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Establishment of partner preference in male rats: Effect of prenatal letrozole and sexual experience

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ABSTRACT

Repeated testing for masculine sexual behavior influences female sex preference in males. Males perinatally treated with aromatase inhibitors show male preference, but also copulate with the receptive female. Such copulation modifies sex preference most likely because of its rewarding properties. In this study, we intended to equal the incentive value of both stimuli -in the sex preference test- by using receptive females with vaginal occlusion. Vehicle and letrozole-treated (0.56 μg/kg, gestation days 10–21) males were repeatedly tested for sex preference at 40, 55, 70, 85 and 100 days of age. These ages were selected because males of 40 days are unable to copulate, while by 100 days of age almost all males show the complete repertoire of masculine sexual behavior. At 40 days of age, males of all groups fail to show sex preference and none of them was able to copulate. In controls of 100 days of age all males showed female-sex preference and all intromitted the female. A large proportion (44%) of vehicle-treated males that could not copulate the female showed male preference. Twenty to 30% of the prenatally letrozole treated males also had same-sex preference even if they could copulate; and most of them (67%) had a male preference when copulation was precluded. These data support the idea that copulation is crucial for developing a female preference in control animals. The results suggest that brain changes produced by estrogens along early development and stimuli coming from the partner are essential for shaping sex preference.

1. Introduction

Under experimental conditions, partner preference involves the choice of a particular subject of a given sex over another of a different sex; both stimuli should be equally accessible, and the experimental subject must be able to display sexual behavior with either stimuli (Vasey, 2002). The three compartments test fulfills these criteria (Olvera-Hernández et al., 2015) and allows the analysis of copulatory behavior. Male rat copulatory behavior is defined as the series of behavioral mounts, intromissions and ejaculations that the animal performs when exposed to a sexually receptive female (Hull and Rodríguez-Manzo, 2009). It is presumed that copulation in this species is a natural non-learned behavior (Hull and Rodríguez-Manzo, 2009; Larsson, 1956), however, its performance improves after repeated training (Dewsbury, 1969; Larsson, 1959); that is, males exposed few times to receptive females copulate more efficiently than males exposed for the first time.

It has been proposed that during early development male rats must be exposed to testosterone, that should be converted to estradiol by the enzyme, aromatase, to acquire female sex-preference and typical-male copulatory behavior in adulthood (McCarthy, 2008). In agreement, perinatal alterations in the brain levels of aromatase, using 1,4,6-androstatriene-3,17-dione (ATD) or androst-4-ene-3,6,17-trione (ADT), result in animals that prefer other males, show female sexual behavior, but retain their ability to mount, intromit and ejaculate with a sexually receptive female (Bakker et al., 1993a, 1993b; Brand et al., 1991; Booth, 1978). We have recently shown that the prenatal treatment with letrozole, a third generation aromatase inhibitor that lacks actions at steroid receptors (Dutta and Pant, 2008; Misso et al., 2012), causes that some males when adults show same-sex preference, lordosis behavior and non-contact penile erections when exposed to another male (Olvera-Hernández et al., 2015). In line with previous data, these males in addition, display full masculine copulatory behavior at similar levels than those expressed by control animals (Olvera-Hernández et al., 2015).

Rat sexual behavior ontogenetic development appears in late puberty (Goldfoot and Baum, 1972; Sachs and Meisel, 1988). Previous data have shown that usually the first mount occurs when the young male

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rat reaches around 40–50 days of age and the first intromission and ejaculation varies between postnatal days 44–75 (Sachs and Meisel, 1988; Sodersten et al., 1977), but by 70 days all subjects display the complete adult copulatory behavior (Sodersten et al., 1977). It has also been demonstrated that intromission and ejaculation possess strong rewarding properties after several copulatory tests (Coolen et al., 2004; Agmo and Berenfeld, 1990; Hughes et al., 1990; Paredes, 2009; Pfaus et al., 2001; Tenk et al., 2009; Paredes and Fernández-Guasti, 2008). Interestingly, ejaculation is the most rewarding component of rat male sexual behavior, but as early as in 1961, Whalen demonstrated that penile intromissions, even without ejaculation, are sufficient for the maintenance of sexual approach to an estrous female (Whalen, 1961). Furthermore, a previous study revealed that preventing from intromitting (and thereby from ejaculating) caused that males lose their preference for the side paired with the receptive female in a place preference test (Hughes et al., 1990). This rewarding effect of intromission, however, seems to depend upon sexual experience (Tenk et al., 2009).

A research group has explored how sex preference was affected by the repeated execution of sexual behavior in controls and in animals with an altered process of sexual differentiation (Brand et al., 1991; Bakker et al., 1993a, 1993b). They found that control adult males tested in a three-compartment sex preference paradigm (with a receptive female and a sexually active male as stimuli) showed a gradual increase in the preference for the estrous female accompanied by an increase in the number of intromissions and by the last test almost all males ejaculated. Interestingly, in the first tests, the males neonatally treated with ATD had preference scores favoring the stimulus male and some even displayed proceptive behaviors; but by the end of the experiment, virtually all males preferred the estrous female. As in the control group, these males also showed an increase in the number of intromissions with the receptive female, but very few ejaculated (Brand et al., 1991; Bakker et al., 1993b). These data indicate that repeated testing for masculine sexual behavior influences the establishment of female sex preference in control males and may affect the sex preference of males perinatally treated with aromatase inhibitors. In these series of experiments, males were first exposed to females at an age when they could already perform penile intromissions and ejaculate.

In the present investigation we analyzed these factors in males prenatally treated with letrozole and in control subjects. We hypothesize that prenatal aromatization interacts with the rewarding properties of mating to establish sex preferences in males. Thus, sexual preferences were assessed repeatedly between day 40 and 100 in males that were treated prenatally with vehicle or letrozole. Subjects were tested in a 3-chamber apparatus with a receptive female and a sexually active male as stimuli. In order to equalize the incentive value of the stimuli, tests were also conducted using females with occluded vagina. We predicted that the repeated execution of sexual behavior with receptive females would enhance sex preferences for females and reduce preferences for males. On the other hand, blocking penile intromission and ejaculation by occluding the vagina of the female should disrupt the acquisition of a female sex preference. The testing age interval of 40 to 100 days was selected because young males of 40 days are unable to copulate, while by 100 days of age almost all males show the complete repertoire of masculine sexual behavior (vide supra).

2. Material and methods

2.1. Animals

Female and male Wistar rats were used in this study. They were housed in a room with controlled conditions of temperature (22 ± 2 °C) under an inverted 12 h light-dark cycle (lights off at 10:00 h); with ad libitum access to water and food throughout the experiment. All experimental procedures were performed in accordance with the Mexican Official Norm for the use and care of laboratory animals "NOM-062-ZOO-1999" and approved by the local Ethics Committee (CICUAL-Cinvestav).

2.2. Treatment

The procedure of letrozole administration has been reported in detail in Olvera-Hernández et al., 2015. Briefly, female rats in proestrus were time mated (day of mating = day 0 of pregnancy). Prenatal treatment consisted of daily subcutaneous injections to the mothers from day 10 of pregnancy until one day before delivery of vehicle (corn oil) or letrozole at a dose 0.56 μg/kg/ml (Sigma-Aldrich, St. Louis, USA). The day of birth, after 22 or 23 days of gestation, the pups were culled to five males and five females. At the age of 21 days the pups were weaned and housed 6–8 rats with other animals of the same treatment per cage.

2.3. Behavioral testing

2.3.1. Partner preference

The partner preference test has been described in detail by Olvera-Hernández et al., 2015. Briefly, the apparatus consisted of a box made of black Perspex and a transparent front with three compartments with a small opening in both partitions near the front window that could be closed by guillotine doors. Consistently the left compartment contained the sexually experienced male with sawdust coming from cages of sexually active males, and the right one the sexually receptive female with its respective sawdust. These stimuli were restrained with a harness in a way that they had a limited action radius but were able to freely display sexual behavior. A male was considered sexually experienced when its ejaculation latencies did not exceed 15 min in at least 3 out of 5 consecutive (one week apart) tests with sexually receptive females. Receptivity was induced by the sequential administration of estradiol (25 μg/rat) and progesterone (1 mg/rat), injected 48 h and 4 h, respectively, before testing. A series of experiments were made with sexually receptive females with vaginal occlusion that was achieved by covering the genital region with a small cloth diaper. This diaper permitted the female to freely move and to display proceptive and receptive behaviors at levels indistinguishable from those displayed by non-covered females. Such manipulation prevented the male to have penile intromissions indispensable for the occurrence of ejaculation.

Behavioral observations started when the males reached the age of 40 days and were repeated every 15 days until they were 100 days of age. All tests were done between 12:00 and 16:00 h in a room under dim red light. Experimental subjects could freely move around and interact with the stimuli. The tests were videotaped for later analyses using The Observer 5.0 (Noldus ®). During the test, the experimental subjects were placed in the middle compartment and the sliding doors removed. The following behaviors were quantified: time spent in each chamber. We considered a male to be in a compartment when all four limbs had entered it. These data are presented as average percentage testing time in each compartment (total = 15 min) to make data comparable to those previously reported by others using percentage of time and this testing time (Hetta and Meyerson, 1978; Eliasson and Meyerson, 1981; Olvera-Hernández et al., 2015, 2017). A preference index was calculated based on previous work from Slob's group (Brand et al., 1991; Bakker et al., 1993a, 1993b). For these experiments, this index was obtained by dividing the time spent with the female by the time spent with the male. All the males that have a preference index of 0.89 (that indicates that males spend at least 40 s more in the male chamber than in the female compartment) or below were considered with same-sex preference. This limit value was set up because it reveals a clear male preference (to remind, a value of 1 denotes a lack of preference: same time with both stimuli). This criterion has been used to establish same-sex preference in rats (Olvera-Hernández et al., 2015, 2017). We calculated the percent of males with same-sex preference. In this test, we also measured the percentage of males that displayed

mounts, intromissions and ejaculations with the sexually receptive females.

2.4. Statistical analyses

Two-way Repeated Measures (RM) ANOVAs (considering age as the RM factor) followed by Holm-Sidak test were used to compare differences in the percent of time spent with either stimulus (sexually active male and receptive female) between treatments (vehicle or letrozole) and copulating conditions (with control or with females with vaginal occlusion). This parametric test was selected because of the lack of non-parametric equivalent analysis. In addition, an eta square generalized (η^2_G) analysis was made to estimate the effects size. This test was selected because it is the most appropriate for analyzing the effect size for two-way RMANOVAs and the present design (Bakeman, 2005; Trigo Sánchez and Martínez Cervantes, 2016). The proportion of rats showing each behavior was compared using the Fisher exact test. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Percentage of subjects showing same-sex preference along development

Table 1 shows the percentage of subjects that displayed same-sex preference along development. The table also shows the mean preference index ± SE of those males that preferred the other male. To remind, a male was considered to have same sex preference if it showed a preference index of 0.89 or less that was calculated by dividing the time spent with the female by the time spent with the male. Interestingly, in the vehicle group, when copulation was permitted, a female preference was established at the age of 70 days. Whereas if intromission and ejaculation were prevented, some males made repeated mounting attempts with palpation and thrusting; females remained proceptive and readily displayed lordosis in response to these mounts. The percentage of subjects that displayed male preference was around 33 to 56% along the whole test (days 40 to 100). In control animals that could not copulate the mean preference index of those males that had same-sex preference were strikingly similar in the first (40 days) and last (100 days) tests.

Remarkably, in the group of males prenatally treated with letrozole, where copulation was allowed, the percentage of males with same-sex preference was kept constant, between 45 and 20% along development (from 40 to 100 days). At 70 days of age there was a clear trend (Fisher exact test, p = 0.06) to increase the proportion of subjects with same-sex preference. Again, the mean values of the preference index were very similar between the first and last tests. When the intromission and ejaculation were precluded in males prenatally treated with letrozole there was a significant increase in the percentage of subjects that showed same-sex preference during the last two tests (at 85 and 100 days), in comparison with the males that were able to copulate (Fisher exact test, $p < 0.05$), and their mean preference indexes were the lowest.

3.2. Development of sex preference in control males

Fig. 1 shows the percentage of time spent with each stimulus in the four groups studied: controls allowed to copulate (Vehicle + copulation, panel A), controls not allowed to copulate (Vehicle – copulation, panel B), males prenatally treated with letrozole allowed to copulate (Prenatal letrozole + copulation, panel C) and males prenatally treated with letrozole not allowed to copulate (Prenatal letrozole – copulation, panel D) along five sex-preference tests performed at different ages: from 40 to 100 days. White bars show the percentage of time spent with the receptive female, while gray bars show percentage of time passed with the sexually active male (the sum of these bars not necessarily is 100%, because this figure excludes the time in the middle compartment).

In males treated with vehicle (panels A and B) the two-way RM ANOVA for the percentage of time in the male's chamber (gray bars) revealed a significant effect of age ($F_{4,88} = 13.33$, $p < 0.001$, $\eta^2_G = 0.12$). In males that were allowed to copulate (gray bars, panel A) there was a lack of sex preference at 40 days of age; that is, males spent a similar time with either stimuli. The time spent by these animals in the male's chamber was of 39%. At 55 days of age there was a drastic reduction in the percentage of time spent in the male's compartment (23%) that continues to decrease in successive tests to 14–8%. Indeed, the percentage of time spent with the male at 70, 85 and 100 days of age of control animals was significantly lower than that found at 40 and 55 days of age (Holm-Sidak differences shown by asterisks below brackets in gray bars). Interestingly, in control males that were allowed to copulate (panel A) the percentage of time spent with the female (white bars) did not vary along the different ages and was maintained at around 51 to 63%. Thus, the factor age of the two-way ANOVA of the time spent in the female's area was not significant ($F_{4,88} = 1.41$).

When intromission and ejaculation were precluded (panel B), control young animals of 40 days of age showed remarkably similar percentage of time spent with both stimuli than their counterparts that were exposed to females with no-occluded vaginas (comparison at 40 days of age between panels A and B). This profile was retained all over the tests. That is, the males that were not allowed to intromit (and thereby could not ejaculate) with the stimulus female passed a higher percentage of time in the male's chamber that was statistically different from that spent by males that could copulate (comparison between gray bars of panels A and B) (Holm-Sidak comparisons shown by & symbol). Thus, the two-way RM ANOVA for the percentage of time in the male's chamber revealed a significant effect of permitting or precluding copulation ($F_{1,22} = 73.66$, $p < 0.001$, $\eta^2_G = 0.49$) and an interaction between both factors, age and copulation ($F_{4,88} = 10.73$, $p < 0.001$, $\eta^2_G = 0.12$). Interestingly, in these animals that were not allowed to

Table 1
Percentage (proportion in parentheses) of subjects with same-sex preference and their mean ± SE preference index.

Condition	Age				
	40 days	55 days	70 days	85 days	100 days
Vehicle + copulation	23% (3/13) 0.84 ± 0.02	8% (1/13) 0.87	0% (0/13)	0% (0/13)	0% (0/13)
Vehicle – copulation	56% (5/11) 0.66 ± 0.04	33% (3/11) 0.79 ± 0.06	33% (3/11) 0.72 ± 0.08	44%* (4/11) 0.67 ± 0.08	44%* (4/11) 0.65 ± 0.04
Letrozole + copulation	45% (9/20) 0.79 ± 0.03	25% (5/20) 0.74 ± 0.03	30% (6/20) 0.65 ± 0.06	20% (4/20) 0.81 ± 0.02	20% (4/20) 0.75 ± 0.06
Letrozole – copulation	33% (3/9) 0.71 ± 0.07	0% (0/9)	22% (2/9) 0.75 ± 0.03	67%* (6/9) 0.63 ± 0.08	67%* (6/9) 0.55 ± 0.04

Fisher exact test, *$p < 0.05$ vs. same treatment + copulation.

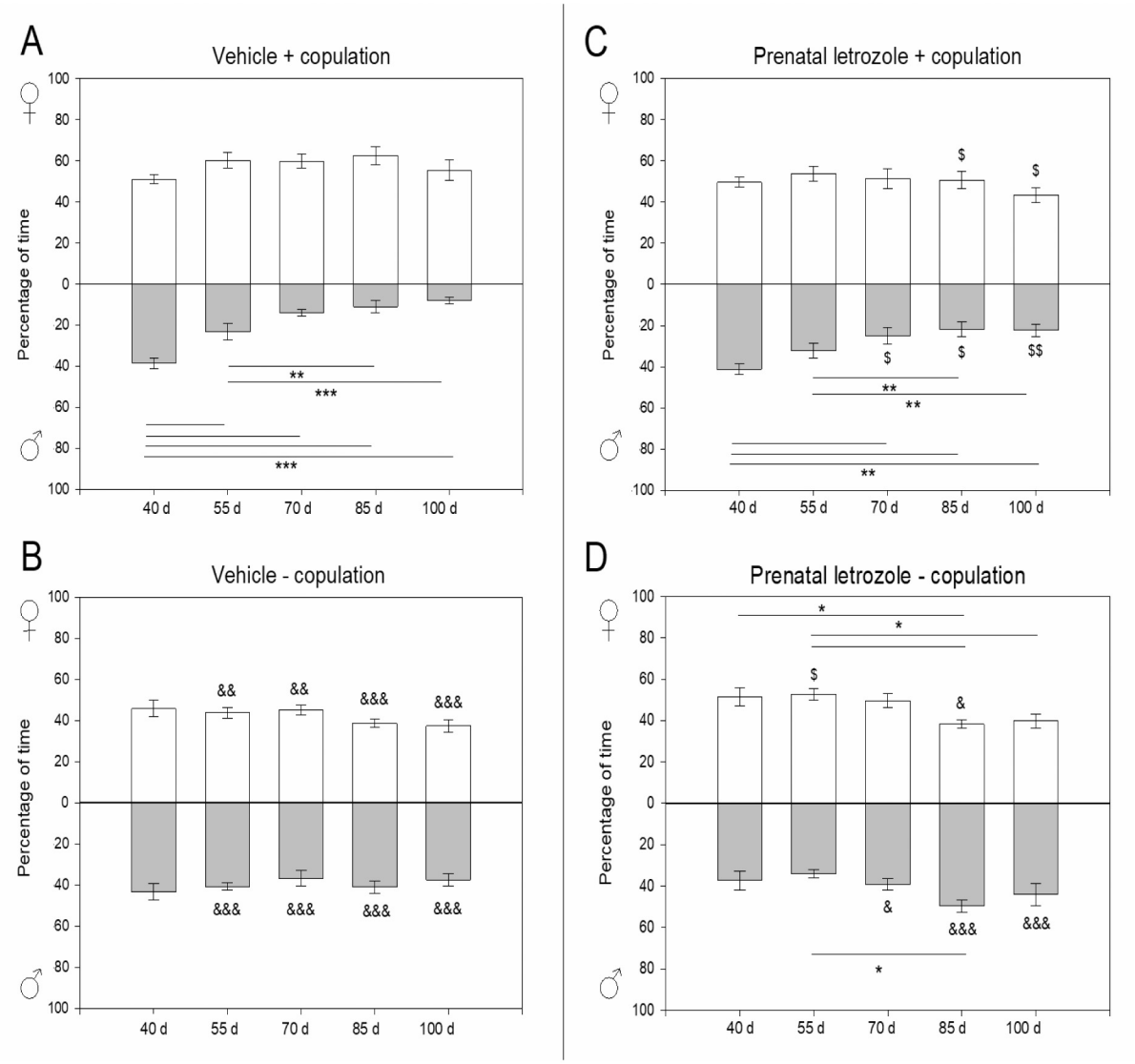

Fig. 1. Sex preference tested in males of 40, 55, 70, 85 and 100 days of age. Panel A: control (vehicle-treated) males exposed to a sexually active male or a receptive female without vaginal occlusion. Panel B: control (vehicle-treated) males exposed to a sexually active male or a receptive female with vaginal occlusion. Panel C: prenatally-letrozole-treated males exposed to a sexually active male or a receptive female without vaginal occlusion. Panel D: prenatally-letrozole-treated males exposed to a sexually active male or a receptive female with vaginal occlusion. Two-way ANOVAs (see text) followed by Holm-Sidak poshoc test: *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$ vs. males of 40 or 55 days of age (brackets). &$p < 0.05$, &&$p < 0.01$, &&&$p < 0.001$ vs. with copulation within the same treatment. \$$p < 0.05$; \$\$$p < 0.01$ vs. vehicle within same condition (with or without copulation). White bars show percentage of time spent with the receptive female, while gray bars show percentage of time passed with the sexually active male (the sum of these bars not necessarily is 100%, because this figure excludes the time in the middle compartment).

copulate, tested at 55 days and onwards, there was also a statistically significant reduction in the percentage of time they spent in the female's compartment (white bars, panel B), when compared with the time spent by males that could intromit and ejaculate (white bars, panel A) (Holm-Sidak comparisons shown by & symbol). In accordance, the two-way RM ANOVA of the percentage of time in the female's arena of vehicle-treated animals revealed an effect of copulation ($F_{1,22} = 22.06$, $p < 0.001$, $\eta^2_G = 0.28$) and a significant interaction between the factors age and copulation ($F_{4,88} = 2.50$, $p < 0.05$, $\eta^2_G = 0.06$).

3.3. Development of sex preference in letrozole-treated males

In animals treated with letrozole (panels C and D, gray bars) the two-way RM ANOVA for the percentage of time in the male's chamber revealed a significant effect of copulation ($F_{1,27} = 9.54$, $p < 0.01$, $\eta^2_G = 0.13$), no effect of age ($F_{4,108} = 1.76$) and an interaction between both factors ($F_{4,108} = 9.23$, $p < 0.001$, $\eta^2_G = 0.19$). As in control vehicle treated males, in young 40 days old males that had the possibility to copulate and that were prenatally treated with letrozole (panel C) there was no preference for either stimuli. After the third, fourth or fifth test, these males also showed a reduction in the percentage of time in the male's compartment (Holm-Sidak differences shown by asterisks below brackets in gray bars). As expected, the most affected group was that of males prenatally treated with letrozole that could not intromit or ejaculate with the female (gray bars, panel D). At 40 days, these males show the same profile than the other groups tested at this age: no preference for either stimuli. However, after repeated testing, these animals showed a significant increased percentage of time in the male's

area that was significantly higher than the time spent by letrozole-treated males that could copulate (Holm-Sidak comparisons between gray bars of panels C and D shown by & symbol). Indeed, the males unable to intromit and ejaculate that were prenatally treated with letrozole, when tested at 85 and 100 days, were the only ones showing a larger preference for the male than for the receptive female. The two-way RM ANOVA of the time spent in the female's compartment for the letrozole-treated males showed no effect of permitting or precluding copulation ($F_{1,27} = 0.66$), a significant effect of age ($F_{4,108} = 3.65$, $p < 0.01$, $\eta^2_G = 0.07$) and a lack of interaction between factors ($F_{4,108} = 1.13$). In these groups of males there was a decrease in the percentage of time spent with the female between ages 40 and 55 and the last two tests (panel D, white bars, asterisk over brackets).

The two-way RM ANOVA for the percentage of time spent in the male's chamber considering the factors treatment (vehicle or letrozole) and age within animals that could copulate revealed a significant effect of treatment ($F_{1,31} = 7.58$, $p < 0.01$, $\eta^2_G = 0.12$) and age ($F_{4,124} = 33.40$, $p < 0.001$, $\eta^2_G = 0.30$), but a lack of interaction between factors ($F_{4,124} = 1.49$) (Holm-Sidak comparison between gray bars of panels A and C shown by $ symbol). Animals treated with letrozole of 70, 85 and 100 days showed a higher percentage of time in the male's chamber (25–22%) as compared with the time spent by vehicle controls in the same area (14–8%). Furthermore, only at 85 and 100 days of age, the letrozole-treated males also showed a marginal statistical significant reduction in the percentage of time they spent in the female's area, even if they were permitted to copulate (two-way RM ANOVA, treatment, $F_{1,31} = 4.15$, $p < 0.05$, $\eta^2_G = 0.06$; age, $F_{4,124} = 2.55$, $p < 0.05$, $\eta^2_G = 0.04$ and interaction, $F_{4,124} = 0.94$).

The results of the two-way RM ANOVA of the percentage of time in the male's area considering those animals that were unable to intromit and ejaculate (comparison between grays bars of panels B and D) did not show statistically significant differences (treatment, $F_{1,18} = 0.24$; age, $F_{4,72} = 1.68$; interaction, $F_{4,72} = 2.07$). However, for the time spent in the female's area, the two-way RM ANOVA revealed a difference for treatment (letrozole or vehicle) ($F_{1,18} = 4.41$, $p < 0.05$, $\eta^2_G = 0.05$) and age ($F_{4,72} = 5.81$, $p < 0.001$, $\eta^2_G = 0.19$), but a lack of interaction ($F_{4,72} = 0.67$) (white bars of panels B and D, Holm-Sidak comparisons shown by $ symbol). The only difference was a higher percentage of time in the female's area at 55 days of age of those males that were not allowed to intromit and ejaculate as compared with those that could copulate.

3.4. Male sexual behavior along development in vehicle or letrozole prenatally-treated males

Table 2 compares the percentage of males that displayed mounts, intromissions and ejaculations, registered within the sex preference test, in all four groups studied. Importantly, at 40 days, regardless of their prenatal treatment or copulating condition, no male displayed an intromission and very few mounted the female. In the vehicle-treated group, the percentage of males that mounted increased from 15% at 40 days to 100% at 100 days of age. In this group, around half of the animals (54%) showed an intromission at the age of 55 days and this percentage increased to 69% at 70 days and to 100% at 100 days of age. Behavioral ejaculation followed a similar pattern as intromission, beginning in some animals when aged 55 days and reaching 69% at the end of the tests. Conversely, in the vehicle-treated group where intromission and ejaculation were precluded, the percentage of males that displayed mounts was kept very low (below 37%) at all testing times and never reached the percentages observed in its counterpart where copulation was permitted.

In relation with the group of males that were prenatally treated with letrozole and allowed to copulate, we found similar percentages of males displaying mounts and intromissions than in the vehicle control group. Interestingly, behavioral ejaculation also began at day 55 and remained between 5 and 35%, these percentages were lower in comparison with those of the vehicle group. Finally, in the letrozole group where intromission and ejaculation were not permitted few animals began to mount at the age of 85 days. This percentage of animals was lower than that found in the letrozole-treated males that could copulate.

4. Discussion

The main findings of the present study are:

1. In control males, the female preference is established at 70 days of age, once most of the animals (70%) have performed penile intromissions with the female. A large proportion (44%) of control males that were not allowed to intromit or ejaculate did not develop a female preference, indicating that copulation plays a crucial role for the establishment of female partner preference and suggesting that this phenomenon has a component of experience.
2. The alteration of the sexual differentiation process (by prenatal letrozole administration) predisposes some males (20–30%) to have a male sex preference even if they could copulate with the female. Males prenatally treated with letrozole that could not intromit or ejaculate showed a clear male preference.

4.1. Partner preference in control males

The results of the present investigation showed that the female preference was established at 70 days of age, because from this age onwards no control male showed same-sex preference and there was a drastic reduction in the time they spent in the area of the stimulus male. Remarkably, a similar result was reported in a previous study (Eliasson and Meyerson, 1981) where the sex preference was analyzed in males of

Table 2
Percentage of males showing mounts, intromissions and ejaculation during the partner preference test.

Condition		Age				
		40 days	55 days	70 days	85 days	100 days
Vehicle + copulation	Mount	15%	54%	77%	92%	100%
	Intromission	0%	54%	69%	92%	100%
	Ejaculation	0%	33%	54%	61%	69%
Vehicle - copulation	Mount	18%	9%*	18%*	36%**	27%**
Letrozole + copulation	Mount	15%	45%	70%	70%	75%
	Intromission	0%	35%	55%	65%	75%
	Ejaculation	0%	5%+	35%	25%+	10%+
Letrozole - copulation	Mount	0%	0%*	0%***	22%**	22%*

Fisher exact test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ within same treatment between copulation allowed and copulation not allowed; +$p < 0.05$ between treatments: vehicle vs. letrozole.

a similar age range, but under conditions where they could not interact with the stimuli, a test similar to the sexual motivation incentive test. Surprisingly, the results of that investigation revealed that in all males, regardless of their previous sexual experience and even if tested after neonatal castration, there was a female preference at 70 days of age, that also did not change with repeated testing at older ages. However, in tests where physical interaction is precluded it is difficult to distinguish between sexual or social preference (Vasey, 2002; Henley et al., 2011; Olvera-Hernández et al., 2015). By contrast, when interaction is permitted, it is possible to evaluate consummatory aspects of sexual behavior -such as mounts, intromissions and ejaculation- that permit to conclude that the preference is indisputably sexual (Henley et al., 2011). In the present investigation we measured the time the experimental male invested in each compartment (Fig. 1) as well as the sexual behavior it displayed towards the sexually receptive female (Table 2).

From the present series of results, we propose that the establishment of sex preference at 70 days of age in control males is strongly influenced by penile intromissions and ejaculation. Two evidences support this proposition: a) young males, of 40 days of age, did not show penile intromissions (Table 2) and fail to have a clear preference for either stimuli (Fig. 1, panel A), and b) control males that could not intromit or ejaculate along repeated testing (aged 40 to 100 days) spent more time in the male's chamber and less in the female's compartment as compared with controls that could copulate (Fig. 1, panel B). In favor of the idea that copulatory behavior shapes sexual preference, it has been reported that impeding intromission and ejaculation provoked that males lose their preference for the side paired chamber associated with the receptive female in a place preference test (Hughes et al., 1990); furthermore, intromission itself (without ejaculation) increases the motivation of the male to investigate the female (Whalen, 1961). As aforementioned, intromissions, and to a higher extent ejaculation, are strong rewarding aspects of sexual behavior (Coolen et al., 2004; Agmo and Berenfeld, 1990; Hughes et al., 1990; Paredes, 2009; Pfaus et al., 2001; Tenk et al., 2009; Paredes and Fernández-Guasti, 2008), putatively because during their occurrence there is a large central release of dopamine and oxytocin, both neurotransmitters involved in the neurobiology of reward (Paredes, 2009). It will be interesting to analyze if control males fail to develop a female preference -even if they can copulate- after blocking the action of these neurotransmitters. Interestingly, the females that had vaginal occlusion displayed lordosis, proceptive behavior and other cues that did not seem important for the induction of sex preference, further suggesting the imperative role of copulation. However, the diaper used to occlude the vaginal opening avoids the perception of genital odors presumably important for the establishment of sex preference (Quintana et al., 2018; Edwards et al., 1990); despite this limitation, in this test the experimental male could perceive the olfactory cues coming from the bedding material scented with odors of sexually receptive females.

Against an absolute association between copulation and female sex preference is the present observation that at 70 days of age all males have a female preference, while only around 70% have performed penile intromissions with the female (comparison of Tables 1 and 2). The cues that determined the female preference in the remaining 30% are unknown, but there might be an inherent predisposition of control males to prefer females as suggested the results of Eliasson and Meyerson (1981) and Brand et al. (1991), who reported the development of a female preference in males under conditions where the animals could not interact (vide supra).

The analysis of the time the control males spent with each stimuli during the sex preference test (Fig. 1, panel A) offers interesting inferences. Firstly, it is of notice that the time spent with the female was strikingly similar along all tests at different ages; however, the sexual behavior displayed towards the female drastically changed, i.e., during the first tests the males were too young to exhibit intromissions and ejaculations, while during the last trials, they invested in the female's chamber the same amount of time, but performing the complete repertoire of masculine sexual behavior (Table 2). Secondly, the time spent in the stimulus male's area was drastically reduced along repeated testing. Thirdly, when intromission and ejaculation were not permitted, rendering both stimuli with a similar incentive value, the experimental male invested the same time with the sexually receptive female and the active male during all tests, from 40 to 100 days of age (Fig. 1, panel B). Under this condition (without copulation) the percentage of mounting was reduced as compared with that shown by males that could copulate (Table 2). These findings, taken together, suggest that when copulation is permitted, the attractiveness of the stimulus male decreases, putatively because the experimental subject cannot perform sexually rewarding behaviors with another male. In other words, when both stimuli have a similar incentive value no sex preference is developed. The inviting experiment to confirm this idea is to increase the male's incentive value by making possible the occurrence of intromissions between males; such manipulation, however, is anatomically unfeasible. Recent experiments have revealed that learning, under Pavlovian conditioning, influences the selection of sexual partners because sexually naïve males that cohabitated with a scented male under the treatment with the dopamine agonist, quinpirole, had sex preference for that male (Triana-Del Rio et al., 2011; Ramírez-Rodríguez et al., 2017). Such manipulation apparently raises the incentive value of a male, without altering its anatomy; therefore, it would be interesting to analyze the preference of prenatally-letrozole-treated males under this form of conditioning.

Another interesting proposal of these series of experiments is the important role of sexual experience in the development of sex preference. Since the pioneer studies of Knut Larsson in the fifties, many others, including the present report, have confirmed that male sexual behavior improves after repeated testing. Thus, sexually experienced male rats show increased genital arousal, more rapid approaches towards the receptive female, initiate copulation faster and display fewer intromissions before ejaculating than inexperienced ones (Larsson, 1956, 1959; Dewsbury, 1969; Hughes et al., 1990). Also, as previously suggested in adults (Brand et al., 1991; Bakker et al., 1993b, 1995), here we show in young rats, that the repeated execution of masculine sexual behavior favors the development of a female sex preference. In line, as aforementioned, males lost their preference for the side paired with the receptive female when they were prevented from intromitting (Hughes et al., 1990); however, such prevention failed to affect the female preference if the adult male rats were sexually experienced (Tenk et al., 2009).

4.2. Partner preference in letrozole-treated males

One of the most interesting findings of this series of experiments is that prenatal letrozole treatment provoked that some males retain a male sex preference even if they could copulate with the female. It could be argued that this way of measuring same-sex preference is veiled by social interaction or simply by the passive staying (without interacting) of letrozole-treated animals in the male's chamber. Against this idea, a previous report has shown that males treated with this aromatase inhibitor increased their time of sociosexual interaction with the stimulus male, as compared with vehicle-controls (Hernández and Fernández-Guasti, 2018). Additionally, treatment with letrozole augmented the proportion of males that show lordosis, proceptive behavior and non-contact penile erections when exposed to another male (Olvera-Hernández et al., 2015). Notwithstanding, it is needed a specific analysis of the sexual interaction between the letrozole-treated and the stimulus males in this test.

As in controls, at 40 days of age there was a relatively high percentage of males treated with letrozole that had a male preference (45%, see Table 1). However, at 70 days of age, when all control rats had developed a female preference, there was a 30% of letrozole-treated males that had sex preference for other males, even if they could copulate with the female. This percentage was of 20% at 85 and

100 days and was accompanied by a higher percentage of time in the male's chamber than their vehicle treated counterparts (see Fig. 1, panels A and C). Interestingly, previous results from our group (Olvera-Hernández et al., 2015; García-Cárdenas et al., 2015) showed that when males -prenatally treated with letrozole- were tested for the first time for sex preference as adults (at around 120 days of age) the percentage of subjects that show same sex preference was also between 20 and 30%. Such similar percentages suggest that in these animals, by contrast with controls, repeated testing has no effect on modifying sex preference. These data invite to think that in some prenatally letrozole-treated males there is not a rewarding effect of copulation or alternatively, that such reward is not enough to influence their preference for the male. Another possible explanation includes the reduced learning produced by this aromatase inhibitor (Zameer and Vohora, 2017; Zhao et al., 2018) that provokes a deficient acquisition of sexual experience. In favor of this idea, in the prenatally letrozole-treated group there was a lower percentage of rats ejaculating than in the control group at 100 days of age after five consecutive tests (see Table 2). The inhibitory effect of the perinatal treatment with the aromatase inhibitor, ATD, on copulatory behavior has been controversial. Thus, while some authors have reported a lack of effect (Brand et al., 1991; Vreeburg et al., 1977; Davis et al., 1979; Whalen and Olsen, 1981), others have shown inhibitory actions, particularly on the percentage of males ejaculating (Brand et al., 1991; Gladue and Clemens, 1980). The nature of this difference is unknown but the dose, route of administration and time of perinatal exposition have been proposed as important factors (see Brand et al., 1991). The differences between our previous findings using letrozole (Olvera-Hernández et al., 2015) and the present results could rely on the testing conditions (cylindrical arena with the receptive female as a single stimulus vs. the three compartments cage including two stimuli) and the duration of the test (a single session of 30 min vs. 15 min repeatedly).

In a thoughtful experiment to analyze the influence of sexual experience in sex preference in ATD-treated males, Brand et al. (1991) exploited the ability of 8-hydroxi-(2-*n*-propyl) amino tetralin (8-OH-DPAT), a 5-HT_{1A} agonist, to stimulate male sexual behavior (Rubio-Casillas et al., 2015). They confirmed that 8-OH-DPAT stimulated copulation (by lowering the number of intromissions preceding ejaculation) and increased the preference scores for an estrous female when sexual interaction was possible in males perinatally treated with ATD. However, the actions of this 5-HT_{1A} agonist on partner preference and male sexual behavior were temporary, because the results of the tests one week after 8-OH-DPAT were similar to those preceding the drug. These interesting data, together with present results, further strength the close relationship between male sexual behavior and female sex preference and suggest that the perinatal effects of aromatase inhibitors cannot be completely overcome by the effects of sexual experience, most likely because they have organized the brain permanently.

Interestingly, in males prenatally treated with letrozole that were unable to intromit and ejaculate with the female, there were high scores of male preference revealed as an increased percentage of males with same sex preference (67% at tests 85 and 100 days, Table 1) and of time invested in the male's area (Fig. 1, panel D). Remarkably, this was the only group where after repeated testing, the receptive female seemed to lose attractiveness, while the stimulus males appeared to develop interest for the experimental male. The signals that provoked such increased attractiveness between males remain unknown.

These series of results, together with previous data, suggest that sex preference in male rats (and possibly in other species including humans, Fisher et al., 2018) is partly shaped by brain changes produced by estrogens/androgens along early development and cues coming from the sexual partner (those produced by copulation for the case of the female). These two factors are not the only ones involved in partner selection (Balthazart, 2011) and interact to produce the final outcome.

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