their offspring (Kleiman and Malcolm, 1981). In biparental mammals, fathers can provide comparable forms and amounts of care as mothers, excluding lactation (Kleiman and Malcolm, 1981), and usually care for unrelated infants during experimental exposure (Gubernick et al., 1987). Paternal care can be important for offspring survival and development in these species (Bales and Saltzman, 2016).

In biparental mammals, the onset of paternal behavior in new fathers is associated with changes in hormones, neuropeptides, and their receptors, which can be triggered by interactions with the pregnant or parturient mate and/or the offspring (Horrell et al., 2021). Reproductively naïve males–i.e., males that have never been housed with an unrelated female and therefore have not copulated or been exposed to pups–however, lack the sexual and parental experience needed to trigger these neuroendocrine changes and the onset of paternal care. Compared to fathers, adult reproductively naïve males exhibit more varied responses when exposed to an unfamiliar pup, performing nurturant, avoidant, or aggressive behavior (Elwood and Stolzenberg, 2020). The sources of this variation in initial responses to pups are not well understood, although reproductively naïve males that behave paternally toward pups may be less anxious than those that do not (Chauke et al., 2012; De Jong et al., 2012). Moreover, repeated exposure to pups can facilitate the onset of paternal care in reproductively naïve males (Horrell et al., 2017; Cai et al., 2020).

Numerous studies in humans, as well as several studies in other biparental mammals, have found that stress can negatively affect parental care and, in humans, can increase the likelihood of child abuse or neglect (Wolfner and Gelles, 1993; Lee et al., 2008). In contrast, virtually nothing is known about the possible effects of stress on responses to infants in reproductively naïve males. Our study aimed to characterize the impacts of stress on the onset of paternal behaviors specifically for reproductively naïve males in a biparental mammal, the California mouse (*Peromyscus californicus*). In this rodent, both parents provide intensive care for their offspring (Dudley, 1974; Gubernick and Alberts, 1987). Fathers spend as much time retrieving, grooming, and huddling their offspring as mothers, and care by fathers is important for the normal behavioral, neuroendocrine, and social development of pups (Bales and Saltzman, 2016).

In the present study, we investigated the long- and short-term effects of stress on the onset of paternal behavior in reproductively naive males. Previous work in our lab has found that in California mouse fathers, acute stress transiently reduces the amount of time spent interacting with the pups and mate (Harris et al., 2013). However, the effects of stress on responses to pups in reproductively naïve males have not been examined. We tested the hypothesis that stressors will inhibit the short-term expression of paternal behavior in reproductively inexperienced males. In addition, we tested the hypothesis that concurrent exposure to a stressor and pups, which constitute an unfamiliar stimulus, will cause reproductively naïve males to form an association between pups and stress, leading to longer-term effects on males' behavioral responses to pups.

METHODS

Animals

California mice were descended from mice purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, USA) and were bred and housed at the University of California, Riverside (UCR). Polycarbonate cages (44 x 24 x 20 cm) were used to house the mice. The animals were kept under standard laboratory conditions (Nguyen et al., 2020), with aspen shavings for bedding and cotton for nesting, and had ad libitum access to food (Purina 5001 Rodent Chow) and water. Animals were removed from their parents' cages at weaning age (27-31 days), before the birth of their younger siblings, and housed in groups of 3-4 same-sex, age-matched mice. At least 2 weeks before being used in the study, groups were split into pairs of reproductively naïve males. All procedures were reviewed and approved by the UCR Institutional Animal Care and Use Committee and were consistent with the recommendations of the *Guide for Care and Use of Laboratory Animals*.

Design and Stimulus Exposure

We used 22 pairs of adult reproductively naïve males (143-168 days of age). Each mouse was tested with an unrelated pup three times at 48-hour intervals. Within each pair, one male was randomly assigned to the stressed group and the other to the control group (**Table 1**). The subjects in the stressed group received an injection of sesame oil immediately before the first and second tests but not the third test; whereas, the subjects in the control group did not undergo any experimental stressors prior to any of the tests. We chose to use oil injection as a stressor because it elicits a stress response in this species (as determined by an increase in plasma concentrations of the adrenocortical stress-responsive hormone corticosterone) beginning within 10 minutes and persisting for at least 40 minutes after injection (Harris et al., 2013) and therefore seemed likely to affect the mice's behavior during the entire test. We considered the entire procedure of injecting the stressed mice with oil (including handling, brief pain from the injection, and more prolonged discomfort from the oil) to constitute the stressor. Therefore, we did not perform any of these procedures on the control mice in order to avoid stressing them.

Group	Day 1: Test 1	Day 3: Test 2	Day 5: Test 3
Stress (N=22)	Acute stress + pup	Acute stress + pup	No stress + pup
Control (N=22)	No stress + pup	No stress + pup	No stress + pup

Table 1. Experimental design. Total sample size N=44.

At the beginning of each test, the subject was placed alone in a clean cage identical to the home cage. After a 20-minute acclimation period, mice in the stressed group were injected with sterile sesame oil (0.2 ml, s.c.); control mice were left undisturbed. An unrelated pup, 2-5 days of age, was then placed in the cage with the subject for 10 minutes. The pup was removed immediately if it was attacked. Cage mates were tested 5 minutes apart and were reunited in their home cage after each test. We conducted tests during the inactive (light) phase of the 24-hour light cycle (9:00-11:00 h).

Behavior Measurements

We video-recorded the mice throughout the 10-minute test period. Behaviors (**Table 2,** next page) were scored from the videos using Behavioral Observation Research Interactive Software (BORIS; Friard and Gamba, 2016). Inter-observer reliability was 91%, and observers were blind to animals' treatment conditions. Because the precise amount of time that pups were present varied slightly across tests, the time spent in each behavior was normalized by dividing the total time of the activity by the duration of the stimulus exposure and multiplying by 100 [Σ behavior (s) / stimulus presentation (s)*100].

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Behavior	Description	Measure
Sniff	Move nose against or in the direction of pup within 1 cm of pup	Duration, latency
Parental Behavior	Stand or lie over or against pup and/or lick pup	Duration, latency
Rest	Sit or lie for ≥2 s while not active and not in contact with the pup	Duration
Jump	Jump up and down	Duration
Proximity	Nose and/or abdomen within 5 cm of pup	Duration
Pick up/Carry	Hold pup in the mouth or hold and locomote	Frequency
Attack	Bite pup	Did or did not occur
Backflip	Flip in a vertical circular motion (a common stereotypical behavior in this species	Frequency
Autogroom	Use hands and/or mouth to pick through, wipe, or scratch its own body	Duration

Table 2. Behaviors scored

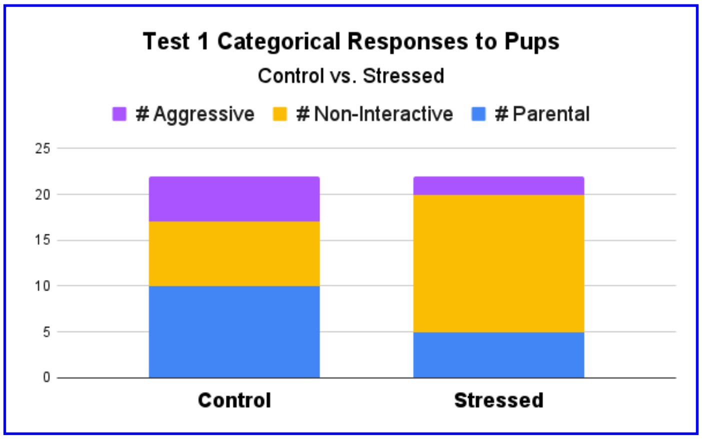
Statistical Analysis

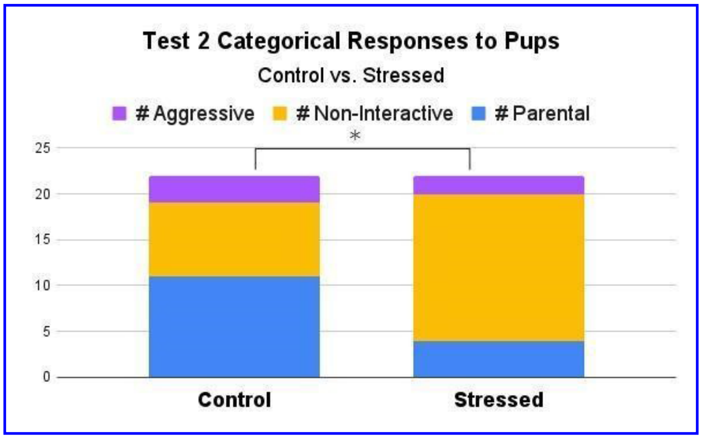
We analyzed data using non-parametric tests because measures did not meet the criteria for parametric analyses. Mann-Whitney U tests were used to compare behavior between stressed and control animals in each test, and Wilcoxon tests were used for within-subjects comparisons between tests 1 and 3 for each treatment group separately. For all analyses, we used a critical P value of 0.05 (2-tailed).

RESULTS

Comparisons of stressed and control groups

The number of reproductively naïve male California mice that exhibited parental (lick, groom, and/or huddle the pup), aggressive (attack the pup), or non-interactive behavior (neither parental nor aggressive) showed a marginally significant difference between the stressed and control mice in test 1 (P=0.053) and differed significantly between the two groups in tests 2 (P=0.047) and 3 (P=0.004; **Fig. 1**). In each test, stressed mice were more likely to be non-interactive and less likely to exhibit parental behavior than controls. Few





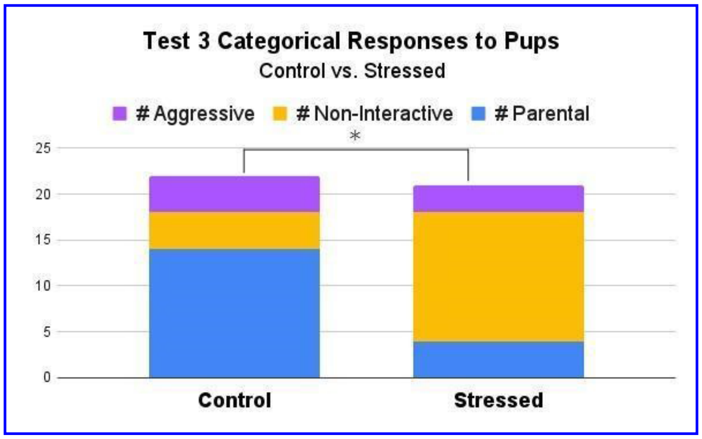
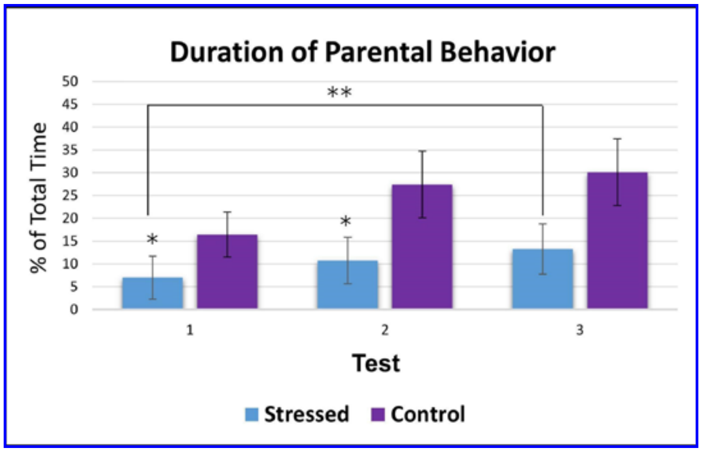


Figure 1. Numbers of stressed and control males that exhibited parental behavior, aggressive behavior, or neither in tests 1, 2, and 3. *P<0.05 for stressed vs. control mice. N=22/group except N=21 stressed males in test 3.

stressed or controlled males attacked the pup in any test.

Mice in the stressed condition spent significantly less time engaging in parental behavior, compared to controls, in test 1 (P=0.044) and test 2 (P=0.037; **Figure 2A**). The same pattern was seen in test 3, but the difference was not statistically significant (P=0.070). Duration of time spent in proximity to the pup did not differ between stressed and control mice in test 1 (P=0.142). However, stressed mice spent significantly less time in proximity to the pup in test 2 (P=0.015) and tended to spend less time in proximity in test 3 (P=0.060), compared to controls (**Figure 2B**). None of the remaining measures (see **Table 2**) differed between the stressed and control groups in any test.



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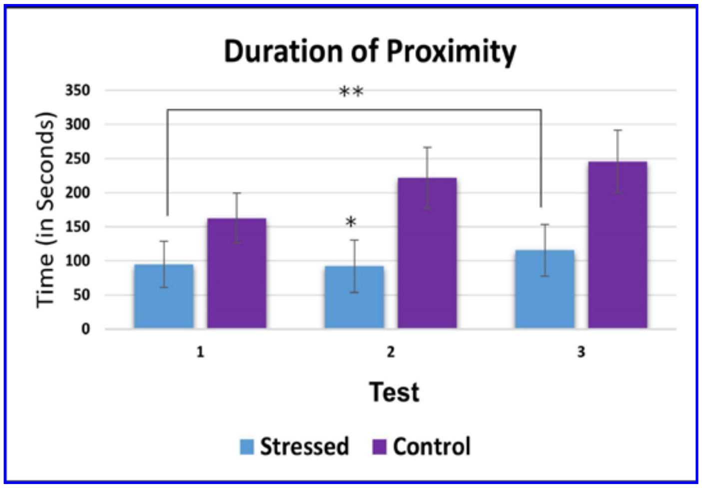

B

Figures 2A and 2B. Duration of time (mean ± SE) spent A) performing parental behavior, and B) in proximity to the pup by stressed and control mice in tests 1, 2, and 3. *P<0.05 for stressed vs. control mice. **P<0.05 for test 1 vs. test 3. N=22/group except N=21 stressed males in test 3.

Comparisons of tests 1 and 3

To determine how responses to pups changed with repeated exposure in mice that were and were not exposed to stress, we compared behaviors between test 1 (acute stress only) and test 3 (previous exposure to stress only) within each treatment group separately. We did not include test 2 in these analyses because, in this test, mice had both acute and previous exposure to stress.

Control mice spent significantly more time engaging in parental behavior (P=0.0014; **Figure 2A**) and more time in proximity to the pup (P=0.028; **Figure 2B**) in test 3 than in test 1, and were quicker to approach the pup (P=0.028; data not shown) and to initiate parental behavior (P=0.036; data not shown) in test 3 than in test 1. In contrast, stressed mice showed a significant change only in the duration of time spent grooming themselves, which decreased from test 1 to test 3 (P=0.0088; data not shown).

DISCUSSION

Fathers in biparental species, such as the California mouse, are strongly attracted to their offspring and actively engage in parental behavior, even toward unrelated infants (Elwood and Stolzenberg, 2020). Reproductively naive male California mice, on the other hand, show highly variable responses to experimentally presented pups, which can include nurturing, avoiding, or attacking the pups (Gubernick et al., 1994; De Jong et al., 2009; Horrell et al., 2017; Nguyen et al., 2020). The sources of this variation are not well understood. In this study, we evaluated the possibility that acute stress can affect reproductively naïve males' initial responses to unfamiliar pups and can have carryover effects on their subsequent behavior toward pups.

Effects of Acute Stress on Initial Responses to Pups

In their first test with a pup, mice that were injected with oil immediately prior to pup exposure spent significantly less time engaging in parental behavior (licking, grooming, huddling) and significantly more time grooming themselves, compared to non-stressed controls. They also tended to be less likely to perform parental behavior and less likely to interact with the pup at all, but these differences were only marginally significant. These results support our hypothesis that stress can have short-term inhibitory effects on parental behavior in reproductively naïve male California mice.

In test 2, as in test 1, oil-injected mice spent significantly less time performing parental behavior and more time grooming themselves, compared to controls. Additionally, in test 2, injected mice spent significantly less time in proximity to the pup and were slower to perform parental behavior than control mice. Oil-injected mice were also significantly less likely to perform parental behavior, or to interact with the pup at all, than non-stressed males in test 2. In this test, however, mice in the stress condition had been exposed to stress both immediately before the presentation of the pup and previously (i.e., in test 1); therefore, the results in test 2 cannot be attributed definitively to either acute or previous stress exposure.

Carryover Effects of Stress on Subsequent Responses to Pups

We found mixed support for our hypothesis that stress can have carryover effects on reproductively naïve males' responses to pups. In test 3, when neither group of males was stressed acutely, the two groups did not differ significantly in the durations or latencies of any behavioral measures. On the other hand, we found a highly significant difference between the stressed and control groups in the number of mice that engaged in parental, non-interactive, and aggressive behavior. As in test 2, stressed males were less likely than controls to engage in parental behavior and more likely to be non-interactive.

Because reproductively naïve male California mice exhibit increasing parental behavior upon repeated pup exposure (Horrell et al., 2017), we compared behavior in test 1 (acute stress only) and test 3 (previous exposure to stress only) to determine if repeated exposure in mice leads to behavioral responses under stressed and non-stressed conditions. We found that in control mice, repeated exposure to pups resulted in significantly more time spent engaging in parental behavior, as in our previous study (Horrell et al., 2017). Additionally, control mice spent more time in proximity to the pup in their third test with a pup compared to their first test. However, the only change stressed mice showed was a significant decrease in auto-grooming from test 1 to test 3. This could be due to the fact that stressed mice did not receive an injection in test 3 and therefore might have spent less time auto-grooming the region of injection when compared to test 1. Thus, concurrent exposure to stress and a pup blocked the usual effects of repeated pup exposure on parental behavior in reproductively naïve males.

Interestingly, very few mice attacked the pup in any of the tests, and control mice were slightly more likely to attack than stressed mice. These findings suggest that stress does not have either acute or carryover effects on pup-directed aggression in reproductively inexperienced male California mice. Moreover, the lack of interest by the stressed reproductively naïve males towards the infants begs the question of whether the effect is pup-specific or reflects a general lack of interest in stimuli caused by the oil injections. To test this question, we are currently replicating the study using virgin females as stimuli instead of pups.

CONCLUSIONS

In conclusion, this study is the first, to our knowledge, to experimentally investigate the effects of stress on reproductively naïve males' responses to pups in a biparental mammal. We found that acute stress reduces paternal behavior and increases non-interactive behaviors in reproductively inexperienced adult males, in both the short and longer terms. Our findings provide new insights into the possible sources of variation in reproductively naïve males' responses to pups and, potentially, into the onset of parental care in new fathers.

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