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Developmental exposure to low levels of ethinylestradiol affects play behavior in juvenile female rats.

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Developmental exposure to low levels of ethinylestradiol affects play behavior in juvenile female rats.

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Abstract

Juvenile social play contributes to the development of adult social and emotional skills in humans and non-human animals, and is therefore a useful endpoint to study the effects of endocrine disrupters on behavior in animal models. Ethinylestradiol (EE_2) is a widely produced, powerful synthetic estrogen that is widespread in the environment mainly because is a component of the contraceptive pill. In addition, fetuses may be exposed to EE2 when pregnancy is undetected during contraceptive treatment. To understand whether exposure to EE_2 during gestation or lactation affects social play, we exposed 72 female Sprague-Dawley rats to EE_2 or vehicle either during gestation (gestation day (GD) 5 through GD 20) or during lactation (from postnatal day (PND) 1 through PND 21). Two doses of EE_2 were used to treat the dams: a lower dose in the range of possible environmental exposure (4 ng/kg/day) and a higher dose equivalent to that received during contraceptive treatment (400 ng/kg/day). Behavioral testing was carried out between PND 40 and 45. A Principal Component Analysis of frequencies of behavioral items observed during play sessions identified 3 main components: Defensive-like play, Aggressive-like play, and Exploration. Aggressive-like play was significantly increased by both doses of EE_2, and the gestational administration was in general more effective that the lactational one. Defensive-like play and Exploration were not significantly affected by treatment. This research showed that low and very low doses of EE_2 that mimic clinical or environmental exposure during development can affect important aspects of social behavior even during restricted time windows.

Keywords: Endocrine disrupters, ethinylestradiol, xenoestrogens, social play, play fighting, exploration, developmental windows, cross-fostering, rat.

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Abbreviations

AFP	α-fetoprotein
AGD	Anogenital distance
ANOVA	Analysis of variance
CNS	Central nervous system
EE_2	17α-ethinylestradiol
ER	Estrogen receptor
GD	Gestation day
GLM	General linear model
PCA	Principal Component Analysis
PCs	Principal Components
PND	Post natal day
SHBG	Sex hormone-binding globulin
SD	Sprague-Dawley

Introduction

Juvenile social play contributes to the development of adult social and emotional skills in humans and non human animals (Bekoff 1974, van den Berg et al. 1999, Pellis et al. 2010, Veenema et al. 2013, Paul et al. 2014, Vanderschuren and Trezza 2014) and is ideal for studying the neurobiology of social development (Paul et al. 2014). Rat juvenile social play is sensitive to chemical factors such as prenatal and neonatal hormones and is a useful behavioral marker of neurodevelopment as severe deficits in this behavior are associated with neurodevelopmental disorders (Blake and McCoy 2015). It is well known that estrogen can exert an organizational effect on CNS and behavior in higher vertebrates during early stages of development (see Phoenix et al. 1959, McEwen 2002, McCarthy 2008, McCarthy and Arnold 2011). A perinatal exposure to estrogen is able to modify behavioral developmental trajectories. In fact, a role for early estrogen in determining the sexual phenotype of the adult rodent brain was clearly established by classical studies that illustrated how exposure to aromatizable androgens is responsible for brain masculinization, whereas a lack of it leads to normal female brain development (McCarthy 2008). Thus, the mammalian brain is essentially feminine in absence of early exposure to gonadal steroid (Gorsky 2002) and is susceptible to the organizational action of sex hormones or of their mimics. The female rat brain has been established as a useful model to study the effects of developmental exposure to estrogen and estrogenic endocrine disrupters (EDC). There is strong evidence that the perinatal period is the most sensible time window for effects of EDCs on brain development, yet few studies have tried to tell apart the effects of gestational from those due to lactational exposure (Gioiosa et al. 2013, Palanza et al. 2016). This is crucial to better understand the time course of developmental EDC action.

The development of play fighting is influenced by perinatal testosterone through its 5α-reduced products (Meaney et al. 1983). Recent research showed however that estrogen are also involved in the development of play behavior in female rats through their action on α receptors (ERα) (Olesen et al. 2005, Ferguson et al. 2014). Moreover, developmental exposure of female rats to the estrogenic substance, bisphenol A, resulted in a slight change in the structure of juvenile play (Porrini et al. 2005). The synthetic estrogen ethinylestradiol (EE_2) is a powerful mimic of natural estrogen and is the active component of most contraceptive pills. Unintentional exposure of the developing human fetus can occur if oral contraception is continued during the early months of undetected pregnancy. Timms et al. (2005) estimated that each year in the USA and Europe almost 2 million women who use oral contraceptives become pregnant accidentally, primarily because of missed pills. Oral contraceptive pills often are taken for months until the unplanned pregnancy is discovered. When taken orally EE_2 is rapidly found in serum (Churchwell et al. 2014). EE_2 binds to

estrogen receptors (ER), in particular ERα, with much higher affinity than endogenous estradiol (Blair et al. 2000). In addition, EE_2 has a low affinity with α-fetoprotein (AFP) (Hong et al. 2012) and with human sex hormone-binding globulin (SHBG) (Hong et al. 2015). As a consequence, EE_2 is able to reach target areas in the brain and to affect physiology and behavior during critical time windows.

Even though oral contraceptives have been used for decades, relatively little research has been conducted in mammals to assess effects of EE2 at or below the clinically relevant dose of 400-800 ng/kg/day on fetuses exposed via the placenta, and on wildlife exposed because of the diffusion of EE2 in the environment (Timms et al. 2005). In addition, EE_2 and other estrogenic compounds are used in hormone replacement therapy and osteoporosis treatment (Lindsay 2015) and as growth enhancement products in veterinary medicine (Arcand-Hoy et al. 1998). Due to its widespread pharmaceutical use and relatively long half-life, EE_2 has been detected in some river systems in the USA and Europe (Kolpin et al. 2002; Nash et al. 2004; Johnson and Williams 2004), and is a matter of concern for public and wildlife health (Wise et al. 2011). Johnson and William (2004) suggested that 40% of the ingested EE_2 is found free (deconjugated) in the environment. The disrupting effects of EE_2 at environmentally relevant levels on fish reproduction and behavior are well known (Nash et al. 2004; Parrot and Blunt 2005; Saaristo et al. 2010; Reyhanian et al. 2011; Sumpter and Jobling 2013). These studies suggest that it is urgent to investigate the effects of EE_2 on mammals, both at contraceptive doses and at very low doses comparable with the concentrations found in untreated surface waters.

In mammals, pharmacological levels of EE_2 exert important effects on reproductive physiology and behavior (Arabo et al. 2005, Dugard et al. 2001, Ferguson et al. 2011, Ferguson et al. 2014, Mandrup et al. 2013). However, studies on terrestrial mammals at concentrations similar to the contraceptive dose of 400-800 ng/kg/day or lower are surprisingly scarce. Administration of EE_2 at clinical or subclinical doses during developmental windows is able to affect a variety of reproductive anatomical or physiological endpoints in mice and rats (Delclos et al. 2009, Delclos et al. 2014, Derouiche et al. 2015, Fusani et al. 2007, Latendresse et al. 2009, Howdeshell et al. 2008, Shirota et al. 2012, Shirota et al. 2015, Takahashi et al. 2014, Thayer et al. 2001, Timms et al. 2005; but see a lack of effects of a 500 ng/kg/day dose in Mandrup et al. (2013). Behavior is a critical endpoint of estrogen action, and previous studies in Sprague-Dawley (SD) rats showed significant effects of a developmental administration (GD 5 - PND 32) of low, subclinical doses of EE_2 (4 ng/kg/day or 400 ng/kg/day) on learning and memory (Corrieri et al. 2007), sexual behavior (Della Seta et al. 2006; Della Seta et al. 2008), pain perception (Ceccarelli et al. 2015), and anxiety (Zaccaroni et al. 2016). In female mice, developmental exposition to clinical or subclinical doses of EE_2 produced a disturbed maternal behavior, a higher lordosis response, a lack of discrimination

between gonad-intact and castrated males in sexually experienced females, and an increased anxiety-related behavior (Derouiche et al. 2015).

In the present paper we studied the effects of EE2 on play behavior of female SD rats exposed to thise chemical during their gestational life (GD5 to birth) or during lactation (PND 1 to PND 21). The animals were exposed by treating their dams with a very low, environmentally relevant dose (4 ng/kg/day), or with a clinical dose (400 ng/kg/day).

We studied play behavior in juvenile females maintained and observed in a social context, with cagemates of the same age (e.g. Meaney and Stewart 1981), which is a more naturalistic setting compared to dyadic encounters preceded by social isolation (e.g. van den Berg et al. 1999). Isolation is a strong stressor *per se* (Blanchard et al. 2001) and, although it may enhance the emergence of effects on play (Blake and McCoy 2015), it represents an important confounding factor. Observations were carried out between 40 and 45 days of age: around this age females approach sexual maturity (Ojeda and Urbanski 1988), but their social play is not significantly different from that expressed at 35 days of age (Porrini et al. 2005). Pellis and Pellis (1990) described in Long Evans hooded male and female rats a peak of play fighting around 41-45 days of age in same sex pairs.

Materials and Methods

Animals and treatment procedure

We used 72 juvenile SD female rats born and bred at the Human Physiology Institute, University of Siena (Italy), exposed to EE_2 during gestation or lactation. To obtain the experimental subjects we housed 100 female-male pairs of sexually mature Sprague-Dawley rats in 100 polysulfone cages (Tecniplast, Italy, 60 x 37 x 20 cm). Cages were provided with metal tops and a wire netting floors for daily search of the vaginal plug to detect the day of copulation (defined gestational day 0 or GD 0). On the same day, the male was removed and the female was housed individually. We selected 72 dams that had been fertilized within two days and transferred them in single cages. Half of the dams (N = 36) were daily treated with either 4ng/Kg EE2 (Sigma- Aldrich; EE4, N=12), 400 ng/Kg EE2 (EE400, N = 12), or vehicle (peanut OIL, N=12) from GD 5 until weaning of the pups, the other 36 dams were untreated. The treatment was administered orally with a pipette. This procedure is likely much less stressful than gavage (Vandenberg et al. 2014, Gioiosa et al. 2015). On postnatal day (PND) 1, pups were removed from their dams and gently placed in a cotton nest; each weighed with an analytical scale, and the anogenital (AGD) distance measured with a caliper. On the same day the litters were culled to 4 females and 4 males and then cross-fostered. Pups born from treated dams were fostered to untreated dams so that their exposure to EE_2 or vehicle was confined to the

gestational period (GEST), whereas pups born from untreated dams were fostered to treated dams so to be exposed to EE_2 or vehicle only during lactation (LACT) (Table 1). At weaning (PND 21), all litters were separated from foster-dams and at PND 32 one female for each litter was individually marked with cosmetic dye on the tail and randomly housed with 3 other females that had received the same treatment. No cage contained siblings. Thus, only one female per litter for a total of 72 females was used for the study i.e. each experimental subject came from a different dam. Vaginal opening was checked daily by a person not blind to treatment.

Here Table 1

The animals were housed in polysulfone cages as previously described under an inverted reversed light-dark cycle (dark 07.30-19.30) with a relative humidity of 60 +/- 10%. Food (Harlan Teklad soy-free AIN-76A diet) and water were supplied *ad libitum* throughout the experiment.

Behavioral testing

Observations were carried out during the dark phase, under dim red light combined with low indirect white light. All sessions were recorded with a video camera (Sony AVC – D5CE) and video recordings were analyzed with 'The Observer Video Pro 4.0' software (Noldus Information Technology, The Netherlands) by an observer blind to treatment.

Subjects were tested for social play between PND 40 and 45, an age at which social play is still vigorous and not significantly different from that expressed at 35 days of age (Porrini et al. 2005). The four females from the same housing cage were tested together in a neutral arena (60 x 35 x 35 cm). At the beginning of the observation, just before the introduction of the rats into the arena, a small quantity of sawdust from the home cage was mixed to the clean one of the testing arena to facilitate habituation to the novel environment. After 1 min of familiarization, the behavior of the four animals was video-recorded for 15 mins. Social and non-social behaviors of each individual were identified according to the ethogram described in Table 2, modified from Porrini et al. (2005). A behavior was attributed to the subject initiating the action. Testing of different experimental groups was balanced across time.

Here Table 2

Animal welfare

The experiments described in this research were approved by the Ethical Committee of the Department of Physiology, University of Siena and followed European Community Council Directive 86/609/EEC and institutional guidelines.

Statistical analysis

To reduce the dimensions of the data set (and thus reduce the number of tests to be carried out) and identify correlated behavioral items (and thus eliminating autocorrelation), we carried out a Principal Component Analysis (PCA) (Jolliffe 2014). The Kaiser Meyer Olkin index (a measure of the proportion of variance in common between the different variables) was used to estimate the overall adequacy of the matrix (Cerny and Kaiser 1977), and communalities (a measure of the proportion of variance of each variance explained by the matrix) were calculated to for identifying variable contribution to the correlation matrix (the higher communality, the more the variable is associated to others) (Tabachnick and Fidell 2007).

Once extracted, the Principal Components (PCs) were rotated (with the Equamax procedure to capture the maximal amount of variance) to facilitate the interpretation of the different components, and Kaiser normalization was applied to reduce anomalies in the components loadings. Scores were saved using the Anderson-Rubin method to guarantee orthogonality between components (Jolliffe 2014).

The first three PCs were used as dependent variables into three General Linear Models (GLM) to compare behavior between different treatment groups and different times of exposure (treatment, timing).

Analysis of variance using a GLM approach was also used to test differences between treatment groups for AGD and body weight, and between treatment groups and times of exposure (and their interaction) for vaginal opening and body weight at 21 days of age. For all analyses, we added 'cage' as a random factor to account for the effect of the social group. Post-hoc comparisons were carried out using the LSD test (Sokal and Rohlf 1995). All tests were performed using IBM SPSS 22 (IBM®, Chicago, IL).

Results

Anatomical and physiological variables

Anogenital distance (AGD) and body weight at PND 1 and PND 21 were not significantly affected by gestational administration of EE_2 (for statistical values, see Tab. 3). The treatment significantly

affected vaginal opening (VO), in particular the higher EE_2 dose (EE400) significantly delayed VO with the main effect due to lactational administration (Tab. 3).

Here Table 3

Social activity

We computed Social activity by pooling all behavioral items indicating any social interaction during the 15 min test (Fig. 1). Social activity significantly increased with increasing EE2 dose ($F_{2,64}$=6.59, P=0.002), EE400 vs Oil (Post hoc LSD P=0.001), EE400 vs EE4 (Post hoc LSD P=0.017). Moreover, time of treatment significantly affected total social activity, with gestational treatment being more effective than lactational one ($F_{1,64}$=10.44, P=0.002). No significant interaction of treatment x timing ($F_{2,64}$=0.991, P=0.370) or cage effect ($F_{2,64}$=0.165, P=0.864) were detected. Non-social activity, including all non-social active behaviors, was not affected by treatment or timing of exposure or cage (data not shown).

Here Fig 1

PCA applied to the frequencies of the behavioral items showed 7 components, explaining 73.77% of the variance (Tab. 4). Principal component 1 (PC1) included most elements of defensive play, explained the higher percentage of variance (16.98%) and was labeled as "Defensive-like play". PC2 included most element of aggressive play and was labeled as "Aggressive-like play" (variance explained 15.73%). PC3 (12.59%) included elements of social and non-social exploration and was labeled as "Exploration". PC4 (7.34%), due to its non homogeneous behavioral components, was not labeled. PC5 (7.33%) included a mixture of a bedding material oriented behavior (chewing) and play (Pinning). PC6 (7.15%) included mainly self-grooming. PC7 (6.65%) excluded allo-grooming and included solitary running.

Based on the weight of each component and their internal coherence and their relevance to social behavior, we decided to consider only the first three components, explaining 45.3% of variance. Since PC 4, 5, 6, 7 (explaining only a residual 28.47% of variance) were not internally consistent, were excluded from further analyses.

GLM was applied to each component, using the individual component scores as variables, considering treatment (OIL, EE4, EE400), timing of administration (GEST, LACT), cage and interactions.

Here Table 4

Defensive-like play

Defensive-like play, described by PC1, was not significantly affected by treatment ($F_{2,64}$=2.04, P=0.139), however we found an effect of timing ($F_{1,64}$=9.65, P=0.003). No significant interaction between treatment and timing ($F_{2,64}$=0.12, P=0.880) or a cage effect ($F_{2,64}$=1.291, P=0.282) were present. The lack of a treatment effect in spite of the presence of an effect of timing might be due to the reduced power (=0.4) of the test, which is particularly relevant when considering the individual variability in the response.

Aggressive-like play

Aggressive-like play (Tab. 4, Fig. 2) described by PC2 was significantly affected by treatment ($F_{2,64}$=5.42, P=0.007). Administration of EE_2 increased the frequency of aggressive-like play, and the higher dose (EE400) was more effective than the lower one (EE4) (post hoc LSD, P=0.01). Timing had also a significant effect, with gestational treatment being more effective than lactational one ($F_{1,64}$=6.29, P=0.015). We found no significant effects of the interaction between treatment and timing ($F_{2,64}$=0.379, P=0.686) or of the cage ($F_{2,64}$=0.001, P=0.999).

Here Fig 2

Aggressive neck grooming and Pounce were the most representative behaviors of Aggressive-like play (Tab. 4). In particular, Aggressive neck grooming (Fig. 3a) was significantly increased by EE2 treatment ($F_{2,64}$=3.59, P=0.033, EE400 vs EE4 Post hoc LSD P=0.016, EE400 vs Oil Post hoc LSD P=0.038). Timing of administration was significant, with gestational administration being significantly more effective than lactational one ($F_{1,64}$=9.23, P=0.003). No significant effects of the interaction between treatment and timing ($F_{2,64}$=0.075, P=0.928) or of cage ($F_{2,64}$=1.480, P=0.235) were detected.

Pounce (Fig. 3b) was significantly increased by both doses ($F_{2,64}$=5.50, P=0.006, EE400 vs EE4 Post hoc LSD P=0.010, EE400 vs Oil Post hoc LSD P=0.003) and the timing of administration had a significant effect, in that gestational administration was significantly more effective than lactational one ($F_{1,64}$=11.89, P=0.001). No significant effects of the interaction between treatment and timing ($F_{2,64}$=0.007, P=0.993) or for cage ($F_{2,64}$ =0.753, P=0.475) were detected.

Here Fig 3 (a, b)

Exploration

Social and non-social exploration (Tab. 4) described by PC3 were not significantly affected by treatment ($F_{2,64}$=2.027, P=0.140), timing ($F_{1,64}$=3.599, P=0.062), or interaction treatment x timing ($F_{2,64}$=1.217, P=0.303), or by cage ($F_{2,64}$=0.825, P=0.443).

Pinning

Pinning (Fig. 4), a playful behavior associated with play fighting, (Pellis 2002), was associated to Defensive-like play (PC1), Aggressive-like play (PC2) and PC 5 in our PCA analysis. For this reason, we decided to consider this behavioral item *per se*. It was significantly increased by treatment ($F_{2,64}$=6.2, P=0.003), with the higher dose more effective than the lower one: EE400 vs EE4 (Post hoc LSD P=0.014), EE400 vs Oil (Post hoc LSD P=0.001). No significant effects were observed for timing ($F_{1,64}$=3.044, P=0.086), the interaction between treatment and timing ($F_{2,64}$=0.200, P=0.819) and cage ($F_{2,64}$=2.286, P=0.110).

The frequencies of all behaviors included in PC1, PC2, PC3 of the PCA with an eigenvalue >0.3 are reported in the Supplementary Table A1.

Here Fig 4

Discussion

Our findings showed that developmental exposure of female rats to low or very low doses of the synthetic estrogen EE_2 during gestation or lactation significantly alters social play by increasing its aggressive components. This is a new finding, and is in line with the results of Olesen et al. (2005) and Ferguson et al. (2014), who observed a similar increase in female aggressive-like play after developmental exposure to pharmacological doses of estradiol. Our PCA divided social play in two main components, one including Defensive-like play behavior such as On back, Lateral display, and Withdrawal, and another one (Aggressive-like play), comprising Aggressive neck grooming and Pounce. The observed effect was particularly evident in Aggressive-like play, and its main components Aggressive neck grooming and Pounce were significantly increased by treatment with EE_2. Pinning, a behavior considered by many authors the most representative element of social play (Blake and McCoy 2015), showed a significant increase in frequency at the higher treatment dose and was mildly correlated to both Defensive-like play and Aggressive-like play (Table 4). This is

not surprising as it is commonly recognized that individuals show reciprocity of roles during play activities, (e.g. switching from being pinned to pinning), a peculiar characteristic of play.

Our study integrates the findings by Meaney and Stewart (1981) who showed that play fighting is influenced by 5α-reduced products of testosterone, and suggests that early estrogen exposure can similarly affect juvenile social play, in line with Olesen et al. (2005) and Ferguson et al. (2014), and in accordance with the known role of estrogen in modulating a wide range of socio-sexual behaviors.

Our results showed a significant increase in aggressive components of play behavior suggesting a possible masculinizing effect of EE2 on female brain. In fact, juvenile social play in rats is often described as sexually dimorphic: males show a greater motivation to play and initiate more playful attacks than females do (Pellis et al. 1997, Auger and Olesen 2009, Argue and McCarthy 2015). However, in a parallel study on male rats in which we followed the same experimental design as the one in the present study, we did not observe significant differences in play behavior between control males and control females (Zaccaroni et al. in prep.). A lack of sexual dimorphism in play behavior has been reported by several authors (Panskepp et al. 1984, Flynn et al. 2001, Colbert et al. 2005, Flynn et al. 2005, Veenema et al. 2013). These contrasting results are probably due to a variety of factors, such as conditions of rearing, familiarity of playmates, familiarity of experimental arena, sex and weight of the playmate (Panskepp et al. 1984, Paul et al. 2014, Argue and McCarthy 2015).

In our study, both doses of EE_2 produced significant effects on behavior; an interesting finding given that the environmentally relevant dose was very low (4 ng/kg/day). In general, gestational exposure was more effective than lactational one, which is consistent with studies reviewed by Delclos et al. (2009) reporting a limited transfer of EE_2 to newborns via milk in humans and rats.

It is remarkable that the low doses of EE_2 used in our experiments, while effective on some aspects of play behavior, were unable to affect important non-behavioral endpoints such as weight and AGD at birth. Since AGD is considered a sensitive androgen-dependent developmental marker (Rhees et al. 1997), our results, in line with those of Howdeshell et al. (2008) and Ferguson et al. (2011), suggest that early EE_2 treatment does not interfere with androgen regulation, which is able *per se* to influence development of social play (Meaney and Stewart 1981; Meaney et al. 1983; Thor and Holloway 1986; Pellis and McKenna 1992). Vaginal opening was slightly but significantly delayed by the higher dose of EE_2, a result that contradicts the idea that estrogen accelerate puberty (reviewed in Goldman et al. 2000) but in agreement with findings of significant delays in sexual development after exposures to EE_2 ranging from 500 to 10.000 ng/kg/day (Sawaki et al. 2003, Delclos et al. 2014, Ferguson et al. 2014; but see Derouiche et al. 2015 in mice). Thus, our results add to the number of studies that showed heterogeneous effects of estrogen on vaginal opening (reviewed by Ferguson et al. 2014).

How can such low doses of EE_2 affect behavior? In female rats, the prenatal brain is protected by AFP from maternal circulating estrogen (Bakker et al. 2006), however EE_2 has low affinity with AFP (Hong et al. 2012) and high affinity with ERα (Blair et al. 2000). Therefore, EE_2 could bypass the protective function of AFP and affect sexual differentiation of the brain even at very low doses. Effects of subtle variations in the concentration of hormones on development have been reported previously, as illustrated by the differences in adult behavior depending on the intrauterine position that affects the prenatal hormonal milieu (Ryan and Vandenbergh 2002, vom Saal 2016).

With an experimental protocol designed to mimic an environmental or clinical exposure to EE2, we observed robust effects of very low doses of EE_2 on key traits such as social play, an essential component of the maturation of social behavior. If play is important for the refinement of social skills (van den Berg et al. 1999, Pellis et al. 2010), then a modification of play may have consequences on adult social behavior. An additional contribution of the present study is the demonstration that low doses of a pure estrogen can be used as a tool to increase our understanding of the maturation of socio-sexual behavior. Previous work relying on castration/hormone replacement approaches often highlighted on-off effects of the hormones with a consequent masking of those traits that typically respond in a dose-dependent manner.

Conclusions

Our study showed that low and very low doses of EE_2, mimicking clinical or environmental exposure during development, can affect important aspects of social behavior even in restricted time windows of action, with possible important consequences on adult behavior. The high sensitivity of the behavioral endpoints examined in our study highlights the importance of implementing behavioral tests on females, the sex more prone to be influenced by developmental exposure to estrogenic substances, to study the potential effects of low doses of endocrine disrupters.

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Conflict of interests

The authors declare that there is no conflict of interests associated with this paper.

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CAPTIONS

Fig. 1 Frequency (n/15 min) of Total social activity of female rats exposed to gestational or lactational treatment with 4 ng/kg/day (EE4) or 400 ng/kg/day (EE400) of EE_2. Box-whiskers show median, interquartiles, and range of individual values. Timing ($F_{1,64}$=10.44, P= 0.002); Treatment ($F_{2,64}$=6.59, P=0.002); EE400 vs Oil (Post hoc LSD P=0.001); EE400 vs EE4 (Post hoc LSD P=0.017).

Fig. 2 Principal components scores of Aggressive-like play (PC2) performed by female rats exposed to gestational or lactational treatment with 4 ng/kg/day (EE4) or 400 ng/kg/day (EE400) of EE_2. Box-whiskers show median, interquartiles, and range of individual values. Timing ($F_{1,64}$=6.23 P=0.015); Treatment ($F_{2,64}$=5.42 P=0.007); EE400 vs Oil (Post hoc LSD P=0.003), EE400 vs Oil (Post hoc LSD P=0.012).

Fig. 3a,b. Frequency (n/15 min) of Aggressive neck grooming (**a**) and Pounce (**b**) performed by female rats exposed to gestational or lactational treatment with 4 ng/kg/day (EE4) or 400 ng/kg/day (EE400) of EE_2. Box-whiskers show median, interquartiles, and range of individual values. Aggressive neck grooming (**a**) test results: Timing ($F_{1,64}$=9.23, P=0.003); Treatment ($F_{2,64}$= 3.59, P=0.033); EE400 vs EE4 (Post hoc LSD P=0.016), EE400 vs Oil (Post hoc LSD P=0.038).
Pounce (**b**) test results: Timing ($F_{1,64}$=11.89, P=0.001); Treatment ($F_{2,64}$=5.50, P=0.006); EE400 vs EE4 (Post hoc LSD P=0.01), EE400 vs Oil (Post hoc LSD P=0.003).

Fig. 4 Frequency (n/15 min) of Pinning performed by female rats exposed to gestational or lactational treatment with 4 ng/kg/day (EE4) or 400 ng/kg/day (EE400) of EE_2. Box-whiskers show median, interquartiles, and range of individual values. Timing ($F_{1,64}$=3.04, P=0.086); Treatment ($F_{2,64}$=6.2, P=0.003); EE400 vs EE4 (Post hoc LSD P=0.014), EE400 vs Oil (Post hoc LSD P=0.001).

Table 1. Outline of the experimental groups. The GESTATIONAL animals received the treatment only in utero (GD 5-20) from their treated dams and at birth were fostered to untreated dams. The LACTATIONAL group received no treatment in utero and were then exposed to the treatment only via the milk of their foster treated dams (PND 1-21). EE4= 4ng/kg/day; EE400=400 ng/kg/day.

	OIL	**EE4**	**EE400**
GESTATIONAL (pups from treated dams fostered to untreated foster dams)	12	12	12
LACTATIONAL (pups from untreated dams fostered to treated foster dams)	12	12	12

Table 2. List of social and nonsocial behaviors considered. Each behavior is performed by the focal subject.

Social behaviors
Aggressive neck grooming (vigorous neck allogrooming)
Allo-grooming
Approach (moving toward another)
Bite
Boxing (both rats stand up facing each other and boxing with forepaws)
Chase
Crawl-over (moving over another)
Crawl-under (moving under another)
Flee
Genital sniffing
Jumping and running
Lateral display (the animal orientates itself broadside to another animal)
On back (lying on the back with belly exposed to another)
Pinning (standing over the opponent with its forepaws on the ventral surface)
Pounce (bouncing over another)
Sniff (sniffing another's body except genital area)
Upright (with erect posture the rat exposes its belly to another)
Withdraw (all movements away from another)

Non-social behaviors
Chew substrate
Crouch
Dig
Explore (exploration of the environment)
Rear (animal stands up)
Run
Self-grooming

Table 3. Anogenital distance at birth, body weight at birth, body weight at 21 days and vaginal opening. General linear model with LSD post-hoc test demonstrating significant main effect values are expressed as mean (SD). N/A=not included in the analysis.

Variables	Timing of administration	OIL	EE4	EE400	treatment *F, P*	timing *F, P*	treat x tim *F, P*	OIL vs EE4 *P*	OIL vs EE400 *P*	EE4 vs EE400 *P*
Anogenital distance (mm)	GEST	1.35 (0.15)	1.26 (0.18)	1.28 (0.25)	0.53, 0.59	N/A	N/A	N/A	N/A	N/A
Body weight at birth (grams)	GEST	6.15 (0.45)	6.13 (0.62)	5.77 (0.59)	1.23, 0.3	N/A	N/A	N/A	N/A	N/A
Body weight at 21 days (grams)	GEST	47.05 (6.09)	49.24 (3.03)	45.99 (1.96)	0.62, 0.54	1.60, 0.21	N/A	N/A	N/A	N/A
	LACT	44.87 (4.02)	45.88 (1.60)	47.03 (2.61)						
Vaginal opening (days)	GEST	36.08 (1.93)	35.83 (1.59)	36.75 (1.42)	4.47, **0.015**	2.60, 0.11	1.1, 0.33	0.72	**0.015**	0.09
	LACT	35.92 (1.24)	36.75 (0.87)	37.67 (1.50)						

Table 4. Results of PCA applied to behaviors of female rats. Total variance explained: 73.77%. Only loadings > ± 0.3 are shown. Components 4, 5, 6 and 7 are not labeled since the behaviors identified are not homogeneous. Each behavior is performed by the focal subject.

	Components						
	1 Defensive-like play	2 Aggressive-like play	3 Exploration	4	5	6	7
On back	0.883						
Lateral display	0.863						
Withdraw	0.796	0.325					
Pinning	0.510	0.347			0.456		
Aggressive neck grooming		0.849					
Pounce	0.527	0.737					
Bite		0.634			0.306		
Crawl-over		0.633	0.352				
Flee	0.523	0.632					
Chase	0.426	0.484	0.388				
Explore			0.914				
Sniff			0.731		0.401		
Rear			0.639		-0.301	-0.336	0.327
Genital sniffing			0.631	0.324			
Crawl-under				-0.766			
Approach		0.460		0.576		0.334	
Boxing	0.439			0.490			
Chew substrate					0.894		
Self-grooming						0.860	
Allo-grooming							-0.850
Run						-0.483	0.600
Variance explained	16.98	15.73	12.59	7.34	7.32	7.15	6.65

Developmental exposure to low levels of ethinylestradiol affects play behavior in juvenile female rats.

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Abstract

Juvenile social play contributes to the development of adult social and emotional skills in humans and non-human animals, and is therefore a useful endpoint to study the effects of endocrine disrupters on behavior in animal models. Ethinylestradiol (EE_2~~),~~) is a widely produced, powerful synthetic estrogen~~,~~ that is widespread in the environment mainly ~~due to its use as~~because is a component of the contraceptive pill. In addition, fetuses may be exposed to EE2 when pregnancy is undetected during contraceptive treatment. To understand whether exposure to EE_2 during gestation or lactation affects social play, we exposed 72 female Sprague-Dawley rats to EE_2 or vehicle either during gestation (gestation day (GD) 5 through GD 20) or during lactation (from postnatal day (PND) 1 through PND 21). Two doses of EE_2 were used to treat the dams: a lower dose in the range of possible environmental exposure (4 ng/kg/day) and a higher dose equivalent to that received during contraceptive treatment (400 ng/kg/day). Behavioral testing was carried out between PND 40 and 45. A Principal Component Analysis ~~on~~of frequencies of behavioral items observed during play sessions identified 3 main components: Defensive-like play, Aggressive-like play, and Exploration. Aggressive-like play was significantly increased by both doses of EE_2, and the gestational administration was in general more effective that the lactational one. Defensive-like play and Exploration were not significantly affected by treatment. This research ~~shows~~showed that low and very low doses of EE_2~~, mimicking~~ that mimic clinical or environmental exposure during development~~,~~ can affect important aspects of social behavior even during restricted time windows.

Keywords: Endocrine disrupters, ethinylestradiol, xenoestrogens, social play, play fighting, exploration, developmental windows, cross-fostering, rat.

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Abbreviations

AFP	α-fetoprotein
AGD	Anogenital distance
ANOVA	Analysis of variance
CNS	Central nervous system
EE_2	17α-ethinylestradiol
ER	Estrogen receptor
GD	Gestation day
GLM	General linear model
PCA	Principal Component Analysis
PCs	Principal Components
PND	Post natal day
SHBG	Sex hormone-binding globulin
SD	Sprague-Dawley

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Introduction

Juvenile social play contributes to the development of adult social and emotional skills in humans and non human animals (Bekoff 1974, van den Berg et al. 1999, Pellis et al. 2010, Veenema et al. 2013, Paul et al. 2014, Vanderschuren and Trezza 2014) and is ideal for studying the neurobiology of social development (Paul et al. 2014). Rat juvenile social play is ~~therefore a useful behavioral marker of neurodevelopment, and is~~ sensitive to chemical factors such as prenatal and neonatal hormones~~:~~ and is a useful behavioral marker of neurodevelopment as severe deficits in this behavior are associated with neurodevelopmental disorders (Blake and McCoy 2015). It is well known that estrogen ~~during early stages of development~~ can exert an organizational effect on CNS and behavior in higher vertebrates during early stages of development (see Phoenix et al. 1959, McEwen 2002, McCarthy 2008, McCarthy and Arnold 2011). A perinatal exposure to estrogen is able to modify behavioral developmental trajectories. In fact, a role for early estrogen in determining the sexual phenotype of the adult rodent brain was clearly established~~:~~ by classical studies that illustrated how exposure to aromatizable androgens is responsible ~~of~~for brain masculinization, ~~while~~whereas a lack of it ~~is essential for~~leads to normal female brain development (McCarthy 2008). Thus, ~~since~~, the mammalian brain is essentially feminine in absence of early exposure to gonadal steroid (Gorsky 2002) and is susceptible to the organizational action of sex hormones or of their mimics~~, the~~. The female rat brain ~~is~~has been established as a useful model to study the effects of developmental exposure to estrogen and estrogenic endocrine disrupters (EDC). There is ~~a~~ strong evidence that ~~in the rat~~ the perinatal period is the most sensible time window for effects of EDCs on brain development, yet few studies have tried to tell apart the effects of gestational ~~vs.~~from those due to lactational exposure (Gioiosa et al. 2013, Palanza et al. 2016). This is crucial to better understand the time course of developmental EDC action.

~~It is known that the~~The development of play fighting is influenced by perinatal testosterone through its 5α~~-~~reduced products (Meaney et al. 1983). ~~However, recent~~Recent research showed however that estrogen~~, through their α receptors (ERα),~~ are also involved in the development of play behavior in female rats through their action on α receptors (ERα) (Olesen et al. 2005, Ferguson et al. 2014). Moreover, developmental exposure of female rats to the estrogenic substance, bisphenol A, resulted in a slight change in the structure of juvenile play (Porrini et al. 2005). The synthetic estrogen ethinylestradiol (EE_2) is a powerful mimic of natural estrogen and is the active component of most contraceptive pills~~: unintentional~~. Unintentional exposure of the developing human fetus can occur if oral contraception is continued ~~through~~during the early months of undetected pregnancy. ~~In fact,~~ Timms et al. (2005) estimated that each year in the USA and Europe almost 2 million women ~~in the United States and Europe~~ who use oral contraceptives become pregnant accidentally, primarily

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because of missed pills. Oral contraceptive pills often are taken for months until the unplanned pregnancy is discovered. When taken orally EE_2 is rapidly found in serum (Churchwell et al. 2014~~);~~ ~~moreover,~~). EE_2 binds to estrogen receptors (ER), in particular ERα, with much higher affinity than ~~the~~ endogenous estradiol (Blair et al. 2000). In addition, EE_2 has a low affinity with α-fetoprotein (AFP) (Hong et al. 2012) and with human sex hormone-binding globulin (SHBG) (Hong et al. 2015). As a consequence, EE_2 is able to reach target areas in the brain and to affect physiology and behavior ~~in~~during critical time windows ~~of action and doses~~.

Even though oral contraceptives have been used for decades, relatively little research has been conducted in mammals to assess effects of EE2 at or below the clinically relevant dose of 400-800 ng/kg/day on fetuses exposed via the ~~placental~~placenta, and on wildlife exposed because of the diffusion of EE2 in the environment (Timms et al. 2005). In addition, EE_2 ~~is also~~and other estrogenic compounds are used in hormone replacement therapy and osteoporosis treatment (Lindsay 2015~~).~~ ~~Estrogenic substances are also used~~) and as growth enhancement products in veterinary medicine (Arcand-Hoy et al. 1998). Due to its widespread pharmaceutical use and relatively long half-life, EE_2 has been detected in some river systems in the ~~United States~~USA and Europe (Kolpin et al. 2002; Nash et al. 2004; Johnson and Williams 2004), and is a matter of concern for public and wildlife health ~~and fauna~~ (Wise et al. 2011). Johnson and William (2004) suggested that 40% of the ingested EE_2 is found free (deconjugated) in the environment. ~~Thus it is of great interest to study the effects of the contraceptive dose assumed on a daily basis with the pill and of a very low dose of EE_2 representing possible environmental exposure, i.e. comparable with the concentrations found in untreated surface waters.~~ The disrupting effects of EE_2 at environmentally relevant levels on fish reproduction and behavior are well known (Nash et al. 2004; Parrot and Blunt 2005; Saaristo et al. 2010; Reyhanian et al. 2011; Sumpter and Jobling 2013). These studies suggest that it is urgent to investigate the effects of EE_2 on mammals, both at contraceptive doses and at very low doses comparable with the concentrations found in untreated surface waters.

In mammals, pharmacological levels of EE_2 exert important effects on reproductive physiology and behavior ~~at pharmacological relevant dosage levels of EE_2 are known~~ (Arabo et al. 2005, Dugard et al. 2001, Ferguson et al. 2011, Ferguson et al. 2014, Mandrup et al. 2013). However, studies on terrestrial mammals at concentrations similar to the ~~clinical~~contraceptive dose of 400-800 ng/kg/day~~,~~ or lower~~,~~ are surprisingly scarce.

Administration of EE_2 at clinical or subclinical ~~dosages~~doses during developmental windows is able to affect a variety of reproductive anatomical or physiological endpoints in mice and rats (Delclos et al. 2009, Delclos et al. 2014, Derouiche et al. 2015, Fusani et al. 2007, Latendresse et al. 2009, Howdeshell et al. 2008, Shirota et al. 2012, Shirota et al. 2015, Takahashi et al. 2014, Thayer et al. 2001, Timms et al. 2005~~);~~; but see a lack of ~~effect in Mandrup et al. (2013)~~effects of a 500 ng/kg/day

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dose~~.~~ in Mandrup et al. (2013). Behavior is a critical endpoint of estrogen action~~: some~~, and previous studies in Sprague-Dawley (SD) rats showed significant effects of a developmental administration (GD 5 - PND 32) of low, subclinical doses of EE_2 (4 ng/kg/day or 400 ng/kg/day) ~~to Sprague-Dawley (SD) rats~~ on learning and memory (Corrieri et al. 2007), sexual behavior (Della Seta et al. 2006; Della Seta et al. 2008), pain perception (Ceccarelli et al. 2015), and anxiety (Zaccaroni et al. 2016). ~~Interestingly, in~~In female mice, developmental exposition to clinical or subclinical doses of EE_2 produced a disturbed maternal behavior, a higher lordosis response, a lack of discrimination between gonad-intact and castrated males in sexually experienced females, and an increased anxiety-related behavior (Derouiche et al. 2015).

~~Considering the importance of social play in the maturation of adult behavior and the developmental sensitivity of female brain to hormones, in~~In the present ~~study~~paper we ~~focused on~~studied the effects of EE2 on play behavior of female SD rats exposed to ~~the~~thise chemical during their gestational life (GD5 to birth) or during lactation (PND 1 to PND 21). The animals were exposed by treating their dams ~~to~~with a very low, environmentally relevant dose (4 ng/kg/day), or ~~to the~~with a clinical ~~one~~dose (400 ng/kg/day).

We studied play behavior in juvenile females maintained and observed in a social context, with cagemates of the same age (e.g. Meaney and Stewart 1981), which is a more naturalistic setting compared to dyadic encounters preceded by social isolation (e.g. van den Berg et al. 1999). Isolation is a strong stressor *per se* (Blanchard et al. 2001) and, although it may enhance the emergence of effects on play (Blake and McCoy 2015~~)~~), it represents an important confounding factor. Observations were carried out between 40 and 45 days of age: around this age females approach sexual maturity (Ojeda and Urbanski 1988), but their social play is not significantly different from that expressed at 35 days of age (Porrini et al. 2005). Pellis and Pellis (1990) described in Long Evans hooded male and female rats a peak of play fighting around 41-45 days of age in same sex pairs.

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Materials and Methods

Animals and treatment procedure

We used 72 juvenile SD female rats born and bred at the Human Physiology Institute, University of Siena (Italy), exposed to EE_2 during gestation or lactation. To obtain ~~these~~the experimental subjects we ~~paired~~housed 100 ~~females and 100 males~~ female-male pairs of sexually mature Sprague-Dawley rats in ~~single~~100 polysulfone cages (Tecniplast, Italy, 60 x 37 x 20 cm~~)~~). Cages were provided with metal tops and a wire netting ~~floor to allow the~~ floors for daily search of the vaginal plug~~: after its detection, at~~ to detect the day of copulation (defined gestational day 0 ~~(~~or GD 0~~),~~). On the same day, the male was removed~~,~~ and the female was housed individually. We ~~then~~ selected 72 dams that had

been fertilized ~~on the same~~within two days and transferred them in single cages. Half of the dams (N = 36) were daily treated with either 4ng/Kg EE2 (Sigma- Aldrich; EE4, N=12~~) or~~), 400 ng/Kg EE2 (EE400, N = 12~~)~~), or vehicle (peanut OIL, N=12) from GD 5 until weaning of the pups, the other 36 dams were untreated. The treatment was administered orally with a pipette. This procedure is likely much less stressful than gavage (Vandenberg et al. 2014, Gioiosa et al. 2015). On postnatal day (PND) 1, pups were removed from their dams and gently placed in a cotton nest; each ~~animal was~~ weighed with an analytical scale, and the anogenital (AGD) distance ~~was~~ measured with a caliper. On the same day the litters~~,~~ were culled to 4 females and 4 males~~, were~~ and then cross-fostered~~:~~. Pups born from treated dams were fostered to untreated dams so that their exposure to EE_2 or vehicle was confined to the gestational period (GEST), whereas pups born from untreated dams were fostered to treated dams so ~~that they were~~to be exposed to EE_2 or vehicle only during lactation (LACT) (Table 1). At weaning (PND 21~~)~~), all litters were ~~isolated~~separated from foster-dams and at PND 32 one female for each litter was individually marked with cosmetic dye on the tail and randomly housed with 3 other ~~3~~ females that had received the same treatment ~~so that no~~. No cage contained siblings. Thus, only one female per litter~~,~~ for a total of 72 females~~,~~ was used for the study i.e. each experimental subject came from a different dam. Vaginal opening was ~~controlled~~checked daily by a person not blind to treatment.

Here Table 1

The animals were housed in polysulfone cages ~~(Tecniplast, Italy, 60 x 37 x 20 cm),~~as previously described under ~~a~~an inverted reversed light-dark cycle (dark 07.30-19.30) with a relative humidity of 60 +/- 10%. Food (Harlan Teklad soy-free AIN-76A diet~~,~~) and water were supplied *ad libitum* throughout the experiment~~) and water were available ad libitum~~.

Behavioral testing

Observations were carried out during the dark phase, under dim red light combined with low indirect white light. All sessions were recorded with a video camera (Sony AVC – D5CE~~);~~) and video recordings were analyzed with 'The Observer Video Pro 4.0' software (Noldus Information Technology, The Netherlands) by an observer blind to treatment.

Subjects were tested for social play between PND 40 and 45~~:~~, an age at ~~this age~~which social play is still vigorous and not significantly different from that expressed at 35 days of age (Porrini et al. 2005). The four females ~~of~~from the same housing cage were tested together in a neutral arena (60 x 35 x 35 cm). At the beginning of the observation, just before the introduction of the rats into the arena, a small quantity of sawdust from the home ~~cage's sawdust~~ cage was mixed to the clean ~~sawdust~~one of the

testing arena to facilitate habituation to the novel environment. After 1 min of familiarization, the behavior of the four animals was video-recorded for 15 ~~min~~mins. Social and non-social behaviors of each individual were identified according to the ethogram described in Table 2, modified from Porrini et al. (2005). A behavior was attributed to the subject initiating the action. Testing of different experimental groups was balanced across time.

Here Table 2

Animal welfare

The experiments described in this research were approved by the Ethical Committee of the Department of Physiology, University of Siena and followed European Community Council Directive 86/609/EEC and institutional guidelines.

Statistical analysis

To reduce the dimensions of the data set (and thus reduce the number of tests to be carried out) and identify correlated behavioral items (and thus eliminating autocorrelation), we carried out a Principal Component Analysis (PCA) (Jolliffe 2014). The Kaiser Meyer Olkin index (a measure of the proportion of variance in common between the different variables) was used to estimate the overall adequacy of the matrix (Cerny and Kaiser 1977), and communalities (a measure of the proportion of variance of each variance explained by the matrix) were calculated to for identifying variable contribution to the correlation matrix (the higher communality, the more the variable is associated to others) (Tabachnick and Fidell 2007).

Once extracted, the Principal Components (PCs) were rotated (with the Equamax procedure to capture the maximal amount of variance) to facilitate the interpretation of the different components, and Kaiser normalization was applied to reduce anomalies in the components loadings. Scores were saved using the Anderson-Rubin method to guarantee orthogonality between components (Jolliffe 2014).

The first three PCs were used as dependent variables into three General Linear ~~Model~~Models (GLM) to compare behavior ~~in~~between different treatment groups and different times of exposure (treatment, timing).

Analysis of variance using a GLM approach was also used to test differences ~~in biometry (AGD, body weight and vaginal opening)~~ between~~,~~ treatment groups for AGD and body weight, ~~or~~and between treatment groups and ~~between timing~~times of exposure (and their interaction) for vaginal opening and body weight at 21 days of age. For all analyses, we added 'cage' as a random factor to

account for the effect of the social group. Post-hoc comparisons were carried out using the LSD test (Sokal and Rohlf 1995). All tests were performed using IBM SPSS 22 (IBM®, Chicago, IL).

Results

Anatomical and physiological variables

Anogenital distance (AGD) and body weight at PND 1 and PND 21 were not significantly affected by gestational administration of EE_2 (for statistical values, see Tab. 3). The treatment significantly affected vaginal opening (VO), in particular the higher EE_2 dose (EE400) significantly delayed VO with the main effect due to lactational administration (Tab. 3).

Here Table 3

Social activity

We ~~measured~~computed Social activity by pooling all behavioral items indicating any social interaction during the 15 min test (Fig. 1~~);~~). Social activity significantly increased with ~~treatment~~increasing EE2 dose ($F_{2,64}$=6.59, P=0.002), EE400 vs Oil (Post hoc LSD P=0.001), EE400 vs EE4 (Post hoc LSD P=0.017). ~~Also, the~~Moreover, time of treatment significantly affected total social activity, with gestational treatment being more effective than lactational one ($F_{1,64}$=10.44, P=0.002). No significant interaction of treatment x timing ($F_{2,64}$=0.991, P=0.370) or cage effect ($F_{2,64}$=0.165, P=0.864) were detected. Non-social activity, including all non-social active behaviors, was not affected by treatment or timing of exposure or cage (data not shown).

Here Fig 1

PCA applied to the frequencies of the behavioral items ~~shows~~showed 7 components, explaining 73.77% of the variance (Tab. 4). Principal component 1 (PC1) ~~includes~~included most elements of defensive play, ~~explains~~explained the higher percentage of variance (16.98%) and ~~is~~was labeled as "Defensive-like play". PC2 ~~includes~~included most element of aggressive play and ~~is~~was labeled as "Aggressive-like play" (variance explained 15.73%). PC3 (12.59%) ~~includes~~included elements of social and non-social exploration and ~~is~~was labeled as "Exploration". PC4 (7.34%), due to its non homogeneous behavioral components, ~~is~~was not labeled. PC5 (7.33%) ~~includes~~included a mixture of a bedding material oriented behavior (chewing) and play (Pinning). PC6 (7.15%) ~~includes~~included

mainly self-grooming. PC7 (6.65%) ~~excludes~~excluded allo-grooming and ~~includes~~included solitary running.

Based on the weight of each component and their internal coherence and their relevance to social behavior, we decided to consider only the first three components, explaining 45.3% of variance. Since PC 4, 5, 6, 7 (explaining only a residual 28.47% of variance) were not internally consistent, ~~we decided not to consider them~~were excluded from further analyses.

GLM was applied to each component, using the individual component scores as variables, considering treatment (OIL, EE4, EE400), timing of administration (GEST, LACT), cage and interactions.

Here Table 4

Defensive-like play

Defensive-like play, described by PC1, was not significantly affected by treatment ($F_{2,64}$=2.04, P=0.139), however we found an effect of timing ($F_{1,64}$=9.65, P=0.003). No significant interaction between treatment ~~x~~ and timing ($F_{2,64}$=0.12, P=0.880) or a cage effect ($F_{2,64}$=1.291, P=0.282) were present. The lack of a treatment effect ~~despite~~ in spite of the presence of an effect of timing might be due to ~~a~~the reduced power ~~(power =~~(=0.4~~),~~) of the test, which is particularly relevant when considering the individual variability in the response.

Aggressive-like play

Aggressive-like play (Tab. 4, Fig. 2~~),~~) described by PC2~~,~~ was significantly affected by treatment ($F_{2,64}$=5.42, P=0.007~~): administration~~). Administration of EE_2 increased ~~its~~the frequency of aggressive-like play, and the higher dose (EE400) was more effective than the lower one (EE4) (post hoc LSD, P=0.01). Timing ~~was~~had also a significant effect, with gestational treatment being more effective than lactational one ($F_{1,64}$=6.29, P=0.015). We found no significant effects of the interaction between treatment ~~x~~and timing ($F_{2,64}$=0.379, P=0.686) or of the cage ($F_{2,64}$=0.001, P=0.999).

Here Fig 2

Aggressive neck grooming and Pounce ~~are~~were the most representative behaviors of Aggressive-like play (Tab. 4). In particular, Aggressive neck grooming (Fig. 3a) was significantly increased by EE2 treatment ($F_{2,64}$=3.59, P=0.033, EE400 vs EE4 Post hoc LSD P=0.016, EE400 vs Oil Post hoc LSD P=0.038). Timing of administration was significant, with gestational administration being

significantly more effective than lactational one ($F_{1,64}$=9.23, P=0.003). No significant effects of the interaction between treatment ~~x~~and timing ($F_{2,64}$=0.075, P=0.928) or of cage ($F_{2,64}$=1.480, P=0.235) were detected.

Pounce (Fig. 3b) was significantly increased by both doses ($F_{2,64}$=5.50, P=0.006, EE400 vs EE4 Post hoc LSD P=0.010, EE400 vs Oil Post hoc LSD P=0.003~~);~~) and the timing of administration ~~was~~had a significant~~,~~ effect, in that gestational administration ~~being~~was significantly more effective than ~~the~~ lactational one ($F_{1,64}$=11.89, P=0.001~~); no~~). No significant effects ~~for~~of the interaction between treatment ~~x~~and timing ($F_{2,64}$=0.007, P=0.993) or for cage ($F_{2,64}$ =0.753, P=0.475) were detected.

Here Fig 3 (a, b)

Exploration

Social and non-social exploration (Tab. 4~~),~~) described by PC3~~,~~ were not significantly affected by treatment ($F_{2,64}$=2.027, P=0.140), timing ($F_{1,64}$=3.599, P=0.062), or interaction treatment x timing ($F_{2,64}$=1.217, P=0.303), or by cage ($F_{2,64}$=0.825, P=0.443).

Pinning

Pinning (Fig. 4), a playful behavior associated with play fighting, (Pellis 2002), ~~in our PCA analysis~~ was associated to Defensive-like play (PC1), Aggressive-like play (PC2) and PC 5 in our PCA analysis. For this reason, we decided to consider this behavioral item *per se*. It was significantly increased by treatment ($F_{2,64}$=6.2, P=0.003), with the higher dose more effective than the lower one: EE400 vs EE4 (Post hoc LSD P=0.014), EE400 vs Oil (Post hoc LSD P=0.001). No significant effects were observed for timing ($F_{1,64}$=3.044, P=0.086), the interaction between treatment ~~x~~and timing ($F_{2,64}$=0.200, P=0.819) and cage ($F_{2,64}$=2.286, P=0.110).

~~Frequencies~~The frequencies of all behaviors included in PC1, PC2, PC3 of the PCA~~,~~ with an eigenvalue >0.3 are reported in the Supplementary ~~material~~ Table A1.

Here Fig 4

Discussion

~~The~~Our findings ~~of our study show~~showed that developmental exposure of female rats to low or very low doses of the synthetic estrogen EE_2 during gestation or lactation significantly alters social play by increasing its aggressive components. This is a new finding, and is in line with the results of Olesen et al. (2005) and Ferguson et al. (2014), who observed ~~in females~~ a similar increase in female aggressive-like play after developmental exposure to pharmacological doses of estradiol. Our PCA divided social play in two main components, one including Defensive-like play behavior such as On back, Lateral display, and Withdrawal, and another one (Aggressive-like play), comprising Aggressive neck grooming and Pounce. The observed effect ~~is~~was particularly evident in ~~particular on Aggressive-like play. In fact,~~ Aggressive-like play, and its main components Aggressive neck grooming and Pounce~~,~~ were significantly increased by treatment with EE_2. ~~Also Pinning showed a significant increase in frequency at the higher dose. Our PCA showed that~~ Pinning, a behavior considered by many authors the most representative element of social play (Blake and McCoy 2015), showed a significant increase in frequency at the higher treatment dose and was mildly correlated to both ~~on~~ Defensive-like play and Aggressive-like play (Table 4). This is not surprising as it is commonly recognized that~~, during play activities,~~ individuals show reciprocity of roles during play activities, (e.g. switching from being pinned to pinning), a peculiar characteristic of play.

Our study integrates the findings by Meaney and Stewart (1981~~),~~) who showed that play fighting is influenced by 5α-~~-~~reduced products of testosterone, and suggests that early estrogen exposure ~~is~~can similarly ~~able to~~ affect juvenile social play, in line with Olesen et al. (2005) and Ferguson et al. (2014~~). This is not surprising due to~~), and in accordance with the known role of estrogen in modulating a wide range of socio-sexual behaviors.

Our results ~~show~~showed a significant increase in aggressive components of play behavior suggesting a possible masculinizing effect of ~~treatment~~EE2 on female brain. In fact, juvenile social play in rats is often described as sexually dimorphic: males show a greater motivation to play and initiate more playful attacks than females do (Pellis et al. 1997, Auger and Olesen 2009, Argue and McCarthy 2015). However, in a parallel study on male rats in which we followed the same experimental design ~~of~~as the one in the present ~~experiment~~study, we did not observe significant differences ~~of~~in play behavior between control males and control females (Zaccaroni et al. in prep.). A lack of sexual dimorphism in play behavior has been reported by several authors (Panskepp et al. 1984, Flynn et al. 2001, Colbert et al. 2005, Flynn et al. 2005, Veenema et al. 2013). These contrasting results are probably due to a variety of factors, such as conditions of rearing, familiarity of playmates, familiarity of experimental arena, sex and weight of the playmate (Panskepp et al. 1984, Paul et al. 2014, Argue and McCarthy 2015).

~~Both~~In our study, both doses of EE_2 produced significant effects on behavior~~,~~; an interesting finding given that the environmentally relevant dose was very low (4 ng/kg/day~~) was very low.~~). In general,

gestational exposure was more effective than lactational one, which is consistent with studies reviewed by Delclos et al. (2009) reporting a limited transfer of EE_2 to newborns via milk in humans and rats.

It is remarkable that the low doses of EE_2 used in our experiments, while effective on some aspects of play behavior, were unable to affect important non-behavioral endpoints such as weight and AGD at birth. Since AGD is considered a sensitive androgen-dependent developmental marker (Rhees et al. 1997), our results, in line with those of Howdeshell et al. (2008) and Ferguson et al. (2011), suggest that early EE_2 treatment does not interfere with androgen regulation, which ~~per se~~ is able *per se* to influence development of social play (Meaney and Stewart 1981; Meaney et al. 1983; Thor and Holloway 1986; Pellis and McKenna 1992). ~~Interestingly, vaginal~~Vaginal opening was slightly but significantly delayed by the higher dose of EE_2~~. This~~, a result that contradicts the idea that estrogen accelerate puberty (reviewed in Goldman et al. 2000~~),)~~ but ~~is~~ in agreement with ~~the~~ findings of ~~Sawaki et al. (2003), Delclos et al. (2014), and Ferguson et al. (2014) who observed~~ significant delays in sexual development after exposures to EE_2 ranging from 500 to 10.000 ng/kg/day (Sawaki et al. 2003, Delclos et al. 2014, Ferguson et al. 2014; but see Derouiche et al. 2015 in mice). Thus, our results add to the number of studies that showed heterogeneous effects of estrogen on vaginal opening (reviewed by Ferguson et al. 2014).

How can such low doses of EE_2 affect behavior? In female rats, the prenatal brain is protected by AFP from maternal circulating estrogen (Bakker et al. 2006), however EE_2 has low affinity with AFP (Hong et al. 2012) and high affinity with ERα (Blair et al. 2000). Therefore, EE_2 could bypass the protective function of AFP and affect sexual differentiation of the brain even at very low doses. Effects of subtle variations in the concentration of hormones on development have been reported previously, as illustrated by the differences in adult behavior depending on the intrauterine position that affects the prenatal hormonal milieu (Ryan and Vandenbergh 2002, vom Saal 2016).

~~We have shown that using~~With an experimental protocol designed to mimic an environmental or clinical exposure~~,~~ to EE2, we observed robust effects of very low doses of EE_2 ~~can be observed~~ on key traits such as social play, an essential component of the maturation of social behavior. If play is important for the refinement of social skills (van den Berg et al. 1999, Pellis et al. 2010), then a modification of play may have consequences on adult social behavior. An additional contribution of the present study is the demonstration that low doses of a pure estrogen can be used as a tool to increase our understanding of the maturation of socio-sexual behavior. Previous work relying on castration/hormone replacement approaches often highlighted on-off effects of the hormones with a consequent masking of those traits that typically respond in a dose-dependent manner.

Conclusions

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Our study ~~shows~~showed that low and very low doses of EE_2, mimicking clinical or environmental exposure during development, can affect important ~~aspect~~aspects of social behavior even in restricted time windows of action, with ~~subsequent~~ possible important consequences on adult behavior. The high sensitivity of the behavioral endpoints examined in our study highlights the importance of implementing behavioral tests on females, the sex more prone to be influenced by developmental exposure to estrogenic substances, to study the potential effects of low doses of endocrine disrupters.

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Conflict of interests

The authors declare that there is no conflict of interests associated with this paper.

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699
700 **CAPTIONS**
701

Fig. 1 Frequency (n/15 min) of Total social activity of female rats exposed to gestational or lactational treatment with 4 ng/kg/day (EE4) or 400 ng/kg/day (EE400) of EE_2. Box-whiskers show median, interquartiles, and range of individual values. Timing ($F_{1,64}$=10.44, P= 0.002); Treatment ($F_{2,64}$=6.59, P=0.002); EE400 vs Oil (Post hoc LSD P=0.001); EE400 vs EE4 (Post hoc LSD P=0.017).

Fig. 2 Principal components scores of Aggressive-like play (PC2) performed by female rats exposed to gestational or lactational treatment with 4 ng/kg/day (EE4) or 400 ng/kg/day (EE400) of EE_2. Box-whiskers show median, interquartiles, and range of individual values. Timing ($F_{1,64}$=6.23 P=0.015); Treatment ($F_{2,64}$=5.42 P=0.007); EE400 vs Oil (Post hoc LSD P=0.003), EE400 vs Oil (Post hoc LSD P=0.012).

Fig. 3a,b. Frequency (n/15 min) of Aggressive neck grooming (**a**) and Pounce (**b**) performed by female rats exposed to gestational or lactational treatment with 4 ng/kg/day (EE4) or 400 ng/kg/day (EE400) of EE_2. Box-whiskers show median, interquartiles, and range of individual values. Aggressive neck grooming (**a**) test results: Timing ($F_{1,64}$=9.23, P=0.003); Treatment ($F_{2,64}$= 3.59, P=0.033); EE400 vs EE4 (Post hoc LSD P=0.016), EE400 vs Oil (Post hoc LSD P=0.038).
Pounce (**b**) test results: Timing ($F_{1,64}$=11.89, P=0.001); Treatment ($F_{2,64}$=5.50, P=0.006); EE400 vs EE4 (Post hoc LSD P=0.01), EE400 vs Oil (Post hoc LSD P=0.003).

Fig. 4 Frequency (n/15 min) of Pinning performed by female rats exposed to gestational or lactational treatment with 4 ng/kg/day (EE4) or 400 ng/kg/day (EE400) of EE_2. Box-whiskers show median, interquartiles, and range of individual values. Timing ($F_{1,64}$=3.04, P=0.086); Treatment ($F_{2,64}$=6.2, P=0.003); EE400 vs EE4 (Post hoc LSD P=0.014), EE400 vs Oil (Post hoc LSD P=0.001).

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732 **Table 1.** Outline of the experimental groups. The GESTATIONAL animals received the treatment
733 only in utero (GD 5-20) from their treated dams and at birth were fostered to untreated dams. The
734 LACTATIONAL group received no treatment in utero and were then exposed to the treatment only
735 via the milk of their foster treated dams (PND 1-21). EE4= 4ng/kg/day; EE400=400 ng/kg/day.
736

	OIL	**EE4**	**EE400**
GESTATIONAL (pups from treated dams fostered to untreated foster dams)	12	12	12
LACTATIONAL (pups from untreated dams fostered to treated foster dams)	12	12	12

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Table 2. List of social and nonsocial behaviors considered. Each behavior is performed by the focal subject.

Social behaviors
Aggressive neck grooming (vigorous neck allogrooming)
Allo-grooming
Approach (moving toward another)
Bite
Boxing (both rats stand up facing each other and boxing with forepaws)
Chase
Crawl-over (moving over another)
Crawl-under (moving under another)
Flee
Genital sniffing
Jumping and running
Lateral display (the animal orientates itself broadside to another animal)
On back (lying on the back with belly exposed to another)
Pinning (standing over the opponent with its forepaws on the ventral surface)
Pounce (bouncing over another)
Sniff (sniffing another's body except genital area)
Upright (with erect posture the rat exposes its belly to another)
Withdraw (all movements away from another)

Non-social behaviors
Chew substrate
Crouch
Dig
Explore (exploration of the environment)
Rear (animal stands up)
Run
Self-grooming

Table 3. Anogenital distance at birth, body weight at birth, body weight at 21 days and vaginal opening. General linear model with LSD post-hoc test demonstrating significant main effect values are expressed as mean (SD). N/A=not included in the analysis.

Variables	Timing of administration	OIL	EE4	EE400	treatment *F, P*	timing *F, P*	treat x tim *F, P*	OIL vs EE4 *P*	OIL vs EE400 *P*	EE4 vs EE400 *P*
Anogenital distance (mm)	GEST	1.35 (0.15)	1.26 (0.18)	1.28 (0.25)	0.53, 0.59	N/A	N/A	N/A	N/A	N/A
Body weight at birth (grams)	GEST	6.15 (0.45)	6.13 (0.62)	5.77 (0.59)	1.23, 0.3	N/A	N/A	N/A	N/A	N/A
Body weight at 21 days (grams)	GEST	47.05 (6.09)	49.24 (3.03)	45.99 (1.96)	0.62, 0.54	1.60, 0.21	N/A	N/A	N/A	N/A
	LACT	44.87 (4.02)	45.88 (1.60)	47.03 (2.61)						
Vaginal opening (days)	GEST	36.08 (1.93)	35.83 (1.59)	36.75 (1.42)	4.47, **0.015**	2.60, 0.11	1.1, 0.33	0.72	**0.015**	0.09
	LACT	35.92 (1.24)	36.75 (0.87)	37.67 (1.50)						

Table 4. Results of PCA applied to behaviors of female rats. Total variance explained: 73.77%. Only loadings > ± 0.3 are shown. Components 4, 5, 6 and 7 are not labeled since the behaviors identified are not homogeneous. Each behavior is performed by the focal subject.

	Components						
	1 Defensive-like play	2 Aggressive-like play	3 Exploration	4	5	6	7
On back	0.883						
Lateral display	0.863						
Withdraw	0.796	0.325					
Pinning	0.510	0.347			0.456		
Aggressive neck grooming		0.849					
Pounce	0.527	0.737					
Bite		0.634			0.306		
Crawl-over		0.633	0.352				
Flee	0.523	0.632					
Chase	0.426	0.484	0.388				
Explore			0.914				
Sniff			0.731		0.401		
Rear			0.639		-0.301	-0.336	0.327
Genital sniffing			0.631	0.324			
Crawl-under				-0.766			
Approach		0.460		0.576		0.334	
Boxing	0.439			0.490			
Chew substrate					0.894		
Self-grooming						0.860	
Allo-grooming							-0.850
Run						-0.483	0.600
Variance explained	16.98	15.73	12.59	7.34	7.32	7.15	6.65

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Figure1

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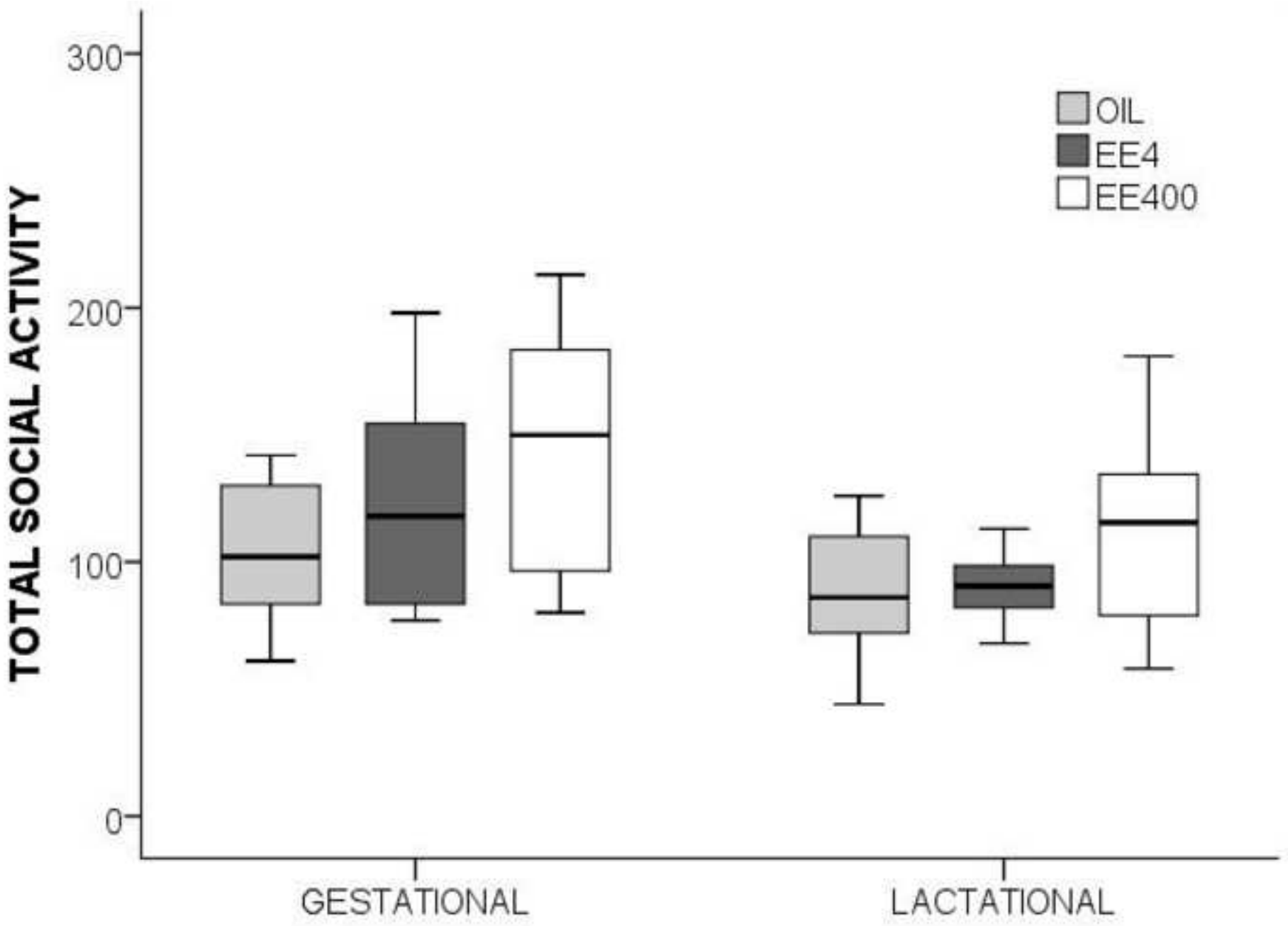

Figure3B

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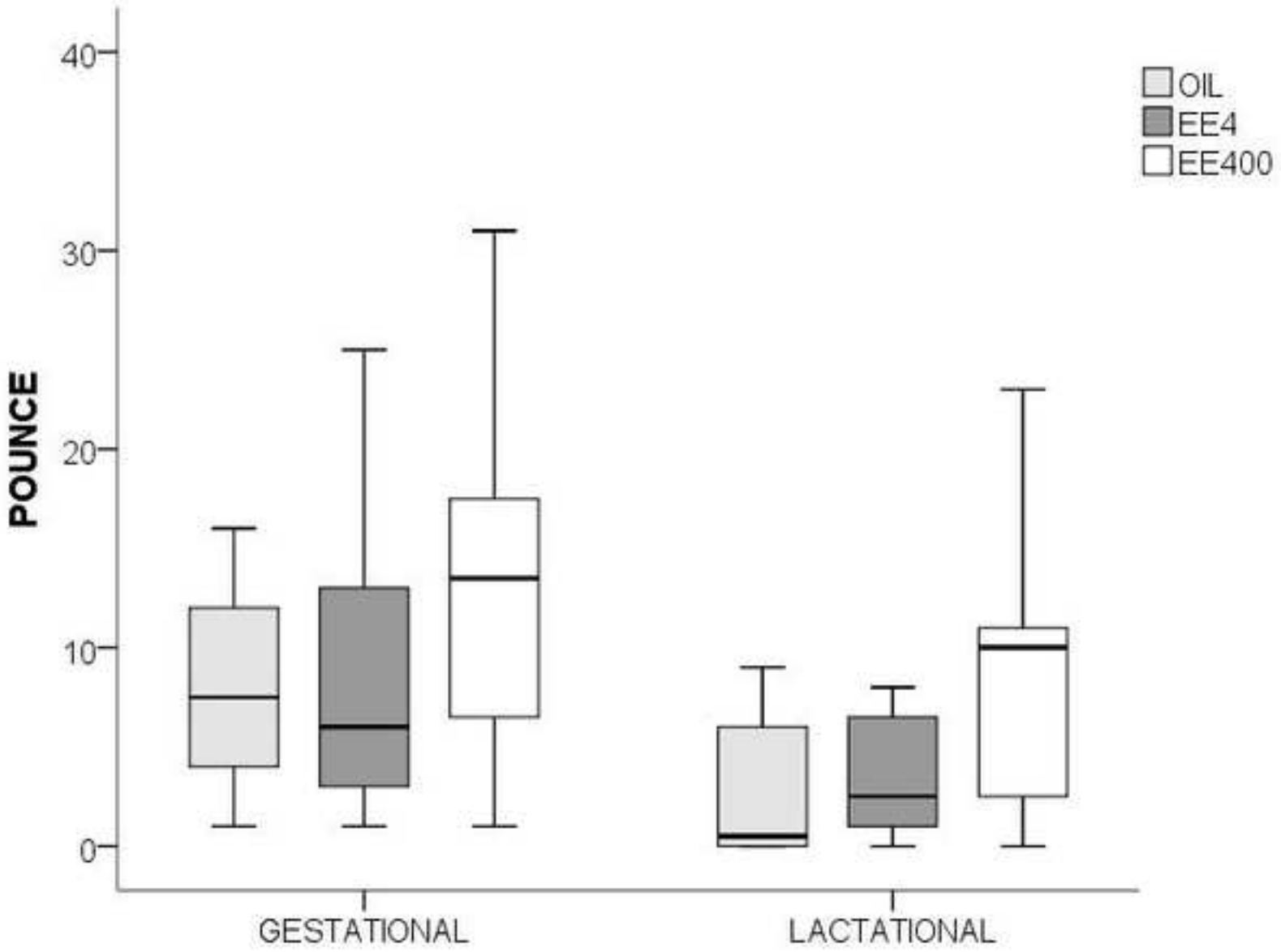

Figure4

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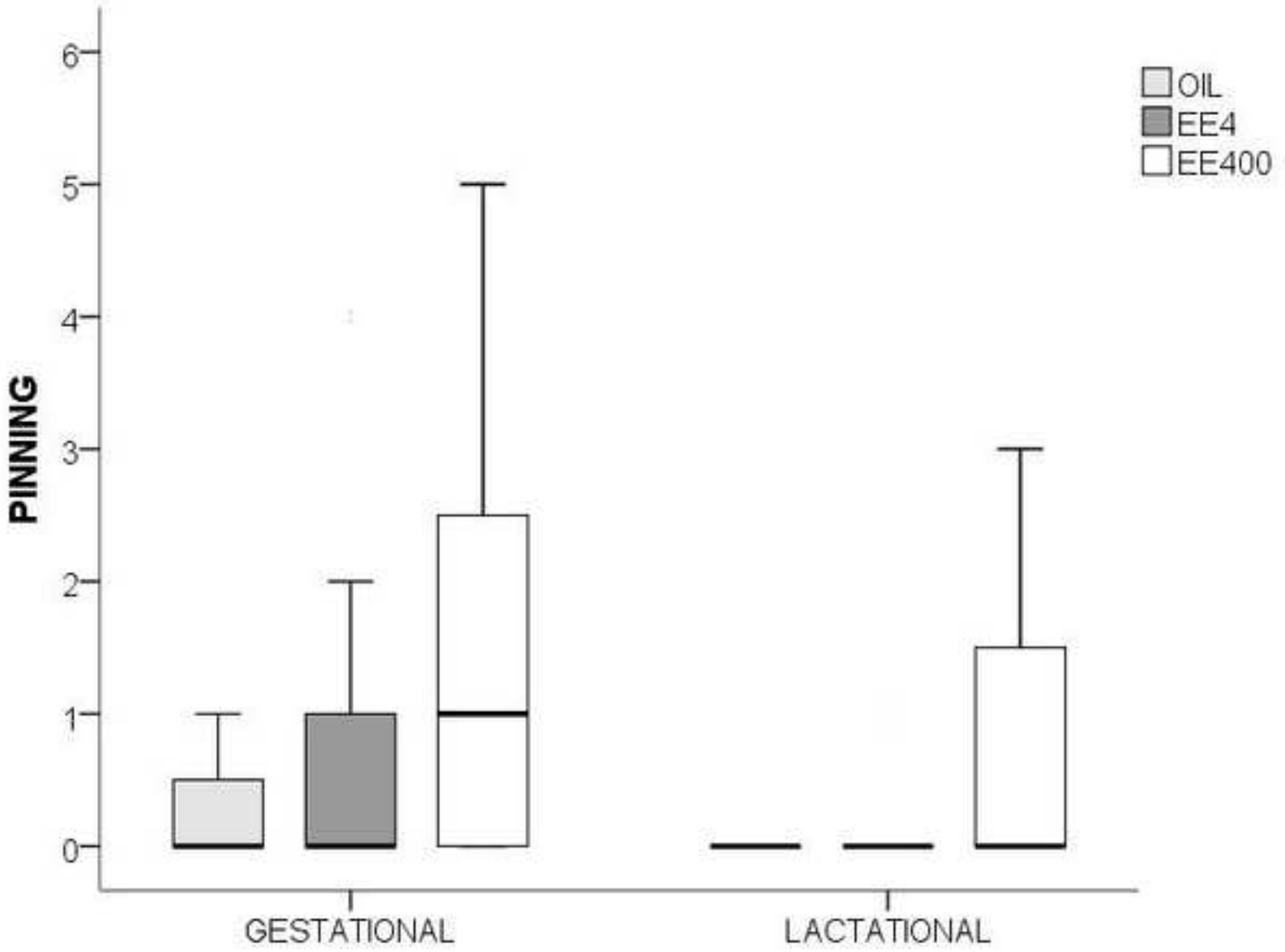

Figure2

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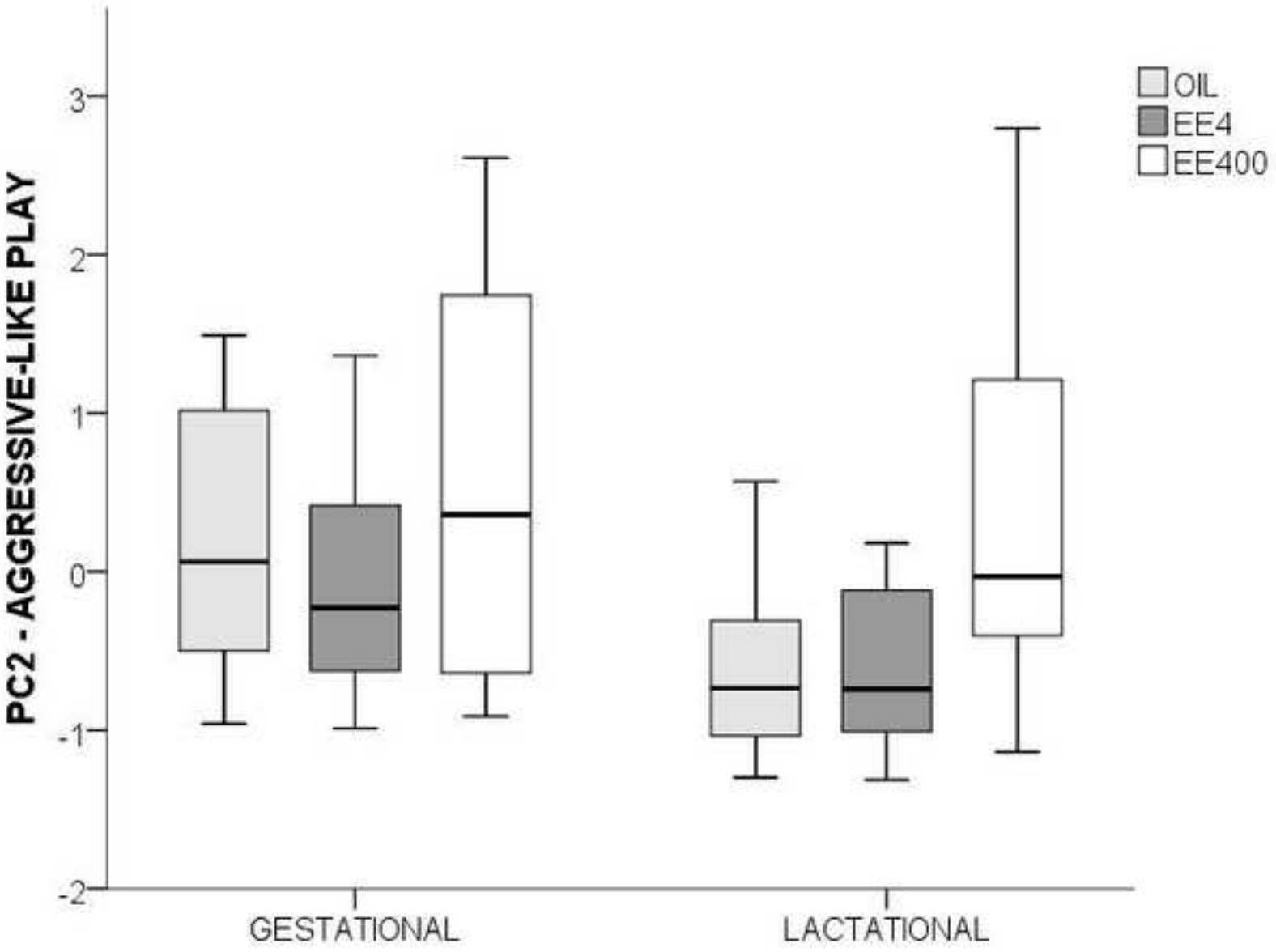

Figure3A

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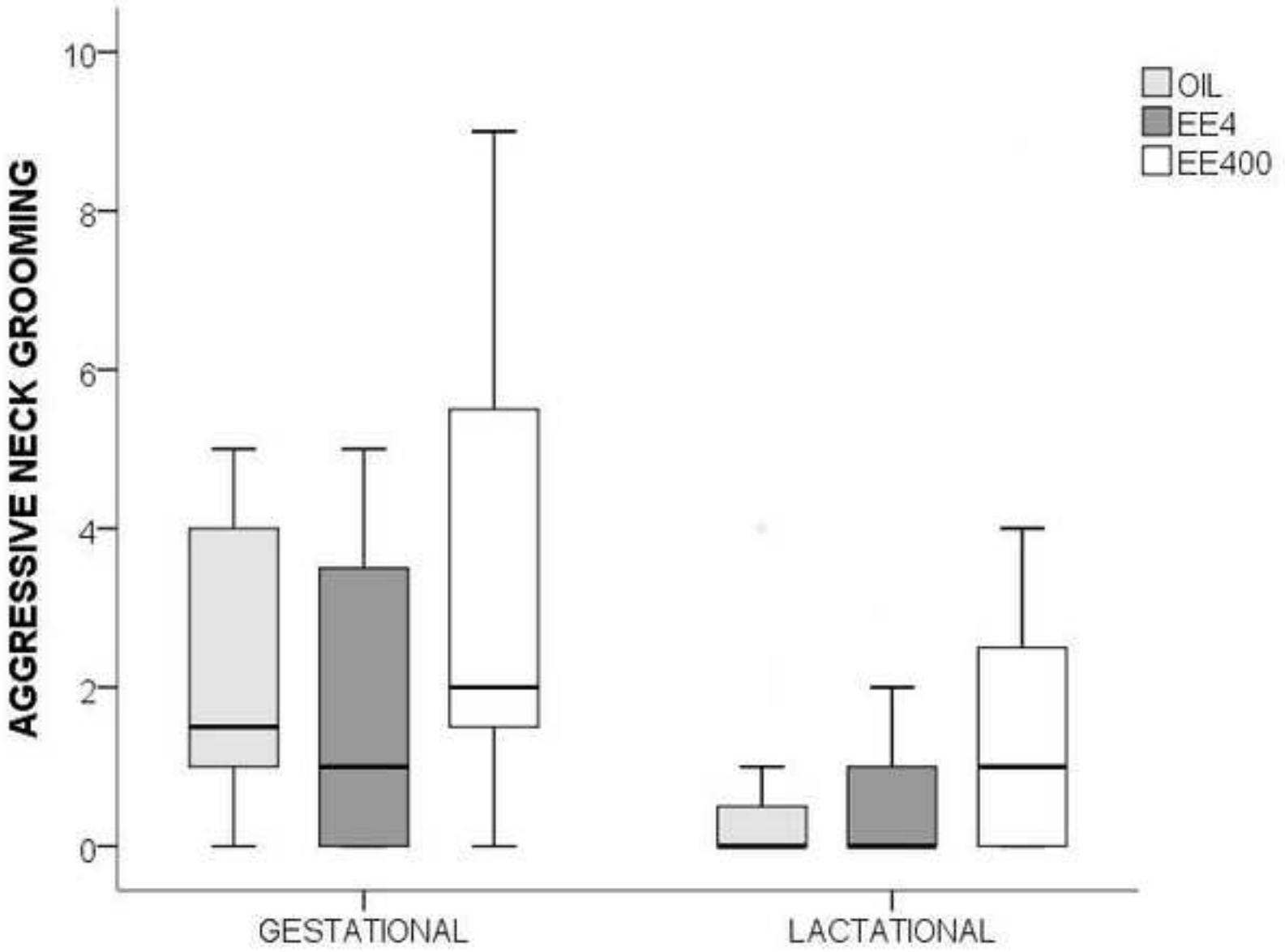

Table1 A