Individual Variation in Paternal Responses of Virgin Male California Mice (*Peromyscus californicus*): Behavioral and Physiological Correlates*

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Accepted 3/18/2012; Electronically Published 4/24/2012

ABSTRACT

California mice *Peromyscus californicus* are a rodent species in which fathers provide extensive paternal care; however, behavioral responses of virgin males toward conspecific neonates vary from paternal behavior to tolerance to infanticide. Indirect evidence suggests that paternal responses might be influenced by social status potentially through increased stress and anxiety in subordinate males. To test this hypothesis, we housed 12 virgin male California mice in same-sex dyads on weaning and assessed their within-dyad subordinate or dominant status using food-competition and urine-marking tests. In addition, behavioral responses to an unrelated pup, expression of vasopressin (AVP) and corticotropin-releasing hormone (CRH) mRNA in the paraventricular hypothalamic nucleus (PVN), basal plasma levels of testosterone and corticosterone, and body mass were measured. Food-competition and urine-marking tests did not reveal strong or stable dominance-subordination relationships in male-male dyads. Latency to sniff a newborn pup was correlated negatively with urine marking in the center of a novel environment and positively with expression of AVP mRNA in the PVN. Because these three parameters are all associated with state anxiety in other rodent species, these results suggest that individual differences in paternal responsiveness may be influenced by individual differences in anxiety but not necessarily by social status in virgin male California mice.

Introduction

Adult male mammals show a wide variety of behavioral responses toward conspecific neonates: they may attack and kill them (nonparental infanticide), tolerate but ignore them, or care for them (paternal behavior). A broadly accepted hypothesis states that males tend to kill or ignore unrelated neonates, whereas they are inclined to tolerate or even actively care for genetically related pups (Hrdy 1979; vom Saal and Howard 1982; Brown 1993; Ebensperger and Blumstein 2007). In altricial nest-dwelling species (such as most rodents), parents generally cannot distinguish their own newborn offspring from unrelated newborns (Lévy et al. 2004) and need to rely on other cues to select the most appropriate or beneficial response. These cues are mostly associated with an increased likelihood that encountered neonates are the male's own offspring (e.g., the male has had a recent mating experience or has formed a pair bond) or an increased likelihood that the neonates are not the male's own offspring (e.g., the male has recently conquered another male's territory; Elwood 1977; vom Saal and Howard 1982; Soroker and Terkel 1988; Perrigo et al. 1991; Brown 1993).

California mice *Peromyscus californicus* are one of the few biparental rodent species. In the field, young males are weaned at 5–6 wk of age (Merritt 1978) and disperse several weeks later at an average age of 77.5 d to establish a territory and to find a mate (Ribble 1992). Once a male-female pair has formed, copulation occurs exclusively within the pair (Ribble 1991), and the male spends as much time as the female caring for their offspring (Ribble and Salvioni 1990). Laboratory experiments have shown that sexually experienced pair-bonded adult males consistently care for any pup to which they are exposed, presumably because under natural circumstances these males would virtually never encounter an unrelated pup in their own territory or nest (Gubernick and Alberts 1987; Gubernick et al. 1994; de Jong et al. 2009, 2010).

Reproductively inexperienced male California mice, on the other hand, may attack, ignore, or care for encountered pups. Percentages differ among studies: Gubernick et al. (1994) reported that the majority of virgin males attacked or ignored a

* This paper was submitted in response to a call for papers for a Focused Issue on "Intraspecific Variation in Physiology and Behavior."

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Physiological and Biochemical Zoology 85(6):740–751. 2012. 1522-2152/2012/8506-1156$15.00. DOI: 10.1086/665831

stimulus pup (both 40%–50%) and <10% behaved paternally, whereas in our lab (de Jong et al. 2009; T. R. de Jong, M. Chauke, B. N. Harris, and W. Saltzman, unpublished observations) the majority of virgin males behave paternally or ignore a stimulus pup (both 40%–50%) and <10% attack the pup. The causes of individual variation in paternal responses among these virgin males are yet unknown. In other rodent species, including promiscuous uniparental house mice (*Mus musculus*) and monogamous biparental Mongolian gerbils (*Meriones unguiculatus*), socially dominant virgin males are significantly more likely to commit infanticide than their subordinate virgin male cage mates (Huck et al. 1982; vom Saal et al. 1982; Elwood and Ostermeyer 1984). Interestingly, in a recent study we found that in six male-male dyads of virgin California mice (three pairs of littermates and three pairs of unrelated age-matched males), each dyad contained one male that responded paternally to a newborn pup and one male that ignored the pup (males were tested individually in novel cages; de Jong et al. 2009). We hypothesized that dominance status within a dyad influenced the behavioral response to pups.

The first aim of this experiment was therefore to examine whether virgin male California mice form dominant-subordinate relationships and whether social status correlates with paternal and/or infanticidal responses to newborn pups. Although it is well known that male California mice aggressively defend their territories and that winning territorial fights induces a strong "winner effect" (i.e., an increased chance of winning future fights) in this species (Fuxjager and Marler 2010), it is not yet known whether cohabiting unrelated adult virgin male California mice form a stable dominant-subordinate relationship. To investigate this, we selected and adapted two behavioral tests commonly used in studies of rodent social hierarchies: competition for a highly palatable food item (Scott 1966; Price et al. 1976; File 1986; Gentsch et al. 1988; Askew et al. 2006) and urine marking in a novel environment (Desjardins et al. 1973; Hurst 1990; Drickamer 2001; Arakawa et al. 2008). We expected to elicit quantifiable behaviors in these tests, because male California mice have been described as competing actively for food and using urine marking to claim ownership of runways that cross multiple territories (Eisenberg 1962). Each behavioral test was performed twice, once before and once after the average age of dispersal (77.5 d), in an attempt to determine the presence and stability of a dominant-subordinate relationship in each dyad.

The second aim of the experiment was to investigate whether and how social status might influence behavioral responses to newborn pups. Long-term cohabitation with a dominant cage mate is known to cause numerous physiological changes in subordinate rodents, including decreases in circulating testosterone and increases in circulating corticosterone levels, alterations in expression of vasopressin (AVP) and corticotropin-releasing hormone (CRH) mRNA in the paraventricular hypothalamic nucleus (PVN), and decreased body mass gain (Albeck et al. 1997; Bartolomucci et al. 2001; Pohorecky et al. 2004; Tamashiro et al. 2004; Reber and Neumann 2008; Erhardt et al. 2009). Changes in testosterone and AVP levels can directly influence the occurrence of paternal or infanticidal behaviors. Castration of California mice decreases the expression of paternal behavior, whereas testosterone replacement reinstates it (Trainor and Marler 2001, 2002), an effect that contrasts with the castration-induced facilitation of paternal care and inhibition of infanticide in rats and house mice (Rosenberg and Herrenkohl 1976; Svare and Mann 1981). Central infusion of AVP facilitates paternal care and reduces infanticide in meadow voles *Microtus pennsylvanicus* (Parker and Lee 2001), and central infusion of AVP increases, whereas an AVP receptor antagonist inhibits, paternal behavior in prairie voles *Microtus ochrogaster* (Wang et al. 1994; Bales et al. 2004). The role of AVP in paternal care in California mice is less well understood, but AVP-immunoreactive staining in the bed nucleus of the stria terminalis correlated positively with paternal behavior in *P. californicus* and white-footed mice *Peromyscus leucopus* (Bester-Meredith and Marler 2003). Decreased body mass gain and increased plasma corticosterone and hypothalamic CRH mRNA levels would most likely be signs of chronic stress. Although the direct effects of stress on paternal behaviors are not yet understood in California mice or other species (as discussed in Harris et al. 2011), chronic social stress is known to increase anxiety (Reber and Neumann 2008; Erhardt et al. 2009), which in turn may cause an increased avoidance of novel stimuli, such as pups (Belzung and Le Pape 1994).

Taken together, in this experiment we tested the hypothesis that individual variation in dominance status underlies individual variation in behavioral responses toward unrelated conspecific pups in adult virgin male California mice. In addition, we tested the hypothesis that physiological changes associated with social status, including changes in circulating basal testosterone and corticosterone levels, expression of AVP and CRH mRNA in the PVN (and as a negative control, expression of AVP mRNA in the supraoptic nucleus [SON]), and body weight, correlate with behavioral responses toward an unrelated pup.

Material and Methods

Animals

Male California mice *Peromyscus californicus* ($N = 12$) were born in the breeding colony at the University of California, Riverside (UCR), which consists of descendants of mice purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia). All males in this experiment were from generation F3 or F4 within our breeding colony (F1 being born to parents purchased from the stock center), and cage mates did not have any common ancestors in the F1–3 generations. On weaning at postnatal day (PND) 30–32 (before the birth of the next litter in the home cage), males were paired with one another in a total of six dyads of unrelated age-matched mice. Mice were housed in polycarbonate home cages (44 cm × 24 cm × 20 cm) containing wood-chip bedding and cotton for nest building. Mice were maintained on a 14L : 10D cycle (lights on at 0500 hours) in an ambient temperature of approximately 23°C and approximately 65% hu-

midity and had ad lib. access to chow (Purina 5001 Rodent Diet) and water throughout the experiment.

All experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the UCR Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering. UCR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Experimental Design

Males were weighed weekly from weaning (week 1 of the experiment) until the end of the behavioral tests (PND 95–98, week 11 of the experiment) in order to monitor health and body mass gain. The experiment was divided into two phases: in weeks 1–8 of the experiment (phase 1), the males were younger than 77.5 d (i.e., the average age of natal dispersal by male California mice; Ribble 1992), and in weeks 9–11 of the experiment (phase 2), the males were older than 77.5 d. In both phases, all males underwent two types of behavioral tests to assess social status: a series of food-competition tests (one at PND 53–56, in week 5 of the experiment, and one at PND 81–84, in week 9 of the experiment) and a urine-marking test (one at PND 60–63, in week 6 of the experiment, and one at PND 89–91, in week 10 of the experiment). In addition, all males underwent one paternal-behavior test at PND 95–98, in week 11 of the experiment. Finally, all males were decapitated at PND 120–140, and brains and trunk blood were collected for neuropeptide (AVP, CRH) mRNA and steroid hormone (testosterone, corticosterone) analyses, respectively.

Food-Competition Test

In a pilot study using a different set of animals, various food types were tested for their attractiveness to *P. californicus*. Organic cornflakes (various brands) appeared to be the most palatable and were selected to determine which individual within a dyad secured the most access to a rewarding food item.

All food-competition trials were performed in the room in which the mice were housed. An investigator dropped a cornflake into the home cage through the wire-mesh cage lid, aiming for a spot at an equal distance from both mice. The behavior of the two resident mice was then videotaped until the cornflake was fully eaten. The test was terminated (after 10 min) if the cornflake was never investigated or if the cornflake was initially approached and touched but then ignored for more than 1 min. Each dyad underwent three such trials in week 5 of the experiment (one trial per day on three consecutive days between 1330 and 1500 hours) and three trials in week 9 of the experiment (one trial per day for three consecutive days between 1000 and 1200 hours; the different time of day was chosen for logistical reasons and was not expected to influence the behavioral data). Tests were videotaped, and behaviors were subsequently scored using the JWatcher event-recorder program (Blumstein and Daniel 2007). The latency to sniff the cornflake was measured for each male in each trial. In addition, the total time a cornflake was being touched or eaten by a single individual (if the two cage mates simultaneously touched a piece of the cornflake, this time was not counted) was determined for each trial, and the percentage of the total time that each individual male was the one touching or eating was calculated; thus, the values for both males in a dyad always added up to 100%. The male within each dyad that had the higher percentage of exclusive cornflake-possession time during a trial was considered the winner.

Urine-Marking Test

All males underwent two urine-marking tests, one in week 6 and one in week 10, between 1420 and 1630 hours, in the room in which the mice were housed. Clean, dry cages were prepared with one layer of filter paper (Whatman no. 1, Whatman, Maidstone, UK) covering the bottom. No wood shavings or other nesting material was present in the cages, and no food or water was provided. The two individuals within a dyad were placed simultaneously into two neighboring test cages for 30 min. At the end of the test period, the mice were reunited in their home cage, and the filter paper from the two test cages was allowed to dry overnight. Fluorescent urine stains were visualized under ultraviolet light by pencil tracing. A clear rectangular plastic sheet with a grid of 8 × 16 squares (2.54 cm × 2.54 cm each) divided into a center rectangle (4 × 10 squares) and a peripheral area (2 squares wide on the long edges, 3 squares wide on the short edges) was placed over the filter paper. Because urination in the center of a novel environment is a marker for dominance in *Mus musculus* (Desjardins et al. 1973; Drickamer 2001), the number of central squares that contained (part of) a urine stain was counted for each individual male.

Paternal-Behavior Test

All males underwent a paternal-behavior test in week 11 of the experiment between 0930 and 1130 hours following the same protocol as described previously (de Jong et al. 2009). Briefly, males were separated from their cage mate and placed in a clean cage in a separate observation room. After 10 min of habituation, an unfamiliar 1–3-d-old pup was placed in the front right corner of the cage. The male's behavior was videotaped for 10 min. If a male attacked the pup, the test was terminated immediately and the pup was returned to its family. Behavioral data were later analyzed using the JWatcher event-recorder program with the following parameters scored: latency to sniff the pup (males that did not sniff the pup were assigned a latency of 600 s for this parameter), total time spent sniffing the pup, and total time spent in paternal behavior (composite score of time spent huddling [with or without kyphosis] and/or licking the pup; de Jong et al. 2009).

Blood and Brain Collection

At least 25 d after the paternal-behavior test, between PND 120 and 140, each mouse was decapitated between 1447 and 1507 hours within 2 min of initial cage disturbance. Immediately on decapitation, trunk blood was collected in heparinized plastic weighing boats, and brains were dissected and flash-frozen in powdered dry ice. Blood was transferred to 1.5-mL Eppendorf tubes and centrifuged for 12 min (13,300 rpm, 4°C), and plasma was collected. Both plasma and brain tissue were stored at −80°C until further processing.

Hormone Measurements

Plasma concentrations of testosterone were measured at the Assay Services Laboratories at the Wisconsin National Primate Research Center (University of Wisconsin–Madison) using an assay previously validated for *P. californicus* (Trainor and Marler 2001). Briefly, samples were extracted with ethyl ether, and steroids were separated using celite chromatography. Total testosterone was analyzed in duplicate using an enzyme immunoassay on a single plate (T antibody R156, UCD, diluted to 1 : 35,000). Assay sensitivity at 90% binding was 0.9 pg, and inter- and intra-assay coefficients of variation (CVs) were 15.5% and 3.9%, respectively ($N = 54$ assays).

Plasma concentrations of corticosterone were determined at UCR as described previously (Chauke et al. 2011). Briefly, samples were analyzed in duplicate using an ^{125}I double-antibody radioimmunoassay kit (07120102, MP Biomedicals, Costa Mesa, CA) that our lab has validated for *P. californicus* plasma using standard biochemical and biological validation procedures (Chauke et al. 2011; M. Chauke, B. N. Harris, and W. Saltzman, unpublished data). Samples from all mice in the experiment were analyzed in a single assay run. Assay sensitivity at 90% binding was 17.4 ng, and inter- and intra-assay CVs were 11.2% and 4.7%, respectively ($N = 45$ assays).

In Situ Hybridization

Frozen brains were sliced on a cryostat into five series of 20-μm-thick coronal sections containing the PVN and thaw mounted on gelatin/chrome-alum-coated glass slides. The first series of each brain was thawed, air-dried, and stained with Quick Stain (American MasterTech, Lodi, CA) to determine the exact locations of the relevant brain areas; the remaining series were kept frozen until in situ hybridization procedures were started. Unfortunately, the brain of one male was damaged during slicing and could not be processed further.

Expression levels of AVP and CRH mRNA were quantified using ^{35}S-labeled deoxyoligonucleotide probes synthesized by Sigma Genosys (Woodlands, TX) that were complementary to the 3′ end of the glycoprotein sequence in the *Rattus norvegicus* AVP gene (48-bp oligomer: GTAGACCCGGGGCTTGGCAGAA TCCACGGACTCTTGTGTCCCAGCCAG) or to the 3′ end of the CRH peptide sequence in the *R. norvegicus* CRH gene (60-bp oligomer: GATAATCTCCATCAGTTTCCTGTTGCTGT-GAGCTTGCTGAG CTAACTGCTCTGCCCTGGC). The probes and procedures were previously validated for *R. norvegicus* and *M. musculus* in the laboratory of T. Z. Baram at the University of California, Irvine (Baram and Lerner 1991; Ivy et al. 2008; Rice et al. 2008), and pilot studies yielded signals in the PVN of *P. californicus* that were highly similar to the signals found in *R. norvegicus* in terms of strength and location.

Frozen sections were fixed in freshly made 4% buffered paraformaldehyde for 20 min followed by dehydration and rehydration through graded ethanols. Sections were exposed to 0.25% acetic anhydride and 0.1 M triethanolamine (pH 8) for 8 min and were dehydrated through graded ethanols. Sections were hybridized overnight (20 h) in a humidified chamber at 42°C with 0.20×10^6 CPM of labeled probe dissolved in a buffer solution (50% formamide, 5X SET, 0.2% SDS, 5X Denhart's, 0.5 mg/mL salmon sperm DNA, 0.25 mg/mL yeast tRNA, 100 mM dithiothreitol, and 10% dextran sulfate; 30 μL per section). After hybridization, sections underwent serial washes of saline sodium citrate (SSC): 4X SSC for 5 min at room temperature (RT), 2X SSC for two times 30 min at 55°C, and 1X SSC and 0.3X SSC for 30 min each at RT. Sections were then dehydrated through graded ethanols containing 0.3 M ammonium acetate followed by 95% and 100% ethanol and air-dried.

Sections were placed in autoradiography cassettes and apposed to film (Kodak BioMax MR Film, Eastman Kodak, NY). Sections containing the PVN ($N = 4$ or 5 per animal per probe) were initially spread over two cassettes per probe, and films were developed after 1 d (AVP) or 6 d (CRH). Subsequently, two sections displaying the strongest signal of each probe were selected for each animal, were placed in one autoradiography cassette per probe, and were reapposed to film together with a ^{14}C-standard (American Radiolabeled Chemicals, St. Louis) for 1 d (AVP) or 14 d (CRH). Developed films were digitized and analyzed using the ImageJ software program from the National Institutes of Health. Gray levels of the ^{14}C-standard on each film were measured and fitted to a curve expressed in nCi/g, and hybridization and background signals on the same film were quantified using that curve. Each positive signal in the PVN was outlined five times, and a neutral area immediately adjacent to the PVN was outlined three times, in order to obtain reliable measurements of signal and background optical densities. For AVP, positive signals in the SON were outlined in the same sections as the PVN. Following the subtraction of average background densities from average signal densities, the two highest values were averaged for each animal in order to calculate the final optical densities.

Statistical Analyses

All statistical analyses were performed using SPSS 16.0. Nonparametric correlation coefficients (Spearman's ρ) were calculated between behavioral and physiological parameters. Correlations for which the correlation coefficient was above 0.6

and the *P* value was below 0.05 were considered biologically significant (Nakagawa 2004).

Results

Behavioral Tests

Of the six dyads, one pair had the same winner in all six food-competition trials, three pairs had the same winner in four out of six trials, and in two pairs each male won half the trials (table 1). In 68% of the trials, the individual that was first to sniff the cornflake also had the highest percentage of cornflake-possession time. However, latency to sniff the cornflake did not correlate significantly with the percentage of cornflake possession in any of the six trials (data not shown).

In the urine-marking tests, males urinated on an average ± SEM of 9.58 ± 2.08 (out of 40) central squares in week 6 and on 13.50 ± 3.90 central squares in week 10 (table 1). Each male urinated at least once in each test. In four out of six dyads, the same male urinated on more central squares in week 6 and week 10. In one dyad the order was reversed, and in one dyad the two males' scores were exactly equal in week 10 (table 1).

In the paternal-behavior test, males showed an average latency to sniff the pup of 71.41 ± 24.22 s and spent an average 169.46 ± 42.70 s behaving paternally toward the pup. Four of the 12 males did not show any paternal behavior: two males ignored the pup, one male immediately and aggressively attacked the pup, and one male nibbled on the pup (no aggressive attack; fig. 1).

Physiological Measurements

Males weighed 20.93 ± 1.04 g at the time of weaning (week 1) and 38.15 ± 1.65 g at the end of behavioral testing (week 11). Average body mass gain between week 1 and week 11 was 85.00% ± 8.39%. Blood plasma collected between 1447 and 1507 hours (several hours before lights off) contained an average of 729.17 ± 217.77 pg/mL testosterone and 414.03 ± 100.38 ng/mL corticosterone, similar to previously reported findings in adult virgin male California mice at approximately the same time of day (Oyegbile and Marler 2005).

Both AVP and CRH showed positive hybridization signals in the PVN in each male (see fig. 2 for representative photographs). The average signal intensity was 1,947.11 ± 162.87 nCi/g for AVP mRNA and 152.71 ± 10.54 nCi/g for CRH mRNA, similar to measurements made in adult female rats using the same protocol (Ivy et al. 2008). AVP also showed a positive hybridization signal in the SON with an average signal intensity of 2,231.83 ± 261.21 nCi/g.

Table 1: Percentage of exclusive cornflake possession in the food-competition test and number of urine-positive center squares in the urine-marking tests in 12 adult virgin male California mice housed in six dyads

	Food competition (week 5)				Food competition (week 9)			
Dyad and male	T1	T2	T3	Urine (week 6)	T4	T5	T6	Urine (week 10)
1:								
1	4.44[a]	44.29	51.15[a]	21	39.70[a]	78.46[a]	57.57	8
2	95.56	55.71[a]	48.85	18	60.30	21.54	42.43[a]	30
2:								
3	69.79[a]	74.35[a]	100.00[a]	9	32.96[a]	49.76[a]	86.05[a]	40
4	30.21	25.65	.00	6	67.04	50.24	13.95	0
3:								
5	7.81	12.71[a]	.00	7	35.57	76.44	61.06[a]	0
6	92.19[a]	87.29	100.00[a]	21	64.43[a]	23.56[a]	38.94	9
4:								
7	100.00[a]	100.00[a]	100.00[a]	10	100.00	100.00	100.00[a]	33
8	.00	.00	.00	4	.00[a]	.00[a]	.00	7
5:								
9	41.06	64.51[a]	60.15[a]	13	37.14[a]	83.17[a]	100.00[a]	13
10	58.94[a]	35.49	39.85	3	62.86	16.83	.00	0
6:								
11	NA	NA	95.51	0	39.83	15.80	49.60	11
12	NA	NA	4.49[a]	3	60.17[a]	84.20[a]	50.40[a]	11

Note. T1–3 were the three trials in week 5; T4–6 were the three trials in week 9 of the experiment. Urine-marking tests were performed in week 6 and week 10. NA = did not approach the cornflake during that trial.

[a]An individual that sniffed the cornflake first during the trial.

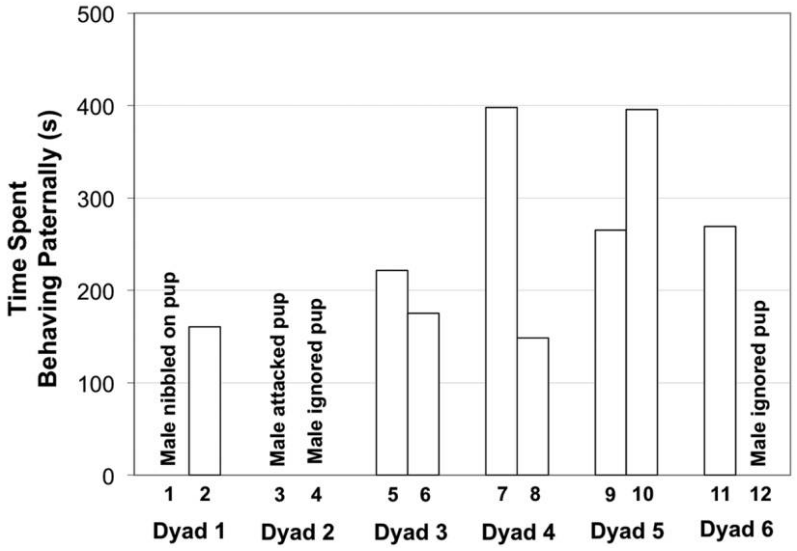

Figure 1. Total duration of paternal behavior toward an unfamiliar newborn pup in week 11 of the experiment of 12 adult virgin male California mice housed in six dyads. Responses of nonpaternally behaving males are noted in the graph as well.

Correlations

A correlational analysis of 10 behavioral parameters was performed: percentage of cornflake possession in each of trials 1–6 of the food-competition test, number of urine-positive central squares in each of the urine-pattern tests, and latency to sniff the pup and time spent behaving paternally in the paternal-behavior test (table 2). Percentages of cornflake possession correlated positively among the first three trials ($\rho > 0.677$, $P < 0.031$ for all three correlations) but were less consistent among the second three trials and between the first and second set of trials. The first three trials also correlated well with the results from the second urine-marking test ($\rho > 0.644$, $P < 0.044$ for all three correlations; see fig. 3*A* for scatterplot) but not with the results from the first urine-marking test, and the two urine-marking tests did not correlate strongly with one another. The time spent behaving paternally in the paternal-behavior test did not correlate with any result from the food-competition or urine-marking tests, but the latency to sniff a pup correlated strongly and negatively with the number of urine-positive center squares in the second urine-marking test ($\rho = -0.736$, $P = 0.006$; see fig. 3*B* for scatterplot).

In addition, correlational analyses were performed among the four most relevant behavioral parameters measured in phase two of the experiment, thus in adult animals (average percentage of cornflake possession in trials 4–6 of the food-competition test, number of urine-positive central squares in the second urine-marking test, latency to sniff a pup, and time spent behaving paternally in the paternal-behavior test), and

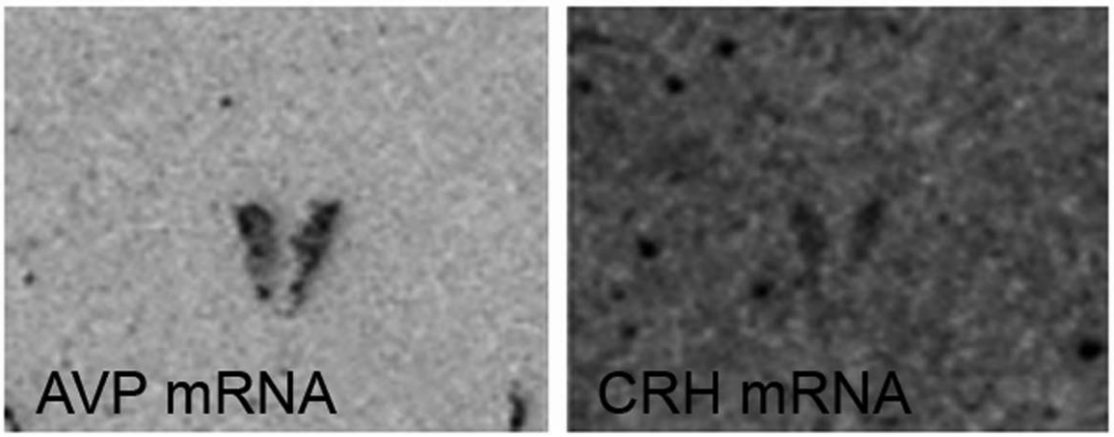

Figure 2. Representative digital images of vasopressin (*left*) and corticotropin-releasing hormone (*right*) mRNA expression as shown by in situ hybridization in the paraventricular hypothalamic nucleus (PVN; the PVN of the same animal is shown in both images).

Table 2: Spearman's correlations among 10 behavioral parameters in the paternal-behavior test

	FC1	FC2	FC3	FC4	FC5	FC6	UM1	UM2	LSP
FC2:									
ρ	**.818**	...	...	...	...	...	...	...	...
P	**.004**	...	...	...	...	...	...	...	...
N	**10**	...	...	...	...	...	...	...	...
FC3:									
ρ	**.677**	**.963**	...	...	...	...	...	...	...
P	**.031**	**.000**	...	...	...	...	...	...	...
N	**10**	**10**	...	...	...	...	...	...	...
FC4:									
ρ	.567	.442	.206	...	...	...	...	...	...
P	.082	.200	.521	...	...	...	...	...	...
N	10	10	12	...	...	...	...	...	...
FC5:									
ρ	.127	.430	.156	.238	...	...	...	...	...
P	.726	.214	.628	.457	...	...	...	...	...
N	19	10	12	12	...	...	...	...	...
FC6:									
ρ	.348	.604	.484	−.161	**.744**	...	...	...	...
P	.325	.065	.111	.616	**.006**	...	...	...	...
N	10	10	12	12	**12**	...	...	...	...
UM1:									
ρ	.322	.614	.416	.102	.323	.343	...	...	...
P	.364	.059	.178	.753	.306	.275	...	...	...
N	10	10	12	12	12	12	...	...	...
UM2:									
ρ	**.644**	**.804**	**.733**	−.018	.279	**.619**	.296	...	...
P	**.044**	**.005**	**.007**	.957	.380	**.032**	.351	...	...
N	**10**	**10**	**12**	12	12	**12**	12	...	...
LSP:									
ρ	−.413	−.419	−.350	.125	.004	−.234	−.114	**−.736**	...
P	.236	.228	.264	.700	.991	.464	.724	**.006**	...
N	10	10	12	12	12	12	12	**12**	...
TSP:									
ρ	.436	.288	.296	.295	.068	.132	−.136	.052	−.188
P	.208	.419	.350	.351	.835	.682	.647	.872	.558
N	10	10	12	12	12	12	12	12	12

Note. FC1–6: percentage of exclusive cornflake-possession time in each of trials 1–6 of the food-competition test; UM1–2: number of urine-positive central squares in each of the two urine-marking tests; LSP: latency to sniff a pup; TSP: time spent behaving paternally. Results in bold are considered biologically significant ($\rho > 0.6$, $P < 0.05$).

six physiological measurements (body mass gain from week 1 to week 11 of the experiment, basal circulating levels of corticosterone and testosterone, and mRNA expression of AVP and CRH in the PVN and AVP in the SON; table 3). One biologically significant correlation was found: expression of AVP mRNA in the PVN was associated positively with the latency to sniff a pup ($\rho = 0.744$, $P = 0.009$; see fig. 3*C* for scatterplot).

Discussion

This experiment was designed to investigate whether dominant-subordinate relationships can be identified in dyads of unrelated virgin male California mice and, if so, whether individual variation in behavioral responses toward newborn pups is associated with social status. Additionally, we aimed to determine which physiological parameters might underlie an association between social status and paternal responsiveness.

In six male-male dyads of virgin California mice, the social relationship between cage mates was assessed using food-competition tests and urine-marking tests. Males underwent these tests at two different ages: once before and once after PND 77.5, which is the average age of male dispersal in this species in the field (Ribble 1992). In the food-competition test, cage mates were found to compete actively for a cornflake in the

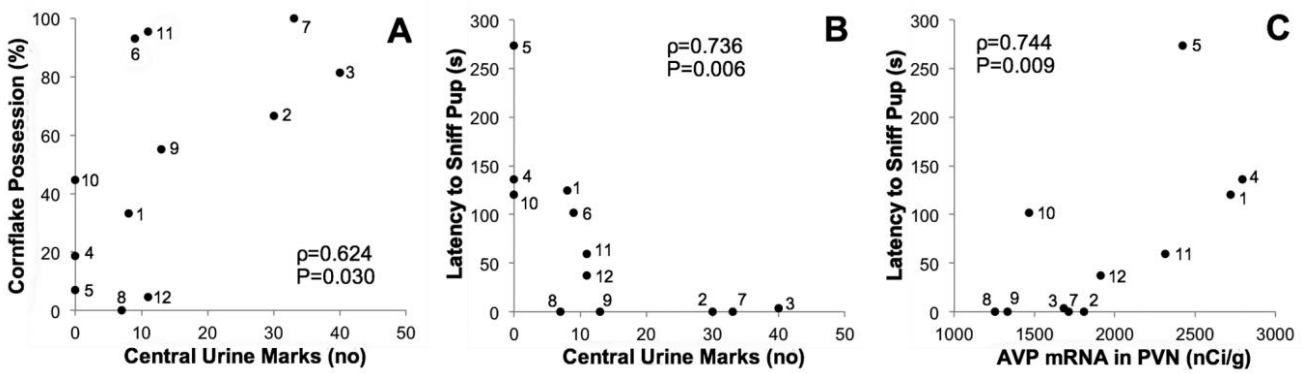

Figure 3. Scatterplots depicting the relationship between selected behavioral and physiological data in individual virgin male California mice (numbers in plots correspond to mice, the same as in table 1). *A*, *Y*-axis: percentage of exclusive cornflake possession averaged over the three trials in the food-competition test performed in week 5. *A*, *B*, *X*-axis: number of urine-positive central squares measured in the urine-marking test performed in week 10. *B*, *C*, *Y*-axis: latency to sniff the pup. *C*, *X*-axis: expression of AVP mRNA in the PVN. Note that dots correspond to real values, whereas statistics (ρ and *P* values) were performed on ranks.

majority of trials. However, the difference in exclusive cornflake-possession time between the "winner" and "loser" was often small within a trial, and in most dyads no consistent winner emerged over the six trials (table 1). Cornflake possession was not strongly influenced by novelty seeking: although in the majority of trials (23 out of 34), the male that first sniffed the cornflake also "won" the trial, latency to approach the cornflake and time spent in exclusive possession were not significantly correlated with one another in any of the trials (data not shown). In male rats, comparable food-competition tests have revealed stable dominance relationships (Joly and Sanger 1991; Millard and Gentsch 2006), although some researchers have argued for stricter criteria and have proposed that as few as 25% of rat dyads form stable dominance relationships (Malatynska and Knapp 2005). Interestingly, in our experiment, results from the first three trials (performed when animals were 53–58 d old) correlated strongly and positively with one another, whereas the second three trials (performed when animals were 81–86 d old) show less consistent results (table 2). It is possible that food competition is a better measure of social status in younger California mice, which would be in concordance with their natural behavior of competing for food while still in the parental nest (Eisenberg 1962); once a male has established its own territory, direct food competition with other males may be unlikely to occur.

Previous research has shown that dominant male house mice placed in a novel environment make multiple small urine marks that are spread out over the test area, whereas subordinate males tend to urinate in a few peripheral pools (Desjardins et al. 1973; Hurst 1990; Drickamer 2001; Arakawa et al. 2008). In this experiment, urine-marking tests were performed twice (once before and once after the average age of dispersal) to assess which male in a dyad was the more dominant. Similar to the food-competition test, there were no strong correlations between the results obtained in the first ("predispersal") and the second ("postdispersal") urine-marking tests, possibly because urine marking becomes a biologically relevant behavior only when males are older and more likely to establish their own territory.

The finding that behavioral differences between the two cohabiting males varied widely from dyad to dyad suggests that dominant-subordinate relationships are not formed reliably in pair-housed unrelated age-matched virgin male *Peromyscus californicus*. It is possible that dominant-subordinate relationships do appear under more natural experimental/housing conditions, for example, when unrelated males are allowed to interact intermittently rather than constantly (simulating encounters at territorial boundaries; Bartolomucci et al. 2001) or when young males are pair-housed with their father or male littermate (simulating a communal nest). Future experiments should explore these possibilities in addition to using a larger sample size and more extensive behavioral testing.

Although dominance-subordinate relationships were not formed reliably within dyads, individual males may still show more or less dominant or subordinate personalities compared with the other males in the study independent of the personality of their cage mate. A correlation analysis showed that behaviors in food-competition and urine-marking tests did not correlate significantly with one another either in weeks 5 and 6 or in weeks 9 and 10. However, percentage of cornflake possession in week 5 (simulating food competition in young males living in the communal nest) correlated significantly and positively with central urine marking in week 10 (simulating the marking of a territory in adult males), suggesting that these age-dependent behavioral parameters are both markers of a certain (potentially dominant/subordinate) personality type. It should be noted here that data from the food-competition tests are dyad dependent (i.e., the percentages of exclusive cornflake possession of two individuals in a dyad always summed to 100%), whereas the other behavioral and physiological parameters are dyad independent. Because both the food-competition and urine-marking tests were selected to measure social status, it

Table 3: Spearman's correlations among four behavioral parameters measured in phase two of the experiment in adult males

	FC4–6	UM2	LSP	TSP
BMG:				
ρ	.210	.303	−.495	−.021
P	.513	.338	.133	.947
N	12	12	12	12
T:				
ρ	.112	.229	−.281	−.345
P	.729	.473	.376	.272
N	12	12	12	12
CORT:				
ρ	.140	.332	−.164	−.210
P	.665	.292	.611	.512
N	12	12	12	12
AVP PVN:				
ρ	−.355	−.510	**.744**	.055
P	.285	.109	**.009**	.872
N	11	11	**11**	11
AVP SON:				
ρ	−.109	−.230	.461	−.110
P	.750	.496	.154	.747
N	11	11	11	11
CRH PVN:				
ρ	−.245	−.556	.428	.000
P	.467	.075	.189	.999
N	11	11	11	11

Note. FC4–6: average percentage of cornflake possession over trials 4–6 in the food-competition test; UM2: number of urine-positive central squares in the second urine-marking test; LSP: latency to sniff a pup; TSP: time spent behaving paternally; BMG: body mass gain between week 1 and week 11; T: basal circulating plasma levels of testosterone; CORT: basal circulating plasma levels of corticosterone; PVN: paraventricular hypothalamic nucleus; AVP PVN: expression of vasopressin (AVP) mRNA in the PVN; AVP SON: expression of AVP mRNA in the supraoptic nucleus (SON); CRH PVN: expression of corticotropin-releasing hormone (CRH) mRNA in the PVN. Results in bold are considered biologically significant ($\rho > 0.6$, $P < 0.05$).

can be assumed that in this particular case, the significant positive correlation reflects a true association between the behaviors. However, significant correlations (or in this case lack thereof) between food-competition values and all other dyad-independent data should be interpreted with care.

Neither percentage of cornflake possession nor central urine-marking in younger or older animals correlated significantly with the time spent behaving paternally toward an unfamiliar pup, indicating that social status is not a strong determinant of paternal or infanticidal responses. However, central urine marking in adult males did correlate significantly and negatively with the latency to sniff the pup (thus, males that approached the pup more quickly also performed more urine marking in the central part of the test cage). It seems likely that anxiety rather than social status links these two behavioral parameters: increased time spent in the center of a novel environment (reflected here by increased urine marking) as well as a rapid approach to novel objects (reflected here by reduced latency to sniff the pup) are both indicators of low levels of anxiety (Belzung and Le Pape 1994; Numan 2007). Consistent with these findings, a recent article reported that female mice that behaved anxiously in an open field test (high levels of immobility, low number of lines crossed) as virgins showed an increased latency to retrieve their own pups as mothers (Curley et al. 2012). Anxiety levels did not predict any other differences in maternal behavior in these females, leading the authors to hypothesize that differences in trait anxiety specifically affect aspects of maternal care that are influenced by neophobia, such as retrieving pups from an unfamiliar environment.

In this study, the latency to sniff a pup correlated strongly and positively with the expression of AVP mRNA in the PVN (but not in the SON): males with lower AVP mRNA expression in the PVN approached the pup more quickly. AVP and CRH mRNA were initially measured to assess whether subordinate animals had alterations in expression of these neuropeptides as a result of chronic social stress, which in turn may affect behavioral responses to newborn pups. Because the animals could not be identified reliably as dominant or subordinate, individual variation in AVP mRNA levels must have been caused by other yet unknown factors. The positive association between AVP mRNA expression in the PVN and latency to sniff a pup could mean that AVP inhibits paternal behavior; however, we did not find a strong correlation between expression of AVP mRNA and time spent behaving paternally. In addition, previous research has found a positive relationship between AVP immunoreactivity in the bed nucleus of the stria terminalis (which is functionally different from AVP in the PVN) and paternal behavior in male California mice (Bester-Meredith et al. 1999; Bester-Meredith and Marler 2001, 2003). Rather than paternal behavior itself, AVP may be associated with the anxiety aspect of the latency to sniff a pup. Positive correlations between AVP expression in the PVN and anxiety have been found in other rodent species (Wigger et al. 2006; Pan et al. 2009). Expression of AVP mRNA did not correlate strongly with central urine marking, which we assumed was an inverse measure of anxiety, but it is possible that AVP is particularly involved in anxiety related to social stimuli such as a conspecific pup (Murakami et al. 2011).

The finding of a nonsignificant negative correlation between time spent in paternal behavior and basal circulating testosterone levels contrasts with a previously reported nonsignificant positive correlation between the time spent huddling pups and basal circulating testosterone levels in the same species (Trainor and Marler 2001). The latter study used experienced fathers rather than virgin males, which could account for the difference in direction of the correlation. In addition, use of an assay measuring free rather than total testosterone in *P. californicus* plasma would provide a more sensitive method to assess the relationship between testosterone and behavior (Lynn 2008).

In conclusion, the results of this study indicate that pairs of

unrelated virgin male California mice do not form strong or stable dominant-subordinate relationships and that behaviors thought to be indicative of social dominance or subordination are not associated with behavioral responses to pups. Latency to sniff a newborn pup was negatively correlated with urine marking in the center of a novel environment and positively correlated with expression of AVP mRNA in the PVN. These three parameters are all associated with anxiety, suggesting that anxiety may influence the initiation of paternal behavior more strongly than social status.

Acknowledgments

The authors would like to thank Leslie Karpinski, Jim Sinclair, and John Kitasako for their excellent caretaking of the mice; Dr. Chris Lytle for the use of and assistance with his cryostat; Professor Tallie Z. Baram for the outstanding help with in situ hybridizations; and Miyetani Chauke, Marian Antonious, Julia Cho, and Saif Hossain for their assistance in the lab. This work was supported in part by National Institutes of Health grant R21MH087806.

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