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Do sires and juvenile male mice (C57BL/6) contribute to the rearing of the offspring?

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Abstract

Copulation and/or cohabitation with a pregnant female facilitate paternal behavior in male mice. However, their contribution to the rearing of the offspring is still not well understood. Our aims were to investigate the behavior of sires toward own or alien pups; the immediate consequences of the presence of fathers on the offspring and the behavior of the mother; and whether the exposure of juvenile males to newborn siblings, in an overlapping litters context, facilitates paternal behavior in C57BL/6 mice. We found that sires behaved paternally toward alien pups at both postpartum days 3 and 7; did not affect the behavior of the mother (e.g., licking and grooming, retrieval behavior, time in the nest, and crouching postures); and reduced the time offspring stayed alone in the nest. The exposure to newborn siblings did not promote paternal behavior in juvenile males. Therefore, sires are more paternal than usually described in the literature for laboratory mice, suggesting a facultative role in the rearing of the offspring. However, juvenile male mice, in contrast to juvenile females, could be adapted to leave the nest earlier without major contribution to the offspring.

Keywords C57BL/6 · Development · Contribution · Paternal · Infanticidal · Recognition · Juveniles · Overlapping litters

Introduction

Males from several species of rodents, including laboratory mice (*Mus musculus*), display parental behavior (i.e., crouching postures, licking and grooming, retrieval, and nest building, Brown 1993; Dewsbury 1985; Elwood 1986; Gubernick et al. 1994; Leblond 1938; Lonstein and De 2000; Noirot 1969; Roberts et al. 1998; Waring and Perper 1979, 1980). However, naïve (no sexual or paternal experience) adult males from most laboratory mouse strains (129S, Balb, C57BL/6, CBA, DBA) are generally described as infanticidal. Recently, we also found that the behavioral response of virgin inexperienced male mice (CB57BL/6) exposed to donor pups was mostly infanticidal or non-parental (Olazábal and Alsina-Llanes 2016). However, a few studies have found that a small percentage of inexperienced adult males displayed paternal behavior immediately after the first exposure to pups (Kennedy and Elwood 1988 in CBA strain; McCarthy and vom Saal 1986 in CF-1 strain; Kuroda et al. 2007 in C57BL/6 strain; Gandelman 1973 in Rockland-Swiss strain).

Several ultimate and proximal mechanisms have been proposed to explain cessation of infanticidal behavior and induction of paternal behavior in the mouse (Cicirello and Wolff 1990; Ebensperger 1998; Wynne-Edwards and Timonin 2007; Bales and Saltzman 2007). The hypothesis of individual recognition proposes a genetic label among individuals (Cheetham et al. 2007). Males would recognize in that way their own pups and inhibit infanticidal behavior. Indeed, several studies found that males laboratory mice would kill alien, but not their pups at the early postpartum period (Brooks and Schwarzkopf 1983; Huck et al. 1982). In our laboratory, we also observed that adding an alien pup to complete a litter, or partially replacing a few own by alien pups at the early postpartum period (postpartum day 0 or 1), induced pup killing in males (unpublished). Others proposed that an association between the odor of pups and the previous sexual partner could also inhibit the attacks (Elwood 1985; Huck et al. 1982; Labov 1980). However, an alternative hypothesis proposed that long-lasting physiological and behavioral changes, not related to recognition, could inhibit infanticidal and promote parental

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behavior in males (Brooks and Schwarzkopf 1983; Elwood 1985; Gubernick et al. 1994; Huck et al. 1982; Kennedy and Elwood 1988; Matsumoto and Kimura 1995; Soroker and Terkel 1988; Tachikawa et al. 2013). Several authors suggested that once paternal behavior was established, males could tolerate or/and take care of alien pups (Brooks and Schwarzkopf 1983; Brown 1993; Huck et al. 1982; Labov 1980; Ostermeyer and Elwood 1983; Tachikawa et al. 2013). Additionally, subordination of males to the lactating female has also been proposed as a mechanism to inhibit infanticidal behavior at the early postpartum period. Nevertheless, inhibition of infanticidal behavior is not sufficient to trigger parental behavior. Males must also show paternal motivation and be attracted to pups to become fully parental. Therefore, long-term neural changes must occur in males after copulation, or around female pregnancy/parturition, to motivate paternal behavior, rather than indifference. Generally, *Mus musculus* mice are considered "promiscuous" and "uniparental" species where females would be adapted to rear their offspring without significant paternal investment. However, several studies have found significant paternal contribution to the rearing of the offspring in promiscuous species (Schradin and Pillay, 2003). In the first experiment, we tested the hypothesis that once the behavior was established, male mice behaved paternally toward own or alien pups, did not affect the behavior of the lactating mother, and could eventually contribute to the rearing of the offspring.

Juvenile naïve (no sexual or pup exposure) males from various species can also take care of pups (Gubernick and Laskin 1994; Olazábal and Young 2006; Roberts et al. 1998; Stern and Rogers 1988). For example, some studies showed the occurrence of paternal care in juvenile male mice (Gandelman 1973; Leblond 1938). However, in the study of Leblond (1938), it is unclear if animals were naïve because they mentioned that juveniles required about 4 days of exposure to pups (sensitization) to display parental behavior. Moreover, in the study of Gandelman (1973), the subjects were considered paternal when only one of the behavioral components of parental behavior was observed (retrieval, licking or crouching). Recent results from our laboratory showed that even though most juvenile males did not attack the pups, they mostly ignored them (Olazábal and Alsina-Llanes 2016). Therefore, if naïve juvenile males contribute to the rearing of their newborn siblings before leaving the nest is also still unclear. We previously found that juvenile female mice were more prone to display parental behavior if previously exposed to their parturient mother and newborn siblings for 12–24 h (Alsina-Llanes et al. 2015). In the present study, we tested the hypothesis that cohabitation with their parturient mother and newborn siblings induced parental behavior in weanling males, as it did in weanling females (Alsina-Llanes et al. 2015). Although parental behavior in the context of overlapping litters have been somewhat studied in rats (Harding and Lonstein 2016; Uriarte et al. 2008), to our knowledge, parental behavior in juvenile male laboratory mice has not been studied in the context of overlapping litters.

In summary, in the present work, we used one of the most common strains of laboratory mice (CB57BL/6) to investigate: first, the contribution of sires to the rearing of the offspring; second, the impact of the presence of sires in the nest on the behavior of the lactating mother (e.g., licking and grooming, retrieval behavior, nest building and time in the nest, crouching postures); third, the behavioral response of those males toward alien pups at postpartum days 3 and 7; fourth, the effect of cohabitation of juvenile males with their pregnant/parturient mothers and newborn siblings on their behavioral response toward pups. We predicted that sires would contribute to the rearing of the offspring (including alien pups), would not negatively affect maternal behavior, and cohabitation of juveniles with their pregnant/parturient mothers and newborn siblings would facilitate the induction of juvenile paternal behavior.

Materials and methods

Subjects

We used C57BL/6 mice originally obtained from Jackson Laboratory and inbred at the animal facility of the Facultad de Medicina (UdelaR, Montevideo, Uruguay). All animals were weaned at age 20–21 days and maintained in same-sex groups of 6–7 individuals per cage. Cages were 45 cm × 25 cm × 15 cm, with transparent Plexiglas walls and wood shaving as bedding. Animals were kept under a 13:11 h light-dark cycle (light on from 6:00 am), at 22 °C, with ad libitum access to food (PMI nutrition international LabDiet®, Shoreview, Minnesota, USA) and water. Cages were regularly changed once a week. All procedures carried out in this study were approved by the local committee of ethics in animal research (CHEA, N° 071140, December 26th, 2011) and followed the recommendations of the "Guide for the Care and Use of Laboratory Animals" of the National Institutes of Health (2011), the "Guidelines for Ethical Conduct in the Care and Use of Animals" (APA, Board of Scientific Affairs, Committee on Animal Research and Ethics, 2012).

Experiment I: Parental behavior during the postpartum period

Thirty-six females were mated with males. Date of birth was registered, and all litters were culled to five pups per dam on postpartum day 1 to reduce variability among groups (Agnish and Keller 1997). In the first group ($n = 17$), males were removed from the cage of the females 5 days after mating. In the second group ($n = 19$), males cohabitated with females from the first day of mating until postpartum day 24. Lactating females and their partners (if it was present) were tested for parental behavior in their homecage at postpartum days 7 and 14. On each of these days, parental behavior was assessed in two different ways. First, we removed the pups for 5 min and later returned them to the cage to record the behavior of the parents for 15 min. Second, we also recorded the behavior of parents in three 1-h daily behavioral observations without disturbing the cage.

Parental behavior during a 15-min test

On the morning (10:00 a.m.–12:00 a.m.) of the day of the test, the complete litter was taken out of the cage during 5 min and later placed again in the corner of the cage opposite to where parents had their nest. 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). The behavior displayed by the mother and the father was simultaneously recorded during 15 min. We recorded latency to approach to the pups, duration of sniffing, frequency and duration of licking and grooming, nest building, and crouching postures, and frequency of retrieval or pup transport. The male was considered paternal if he retrieved the pups or licked them (≥ 30 s) and crouched over the pups (≥ 30 s). This minimal criterion for parental behavior was chosen considering the presence of the lactating female that might interfere with the performance of the male. Data did not pass normality and homogeneity of variance tests and were analyzed by non-parametric statistics. Data are expressed as median (semi-interquartile range, SIQR). All behavioral observations were recorded using the free software StopWatch http://www.cbn-atl.org/research/stopwatch.shtml.

Parental behavior during three 1-h observational sessions

In a different set of animals, we performed behavioral observations during three 60-min sessions at postpartum days 7 and 14 (two different developmental stages of pups). The observations were performed during the light phase (10:00 a.m., 14:00 p.m., 18:00 p.m). In each session of 60 min, the behavior was scored every 3 min. Therefore, 21 observations were registered during three daily sessions for each animal resulting in a total of 63 instantaneous observations. Data are shown as means ± SE of the frequency from a total of 63 observations for each animal.

In each session, we registered the behavior displayed by both parents, or just the mother when the male was not present: retrieving or transport of the pups to the nest, licking of the pups (anogenital region or body surface), nest building (gathering of nest material that consisted of paper towels cut in small pieces), high crouching posture (the mother adopts an arched-back position), flat crouching posture (the mother lies over the pups), supine posture (the female nurse the pups lying on her back), mother/father off (mother/father away [> 10 cm] from the pups), attack (pups are bitten). In addition, we recorded when fathers remained in the nest in contact with the pups but did not display crouching posture or other paternal behaviors.

Experiment II. Male behavioral response toward alien pups at postpartum days 3 and 7 in the presence of their paired lactating female

We used 6 pairs of males and females that cohabitated from mating to postpartum day 3. The dates of delivery were recorded, and all litters were culled to 5 pups on postpartum day 1. On the morning (10:00 a.m.–12:00 p.m.) of postpartum day 3, the complete litter was taken out of the cage. Five minutes later, two alien pups (same age as those removed previously) from donor mothers (paired with a different male) were placed in the corner of the cage opposite to where parents had their nest. The behavior of the father ($n = 6$) and the mother ($n = 6$) was simultaneously recorded during 15 min using the variables described before.

In a second instance, we used 18 pairs of males and females that cohabitated from mating to postpartum day 7. On the morning (10: 00 a.m.–12: 00 p.m.) of postpartum day 7, nine pairs were tested in their homecages for paternal behavior toward alien pups, as described in the previous paragraph, but in this case, we considered also interesting to compare their paternal behavior with nine pairs tested in the same conditions with their own pups. The litter was taken out of the cage and two of their own pups or alien newborns (same age) taken from a donor lactating female were placed in the cage. The behavior of the parents was simultaneously recorded during 15 min as described above in Exp I.

Experiment III. Behavioral response toward pups by juvenile males from single or overlapping litters

Twelve females were mated with males. Five males remained with females during all the gestational period, and beyond parturition and postpartum estrous (Overlapping litters group, OvL), while the other males ($n = 7$) were removed 7 days after mating (Single litter group, SL). Date of the first delivery was recorded and all litters were culled to five pups per mother.

We used 17 juvenile males that were reared with both parents (father was removed 1–2 days before delivery of second litter) and cohabitated continuously only with their mothers until shortly after (12–24 h) the second litter was born (OvL), and 20 juvenile males from SL. Animals were weaned at 25–30 days, individually housed, and habituated to the cage (27 cm × 21 cm × 14 cm, floor area of 370 cm^2) during 45–

60 min before testing. After that, two pups (1–3 days of age) were added to the cage and the subject's behavioral response toward the pups recorded during 15 min. Juvenile males from OvL were tested with their sibling pups, while those from SL (no second delivery) with alien pups from donor mothers.

The parental test consisted in placing two newborns and nest material scattered in the side opposite to where the subject was located before opening the cage. We recorded the latency to approach to the pups; duration and frequency of sniffing, licking, grooming, nest building, and crouching postures; and frequency of retrieval or pup transport. In case that the subject attacked the pups, the test was immediately stopped and pups sacrificed. Animals were then included in one of four categories: full parental behavior (FPB) if they displayed all components of parental behavior: pup retrieval, licking (≥ 60 s), and crouching over at least one pup (≥ 30 s); partial parental behavior (PPB) if they showed two of the three main components of parental behavior; non-parental (NP) if they showed only one or none of the main components of parental behavior; infanticidal (I) if they attacked the pups. All behavioral observations were recorded using the free software StopWatch.

Statistical analysis

Behavioral data was analyzed using the statistical package StatView (SAS Institute Inc., Cary, NC). All experimental data was tested for normality (Kolmorgorov-Smirnov test) and homogeneity of variance (Bartlett test). In case data did not pass the requirements of normal distribution and homogeneity of variance, they were analyzed by non-parametric Kruskal-Wallis test followed by Mann-Whitney *U* for independent data, or Friedman one-way analysis of variance followed by Wilcoxon's matched-pair test for dependent data. Parametric tests (*T* test) were applied if data passed those criteria. Chi-square test was applied to analyze the frequency of behaviors between groups. Statistical significance level was $p < .05$ (two-tailed). Data are expressed as mean ± SE if analyzed by parametric tests, or median (SIQR) if analyzed by non-parametric tests.

Results

Experiment I: parental behavior during the postpartum period

Parental behavior during a 15-min test

None of the males attacked their own pups and 66.6 and 16.7% of them reached the criterion for paternal behavior at postpartum days 7 and 14, respectively. Higher percentage of fathers retrieved ($\chi^2 = 4.8$, $p < .05$) and reached the parental criteria for crouching posture ($\chi^2 = 3.8$, $p < .05$) at postpartum day 7 (33 and 58.3% respectively) than at day 14 (0 and 18.1% respectively). Behavioral data for the 15-min test did not pass the test for normality and were analyzed using non-parametric statistic. At postpartum day 7, the father also adopted crouching posture ($Z = 1.95$, $p = 0.05$) and licked and groomed the pups ($Z = 1.95$, $p = 0.05$) for longer period of time with respect to postpartum day 14 (Table 1). No difference was observed in frequency (2.0(2) vs 3.5(2)) or duration (3.5(3.6) vs 3.25(3.5) s) of sniffing behavior, respectively, for postpartum days 7 and 14. Crouching was always active during this 15-min behavioral test; therefore, no distinction was made between flat and high crouching.

There was no major change in maternal behavior when lactating females were tested in the presence or absence of the father in her homecage, either at postpartum day 7 or 14 (Table 2). The only exception was lower nest building found in lactating females without the father at postpartum day 7 ($U = 20.5$, $p < .05$, Table 2).

Parental behavior during three 1-h observational sessions

Behavioral data for these repeated 1-h observations passed the requirements for normality and homogeneity of variance and were analyzed using parametric tests. No significant differences in paternal behavior were found among the three daily observations (data no shown), or at postpartum day 7 or 14. Therefore, the total frequency of the behavior observed for each animal and day was averaged and used to analyze the data. Sires displayed low frequency of licking (0.3 ± 0.2; 1.0 ± 0.4 in a total of 63 observations), high crouching posture (3.4 ± 1.8; 1.7 ± 1.4), flat crouching posture (10.7 ± 3.6; 11.0 ± 4.0), and nest building (1.0 ± 0.8; 0.4 ± 0.3) at postpartum day 7 or 14, respectively. Most high crouching posture in sires occurred when females were away from the nest (92.4% of the time females were off the nest at postpartum day 7, and 83.3% at postpartum day 14), while flat crouching posture was rather similar when the females were in or off the nest (34.7% at postpartum day 7 and 59.7% at postpartum day 14). The frequency that fathers stayed away from the pups (16.1 ± 3.8; 14.3 ± 2.4 in a total of 63 observations), or in the nest in contact with them, but not performing any paternal behavioral components (31.9 ± 3.2; 35.3 ± 4.4), was not different at postpartum day 7 or 14 respectively.

As in the 15-min test, the presence of the father did not affect the behavior of the mothers at postpartum day 7 or 14 (Fig. 1). Licking and grooming and nest building did not differ between the groups and were almost absent in this test (data not shown). However, mothers stayed off of the nest more often than did their partners at postpartum days 7 ($t = 2.0$, DF = 12, $p = 0.05$) and 14 ($t = 2.9$, DF = 12, $p < 0.05$). The frequency that pups stayed alone in the nest was lower ($t = 4.6$, DF = 12, $p < .05$) when both parents remained in the

Table 1 Exp I: Paternal behavior at postpartum days 7 and 14 in presence of the mother (15 min test)

		Postpartum day ($n = 12$)	Postpartum day (n = 12)
Percentage	Retrieval	33.3%*	0.0%
Frequency	Licking and grooming bouts	4.0 (2.0)	1.5 (2.0)
	Crouching postures	2.5 (2.5)	.5 (1.3)
	Nest building	5.0 (2.0)	4.0 (2.0)
Time (s)	Licking and grooming	13.4 (17.3)#	2.9 (4.1)
	Crouching postures	27.9 (27.2)#	.9 (12.3)
	Nest building	353.7 (174.9)	259.1 (226.3)

Data are expressed as median (SIQR), Wilcoxon´s matched-pair test

*p<.05, #p=.05, postpartum day 7 vs. 14

homecage (5.8 ± 2.3) than when only the mother stayed (30.1 ± 5.8) at postpartum day 14, but not at postpartum day 7 (11.2 ± 2.0, 21.0 ± 6.5, $p = 0.08$; respectively).

Experiment II. Male behavioral response toward alien pups at postpartum days 3 and 7 in the presence of their paired lactating female

Replacing the litter with two alien pups at postpartum day 3 or 7 did not inhibit paternal behavior (Table 3). None of the males exposed to alien or their own pups attacked them. Paternal behavior was observed in 60.0% (4/6) and 66.7% (6/9) of males exposed to alien pups at postpartum days 3 and 7, respectively. There was no major behavioral difference when the behavior of the fathers toward alien or own pups was compared at postpartum day 7 (no data for day 3). Fathers tested with alien pups only showed shorter latency ($U = 64.5$, $p < .05$) to crouch over them [135.6 (49.6)] than fathers tested with their own pups [561.8 (236.6)]. No difference was found in the behavior of mothers tested with alien or own pups in the presence of the males (data not shown).

Experiment III. Behavioral response toward pups by juvenile males from single or overlapping litters

The percentage of males from OvL or SL that displayed FPB (5.8, 5.0%), PPB (29.4, 25.0%), NPB (64.7, 65.0%), or IB (0.0, 5.0%), respectively, was not significantly different ($\chi^2 = .93$, $p = .8$, Fig. 2). The percentage of subjects that retrieved (11.7, 10.5%, $\chi^2 = .01$, $p = 0.9$), licked (17.6, 40.0%, $\chi^2 = 2.1$, $p = 0.1$), or adopted crouching posture (29.4, 25.0%, $\chi^2 = .09$, $p = 0.7$) did not show significant differences between juvenile males from OvL and SL respectively. Parental (FPB and PPB) males from OvL ($n = 6$) licked the pups less often [$U = 31.5$, $p < .05$; OvL 8.0 (1.0) vs. SL 13.5 (1.5)] and for less time [$U = 30.0$, $p = .054$; OvL 46.2 (28.9) vs. SL 123.6 (35.9)] than parental males from SL ($n = 6$).

Discussion

The present study made a detailed analysis of the paternal behavior of laboratory mice (CB57Bl/6) at different contexts. First, we showed that sire mice displayed paternal behavior

Table 2 Exp I: Maternal behavior at postpartum days 7 and 14 in presence or not of the father (15 min test)

		Postpartum day 7		Postpartum day 14	
		♀ + ♂ ($n = 12$)	♀ ($n = 10$)	♀ + ♂ ($n = 12$)	♀ ($n = 10$)
Percentage	Retrieval	100.0%	100.0%	58.3%	46.2%
Frequency	Licking and grooming bouts	4.0 (3.0)	4.0 (3.0)	4.0 (2.5)	4.0 (1.0)
	Crouching postures	6.5 (2.5)	6.5 (3.5)	3.5 (3.2)	3.0 (1.0)
	Nest building	7.0 (1.3)*	4.5 (1.0)	7.0 (1.7)	5.5 (1.0)
Time (s)	Licking and grooming	20.0 (39.1)	31.8 (23.9)	14.7 (18.7)	13.0 (11.3)
	Crouching postures	116.9 (49.8)	51.0 (78.5)	17.2 (50.0)	16.9 (23.0)
	Nest building	511.4 (72.5)	393.7 (233.8)	356.3 (117.8)	503.7 (86.7)

Data are expressed as median (SIQR), Mann-Whitney U test

*p<.01 vs. female without male

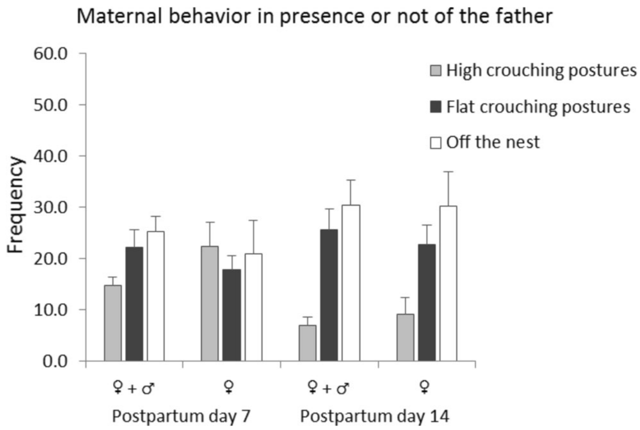

Fig. 1 Maternal behavior at postpartum day 7 and 14 in presence or not of the father (three 60-min observation sessions). Data are expressed as mean ± SE of frequencies from a total of 63 observations. Behaviors were scored every 3 min, during three 60-min periods = 63 observations/subject/day. *N* of each group = 7

toward either own or alien pups without disturbing or affecting the behavior of the mother. Although this species is not considered biparental, males are able to show parental behavior, and their presence can benefit the offspring reducing the time pups stay alone in the nest. Second, in contrast to other species (e.g., rats and prairie voles), and to juvenile female mice, juvenile males (25–30 days of age) participated minimally in the caring activities of newborns and their behavioral response did not improve after a short exposure to a parturient mother and newborn siblings. This last finding suggested that in contrast to female CB57BL/6 mice (Olazábal and Alsina-Llanes 2016), juvenile males could be adapted to disperse from their natal nest without major contributions to the rearing of their younger siblings.

The lower frequency of crouching displayed by fathers compared with mothers, agreed with a previous study in the same strain of mice (Gandelman et al. 1970). However, other studies in albino mice (Ostermeyer and Elwood 1983; Priestnall and Young 1978; Wright and Brown 2000) found no difference in parental behavior in mothers and fathers. We must note that all those early studies grouped all behavioral components in a parental score or averaged them throughout the whole postpartum period. In the case of Ostermeyer and Elwood (1983), our results partially agreed with their findings because they only showed the results of sniffing and licking behavior, two behavioral components that, in our study, did not differ between males and females. Given that previous studies did not provide detailed information on the different behavioral components or their changes in the different postpartum days, many differences could have remained undetected.

The present study also suggests that when males are challenged to retrieve the pups previously removed from the nest, as in our 15-min test on postpartum day 7, most of them behave parentally even though the mother is also present. However, when animals are left undisturbed, the males show minimal parental behavioral components, as also shown by Wright and Brown (2000), except for crouching or thermoregulation at the nest. In the present study, we did not find any negative effect of the presence of the fathers on the behavior of the mothers. Previous studies in albino mice (Priestnall and Young 1978; Wright and Brown 2000) also showed that the presence of the males had no negative effect on the development or survival of the offspring. Although several studies in the laboratory using laboratory, house mice, and *Peromyscus californicus* have failed to show any effect of the presence of the father on the survival of the offspring (Brown 1993; Cantoni and Brown 1997; Gerlach 1990; Wright and Brown 2000; Priestnall and Young 1978), we cannot exclude the possibility that males play an important role in natural and more challenging conditions (Brown et al. 1999; Gubernick et al. 1993). For example, the contribution of males could be revealed more clearly as part of a strategy to defend the nest. Some authors have previously proposed that, in the wild, fathers could mainly engage in defending the female/harem and pups against danger (McCarthy 2010). In our study, the presence of the male resulted in a reduction of the time pups were left alone, what can be considered an advantage in terms of thermoregulation, contact, or/and defense of the newborns. Previous studies in *Peromyscus californicus* showed that the

Table 3 Exp II: Paternal behavior toward alien or own pups during postpartum day 3 or 7 (15 min test)

		Postpartum day 3 (alien pups) $n = 6$	Postpartum day 7 (alien pups) $n = 9$	Postpartum day 7 (own pups) $n = 9$
Percentage	Retrieval	33.3%	22.2%	33.3%
Frequency	Licking and grooming bouts	7.0 (1.5)	4.0 (2.1)	3.0 (2.0)
	Crouching postures	4.5 (1.5)	4.0 (1.2)	2.0 (1.7)
	Nest building	7.5 (3.0)	5.0 (1.3)	5.0 (2.3)
Time (s)	Licking and grooming bouts	67.2 (47.4)	29.3 (16.0)	4.6 (13.0)
	Crouching postures	66.1 (54.8)	46.0 (28.7)	23.5 (19.6)
	Nest building	202.1 (77.8)	255.4 (192.8)	399.8 (196.7)

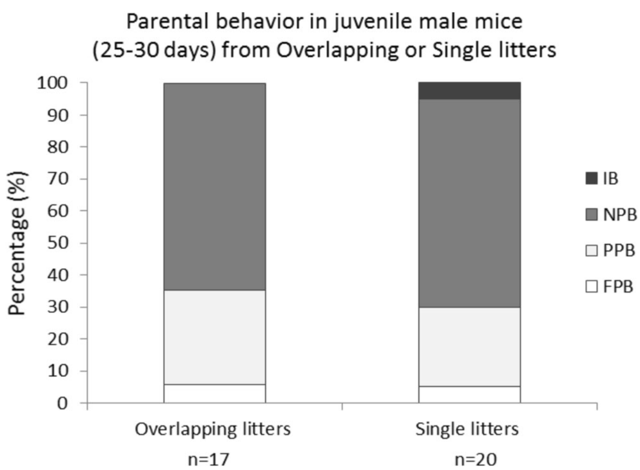

Fig. 2 Percentage of juvenile males 25–30 days old that displayed infanticidal behavior (IB), non-parental behavior (NPB), partial parental behavior (PPB), or full parental behavior (FPB) when tested (15 min test) in a novel cage. Groups: OvL ($n = 17$), juveniles from overlapping litters exposed to their pregnant/parturient mother and newborn siblings during ~ 12–24 h; SL ($n = 20$), juveniles from single litters with no previous exposure to pups

physical contact between siblings was also greater if the father was present (Vieira and Brown 2003).

The absence of male aggressiveness toward alien pups suggested that either males were already subordinated to the mothers at postpartum days 3 and 7 or they underwent long-term changes after the induction of paternal behavior that resulted in a non-selective parental behavioral response. Gandelman et al. (1970) also found that male mice (C57BL/6 and Rockland-Albino Swiss) that had cohabited (but no copulated) with a pregnant/lactating female and her litter were capable to display parental behavior when tested with those alien pups in isolation. However, they did not test the males with pups from a different female to those they cohabitated with, as in the current study. Also, in agreement with the current study, Brooks and Schwarzkopf (1983) found that males (C57BL/6 and DBA) that copulated and cohabitated only 1 week with a pregnant female and were placed (14–21 days later) in the cage of an unknown lactating female (same or different strain) and their litters on postpartum day 1 did not attack alien pups. Besides, those males did not attack when tested isolated in a new cage. Besides, other authors found that adult virgin males introduced to lactating females at postpartum day 7 rarely killed the pups (McCarthy and vom Saal 1986). These authors, who tested the CF-1 albino strain and the wild house mouse, proposed that females inhibited infanticidal behavior in males by attacking and subordinating them (see also Elwood 1986). For example, at postpartum day 2, males killed the pups (2 or 7 days of age) if the lactating female did not show maternal aggression toward them (Vom Saal and Howard 1982; McCarthy and vom Saal 1986). We have also observed males attacking pups when we tried to complete a litter adding alien pups to the cage on postpartum day 1. However, in the present work, males were capable to tolerate and take care of alien pups as early as postpartum day 3. The current findings cannot be explained by the hypothesis that males recognize some genetic label that avoids infanticidal behavior because males had copulated with the females and had already been exposed to their own pups. An alternative explanation might be related to the presence of the postpartum estrous on the first 2 days after parturition that might also contribute, in some way, to pup-killing (Mennella and Moltz 1988a, 1988b). Therefore, our results agreed with the hypothesis that once paternal behavior was well established and/or males subordinated to females, something that might consolidate during the first two postpartum days, the strange odor of alien pups did not necessarily trigger infanticidal behavior. In fact, our results suggest that males initially inhibit infanticidal behavior likely by chemical and behavioral factors associated with the female and the territory they share, but later the continuous exposure to the multiple sensory stimulation emitted by pups stimulate attraction to the pups and high motivation to take care of them.

Finally, our results indicated that unlike juvenile females, juvenile males did not improve their behavioral response toward pups after the exposure, in their homecage, to their pregnant/parturient mother, birth fluids, and newborn sibling for 12–24 h. This agrees with the fact that while most male mice disperse from the nest, most females remain for longer period of time (Gerlach 1990). Thus, although variability in nature exists and either some females might disperse early and males stay near the nest for longer period of time, males could be better adapted to disperse without major or any contribution to the rearing of their newborn siblings. As males grow, most of them will develop aggressiveness toward pups (Olazábal and Alsina-Llanes 2016) that will be overcome by copulation and/or cohabitation with a pregnant female. However, it is still interesting to explore why a few naïve virgin, in particular young, males (in the current study 30% of juveniles in both groups) show some behavioral components of parental behavior (Olazábal and Alsina-Llanes 2016). Previous studies (Leblond 1938) found high levels (50%) of paternal behavior in males 22–28 days of age. However, in that classic study, it is unclear if males were naïve because the authors also reported that juveniles required about 4 days of exposure to pups (sensitization) to display parental behavior. Gandelman (1973) also reported that around half of Rockland-Swiss juvenile mice (22 days of age) displayed some components of parental behavior (retrieval and licking or crouching posture). Similarly, Svare and Mann (1981) reported that 15 and 20% of 25-day-old male mice (C57BL/6 and DBA strain respectively) retrieved a pup. Finally, McCarthy and vom Saal (1986) also found that 20% of adolescent males hovered over the pups in the nest during a test of 30 min. The variability in the incidence of parental behavior observed in juveniles might then be consequence of strain differences (McCarthy and vom

Saal 1986; Svare and Mann 1981) or different criteria to consider an animal as parental, among other minor methodological differences (Alsina-Llanes et al. 2015; Priestnall and Young 1978; Svare and Broida 1982). For example, licking is usually a behavior used to consider an animal as parental. However, in our results, juvenile parental males exposed to alien pups licked them for longer period of time and more often than juveniles tested with their newborn siblings, supporting the idea that, in this context, licking plays an important role in pup recognition rather than in pup stimulation. We have also previously shown that lactating and juvenile females showed higher licking behavior toward alien compared to their own pups (Alsina-Llanes et al. 2015).

We want to note that we cannot exclude the possibility that longer exposure to pups (3–4 days compared to 24 h) could have facilitated the onset of parental behavior in juvenile CB57Bl/6 males. For example, in the classic study of Gubernick and Laskin (1994) in *Peromyscus californicus* (considered a biparental and socially monogamous species), ~ 70% of 35–40-day-old mice (males and females pooled together) that had been housed with their parents, and cohabitated with younger siblings during 3 days, were induced to show parental behavior. However, when they cohabitated with younger siblings by shorter period of time (4 h) only 40% displayed paternal behavior (licking or crouching posture in 10 min). Therefore, the duration of the period of cohabitation with pups in an overlapping litter context impacted in the incidence of parental behavior in that species. However, as we already mentioned, *Peromyscus californicus* shows a different reproductive strategy and, unlike naïve adult male CB57BL/6 mice, *Peromyscus californicus* males can be rapidly induced to display paternal behavior (Horrell et al. 2017).

In summary, despite the enormous variability reported in the literature, generally attributed to strain differences, handling, or housing conditions, a careful analysis of behavioral data showed some clear pattern of behavioral performance in laboratory male mice. Although most naïve males are not parental, they show a rather flexible behavioral response toward pups suggesting that under certain conditions, paternal behavior in virgin males could gain biological significance. For example, the so called promiscuous or polygamous species are generally associated with lower levels of paternal behavior than those called socially monogamous species. However, sires from promiscuous species generally take care of pups and are capable to contribute to the rearing of the offspring resulting in a reduction in the time pups remain alone. Besides, in other non-monogamous rodents (i.e., *Rhabdomys pumilio*, Schradin and Pillay, 2003) is not uncommon to find high levels of paternal behavior, suggesting that the identification of species as biparental could be in some cases underestimated and biased by their reproductive strategy (monogamous or non-monogamous) rather by the actual behavior observed in the sires. Although the benefit of paternal care in laboratory mice is not clear, we cannot rule out the possibility that under certain ecological or stressful conditions, the presence of the father in the nest will be favored resulting in long-lasting effects on the offspring. Thus, the presence of male mice in the nest and its contribution to pups will likely be context specific. Future field and laboratory studies in non-traditional rodent species with different social and reproductive strategies, or at different ecological conditions, might contribute to further advance our understanding of the biological function of paternal behavior.

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Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare that they have no conflict of interest.

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