INCREASE IN SEXUAL MOTIVATION THROUGHOUT ADOLESCENCE IN THE CYCLING FEMALE RAT

Running title: Sexual motivation in adolescent female rats

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CONFLICT OF INTEREST

Funding sources had no involvement in the design of this study, or any other aspect related to it. Authors report no conflict of interest.

DATA AVAILABILIY STATEMENT

The data that support the findings of this study are available fromthe corresponding author upon reasonable request.

ABSTRACT

Sexual behavior in the female rat is a highly motivated behavior first displayed during adolescence, a developmental period when neural circuits underlying motivation are not mature. This study characterizes the natural development of sexual motivation and behavior of female rats. We compared the incentive value of the male for mid-adolescent (PNDs:39-43), late adolescent (PNDs:49-53) and adult (PNDs:90-115) cycling females, using a male-female preference task and an ultrasonic vocalization emission test following exposure to a male or female stimulus animal. Furthermore, display of sexual and social behaviors during an interaction with a male or a non-receptive female was assessed. Mid-adolescent rats exhibited a reduced preference for the male than adults and performed less attempts to access the male. Unlike late adolescent and adult females, mid-adolescent rats did not increase their ultrasonic vocalization emission after interacting with a male relative to a female. Although most of the sexual behavior did not differ between groups, mid-adolescent females showed lower lordosis magnitude and higher levels of play and social investigation during a sexual interaction, giving rise to a unique behavioral profile. Present results indicate that the sexual behavior repertoire is fully displayed by mid-adolescence, but sexual motivation is low and increases into late adolescence.

Key words: adolescence, female rat, sexual behavior, sexual motivation, ultrasonic vocalization

1 INTRODUCTION

In the female rat, sexual behavior is a highly motivated behavior(Agrati, Fernández-Guasti, & Ferreira, 2008; Clark, Kelton, Guarraci, & Clyons, 2004; Cummings & Becker, 2012) mediated, in naturally cycling animals, by the rise in steroid hormone levels that occurs in the proestrous stage of the estrous cycle(Pfaus, Jones, Flanagan-Cato, & Blaustein, 2015). During sexual interactions, adult female rats display proceptivebehaviors, including solicitations and paracopulatory displays (such as hops & darts, presentation postures and ear wiggling), that encourage mounting by the male, and lordosis postures in response to suchmounts(Beach, 1976; Blaustein & Erskine, 2002; Erskine, 1989; Pfaus et al., 2015). This display of proceptive behaviors has traditionally been used to assess female sexual motivation(Heijkoop, Huijgens, & Snoeren, 2018).However, the use of specific motivation-assessing teststhat do not involve copulation but are based on learning tasks (e.g. lever-pressing, place preference conditioning or nose poking) (Bermant, 1961; Paredes & Vazquez, 1999; Uphouse, Pinkston, Baade, Solano, & Onaiwu, 2015)or on unconditioned approaching behaviors (e.g. partner preference or multiple partner choice arena)(Ågmo, Turi, Ellingsen, & Kaspersen, 2004)haveprovided further evidence on the motivational nature of this behavior(Ventura-Aquino & Paredes, 2017).

Sexual behavior in female rats is first displayed during adolescence(Holder & Blaustein, 2014; Södersten, 1975), a developmental period when maturation of multiple neural circuits and neuroendocrine systems, including those known to be involved in reward and sexual behavior, is not yet complete (Brenhouse & Andersen, 2011). In line with such changes, adolescence is also characterized by a specific behavioral profile. For instance, social play behavior is highly expressed by juvenile rats, but negligible in adults (Panksepp, 1981). On the other hand, female sexual behavior appears after vaginal opening

(approximately postnatal days (PNDs) 35-36) (Hashizume & Ohashi, 1984). However, the development of sexual motivation from adolescence to adulthood remains uncharacterized.

In the rat, adolescence is typically divided in three stages termed *early* (PNDs 21-34), *mid* (PNDs 34-46) and *late* (PNDs 46-59) adolescence (Tirelli, Laviola, & Adriani, 2003). During late adolescence, as during adulthood, naturally cycling females in late proestrous display the full repertoire of sexual behaviors and express a strong preference for a male over a sexually active female (Agrati et al., 2018). On the other hand, studies on ovariectomized early adolescent females suggest that sexual behavior is not fully developed at this time but undergoes a further maturation process. When exogenous cyclicity is imposed by an estradiol and progesterone treatment, females increase the display of both receptive and proceptive components during a sexual encounter until the sixth induced cycle, when their expression equals that displayed by adults (Hliňák, 1986). Given that in naturally cycling females the sixth cycle approximately coincides with late adolescence (Tirelli et al., 2003), the exposure of late adolescent rats to several estradiol and progesterone cycles could underly the high incentive value of the male and the full expression of sexual behavior we previously observed (Agrati et al., 2018). Because mid-adolescent rats only experienced one or two ovarian cycles, their sexual behavior and motivation may not yet be fully developed.

During adolescence sexual and other social motivations typical of this developmental period can be intermingled. For instance, during sexual interaction, late adolescent females exhibit more social investigation than adult rats and display particular behaviors that resemble juvenile play (Agrati et al., 2018). Play behavior is highly rewarding (Achterberg et al., 2016; Achterberg & Vanderschuren, 2020) and has been previously shown to increase from the juvenile to the mid-adolescence stage, being maximum between PNDs 32 and 40

and declining to a negligible amount by PND 60 (Panksepp, 1981). Interestingly, some behavioral components are shared between typical play and sexual behaviors: for example, play and sexual solicitations resemble each other (Thor & Holloway, 1984) and a crouching or presentation posture is typically displayed during both types of interactions (Poole & Fish, 1975). Therefore, if adolescent females display play behavior while sexually interacting with a male, it would be difficult to discern the sexual or social nature of the observed behavior in this context. Consequently, when assessing female sexual behavior during adolescence, it seems essential to consider the display of play elements and social investigation and to compare the expression of these components with those exhibited in a social interaction, in the absence of a sexual incentive. Moreover, sexual motivation in adolescent females should be assessed with specific motivational tests in order to avoid a possible confusion rising from the co-expression of sexual and play behaviors.

Unconditioned teststo assess sexual motivationthatdo not require prior training, as is the case of the male-female preference tasks (Ågmo et al., 2004; Agrati et al., 2018), provide an advantage when studying a highly dynamicperiod like adolescence.McGinnis & Vakulenko (2003)proposed an unconditioned sexual motivation-assessing test based on the emission of 50kHz ultrasonic vocalizations (USV) after a brief interaction with a male or a female. This type of USV, which has been associated to a positive hedonic stateandis commonly observedduring rewarding social interactions(Knutson, Burgdorf, & Panksepp, 2002),is emitted by both the male and the female rat during asexual encounter (Barfield, Auerbach, Geyer, & Mcintosh, 1979; Sales, 1972). After a brief interaction without physical contact with a male, sexually active female rats continue to emit 50kHz USVin different extent (McGinnis & Vakulenko, 2003). Both ovariectomized female rats with induced sexual receptivity (McGinnis & Vakulenko, 2003) and naturally cycling adult females(Börner,

Hjemdahl, Götz, & Brown, 2016), emit a higher number of 50kHz USV after interacting with a testosterone-treated male than after interacting with a castrated male or a female. Thus, the higher emission of 50kHz USV after a brief interaction with no direct physical contact with a male (sexual stimulus)in relation to a female (social stimulus) has been proposed to provide a sensitive and reliable measure of female sexual motivation (McGinnis & Vakulenko, 2003).

We hypothesized that sexual motivation in the female rat increases from mid to late adolescence, when it reaches adult levels. Furthermore, we predicted that mid-adolescent females display a unique behavioral pattern during a sexual encounter by combining the expression of social behaviors typical of this developmental period with components of female sexual behavior. To test these hypotheses, we compared the incentive value of a sexual and a social stimulus for naturally cycling mid-adolescent, late adolescent and adult female rats in late proestrous using two different tests, as is suggested for studies assessing sexual motivation (Ventura-Aquino & Paredes, 2017): a male versus female preference task(Agrati et al., 2018)and a USV emission test after a brief contact with a male or a female (McGinnis & Vakulenko, 2003). Furthermore, we compared the expression of sexual and social behaviors of sexually active females of these age-groups during an interaction with a sexually active male (sexual interaction)ora non-receptive adult female (social interaction).

2 METHODS

2.1 Animals

Female and male rats (*Rattus norvegicus*, Wistar strain) were housed in a temperature and humidity-controlled environment (21 ± 1°C and 50–70%, respectively) under a 12-h light–dark cycle (lights on at 03.00 h). Adolescent and adult females were housed in groups of 5

and 4, respectively. Vaginal opening was checked in adolescent females every morning from PND 33 (Caligioni, 2009). The oestrous cycle of all females was monitored by daily vaginal smears in the morning (Cora, Kooistra, & Travlos, 2015; Marcondes, Bianchi, & Tanno, 2002) starting the day of vaginal opening for mid-adolescent and late adolescent females, and onPNDs 80-90 for adults. Animal care and experimental procedures were performed following the Guide for the Care and Use of Laboratory Animals of the NIH and the Uruguayan law (number 18 611) for the Care and Use of Laboratory Animals. The experimental protocol employed was approved by the Ethical Committee on Animal Care and Protocols of Facultad de Ciencias, Universidad de la República (protocol number 240011 -000941-17).

2.2 Male versus non-receptive female preference test

A preference test(Agrati et al., 2018) between a sexually active male and a non-receptive female was performed in a transparent Y-shaped maze with three equal-sized chambers (25cm wide × 30cm long × 18cm high) at the end of each arm (10cm wide × 30cm long × 10cm high). One of the chambers contained a sexually active male (trained to perform one ejaculation in less than 15 minutes), a second chamber contained a non-receptive cycling female, and the third chamber (neutral chamber) remained empty. The locations of the stimulus animals were counterbalanced in a random manner. Non-sexually receptive females were employed as social stimulus in order to eliminate potential sexual cues related to the endocrine status of sexually receptive females (McGinnis & Vakulenko, 2003). Each chamber is divided in two halves by a perforated acrylic transparent division. The stimulus is located in the outer half, and the experimental female can access the chamber to its inner half.

The perforated division allows the females to see, hear and smell the stimulus animals while preventing direct contact.

Experimental females were placed in the center and allowed to explore the empty maze for a 15-minute acclimatization period. Immediately after, the stimulus animalswere placed in the chambers and the experimental female's behavior was registered during a 20-minute testing period. The measuredvariables included: the number of entries to each arm and chamber,thecumulative time spent in each chamber, the number of sniffings to the inner chamber walls, and the number of attempts to access each stimulus animal (defined as the act of biting or scratching the inner chamber wall). The entire maze was cleaned with a 50% ethanol solution and dried thoroughly between test sessions.

Two complementary measures of preference were determined: 1) number of females in each group exhibiting preference for the male, the female or the neutral chambers or no preference, and 2) the total time that females spent in each chamber. Preference for a particular chamber was assigned if the female spent in this chamber more than 50% of the whole time spent in the three chambers, being this time at least 25% greater than the time spent in either of the two remaining chambers (Agrati et al., 2016, 2018; Pereira & Ferreira, 2006).In addition, locomotion was assessed by counting the total number of entries in the arms and the chambers of the maze. Because the total number of entries to the chambers differed between groups, the percentage of entries to each chamber was used for comparisons.

2.3 Ultrasonic vocalization emission test after a brief male or female interaction

The emission of USV by females after a brief sexual or social interaction with limited contactwas assessed using the test proposed by McGinnis & Vakulenko (2003). The settingconsists of an acrylic enclosure (38cm wide x38cm long x40cm high) with bedding floor and a vertically sliding wire mesh dividing the enclosure in two equal compartments.

Females were acclimatized to the setting for 15 minutes during the 4 consecutive days prior to testing. On the testing day, the non-receptive female (social stimulus) or the sexually active male (sexual stimulus) was placed inside one compartment and allowed to explore. After 2 minutes, the experimental female was placed in the adjacent compartment. The wire mesh allowed visual, olfactory, auditory and mild-tactile interaction between the two animals, preventing direct body-on-body contact. After a 5-minute period of interaction, the stimulus animal was removed from the experimental room so that the experimental female was left alone in the acrylic enclosure, and the wire mesh removed. The emission of USV by the experimental female was recorded for5 minutes (without stimulus animal) using an UltraSoundGate microphone (Avisoft Recording Software) placed on top of the acrylic enclosure.

USV emission recordings were analyzed using the Avisoft-SASLab Pro (Avisoft Bioacustics) software. USV were classified according to Brudzynski (2013)into two main groups (50kHz and 22 kHz) depending on their average frequency, and into two subtypes (50kHz FM and 50kHz flat) according to the degree of frequency modulation. Based on (Börner et al., 2016), the proportion of FM over the total number of 50 kHz USV emittedwas calculated.

Because the basal emission of USV might vary throughout adolescence and adulthood(Willey & Spear, 2012; Willey, Varlinskaya, & Spear, 2009), we considered the difference between the emission of USV after an interaction with a male (sexual stimulus) vs. a female (social stimulus) within an age group as ameasure of the male's sexual incentive value(McGinnis & Vakulenko, 2003).

2.4 Social/sexual interactiontest

Social and sexual interactions were assessed in a circularplexiglas arena (90 cm in diameter).The experimentalfemalewasplaced in the arena and allowed to explore for 5 minutes, after which a sexually active male (for sexual interaction) or a non-sexually receptive female (for social interaction)was introduced, and behavior was recorded for 10 minutes.According to Agrati et al. (2018)female behavior was assessed for the following components of sexual behavior: number of lordosis postures in response to mounting,hops & darts (short distance hopping and/or running near the stimulus animal, usually with a rigid body posture), and presentation or crouching postures (freezing in a crouch posture usually with ears facing back). In addition, latency to the display of the first hop&dart and lordosis posture was measured. In order to assess basal levels of sexual receptivity,the lordosis quotient (LQ) was consideredas the number of lordosis divided bythe number of mounts (with or without intromission or ejaculation). In addition, the intensity of lordosis was assessed using the three-point scale (1= marginal lordosis, 2 = lordosis with head at an approximate angle of $30°$ from horizontal and 3 = lordosis with head at an approximate angle of $45°$ or more from horizontal) proposed byHardy & DeBold(1972) and amean lordosis intensity*per* female (sum of lordosis intensity points/ total number of lordosis) was calculated.

Furthermore, on the basis that adolescent females might display play behavior and high social investigation during both types of interactions,the number of corporal sniffings (on the nape, back orventralregions of the stimulus animal) and anogenital sniffings (on the anogenital region of the stimulus animal), as well asthe number ofcrawling over (explore the stimulus animal by mounting with at least two legs or cross above) and crawling under (explore or cross below the ventral region of the stimulus animal)(Vanderschuren, Niesink, & Van Ree, 1997; Willey & Spear, 2012)behaviors were measured.Probably due to size and age differences between the experimental and social stimulusanimal (an adult female),we observed that characteristic parameters of juvenile play behavior like pinning and pouncing(Trezza, Baarendse, & Vanderschuren, 2010) were less frequent or absent. Therefore,based on a preliminary analysis of social interactions, weevaluatedcrawling over and underas these were the components of playbehavior most frequently exhibited.Because in all age groups and in both conditions (social and sexual interaction) corporal sniffings and anogenital sniffings followed the same expression pattern, we refer to their sum as social investigation.

Male sexual behavior was assessedfor the number of mounts, intromissions and ejaculations, and their respective latencies. In both, social and sexual interactions, the number of anogenital sniffings performed by the stimulus animal was alsomeasured.

2.5 Experimental procedure

In order to assess the expression of sexual motivation throughout adolescence, mid-adolescent, late adolescent and adult naturally cycling virgin females were tested either in the male versus non-receptive female preference test (mid-adolescent: n=9; late adolescence: n=9

and adults: n=9), or in the USV emission test after a brief interaction with a non-receptive female as social stimulus, or a male as sexual stimulus. To characterize the display of sexual behavior during adolescence, as well as the expression of other social behaviors during a sexual interaction, all mid-adolescent, late adolescent and adult females previously tested in the USV emission test were submitted to a social or sexual behavioral test with the same stimulus animal employed in the USV emission test. Due to technical problems with recordings fromone mid-adolescent and two adults exposed to a social stimulus, and fromtwo mid-adolescents exposed to a male, these animals were not included in the final USV analyzes. Moreover, one late adolescent and one adult female in male exposure groups did not complete the sexual behavior test, and the sexual behavior test from one mid-adolescent female was discarded due to absence of mount behavior by the male in the first three minutes of the test. Final number of subjects per group and test was as follow: *USV male*: mid-adolescent: n=10; late adolescence: n=11 and adults: n=13 and *male interaction*: mid-adolescent: n=11; late adolescence: n=10 and adults: n=12; and *USV female*: mid-adolescent: n=9; late adolescence: n=9 and adults: n=9 and *female interaction*: mid-adolescent: n=10; late adolescence: n=9 and adults: n=11.

In an attempt to reduce the difference in physical sizes of the male stimulus and the experimental adolescent females, the age of the males used for USV and sexual behavior tests varied according to the experimental groups, with lower weighing males (375g±48g) used as sexual stimulus for adolescent females, and higher weighing males (511g±49g) used for adults.

All tests were performed during the night of the proestrous stage of the oestrous cycle, 2 to 3 hrs after lights were switched off, when the age of the animals was appropriate to each

experimental group: mid-adolescence PNDs39-43 (41.7±1.3 days), late adolescence PNDs 49-53 (50.6±1.4 days) and adults PNDs 90-115 (101.3±20.6 days). These time windows were defined in order to assure that subjects were in the appropriate developmental period according to Tirelli et al. (2003).

As oestrous cycle regularity is not well-established during adolescence (Södersten, 1975) 2 to 3 h after lights were switched off, all potentially receptive females were briefly tested with a male in order to confirm sexual receptivity. Only females that displayed a lordosis posture in response to a mount were considered to be sexually receptive and used for testing.

2.6 Statistical analysis

The behavioral data obtained was tested for variance homogeneity using Levene's test and for normality using the Kolmogorov-Smirnov test. Most of the data did not show normal distribution or variance homogeneity and was, therefore, analyzed using non-parametric statistical tests and expressed in medians and semi-interquartile ranges. To compare dependent data within groups a Friedman analysis of variance by ranks test, followed by Wilcoxon matched-pairs test, were performed, while for comparisons between independent groups a Kruskal-Wallis analysis of variance, followed by a Mann–Whitney U-test, were used (Siegel & Castellan, 1988). 3x2 exact one-tailed contingency tables and Fisher Exact Probability test were used to compare the proportion of females from 3 or 2 groups, respectively, showing a particular behavior (Mehta & Patel, 1983). Weight and age data is expressed as mean ± standard errors.

3 RESULTS

3.1 Male versus female preference

Both late adolescent andadult females showed a greater percentage of visits(late adol: $T_{(9)}=1.0$, $p=0.02$andadults: $T_{(9)}=0.0$, $p\leq0.01$; Table 1) and spent more time (late adol: $T_{(9)}=0.0$, $p<0.01$ andadults: $T_{(9)}=0.0$, $p\leq0.01$; Fig. 1a) in the male chamber compared with the female chamber. Moreover, both groups showed higher number of attempts to access the stimulus animal (late adol.: $T_{(9)}=0.0$, $p\leq0.01$ andadult: $T_{(9)}=0.0$, $p\leq0.01$; Fig 1b) and sniffings (late adol.: $T_{(9)}=1.0$, $p=0.01$andadult: $T_{(9)}=1.0$, $p=0.01$; Fig. 1c) in the male, compared to the female,chamber. In contrast, although mid-adolescent females alsospent more time in the male thanin the female chamber ($T_{(9)}=6.0$, $p=0.05$; Fig. 1a), the percentageof visits ($T_{(9)}=14.0$, $p=0.58$; Table 1), number of attempts to access the stimulus animal ($T_{(9)}=8.5$, $p=0.18$; Fig. 1b) and sniffings ($T_{(9)}=10.0$, $p=0.14$; Fig. 1c), did not differ between the male and female chamber.

Time spent in the male chamber($H_{(2)}=6.6$, $p=0.04$) and attempts to access the male stimulus ($H_{(2)}=10.5$, $p\leq0.01$) also differed between age-groups: mid-adolescent females spent less time in the male chamber compared to adults ($U_{(9,9)}=14.0$, $p=0.02$; Fig. 1a), and performed a lower number of attempts to access the male in comparison to both the late adolescent and adult groups (vs.late adol.: $U_{(9,9)}=17.0$, $p=0.04$ andvs. adult: $U_{(9,9)}=6.5$, $p\leq0.01$; Fig. 1b). The proportion of subjects showing preference for the male varied across groups (preference distribution: $p=0.01$, 2x3 Exact Contingency Tables; Table 1), being significantly lower in themid-adolescent than in the adult group (preference distribution: $p=0.04$, Fisher Exact Probability Test; Table 1).

The percentageof visits ($H_{(2)}=1.42$, p=0.49; Table 1) and the time spent ($H_{(2)}=1.44$, p=0.49; Fig. 1a) in the female chamber, as well as the number of sniffings ($H_{(2)}=3.31$, p=0.19; Fig. 1c) and attempts to access the female ($H_{(2)}=2.22$, p=0.33; Fig. 1b)did not differ between groups.

Although locomotion in the Y-maze was significantly higher in late adolescence females compared to adults (number of crosses:152(2.5) vs. 117 (16.1);$U_{(9,9)}=15.0$, p=0.03), and tended to be higher in comparison to the mid-adolescent group (number of crosses:152(2.5) vs. 124 (23.5), $U_{(9,9)}=19.0$, p=0.06), this did not affect the total time spent in chambers, which did not differbetween groups ($H_{(2)}=4.39$, p=0.11).

3.2 USV emission after a brief interaction with a conspecific

Late adolescent and adult females emitted a higher number of 50kHz USV after interacting with the male in comparison with the female stimulus (late adol.: $U_{(11,9)}=14.0$, p≤0.01 and adult: $U_{(13,9)}=23.0$, p=0.02; Fig. 2); but this difference was absent in the mid-adolescent group ($U_{(10,9)}=32.0$, p=0.32; Fig. 2). All three groups emitted more FM than flat USV sub-types after both sexual and social interactions (Mid-adol. w/male: $T_{(10)}=1.0$, p=0.03 and w/female: $T_{(9)}=5.0$, p=0.04;Late adol. w/male: $T_{(11)}=0.0$, p≤0.01 and w/female: $T_{(9)}=1.0$, p=0.01 and Adult w/male: $T_{(13)}=14.0$, p=0.03and w/female: $T_{(9)}=1.5$, p=0.03; Table 2), and no 20kHz USV (0.0 (0.0) for all age-groups).

Comparison between age-groups revealed differences in the total number of 50 kHz and FM subtype USV emitted both after the interaction with the male (Total 50 kHz USV: $H_{(2)}=10.0$, p≤0.01andFM 50 kHz USV: $H_{(2)}=12.6$, p≤0.01)and the female (Total 50 kHz USV:

$H_{(2)}=6.6$, p=0.04 andFM 50kHz USV: $H_{(2)}=6.6$, p=0.04). Thus, after interacting with the male, late adolescent females emitted a higher number of total 50kHz and FM subtype USV in comparison with mid-adolescent and adult rats (Total 50 kHz USV: vs.mid-adol.: $U_{(11,10)}=13.0$, p≤0.01 andvs.adult:$U_{(13,11)}=34.0$, p=0.03; Fig.2; and FM 50kHz USV: vs.mid-adol: $U_{(11,10)}=10.0$, p≤0.01 and vs.adult: $U_{(13,11)}=26.0$, p≤0.01; Table 2). On the other hand, after interacting with the female, the emission of total 50kHz USV, as well as FM and flat USVs sub-types, was higher in mid- and late adolescentratscompared to adults (Total 50 kHz USV: vs.mid-adol.: $U_{(9,9)}=15.5$, p=0.02 andvs.late adol.:$U_{(9,9)}=15.5$, p=0.02; Fig.2, FM 50kHz USV: vs.mid-adol: $U_{(9,9)}=16.0$, p=0.03andvs.lateadol.: $U_{(9,9)}=15.0$, p=0.02andflat 50kHz USV: vs.mid-adol: $U_{(9,9)}=16.5$, p=0.03 and vs.lateadol.: $U_{(9,9)}=16.5$, p=0.03; Table 2).

3.3 Social/sexual interaction test

3.3.1 Sexual behavior with a male

Although when interacting with a male all groups showed high levels of sexual receptivity (LQ = 1.0 (0.0) for all groups), the mean lordosis intensity was significantly reduced in the mid-adolescence group compared to late adolescent and adult females (mid adol: 2.3 (0.3), late adol: 2.7 (0.1) and adult: 2.8 (0.1), mid adol vs. late adol: $U_{(10,11)}=18.5$, p≤0.01 and vs. adult: $U_{(12,11)}=14.0$, p≤0.01). The display of paracopulatory behaviors (presentation postures and hops & darts) did not differ between groups (presentation postures: $H_{(2)} = 3.5$, p= 0.17andhops &darts: $H_{(2)} = 0.35$, p = 0.8; Fig. 3). Mid-adolescents showed a higher latency to lordosis compared to late adolescents and adults (latencies in seconds: mid-adol: 90.3(50.5), late adol: 15.3 (5.3) and adult: 13.5 (6.3), mid-adol vs. late adol: $U_{(10,11)} = 17.0$, p≤0.01 and vs. adults $U_{(11,12)}= 16.0$, p≤0.01), while the latency to display proceptive behaviors did not

differ between groups (latencies in seconds: mid-adol: 26.6 (42.1), late adol:15.5 (7.1) and adult:24.8 (8.6); $H_{(2)}$=2.1, p=0.33).

The number of anogenital sniffings, mounts, intromissions and ejaculations performed by the males did not differ between groups (sniffing $H_{(2)}$=3.9, p=0.14, mounts $H_{(2)}$=4.5, p=0.11, intromissions$H_{(2)}$=4.1, p=0.13 and ejaculations $H_{(2)}$=1.4, p=0.5, Table 3). However, males exhibited a higher latency to mount mid-adolescent rats in comparison to late-adolescent and adult females (vs. late adol: $U_{(10,11)}$= 22.0, p= 0.02 and vs. adults: $U_{(11,12)}$= 19.0, p≤0.01; Table 3)

3.3.2 Sexual behavior with a female

Sexually active females also exhibited paracopulatorybehaviors towards the non-receptive female during the social interaction. However, while 55% of adults (6/11) displayed presentation postures and hops&darts, only 22% (2/9) of late, and 10% (1/10) of mid-adolescent rats expressed these behaviors (mid-adol. vs. adult: p=0.04, Fisher Exact Probability test, one-tail). Nevertheless, the number of hops & darts displayed was very low and did not differ between groups ($H_{(2)}$ = 5.37 p= 0.68), and the expression of presentation postures only tended to be greater in adult than in late adolescent rats (vs.mid-adol: $U_{(11,10)}$ = 31.0, p= 0.11 and vs. late adol: $U_{(11,9)}$ = 26.0, p= 0.08).

The number of anogenital sniffing of the social stimulustowards the experimental female did notdifferbetween age-groups (towards mid-adol:13.5 (8.6), late adol: 18.0 (4.0), and adult: 13.0(5.5), $H_{(2)}$ = 1.4, p = 0.50).

Although females displayed proceptive behaviors in both, sexual and social interactions, all females performed more hops & darts towards the male than towards the non-receptive female (mid-adol.: $U_{(10,11)} = 9.0, p \leq 0.01$; lateadol.: $U_{(9,10)} = 0.0, p \leq 0.01$ and adult: $U_{(11,12)} = 16.5$, $p \leq 0.01$, Fig. 3). Moreover, late adolescent females exhibited a greater number of presentation postures towards a male than towards a non-receptive female ($U_{(9,10)} = 15.5$, $p = 0.01$, Fig. 3).

3.3.3 Social and play behavior with a male

Mid-adolescent females displayed more social investigation (sum of anogenital and corporal sniffings) towards the male than adult females ($U_{(11,12)} = 10.5$, $p \leq 0.01$; Fig. 3c), while the expression of this behavior did not differ between late-adolescent females and the other two groups (vs.mid-adol: $U_{(10,11)} = 31.5$, $p = 0.1$ and vs.adults: $U_{(10,12)} = 35.0$, $p = 0.1$).

In a similar way, the expression of play behavior during a sexual interaction was greater in mid-adolescentcompared to adult females ($U_{(11,12)} = 12.0, p \leq 0.01$; Fig. 3d), while late-adolescent females showed an intermediate profile as their play behavior did not differ from mid-adolescent ($U_{(10,11)} = 34.5$, $p = 0.15$)oradult($U_{(10,12)} = 36.0$, $p = 0.14$) rats. However,the number of females displaying play behavior in this group was greater than that of the adult group (4/10 lateadol. vs. 0/12 adult; p=0.03, Fisher Exact Probability Test) and tended to be lower than that of the mid-adolescent group (4/10 lateadol. vs.9/11 mid-adol., p=0.08, Fisher Exact Probability Test).

3.3.4 Social and play behaviors with a female

During a social interaction, both adolescent groups exhibited more play behaviorand social investigation than adults (social investigation: vs.mid-adol.: $U_{(11,10)} = 17$, p= 0.02andvs. lateadol:$U_{(11,9)} = 1$, p≤0.01, Fig. 3c; and play behavior: vs.mid-adol: $U_{(11,10)} = 0$, p≤0.01 and vs. lateadol.:$U_{(11,9)} = 1.5$, p≤0.01; Fig. 3d). Both behaviors were higher in the mid-adolescentcompared to the late-adolescentgroup(social investigation: $U_{(10,9)} = 9$, p≤0.01andplay behavior: $U_{(11,9)} = 16.5$, p= 0.02).

Social investigation and play behavior were significantly greater when females of all age-groups interacted with the non-sexually receptive female compared with a sexually active male (social invest: mid-adol $U_{(10,11)} = 0$, p≤0.01; late adol. $U_{(9,10)} = 0$, p≤0.01 and adults $U_{(11,12)} = 2,5$, p≤0.01 and play: mid-adol $U_{(10,11)} = 0$, p≤0.01;late adol $U_{(9,10)} = 3,5$, p≤0.01; adults $U_{(11,12)} = 24$, p≤0.01).

4 DISCUSSION

Although in naturally cycling female rats sexual behavior is first expressed during the middle stage of adolescence, the present study shows that sexual motivation at this developmental timeis lower than that observed in later adolescent and adult phases. Unlikethe females in laterdevelopmental stages,mid-adolescent females did not perform a greater number of sniffings or attempts to access the male compared to the female stimulus in the preference task, nor was their USV emission higher after interacting witha male relative to a female.

Despite this reduced sexual motivation, we found that, during a sexual interaction, naturally cycling mid-adolescent females showed maximal expression of proceptive paracopulatory behaviors. Nevertheless, although sexual receptivity was also maximal, the reduction in the magnitude of the lordosis response suggests a lower sensitivity to male's mounting stimulation. Interestingly,this adult-like sexual behavior of mid-adolescent females wasaccompaniedbythe expression of behavioral components typical of juvenile play and high social investigation, giving rise to a unique behavioral repertoire of younger adolescent rats during a sexual interaction.

4.1 Sexual motivation throughout adolescence

As expected, late proestrous females from allage groups spent a greater time in the male chamber than in the female chamberin the preference test, highlighting the sexual incentive value of a male for a sexually active female(Ågmo et al., 2004; Clark et al., 2004). In line with previous results from our group (Agrati et al., 2018), the behavior of late adolescent and adult females in this test did not differ.However,thereduced preference for the male of mid-adolescent rats when compared to adults, as well as their lower effort to access the male, indicates that the male has a lower incentive value for a sexually active female in this early developmental stage. This idea is further supportedbythesimilaremission of 50kHz USV by mid-adolescent females after interacting with a sexual or socialstimulus.Forlate adolescent and adult rats, however,theemission of 50kHz USV was higher after interaction with the sexual than a social stimulus; aresultthat is in line with previous studies employing adult ovariectomized hormonally primedfemales(McGinnis & Vakulenko, 2003).

Although it has been suggested that the subtype of 50 kHz USV emitted by females may differ according to the sex of the stimulus animal(Börner et al., 2016), in the present study all females emitted a higher number of FM than flat USV, regardless ofthe nature of the stimulusanimal.A higher emission of FM compared to flat USVs aftersocial and sexual interactions has been previously reported (Mulvihill & Brudzynski, 2018). However, authors from this study report that the emission of trill-shaped 50kHz FM USV washigherfor the sexual condition, suggesting that other characteristics of the USV emission may differ between the exposure to sexual and social incentives.

Interestingly, both adolescent groups emitted more USV after interacting with a female than the adult group; a result that could be indicative of a higher incentive value of a social stimulus for a sexually receptive female during this period. In this line of thought, it has been reported that the tendency to engage in play behavior with a same-age conspecific is maximum during mid-adolescence and decreases to negligible levels in adult rats (Panksepp, 1981). Our present results extend this idea, showing a similar tendency when sexually active females of different developmental stages interact with a non-receptive adult female (see discussion below). Moreover, Douglas, Varlinskaya, & Spear (2004) reported that adolescent, but not adult, male rats develop place preference conditioning for an environment associated with a social interaction. Thus, our results regarding 50 kHz USV emission of sexually active females after interacting with a social stimulus suggest that social motivation shows a decreasing pattern from adolescence to adulthood for sexually active cycling females. This opposite developmental pattern of social and sexual motivations supports the idea that the increase in the incentive value of the male for sexually-active female throughout adolescence represents a selective increment of sexual -rather than social- motivation.

4.2 Sexual and social behaviors throughout adolescence

Despite showing lower sexual motivation, mid-adolescent females exhibited asimilarexpression of receptive and proceptive paracopulatory components from those of late adolescent and adult rats during the sexual interaction, indicating that on this early stage of development females are able to display the complete sexual behavioral repertoire. This was previously suggested bySödersten(1975) and Hansen (1977), who observed that cycling Wistar rats exhibit their first behavioral estrus in response to manual stimulation around day 40 of life. At this same age, female rats raised together with males begin to displaylordosis and proceptive behaviors(Meaney & Stewart, 1981).Despite been highly receptive, mid-adolescent rats expressed lordosis response of lower magnitude, a result that could be relatedto a reduced responsiveness of these females to the flank and perineal pressure exerted by themale´s mounting (Pfaff, Montgomery, & Lewis, 1977). This could bea consequence of a differentialsensitivity to ovarian steroids of the neural circuitry regulating this response(Kow & Pfaff, 1998), as several studies show that ovarian steroids modulate the magnitude of lordosis (Domínguez-Ordóñez et al., 2016; Olster & Blaustein, 1988; Pfaff et al., 1977).

Males exposed to mid-adolescent females exhibited a higher latency to mount, and tended to express a lower number of mounts, while interacting with mid-adolescent females. Given that the number of proceptive behaviors and the latency to display them did not differ between age groups, changes in male sexual response could be due to differences in female attractivity, which is known to be modulated by different physiological variables (Brown, 1977; Lucas, Donohoe, & Thody, 1982; Lydell & Doty, 1972). Furthermore, differences in attractivity could also be due to a differential emission of50 kHz USV by the female during

sexual interaction (Agmo & Snoeren, 2017; Barfield et al., 1979; White & Barfield, 1987, 1989).

A higher percentage of adult females displayedhops&darts and presentation postures during the social interactionwhen compared to mid-adolescent rats. As these paracopulatory behaviors are commonly displayed in response to physical stimulation(Blaustein, 2008),it is possible that they were expressed in response to social investigation and physical contact bythe stimulus animal. Given that social investigation by the social stimulus was equal towards all groups, the higher expression of paracopulatory behaviors by adult females couldbe a consequence of a higher sensitivity to such physical stimulation that is not yet fully mature during mid-adolescence. The reduced magnitude of the lordosis response of mid-adolescent females to male mounting stimulation support this idea, yetfuture experiments should be conducted to explore this possibility.

Adult-like sexual behavior in mid-adolescent rats was expressed togetherwithhighersocialinvestigation and play behavior, indicating that while sexually interacting with a male, these females express other social motivations characteristic of their developmental stage (Meaney & Stewart, 1981; Thor & Holloway, 1984; Vanderschuren et al., 1997). The expression of these behaviors did not seem to interfere with the display of sexual behavior, as no differences were observed between age groups in sexual proceptive components. Although it could be considered that the expression of hops &darts by mid-adolescent females during the sexual encounter is part of their play behavioral repertoire(Thor & Holloway, 1984), the absence of these behavioral elements in their social interaction with a non-receptive femaledoes not support this claim. Thus, our results indicate a unique behavioral profile of mid-adolescent females during sexual interaction characterized

by both, the expression of a full sexual behavior repertoire, andofsocial investigation and play.

Late adolescent females showed an intermediate behavioral profile between mid-adolescent and adult rats, as they exhibited similar levels of sexual motivation as adults, but an intermediate expression of other social motivations.The gradual reduction in play behavior and social investigation from mid to late adolescence, is in agreement with several studies reporting that the expression of these behaviors declines from mid to late adolescence and from late adolescence to adulthood(Klein, Padow, & Romeo, 2010; Panksepp, 1981). Moreover, as previously discussed, a greater social interest of adolescent rats is also suggested by ahighemissionof 50 kHz USV after a brief social interaction. Thus, when sexual and social interactions are considered together, late adolescence in naturally cycling females appears to be a particular developmental periodwith high expression of both sexual and social motivations.

4.3 Conclusions and final remarks

We reportanincrease in sexual motivation in the virgin cycling female rat throughout adolescence; a motivational change that seems to bespecificofsexual motivation, as social motivation showsan opposing developmentalpattern.Although the sexual behavior repertoire was completed by mid-adolescence, during a sexual interaction adolescent females presented a unique behavioral profile that included the expression of other behaviors typical of their developmental period.The full expression of sexual behavior of mid-adolescentfemalesseems to indicate that the neural circuits that underlie its expression are fully operating. However the lower sexual motivation and possible lower sensitivity to mounting stimulationfound in

this study, together with previous work reporting agradual increase in estrogen sensitivity to induce proceptivity throughout adolescence (Södersten, 1975), suggest that the neuroendocrine mechanisms underlying sexual motivation are still maturing during this period. Moreover, when sexual motivation and behavior are considered together, differences between mid and late adolescents arise. Thishighlightsthe importance of considering adolescence not only as different from infancy and adulthood, but asanheterogeneous period itself. Hence, we suggest that the fragmented studyof adolescence isessentialforthe full comprehension of behavioral developmental dynamics.

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Table 1. Percentage of chamber entries [median (SIQR)]and group preferences (number of animals showing preference for stimulus chamber/total number of animals in the group) in the male-female preference task

	Percentageof entries to the chambers			Preference		
	Male	Female	Neutral	Male	Female	No preference
Mid-adol	39.7% (10.1)	35.0% (7.0)	18.5% (3.4)[a, bb]	5/9[*]	1/9	3/9
Late adol	50.9% (6.3)[a]	38.8% (1.7)	13.7% (4.3)[aa, bb]	8/9	0/9	1/9
Adult	44.4% (4.4)[aa]	35.6% (3.3)	19.4% (3.1)[aa, bb]	9/9	0/9	0/9

[a] $p \leq 0.05$, [aa] $p \leq 0.01$ vs. female; and [b] $p \leq 0.05$, [bb] $p \leq 0.01$ vs. male within group comparison, Wilcoxon matched-paired test.[*] $p \leq 0.05$ vs. adult in male preference, Fisher Exact Probability test. n= 9/group

Table 2.Median (SIQR) of the number of FM and flat 50kHz USV sub-types and proportion of FM USV emitted by females after an interaction with a female or male stimulus

	Female stimulus			Male stimulus		
	FM	Flat	$\dfrac{\text{FM}}{\text{FM + flat}}$	FM	Flat	$\dfrac{\text{FM}}{\text{FM + flat}}$
Mid-adol	81.0 (40.0)[*]	40.0 (10.5)[*a]	0.66 (0.05)	33.0 (39.5)[††]	25.0 (24.6)[a††]	0.59 (0.07)
Late adol	62.0 (54.5)[*]	35.0 (22.5)[*aa]	0.64 (0.04)	217.0 (95.0)[##**]	109.0 (48.0)[aa*]	0.72 (0.06)
Adult	6.0 (10.0)	2.0 (5.0)[a]	0.66 (0.07)	78.0 (69.0)[#]	27.0 (48.5)[a, #]	0.66 (0.14)

[a] p≤ 0.05, [aa] p≤ 0.01 vs. FM within a group with same stimulus, Wilcoxon matched pairs test; [#] p≤ 0.05, [##]p≤ 0.01 vs. female in the same age group; and [*]p≤ 0.05, [**]p≤ 0.01 vs. adult and [†] p≤ 0.05,[††] p≤ 0.01vs. late adolescent with same stimulus, Mann-Whitney U-test. For FM proportion p=ns, Mann-Whitney U-test. Female stimulus: mid adol n=9; late adol n=9; adult n=9. Male stimulus: mid adol n=10; late adol n=11; adult n=13

Table 3.Male sexual behavior during sexual interaction[median (SIQR)] with mid-adolescent, late adolescent or adult females

	Number				Latency (s)	
	Sniffing	Mount	Intromission	Ejaculation	Mount	Ejaculation
Mid-adol	16.0 (3.5)	3.0 (1.5)	6.0 (0.8)	1.0 (0.0)	52.2 (56.5)[**†]	328.2 (119.7)
Late adol	10.5 (6.0)	4.5 (0.9)	8.0 (1.1)	1.0 (0.8)	15.0 (5.3)	260.9 (221.9)
Adult	18.0 (8.9)	4.5 (1.9)	8.5 (1.6)	1.0 (0.5)	13.2 (5.4)	508.0 (179.7)

**p≤0.01 vs. Adult and † ≤ 0.05 vs. Late adol, Mann-Whitney U-test. Mid-adol n=11, late adol n=10 and adult n=12.
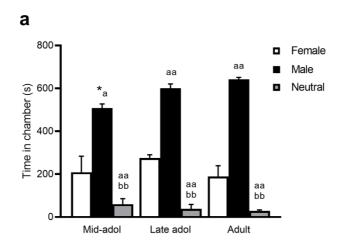
Figure legends

Figure 1. Time (in seconds)spent in female (white bars), male (black bars) and neutral (grey bars) chambers (**panel a**), number of attempts to access the female (white bars) and male (blackbars) stimulus (**panel b**), and number of sniffings in the female (white bars) andmale(black bars) chambers(**panel c**), displayed bymid-adolescent, late adolescent and adult females (n=9/group) in the Y maze.Data is expressed in medians (semi-interquartile range). [a] $p \leq 0.05$, [aa] $p \leq 0.01$ vs. female within a group;and[b]$p \leq 0.05$, [bb] $p \leq 0.01$ vs. male within a group, Wilcoxon matched-pairs test. * $p \leq 0.05$, ** $p \leq 0.01$ vs adultand[†]$p \leq 0.05$ vs late adolescent, Mann-Whitney U-test.
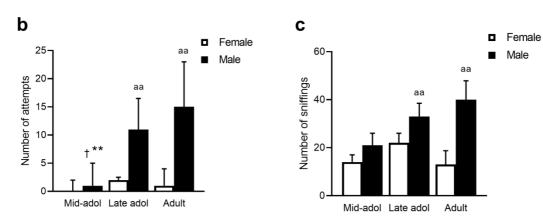
Figure 2. Total number of 50 kHz USV emitted by mid-adolescent, late adolescent and adult femalesafterinteraction with a female (white bars – mid adol n=9; late adol n=9; adult n=9) or a male (black bars – mid adol n=10; late adol n=11; adult n=13) stimulus.Data is expressed as medians (semi-interquartile range).[a] $p \leq 0.05$, [aa] $p \leq 0.01$ vs. female within a group and* $p \leq 0.05$ vs. adults,and † $p \leq 0.05$, †† $p \leq 0.01$ vs. late adolescent with same stimulus, Mann-Whitney U-test.

Figure 3. Total number of presentation postures (**panel a**),hops & darts (**panelb**),social investigation (**panel c**) and play behavior (**panel d**)displayedbymid-adolescent, late adolescent and adult females during an interaction with a non-receptive adult female (white bars – mid adol n=10; late adol n=9; adult n=11) or amale (black bars – mid adol n=11; late adol n=10; adult n=12). Data is expressed as medians (semi- interquartile range). [a]$p \leq 0.05$,

[aa]p≤0.01 vs.female; [*]p≤ 0.05, [**]p≤ 0.01 vs.adults; and [†]p≤0.05, [††]p≤0.01 vs.lateadolescent,
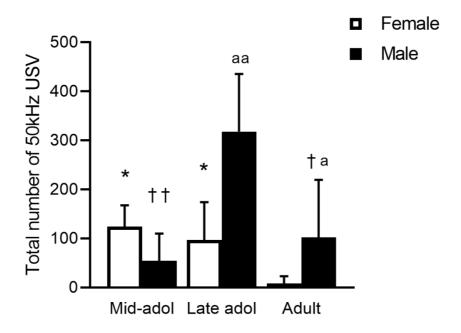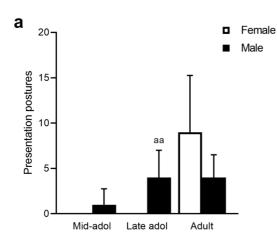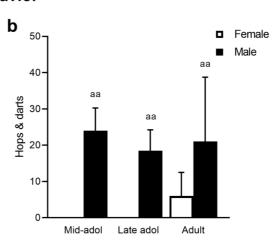
Mann–Whitney U-test.

FIGURE 1

FIGURE 2

FIGURE 3

Sexual behavior

Social behavior