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Fatherhood increases attraction to sensory stimuli from unrelated pups in male California mice, *Peromyscus californicus*

April M. Arquilla [a], Kerianne M. Wilson [a, 1], Khaleel A. Razak [a, b, c], Wendy Saltzman [a, c, *]

[a] Department of Evolution, Ecology, and Organismal Biology, University of California, Riverside, CA, U.S.A.
[b] Department of Psychology, University of California, Riverside, CA, U.S.A.
[c] Neuroscience Graduate Program, University of California, Riverside, CA, U.S.A.

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When becoming parents, female mammals undergo extensive physiological and behavioural changes that facilitate the onset of parental care. These include increased attraction to sensory stimuli from neonates, mediated by hormonal and neural shifts accompanying pregnancy and parturition. In some biparental species, new fathers also become more attracted to neonates; however, the underlying mechanisms are not clear. We examined the effects of becoming a father on males' behavioural responses to two sensory stimuli from unrelated pups – odour and vocalizations – in the biparental California mouse. First-time fathers and age-matched virgin males were exposed for 10 min to a pup-related stimulus (pup odour, pup vocalizations or both) and, simultaneously, to a control stimulus (unscented cotton and white noise) in a 1 × 1 m open-field arena. Fathers spent significantly more time in proximity to and in contact with pup-related stimuli, regardless of sensory modality, than control stimuli, while virgins showed no differences in their responses to pup-related and control stimuli. Neither fathers nor virgins responded differentially to pup odours, pup vocalizations or the combination of these stimuli; however, males exposed simultaneously to pup odours and vocalizations spent less time near the arena walls compared to males in all other stimulus conditions and spent more time in the empty corners of the arena compared to males presented with only pup odour. These results suggest that fathers are attracted to pup stimuli but do not show differential attraction to specific sensory modalities. Virgin males, in contrast, do not demonstrate either attraction or aversion to these stimuli. Our findings will help to elucidate the mechanisms underlying the onset of parental behaviour in new fathers, with particular emphasis on sensory plasticity.

In mammals, successfully raising offspring can require extensive and complex communication between parents and their young. Offspring elicit care from their parents through sensory cues such as vocalizations, odours and tactile stimulation (Hofer et al., 2001; Lévy et al., 2004; Lévy & Keller, 2009; Shair, 2018), and parents must be adept at detecting and responding to such cues in order to meet their offspring's needs. Neural circuits underlying the detection of and responses to sensory stimuli from young can be plastic, with neural and behavioural responses changing during the transition to parenthood (Kinsley et al., 2007; Kinsley & Lambert, 2006; Kohl & Dulac, 2018; Lambert, 2012; Leuner et al., 2010; Numan, 2012; Rogers & Bales, 2019). This plasticity may play an important role in the onset of parental care.

In mammalian females, plasticity in responses to infant-related sensory stimuli appears to be mediated largely by neuroendocrine changes associated with pregnancy and lactation (Dunlap & Liu, 2018; Lévy, 2016; Lévy et al., 2004; Miranda & Liu, 2009). For example, nulliparous female rats, *Rattus norvegicus*, and house mice, *Mus musculus*, are averse to pups and pup-related sensory stimuli, especially pup odours, and exposure to such odours leads to the females avoiding or attacking pups (Lévy et al., 2004; Liu et al., 2006; Rosenblatt et al., 1988). Near parturition, however, pup odours and vocalizations instead elicit maternal responses through stimulation of the reward circuitry in the brain (Lévy et al., 2004; Lévy & Keller, 2009; Liu et al., 2006; Rosenblatt et al., 1988). Moreover, when female house mice are exposed to broadband acoustic stimuli, sound-processing regions in the brainstem (i.e.

* Corresponding author.
E-mail address: saltzman@ucr.edu (W. Saltzman).
[1] K. M. Wilson is now at the Department of Biology, Pomona College, Claremont, CA, U.S.A.

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auditory nerves and cochlear nuclei) are significantly slower to respond in nulliparous, pup-naïve females than in both mothers and nulliparous females that have previously interacted with pups (Miranda et al., 2014). This suggests that interaction with pups, with or without pregnancy, may increase the processing speed of early subcortical auditory structures, an effect that may be modulated by oestrogen and oxytocin (Charitidi et al., 2012; Miranda & Liu, 2009; Valtcheva & Froemke, 2019).

The olfactory system, too, undergoes plasticity near the time of parturition. In female house mice, mothers exhibit increased activity in the main olfactory bulbs, compared to sexually naïve females, when exposed to behaviourally relevant odours such as odour from their nest, food odours and urine from other adult mice; however, these mothers also exhibit decreased responsiveness to pure synthesized odorants (control odours) that are used to reliably activate the olfactory bulbs (Vinograd et al., 2017). This suggests that, in female mice, plasticity in the main olfactory system associated with motherhood dampens the response to nonsalient environmental stimuli while enhancing sensitivity to biologically important stimuli, including pup odours. Additionally, in virgin male house mice, which generally do not exhibit parental care under natural conditions, activation of the accessory olfactory pathway by pup odours leads to aggression towards pups (Clancy et al., 1984), but males that have recently cohabitated with a female exhibit significantly reduced activation of the vomeronasal organ in response to pup odours and reduced aggression towards pups (Tachikawa et al., 2013). The dampening of this response, which is also seen in the biparental prairie vole, *Microtus ochrogaster*, likely ensures that males do not attack pups that they may have fathered (Jean-Baptiste et al., 2008).

Although species often rely on one sensory modality more heavily than others, integration of sensory input from different modalities can be important for processing complex information and can modulate responses in the receiver. For example, female house mice are quicker to retrieve and care for pups when simultaneously exposed to both pup calls and pup odours, compared to females exposed to only one of these stimuli (Okabe et al., 2013). Similarly, female Australian sea lions, *Neophoca cinerea*, more frequently approach and sniff a model of a conspecific pup when it is swabbed with pup odours (saliva, nasal mucous, ocular secretions and skin secretions) and presented alongside pre-recorded pup calls compared to females presented with pup calls alone or a pup model paired with only pup calls and no odour component (Wierucka et al., 2018). The central olfactory and auditory pathways interact at several levels, and stimuli from both modalities are processed in brain regions involved in parental care, consistent with the possibility that neural responses to odours and vocalizations from infants modulate one another (Choi et al., 2018; Cohen et al., 2011). However, the role of multisensory integration in parental care has received little attention.

In the 5–10% of mammalian species that are biparental (i.e. both sexes provide care for their offspring), males, like females, may exhibit increased attraction to infants and infant-related stimuli as they become parents. For example, common marmoset, *Callithrix jacchus*, fathers spend significantly more time investigating pre-recorded infant calls compared to mated males without offspring of their own (Ziegler & Sosa, 2016), and human fathers exhibit higher alertness and levels of sympathy in response to infant cries than nonfathers (Fleming et al., 2002). Although effects of fatherhood on males' behavioural responses to infant odours have not been examined extensively, studies in the prairie vole have shown that combined ablation of both the main and accessory olfactory bulbs reduces paternal behaviour in virgin males when exposed to unfamiliar pups (Kirkpatrick et al., 1994). Moreover, in common marmoset fathers, exposure to odours from their own infants significantly reduces testosterone levels (Ziegler, 2013), which typically are inversely correlated with paternal behaviour (Saltzman & Ziegler, 2014). These findings suggest that becoming a father involves sensory plasticity within the central nervous system, the peripheral sensory structures, or both, which alters neural and behavioural responses to infant stimuli (Wilson et al., 2022). However, the relative importance of offspring stimuli in different sensory modalities and possible interactions among modalities require additional study.

In this study, we tested the hypothesis that fatherhood alters behavioural responses to both pup chemosensory and pup vocal stimuli in male California mice, a monogamous, biparental rodent. Fathers in this species provide comparable amounts of care for their pups (i.e. grooming, huddling, retrieving, nest building) to mothers, with the exception of lactation (Dudley, 1974; Gubernick & Alberts, 1987). Pup-naïve virgin males, in contrast, show highly variable responses to experimentally presented pups, ranging from grooming and huddling (i.e. paternal behaviour) to avoidance and to aggression (Chauke et al., 2012; de Jong et al., 2012). We predicted that fathers would spend more time investigating sensory stimuli from pups compared to neutral stimuli, whereas pup-naïve virgin males would not consistently be attracted to pup stimuli and might avoid them altogether. We also tested the hypothesis that simultaneous exposure to chemosensory and acoustic stimuli from pups would enhance behavioural responses in fathers. We predicted that fathers, but not pup-naïve virgin males, would spend more time interacting with a multimodal stimulus compared to either pup odours or pup vocalizations alone. Finally, we predicted that virgins would show more anxiety-like behaviour than fathers during exposure to pup-related sensory stimuli.

METHODS

Animals

California mice were descended from animals obtained from the *Peromyscus* Genetic Stock Center (University of South Carolina, Columbia, SC, U.S.A.) and were housed in our colony at the University of California, Riverside (UCR). All animals were housed in $44 \times 24 \times 20$ cm polycarbonate cages with aspen shavings for bedding and cotton for nesting material; subjects remained under a 14:10 h reversed light:dark cycle (lights off at 1300 hours and on at 2300 hours) and were provided with food (Purina 5001 Rodent Chow, LabDiet, U.S.A.) and water ad libitum. Average room temperature was 22.7 °C, and average humidity was 65%. All animals were checked twice daily, and their cages were changed weekly.

Ethical Note

Procedures were performed under the supervision of the UCR veterinary staff in accordance with the *Guide for the Care and Use of Laboratory Animals* and were reviewed and approved by the UCR Animal Care and Use Committee (animal use protocol 20180068). To minimize the number of animals used, we established our target sample sizes based on a prospective power analysis using data from previous studies in our laboratory (de Jong et al., 2009). Animals that showed signs of distress, such as fighting with their cagemates, were removed from the study. We found no evidence that aggression differed between breeding pairs and virgin pairs. For our control group, we used males housed with ovariectomized females, rather than males housed with other males, to better control for the subjects' social environment. This was also done to reduce stress and potential injury; adult males are prone to fighting with male cagemates, which

would negatively affect animal welfare and the experiment's results. UCR is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Experimental Design

Animals were weaned into same-sex groups of two to four mice between 27 and 31 days of age. At 83–135 days of age, each male was randomly assigned to either the virgin male group or the father group and was paired with an age-matched, unrelated female that was either bilaterally ovariectomized or sham-ovariectomized, respectively (see below: Ovariectomies and Sham Surgeries). (In rodents, ovariectomized females typically do not engage in sexual behaviour unless primed with ovarian steroid hormones (Pfaus et al., 2015), and prior research in our laboratory found the same pattern in California mice (Zhao et al., 2018). However, sexual behaviour was not explicitly monitored in the present study.) Each male underwent a single behavioural preference test (see below: Preference Tests). Fathers (mean ± SE = 104.03 ± 2.19 days old, $N = 39$) were tested 1–3 days after the birth of their first litter, and virgin males (mean ± SE = 99.67 ± 2.22 days old, $N = 41$) were tested at comparable time points to the fathers, with respect to age and time elapsed since pairing.

Ovariectomies and Sham Surgeries

Surgeries were performed under aseptic conditions using standard procedures, as previously described by our laboratory (Zhao et al., 2018). Females (90–120 days old) were anaesthetized with 2.5% isoflurane gas and underwent either bilateral removal of the ovaries or a sham surgery, which followed the ovariectomy protocol but involved only lifting the ovaries briefly from the abdominal cavity before returning them, instead of removing them, through a 0.5 cm lower-abdominal incision. Animals received 5 mg/kg Carprofen subcutaneously (1:50 dilution; Carprieve Injection, Norbrook Laboratories, Lenexa, KS, U.S.A.) every 12 h for 48 h following surgery and were housed individually for 1 week to facilitate recovery. They were then returned to their original groups of two to four females for an additional 3 days before being paired with an age-matched male. Male and female pair mates were no more closely related than first cousins.

Stimulus Preference Tests

Tests were conducted during the dark (active) phase of the light cycle, between 1400 and 1600 hours, under two red-light lamps placed above the sides of the arena angled towards the centre, in a sound-attenuated room lined with anechoic foam. Tests were performed in a 1 × 1 × 0.5 m open-field arena constructed of opaque black acrylic with clusters of 25 small holes arranged in a circle (Ø = 6.5 cm) punched in each corner of the arena floor. The arena was raised 10 cm off the ground to allow speakers to be placed under the holes, and the floor was covered with white butcher paper to obscure the holes and increase visual contrast under red light. Each male was exposed simultaneously to two stimulus pairs, each consisting of an odour stimulus and an acoustic stimulus; the two stimulus pairs were placed in opposite (diagonal) corners of the arena (Fig. 1).

In each test, one stimulus pair ('control') consisted of a stainless-steel mesh tea ball (Ø = 6 cm) containing clean cotton and a speaker (UltraSoundGate BL Pro, Avisoft Bioacoustics, Nordbahn, Germany) beneath the arena floor, approximately 3 cm below the ball, playing pre-recorded white noise. The second stimulus pair consisted of one of the following presented together in the same corner opposite the control stimulus: (1) pup odour (stainless-steel mesh tea ball containing pup-scented cotton + speaker playing white noise); (2) pup calls (ball containing clean cotton + speaker playing pre-recorded pup calls); (3) pup odour + pup calls (ball

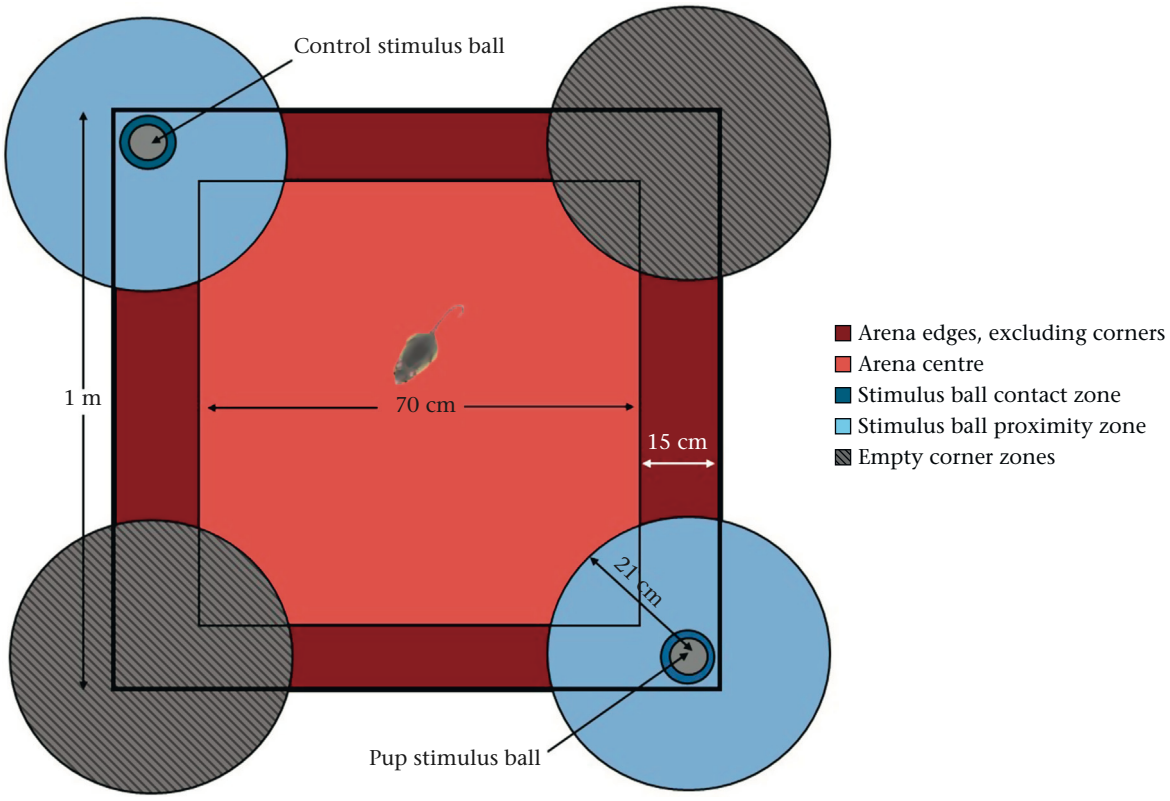

Figure 1. Open-field arena used for preference tests. The arena was separated into measurement zones as shown. Times spent in each zone were mutually exclusive, with the exception that time spent in the ball contact zones also counted towards the time spent in the corresponding stimulus proximity zones. Time spent in the two empty (nonstimulus) corners was averaged for final analysis.

containing pup-scented cotton + speaker playing pup calls); (4) control (ball containing clean cotton + speaker playing white noise). Thus, in three of the four stimulus conditions, one corner of the arena contained pup odour and/or calls and the opposite corner contained only control stimuli. In the fourth (null) stimulus condition, the two opposite corners both contained control stimuli. Males within each parental group were randomly assigned to one of the four stimulus conditions (Table 1), with the exception that any littermates were assigned to different conditions.

Pup-scented cotton was prepared by rubbing a clean cotton ball on a litter of two or three pups (3–7 days old), unrelated to the male subject, for 2 min, emphasizing the pups' anogenital regions to maximize odour accumulation. Pup separation calls (repeated cycles of two to six call bursts, averaging about 18 kHz) presented during tests were previously obtained from a 4-day-old pup, unrelated to the subjects, that was isolated and recorded for 2 min using a BAT miniMIC, an ultrasonic-capable microphone (Binary Acoustic Technology, Tucson, AZ, U.S.A.) and Spectr III software (Spectral Analysis, Digital Tuning, and Recording Software; Binary Acoustic Technology). Pre-recorded white noise was altered to replicate the pattern of a 4-day-old pup's average vocalizations (i.e. six bursts of white noise followed by a 1 s pause). The final pup call and white-noise recordings were compared to calling patterns found in previous characterizations of California mouse pup vocalizations to ensure their accuracy (Johnson et al., 2017; Vieira & Brown, 2002). Pup calls and white noise were each played on a loop for 10 min at 68 dB (measured from the arena centre, approximately 50 cm from each speaker) using UltraSoundGate Avisoft-RECORDER USGH version 4.30.01 playback software (Avisoft Bioacoustics, Nordbahn, Germany).

For testing, each male was transported to the test room, placed individually in the test arena and allowed to acclimate for 10 min. The two stimulus pairs – one control stimulus pair and one of the four experimental pairs described above (Table 1) – were then randomly assigned to opposite, diagonal corners. The balls were placed in the corners, and the speakers were turned on simultaneously. Subjects were allowed to explore the arena for an additional 10 min, after which they were returned to their home cage. The arena was cleaned thoroughly with Vikron disinfectant solution after each test. Tests were recorded under red light using a camcorder suspended 2 m above the arena floor.

Video Scoring

Videos were scored using TopScan Lite behavioural tracking software (CleverSys, Reston, VA, U.S.A.). We generated two concentric circular zones around the centre point of each stimulus ball in TopScan as well as identical zones in each nonstimulus corner for comparison (Fig. 1). The smaller circle (radius 3 cm, referred to henceforth as the 'contact zone') around each stimulus ball encompassed an area slightly larger than the ball (0.75 cm from the outer edge of the ball); duration of time spent in each of these zones was used as an index of time spent sniffing/interacting with each stimulus ball. Durations of time spent in the larger zones (radius 21 cm from the centre of the ball to the outer edge of the larger zones, referred to henceforth as the 'proximity zones') were used to measure time spent in proximity to each stimulus. Each proximity zone encompassed roughly 6.25% of the total area of the arena floor. We recorded the subjects' latencies to contact the balls (i.e. latency to enter the contact zone around each ball) and the durations of time spent in contact with and proximity to each stimulus ball, in the centre square of the arena (70 × 70 cm) and in the outer edges of the arena near the walls (extending 15 cm inward from the walls around the arena perimeter), excluding any areas where they overlapped with the circular zones.

Statistical Analyses

Data were analysed with SPSS version 27.0 statistical software (IBM Corporation, Armonk, NY, U.S.A.). Residuals were evaluated for each measure, and data were transformed as necessary to achieve normality and homogeneity of variance (see Results). Extreme outlier values were excluded on a case-by-case basis. Three sets of analyses were performed. First, we examined effects of parental group and stimulus condition (Table 1) on behavioural responses to pup-related stimuli (latency to contact pup stimulus ball, duration of time in contact with pup stimulus ball and duration of time in proximity to pup stimulus ball). For these analyses, we performed two-way ANCOVAs (parental group × stimulus condition) with age as a covariate, using only the mice tested with pup-related stimuli (i.e. mice tested in the null condition were excluded).

Next, we analysed males' responses to the two simultaneously presented stimuli (pup-related and control stimuli) using between- and within-subjects approaches, both of which excluded mice tested in the null condition. In the first approach, we calculated a delta score for each mouse's response to the two stimuli (e.g. duration of contact with the pup stimulus ball minus duration of contact with the control stimulus ball) and compared the delta scores using the same two-way ANCOVA set-up as the first set of analyses. In the within-subjects approach, which was performed for fathers and virgin males separately, we used paired *t* tests to compare each male's responses to the two simultaneously presented stimuli. Because the previous analyses did not reveal significant differences in males' responses across the pup stimulus conditions, we pooled data from all three conditions.

Finally, we evaluated effects of parental group and stimulus condition on males' use of arena space apart from the stimulus zones, using two-way (parental group × stimulus condition) ANCOVAs with age as a covariate. These analyses included mice from all four stimulus conditions. For all significant ANCOVA results (two-tailed alpha level $P < 0.05$), post hoc *t* tests with Bonferroni corrections were used to detect pairwise differences.

RESULTS

Responses to Pup Stimuli Across Stimulus Conditions

We performed two-way ANCOVAs to compare responses to the stimuli between fathers and virgin males across the pup odour, pup calls and pup odour + pup calls conditions ($N = 64$). None of the behavioural measures examined (latency to contact pup stimulus ball, duration of time contacting pup stimulus ball, duration of time in proximity to pup stimulus ball) differed significantly between fathers and virgins or across the stimulus conditions, and we did

Table 1
Stimulus pairs presented simultaneously in each stimulus condition

Stimulus condition	Experimental stimulus pair	Control stimulus pair	Fathers (*N*)	Virgin males (*N*)
Pup odour	Pup-scented cotton + white noise	Clean cotton + white noise	9	12
Pup calls	Clean cotton + pup calls	Clean cotton + white noise	10	10
Pup odour + pup calls	Pup-scented cotton + pup calls	Clean cotton + white noise	11	11
Null	Clean cotton + white noise	Clean cotton + white noise	8	9

not find any significant interactions between the factors (Table 2). However, the duration of time males spent in contact with the pup stimulus ball was negatively influenced by age ($F_{1,57} = 5.429$, $P = 0.023$, $\eta_p^2 = 0.087$).

Responses to Simultaneously Presented Pup-related and Control Stimuli

Two-way ANCOVAs examining delta scores in animals' responses to simultaneously presented stimuli revealed that difference in latency to contact the pup stimulus and control stimulus balls differed marginally among stimulus conditions ($F_{2,27} = 3.193$, $P = 0.057$, $\eta_p^2 = 0.191$): mice tested in the pup odour condition tended to contact the pup stimulus ball more quickly relative to the control stimulus ball than mice in the pup odour + pup calls condition. Delta scores for latency to contact the stimulus balls did not differ significantly between fathers and virgin males but showed a marginal effect of the interaction between parental group and pup stimulus condition ($F_{2,27} = 2.980$, $P = 0.068$, $\eta_p^2 = 0.181$). We found no significant effects of group, stimulus condition or age on delta scores for duration in contact with the pup stimulus ball versus control stimulus ball or on delta scores for duration in proximity to the pup stimulus versus control stimulus balls (Table 2).

Because we found only marginally significant differences when comparing behaviour in the pup odour, pup calls and pup odour + pup calls conditions, we next pooled the data from the three conditions and performed paired *t* tests for fathers and virgins separately to determine whether males responded differently to simultaneously presented stimuli. Fathers did not show a significant difference in latencies to contact the pup stimulus ball and the control stimulus ball (paired *t* test: $t_{25} = 0.559$, $P = 0.581$, Cohen's $D = 0.110$). Fathers did, however, spend significantly more time in contact with the pup stimulus ball than with the control stimulus ball ($t_{30} = -2.097$, $P = 0.047$, $D = 0.377$; Fig. 2a) and significantly more time in proximity to the pup stimulus ball compared to the control stimulus ball ($t_{30} = 3.367$, $P = 0.002$, $D = 0.605$; Fig. 3a). In contrast, virgin males showed no differences in latency to contact the pup stimulus ball versus the control stimulus ball ($t_{24} = 0.148$, $P = 0.883$, $D = 0.030$), duration in contact with the pup stimulus ball versus the control stimulus ball ($t_{32} = -1.644$, $P = 0.110$, $D = 0.286$; Fig. 2b) or duration in proximity to the pup stimulus ball versus the control stimulus ball ($t_{32} = 0.782$, $P = 0.440$, $D = 0.136$; Fig. 3b).

Use of Arena Space

Finally, we examined the amount of time subjects spent in each area of the arena, excluding the corners that contained the stimuli (Fig. 1). Animals from all four stimulus conditions were used ($N = 86$). Time spent in the outer edges of the arena (i.e. within 15 cm of the walls, excluding the four corners) differed significantly among the four stimulus conditions (two-way ANCOVA: $F_{3,76} = 7.517$, $P < 0.0001$, $\eta_p^2 = 0.229$). Mice exposed to a combination of pup odour and pup calls spent significantly less time in the edges of the arena than those exposed to pup odour ($P = 0.018$), pup calls ($P < 0.0001$) or control stimuli ($P = 0.001$); no differences were found among the latter three conditions (Fig. 4). Time spent in the arena edges was also positively influenced by age ($F_{1,76} = 6.102$, $P = 0.016$, $\eta_p^2 = 0.074$). We did not find a significant effect of parental group or a group∗stimulus condition interaction on the time spent in the edges of the arena (Table 2).

Total duration of time spent in the centre of the arena did not differ between fathers and virgin males or across the four stimulus conditions, nor was there a significant interaction (Table 2). However, age tended to positively affect time spent in the arena centre ($F_{1,77} = 3.310$, $P = 0.073$, $\eta_p^2 = 0.041$). Total duration of time spent in the empty corners of the arena differed significantly across the four stimulus conditions ($F_{3,76} = 3.008$, $P = 0.035$, $\eta_p^2 = 0.079$): males exposed to both pup odour and pup calls spent more time in the empty corners than males exposed to only pup odour ($P = 0.041$). Time in the empty corners was not influenced significantly by parental group or by an interaction between parental group and stimulus condition (Table 2) but showed a near-significant positive effect of age ($F_{1,76} = 3.606$, $P = 0.061$, $\eta_p^2 = 0.045$). Lastly, the total distance travelled in the arena did not differ between fathers and virgins or across stimulus conditions, nor was there an interaction between these factors (Table 2); however, age tended to have a positive effect on distance travelled ($F_{1,75} = 3.736$, $P = 0.057$, $\eta_p^2 = 0.028$).

DISCUSSION

In the present study, we examined the effects of parental status on males' behavioural responses to sensory stimuli from pups in the biparental California mouse. We tested the hypotheses that becoming a father increases males' attraction to pup calls and odours and that fathers are most attracted to these stimuli when they are presented together. We found mixed support for our first hypothesis. Although we did not find significant differences between fathers and virgins across the four stimulus conditions, the two groups differed in their responses to pup-related stimuli compared to simultaneously presented control stimuli: fathers spent more time in proximity to, and in contact with, any type of pup stimulus (i.e. pup odour, pup calls or pup odour + pup calls) compared to control stimuli, while virgin males spent equal amounts of time with the pup-related and control stimuli. Interestingly, between-subjects comparisons found no differences in behavioural responses to pup-related stimuli among males tested with pup odour, pup calls or pup odour + pup calls, suggesting that

Table 2
Two-way ANCOVA results for effects of parental group, stimulus condition and age on all behavioural measures

Behavioural measures	Trans.	*df*	Group			Condition			Group∗condition			Age		
			F	*P*	η_p^2	*F*	*P*	η_p^2	*F*	*P*	η_p^2	*F*	*P*	η_p^2
Latency (s) to pup ball	$x^{0.3}$	55	1.568	0.217	0.031	1.723	0.189	0.066	0.219	0.804	0.009	0.387	0.537	0.008
Duration (s) in contact with pup ball	$x^{0.35}$	63	0.563	0.456	0.010	0.241	0.786	0.008	1.987	0.146	0.065	5.429	**0.023**	0.087
Duration (s) in proximity to pup ball	$x^{0.8}$	63	2.856	0.096	0.048	0.041	0.960	0.001	1.471	0.238	0.049	1.560	0.217	0.027
Delta scores: Latency (s) to pup ball/null ball	Log	33	0.676	0.418	0.024	3.193	*0.057*	0.191	2.980	*0.068*	0.181	0.003	0.959	<0.0001
Delta score: Duration (s) in contact (pup ball minus null ball)	None	62	0.385	0.538	0.007	0.811	0.449	0.028	0.257	0.774	0.009	2.570	0.115	0.044
Delta score: Duration (s) in proximity (pup ball minus null ball)	None	62	2.547	0.116	0.044	0.168	0.846	0.006	0.045	0.956	0.002	0.084	0.772	0.002
Duration (s) in arena edges	None	84	0.113	0.738	0.001	7.517	**<0.0001**	0.229	0.492	0.689	0.019	6.102	**0.016**	0.074
Duration (s) in arena centre	$x^{0.5}$	85	1.186	0.280	0.015	1.747	0.164	0.064	0.603	0.615	0.023	3.310	*0.073*	0.041
Total duration (s) in empty corners	Log	84	2.035	0.158	0.026	3.008	**0.035**	0.106	2.160	0.100	0.079	3.606	*0.061*	0.045
Distance travelled (m)	None	85	0.275	0.601	0.004	1.137	0.340	0.044	0.895	0.448	0.035	3.736	*0.057*	0.047

Trans.: transformation. Significant *P* values (<0.05) are shown in bold. Trends ($P < 0.1$) are shown in italics.

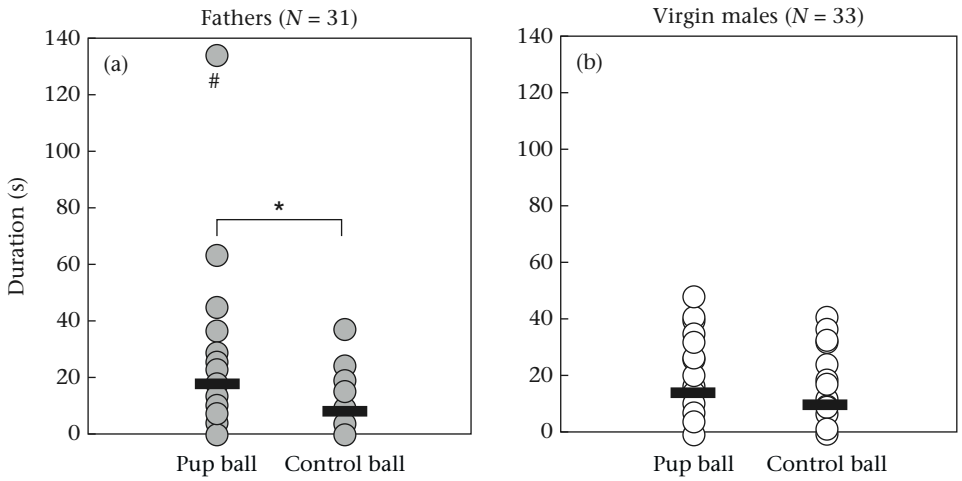

Figure 2. Duration of time that (a) fathers and (b) virgin males spent in contact with the simultaneously presented pup stimulus ball and control ball. Paired *t* tests were performed on nontransformed data. Results from the pup odour, pup calls and pup odour + pup calls conditions were pooled for each parental group; data from animals in the null treatment condition were excluded. Circles represent individual animals; bars represent means of raw data for each group and stimulus ball. One positive outlier in the father group (#) was excluded from the analysis. $^*P < 0.05$.

the specific stimulus modality was not a highly salient determinant of behavioural responses in this test paradigm.

Behavioural Responses to Simultaneously Presented Pup and Control Stimuli

Our findings (i.e. that fathers spent more time in proximity to and in contact with pup-related stimuli compared to control stimuli) are consistent with previous findings in biparental primates, including humans, that fathers show increased attraction to sensory cues from infants compared to control stimuli (Fleming et al., 2002; Ziegler, 2013; Ziegler & Sosa, 2016). Correspondingly, fathers in several rodent species, including California mice, exhibit greater activation in brain regions associated with parental motivation, primarily the medial preoptic area of the hypothalamus (MPOA), as well as regions associated with reward, in response to infants or infant-related stimuli, compared to nonfathers (reviewed in Horrell et al., 2021; Saltzman et al., 2017). For example, compared to pup-naïve males, California mouse fathers show increased activation in these regions when exposed to either related or unrelated pups, while also showing reduced activity in regions associated with fear and aggression (de Jong et al., 2009; Lambert, 2012; Wilson et al., 2022).

In a recent experiment, we characterized both behaviour and neural activation (via expression of Fos, the protein product of the immediate early gene *c-fos*) in male California mice following exposure to one of the four stimulus conditions used in the present study (Wilson et al., 2022). In contrast to the current study, the previous one found no differences between fathers and virgin males in their behavioural responses to the stimuli, likely as a result of methodological differences between the two experiments: stimulus exposure tests in the previous study were performed during the lights-on period, used much smaller cages that limited animals' ability to avoid the stimuli and did not allow the mice a choice between pup stimuli, control stimuli and empty areas of the cage. Nevertheless, fathers had significantly greater neural activation in the MPOA than virgin males (Wilson et al., 2022). The MPOA interacts extensively with the brain's reward circuitry and plays a central role in positive reinforcement of parental behaviour in mothers (Numan, 2012, 2020; Olazábal et al., 2013). As such, the stronger activation of the MPOA in California mouse fathers,

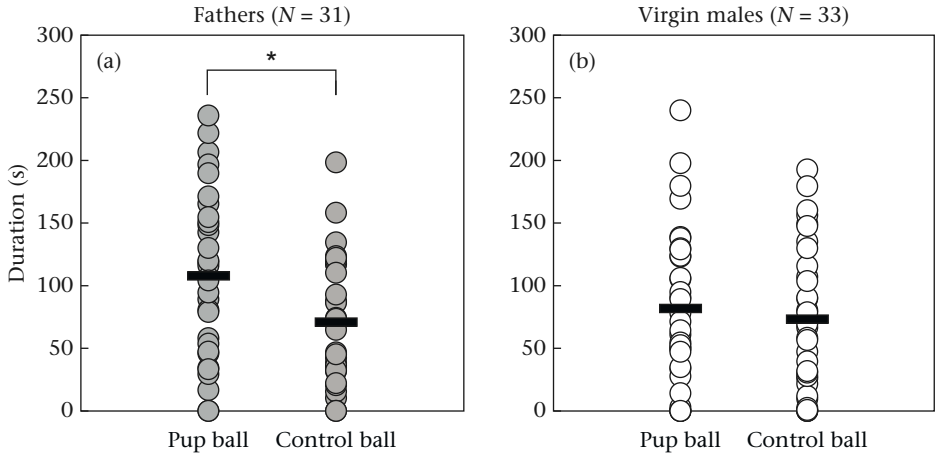

Figure 3. Duration of time that (a) fathers and (b) virgin males spent in proximity to the simultaneously presented pup stimulus ball and control ball. Data from the pup odour, pup calls and pup odour + pup calls conditions were pooled for each parental group; data from animals in the null treatment condition were excluded. Circles represent individual animals; bars represent means of raw data for each group and stimulus ball. Paired *t* tests were performed on nontransformed data. $^*P = 0.002$.

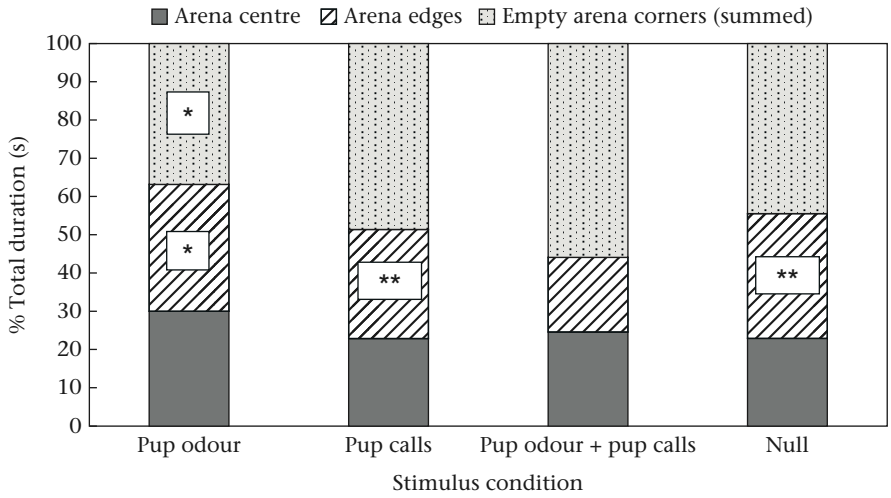

Figure 4. Average duration of time mice in each stimulus condition (fathers and virgin males combined) spent in nonstimulus regions of the arena: the arena centre ($x^{0.5}$ transformed), the arena edges (no transformation) and the two empty arena corners (log-transformed). Asterisks indicate which groups differed significantly from mice exposed to pup odour + pup calls: $*P < 0.05$; $**P \leq 0.001$. See text for additional statistical information.

compared with virgin males, might contribute to their increased attraction to pup stimuli, which may be positively reinforced through the same neural pathways that are important in mothers. Thus, our previous findings on neural responses to pups reinforce the behavioural results of the current study.

In contrast to fathers, the virgin males in our study spent similar amounts of time both in proximity to and in contact with pup-related and control stimuli. As such, we found no evidence that virgin males were attracted to pup odours and/or pup calls. Interestingly, they also did not appear to avoid these stimuli. In several uniparental species, such as house mice and rats, and in some biparental species, such as Mongolian gerbils, *Meriones unguiculatus*, reproductively naïve adult males avoid or attack immature conspecifics (Elwood & Stolzenberg, 2020; Isogai et al., 2018). Virgin male California mice, however, show considerable variation in their behaviour towards unrelated pups prior to becoming fathers, ranging from aggression to parental behaviour (Chauke et al., 2012; de Jong et al., 2009, 2012). Consequently, we did not expect the virgin males to consistently avoid the pup stimuli.

Note that the subjects' behaviour in their home cages was not monitored systematically. Based on extensive findings on female rodents (Pfaus et al., 2015) and previous observations in California mice (Zhao et al., 2018), it is highly unlikely that males paired with ovariectomized females engaged in any sexual behaviour; nevertheless, we cannot completely rule out the possibility that such sexual behaviour did occur. Even if 'virgin' males did mate, however, this is unlikely to have significantly affected their responses to pup stimuli, as indicated by findings from a recent study on California mice (Khadraoui et al., 2022).

Relative Importance of Auditory and Olfactory Modalities

Detecting stimuli in multiple modalities increases the amount of information an animal can synthesize about its environment, potentially modulating its behavioural responses to these stimuli (Alais et al., 2010; Choi et al., 2018; Cohen et al., 2011; Wierucka et al., 2018). Multimodal stimuli from infants can work additively or synergistically to elicit care from mothers (Sèbe et al., 2008; Stein & Stanford, 2008), and in some species, a mother's perception of multimodal sensory cues can help her distinguish between signals from unrelated young and those produced by her own offspring (Stern, 1990; Wierucka et al., 2018). Rodents rely heavily on olfaction, especially during close-range communication. On the other hand, vocalizations, particularly ultrasonic calls, may be associated with long-distance communication in adult rodents and have been studied heavily with respect to pups' communication with their parents during early life (Hofer et al., 2001; Kalcounis-Rueppell et al., 2018).

As mentioned above, mothers in at least some mammalian species, such as house mice, domestic sheep, and Australian sea lions, respond more quickly when presented simultaneously with infant stimuli in multiple sensory modalities than when exposed to infant stimuli in only a single modality; as such, we predicted that male California mice, too, would spend more time investigating pup stimuli, and would contact these stimuli more quickly, when odours and calls were presented simultaneously. This pattern, however, was not observed. Neither fathers nor virgin males showed significant differences in the amount of time they spent in proximity to or in contact with the stimuli across stimulus conditions (i.e. pup odour, pup calls and pup odour + pup calls).

Importantly, the mice in our study were unable to physically contact the pup odour stimulus (pup-scented cotton) because it was confined in a steel mesh ball. In rodents and other mammals, volatile compounds are detected primarily in the nasal epithelium and subsequently processed in the main olfactory pathway (Mucignat-Caretta, 2010). In contrast, comparatively heavy, liquid-borne compounds are drawn in at close range through the mouth and/or nose into the vomeronasal organ (VNO) and processed in the accessory olfactory pathway (Clancy et al., 1984; Tachikawa et al., 2013). Because this pathway requires direct contact of these compounds with the VNO, the steel mesh ball used in the study likely prevented the mice from detecting such compounds. Therefore, it is possible that the fathers and virgin males in this study did not react to the pup odours in the same manner as they would in a natural setting.

Although both the main and accessory olfactory pathways play roles in detecting infant odours in male rodents (Horrell et al., 2019; Saltzman et al., 2017), the reduced emphasis on the accessory olfactory pathway in our study is significant because this pathway has been implicated in the transition from avoidance to parental behaviour in several species (Kohl & Dulac, 2018; Numan, 2020). Lesioning the VNO leads to a marked reduction of aggression and an increase in parental behaviour towards unrelated pups in virgin male house mice and rats (Elwood & Stolzenberg, 2020; Isogai et al., 2018; Tachikawa et al., 2013). In view of this relationship

between the VNO and aggression, it is possible that the virgin male California mice in our study were less inclined to avoid the pup odour and pup odour + pup calls stimuli because they were not able to detect some of the odorants.

Use of Arena Space

Finally, we analysed the time spent in the areas of the arena without either pup-related or control stimuli, as well as the total distance travelled during each trial. Although none of these behaviours differed significantly between fathers and virgin males, males exposed to the pup odour + pup calls stimulus spent less time near the walls of the arena compared to males in the remaining three stimulus conditions. They also spent more time in the empty corners of the arena compared to males exposed only to the pup odour. In open-field tests, rodents that spend more time near the walls are generally classified as being more anxious than animals that spend more time in the centre of the arena (Kraeuter et al., 2019). It is not clear if the males in the pup odour + pup calls condition were either more or less anxious than those in the other stimulus conditions; the tests were run in the dark phase under red light, whereas typical open-field tests are conducted under bright light to induce anxiety (Kulesskaya & Voikar, 2014). Additionally, although mice in the pup odour + pup calls condition spent less time near the arena walls compared to the mice in the other three stimulus conditions, they spent more time in the empty corners compared only to males that were exposed to pup odour alone. These findings are difficult to interpret, as it is unclear whether differences in use of space across stimulus conditions reflect differences in males' anxiety levels and/or differences in motivation to explore the environment when facing the potential to encounter an unknown pup.

Conclusions

In female mammals, the transition into parenthood is often associated with increased attraction to infants, mediated in part by changes in the neural processing of infant-related sensory stimuli (Okabe et al., 2013; Tachikawa et al., 2013; Wierucka et al., 2018). Males, too, may show enhanced attraction to infants when becoming parents, but the sensory basis of this change has received relatively little attention (Horrell et al., 2019; Saltzman et al., 2017). Our study provides some of the first evidence of fatherhood-induced sensory plasticity in males and, to our knowledge, the first evidence in a naturally biparental species. Elucidation of the experiential, endocrine and neural mechanisms underlying this plasticity will provide new insights into the proximate basis of paternal care.

Author Contributions

A. M. Arquilla: Methodology, Formal Analysis, Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing, Visualization. **K. M. Wilson**: Conceptualization, Methodology, Investigation, Analysis, Writing – Original Draft, Writing – Review & Editing. **K. A. Razak**: Methodology, Software, Resources. **W. Saltzman**: Conceptualization, Methodology, Formal Analysis, Writing – Original Draft, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition.

Data Availability

The data from this study are available on Figshare (https://figshare.com/s/5c7ecdf8aea90947fb49).

Declarations of Interest

None.

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APPENDIX

Table A1
Means and standard errors by parental group and treatment condition for all behavioural measures

Behavioural measure	Parental group	Pup odour ($N = 9$ fathers, 12 virgin males)		Pup calls ($N = 10$ fathers, 10 virgin males)		Pup odour + pup calls ($N = 11$ fathers, 11 virgin males)		Null ($N = 8$ fathers, 9 virgin males)	
		Mean ± SE	EMM	Mean ± SE	EMM	Mean ± SE	EMM	Mean ± SE	EMM
Latency (s) to pup ball	Fathers	49.66±18.08	2.88	82.90±35.80	3.00	160.34±64.20	4.02	—	—
	Virgin males	152.37±54.61	3.78	124.90±47.95	3.45	154.66±46.86	4.24	—	—
Duration (s) in contact with pup ball	Fathers	27.53±12.68	2.79	14.12±2.58	2.30	14.93±6.77	1.82	—	—
	Virgin males	11.99±4.55	1.89	13.61±3.63	1.96	18.58±4.64	2.39	—	—
Duration (s) in proximity to pup ball	Fathers	121.62±19.27	47.05	112.49±17.32	41.90	88.81±25.87	33.78	—	—
	Virgin males	67.83±15.71	27.64	80.77±16.14	30.50	96.72±23.72	37.11	—	—
Delta score: Latency (s) to contact (pup ball minus null ball)	Fathers	6.82±8.15	1.14	5.25±46.63	1.97	36.54±58.71	1.74	—	—
	Virgin males	−38.55±56.00	1.67	−9.24±37.11	1.60	4.14±36.79	1.98	—	—
Delta score: Duration (s) in contact (pup ball minus null ball)	Fathers	15.01±13.47	4.66	6.13±3.36	5.22	9.30±6.00	9.67	—	—
	Virgin males	5.76±3.88	5.60	0.76±5.00	−0.88	6.36±4.86	7.34	—	—
Delta score: Duration (s) in proximity (pup ball minus null ball)	Fathers	45.96±20.13	36.43	26.73±15.66	26.07	39.14±22.72	39.41	—	—
	Virgin males	4.45±11.28	4.33	6.74±16.34	5.56	14.37±27.22	15.08	—	—
Duration (s) in arena edges	Fathers	153.90±16.37	148.20	106.43±5.39	111.16	64.11±14.37	62.77	123.48±26.89	123.14
	Virgin males	118.83±18.05	120.00	135.12±32.04	118.13	63.09±11.65	58.82	133.98±19.08	132.04
Duration (s) in arena centre	Fathers	139.68±26.76	10.10	123.79±23.99	10.78	84.92±35.29	7.29	84.95±24.07	8.10
	Virgin males	107.18±25.91	9.29	69.74±15.11	7.98	75.71±21.09	7.01	95.40±23.68	8.67
Total duration (s) in empty corners	Fathers	54.58±7.72	1.72	85.77±13.65	1.86	156.25±30.84	2.10	89.10±16.46	1.96
	Virgin males	121.40±23.19	2.02	120.17±18.70	1.96	141.06±27.74	2.09	87.18±18.80	1.88
Distance travelled (m)	Fathers	751.57±129.29	617.27	530.99±46.60	547.39	453.76±140.63	341.96	459.50±78.81	455.84
	Virgin males	451.50±96.59	453.85	454.25±109.33	484.37	472.13±100.24	452.91	459.56±49.25	449.55

Means and associated standard errors calculated for nontransformed data; estimated marginal means (EMM) calculated for the transformed data (see Table 2 for transformations used). Data from subjects in the null condition are excluded from analyses of stimulus-related behaviours.