

Marcela Alsina-Llanes

Victoria De Brun
Daniel E. Olazábal

Departamento de Fisiología
Facultad de Medicina
UdelaR. Gral Flores 2125, Montevideo,
18000 Uruguay
E-mail: dolazabal@fmed.edu.uy

Development and Expression of Maternal Behavior in Naïve Female C57BL/6 Mice

ABSTRACT: Naïve female mice are usually described as spontaneously maternal. We investigated how many exposures to pups (15 min vs. 1 hr) were needed to induce full maternal behavior (FMB) in 20–22, 30–35, 60–65-days-old naïve female mice (C57BL/6), and how cohabitation with the parturient mother and newborn siblings facilitated juvenile maternal behavior (MB). Only 20% of the adults displayed FMB immediately after the first exposure to pups. Incomplete MB was present in 11%, 20%, and 30% of juveniles, adolescents and adults, respectively. Three-sixty minute exposures to pups induced FMB in all adult subjects. All naïve juveniles that were not exposed to their siblings and maternal fluids failed to show maternal behavior. In contrast, more than half of the juveniles present at their homecage during delivery of a second litter showed incomplete MB (34.5%) or FMB (21.5%) when tested individually housed in a novel cage. This study suggests that most adult female mice are not spontaneously maternal but gradually sensitized. Besides, naïve juveniles could be inhibited or not motivated to show MB, but display adult-like behavior toward pups if previously exposed to newborn siblings and maternal fluids. © 2015 Wiley Periodicals, Inc. *Dev Psychobiol* 57:189–200, 2015.

Keywords: development; juvenile; maternal behavior; *Mus musculus*; C57BL/6; ontogeny; overlapping litters; sensitization

Maternal behavior in altricial rodents consists of several behavioral components such as pup retrieval, licking and grooming, crouching postures, and nest building (Noirot, 1969; Roberts, Williams, Wang, & Carter, 1998; Rosenblatt, 1967). In many species, females can also show maternal behavior in non-reproductive contexts (Noirot, 1972; Roberts et al., 1998; Rosenblatt, 1967). For example, most naïve juvenile and adult female prairie voles (*Microtus ochrogaster*) display parental behavior immediately after the first exposure to pups (Olazábal & Young, 2006a, 2006b; Roberts et al., 1998). In contrast, juvenile and adult female rats require 1–2 versus 4–8 days of exposure to newborns, respectively, to display all components of parental

behavior (Bridges, Zarrow, Goldman, & Denenberg, 1974; Mayer, 1983; Rosenblatt, 1967). Unlike most prairie voles, rats develop a neophobic response toward pups from age 20 to 24 days to adulthood.

In mice, naïve females are usually described as “spontaneously maternal” (Calamandrei & Keverne, 1994; Gandelman, 1973b, 1973c; Leussis, Bond, Hawken, & Brown, 2008; Lonstein & De Vries, 2000; Noirot, 1969, 1972; Stolzenberg & Rissman, 2011), displaying maternal behavior during the first 15 or 60 min period of exposure to pups. However, the behavior of mice is extremely variable and several studies found that most mice became fully maternal only after 2 days of cohabitation with newborns (Brown, Ye, Bronson, Dikkes, & Greenberg, 1996; Hamaguchi-Hamadaa, Sanbo, Hamadaa, & Yagi, 2004; Lucas, Ormandy, Binart, Bridges, & Kelly, 1998; Pedersen, Vadlamudi, Boccia, & Amico, 2006). For example, Brown et al. (1996) showed high levels of maternal behavior in at least half of the subjects by the second 30 min exposure; but several days were needed for greater performance. Differences in strain, age, sex,

Manuscript Received: 19 August 2014

Manuscript Accepted: 2 December 2014

Correspondence to: Daniel E. Olazábal

Contract grant sponsor: Comisión Sectorial de Investigación Científica (CSIC), UdelaR

Article first published online in Wiley Online Library (wileyonlinelibrary.com): 21 January 2015

DOI 10.1002/dev.21276 • © 2015 Wiley Periodicals, Inc.

experience, testing, housing and physiological condition, among other factors, might explain the variability in findings reported in the literature (Brown, Mathieson, Stapleton, & Neumann, 1999; Kuroda, Tachikawa, Yoshida, Tsuneoka, & Numan, 2011; McCarthy & vom Saal, 1985; Numan & Insel, 2003; Svare & Broida, 1982; vom Saal, 1984).

However, a detailed analysis of the literature revealed also some inconsistencies in the definition or the criteria used by the authors to consider an animal as maternal. Some studies did not clarify which behavioral components of maternal behavior were used to consider an animal as maternal, or used only one of them (usually retrieval or licking and grooming, Calamandrei & Keverne, 1994; Gandelman, 1973b; Gandelman, Paschke, Zarrow, & Denenberg, 1970; Jin, Blendy, & Thomas, 2005; Kuroda, Meaney, Uetani, & Kato, 2008; Li, Keverne, Ishino, Barton, & Surani, 1999; Ragnauth, Devidze, Moy, Finley, Goodwillie, Kow, Muglia, & Pfaff, 2005; Pedersen et al., 2006). In addition, some studies had pre-exposed animals to pups before the actual test took place, adding still more variability (Gandelman and vom Saal, 1975; Leussis et al., 2008; Stolzenberg & Rissman, 2011). Surprisingly, most studies agreed that juvenile mice did not show rapid induction of maternal behavior (Gandelman, 1973b; Noirot, 1972). However, to our knowledge, juvenile maternal behavior in overlapping litters had never been studied in mice.

In Experiments I and II, we investigated at what age female mice (C57BL/6) started to display full maternal behavior (retrieval, licking, crouching posture, and nest building), and how many single or repeated short (15 min) or long (1 hr) exposures to pups were needed to induce full maternal behavior. In Experiment III, we investigated if juveniles could be induced to display maternal behavior by 12–24 hr of cohabitation with their newborn siblings (overlapping litters) and parturient mothers in their home cage.

GENERAL MATERIALS AND METHODS

Subjects

All C57BL/6 mice were originally obtained from Jackson laboratory and maintained at our animal facility at the Facultad de Medicina, UdelaR, Montevideo, Uruguay. No more than two litter mates were used for each group. Animals were weaned at age 20–21 days, and maintained in same-sex groups of 6–7 individuals per cage. Cages were 45 cm x 25 cm x 15 cm, with transparent plexiglas walls and wood shaving as bedding. Animals were kept under a 12:12-hr light-dark cycle (light on from 6:00 am), at 22°C, with ad libitum access to food (Vitaron, Montevideo, Uruguay) and water. Cages were

regularly changed once a week. Animal care and experimental procedures were performed in accordance with the “Guide for the Care and Use of Laboratory Animals” of the National Institutes of Health (2011) and the “Guidelines for Ethical Conduct in the Care and Use of Animals” (APA, Board of Scientific Affairs, Committee on Animal Research and Ethics, 2012). These experiments were also approved by the local Ethical Committee on use of Animal Experimentation (CHEA) of the Facultad de Medicina, UdelaR, Uruguay (N° 071140, December 26, 2011).

Maternal Behavior Test

All females were individually housed in a testing cage (27 cm x 21 cm x 14 cm, floor area of 370 cm²) and allowed to habituate to the cage for 45–60 min before testing. Maternal behavior test consisted in placing two pups (1–3 days of age, taken from donor lactating mothers) and nest material scattered in the side opposite to where the subject was located before opening the cage. Immediately after, we recorded (during 15 min) all components of maternal behavior, including frequency of retrieval and attacks; latency to first retrieval or attack; time distant from pups (>10 cm); duration and frequency of sniffing, licking, crouching over at least one pup (immobile or active), and nest building. Pups were removed from the cage at the end of each test, or immediately after the first attack. In that case, pups were immediately sacrificed.

We categorized animals as showing full maternal behavior (FMB), partial maternal behavior (PMB), non-maternal behavior (NMB), or infanticidal behavior (IB) according to the following criteria:

FMB: animals that performed all main behavioral components of maternal behavior: pups retrieval, licking (≥ 60 s), and crouching over at least one pup (≥ 30 s).

PMB: animals that performed only two of the behavioral components described above.

NMB: animals that performed only one or none of the maternal behavioral components.

IB: Infanticidal behavior; animal that attacked the pups. That is female bit a pup, and pup squealed. The attack was confirmed by inspecting the pup for wounds, and bleeding after female was removed.

Maternal Behavior test was carried out during the light phase because previous studies (also confirmed in our laboratory) showed no difference in the incidence of maternal or non-maternal animals during the light-dark cycle (Kuroda et al., 2011; Lucas et al., 1998). All behavioral variables were recorded using the program Stopwatch <http://www.cbn-atl.org/research/stopwatch.shtml>.

Statistical Analysis

Behavioral data were analyzed using the statistical package StatView (SAS Institute Inc, Cary, NC). The data were checked for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Bartlett test). In most cases, data did not pass the requirements of normal distribution and homogeneity of variance and were analyzed by non-parametric Kruskal–Wallis test followed by Mann–Whitney U for

independents data, or Friedman one-way analysis of variance followed by Wilcoxon's matched-pair test for dependent data. Chi-square test was applied to analyze the frequency of behaviors among groups. Statistical significance was $p < .05$. Data are expressed as median (SIQR).

EXPERIMENT I

Ontogeny of Maternal Behavior in Female Mice (C57BL/6)

Experiment I was designed to determine at what age female mice (C57BL/6) started to show spontaneous FMB (retrieval, licking, and crouching posture). Previous studies suggested that adult, but not juvenile, females displayed immediate maternal behavior (Noirot, 1969, 1972). Preliminary not published studies carried out by Olazábal DE in two different institutions/animal facilities (Emory and Vanderbilt University), and several strains (C57BL/6; BALB/c) suggested that only a very small percentage of adult mice tested with pups for the first time showed all behavioral components of maternal behavior. Therefore, we investigated if two 15 min repeated exposure to pups were sufficient to induce FMB in juvenile, adolescent and adult mice.

Methods

Naïve females 20–22 ($n = 9$), 30–35 ($n = 10$), and 60–90 ($n = 10$) days of age were tested for maternal behavior as described in the general methods section. Animals were exposed to pups for 15 min twice a day (approximated testing interval was 10–12 hr). Maternal behavior was recorded as described in general methods. Exploratory and general locomotor activity (rearing, climbing, immobility, and autogrooming) inside the cage was also recorded.

Results

Higher incidence of behavioral components of maternal behavior was found in adult females, but there was no significant difference ($\chi^2 = 5.7$, $p = .2$) among age in the percentage of maternal or non-maternal animals (Fig. 1). No significant difference was found in the percentage of maternal or non-maternal animals tested at the first or second exposure to pups ($\chi^2 = 2.62$, $p = .26$). Combining all age groups, 2, 6, 21 animals displayed FMB, PMB, or NMB at the first 15 min exposure to pups, while 0, 10, 19 animals displayed FMB, PMB, or NMB at the second exposure to pups.

Table 1 shows the analysis of the different behavioral components of maternal behavior (retrieving,

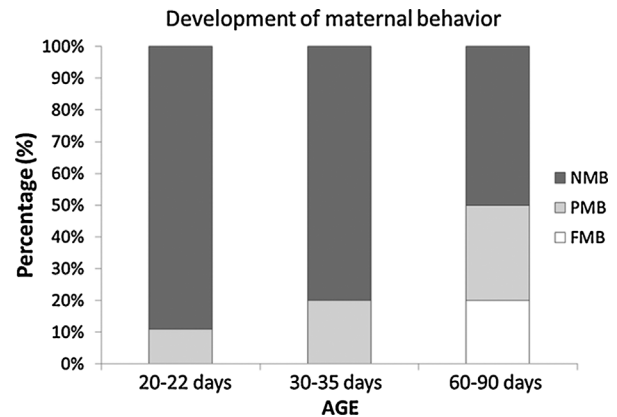


FIGURE 1 Percentage of maternal or non-maternal females (juveniles, adolescents, and adults) after the first 15 min exposure to pups. $\chi^2 = 5.7$, $p = .2$. FMB, full maternal behavior; PMB, partial maternal behavior; NMB, non-maternal behavior.

licking, crouching posture, and nest building). Adult females licked the pups more often ($U = 83.5$, $p < .01$ vs. 20 days; $U = 98.5$, $p < .01$ vs. 30 days) and for longer period of time ($U = 76.0$, $p < .05$ vs. 20 days; $U = 88.0$, $p < .01$ vs. 30 days) than the other two groups. Adults also crouched more often ($U = 78.5$, $p < .01$) and for longer period of time ($U = 75.5$, $p < .05$) than juveniles. Neither difference in the frequency nor the period of time performing crouching postures was found between 30- and 60-days-old animals ($U = 73.0$, $p = .08$; $U = 59.0$, $p = .4$, respectively). Low incidence of retrieval and nest building was observed at all ages.

At 60 days of age, the percentage of subjects that licked the pups (90%) was significantly higher than at 20 (30%; $\chi^2 = 8.9$, $p < .01$) and 30 (22.2%; $\chi^2 = 7.5$, $p < .01$) days of age. The percentage of subjects that retrieved (see Tab. 1, $\chi^2 = 3.0$, $p = .2$) or adopted crouching posture (11%, 30%, 40% in 20, 30 or 60 days of age respectively, $\chi^2 = 2.0$, $p = .3$) did not differ among the groups. In this experiment, none of the animals showed immobile crouching posture.

Results of exploratory and locomotor activity revealed that adults smelt the pups less ($U = 88.0$, vs. juveniles; $U = 87.0$, vs. adolescent, $p < .05$) and for shorter period of time ($U = 79.0$, vs. juveniles; $U = 78.0$, vs. adolescent, $p < .05$) than the other two groups (Tab. 2). Juveniles displayed rearing behavior less often and for shorter period of time than the other two groups ($p < .05$). Frequency, but not duration, of climbing up was also lower in juveniles compared to adolescents. Time spent climbing up was lower in adults compared to adolescents (Tab. 2). Juveniles were more often and for longer period of time immobile

Table 1. Maternal Behavioral Components at Different Ages

Age	20–22 Days (<i>n</i> = 9)	30–35 Days (<i>n</i> = 10)	60–90 Days (<i>n</i> = 10)
Percentage			
Retrieval	.0%	20.0%	30.0%
Frequency			
Licking Bouts	5.0 (5.8)**	14.4 (4.0)**	44.0 (8.0)
Crouching Postures	.0 (0.1)**	2.0 (5.0)	9.0 (1.5)
Nest Building	.0 (.0)	.0 (.0)	.0 (3.0)
Time (s)			
Licking	23.9 (23.3)*	37.6 (13.9)**	120.1 (27.3)
Crouching Postures	.0 (.2)*	3.0 (28.5)	22.4 (14.8)
Nest Building	.0 (.0)	.0 (.0)	.0 (43.5)

Data are expressed as median (SIQR).

Kruskal–Wallis analysis of variance followed by Mann–Whitney U test.

* $p < .05$.

** $p < .01$ vs. female 60 days of age.

compared to the other groups ($U = 75.0$, $p < .05$ vs. 30 and 60 days). They also spend more time ($U = 73.5$, $p < .05$), and were more often ($U = 72.0$, $p < .05$) apart from the pups a distance larger than 10 cm compared to adults. Autogrooming was not different among the different age groups.

EXPERIMENT II

Induction of Full Maternal Behavior During 1 hr Repeated Exposures to Pups

In Experiment I, we found that naïve adult females failed to display FMB at the first exposure (15 min) to pups. Some of the previous studies had pre-exposed the

animals to pups (e.g., 15–45 min) before testing the animals for maternal behavior (Gandelman and vom Saal, 1975; Leussis et al., 2008; Stolzenberg & Rissman, 2011). Then, in experiment II, we determined how many 1 hr exposures to pups were needed to induce full maternal behavior in naïve adult female C57BL/6 mice. In this experiment, we exposed the animals to pups twice a day until all subjects were induced to show FMB.

Methods

Eight adult females were individually housed, habituated to the cage for 60 min, and later exposed to two pups (1–3-days-old) for 1 hr. Exposures to pups were repeated twice a day in intervals of 10–12 hr, until all

Table 2. Exploration and Locomotor Activity at Different Ages

Age	20–22 Days (<i>n</i> = 9)	30–35 Days (<i>n</i> = 10)	60–90 Days (<i>n</i> = 10)
Frequency			
Sniffing	10.0 (4.0) ^a	8.0 (14.5) ^a	1.0 (1.5) ^b
Rearing	28.0 (12.6) ^a	77.5 (14.5) ^b	75.0 (18.0) ^b
Distant 10 cm	8.0 (5.6) ^a	13.0 (4.0) ^{a,b}	18.0 (3.0) ^b
Immobile	3.0 (2.1) ^a	.0 (.0) ^b	.0 (.0) ^b
Autogrooming	5.0 (1.3)	5.5 (2.0)	8.0 (2.0)
Climbs	1.0 (1.1) ^a	5.0 (2.0) ^b	3.0 (2.0) ^{a,b}
Time (s)			
Sniffing	22.3 (7.6) ^a	19.1 (14.6) ^a	5.8 (4.8) ^b
Rearing	37.2 (25.3) ^a	95.6 (20.3) ^b	109.0 (25.9) ^b
Distant 10 cm	405.9 (173.3) ^a	265.5 (86.6) ^{a,b}	115.5 (44.5) ^b
Immobile	76.3 (92.5) ^a	.0 (.0) ^b	.0 (.0) ^b
Autogrooming	80.1 (42.1)	42.3 (42.6)	43.6 (35.6)
Climbs	68.2 (80.2) ^{a,b}	67.4 (44.7) ^a	34.4 (24.5) ^b

Data are expressed as median (SIQR).

Different letters (a and b) indicate statistical differences ($p < .05$) among groups.

Kruskal–Wallis analysis of variance followed by Mann–Whitney U test.

females displayed FMB for two consecutive tests. Pups were returned to the donor lactating mothers after each 1 hr exposure. We recorded maternal behavior during the first 15 min of each 1 hr of cohabitation with pups as described in Experiment I. The location of the females and pups (inside or outside the nest), and the occurrence of crouching posture were also recorded at the end of each 1 hr period before removing the pups. Animals were categorized according to the criteria described in general methods (FMB, PMB, NMB, and IB). In addition, flow diagrams of frequencies among six maternal behavioral components and its main transitions were created. We used the observed sequence of behavioral components to construct a matrix that listed the number of times that each behavior followed another. We calculated the transition frequency for each behavioral sequence. Matrixes containing all behavioral records obtained in the 15 min tests were used to make the flow diagrams for the eight animals when they were rated as non-maternal, partially maternal or fully maternal. An additional group of lactating animals tested (in this case with five pups) at postpartum day 7 was also used to compare the flow diagram of FMB naïve and lactating female.

Results

The percentage of FMB, PMB, NMB, and IB changed in the consecutive exposures. More animals displayed FMB at the fourth ($\chi^2 = 8.5, p < .05; \chi^2 = 7.4, p < .05$) and fifth ($\chi^2 = 11.4; p < .01, \chi^2 = 10.5, p < .01$), compared to the first and second exposure to pups (Fig. 2). At least three 60-min repeated exposures to pups were needed to induce FMB or PMB in all subjects. At the first exposure to pups, one female showed infanticidal behavior and was removed from the subsequent behavioral analysis.

A detailed analysis of the 15 min maternal behavior test at the first three exposures to pups revealed a non-significant increase in retrieval and a significant increase in nest building (Tab. 3). Animals built nest more often ($Z = 1.9, p = .057; Z = 2.3, p < .05$) and for longer period of time ($Z = 2.2, p < .05; Z = 2.1, p < .05$) at the second and third compared to the first exposure to pups. Besides, time spent building a nest was higher in the third than in the second exposure to pups ($Z = 2.3, p < .05$). Data of fourth and fifth exposure to pups are not shown in the table because animals (2) that showed FMB for two consecutive days during the first three exposures were no longer tested and the n decreased subsequently. Females also smelt the pups less and spent less time away from pups at the second ($Z = 2.2, p < .05; Z = 2.36, p < .05$) and third ($Z = 2.19, p < .05; Z = 2.36, p < .05$) compared to the

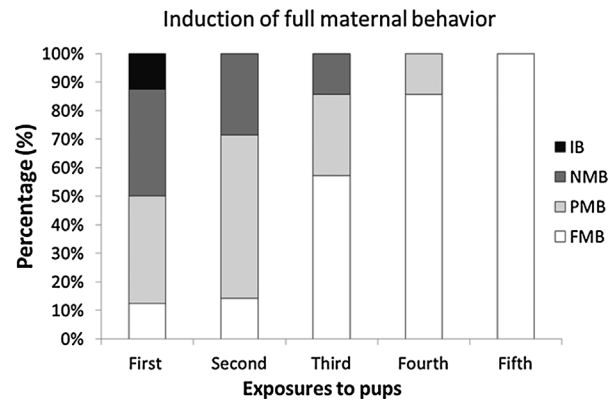


FIGURE 2 Percentage of adult (60–90 days) females that showed maternal or non-maternal behavior during the first 15 min of a series of 60 min repeated exposures to pups. FMB, full maternal behavior; PMB, partially maternal behavior; NMB, non-maternal behavior; IB, infanticidal behavior.

first exposure to pups respectively. Females were also away from pups for longer period of time at the second compared to the third exposure to pups ($Z = 2.3, p < .05$). Maternal behavior observations at the end of each 60 min exposure (Fig. 3), revealed that at the end of the third 60 min exposure to pups all subject engaged in nest building, gathering, and crouching over the pups ($\chi^2 = 17.2, p < .05$).

The sequence of maternal behavioral components and other related behaviors (sniffing, licking, retrieval, transport, crouching postures, and nest building) were analyzed in successive repeated exposures to pups when the females displayed FMB, PMB, and NMB (Fig. 4). The flow diagrams show the frequencies of each behavioral component and the probability of their transitions. The typical maternal behavior sequence displayed by lactating females was similar to the sequence shown by naïve sensitized females when they were considered showing FMB. When animals displayed NMB or PMB, both the occurrence of the behavioral components and several of the transitions were qualitatively different.

EXPERIMENT III

Juvenile Parental Care

Findings in Experiment I showed that juvenile failed to display maternal behavior in two 15-min repeated exposure to pups. That result agreed with previous studies (Gandelman, 1973b) that failed to find maternal behavior in juvenile mice. However, those findings were surprising considering the attraction to pups that

Table 3. Maternal Behavior During First 15 min of Three 60-min Repeated Exposure

Exposure	First 15 min of Test		
	First	Second	Third
Percentage			
Retrieval	25.0%	28.5%	57.1%
Frequency			
Sniffing	14.0 (5.1)	2.0 (1.7)*	1.0 (1.8)*
Licking Bouts	24.0 (11.2)	23.0 (12.1)	15.0 (5.6)
Crouching Postures	10.0 (8.1)	9.0 (4.0)	8.0 (2.2)
Nest Building	.0 (.8)	11.0 (2.3)	10.0 (2.3)*
Distant 10 cm	23.0 (4.3)	5.0 (1.2)*	2.0 (.5)*,#
Time (s)			
Sniffing	3.8 (1.7)	1.4 (.8)*	.8 (.7)*
Licking	52.0 (25.8)	81.4 (44.1)	14.7 (24.3)
Crouching Postures	26.9 (24.0)	44.4 (17.2)	31.0 (18.1)
Nest Building	.0 (8.6)	294.8 (78.6)*	566.2(68.0)*,#
Distant 10 cm	190.0 (146.4)	63.2 (18.0)*	16.6 (5.4)*,#

Data are expressed as median (SIQR).

* $p < .05$, vs. first exposure.

$p < .05$, vs. second exposure.

juvenile rats and prairie voles showed when exposed to them for the first time (Bridges et al., 1974; Mayer, 1983; Olazábal & Young, 2006a). Besides, juveniles from several species displayed maternal behavior toward younger siblings when two litters overlapped (Barbosa and Da Silva Mota, 2013; Gubernick & Laskin, 1994; Stern & Rogers, 1988; Uriarte, Ferreira, Rosa, Sebben, & Lucion, 2008). There was no study analyzing mouse juvenile behavior toward newborns in overlapping litters. Therefore, in the Experiment III, we investigated if juveniles could be induced to display maternal behavior after being exposed to their parturient mother and their sibling pups by 12–24 hr.

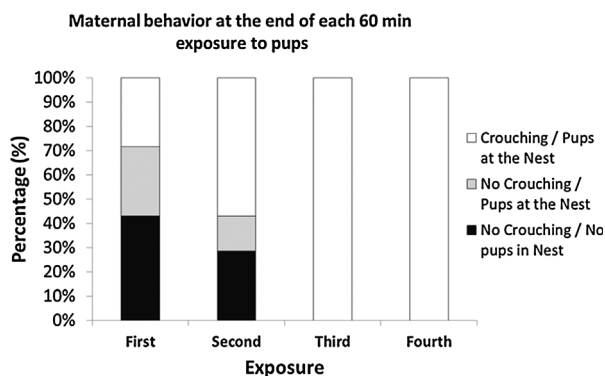


FIGURE 3 Percentage of adult (60–90 days) females with pups in or outside the nest, and adopting, or not, crouching posture at the end of each 60 min exposure to pups. Data are expressed as percentages (number of animals in that condition / total number of animals \times 100).

Methods

Multiparous females ($n = 31$) were mated with males. In 23 of them, the male remained with female beyond the first postpartum estrous (Overlapping Litter, OvL), and in 8 females, the males were removed 1 week after mating (Single Litter, SL). Date of birth was registered, and all litters were culled to five pups per dam. Female mice exhibit a postpartum estrous 48 hr after delivery, which enables them to nurse the first litter while pregnant of a second litter (Elwood, Nesbitt, & Kennedy, 1990; Ostemeyer, 1983).

At the postpartum day 22–25, 23 juvenile females that cohabitated with their parturient/lactating mother and sibling newborns were tested for maternal behavior (15 min test) with five pups, and their mothers in their homecage (TOvL group). Immediately after, these juveniles were weaned and individually housed for 1 hr and re-tested for maternal behavior with two pups. Juveniles from SL ($n = 16$) were either identically tested for maternal behavior as the TOvL group (using donor pups, TSL group); or directly weaned, individually housed and tested only once without a previous 15 min test in their homecage (SL group, see Tab. 4).

Results

The analysis of maternal behavior of the juveniles and their lactating mothers during the first 15 min test in their homecage showed significant differences. Lactating mothers tested in an overlapping context with their own pups (TOvL), spent less time licking the pups than lactating mothers (TSL) tested with alien pups (22.1

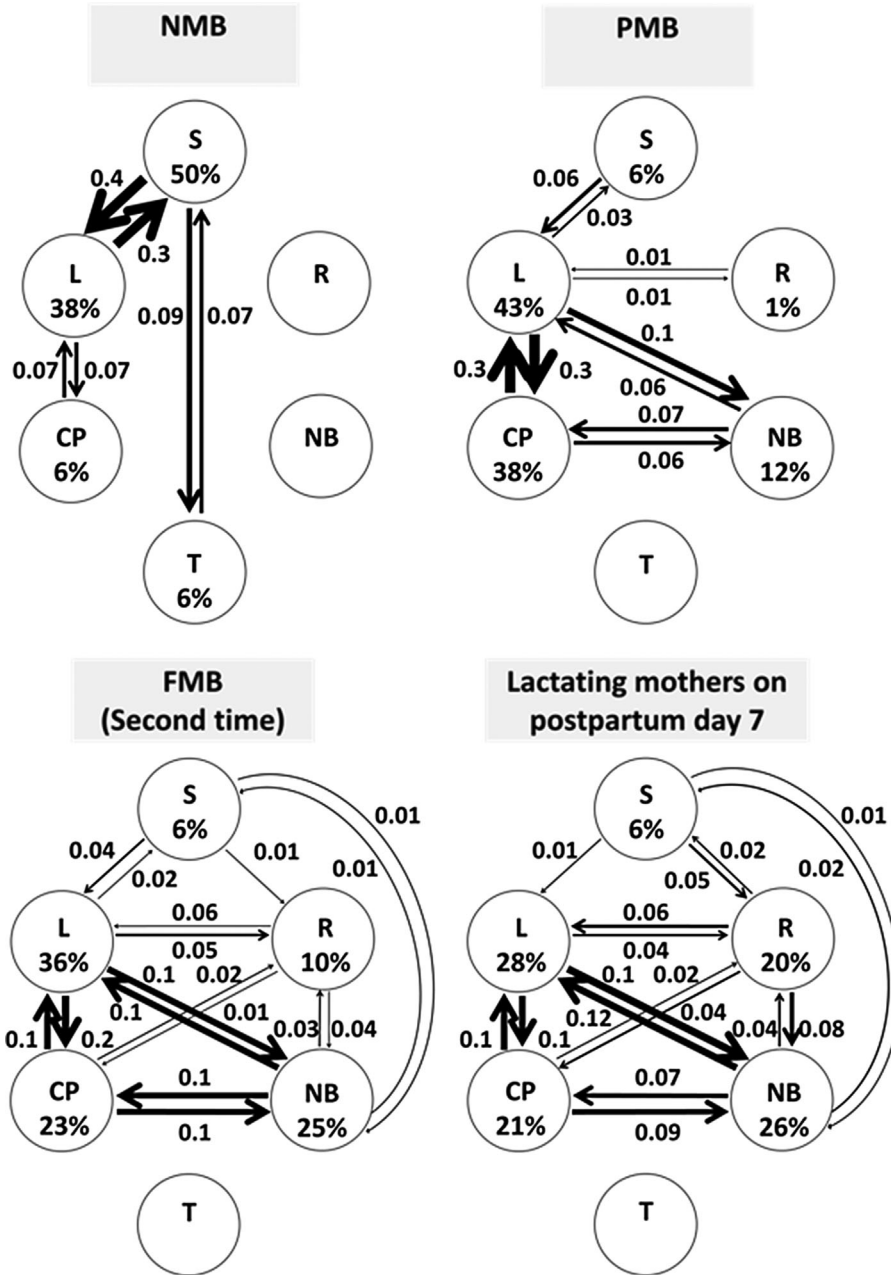


FIGURE 4 Flow diagram of frequencies of behavioral components and their transitions when inexperienced adult females display NMB (non-maternal behavior, $n = 4$), PMB (partial maternal behavior, $n = 6$), FMB (full maternal behavior, by second time, $n = 5$) or at 7 day postpartum ($n = 10$). Frequency of behavioral components is shown as percentage. Probabilities of transitions are shown as numbers in the arrows and represented (approximately) as the wide of the arrow. The direction of the arrows indicates which behavior leads and which follows. S: sniffing, L: licking, R: retrieval, C: crouching posture, NB: Nest building, T: Transport.

[22.0], 347.6 [113.7], respectively, $U = 31.0$, $p < .05$). Similarly, juveniles tested in an overlapping context with their younger siblings (TOvL), spent less time licking the pups than juveniles from single litters (TSL) tested with alien pups (1.15 [2.0], 36.7 [47.22],

respectively, $U = 32.0$, $p < .01$). However, the time spent building a nest (460.5 [138.0]) was higher ($U = 32.0$, $p < .01$) in mothers from TOvL than in mothers from TSL (234.5 [135.16]). No other difference was found between TOvL and TSL in mothers or

Table 4. Groups of Experiment III

Groups	Juveniles From Overlapping litters (Cohabitated With Parturient Mother And Newborn Siblings)	Previously Tested for MB In Their homecage With Their Mothers
TOvL	+	+
TSL	–	+
SL	–	–

+/- sign represent presence or not of the specific experience.

juveniles tested with own or alien pups respectively (data not shown).

The percentage of individually housed juveniles that displayed maternal behavior in the different groups was also significantly different ($\chi^2 = 16.8$, $p < .01$, Fig. 5). The group TOvL displayed higher maternal behavior than TSL ($\chi^2 = 7.8$, $p < .05$) and SL ($\chi^2 = 10.2$, $p < .05$). There was no difference between TSL and SL (complete absence of PMB or FMB animals) ($\chi^2 = 1.3$, $p = .2$). The average age among the groups (TOvL, TSL, and SL) was not different (23.2 ± 0.92 , 23.0 ± 0.75 , 22.7 ± 0.70 , $p > .05$).

TOvL females licked the pups for longer period of time than TSL ($U = 142.0$, $p < .05$) and SL ($U = 148.0$, $p < .05$) females when tested individually housed. Moreover, frequency and time spent in crouching posture was higher in juveniles of TOvL than TSL ($U = 169.0$, $U = 169.5$, $p < .01$) and SL ($U = 159.5$; $U = 163.5$, $p < .01$) groups. Nest building was also observed to occur with higher frequency and duration in juveniles TOvL than in TSL and SL ($U = 136.0$, $p < .05$, Table 5).

DISCUSSION

In the present study, we showed that inexperienced adult, but not juvenile, females can be rapidly induced to display full maternal behavior. However, maternal behavior is not spontaneous in mice but the outcome of a sensitization process similar to that well described in rats. Interestingly, in contrast to rats or prairie voles (Bridges et al., 1974; Mayer, 1983; Olazábal & Young, 2006a, 2006b), naïve juvenile mice show an initial inhibitory or neutral behavioral response toward newborns that can be overcome by cohabitation with their newborn siblings, and maternal fluids of parturition in an overlapping litter context.

A higher incidence of maternal behavior in adult mice was also found in previous studies (Gandelman, 1973a, 1973b, 1973c; Leblond, 1938; Noiro, 1964a, 1964b, 1969, 1972). However, in contrast to several studies who found spontaneous or FMB in most or all naïve females (70–100%; Calamandrei & Keverne, 1994; Gandelman, 1973b, 1973c; Gandelman and vom Saal, 1975; Leussis et al., 2008b, 1969, 1972; Noiro, 1964a; Stolzenberg & Rissman, 2011), we failed to find FMB on the first exposure to pups. In fact, two repeated exposures to pups of 15 min also failed to induce FMB in most animals. Although 50% of animals displayed some behavioral component of maternal behavior immediately, increasing the time and frequency of exposures to pups improved the behavioral performance of 80% of the animals. Some of the apparent discrepancies in the incidence of maternal behavior in adult female mice found in the literature might be consequence of different criteria used to consider an animal as maternal. Several authors have considered that an animal was maternal when only one of the behavioral components of maternal behavior (e.g.,

Table 5. Maternal Behavior of Juvenile Females (22–25 Days Old) in Overlapping or Single Litters

Groups	TOvL ($n = 23$)	TSL ($n = 8$)	SL ($n = 8$)
Percentage			
Retrieval	34.8%	12.5%	0.0%
Frequency			
Sniffing	1.0 (.85)	2.5 (1.0)	2.5 (1.7)
Licking Bouts	8.0 (3.5)	6.0 (2.7)	5.0 (3.2)
Crouching Postures	5.0 (1.5) ^a	.0 (.7) ^b	.0 (1.0) ^b
Nest Building	.0 (1.8) ^a	.0 (.0) ^b	.0 (.0) ^b
Time (s)			
Sniffing	2.4 (2.3)	2.9 (1.8)	2.2 (3.8)
Licking	60.7 (44.3) ^a	16.1 (21.5) ^b	8.4 (19.9) ^b
Crouching Postures	36.0 (36.25) ^a	.0 (1.9) ^b	.0 (3.2) ^b
Nest Building	.0 (26.7) ^a	.0 (.0) ^b	.0 (.0) ^b

Data are expressed as Median (SIQR).

Different letters (a and b) indicate statistical differences ($p < .05$) among groups.

Kruskal–Wallis analysis of variance followed by Mann–Whitney U test.

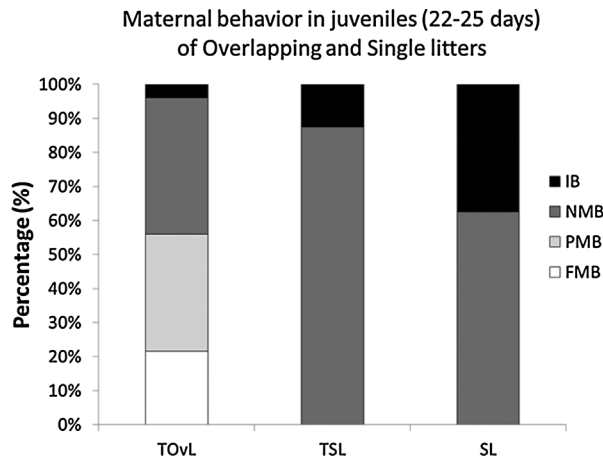


FIGURE 5 Percentage of maternal or non-maternal juveniles tested (15 min test) in a novel cage. Groups TOVL: Juveniles from overlapping litters exposed to sibling pups during ~12–24 hr and previously tested for maternal behavior in their homecage in presence of their mothers; TSL: Juvenile from single litters previously tested for maternal behavior with donor pups in their homecage in presence of their mothers; SL: Juvenile from single litters with no previous exposure to pups. FMB, full maternal behavior; PMB, partial maternal behavior; NMB, non-maternal behavior; IB, infanticidal behavior. Data are expressed as percentage, $\chi^2 = 16.8$, $p < .01$.

retrieval, licking and grooming, crouching postures, or nest building) was observed (Gandelman, 1973b; Gandelman et al., 1970; Jin et al., 2005; Li et al., 1999; Pedersen et al., 2006; Ragnauth et al., 2005). The disadvantages of considering an animal as maternal by just observing one or some of the components of maternal behavior have been already discussed by Priestnall and Young (1978). In contrast to most previous studies, we presented in the current investigation a detailed analysis of all maternal behavioral components and their changes after repeated exposures to pups. Another potential source of variability could be the treatment that some investigators gave to their animals before starting the test. For example, while in our study all females were completely naïve, some authors performed previous exposures to pups (15–45 min to 24 hr) before applying the test (Gandelman, 1973c; Leussis et al., 2008; Stolzenberg & Rissman, 2011).

Variability of the behavioral response toward newborns in female mice has been previously explained by differences in the testing procedures (e.g., homecage or novel cage), time of habituation to the novel cage, type of bedding, food, among others (Kuroda et al., 2011). However, several studies have also found that the testing conditions, the time of habituation, rearing conditions, or origin of the animals do not necessarily

induce differences in maternal or other behaviors (Crawley, Chen, Puri, Washburn, Sullivan, Hill, Young, Nadler, Moy, Young, Caldwell, & Young, 2007; see results for C57BL in Svare & Broida, 1982). The absence of spontaneous maternal behavior in mice, and the need for a sensitization period have been previously suggested by other authors (Brown et al., 1996; Gandelman, 1973b; Kuroda et al., 2011; Lucas et al., 1998; among others). In Experiment II, we confirmed that most animals required at least three 60-min exposure to pups to display full maternal care.

The analysis of the flow diagrams confirmed that our definition was appropriate and animals in each group behaved differently. The detailed analysis of the behavioral sequence at the different stages of the sensitization process clearly showed a change in the occurrence of the main components of maternal behavior and their transitions. The most common behavioral sequence displayed by non-maternal females consisted of smelling the pups followed by licking behavior. However, smelling and licking behaviors were not followed by retrieval behavior or nest building. In the case of animals that showed PMB, the sequence analysis showed that the most common behavioral transition was licking the pups followed by placing them in contact with the ventral region of the female (active crouching posture). Females investigated less and engaged quickly in licking behavior followed, in some cases, by nest building, but rarely by retrieval behavior. In contrast to other studies (Calamandrei & Keverne, 1994; Noirot, 1964a, 1964b, 1969; Stolzenberg & Rissman, 2011), we observed retrieval behavior in most animals only after the second or third 1-hr exposure to pups. Similarly to Gandelman (1973b), we found that initially animals mostly smelt and licked the pups, while retrieval behavior appeared only after the animal was clearly maternal. In our study, nest building also appeared more frequently and for longer period of time in maternal animals. However, licking behavior was not a good predictor of maternal behavior and rather was associated with recognition of the newborn as previously suggested by Ostermeyer and Elwood (1983). Similarly, juveniles tested with alien pups in their homecage, in the presence of their mothers, licked the alien pups more and for longer period of time than juveniles tested with their newborn siblings in the same conditions. However, when individually housed, those juveniles that licked the alien pups more often and for longer period of time failed to show maternal behavior.

When females showed FMB, retrieval behavior also followed all the other behavioral components, and occurred, in some cases, after the first investigation (e.g., sniffing the pups). In some cases, animals that showed FMB also engaged in nest building immediately after sniffing the pups and before retrieving them.

In FMB, but not PMB, animals all behavioral transitions between the behavioral components of maternal behavior occurred. In contrast, NMB animals did not continue their behavioral sequence toward retrieval or nest building, and instead, most common behaviors were licking and sniffing the pups. Investigation (sniffing and licking the pups) played a more critical role in the NMB and PMB than in FMB animals. It is interesting to note that in the present study only active, but not quiescent, crouching posture was observed. Significant amount of the total time spent adopting crouching postures during the first 15 min of interaction with the pups was consequence of the posture adopted by females while licking the pups, and not a posture of protection or thermoregulation. However, crouching was clearly associated with FMB when females stayed in the nest with the pups at the end of the 1 hr exposure to them.

Previous studies (Gandelman, 1973b; Noirot, 1972) suggested that the lack of a pup-oriented behavior in juvenile mice could be consequence of the separation from their mothers and littermates, or their physical condition. That possibility was evaluated by Gandelman (1973b) in mice, and others in rats (Mayer & Rosenblatt, 1979), showing that maternal behavior in juveniles was not affected by weaning or mediated by contact with same age littermates. In the present study, we showed that juveniles exposed to maternal fluids and newborn siblings, at least for 12 hr, displayed adult-like behavioral responses toward pups. That finding confirmed that juveniles did not lack the physical capability to perform maternal behavior, and also that the novel cage by itself could not inhibit maternal behavior. Naïve juvenile and pubertal females smelt the pups more often and for longer period of time than adults, showing that the presence of the pups was detected at those ages. However, that initial approach and exploration was not followed by the display of MB.

The analysis of the behavioral sequence of NMB, PMB and FMB animals clearly indicated that smelling is an important behavioral response during the initial stages of investigation of the pups, when the animal is still non-maternal. Perhaps, as a consequence of this initial exploration, juveniles stayed apart from the pups. In contrast, adult females, more prone to interact with pups, investigated (i.e., smelt) the pups less, and spent more time closer to them. In the current testing conditions, juveniles were also less exploratory (less rearing) and spent more time immobile than the other two groups, suggesting a possible aversive behavioral response toward pups. However, the behavioral response of juveniles might also be due to their age, not to the presence of the pups. In the case of pubertal animals, they did not show higher immobility or

reduced exploration, but also failed to display maternal behavior. Therefore, whether juveniles showed or not an inhibitory behavioral response must be studied in more detail. Pups might not elicit maternal behavior at that age. Climbing was observed at all ages, showing that the motor activity and capability of the animals were good, and they were not slept or doing freezing during the test.

In summary, inexperienced juveniles smelt and investigated the pups but were not motivated to take care of them. Therefore, one interpretation could be that maternal behavior in juvenile mice was inhibited by olfactory mechanisms, what increased the time spent immobile, or that pups did not have a motivational value at this age. The inhibition could be removed during the period of cohabitation of juveniles with their mothers around the time of delivery of a new litter or the behavior elicited by the association of pups with maternal fluids, among other possibilities. Gubernick and Laskin (1994) had already shown the importance that cohabitation with newborn siblings had for the induction of maternal behavior in *Peromyscus californicus* juveniles. The authors found that both female and male 40-days-old *Peromyscus californicus*, which cohabitated with their newborn siblings and parents by 4 hr, failed to show parental behavior. However, if they cohabitated with their sibling pups by 5 days, 70% displayed parental behavior. In that study, they speculated whether parental behavior of adults could elicit parental responses from juveniles. We tested that possibility and found that testing juveniles and their lactating mothers for parental behavior simultaneously had no effect on juvenile behavioral response toward pups after individually housed. However, exposure to pups and maternal fluids during a time longer than 4 hr (12–24 hr), in overlapping litter context, was sufficient to enhance parental responses in juvenile mice, younger (22–25-days-old) than those tested in Gubernick and Laskin (1994). Gerlach (1990) proposed that in semi-natural conditions, only the oldest females, within a family of wild house mice, was capable to reproduce. They suggested that, under high population density, adult females were pressed to disperse. Therefore, absence of maternal behavior in juvenile mice (unless they remained in the nest with the mother) might be an adaptation of the species for rapid dispersal. The induction of maternal behavior in those juveniles that stayed in the nest until a second delivery might facilitate the possibility of some juvenile females to form, in the future, a communal nest with her mother, contributing with the caring of their own and also their sibling newborns (Hayes, 2000).

Therefore, we propose that maternal behavior in adult naïve female mice differs from the behavior shown by

rats and prairie voles, and those differences are likely consequence of different social and reproductive strategies among these species. Maternal behavior in mice is not immediate, but it is clearly induced faster than in adult rats (Olazábal & Young 2006a; Rosenblatt, 1967), what might also be necessary to join other adult females to form new communal nests or plural breeding systems (Hayes, 2000).

NOTES

The authors want to thank the staff from the animal facility of the Facultad de Medicina who took care of the mice and provided appropriate conditions to carry out these experiments. D.E.O. and M.A. received funding support from the Comisión Sectorial de Investigación Científica (CSIC), UdelaR.

REFERENCES

- Barbosa, M. N., & Da Silva Mota, M. T. (2013). Alloparental responsiveness to newborns by nonreproductive, adult male, common marmosets (*Callithrix jacchus*). *American Journal of Primatology*, 75, 145–152.
- Bridges, R. S., Zarrow, M. X., Goldman, B. D., & Denenberg, V. H. (1974). A developmental study of maternal responsiveness in the rat. *Physiology & Behavior*, 12, 149–151.
- Brown, R. E., Mathieson, W. B., Stapleton, J., & Neumann, P. E. (1999). Maternal behavior in female C57BL/6J and DBA/2J inbred mice. *Physiology & Behavior*, 67, 599–605.
- Brown, J. R., Ye, H., Bronson, R. T., Dikkes, P., & Greenberg, M. E. (1996). A defect in nurturing in mice lacking the immediate early gene *fosB*. *Cell*, 86, 297–309.
- Calamandrei, G., & Keverne, E. B. (1994). Differential expression of Fos protein in the brain of female mice dependent on pup sensory cues and maternal experience. *Behavioral Neuroscience*, 108, 113–120.
- Crawley, J. N., Chen, T., Puri, A., Washburn, R., Sullivan, T. L., Hill, J. M., ... Young, W. S. (2007). Social approach behaviors in oxytocin knockout mice: Comparison of two independent lines tested in different laboratory environments. *Neuropeptides*, 41, 145–163.
- Elwood, R. W., Nesbitt, A. A., & Kennedy, H. F. (1990). Maternal aggression in response to the risk of infanticide by male mice, *Mus domesticus*. *Animal Behavior*, 40, 1080–1086.
- Gandelman, R. (1973a). Induction of maternal nest building in virgin female mice by presentation of young. *Hormones & Behavior*, 4, 191–197.
- Gandelman, R. (1973b). The ontogeny of maternal responsiveness in female Rockland-Swiss albino mice. *Hormones & Behavior*, 4, 257–268.
- Gandelman, R. (1973c). Maternal behavior in the mouse: Effect of estrogen and progesterone. *Physiology & Behavior*, 10, 153–155.
- Gandelman, R., & vom Saal, F. (1975). Pup-killing in mice: The effects of gonadectomy and testosterone administration. *Physiology & Behavior*, 15, 647–651.
- Gandelman, R., Paschke, R., Zarrow, M. X., & Denenberg, V. H. (1970). Care of young under communal conditions in the mouse (*Mus Musculus*). *Developmental Psychobiology*, 3, 245–250.
- Gerlach, G. (1990). Dispersal mechanisms in a captive wild house mouse population (*Mus domesticus* Ruddy). *Biological Journal of the Linnean Society*, 41, 271–277.
- Gubernick, D. J., & Laskin, B. (1994). Mechanisms influencing sibling care in the monogamous biparental California mouse, *Peromyscus californicus*. *Animal Behavior*, 48, 1235–1237.
- Hamaguchi-Hamadaa, K., Sanbo, C., Hamadaa, S., & Yagi, T. (2004). Exposure to hexanal odor influences maternal behavior and induces neonatal death in Fyn tyrosine kinase-deficient mice. *Neuroscience Research*, 48, 259–267.
- Hayes, L. (2000). To nest communally or not to nest communally: A review of rodent communal nesting and nursing. *Animal Behaviour*, 59, 677–688.
- Jin, S., Blendy, J. A., & Thomas, S. A. (2005). Cyclic AMP response element-binding protein is required for normal maternal nurturing behavior. *Neuroscience*, 133, 647–655.
- Kuroda, K., Meaney, M. J., Uetani, N., & Kato, T. (2008). Neurobehavioral basis of the impaired nurturing in mice lacking the immediate early gene *FosB*. *Brain Research*, 1211, 57–71.
- Kuroda, K. O., Tachikawa, K., Yoshida, S., Tsuneoka, Y., & Numan, M. (2011). Neuromolecular basis of parental behavior in laboratory mice and rats: With special emphasis on technical issues of using mouse genetics. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, 35, 1205–1231.
- Leblond, C. P. (1938). Extra-hormonal factors in maternal behavior. *Proceedings of the Society for Experimental Biology and Medicine*, 38, 66–70.
- Leussis, M. P., Bond, T., Hawken, C. M., & Brown, R. E. (2008). Attenuation of maternal behavior in virgin CD-1 mice by methylphenidate hydrochloride. *Physiology & Behavior*, 95, 395–399.
- Li, L., Keverne, E. B., Aparicio, S. A., Ishino, F., Barton, S. C., & Surani, M. A. (1999). Regulation of maternal behavior and offspring growth by paternally expressed *Peg3*. *Science*, 284(5412), 330–333.
- Lonstein, J. S., & De Vries, G. J. (2000). Sex differences in the parental behavior of rodents. *Neuroscience & Biobehavioral Reviews*, 24, 669–686.
- Lucas, B. K., Ormandy, C. J., Binart, N., Bridges, R. S., & Kelly, P. A. (1998). Null mutation of the prolactin receptor gene produces a defect in maternal behavior. *Endocrinology*, 139, 4102–4107.
- Mayer, A. D. (1983). The ontogeny of maternal behavior in rodents. In: R. W. Elwood, (Ed.), *Parental behavior of rodents* pp. 1–20. Chichester, England: Wiley.
- Mayer, A. D., & Rosenblatt, J. S. (1979). Ontogeny of maternal behavior in the laboratory rat: Early origins in 18- to 27-day-old young. *Developmental Psychobiology*, 12, 407–424.

- McCarthy, M. M., & vom Saal, F. S. (1985). The influence of reproductive state on infanticide by wild female house mice (*Mus musculus*). *Physiology & Behavior*, 35, 843–849.
- Noirot, E. (1964a). Changes in responsiveness to young in the adult mouse: The effect of external stimuli. *Journal of comparative and Physiological Psychology*, 57, 97–99.
- Noirot, E. (1964b). Changes in responsiveness to young in the adult mouse. IV. The effect of an initial contact with a strong stimulus. *Animal Behaviour*, 12, 442–445.
- Noirot, E. (1969). Serial order of maternal responses in mice. *Animal Behaviour*, 17, 547–550.
- Noirot, E. (1972). The onset and development of maternal behavior in rat, hamster and mice: A selective review. *Advances in the Study of Behavior*, 4, 107–145.
- Numan, M., & Insel, T. R. (2003). *The neurobiology of parental behavior*. New York: Springer-Verlag.
- Olazábal, D. E., & Young, L. J. (2006a). Species and individual differences in juvenile female alloparental care are associated with oxytocin receptor density in the striatum and the lateral septum. *Hormones & Behavior*, 49, 681–687.
- Olazábal, D., & Young, L. (2006b). Oxytocin receptor in the nucleus accumbens facilitate Spontaneous” maternal behavior in adult female prairie voles. *Neuroscience*, 141, 559–568.
- Ostermeyer, M. C. (1983). *Maternal aggression*. pp. 151–179. Chichester: John Wiley.
- Ostermeyer, C. M., & Elwood, R. W. (1983). Pup recognition in *Mus musculus*: Parental discrimination between their own and alien young. *Developmental Psychobiology*, 16, 75–82.
- Pedersen, C. A., Vadlamudi, S. V., Boccia, M. L., & Amico, J. A. (2006). Maternal behavior deficits in nulliparous oxytocin knockout mice. *Genes, Brain & Behavior*, 5, 274–281.
- Priestnall, R., & Young, S. (1978). An observational study of caretaking behavior of male and female mice housed together. *Developmental Psychobiology*, 11, 23–30.
- Ragnauth, A. K., Devidze, N., Moy, V., Finley, K., Goodwillie, A., Kow, L., ... Pfaff, D. W. (2005). Female oxytocin gene-knockout mice, in a seminatural environment, display exaggerated aggressive behavior. *Genes, Brain and Behavior*, 4, 229–239.
- Roberts, R. L., Williams, J. R., Wang, A. K., & Carter, C. S. (1998). Cooperative breeding and monogamy in prairie voles: Influence of the sire and geographical variation. *Animal Behaviour*, 55, 1131–1140.
- Rosenblatt, J. S. (1967). Nonhormonal basis of maternal behavior in the rat. *Science*, 156, 1512–1514.
- Stern, J. M., & Rogers, L. (1988). Experience with younger siblings facilitates maternal responsiveness in pubertal Norway rats. *Developmental Psychobiology*, 21, 575–589.
- Stolzenberg, D. S., & Rissman, E. F. (2011). Oestrogen-independent, experience-induced maternal behaviour in female mice. *Journal of Neuroendocrinology*, 23, 345–354.
- Svare, B., & Broida, J. (1982). Genotypic influences on infanticide in mice: Environmental, situational and experiential determinants. *Physiology & Behavior*, 28, 171–175.
- Uriarte, N., Ferreira, A., Rosa, X. F., Sebben, V., & Lucion, A. B. (2008). Overlapping litters in rats: Effects on maternal behavior and offspring emotionality. *Physiology & Behavior*, 93, 1061–1070.
- vom Saal, F. S. (1984). Proximate and ultimate causes of infanticide and parental behavior in male house mice. pp. 401–424. New York: Aldine.