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Mouse females devoid of exposure to males during fetal development exhibit increased maternal behavior

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Summary Many sex differences can be found in the expression of aggression and parental nurturing behaviors. It is important to determine if these are modulated by prenatal conditions. Here, using assisted reproduction technologies, we generated females that were (mixed-sex) or were not (same-sex) exposed to males during fetal development, raised them by cross fostering among fosters' own female only pups to control for effects of postnatal environment, and compared their reproductive abilities and behavior. There were no differences between females from the two prenatal conditions in estrus cycle length and length of time spent at individual estrus cycle stages. Both types of females had similar ovulation efficiency and bred equally well yielding comparable litter size and progeny sex ratio. Females from the two prenatal conditions were also indistinguishable in social behavior and exhibited normal social responses towards unfamiliar females in the three-chamber social approach and social proximity tests. When urine was collected from both types of females and used as a point source in a scent-marking paradigm, exposed males showed a similar distribution and extent of urinary scent marking in response to urine from each type of female but tended to engage in higher durations of sniffing the urine from same-sex females. When females were tested in a resident-intruder paradigm 3 days after giving birth, same-sex females exhibited enhancement of pup grooming and an overall decrease of non-pup activity prior to male intruder introduction, and after introduction were more defensive as evidenced by higher rates of burying, open-mouth threat/lunges, and attacks towards the male, and decreased latencies to display these defensive behaviors. Our results suggest that females devoid of male exposure during fetal development have reproductive abilities similar to those of

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females from mixed-sex pregnancies, and have normal social interactions with other females. However, they exhibit hyper-maternal behavior both in terms of the care and defense of pups in front of a male intruder, and potentially produce a pheromonal milieu that renders them more attractive to males during olfactory investigations.
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1. Introduction

In mammals, the most obvious sex differences are in external and internal genitalia, development of which depends on gonadal secretions during fetal development (Goodfellow and Lovell-Badge, 1993). Gonadal secretions are therefore thought to be primary causal agents of sexual dimorphism, also important for reproduction-linked abilities and behaviors of both sexes (Goy and McEwen, 1980; Morris et al., 2004). The effects of gonadal secretions on reproduction-related phenotype has primarily been studied using 'intrauterine positioning' analyses, which compared females that as fetuses occupied uterine positions adjacent to one or two males (1M and 2M females) or not adjacent to males (0M females). The concept was that close proximity of males would lead to androgenization of females during prenatal development, which in turn would modulate androgen-dependent anatomical, physiological and behavioral events in adulthood (Ryan and Vandenbergh, 2002; Morley-Fletcher et al., 2003; Banszegi et al., 2009; Mori et al., 2010). Indeed, in a classic experiment in 1959 Phoenix et al. (Phoenix et al., 1959) demonstrated that exposing female guinea pigs to testosterone in the fetal period permanently masculinized and defeminized their sexual behavior. Since then many other experiments supported that testicular secretions released during fetal and neonatal life have permanent organizational effects (reviewed in Arnold and Gorski, 1984; Ryan and Vandenbergh, 2002).

Thus far, studies on the effects of gonadal secretions acting during fetal development have utilized three approaches: (1) exposure of developing fetuses to hormones administered during pregnancy; (2) analysis of animals from different intrauterine positions in respect to their opposite sexes; and (3) analysis of animals from spontaneously arising single-sex litters. Although these methods provided a wealth of knowledge on the subject, they are not free from limitations. Experimental manipulation of hormone levels is a rather crude and not particularly physiological approach. Intrauterine positioning reflects naturally occurring subtle hormonal variations but the effects of siblings residing farther in the uterus cannot be excluded. Finally, presence of single sex progeny in a naturally delivered litter does not preclude the possibility that opposite sex fetuses were present during gestation but lost prior to delivery.

Here, we used a novel approach and employing assisted reproduction technologies and molecular methods we manipulated prenatal gender composition to create female-only and mixed-sex pregnancies. We applied in vitro fertilization (IVF) to generate embryos, which were subsequently subjected to single blastomere biopsy and biopsied cell sexing. Blastomere biopsy is commonly used in human assisted reproduction technology (ART) clinics as part of preimplantation genetic diagnosis (PGD), a genetic screening of embryos to identify those carrying genetic defects. In humans, sexing of embryos is primarily performed to avoid transmission of sex chromosome linked defects (Handyside et al., 1990). Here we applied the same principle to address a question on male intrauterine influence on reproductive abilities and behavior of females. The benefits of this method is that it is physiological (i.e. there are no external delivery of hormones) and provides a clean experimental model enabling analyses of females developed in utero without *any* male influence.

We demonstrated that females developed prenatally in a complete absence of males were similar to those from mixed-sex pregnancies in respect to their reproductive phenotype but differed in certain behavioral traits, and particularly maternal aggression.

2. Methods

2.1. Animals

B6D2F1 mice (C57BL/6 × DBA/2) and CD-1 were obtained at 6 weeks of age from National Cancer Institute (Raleigh, NC) and Charles River Laboratories (Wilmington, MA), respectively. B6D2F1 mice were used as sperm and oocytes donors for in vitro fertilization (IVF), and CD-1 mice were used as surrogate mothers and vasectomized males for embryo transfer. Mice were fed ad libitum with a standard diet and maintained in a temperature and light-controlled room (22 °C, 14 h light/10 h dark), in accordance with the guidelines of the Laboratory Animal Services at the University of Hawaii and guidelines presented in National Research Council's (NCR) ''Guide for Care and Use of Laboratory Animals'' published by the Institute for Laboratory Animal Research (ILAR) of the National Academy of Science, Bethesda, MD, 2011. The protocol for animal handling and treatment procedures was reviewed and approved by the Animal Care and Use Committee at the University of Hawaii. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

2.2. Assisted reproduction techniques

2.2.1. Reagents

Mineral oil was purchased from Squibb and Sons (Princeton, NJ); pregnant mares' serum gonadotrophin (eCG), human chorionic gonadotrophin (hCG) from Calbiochem (San Diego, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

2.2.2. Media

Medium T6 (Quinn et al., 1982) or HTF (Quinn et al., 1985) was used for IVF and HEPES-buffered CZB medium (HEPES-CZB; Chatot et al., 1989; Kimura and Yanagimachi, 1995) was used for gamete handling and embryo micromanipulation. Medium CZB (Chatot et al., 1989) was used for embryo

culture. Media CZB, T6 and HTF were maintained in an atmosphere of 5% CO_2 in air, and HEPES-CZB was maintained in air.

2.2.3. In vitro fertilization

Sperm capacitation and IVF were performed as reported by us before (Ajduk et al., 2006). Briefly, the oocytes were collected from females induced to superovulate with injections of 5 IU eCG and 5 IU hCG given 48 h apart. Epididymal sperm were collected by release from cauda epididymis directly into T6 medium, and were capacitated for 1.5 h at 37 °C in a humidified atmosphere of 5% CO_2. The gametes were co-incubated for 4 h. After gamete co-incubation, the oocytes were washed with HEPES-CZB, followed by at least one wash with CZB medium. Only morphologically normal oocytes were selected for culture.

2.2.4. Embryo culture, biopsy, and transfer

Fertilized oocytes (oocytes with two well developed pronuclei and extruded 2nd polar body) were cultured in 50 μl drops of CZB medium pre-equilibrated overnight with humidified 5% CO_2 in air. After ~48 h of culture, four-cell embryos were transferred into Ca^{2+}- and Mg^{2+}-free CZB for 10–20 min to disrupt cell adhesion, and were then transferred to microdrops of Ca^{2+}- and Mg^{2+}-free HEPES-CZB on the micromanipulation dish. Blastomere biopsy was performed using Eppendorf Micromanipulators (Micromanipulator TransferMan, Eppendorf, Hamburg, Germany) with a Piezo-electric actuator (PMM Controller, model PMAS-CT150, PrimeTech, Tsukuba, Japan). The zona pellucida was penetrated with a micropipette (20 μm ID) and one blastomere was aspirated. Isolated blastomeres were washed in 0.1% PBS containing 0.1% polyvinylpyrrolidone (PVP) and transferred individually to 0.2 ml PCR tubes (VWR, West Chester, PA) in a minimal amount of medium, (~0.5–1 μl). Samples were either analyzed immediately or stored at −20 °C. Control (non-biopsied) embryos from the same IVF cohorts were pre-incubated in Ca^{2+}- and Mg^{2+}-free CZB medium but were not transferred into Ca^{2+}- and Mg^{2+}-free HEPES-CZB medium, and were not micromanipulated. Biopsied embryos were cultured individually and non-biopsied embryos were cultured in a group (up to 10 embryos) for subsequent 24 h until they developed to morula/early blastocyst stage. The biopsied embryos were then transferred into the oviducts (4–8 per oviduct; in case of mixed sex embryos the same number of males and female embryos were transferred per female) of CD-1 females mated during the previous night with vasectomized CD-1 males. The females were allowed to give birth naturally. Pups were sexed by observing the anogenital distance within 2 days after delivery and by PCR at weaning.

2.2.5. Single blastomere sexing

To define sex of the biopsied embryos multiplex, nested PCR was performed on single blastomeres using primers amplifying the polymorphic X chromosome microsatellite locus (*DXNds-3*) (Love et al., 1990) and Y chromosome encoded gene *Sry* (Gubbay et al., 1990). The primer sequences were as follows: SRY1: GTGAGAGGCACAAGTTGGC; SRY2: TCTTAAACTCTGAAGAAGAGA; SRY3: CTCTGTGTAGGATCTTCAATC; SRY4: GTCTTGCCTGTATGTGATGG; NDS1: ATGCTTGGCCAGTGTACATAG; NDS2: TCCGGAAAGCAGCCATTGGAGA; NDS3: GAGTGCCTCATCTATACTTACAG; NDS4: TCTAGTTCATTGTTGATTAGTTGC. Four microliters of lysis buffer (Krisher et al., 1995) were added to each tube containing a single blastomere. The tubes were heated at 65 °C for 10 min followed by 94 °C for 10 min, and cooled to 4 °C to achieve proteolysis and inactivation of proteinase K. The reaction was carried out in a volume of 15 μl, with 80 nM final concentration of each outer primer (NDS3 and 4, SRY2 and 4) using Go TaqGreen master mix (Promega, Madison, WI). The PCR conditions were: initial denaturation at 97 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. One microliter of amplified DNA was transferred to a new reaction tube containing inner primers (NDS1 and 2, SRY1 and 3) at final concentration of 0.2 μM and Go TaqGreen master mix, to give a reaction volume of 10 μl. The reaction conditions were as before. Ten microliters of amplified DNA was electrophoresed for 20 min at 100 V through a 3% NuSieve GTG agarose (Lonza, Basel, Switzerland) gel containing 0.4 μg/ml ethidium bromide. Bands (NDS – 111 bp and SRY 147 bp) were visualized with a UV transilluminator (Vilber Lourmat, Marne-la-Vallée, France) and photographed (Kodak, Los Angeles, CA).

2.2.6. Fostering conditions

Both prenatal and postnatal environments have considerable influences of on the development of behavior in laboratory rodents (Francis et al., 2003). To eliminate the postnatal influence of distinct developmental environments and to warrant that any changes in behavior or physiology result from the uterine environment of the pups themselves we used cross fostering. Same-sex pregnancy females and mixed-sex pregnancy females were marked by toe clipping to enable identification and were raised by an unrelated lactating foster mothers among foster's own female only pups. The litter size was maintained between 5 and 8 pups per foster (average 6.8 ± 0.5; mean ± SEM), and experimental pups constituted ~50% (51.3% ± 7.4; mean ± SEM) of pups raised by each foster.

2.2.7. Classification of the estrous cycle

To examine the influence of intrauterine environment on the length of the estrous cycle, vaginal discharges from same-sex, mixed-sex, and naturally bred B6D2F1 females at 12–18 weeks of age were acquired daily between 12:00 h and 13:00 h and smeared on the slides. The smears were classified into one of four phases of estrus: a thin smear of leukocytes and elongated nucleated epithelium indicated proestrus; large cornified epithelial cells were exclusively found in estrus; metestrus was marked by a thick smear composed of equal numbers of nucleated epithelial cells and leukocytes; and a smear consisting almost exclusively of leukocytes depicted diestrus (Hubscher et al., 2005). Cycle length was determined as the length of time between two consecutive occurrences of estrus. After the mice had progressed through three consecutive estrous cycles, the length of the estrous cycle and the number of days spent at each stage of the cycle were evaluated (Jablonka-Shariff et al., 1999).

2.2.8. Fecundity testing

Same-sex and mixed-sex females were paired with B6D2F1 stud males. On detection of a copulatory plug, the females were transferred back to their respective cages. On completion of the 15th day after detection of vaginal plug, the pregnant females were separated and housed individually so

that individual litters could be analyzed. Pups were sexed within 3 days of delivery by observing the anogenital distance.

2.2.9. Oocyte retrieval after ovarian hormonal stimulation

Same-sex and mixed-sex females were induced to superovulate with injections of 5 IU eCG and 5 IU hCG given 48 h apart. Oviducts were removed 14–15 h after the injection of hCG and the cumulus-oocyte complexes were released from the oviducts into 0.1% of bovine testicular hyaluronidase (300 USP units/mg) in HEPES-CZB medium to disperse cumulus cells. The cumulus-free oocytes were washed with HEPES-CZB medium and the number of oocytes was counted.

2.3. Behavioral techniques

2.3.1. Three chamber social approach task

Prior to the aforementioned fecundity testing, mice were tested for social approach behavior in a three chamber apparatus, constructed and performed according to published studies (Moy et al., 2004). Initially, mice were placed into the center of the divided 41 cm × 70 cm × 28 cm (H) apparatus, with one empty, inverted wire cup (Galaxy Pencil/Utility Cup, Spectrum Diversified Designs, Inc., Streetsboro, OH) placed in each of the two outer chambers. Empty glass jars of the same diameter were placed on top of the base of the wire cup to prevent movement of the enclosures, or escape by stimulus mice (Fig. 3a). For the habituation phase, the sliding doors were elevated and the mouse was permitted 10 min to explore the three chambers. The duration of time in each compartment was manually scored by one observer with two hand-held stopwatches. At the end of the 10 min habituation session, mice were placed back into the middle of the apparatus, the sliding doors were lowered, an unfamiliar adult female B6D2F1 mouse was placed into the wire cup, and the doors were again lifted and the mouse was permitted to explore the entire apparatus for 10 min; this constituted the sociability phase. The time spent in each compartment was recorded in real-time with a single observer and two stopwatches. During both the habituation and sociability phases, cameras were mounted in front of both outer compartments and connected to a DVD recorder. The duration of contact (with the stimulus cup), stimulus mouse sniff, and nose-to-nose contact were scored using Noldus Observer software (Noldus Information Technology, Wageningen, The Netherlands) for each of the two outer compartments during the sociability phase as described before (Pearson et al., 2010).

2.3.2. Social proximity test

Female subject mice were placed in a 14 cm × 7 cm × 30 cm (H) clear Plexiglas enclosure with an adult female B6D2F1 stimulus mouse (Fig. 4a) for a 20 min session and permitted to freely interact (Defensor et al., 2011). Two digital video cameras collected video from two lateral aspects to ensure all interactions were visible. The following behaviors were manually scored offline by an observer blind to condition. Nose-to-Nose was defined as simultaneous contact between the nose and/or vibrissae of the two mice. Nose-to-Face was scored when the nose or vibrissae of the subject or stimulus contacted the rostral or frontal aspect of the other mouse's head. Nose-to-Anus was scored when one mouse's nose made contact with the base of the tail or anogenital area of the other. Crawl Over required head, forelimb and shoulders to cross the dorsal midline of the other mouse. Finally, Crawl Under was scored when the posterior tip of the ears pass the ventral midline of the other mouse (Defensor et al., 2011).

2.3.3. Urinary scent marking to female urine

Urine was collected from same-sex and mixed-sex females at estrus either by pushing the bladder or by placing females in metabolic cages and checking every hour for presence of urine. Collected urine was stored at 4 °C for up to 2 h before testing. Prior to scent marking testing, urine was brought to room temperature, and 10 μl was applied to the center of a quartered section of filter paper (Whatman 4, 110 mm) taped 4 cm from the base of an inverted polycarbonate cage (46 cm × 24 cm × 21 cm) which was positioned over a 30 cm × 45 cm section of drawing paper (Fig. 5a). An adult male CD-1 mouse was placed within the compartment for 20 min and permitted to explore the stimulus urine and apply scent marks to the paper. Digital video was collected from an angled aspect lateral to the long axis of the cage. At the end of the 20 min session, the paper was removed and dried overnight before fixing and staining with a 6% solution of ninhydrin (S93313, Fisher); then allowed to dry (Fig. 5b). To quantify the amount of urinary scent marking, a 1 cm × 1 cm printed transparency grid was placed over the paper and the number of squares containing a stained mark was counted manually according to previously published methods (Arakawa et al., 2009). Video was scored for the frequency and duration of contact with the filter paper and sniffing of the urine using Noldus Observer software (Noldus Information Technology, Wageningen, The Netherlands) by an observer blind to the condition of the stimulus.

2.3.4. Maternal behavior

Same-sex and mixed-sex females were paired with B6D2F1 stud males for fecundity testing. Additionally, on day 3 post delivery female subjects were individually tested for maternal behavior and defensiveness according to procedures described before (Palanza et al., 1994). Two digital video cameras were oriented to two aspects of the dam's home cage. The nest substrate was removed and a five min presession was recorded; then an unfamiliar adult male B6D2F1 male was introduced into the cage for an additional 5 min. The pups and the dam remained in the cage, but if the male intruder attacked a pup, the session was immediately terminated and the injured pup euthanized. Video collected during the pre-session was scored for the frequency, latency to engage in, and proportion of time the dam was on the nest, contacting pups, and grooming the pups after the disturbance. Additionally, the frequency, latency, and percent time the dam self-groomed, or engaged in other non-pup related activity (such as eating, drinking, or locomoting) was measured. During the intruder session, the rate per min (since not all sessions lasted 5 min due to intruder infanticide) and latency to defensively bury, threat (open mouth lunge behavior), attack, follow, and anogenitally investigate the intruder male was analyzed by an observer blind to the dam's prenatal condition using Noldus Observer Software.

2.4. Statistical analyses

Data on the estrous cycle length and number of days spent in each stage of the estrus cycle were analyzed using Kruskal—Wallis test. Ovulation efficiency, the number of days spent from mating to giving birth to pups, litter size and sex ratio were analyzed using Mann—Whitney's *U*-test. Two-way analysis of variance (ANOVA) with stimulus side as the within-subjects and the prenatal sex condition as the between-subjects variables for the three-chamber sociability chamber and behavior duration values was used. Nose-to-nose behavior is only possible in the social chamber, so an unpaired *t*-test was used to compare mean duration between the two prenatal sex conditions. Unpaired *t*-tests and Mann—Whitney's *U*-test were applied to compare average frequencies of the categories in the social proximity test. Two-way repeated-measures ANOVA was used to compare the number of squares containing scent marks in the half of the arena further from the stimulus, and adjacent to the stimulus. Unpaired *t*-tests were used to compare the duration of contact and sniff during the scent marking session. Two-way repeated-measures ANOVA were used to compare mean values between maternal care and intruder-related variables in the maternal aggression tests. When significant prenatal sex condition main effects or interactions were noted, Bonferroni post-hoc comparisons were conducted.

3. Results

3.1. Generation of single sex pregnancies

We applied in vitro fertilization (IVF) to generate embryos, which were subsequently subjected to single blastomere biopsy, biopsied cell sexing, and embryo transfer. Assisted fertilization rather than flushing of naturally conceived embryos was preferred because the former is more predictable in respect to number of embryos obtained. Because same-sex versus mixed-sex females were compared, both originating from the same IVF cohorts, the use of assisted reproduction did not affect study design.

Six IVF experiments were performed, which yielded a total of 329 four-cell embryos, 217 of which were subjected to blastomere biopsy (Table 1, Fig. 1a—c). Almost all (96%) of biopsied embryos survived the procedure and all but five of those (98%) developed to morula/early blastocyst stage (Fig. 1d). Biopsied blastomeres were sexed by PCR (Fig. 1e) and the majority (93%, 193/208) yielded readable result. Among successfully sexed blastomeres, 57% (109/193) were derived from female embryos. Sexed embryos (130) were transferred into oviducts of 11 surrogates (Fig. 1f and g), and all females became pregnant. A total of 52 (40%) live pups were born (Table 1). The results of embryo transfer were similar when same-sex and mixed-sex embryos were transferred (46%, 30/66 vs. 34%, 22/64, P = NS, for same-sex and mixed-sex, respectively). As expected, six surrogates subjected to transfer with female-only embryos yielded only female pups while five surrogates subjected to transfer of mixed female and male embryos yielded mixed-sex litters. The sex of offspring was determined visually based on urogenital appearance immediately after birth to enable placing pups in correct fostering conditions, followed by PCR.

Non-biopsied embryos from IVF cohorts shown in Table 1 were not transferred so we cannot compare directly live offspring rate with and without biopsy. However, in an independent project focusing on the effect of blastomere biopsy on embryo developmental competence we obtained similar fetal rates after transfer with biopsied and non-biopsied embryos from mice on the same genetic background as those used in this study (our unpublished work).

3.2. Estrus cycle length, ovulation efficiency, and fecundity

To determine whether same-sex and mixed-sex prenatal condition females were undergoing normal estrous cycling, vaginal smears were examined histologically, and compared with those of naturally bred B6D2F1 females. Control B6D2F1 females reached estrus every 6—7 days, and their smear patterns at each stage displayed an expected morphology. Same-sex and mixed-sex females had similar estrus cycle length (7.21 ± 0.52 vs. 7.35 ± 0.52; mean $\pm$ SEM; $P > 0.05$, Fig. 2a), which was comparable to that of B6D2F1 females (6.74 ± 0.81; mean $\pm$ SEM, $n = 5$). When the length of individual stages of the estrus cycles was compared, there were no differences between same-sex and mixed-sex females (Fig. 2b). Same- and mixed-sex females had similar lengths of individual estrus cycle stages as naturally bred B6D2F1 mice suggesting that assisted conception per se does not affect estrus cycle.

When the ovulation efficiency measured by the number of oocytes ovulated after hormonal stimulation was compared, no differences were noted between mixed- and same-sex females (11.9 ± 2.8 vs. 10.6 ± 3.2; mean $\pm$ SEM; $P > 0.05$, Fig. 2c).

Table 1 Production of fetuses after single blastomere biopsy.

No. of oocytes inseminated (no. experiments)	No. of 2-cell embryos obtained (%)[1]	No. of 4-cell embryos developed (%)[2]	No. of 4-cell embryos biopsied	No. of 4-cell embryos non-biopsied	No. of embryos survived biopsy (%)[3]	No. of embryos developed to M/EB (%)[4]	No. of M/EB transferred (No of females)	No. of live offspring (%)[5]
394 (6)	329 (84)	329 (100)	217 n/a	n/a 112	208 (96) n/a	203 (98) 112 (100)	130 (11) n/d	52 (40) n/d

M/EB = morula/early blastocyst stage; n/a = non-applicable; n/d = not done. Percentage calculated from [1]oocytes inseminated; [2]2-cell embryos; [3]4-cell embryos biopsied; [4]no. of survived biopsied or non-biopsied 4-cell embryos; [5]M/EB transferred.

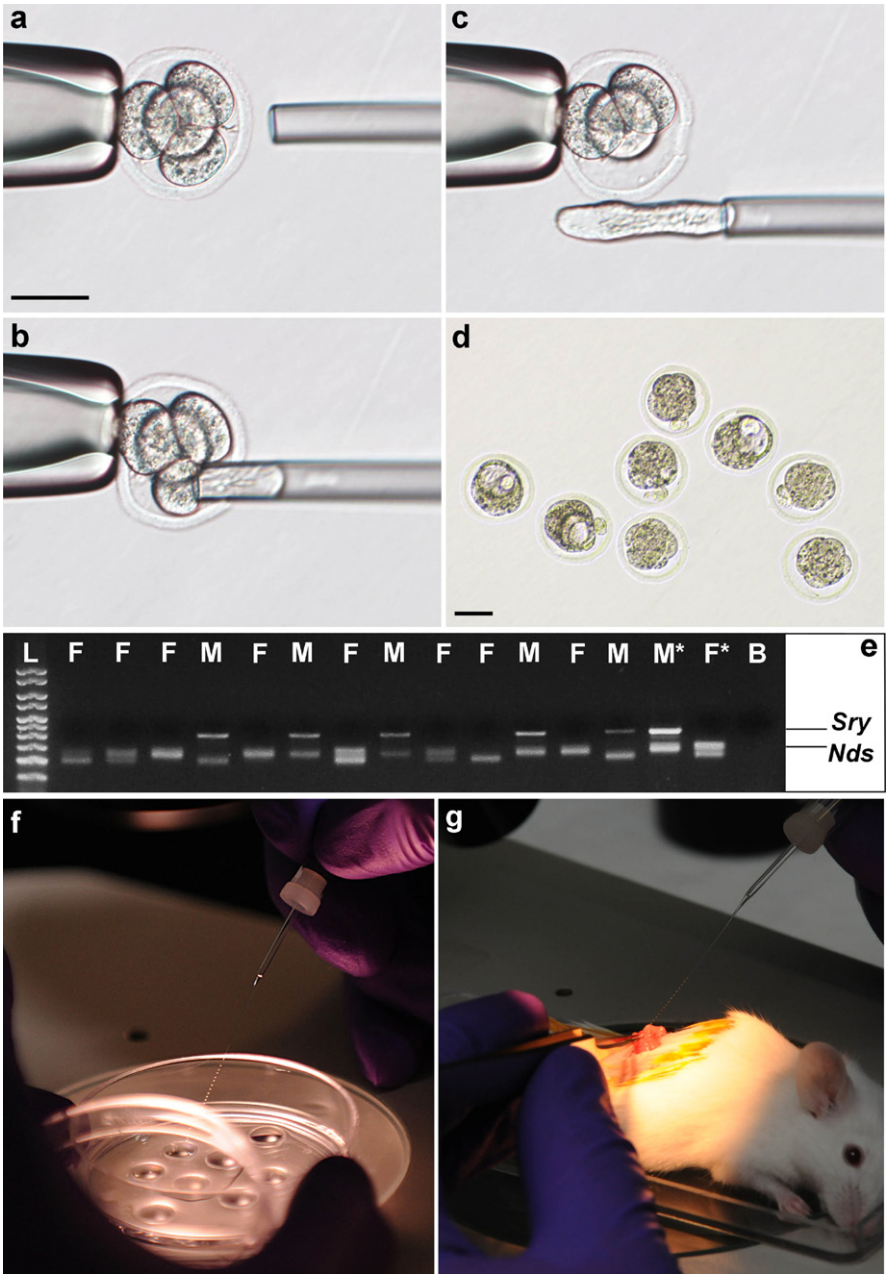


Figure 1 Blastomere biopsy and sexing. Embryos produced by in vitro fertilization (IVF) were biopsied at 4-cell stage (a—c). Biopsied embryos were cultured in vitro until morula/early blastocyst stage (d). Single blastomeres were subjected to multiplex nested PCR using primers specific to X chromosome encoded *Nds* and Y chromosome encoded *Sry* (e) to identify the sex of embryos from which they were derived. Sexed embryos (female only and female + male) were transferred from culture into the oviducts of pseudopregnant females (f and g), and resulted in same-sex and mixed-sex pregnancies, respectively. Presence of double or slightly shifted *Nds* bands is likely due to incomplete extension by the polymerase and/or terminal transferase activity of the enzyme (Love et al., 1990) and does not interfere with identification of male vs. female blastomeres, which is based on presence of *Sry* band. Bar = 50 μm. L = ladder, F = female blastomere, M = male blastomere, F* = female control DNA, M* = male control DNA, B = blank.

There were also no differences in fecundity of mixed- and same-sex females expressed as duration (days) between mating and delivery (23.2 ± 0.9 vs. 21.5 ± 0.3; mean ± SEM; $P > 0.05$, Fig. 2d). Females from mixed and same-sex had similar litter size (7.50 ± 0.72 vs. 6.50 ± 0.74; mean ± SEM; $P > 0.05$, Fig. 2e) and similar progeny sex ratio (% females of total pups; 45.62 vs. 51.04; $P > 0.05$).

3.3. Three-chamber social approach task

There were no differences between females from both prenatal conditions in the habituation phase of the test. Both types of females explored the left and right side of the chamber similarly (Fig. 3b). For the sociability phase, two way ANOVA revealed a significant main effect of Chamber

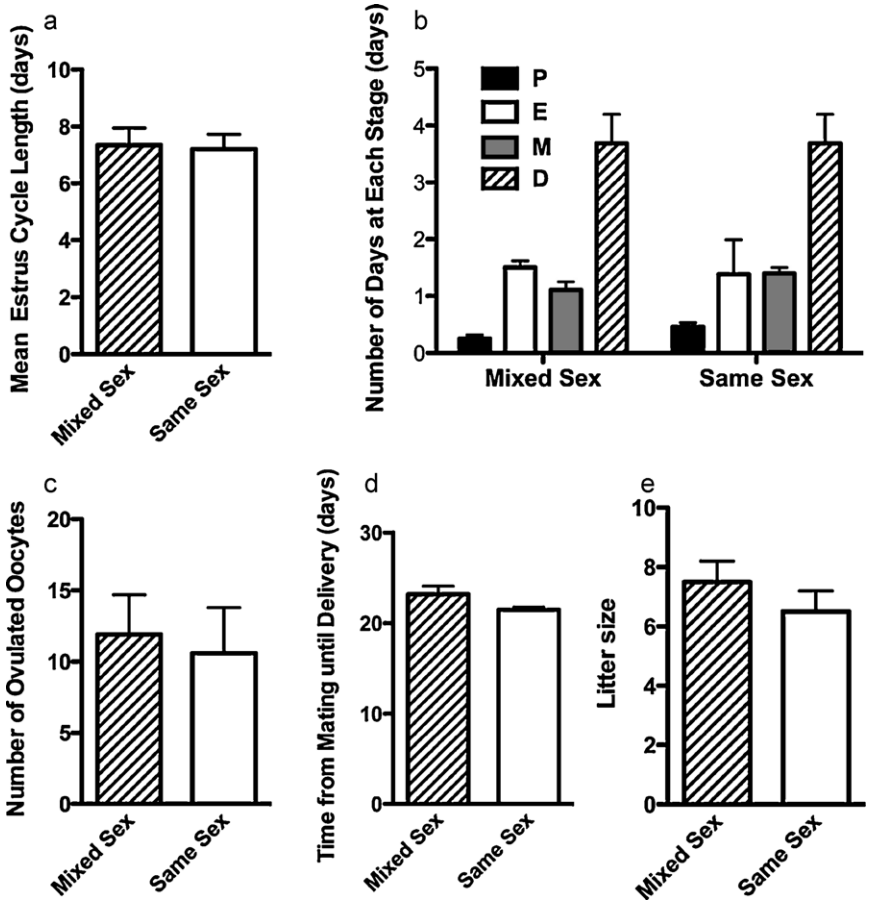


Figure 2 Reproductive characteristics. Females from both prenatal conditions (mixed- and same-sex) were examined in respect to their reproductive abilities expressed as (a) mean estrus cycle length; (b) number of days spent at each stage of the estrus cycle; (c) response to hormonally stimulated ovulation; (d) fecundity measured by the number of days between the initiation of mating to delivery; and (e) litter size. There were no differences between mixed- and same-sex females in either of measured parameters. Each graph represents a mean ± SEM, with the following number of mice tested: (a and b) $n = 16$ same-sex and $n = 12$ mixed-sex; (c) $n = 8$ same-sex and $n = 9$ mixed-sex; (d and e) $n = 14$ same-sex (two females failed to establish pregnancy) and $n = 12$ mixed-sex. Lower number of mice in (c) is due to accidental euthanasia performed before testing. In (b) P = proestrus, E = estrus, M = metaestrus, and D = diestrus.

[$F(1,24) = 22.15$, $P < 0.0001$] indicating that females from both prenatal conditions show a similar, but prominent preference for an unfamiliar female social stimulus (Fig. 3c). Behavioral analyses indicated a significant main effect of chamber for the duration of Contact [$F(1,24) = 9.75$, $P < 0.0046$; Fig. 3d], and Sniffing [$F(1,24) = 82.1$, $P < 0.0001$; Fig. 3e] of the cup containing the unfamiliar stimulus mouse. Finally, for the analyses of nose-to-nose behavior, independent t-test revealed a significant reduction in the duration of nose-to-nose contact with the unfamiliar stimulus by the same sex prenatal condition females (Fig. 3f).

3.4. Social proximity test

No significant differences in the frequencies of the types of contact in the social proximity test were noted (Fig. 4b).

3.5. Urinary scent marking to female urine

Two way repeated-measures ANOVA yielded no significant difference in the number of 1 cm × 1 cm squares containing scent marks for male mice exposed to urine from mixed- or single-sex prenatal females (Fig. 5c). Likewise, there was no significant difference in the duration of contact with the scent stimulus substrate between the two conditions (Fig. 5d). However, behavioral observations of male CD-1 mice in the scent marking apparatus indicated that they spent a greater amount of time directly sniffing the urine from the same-sex females [$t(20) = 2.583$, $P = 0.0178$] (Fig. 5e).

3.6. Maternal behavior

Two-way repeated measures ANOVA revealed that there were no significant differences in the frequency of pup related behavior (On Nest, Pup Contact, Pup Groom) between the dams from two prenatal conditions during the pre-session (Fig. 6a). However, there was a significant Maternal Behavior × Condition interaction [$F(2,23) = 4.246$, $P = 0.0203$] and a main effect for condition which approached statistical significance [$F(1,23) = 4.239$, $P = 0.051$]. Finally, Bonferroni

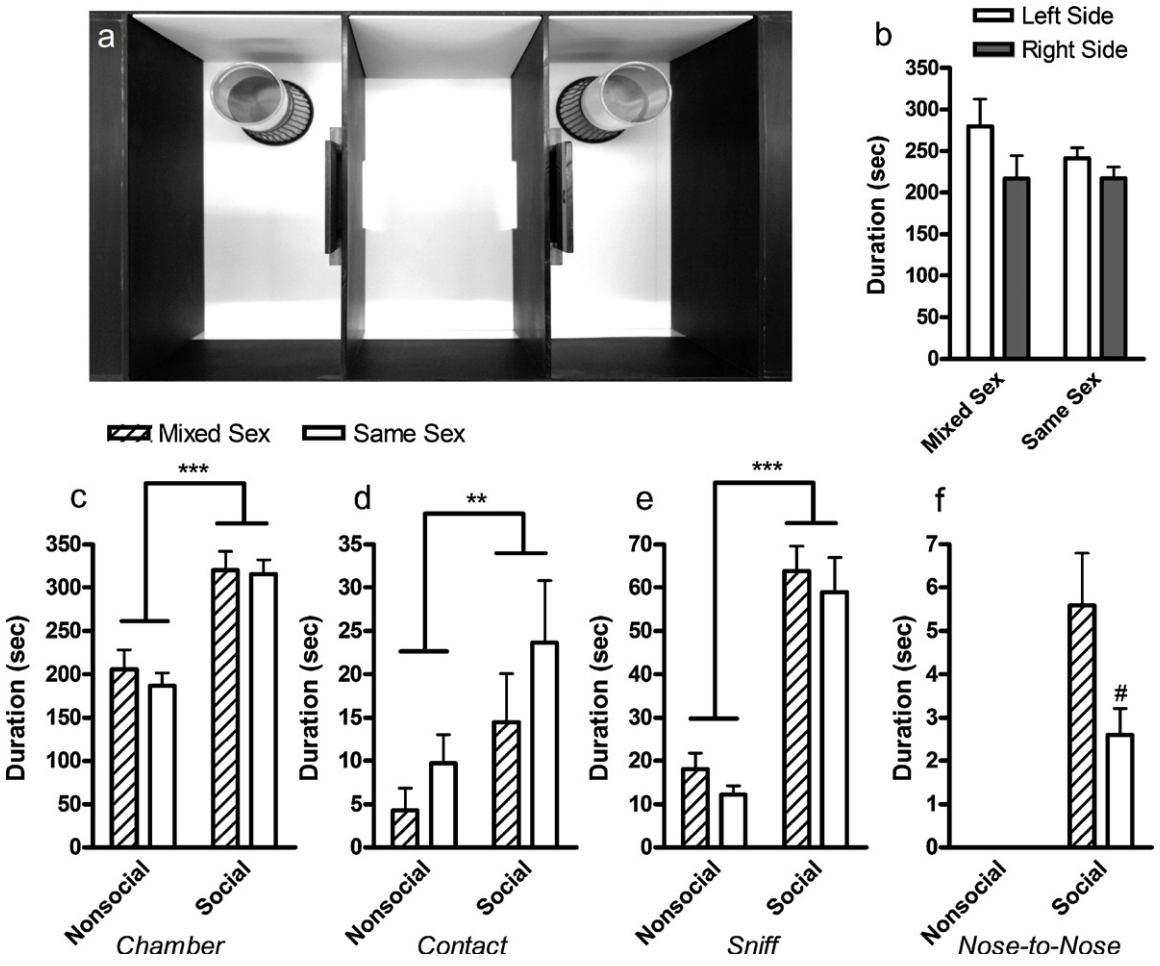


Figure 3 Three-Chamber Sociability Test. Three-chamber test involved placing a subject female in the center chamber of a three-chamber apparatus (a). When females from two prenatal conditions (mixed- and same-sex) were allowed to explore the remaining chambers during 'habituation phase', they spent similar amount of time in left and right side chambers (b). When an unknown female was placed in the wire cup of one of the chambers ('sociability phase'), females from both prenatal conditions showed clear preference towards spending time in the chamber containing stimulus mouse (c). The duration of Contact (d) and Sniff (e) with the cup was always higher in the chamber containing stimulus mouse as compared to chamber without stimulus. Same sex females had decreased duration of nose-to-nose exploration of stimulus cup than mixed sex females (f). Statistical significance: $^{**}P < 0.01$; $^{***}P < 0.0001$ (ANOVA) social vs. non-social and $^{\#}P < 0.05$ (*t*-test) same vs. mixed sex. Social = chamber with a cup containing stimulus mouse; Nonsocial – chamber with a cup without a stimulus mouse. Each graph represents a mean ± SEM, with $n = 16$ (same-sex) and $n = 12$ (mixed sex).

post-hoc comparisons indicated a significantly reduced latency to groom pups by same-sex prenatal condition dams ($P < 0.01$, Fig. 6b). Additionally, there was a significant main effect for Condition for percent time engaged in all maternal behavior categories [$F(1,23) = 7.763$, $P = 0.0105$] indicating that altogether, same-sex mothers display more maternal behavior in the pre-intruder session than do mixed-sex females (Fig. 6c). Analyses of non-maternal behavior in the pre-session revealed no effect of frequency (Fig. 6d) or latency (Fig. 6e) but a significant main effect of Condition for the percent time in non maternal behavior [$F(1,23) = 7.769$, $P = 0.0105$] as well as a significant Behavior × Condition interaction [$F(1,23) = 6.142$, $P = 0.021$] (Fig. 6f). Bonferroni post-hoc test indicated a significant reduction in the percent time in non-pup related activity by same-sex mothers (Fig. 6f), which corroborates the interpretation that they engage in more maternal behavior in the pre-session.

A significant main effect of Condition for the rate of agonistic defensive behavior [$F(1,23) = 9.060$, $P = 0.0062$] with same-sex prenatal females showing a higher rate of all three behaviors (burying, open-mouth threat/lunge, and attacks) during the intruder session was noted (Fig. 6g). There was also a significant main effect of Condition for the latency of aggressive behavior [$F(1,23) = 5.887$, $P = 0.0235$] with same-sex females showing an overall decreased latency to display aggressive behavior (Fig. 6h). There were no significant statistical differences in investigatory behavior during the intruder portion of the test (Fig. 6i and j).

4. Discussion

This study utilized assisted reproduction technologies to manipulate prenatal sex composition and evaluate whether absence of males during fetal development affects female reproduction and behavior. To our knowledge such an approach has never been used before in this scientific context.

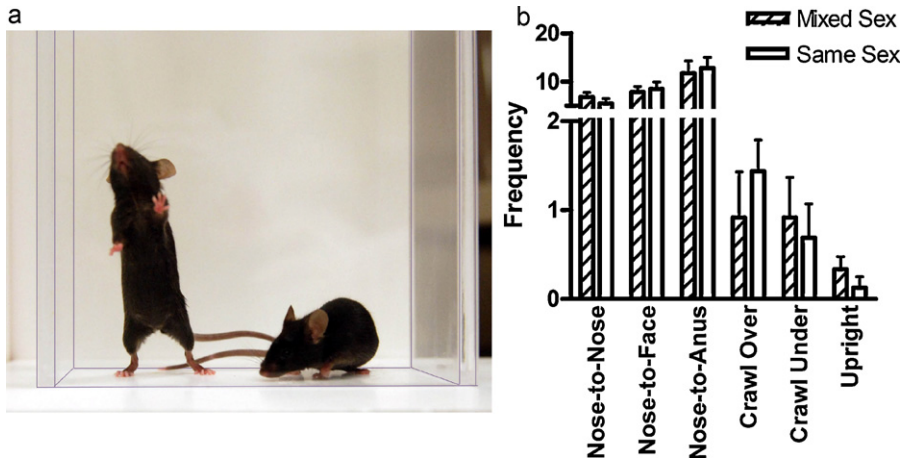


Figure 4 Social Proximity Test. Same-sex and mixed-sex females were placed in an enclosure with an adult female B6D2F1 stimulus mouse (a) and permitted to freely interact. The lines in the image (a) are added to delineate the edges of the clear Plexiglas arena. Six behaviors were scored: Nose-to-Nose, Nose-to-Face, Nose-to-Anus, Crawl Over, Crawl Under, and Upright. No significant differences in frequencies of these behaviors were noted (b). Each graph represents a mean ± SEM, with $n = 16$ (same-sex) and $n = 12$ (mixed sex).

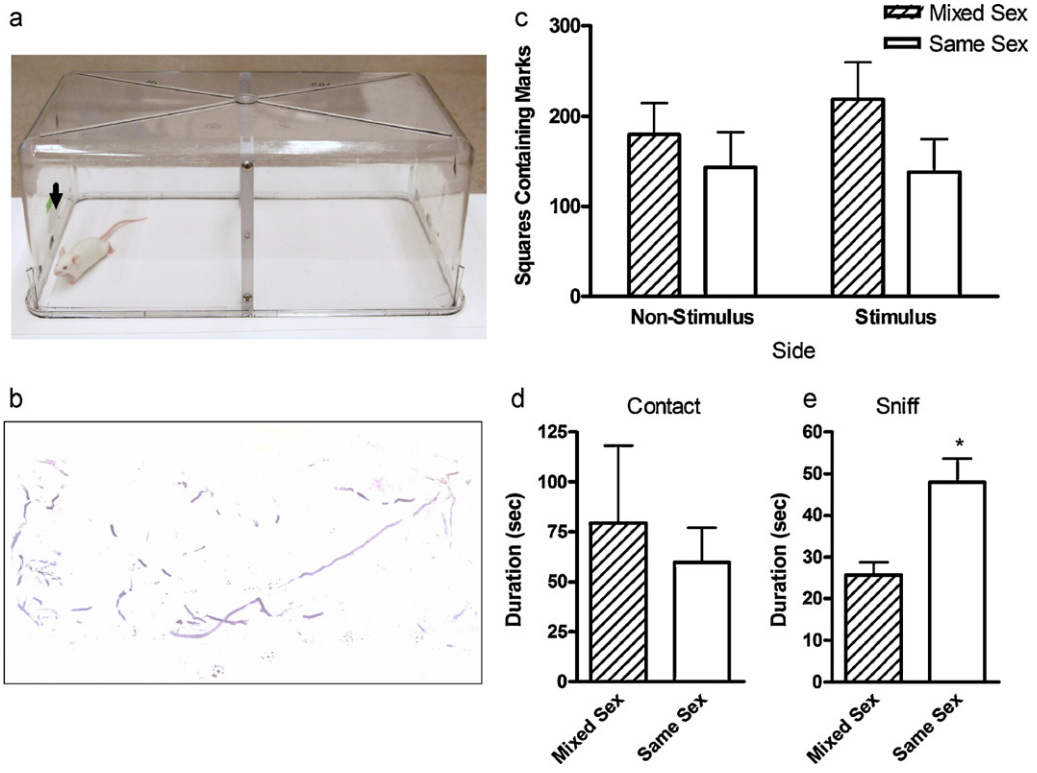


Figure 5 Scent Marking. When adult male CD-1 mice were allowed to explore a cage (a) with stimulus urine from females from two prenatal conditions (mixed- and same-sex), scent marks were left in both stimulus (containing stimulus urine) and non-stimulus parts of the compartment (b), with a similar response to urine from both prenatal conditions (c). The duration of Contact was similar for same- and mixed-sex stimulus urine (d) but males spent longer time Sniffing urine from same-sex females (e). Statistical significance: $^*P < 0.05$ (t-test). The arrow in Fig. 5a indicates the filter paper to which female urine was applied. Each graph represents a mean ± SEM, with $n = 15$ (same-sex) and $n = 7$ (mixed sex). The number of examined mice is lower than in other behavioral tests due to failure in obtaining urine from one same-sex and five mixed-sex females.

The effects of prenatal gonadal secretions have been previously studied using 'intrauterine positioning' (IUP) analyses, which compared females that as fetuses occupied uterine positions adjacent to one or two males (1M and 2M females) or not adjacent to males (0M females). The foundation of IUP studies is that diffusion of androgens from male embryos (contiguity hypothesis) and/or vascular transport of androgens from caudal circulation (caudal male hypothesis)

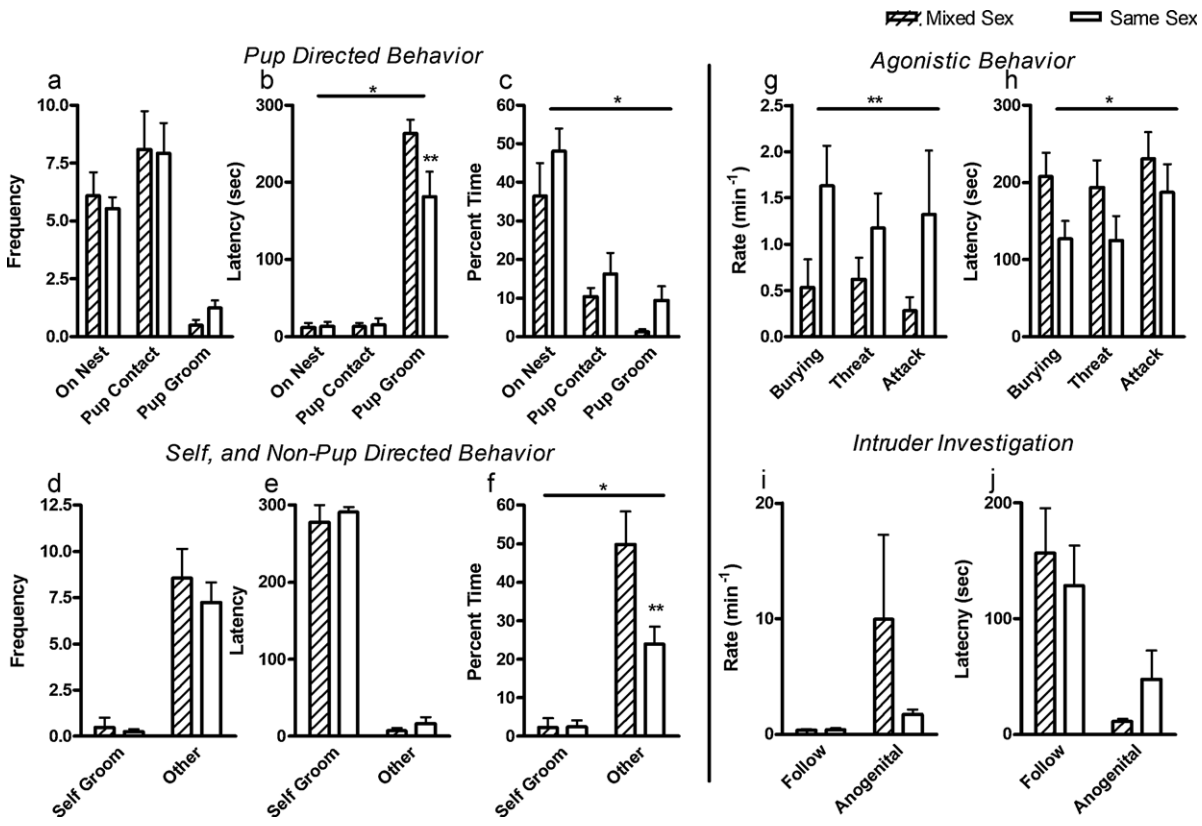


Figure 6 Maternal Behavior and Defensiveness. Females from both prenatal conditions (mixed- and same-sex) were mated with normal wild-type males and tested for maternal behavior/defensiveness 3 days post-partum. Female behavior was recorded during pre session (a—f) and after introduction of male intruder (g—j). The data are shown as frequency (a and d), latency (b, e, h, j), percent time (c and f) and rate/min (g and i) of variable behavioral responses. Females from same sex litters showed decreased latency to groom their pups (b), spent an overall larger proportion of time engaging in maternal care (c) and conversely, less proportional time in activities other than maternal care (f). After introduction of the intruder male, same-sex condition females showed augmented rates (g) and decreased latencies (h) to engage in agonistic responses to the intruders. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$ (ANOVA). Lines indicate a significant main effect of condition (mixed-sex vs. same-sex). Bonferroni post-hoc comparisons are indicated over error bars (mixed-sex vs. same-sex). Each graph represents a mean ± SEM, with $n = 14$ (same-sex) and $n = 12$ (mixed-sex).

cause masculinization and/or de-feminization of female fetuses (Houtsmuller and Slob, 1990). In mice, the downstream effects should be minimal given a bi-directional uterine blood flow (Vom Saal and Dhar, 1992). The myriad effects of IUP on physiology and behavior have been reviewed elsewhere (vom Saal, 1981; Ryan and Vandenbergh, 2002). Physiological changes have been noted with varying degrees of adjacent or upstream male siblings. Having one or more male sibling neighbors in the uterus alters the mu-opiod system which further influences psychostimulant-based conditioned place preference and drug-induced analgesia (Morley-Fletcher et al., 2003). IUP influences estrogen receptor subunit (ERα) expression in ventromedial hypothalamus, ventrolateral region (vlVMH), and this is mediated by epigenetic promoter methylation (Mori et al., 2010). Also, general changes in hypothalamic metabolic capacity have been noted specific to IUP (Jones et al., 1997). IUP relative to males does not appear to influence brain aromatase activity in the forebrain in rats (Tobet et al., 1985) but aromatase is elevated in the whole body (but not in the brain) of female ferrets downstream of males (Krohmer and Baum, 1989). This suggests that the availability of androgens, rather than the amount of aromatase enzyme, is the key factor in the degree of androgen-induced effects in 1M or 2M females (Krohmer and Baum, 1989). Given known effects of having or not having one or two adjacent males in utero in female rodents (Ryan and Vandenbergh, 2002) we might expect hyper-feminization and/or absent masculinization of physiology and behavior of females born from a uterine environment devoid of males.

Sex differences are common in expression of aggression and parental nurturing behaviors, with males being typically more aggressive and less parental than females. These sex differences can be attributed to steroid hormone differences during development and/or adulthood, especially the higher levels of androgens experienced by males depending on activity of the testis-determining Y chromosome encoded gene *Sry,* and to other Y chromosome-encoded genes (Arnold and Gorski, 1984; Gatewood et al., 2006). Our data indicate that females from same-sex pregnancies exhibit more parental behavior than females from the mixed-sex condition. Specifically, they showed enhanced pup grooming and were more aggressive to a male intruder. Interestingly, IUP analysis demonstrated that 2M, and not OM females, were more aggressive during pregnancy and lactation (Kinsley et al., 1986).

Previous findings that 2M females show more maternal aggression (vom Saal and Bronson, 1978) are in contrast to our current findings of increased maternal aggression in females with no male uterine siblings. These apparently contradictory findings may possibly reflect that maternal aggressive behaviors may involve two different patterns of attack, responding differentially to androgens and estrogens. This notion is supported by reports of different targets for attack by postpartum rodent females, in which fearful, defensive attack behaviors, with biting aimed at defensive target sites, were made largely toward male intruders, while offensive attack with offensive attack targets were involved in attack to female intruders (Parmigiani et al., 1988). These differences appear to be mediated by fear of the male, as chlordiazepoxide switched female attack on male intruders from a defensive to an offensive pattern (Palanza et al., 1996). In addition, fluprazine, an inhibitor of offensive attack, reduced female attack toward females more than female attack toward males (Parmigiani et al., 1989). Notably, while the concentrations of testosterone are higher in 2M females, the concentrations of estradiol-17β are higher in 0M females (vom Saal and Bronson, 1978; vom Saal et al., 1990). Thus, it is possible that females from the same-sex prenatal condition may show abnormally large amounts of estradiol, potentiating defensive attack toward the present male intruders. Consonant with this view, estradiol (Mayer and Rosenblatt, 1987, 1993) as well as both arginine vasopression (Bosch and Neumann, 2010) and prenatal testosterone supplementation (Mann and Svare, 1983) have been shown to increase maternal aggression, in studies in which relative components of offensive vs. defensive aggression were not evaluated. In the current study, a formal analysis of target sites was not possible given the quality of videos and since all intruders were male. Future studies might aim to clarify the hypothesis that defensive maternal aggression is modulated by hyperfeminization/de-masculinization, but offensive aggression by lactating dams is directly influenced by pre- and postnatal de-feminization/masculinization.

Previous studies on IUP suggested that it influences reproductive phenotype of females. 2M females were reported as having longer estrus cycles (vom Saal and Bronson, 1980a,b; vom Saal et al., 1981), delayed sexual maturation (Clark and Galef, 1988; Vandenbergh and Huggett, 1995), accelerated decline in reproductive capacity (Vom Saal and Moyer, 1985), and increased incidence of male offspring (Clark et al., 1993; Vandenbergh and Huggett, 1994). Here, we demonstrated that absence of males during fetal development had no effect on estrus cycle, fecundity, offspring sex ratio, and ovulation efficiency in females resulting from single-sex pregnancies. The discrepancy between our study and previous findings was unexpected but could be due to 'global' measure of masculinization of females from the mixed-sex pregnancies, which yielded a weaker effect. We did not check intrauterine position of these females so we do not know whether they developed in an immediate proximity to males, or not. If not, they might have been less affected by androgens than 2M females in IUP studies.

Previous studies in rats demonstrated that females born from spontaneous, all-female litters did not differ in sex steroid-induced mounting and lordosis behavior (Slob and Van der Schoot, 1982; Van de Poll et al., 1982), suggesting that a lack of prenatal androgen does not preclude activation effects of testosterone on sexual behavior. Masculinization of 2M females might affect their social behavior by prompting them to assume a dominant social role. Here, females from both prenatal conditions exhibited similar social behaviors and exhibited normal social responses towards unfamiliar females in the three-chamber social approach and social proximity tests. This suggests that any organizational influences of the differential androgen milieu in utero between these females might only be reflected under specific conditions.

The reproductive success of a female from a particular intrauterine position is influenced not only by its physiological response to social conditions but also by male preference. It has been shown that when allowed to choose, males preferred to associate with and mount OM over 2M females (vom Saal and Bronson, 1978, 1980a,b). Moreover, it has been shown that that males exposed to ovariectomized 0M and 2M females treated with estrogen and progesterone prior to testing, usually inseminated the OM female first (vom Saal and Bronson, 1980a,b). These data imply that 2M females may be less attractive to males, in agreement with our findings in this study. The fact that males spent longer time sniffing urine from same-sex females suggests that these females might have produced a pheromonal milieu that rendered them more attractive to males during olfactory investigations. The exact constituents of urine in female mice, which instigate investigation and onset of appetitive sexual behavior in males is still being investigated. Nevertheless, it has been shown that volatile compounds and their associations with major urinary proteins (MUPs), acting on the accessory olfactory system and vomeronasal organ (VNO) system (Brennan and Keverne, 2004) elicit physiological and behavioral changes (Johnston and Bronson, 1982; Sipos et al., 1992). The relative production of these molecules appears to be under hormonal control (Finlayson et al., 1963; Johnston and Bronson, 1982; Sampsell and Held, 1985). Future work should determine if absence of male siblings through development acts on the expression of these candidate molecules, particularly of those for the MUPs with known genetic representation (Bennett et al., 1982).

In our study mice were produced by in vitro fertilization followed by blastomere biopsy, embryo culture and embryos transfer. It has previously been shown that in vitro gamete and embryo manipulations can result in aberrant expression of imprinted genes (Fortier et al., 2008; Rivera et al., 2008). Thus, our approach might have interfered with epigenetic marks, which consequently might have influenced the results. This should be kept in mind when interpreting our data in the context of previous IUP reports, in which only naturally conceived mice were used. However, because same-sex and mixed-sex females were produced in exactly the same way, any epigenetic effect caused by methodology would affect both groups similarly. Also, in vitro fertilization and blastomere biopsy were previously shown to be neutral for embryo development and global patterns of gene expression (Duncan et al., 2009).

To summarize, we utilized a unique approach, creating female-only pregnancies, to validate and extend studies on intrauterine positioning effect on physiology and behavior. The importance of our study is twofold. First, because IUP can alter susceptibility of fetuses to endogenous hormones

and endocrine-disrupting chemicals (EDC) failure to account for its effects could lead to false-negative results in toxicology studies (Howdeshell et al., 1999; Welshons et al., 1999). Our proposed model, creation of single sex pregnancies, can offer a useful alternative for toxicology field. Second, there is an increasing concern about the potential effects of endocrine-disrupting chemicals, and particularly environmental estrogens, on mammalian sexual differentiation. Our finding that females developed in utero in absence of males, and potentially estrogenized, exhibit hyper-maternal behavior put these concerns in a new light and opens a door for future investigations on estrogen's role in behavior.

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Conflict of interest

All authors declare they have no conflict of interest.

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