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Environmental-Like Exposure to Low Levels of Estrogen Affects Sexual Behavior and Physiology of Female Rats

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Xenoestrogens are endocrine-disrupting chemicals that mimic the action of endogenous estrogen hormones. Effects of xenoestrogen on aquatic wildlife are well documented, whereas the experimental evidence for impairment of reproductive behavior and physiology in mammals after exposure to xenoestrogens has been debated. The strongest arguments against such studies have been that the route, time course, and intensity of exposure did not simulate environmental exposure and that the chemicals tested have additional nonestrogenic toxic effects, hindering generalization of actual xenoestrogenic effects. Here we show that environmental-like exposure to the pure estrogen 17α-ethinylestradiol during development alters reproductive behavior and physiology in adult female Sprague-Dawley rats. We simulated environmental exposure by giving low doses (0.4 and 0.004 μg/kg·d) of 17α-ethinylestradiol orally to pregnant females from conception to weaning of the pups, which continued to receive the treatment until puberty. We studied the sexual behavior, estrous cycle, and estradiol plasma levels of intact female rats when they reached 3 months of age. Exposure to the higher dose strongly affected female sexual behavior and physiology, with suppression of lordosis and the estrous cycle and enhanced aggression toward males. The lower dose disrupted appetitive components of sexual behavior that influence the rate of copulation. Estradiol plasma levels were not affected by the treatment. Our study revealed that exposure to low oral doses of a pure estrogen during development alters female sexual behavior and physiology. These results suggest potential risks of reproductive failure from xenoestrogen exposure in realistic ecological conditions. (*Endocrinology* 149: 5592–5598, 2008)

DURING THE LONG developmental period from conception to the attainment of sexual maturity, sex hormones strongly influence the anatomical and functional organization of the central nervous system (CNS), sex organs, and skeletal and muscular structure, thus playing a key role in sexual differentiation (1, 2). In particular, the presence of estradiol in the perinatal period, from conception to puberty, determines the male organization of the CNS, whereas its absence results in female organization (3–8). The female is protected from the masculinizing action of estrogens by α-fetoproteins, which reduce the amount of active estrogen (9–11). In litter-bearing mammals, even small physiological changes of the hormonal milieu during pregnancy can affect adult sociosexual behavior and morphological reproductive parameters of the offspring (intrauterine position phenomenon) (12).

Studies on wildlife have shown that prenatal exposure to synthetic chemicals can interfere with the endocrine system and other vital systems during development (13). Some of the environmental chemicals associated with adverse reproductive and developmental effects in animals mimic the action of estrogenic hormones. It has been hypothesized that exposure to these compounds, generically referred to as xenoestrogens, can produce similar adverse effects on human reproduction and development (14, 15). In addition, environmental agents with estrogenic or antiestrogenic effects may play a role in reported declines in sperm counts, increased incidences of testicular and prostate cancer, and observable abnormalities (16, 17).

Behavior is the final point of confluence of complex integrated systems, which can be influenced by subtle environmental alterations. The study of behavior, supplemented by the analysis of neuroendocrine parameters, can provide indications about the effects of xenoestrogens in the phases of development in which behavioral circuits are organized (18). During the period when the reproductive organs and the CNS are undergoing rapid and irreversible developmental changes, xenoestrogens at environmentally relevant concentrations, *i.e.* within the measured range of exposure for human and wildlife populations, can lead to irreversible alterations of development and ultimately behavior (19).

In the rat, as in most mammals, behavior and neuroendocrine function are reciprocally linked in the control of reproduction. The neuroendocrine events necessary for ovulation, ejaculation, and implantation are triggered and modulated by specific patterns of copulatory behavior (20). During estrus, the female rat displays receptive behavior together with a varied and complex pattern of proceptive behavior, which trigger copulatory mounts by the male (21). Receptive behavior is mainly lordosis, the reflexive posture of the consummatory phase (stereotyped copulatory response) (22, 23), whereas proceptive components reflect the appetitive and precopulatory aspects of female sexual behavior (flexible behaviors such as solicitation and courtship) (23–25). In a previous study, we showed that perinatal exposure to the xenoestrogen bisphenol A affects female sexual behavior, increasing motivation and receptivity but not pro-

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Abbreviations: CNS, Central nervous system; EE2, ethinylestradiol; d, duration; f, frequency; l, latency; PND, postnatal day.

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ceptivity (26). Nevertheless, some of the reported effects of xenoestrogens at low doses, as well as the direction of observed behavioral modifications, cannot be fully explained by a classical estrogenic action involving binding to estrogen receptors (14, 27, 28).

In this experiment, we studied sexual behavior of intact female rats exposed to low doses of a pure estrogen from conception to puberty. We selected ethinylestradiol (EE2) because it is a pure synthetic estrogen, the main estrogenic component of the contraceptive pill. It is estimated that each year almost 2 million women who use oral contraceptives become pregnant, and often pills are taken until the unexpected pregnancy is discovered (29–31). Thus, very many human fetuses are exposed to EE2 in the early phases of development (13). In addition, because of its widespread use, EE2 is also an environmental estrogen and is commonly found in urban sewage water (32–35). EE2 seems to be more resistant to biodegradation than other estrogens and accounts for 35–50% of the estrogenic activity in rivers (35, 36). High doses of EE2 given prenatally disrupt reproductive function in both male and female rodents (37–42) and cause toxicity and elicit behavioral abnormalities in the rat (43–45). Recent studies have shown altered prostate growth and daily sperm production in male mice exposed prenatally to subclinical doses of EE2 (31) due to the disruption of mouse prostate and urethra development (46) as well as reproductive failure in fish exposed to environmental concentrations of EE2 (47). To our knowledge, no study has examined the effects of environmentally relevant doses of EE2 on mammalian sexual behavior.

Materials and Methods

Animals and treatment procedure

Thirty-six mature Sprague Dawley female rats were used. They were born and bred in the Physiology Department, University of Siena, from 36 females and 20 males purchased from Harlan Italy (S. Pietro al Natisone, Italy). Breeding pairs were housed in Plexiglas cages (Tecniplast, Buguggiate, Italy; 60 × 37 × 20 cm) with metal tops and a wire netting floor to allow the daily search of the vaginal plug. After detection of the vaginal plug (gestational d 0), the male was removed and the female was housed individually. On postnatal day (PND) 2, pups were temporarily removed from the mother and gently placed in a cotton nest; each animal was weighed with an analytical scale and the anogenital distance was measured. The litter was reduced to five male and five female pups and left with the mother until weaning on PND 21. On PND 32 juveniles of each litter were individually marked, separated, and randomly housed in groups of four, according to sex, so that no cage contained siblings. Only one female per litter was used in the present study.

All rats were housed in Plexiglas cages (Tecniplast; 60 × 37 × 20 cm) with metal tops and sawdust bedding in an air-conditioned room (temperature 21 ± 1 C, relative humidity 60 ± 10%), with a 12-h light, 12-h dark cycle (lights on from 1930 to 0730 h). Water and food (Diet Harlan Teklad) were available *ad libitum*. All experimental procedures followed European Community Council Directive 86/609/EEC and institutional guidelines.

The mothers of experimental females were trained to receive peanut oil (OIL; Sigma-Aldrich, Milan, Italy) orally via a micropipette for 5 d before pairing. EE2 (Sigma-Aldrich) at 0.4 μg/kg·d (EEH, n = 12), 0.004 μg/kg·d (EEL, n = 12), or vehicle only (OIL, n = 12) were given orally (100 μl/d of solution) to the mothers during gestation (gestational d 5–21) and lactation (PND 0–21). From weaning to puberty (PND 21–32), treatments were given orally directly to the experimental animals. With this procedure, experimental rats received the treatment from conception to puberty: indirectly during the perinatal period (42 d) and directly during the pubertal period (10 d). We consider 0.004 μg/kg·d (EEL) an environmental dose because it matches concentrations of EE2 found in contaminated surface waters (47, 48). In fish, body concentrations of EE2 are about 500-fold higher than water concentrations (49, 50). Therefore, animals or humans that regularly eat fish from contaminated waters would ingest an amount of EE2 relative to body weight similar to the levels found in the water. The 0.4 μg/kg·d dose (EEH) is equivalent to that of most estrogenic or estrogen plus progestin contraceptive pills. We chose this dose because it is estimated that every year 1–2 million human fetuses are exposed to similar doses of EE2 during the first months of development because of undetected pregnancy during contraceptive therapy (missed pill) (51). Sexually mature and experienced male rats, purchased from Harlan Italy, were used as a stimulus in the female sexual activity test. To avoid any bias, males were rotated among females of different treatments.

Behavioral testing

Behavioral testing started after PND 90. Tests were performed during the dark phase (0900–1500 h) under dim red light combined with low indirect white light. All sessions were recorded with a video camera (AVC-D5CE; Sony Italia SpA, Cinisello Balsamo, Italy); the video recordings were later analyzed with The Observer Video Pro 4.0 software (Noldus Information Technology, Wageningen, The Netherlands) by an observer blind to treatment.

Female sexual activity test. Vaginal smears were taken from the experimental females daily 1 h after lights off until estrus was detected, in which case the female underwent behavioral testing on the same day. Each female was tested only once for sexual activity in a black Perspex arena (80 × 80 × 35 cm); a small cage (25 × 12 × 12 cm) of transparent Perspex, with two holes (diameter 5 cm) large enough to allow passage of the female but not the male, was fixed on one side of the arena. In this protocol, females can control the timing of male mounts (pacing behavior). Females were allowed to familiarize with the small cage, including passing through the holes, before the test. At the beginning of the test, the female was put in the small cage and the stimulus male (a sexually mature, experienced subject) was placed in the center of the arena. The frequency (f), duration (d), and/or latency (l) of the following male and female behaviors were recorded for 20 min (20, 25, 26):

Entrance (l, f): the female's first entrance from the small cage into the compartment containing the male, number of entrances; in d: total time spent by the female in the small cage.

Exploration (d): female nonsocial exploration activities.

Introductory behavior (d): female approach, sniffing, grooming, and anogenital sniffing (d) and female anogenital sniffing.

Solicitation (f, l): female jerky run to male, crawl over male head, walk past and runaway.

Hop/dart gait (f): female hopping and darting.

Lordosis (f, l): number of female lordosis, time from the beginning of the test to first lordosis.

Female self-grooming (d).

Retreat (f): female runaway without previous solicitation and/or kickout of the male.

Percent exits: percentage of times the female leaves the compartment containing the male after a partial or complete sexual interaction (mount/intromission/ejaculation).

Return latency: the amount of time the female remains in the small cage avoiding the male after mount/intromission/ejaculation.

Aggression (f): any female aggressive acts (from aggressive grooming to boxing, *etc.*).

On back (f): female submission.

Male mounting (f): male mounts (with or without intromission).

Ejaculation (l): latency to reach first ejaculation.

Solicitation and hop/dart gait represent the proceptive components of sexual behavior. Another proceptive component, ear-wiggling behavior, was not scored because it was difficult to record with our experimental setup. A female was considered receptive if she showed at least two episodes of lordosis and proceptive if she showed at least two episodes of solicitation and/or hop/dart gait. Percent exits and return latencies represent pacing behavior, the female's control of the rate of copulation (26). The lordosis quotient (no. lordosis/no. mounts × 100) and the total social activity were also calculated.

TABLE 1. Physiological and morphological parameters of treated rats at birth and during development

	PND	Sex	OIL (n = 12)	EEL (n = 12)	EEH (n = 12)	F test	*P*
Number of pups	2	M	7.00 ± 0.52	6.75 ± 0.60	6.17 ± 0.52	0.60	0.55
	2	F	6.75 ± 0.54	7.00 ± 0.43	6.75 ± 0.70	0.07	0.94
	2	M+F	13.75 ± 0.66	13.75 ± 0.70	12.92 ± 1.02	0.59	0.56
Anogenital distance	2	M	3.79 ± 0.08	4.09 ± 0.17	4.09 ± 0.13	0.13	0.88
	2	F	1.31 ± 0.04	1.41 ± 0.06	1.40 ± 0.10	1.74	0.19
Litter mass (g)	2	M+F	85.78 ± 4.84	86.94 ± 3.18	81.23 ± 6.02	0.54	0.59
Body mass (g)	2	F	6.04 ± 0.09	6.19 ± 0.15	6.14 ± 0.15	0.36	0.70
	7	F	13.02 ± 0.51	13.34 ± 0.50	12.86 ± 0.45	0.25	0.78
	14	F	27.59 ± 1.06	28.33 ± 0.63	27.49 ± 0.74	0.30	0.74
	22	F	47.41 ± 1.70	48.86 ± 1.14	47.90 ± 1.43	0.26	0.77
	32	F	83.91 ± 2.30	90.26 ± 3.69	86.26 ± 2.87	1.14	0.33

The last two columns report the results of one-way ANOVA among treatment groups (F statistics and significance). OIL, Vehicle only; EEL, EE2, 0.004 μg/kg·d; EEH, EE2, 0.4 μg/kg·d; M, male pups; F, female pups.

Estrous cycle

The stages of the estrous cycle were determined by examining morphological changes in vaginal epithelial cells under light microscopy (52). Vaginal smears were collected using a micropipette filled with 50 μl of saline, inserted into the vagina by a dorsal approach, and flushed until the saline became cloudy. This noninvasive procedure reduces the occurrence of pseudo-pregnancy (53).

Hormonal measurements

At the end of the behavioral tests, the animals were euthanized with an overdose of Nembutal (Sigma-Aldrich). A blood sample was taken with a heparinized syringe from the vena cava after the thoracic cavity of the anesthetized animal was opened. No blood was obtained from 1 EEH and 1 OIL female. The blood was centrifuged to separate plasma, which was collected and stored at −40 C until assayed. The concentration of 17β-estradiol in the plasma was measured in duplicate aliquots (200 μl) with an I^{125}-based RIA kit (DSL-39100; Diagnostic Systems Laboratories, Webster, TX). The assay has insensitivity of 0.6 pg/ml with the lowest standard concentration being 1.5 pg/ml. The intrassay coefficient of variation was less than 10.0%. The measurement of two external controls with known concentrations of 10 ± 2.5 and 30 ± 7.5 pg/ml yielded 12.6 and 29.7 pg/ml, respectively.

Statistical analysis

χ^2 analysis (2 × 2 contingency table) was used to compare the proportions of animals showing behaviors among treatments. Female behavior was analyzed by one-way ANOVA with treatment as the between-subjects factor. Variables that did not have a normal distribution were square root transformed. If transformed data did not have homogeneous variance (Bartlett's test, $P < 0.05$), they were analyzed with nonparametric Kruskal-Wallis ANOVA or Mann-Whitney *U* tests. Multiple comparisons were performed using least significant differences or Dunn tests (54).

Mean body weights of male and female pups at PND 2, 7, 14, 21, and 32 were analyzed by repeated-measures ANOVA, with treatment and sex as between-subjects factors and week as within-subjects factor.

Results

Developmental effects and reproductive physiology

There were no effects of the treatment on physiological parameters at birth (Table 1). Because the animals were not individually marked until PND 32, these data refer to not only the animals used in the present work but also the entire F1 litters, and they have appeared in previous publications (45, 55). At PND 2, no differences were found among groups for the number of pups per litter, sex ratio, and total litter mass. There was no significant effect of treatment on body mass from PND2 to PND 32.

Plasma levels of 17β-estradiol were above the detection limit of the RIA in all samples. The concentration of E2 (± SEM) was 54.55 ± 5.26 pg/ml in EEH (n = 11), 52.27 ± 9.26 pg/ml in EEL (n = 12), and 51.39 ± 9.69 pg/ml in OIL (n = 11) females. There was no significant effect of treatment on E2 plasma levels ($F_{2, 33} = 0.037$, $P > 0.9$).

Female sociosexual behavior

Considering the proportions of animals showing behaviors (Table 2), the 2 × 2 contingency table revealed a significantly lower number of proceptive females in both EEH ($\chi^2 = 14.40$, DF = 1, $P = 0.001$) and EEL ($\chi^2 = 4.80$, DF = 1, $P = 0.02$) than in OIL. The number of receptive females was significantly lower in EEH than in OIL ($\chi^2 = 8.22$, DF = 1, $P = 0.004$).

Table 3 reports the sexual and nonsexual elements of behavior. We performed sexual behavior tests according to sexual receptivity (estrus) as determined by vaginal smears. Most (11 of 12) EEH females were in estrus at the first smear; however, the vaginal cytology often appeared abnormal, with leukocytes mixed with cornified cells, which is typical of persistent estrus (42). These effects were confirmed by a study on the sisters of the experimental females, which showed significant effects of the treatment on the estrous cycle: most EEH females did not cycle and were in persistent estrus, whereas the cycle of EEL females did not differ from that of OIL females (55). We did not expect EEH females to be in permanent estrus when we began sexual tests because such an effect had been reported only for higher doses of EE and at a later age (42). Thus, the behavior of EEH females cannot be compared with that of the other two groups because in these females the vaginal estrus did not correspond to the behavioral estrus. Therefore, we excluded the EEH group from the ANOVA of sexual behavior variables.

TABLE 2. Female sexual activity test: number of rats displaying behavior

	OIL (n = 12)	EEL (n = 12)	EEH (n = 12)
Female proceptive	12	8[a]	3[b]
Female receptive	9	6	2[b]
Stimulus male mount	11	10	3[b]
Stimulus male ejaculation	6	4	0[b]

OIL, Vehicle only; EEL, EE2, 0.004 μg/kg·d; EEH, EE2, 0.4 μg/kg·d.
[a] $P < 0.05$ *vs.* OIL, χ^2 analysis.
[b] $P < 0.01$ *vs.* OIL, χ^2 analysis.

TABLE 3. Female sexual activity test: f, l, and d of behaviors shown by female rats (mean ± SEM)

	OIL (n = 11)	EEL (n = 12)	EEH (n = 12)
First entrance (l)	20.8 ± 5.6	19.7 ± 5.5	34.7 ± 12.2
Entrances (f)	12.8 ± 1.9	10.9 ± 1.6	13.0 ± 1.9
Exploration (d)	662.3 ± 35.9	633.8 ± 34.6[a]	760.7 ± 22.7[b]
Introductory behavior (d)	46.8 ± 6.1	52.9 ± 5.6	66.4 ± 9.9
Anogenital sniffing (d)	60.9 ± 7.4	68.7 ± 8.2	71.5 ± 7.7
Self-grooming (d)	22.6 ± 7.1	17.2 ± 5.1	12.3 ± 2.4
Retreat (f)	20.0 ± 4.9	30.9 ± 6.3	31.2 ± 5.0
Aggression (f)	0.5 ± 0.3	1.8 ± 0.8[c]	7.6 ± 2.0[b]
On back (f)	1.7 ± 1.0	4.2 ± 1.3	1.2 ± 0.4
Solicitation (l)	204.9 ± 50.3	584.0 ± 146.3[c]	962.5 ± 125.2[d]
Solicitation (f)	28.1 ± 4.9	15.1 ± 5.0[c]	2.3 ± 1.3[d]
Hop/dart gait (f)	15.6 ± 5.7	11.2 ± 5.8	0.7 ± 0.3[d]
Lordosis (l)	583.5 ± 140.8	695.2 ± 154.2	1110.6 ± 82.7[d]
Lordosis (f)	14.4 ± 4.7	10.3 ± 3.7	0.7 ± 0.5[d]
Exits (%)	8.7 ± 3.2	2.2 ± 1.4	4.3 ± 4.3[d]
Return latency (l)	11.6 ± 2.6	50.4 ± 19.4[c]	9.3 ± 9.3[d]
Lordosis quotient (%)	51.63 ± 13.50	34.55 ± 11.93	8.33 ± 8.33[d]
Total social activity (f)	154.3 ± 12.6	175.7 ± 12.1	155.3 ± 13.0

$P < 0.05$, ANOVA followed by least significant differences *post hoc* test. OIL, Vehicle only; EEL, EE2, 0.004 μg/kg·d; EEH, EE2, 0.4 μg/kg·d.

[a] EEL *vs.* OIL and *vs.* EEH.

[b] EEH *vs.* OIL and *vs.* EEL.

[c] EEL *vs.* OIL.

[d] Sexual behavior of EEH females was excluded from the ANOVA (see text for details).

In EEL-treated females, there was an overall reduction of sexual activity, compared with OIL (Fig. 1). Proceptive behavior was significantly decreased (solicit latency: $F_{1,21} = 4.73$, $P = 0.041$; solicit frequency: $F_{1,21} = 4.77$, $P = 0.040$), and the return latency component of pacing behavior was significantly increased ($F_{1,7} = 8.54$, $P = 0.022$). All other sexual activities were not significantly modified by the treatment.

Some nonsexual behaviors were affected by the treatment. In particular, we found differences for the duration of exploration ($F_{2,32} = 4.60$, $P = 0.017$) and the frequency of aggression ($F_{2,32} = 7.96$, $P = 0.0016$). The *post hoc* tests showed that EEH females were more explorative and more aggressive toward the stimulus male than OIL and EEL females (Table 3).

Stimulus males were differently active, depending on the treatment of the experimental female (Fig. 2). We extended the analysis of male behavior to EEH females to test whether males could detect the abnormal estrus state of these females. Most males (92%) mounted OIL females, whereas the proportions of males mounting ($\chi^2 = 10.97$, DF = 1, $P = 0.0009$) and reaching ejaculation ($\chi^2 = 8.0$, DF = 1, $P = 0.0047$) were significantly reduced with EEH females. The proportion of males mounting and ejaculating did not differ between OIL and EEL females ($\chi^2 = 0.38$, DF = 1, $P = 0.537$). The qualitative analysis of behaviors showed that the frequency of male mounts did not differ between OIL and EEL females but was significantly reduced for EEH females ($F_{2,32} = 9.19$, $P = 0.0007$) (Fig. 2). Similarly, mount latencies were increased when directed to EEH females ($F_{2,32} = 9.69$, $P = 0.00051$), and no stimulus males reached ejaculation when paired with EEH females (Kruskal-Wallis: $H_{2,35} = 7.71$, $P = 0.021$, Dunn *post hoc* test).

Discussion

This study shows that developmental exposure to low doses of EE2 permanently affects the reproductive behavior of female rats. The higher dose (0.4 μg/kg·d, EEH) strongly affects sexual behavior and physiology, with suppression of lordosis and the estrous cycle. The lower dose (0.004 μg/kg·d, EEL) disrupts appetitive components of sexual behavior, which influence the rate of copulation, but does not hinder the possibility of fecundation.

Reproductive disruption at low doses

Our results with the clinical dose used in the contraceptive pill (EEH) confirm previous reports of suppressive effects of EE2 on the female reproductive organs and cycle (41). However, the latter study found that only females exposed to physiological doses of 50 μg/kg·d showed persistent estrus, whereas we found the same effects with a 100-fold smaller dose. The main difference between the two studies lies in the duration of the treatment, which was interrupted at weaning in the work of Sawaki *et al.* (41) and continued until puberty in our study. This suggests that extending the exposure to xenoestrogens to the pubertal period can amplify its effects and confirms previous reports of organizational effects of estrogen during this developmental period (56–58). Unlike a previous study showing that aged females in persistent estrus were sexually receptive but not proceptive (59), our study revealed that persistent estrus was accompanied by a marked reduction of sexual receptivity and proceptivity. In addition, nonreproductive behaviors such as enhanced exploration and aggression were abnormally high in these females during mating. These results, particularly the latter one, suggest a masculinizing effect of EE2 exposure during the developmental period. Mate choice by males is based on the female odor, ultrasounds, and the type or gait of her solicitation (20). If females are not receptive, males are not motivated to achieve an ejaculation with them (53). In this experiment, EEH females did not simply avoid stimulus males, showing retreat or defending; on the contrary, they were even more aggressive with males that did not attempt to mount them. This behavior is typical of agonistic encounters by males with same-sex intruders (26, 60). Pathological alterations of sexual behavior in the offspring of mothers receiving similar clinical contraceptive doses of EE2 had not been reported previously in mammals. Thus, there is a potential risk of behavioral and physiological alterations in the children of the 3% of women who remain pregnant while using oral contraceptives (30). Although in these cases contraceptive use is interrupted as soon as pregnancy is detected and thus exposure is limited to the first period of development, the possibility of adverse effects should not be ruled out.

Sexual behavior affected by environmental concentrations

The main result of EE2 exposure at the lower environmental dose (EEL = 0.004 μg/kg·d) is a reduction of solicitation. This dose matches concentrations measured in contaminated surface waters, in which EE2 is one of the most common hormonally active pollutants (47), and is only 10

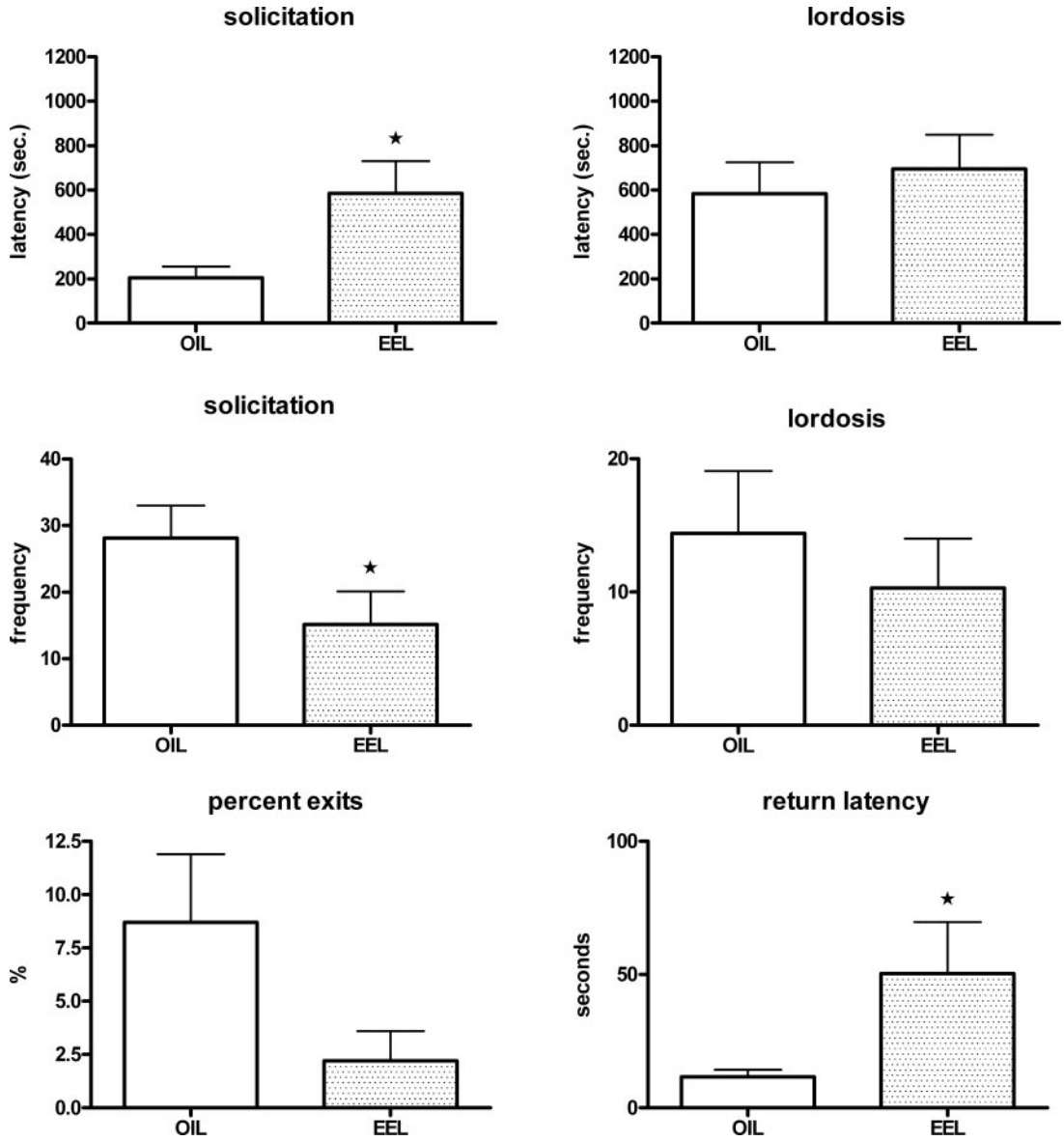

FIG. 1. Effects of developmental exposure to a low dose of EE2 on female proceptive, receptive, and pacing behaviors recorded during sexual activity tests. *, $P < 0.05$, EEL *vs.* OIL.

times higher than the concentration found in drinking water in Western countries (0.35 ng/liter) (61). In addition to the amount of EE2 assumed via water, mammalian (including human) populations with a fish-rich diet will ingest amounts equivalent to or higher than levels found in the water because EE2 is accumulated 500–1000 times in fish tissues (49, 50). EEL females had the same receptive levels than controls and stimulus males did not reduce sexual activity during the tests. The reduced amount of solicitation by EEL rats was paralleled by the increased latency of the same behavior. The use of an arena suitable to measure pacing behavior showed that EEL females spent more time away from the male before they returned to the male after a mount or an intromission (return latencies). This index of the pacing response to the male is positively correlated with the probability that pregnancy will occur (53). Under seminatural conditions, the female is able to use proceptive behavior sequentially through the control of the rate of copulation (pacing behavior) (20). Males prefer regular and rapid intromission to achieve ejaculation, whereas females require longer intervals between intromission to optimize vaginocervical stimulation. This is important to trigger the reflex that promotes implantation of the embryo through activation of the corpus luteum and the consequent release of progesterone (62, 63). Thus, our results indicate an alteration of the timing of appearance of appetitive aspects during the copulatory sequence. Because neuroendocrine changes necessary for initiating pregnancy depend on stimuli received during mating (64), the effects of EEL on proceptive behavior may influence the reproductive function of females. Such an influence does not necessarily have to be negative; indeed, we recently found that pairs of rats that underwent the same EEL treatment as in the present study actually showed increased fecundity (55). These effects could account for

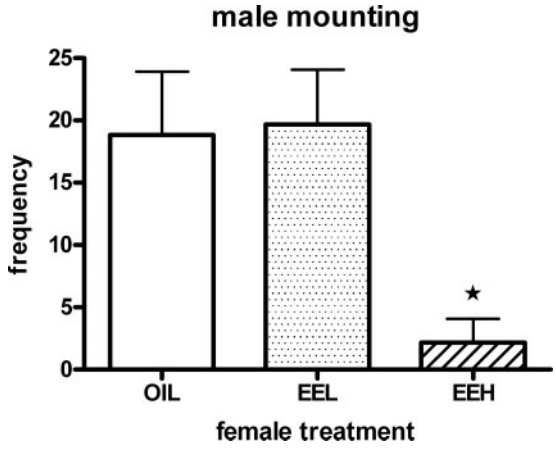

FIG. 2. Frequency (mean ± SE) of mounts of experienced stimulus males paired with experimental females exposed to different doses of EE2 during development. *, $P < 0.05$, EEH *vs.* OIL and EEH *vs.* EEL.

the results of our previous study showing increased motivation and receptivity in females perinatally exposed to low doses of bisphenol A (26).

Permanent effects on differentiation of brain and behavior

In summary, despite the lack of effects on receptive components of behavior, the delay of proceptive behavior that initiates the copulatory sequence and the enhanced control of the rate of copulation suggest an alteration of the female's control of mating to assure reproductive success (65). This suggests that developmental exposure to very low doses of exogenous estrogen affects the differentiation of the central nervous system, in particular the hypothalamic regions involved in the control of appetitive sexual behavior (66–69). On the other hand, developmental exposure to higher EE2 doses disrupts the whole copulatory sequence, at the peripheral level by affecting vaginal cytology and possibly at the central level by acting on neuronal substrates regulating behavior. The latter hypothesis is confirmed by a recent study showing that prepubertal exposure to EE2 at the same dose used in the present study permanently modifies the number of cells expressing estrogen receptors in the ventromedial hypothalamic and medial preoptic area of female rats (70).

We cannot rule out that some of the more subtle effects of the treatment could depend on alterations of maternal behavior, as shown in our previous work (71). However, it is unlikely that the strong effects of the higher dose on the estrous cycle were due to altered maternal behavior. In addition, this work was not aimed at dissecting out the mechanisms of behavioral disruption caused by xenoestrogens but rather at identifying behavioral alterations in a model as close as possible to the real world, *i.e.* environmental-like exposure, intact animals, and sensitive tests.

Conclusions

Previous studies have shown that female rodents influence reproduction through sexual selection (72, 73). Of particular importance in evaluating the possible adverse effects of xenoestrogens are behaviors critical for survival and reproduction, such as sexual behavior. Any disturbance of these behaviors is likely to be of biological significance in both human and animal ecosystems (74). Evidence of xenoestrogen-altered behavior and physiology in laboratory studies does not necessarily mean that such effects occur in the wild. However, such evidence has greater external validity when the laboratory conditions and the treatment closely simulate environmental exposure.

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