

# Altered reproductive success in rat pairs after environmental-like exposure to xenoestrogen

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Endocrine-disrupting compounds (EDCs) have the capacity of altering the normal function of the endocrine system. EDCs have shown dramatic effects on the reproductive biology of aquatic wildlife and may affect human reproduction as well. Studies on EDCs in mammalian species have often investigated the effects of short-term, high doses on male and female reproductive physiology. However, it is difficult to predict from such studies the effects of EDC on populations that are exposed to very low doses throughout their life via contaminated food and water. We studied the effects of EDC on mammalian reproduction with an environmental-like protocol where the endpoint is the reproductive success of exposed pairs. We focused on a subclass of EDC, the xenoestrogens, which mimic the action of natural oestrogen hormones. Male and female rats were exposed to low doses of the pure oestrogen, ethinyloestradiol, during development, by oral administration to their mothers during pregnancy and lactation, and to them until puberty. We evaluated the effects of the exposure on development and reproductive physiology of individuals, and on fertility and fecundity of pairs in which both members had been exposed to the same treatment. We found that low doses caused major reproductive deficits in the experimental animals. Very low, environmentally relevant doses did not have evident effects on exposed animals; however, the fecundity of exposed pairs was substantially altered. Environmentally relevant doses of xenoestrogens which have no evident physiological effects can alter the reproductive success of exposed pairs in natural populations.

**Keywords:** xenoestrogen; reproductive success; fertility; fecundity; environmental exposure

## 1. INTRODUCTION

Among the major environmental concerns nowadays are the endocrine-disrupting compounds (EDCs). EDCs are a heterogeneous class of chemicals, both man-made and natural, which are present in the environment and have the potential of altering the endocrine system of organisms (Colborn *et al.* 1993; Anway *et al.* 2005). Many EDCs are classified as ‘xenoestrogen’ because their action mimics that of oestrogen hormones. The first evidence for the effects of EDC came from observations of reduced fertility and hormonal perturbation in aquatic organisms living in polluted waters (Colborn *et al.* 1993; Guillette & Gunderson 2001). To date, deleterious effects of EDC have been described for a number of vertebrate species including humans (Fox 2001; reviewed by Schantz 1996; Milnes *et al.* 2006).

A number of studies have been conducted to determine the potential dangers of several chemicals for human and wildlife. Most studies have focused on the effects on the reproductive systems, with a particular attention to fertility and fecundity. However, the classical toxicological approach does not always provide the best way to estimate the real dangers of exposure for wildlife populations (Tyler *et al.* 1998; Brown *et al.* 2001). Typically, the chemical to be tested is given at relatively high doses via injections or subcutaneous implants for a limited period of time, either

developmentally or in the adult. Although this design allows the study of the effects of the tested chemical on a range of physiological markers, the step to predicting the consequences for natural populations is a long one.

More recently, several studies have examined the effects of EDC in semi-natural settings, for example, by exposing aquatic organisms to an EDC throughout their life stages and for several generations (Patyna *et al.* 1999; Nash *et al.* 2004; Brown *et al.* 2005; Kristensen *et al.* 2005). Few studies have followed the same approach for terrestrial vertebrates and in particular for mammals (Bogh *et al.* 2001; Oskam *et al.* 2005; Ottinger *et al.* 2005b). Most of the work done on laboratory rodents, in fact, has followed the traditional toxicological approach, although a few laboratories including ours have started using environmental-like exposure protocols to warrant a more realistic interpretation of the results (Farabollini *et al.* 2002; Palanza *et al.* 2002). We define an exposure ‘environmental-like’ if it satisfies three criteria: (i) the key doses must match concentrations of pollutants found in the environment, (ii) the route of exposure should be the actual one, i.e. food and water for a terrestrial mammal, and (iii) the exposure should be prolonged because animals are likely to be exposed the EDC present in their habitat throughout their life. Even following the above criteria, however, it is difficult to predict the long-term effects on natural populations by studying physiological variables in the exposed individuals (Matthiessen 2003; Propper 2005).

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In this paper, we have gone a step further and developed a non-standard protocol for testing the effects of EDC on reproductive success of exposed populations. We have studied the effects of the oestrogen, 17 $\alpha$ -ethynylloestradiol (EE), on the fertility and fecundity of pairs of rats after an environmental-like exposure to this chemical during development. We focused on EE because it is a pure oestrogen, the main component of the contraceptive pill, and at the same time, an environmental oestrogen found in urban waste water owing to its widespread use (Nash *et al.* 2004). Thus, effects of EE are truly xenoestrogenic and allow extrapolation to how other estrogenic substances would act in the same conditions. The animals were exposed to EE by giving it orally to their mothers during pregnancy and lactation and to them from weaning to puberty. In adulthood, we evaluated the fertility and fecundity of pairs in which both animals were from the same treatment group. We found that although environmental doses of EE do not have evident effects on the growth and development of exposed animals, they impact substantially their reproductive success.

## 2. MATERIAL AND METHODS

### (a) *Animals and treatment*

We used 36 male and 36 female Sprague–Dawley rats, which were born and raised in the Physiology Department, University of Siena. They were the F1 of 36 females and 20 males purchased from Harlan Italy. Starting from 5 days before pairing, the mothers were trained to drink the vehicle (peanut oil, Sigma–Aldrich) from a pipette. From gestation day 5 to weaning at postnatal day 21 (PND21), the mothers received orally 17 $\alpha$ -ethynylloestradiol (EE, Sigma–Aldrich) at a dose of 4 ng kg<sup>-1</sup>d<sup>-1</sup> (EE4, *N*=12) or 400 ng kg<sup>-1</sup>d<sup>-1</sup> (EE400, *N*=12), or the vehicle alone (OIL, *N*=12). To avoid confusion, from now on, we will call treated animals the offspring of these mothers. The dose received by the EE4 group matches concentrations actually measured in contaminated European and US surface waters, where EE is one of the most common hormonally active pollutants (Nash *et al.* 2004). Since EE is accumulated in fish tissues (Lange *et al.* 2001; Skillman *et al.* 2006), populations with a diet based on fish may ingest amounts of EE equivalent to levels found in the water (see also §4). The EE400 dose is equivalent to that of most contraceptive oestrogenic or oestrogen plus progestin pills and can be considered a physiological dose because it matches endogenous levels of oestrogens. At PND2, we weighed all pups and one operator measured the anogenital distance, an index of morphological sex differentiation. The litters were then reduced to five male and five female pups to standardize maternal effort. Body mass of all pups was measured again at PND7, -14, -22 and -32. The young rats were separated from the mother at weaning, and received the treatment orally until puberty (PND32). Thus, experimental rats received the treatment indirectly through the placenta and the milk from conception to weaning, and directly until puberty. On PND32, juveniles were individually marked and randomly assigned to unisexual groups of four, so that no cage contained siblings and all animals in a cage had received the same treatment. At seven months of age, we paired one male and one female of the same treatment group but not from the same litter, forming 12 pairs per treatment group, for a total of 36 pairs. We used only one male and one female from each litter, to avoid possible litter effects. These animals had been

used in a spatial learning experiment two months before pairing (Corrieri *et al.* 2007). After pairing, the male and the female were left together for 15 days, after which we removed the male. We recorded the number of pregnant females and the duration of gestation from the day of pairing. We weighed and sexed all F2 pups at PND2 and again at PND10. The experiments were concluded on this day. All rats were housed in Plexiglas cages (Tecniplast, Italy, 60×37×20) with metal tops and a sawdust bedding at 21±1°C, with a relative humidity of 60±10% and a 12L:12D cycle (lights off: 07.30). Water and food (Harlan Teklad rat chow) were available *ad libitum*.

### (b) *Reproductive physiology*

#### (i) *Oestrous cycle*

Six weeks before pairing, we collected non-invasive daily vaginal smears from all 36 experimental females for 15 days to evaluate the regularity of the oestrous cycle. The stage of the cycle was determined by examining the cell type composition of the vaginal smears (as described in Jenkins & Becker 2005). By observing the series of daily smears, we estimated the regularity of the cycle, as follows: (i) regular cycle, oestrus lasts 1–2 days and is repeated every 4–6 days, (ii) irregular cycle, cycle is shorter or longer than 4–6 days, and (iii) persistent oestrus, cytology is permanently oestrus like (more than 8 days) and abnormal (§3). In addition, each daily smear was given a score from 0 (beginning of metoestrus) to 4 (end of oestrus) and we calculated for each individual the average score along the 15 days.

#### (ii) *Male androgen*

After pairing, the males were euthanized by an overdose of anaesthetic and we collected a blood sample with a heparinized syringe. After centrifugation, the plasma was collected and stored at -40°C until assayed. The concentration of the androgen testosterone was measured in a 50  $\mu$ l sample with a coated tube radioimmunoassay system (DSL-4000, Diagnostic Systems Laboratories, Inc., Webster, TX, USA). The sensitivity of the assay is 80 pg ml<sup>-1</sup>, and the intra-assay variation is lower than 10%.

## 3. RESULTS

### (a) *Developmental effects and reproductive physiology*

#### (i) *Development*

There was no evident effect of the treatment on survival and physiological parameters of the treated animals, *i.e.* the F1. There was no difference at PND2 between treatments for the number of pups (males: OIL, 7.00±0.52, EE4, 6.75±0.60, EE400, 6.17±0.52,  $F_{2,35}=0.60$ ,  $p=0.55$ ; females: OIL, 6.75±0.54, EE4, 7.00±0.43, EE400, 6.75±0.70,  $F_{2,35}=0.07$ ,  $p=0.94$ ), total litter mass (OIL, 85.78±4.84 g, EE4, 86.94±3.18 g, EE400, 81.23±6.02 g,  $F_{2,35}=0.54$ ,  $p=0.59$ ) and anogenital distance (males: OIL, 3.79±0.08 mm, EE4, 4.09±0.17 mm, EE400, 4.09±0.13 mm,  $F_{2,35}=0.13$ ,  $p=0.88$ ; females: OIL, 1.31±0.04 mm, EE4, 1.41±0.06 mm, EE400, 1.40±0.10 mm,  $F_{2,35}=1.74$ ,  $p=0.19$ ). We also found no effects of the treatment on the growth rate of the animals from PND2 until they reached puberty at PND32 (table 1). All the above data refer not only to the animals used in the following parts of the experiment but also to the entire F1 litters and, except for female body

Table 1. Body mass growth (g, means  $\pm$  s.e.m.) of the entire F1 litters from postnatal day (PND) 2 to weaning (PND 32). (The last two columns report the result of a one-way ANOVA ( $F$ ) and relative significance ( $p$ ). EE4, 4 ng kg<sup>-1</sup>d<sup>-1</sup> ethnyloestradiol; EE400, 400 ng kg<sup>-1</sup>d<sup>-1</sup> ethnyloestradiol; OIL, vehicle only (peanut oil). These data refer not only to the animals that were paired and used for the remaining parts of the experiment but also to their siblings, and those relative to males have been published previously, Corrieri et al. 2007.)

age	sex	OIL (12)	EE4 (12)	EE400 (12)	$F$	$p$
PND2	M	6.40 $\pm$ 0.12	6.60 $\pm$ 0.20	6.56 $\pm$ 0.13	0.49	0.61
	F	6.04 $\pm$ 0.09	6.19 $\pm$ 0.15	6.14 $\pm$ 0.15	0.36	0.70
PND7	M	13.56 $\pm$ 0.38	13.77 $\pm$ 0.57	13.75 $\pm$ 0.36	0.06	0.94
	F	13.02 $\pm$ 0.51	13.34 $\pm$ 0.50	12.86 $\pm$ 0.45	0.25	0.78
PND14	M	29.72 $\pm$ 0.75	29.05 $\pm$ 0.62	28.97 $\pm$ 0.60	0.39	0.68
	F	27.59 $\pm$ 1.06	28.33 $\pm$ 0.63	27.49 $\pm$ 0.74	0.30	0.74
PND22	M	51.01 $\pm$ 1.35	50.79 $\pm$ 1.18	51.05 $\pm$ 1.12	0.01	0.99
	F	47.41 $\pm$ 1.70	48.86 $\pm$ 1.14	47.90 $\pm$ 1.43	0.26	0.77
PND32	M	97.73 $\pm$ 2.53	99.95 $\pm$ 3.03	96.57 $\pm$ 2.69	0.39	0.68
	F	83.91 $\pm$ 2.30	90.26 $\pm$ 3.69	86.26 $\pm$ 2.87	1.14	0.33

growth, have been published in a previous paper (Corrieri et al. 2007).

#### (ii) Adult males

The treatment did not affect the body mass of the animals once adult (OIL, 571.7  $\pm$  14.4 g; EE4, 559.2  $\pm$  10.8 g; EE400, 546.1  $\pm$  12.5;  $F_{2,32} = 0.995$ ,  $p = 0.382$ ). The circulating levels of testosterone were numerically higher in EE400 animals; however, the difference was not significant (OIL, 1.80  $\pm$  0.34 ng ml<sup>-1</sup>; EE4, 1.80  $\pm$  0.40; EE400, 2.74  $\pm$  0.57;  $F_{2,31} = 1.475$ ,  $p = 0.246$ ).

#### (iii) Adult females

In females, there was an effect of the treatment on adult body mass: EE400 females had a lower body mass than OIL and EE4 females (OIL, 282.2  $\pm$  7.4 g; EE4, 295.1  $\pm$  7.3 g; EE400, 257.3  $\pm$  6.2;  $F_{2,35} = 7.55$ ,  $p = 0.002$ ; Newman-Keuls *post hoc*,  $p < 0.05$ ). The 400 ng EE dose also affected significantly the reproductive physiology of the females. The examination of 15-day serial smears showed that in the EE400 group, there was a higher proportion of females with an irregular oestrous cycle (Pearson  $\chi^2_2 = 21.27$ ,  $p < 0.0001$ ; figure 1a). This was confirmed by the highly significant effect of the treatment on the mean oestrous cycle score (Kruskal-Wallis non-parametric ANOVA,  $\chi^2_3 = 16$ ,  $p = 0.0003$ ; figure 1b). *Post hoc* tests showed that in the EE400 females, the mean oestrus score was significantly higher than in the other two groups (Dunn's multiple comparison test,  $p < 0.01$ ; figure 1b). Most EE400 females showed abnormal vaginal cytology, characterized by permanent oestrous-like smears with leucocytes mixed within a thick layer of cornified cells. This type of condition is called 'persistent oestrus' and is observed in old females after the oestropause and in young females that received oestrogen treatment in the perinatal period (Everett 1939; Gorski 1968; Nass et al. 1984).

#### (b) Fertility and fecundity

The effects of the environmental-like exposure on fertility and fecundity were dramatic. Only one EE400 female remained pregnant, but gave birth to dead pups (live pups: Pearson  $\chi^2_2 = 13.03$ ,  $p < 0.001$ ; figure 2). Thus, no EE400 pair had viable offspring. On the contrary, we found no significant difference in fertility between EE4 and OIL pairs (figure 2). A similar proportion of females in both

groups remained pregnant (Pearson  $\chi^2_1 = 0.750$ ,  $p = 0.386$ ) and gave birth to live pups (Pearson  $\chi^2_1 = 0.178$ ,  $p = 0.673$ ; figure 2) after a 15-day period spent with a male that had undergone the same treatment. The following analyses include only the eight OIL and seven EE4 females that gave birth to live pups. The duration of gestation, calculated from the day of pairing, did not differ between OIL and EE4 pairs (26.7  $\pm$  2.0 versus 25.0  $\pm$  1.2 days,  $t = 0.737$ , d.f. = 13,  $p = 0.47$ , respectively). However, there were differences in the fecundity and reproductive success between fertile EE4 and OIL pairs. EE4 animals gave birth to more live pups ( $t$ -test,  $t = 2.65$ , d.f. = 13,  $p = 0.020$ ; figure 3) than OIL animals. Although the effects were similar for both sexes, only for male pups did the difference reach significance (males:  $t = 2.87$ , d.f. = 13,  $p = 0.013$ ; females:  $t = 1.59$ , d.f. = 13,  $p = 0.136$ ; figure 3). Overall, EE4 pairs had a higher reproductive success than OIL pairs because they generated heavier litters ( $t = 2.46$ , d.f. = 13,  $p = 0.029$ ; figure 4), whereas the average male and female pup mass did not differ between groups ( $t = 0.74$ , d.f. = 13,  $p = 0.472$ ;  $t = 0.61$ , d.f. = 13,  $p = 0.550$ , respectively; figure 4). There was no effect of the treatment on pup mortality until PND10 (OIL, 0.6  $\pm$  0.3 pups; EE4, 0.6  $\pm$  0.4 pups;  $t = 0.11$ , d.f. = 13,  $p = 0.914$ ).

## 4. DISCUSSION

This study shows that developmental exposure to low doses of the synthetic oestrogen, ethnyloestradiol, leads to permanent alterations of the reproductive physiology, fertility and fecundity of pairs of rats. The physiological dose of EE induced obvious changes in female reproductive physiology and no pair exposed to this dose had a successful pregnancy. The environmental dose of EE did not have significant effects on female or male reproductive physiology or on fertility; however, it affected significantly fecundity, i.e. the number of live pups. Thus, our study shows that environmental-like exposure to xenoestrogen has long-lasting effects on the reproductive success of exposed pairs, with the potential of determining major alterations in the growth and survival of mammalian populations.

The effects of the exposure to 400 ng EE per kg d<sup>-1</sup> are in line with previous reports of deleterious effects of xenoestrogens at physiological doses on female and/or male reproductive systems (Delbes et al. 2006; reviewed

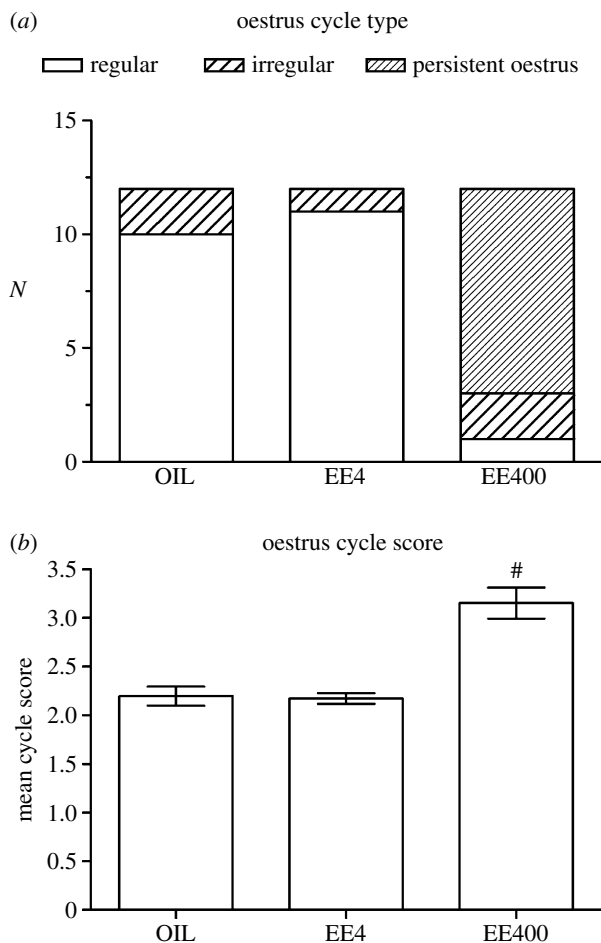


Figure 1. Oestrous cycles of female rats exposed to 4 ng kg<sup>-1</sup>d<sup>-1</sup> (EE4) or 400 ng kg<sup>-1</sup>d<sup>-1</sup> (EE400) EE during development. (a) Oestrous cycle type as determined by observation of a 15-day series of vaginal smears. Cycle was scored as irregular when there was no alternation of dioestrus, pro-oestrus and oestrous with a 4- to 5-day period. Most EE400 females showed irregular oestrous cycle with an abnormal vaginal cytology typical of persistent oestrous of aged and perinatally oestrogen-treated females. Pearson  $\chi^2$ ,  $p < 0.0001$ . (b) The mean oestrous score was higher (4 = oestrous) in EE400 females than in the other two groups. Hash,  $p < 0.01$ , Kruskal–Wallis ANOVA followed by Dunn’s *post hoc* test, EE400 versus OIL and EE4.

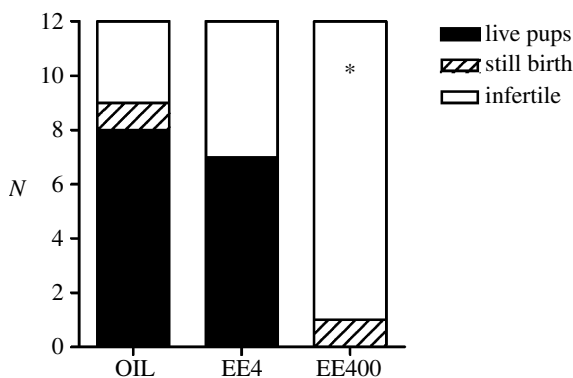


Figure 2. Fertility of pairs of rats which had been exposed during development to 4 ng kg<sup>-1</sup>d<sup>-1</sup> (EE4) or 400 ng kg<sup>-1</sup>d<sup>-1</sup> (EE400) of EE. No EE400 female gave birth to life pups. There was no difference in fertility between OIL and EE4 females. Asterisk,  $p < 0.001$ , Pearson  $\chi^2$ .

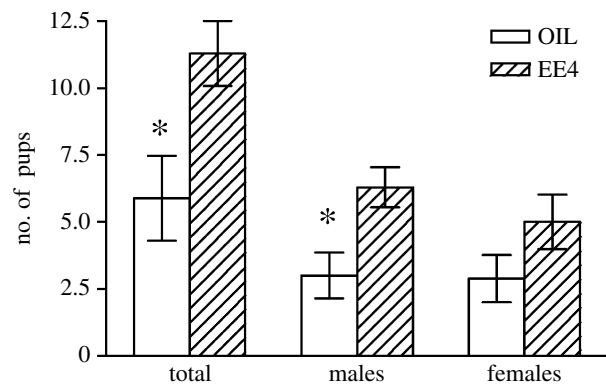


Figure 3. Pairs of rats that were exposed to 4 ng kg<sup>-1</sup>d<sup>-1</sup> of EE (EE4) during development generated more live pups, and particularly more male pups, than control treated pairs. Asterisk,  $p < 0.02$ , *t*-test.

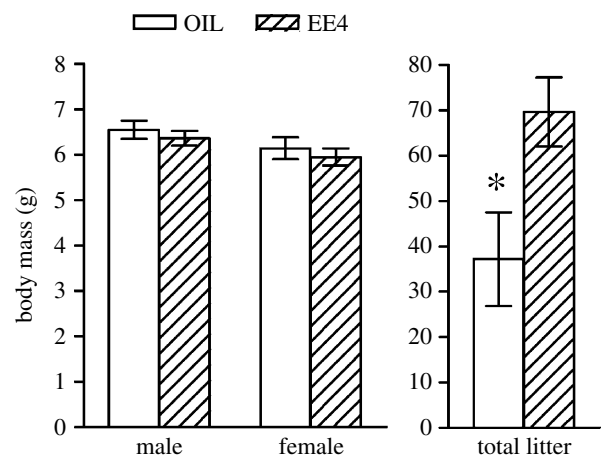


Figure 4. Pairs of rats that were exposed to 4 ng kg<sup>-1</sup>d<sup>-1</sup> of EE (EE4) during development generated pups with the same average body mass as control treated animals. Owing to the higher number of pups per litter (figure 3), however, EE4 pairs had heavier litters. Asterisk,  $p < 0.03$ , *t*-test.

by Sawaki *et al.* 2003a; Milnes *et al.* 2006). In rats, dramatic effects of EE exposure at 0.1–5  $\mu\text{g kg}^{-1} \text{d}^{-1}$  during development have been reported previously, with anomalies of female and male external genitalia and loss of regular oestrous cycle (Sawaki *et al.* 2003a,b; Timms *et al.* 2005). Male reproductive organs were not studied in this work, however, in the brothers of the experimental males which had been euthanized at four months of age there was no difference in testes weight between treatment groups (D. Della Seta & L. Fusani, unpublished data). The presence of persistent oestrous following early exposure to physiological levels of oestrogen is accompanied by loss of female sexual receptivity (D. Della Seta *et al.*, unpublished data), which might explain the very low fecundation success in EE400 pairs. On the contrary, effects of environmental doses of EE have not been reported previously in rats and became evident in our study only when the reproductive outcome of exposed pairs was examined. The exposed-pair approach has been recently used in a few recent studies including goat (*Capra hircus*; Oskam *et al.* 2005), pig (*Sus scrofa*; Bogh *et al.* 2001), Japanese quail, (*Coturnix coturnix japonica*; Ottinger *et al.* 2005a), zebrafish (*Danio rerio*; Nash *et al.* 2004), guppy (*Poecilia reticulata*; Kristensen *et al.* 2005) and Japanese medaka (*Oryzias latipes*; Patyna *et al.* 1999),



but not in laboratory rodents. In fact, reproductive success might be more informative than reproductive physiology when the aim of the study is to determine the potential danger of an EDC for the dynamics of a population.

At first, it may appear surprising that  $4 \text{ ng kg}^{-1} \text{ d}^{-1}$  EE induced an increase in the reproductive success of exposed pairs. However, a review of the literature shows that such effects are in fact very common. Similar doses of EE increase egg viability in the cunner (*Tautoglabrus adspersus*, Gutjahr-Gobell et al. 2006), and increase fecundity, i.e. the mean number of spawned eggs per pair, in the fathead minnow (*Pimephales promelas*, Pawlowski et al. 2004), two species of fish. At present, we do not know the mechanisms that are responsible for the increased fecundity in the EE4 pairs six months after the exposure to EE has been interrupted. Inverted U-shaped dose–response curves where low doses have stimulating effects are in fact commonly observed, a phenomenon called hormesis (Welshons et al. 2003). Hormesis is generally thought to be an adaptive response which may reduce subsequent stress, but in the case of xenoestrogen, the effects are unlikely to be beneficial (Weltje et al. 2005).

The external validity of our results, i.e. the biological relevance of the study for inferring potential effects of xenoestrogens on natural populations, is warranted by the approach followed. Beside the use of reproductive success of exposed pairs as an indicator, we have used an environmental-like exposure protocol that involves a continuous, long-term treatment, a natural, non-stressful way of administering the chemical through the placenta, the milk, or the food, and an environmentally relevant dose. The latter point is particularly important, because higher doses can have different or even opposite effects (Sawaki et al. 2003a; Timms et al. 2005; this study). We defined our dose of  $4 \text{ ng kg}^{-1} \text{ d}^{-1}$  as environmentally relevant because it matches concentrations of EE found in contaminated surface waters (i.e.  $1\text{--}4 \text{ ng l}^{-1}$ , Nash et al. 2004; Parrott & Blunt 2005). In fish, plasma and whole-body concentrations of EE are approximately 500-fold higher when compared with water concentration (Lange et al. 2001; Skillman et al. 2006). Therefore, animals or humans who eat fish regularly from contaminated waters would ingest an amount of EE relative to body weight at levels similar to those found in the water. Comparable low doses of EE have been shown to affect reproductive physiology and/or success of a few fish species (Nash et al. 2004; Pawlowski et al. 2004; Kristensen et al. 2005; Parrott & Blunt 2005; Gutjahr-Gobell et al. 2006), but to our knowledge, no other study has reported effects of similar doses in mammals. This is probably due to the treatment protocols and analysed endpoints rather than to the absence of effects, given that there are theoretical reasons to expect that such doses can have a substantial influence on development (Welshons et al. 2003). For example, the dose that we have used matches pretty closely the concentration of oestradiol in newborn rats at PND2 ( $5.6 \pm 0.6 \text{ ng l}^{-1}$ , Montano et al. 1995), thus it seems logical to expect some effects from a doubling of the physiological concentration.

There is ample scientific evidence for endocrine disruption or, even, alteration in wildlife and humans (Vos et al. 2000; Fox 2001; Colborn 2004); however, several authors point out that the experimental support is scarce, especially for terrestrial vertebrates and humans

(Tyler et al. 1998; Dawson 2000; Brown et al. 2001; Rogan & Ragan 2003). We have shown that using an experimental protocol designed to mimic as closely as possible an environmental exposure, robust effects of very low doses on key traits such as reproductive success can be discovered. Studies of this type are required to understand the real danger of contamination of food and water with endocrine disruptors for mammals and humans.

The experiments described in this work were approved by the Ethical Committee of the University of Siena and by the Italian Ministry of Health, in adherence to EU directive 86/609.

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