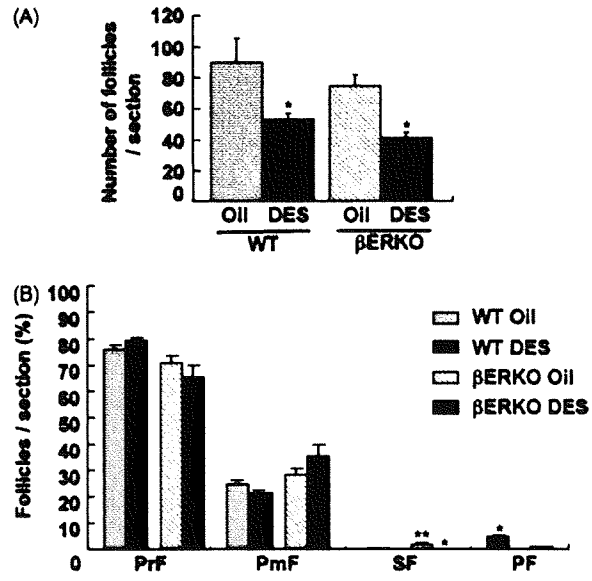


Fig. 6. The number of follicles per section of 5-day-old oil- or DES-treated WT and βERKO mouse ovaries (A). Percentages of PrFs, PmFs, SFs and polyovular follicles (B) per section of oil or DES-treated mouse ovaries. $^{*}p < 0.05$, compared with respective oil-treated mice. $^{**}p < 0.05$, compared with WT oil control mice.

Formation of PrF and an initiation of PrF to PmF transition have been shown without gonadotropins *in vitro* [29,30]. In addition, folliculogenesis of LH or FSH receptor knockout mice is normal until the preantral stage [31,32], suggesting that the early folliculogenesis is independent on gonadotropins, and local factors are thought to be the main regulators in this process. Expression of mRNAs which are associated with PrF and PmF development was not changed by DES exposure in 2-day-old C57BL/6J mice. It suggests that the delay of follicle development by DES exposure in 5- to 10-day-old mice is not accompanied with changes in mRNA expression of Nobox, Figα, Sohlh1, Foxl2, Foxo3a, Kit, KL, NGF and TrkB in 2-day-old mice. There are few PmFs fully formed in 2-day-old mouse ovaries. If any of these factors are expressed only in follicle cells, changes in their expression level may not be detected against the background of the other cell types making up the majority of the ovary. Therefore, further studies are needed to identify ovarian cell types expressing these genes in this stage and to examine expression of these genes at late stage of ovarian development. In addition, other factors may be involved in the disorder of folliculogenesis in DES-exposed neonatal mice. Müllerian inhibiting substance (MIS), also known as anti-Müllerian hormone (AMH), is a member of the TGFβ superfamily and is produced by granulosa cells of small growing follicles [33]. MIS inhibits recruitment of PrFs to PmFs directly and indirectly inhibits FSH action which progresses PmFs to SFs [34]. In 5-day-old DES-exposed mouse ovaries, MIS mRNA is high compared with that in oil-exposed mice [12]. Nagai et al. [35] have shown that the expression of MIS mRNA is increased in PmFs of 7-day-old DES-exposed rats. In addition, MIS mRNA and protein expression are increased but follicular growth is inhibited in estradiol benzoate-exposed rats [36]. Therefore, MIS may play roles in the delay of PmF progression from PrFs and SF progression from PmFs in neonatally DES-exposed mice. SF progression from PmFs requires other local factors such as GDF-9, BMP-4, BMP-7 and activins [37]. In GDF-9 null mice, follicle development beyond PmFs does not occur [38]. Addition of BMP-4 and BMP-7 decreases PrFs, and increases PmFs and preantral follicle number *in vitro* [39,40]. Activin stimulates granulosa cell proliferation in preantral follicles of immature mice [41]. The follicle development is also arrested at the early antral stage in activin type-IIB receptor deficient mice, suggesting further supporting roles of activin in the promotion of granulosa cell proliferation/differentiation [42]. Thus, the disorder of folliculogenesis induced by neonatal DES exposure may be correlated with changes in TGFβ superfamily. In fact, neonatal DES or E2 exposure decreases the number of small antral follicles, activin β-subunit mRNA and protein levels [43]. The expression of TGFβ superfamily genes in neonatally DES-exposed mice should be investigated to elucidate the mechanisms of early folliculogenesis.

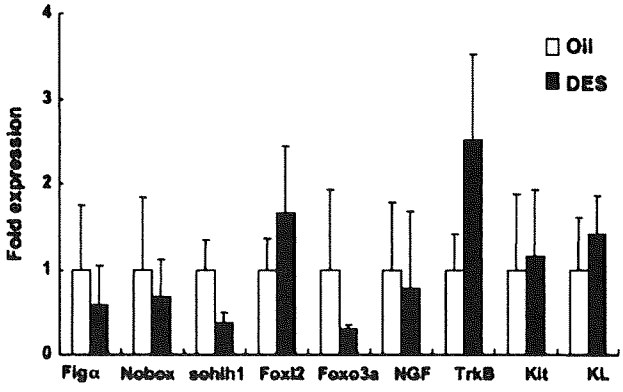


Fig. 7. Changes in mRNA expression of genes related to follicular growth in 2-day-old mouse ovaries.

Studies using ERα and/or ERβ knockout mice showed their roles in the ovary. Adult αERKO mice have large hemorrhagic follicles and absence of corpora lutea in the ovary because of chronically elevated LH levels caused by lack of the negative feedback through ERα in the hypothalamus [44]. Folliculogenesis proceeds normally up to the preantral stage in αERKO mice [27,45]. Therefore, ERα is not thought to be essential for early folliculogenesis. On the other hand, βERKO mice carry less copora lutea in the ovary and show reduced fertility compared to WT mice [27]. In adult βERKO mice, PrFs are increased but PmFs are decreased, and large preantral follicles in immature βERKO mice are increased [46]. Thus, ERβ may play a role in controlling early folliculogenesis. It is suggested by data from aromatase knockout mice that estrogen may regulate the PrF pool [47]. Hegele-Hartung et al. [48] have elucidated that direct effects of estrogen on ovarian follicle development are mediated by ERβ. Furthermore, ERα and ERβ double knockout mouse shows ovarian follicle transdifferentiation to structures resembling seminiferous tubules of the testis [49]. These reports suggest that both ERα and ERβ are involved in early follicle formation and regulation.

In 5-day-old βERKO mice, the number of follicles did not change compared with that in WT mice, suggesting that ERβ is not crucial for PrF formation. Numbers of PrFs and PmFs in immature βERKO mice are similar to those in WT mice [46]. Thus, ERβ is not necessary for PrF formation in either neonatal or immature mice. In 5-day-old WT mice, the number of follicles was decreased by DES exposure as well as in C57BL/6J mice. Similar to WT mice, the number of follicles was also decreased by DES exposure in βERKO mice. These results suggest that DES inhibits the follicle formation and ERβ is not involved in this DES effect. Although an innate role of ERα in the neonatal ovary is not yet known, our results suggest that ERα may mediate DES signals to inhibit PrF assembly. However, there are membrane-associated receptors that also bind estrogen [50]. It is also possible that these non-canonical ERs are involved in folliculogenesis.

While neonatal DES exposure delays PmF progression from PrFs and SF progression from PmFs in 5- and 10-day-old C57BL/6J mice, numbers of PrFs and PmFs in WT mice were not changed by DES

exposure. It seems that effects of DES on follicular development may be dependent on mouse strains. Strain differences in DES effects are also reported in the induction of polyovular follicles [51].

On the other hand, SFs were occasionally observed in 5-day-old βERKO mice. SFs usually appear around 7 days of age in mice [16]. Thus, SF progression from PmFs is promoted in βERKO mice. Since SF progression from PmFs requires granulosa cell proliferation, ERβ may negatively regulate the cell proliferation of neonatal mouse granulosa cells. ERβ plays a role in the inhibition of epithelial cell proliferation in the adult prostate and uterus [52,53]. However, the mechanisms by which ERβ inhibit cell proliferation are unclear. The percentage of SFs in DES-exposed βERKO mice was significantly lower than that in oil-exposed βERKO mice. This suggests that precocious SF progression due to lack of ERβ is cancelled by neonatal DES exposure via ERα. DES may inhibit granulosa cell proliferation through ERα or restore a negative signaling pathway that affects SF progression.

Jefferson et al. [54] have reported that neonatal treatment of genistein induced polyovular follicles in wild-type mice and αERKO mice but not βERKO mice, suggesting ERβ in induction of polyovular follicles. In the present study, neonatal DES treatment induced polyovular follicles in C57BL mice and WT mice but not in βERKO mice, agreeing with previously reported findings [54].

In conclusion, this study shows that the induction of polyovular follicles and the delay of follicle development in neonatal mice were caused by neonatal DES treatment. DES affects follicle formation and suppresses follicular development via ERα but not ERβ. Further study is needed to investigate the molecular mechanism of DES action in the induction of follicular developmental delay in newborn mouse ovaries.

Conflict of interest

There is no conflict of interest.

Acknowledgments

This work was partially supported by a Grant-in-Aid for Scientific Research (B) (T.I.), a Grant-in-Aid for Encouragement of Young Scientists (T.S.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Grants for Support of the Promotion of Research at Yokohama City University (No. K17030 and W18005, to S.H. and T.S.), a Health Sciences Research Grant from the Ministry of Health, Labor and Welfare, Japan (to T.I.).

References

[1] Takasugi N. Vaginal cornification in persistent-estrous mice. Endocrinology 1963;72:607–19.
[2] Forsberg JG. Developmental mechanism of estrogen-induced irreversible changes in the mouse cervicovaginal epithelium. Natl Cancer Inst Monogr 1979;51:41–56.
[3] Newbold RR, McLachlan JA. Diethylstilbestrol associated defects in murine genital tract developmental. In: McLachlan JA, editor. Estrogens in the environment II: influences in development. New York: Elsevier North Holland; 1985. p. 288–318.
[4] Iguchi T. Cellular effects of early exposure to sex hormones and abnormalities of female reproduction. Int Rev Cytol 1992;139:1–57.
[5] Iguchi T, Sato T. Endocrine disruption and developmental abnormalities of female reproduction. Am Zool 2000;40:402–11.
[6] Tenenbaum A, Forsberg JG. Structural and functional changes in ovaries from adult mice treated with diethylstilbestrol in the neonatal period. J Reprod Fertil 1985;73:465–77.
[7] Iguchi T, Takasugi N, Bern HA, Mills KT. Frequent occurrence of polyovular follicles in ovaries of mice exposed neonatally to diethylstilbestrol. Teratology 1986;34:29–35.
[8] Iguchi T, Fukuzawa Y, Uesugi Y, Takasugi N. Polyovular follicles in mouse ovaries exposed neonatally to diethylstilbastrol *in vivo* and *in vitro*. Biol Reprod 1990;43:478–84.
[9] Kuiper GGJM, Carlsson B, Grandien K, Enmarkr E, Haggblad J, Nilsson S, et al. Comparison of ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 1997;138:863–70.
[10] Sar M, Welsch F. Differential expression of estrogen receptor-β and estrogen receptor-α in rat ovary. Endocrinology 1999;140:963–71.
[11] Couse JF, Korach KS. Estrogen receptor-α mediates the detrimental effects of neonatal diethylstylbestrol (DES) exposure in the murine reproductive tract. Toxicology 2004;205:55–63.
[12] Kirigaya A, Sato T, Iguchi T, Hayashi S. Involvement of MIS and ERβ in induction of polyovular follicles (PF) in mouse ovary treated with DES neonatally. Zool Sci 2004;21:1341.
[13] Pepling ME, Spradling AC. Female mouse germ cell form synchronously dividing cysts. Dev Biol 1998;125:3323–8.
[14] Pepling ME, Spradling AC. Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. Dev Biol 2001;234:339–51.
[15] Epifano O, Dean J. Genetic control of early folliculogenesis in mice. Trends Endocrinol Metabol 2002;13:169–73.
[16] Peters H. The development if mouse ovary from birth to maturity. Acta Endocrinol 1969;62:98–116.
[17] Soyal SM, Amleh A, Dean J. FIGα, a germ cell-specific transcription factor required for ovarian follicle formation. Dev Biol 2000;127:4645–54.
[18] Rajkovic A, Pangas SA, Ballow D, Suzumori N, Martzuk MM. Nobox deficiency disrupts early folliculogenesis and oocyte-specific gene expression. Science 2004;305:1157–9.
[19] Pangas SA, Choi Y, Ballow DJ, Zhao Y, Westphal H, Matzuk MM, et al. Oogenesis requires germ cell-specific transcriptional regulaters Sohlh1 and Lhx8. Proc Natl Acad Sci USA 2006;103:8090–5.
[20] Loffler KA, Zarkower D, Koopman P. Etiology of ovarian failure in blepharophimosis ptosis epicanthus inversus syndrome: FOXL2 is a conserved, early-acting gene in vertebrate ovarian development. Endocrinology 2003;144:3237–43.
[21] Schmidt D, Ovitt CE, Anlag K, Fehsenfeld S, Gredsted L, Treier AC, et al. The murine winged-helix transcription factor Foxl2 is required for granulose cell differentiation and ovary maintenance. Development 2004;131:933–42.
[22] Casteillon DH, Miao L, Kollipara R, Horner JW, Depinho RA. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science 2003;301:215–8.
[23] Dissen GA, Romero C, Hirshfield AN, Ojeda SR. Nerve growth factor is required for early follicular development in the mammalian ovary. Endocrinology 2001;142:2078–86.
[24] Ojeda SR, Romero C, Tapia V, Dissen GA. Neurotrophic and cell–cell dependent control of early follicular development. Mol Cell Endocrinol 2000;163:67–71.
[25] Paredes A, Romero C, Dissen GA, DeChiara TM, Reichardt L, Cornea A, et al. TrkB receptors are required for follicular growth and oocyte survival in the mammalian ovary. Dev Biol 2004;267:430–49.
[26] Parrott JA, Skinner MK. Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. Endocrinology 1999;140:4262–71.
[27] Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M. Effects of single and compound knockouts of estrogen receptors α (ERα) and β (ERβ) on mouse reproductive phenotypes. Development 2000;127:4277–91.
[28] Bookout AL, Mangelsdorf DJ. Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. Nuc Receptor Signal 2003;1:e012.
[29] Kezele P, Skinner MK. Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: endocrine model of follicle assembly. Endocrinology 2003;144:3329–37.
[30] Chen Y, Jefferson WN, Newbold RR, Banks EP, Pepling ME. Estradiol, progesterone and genistein inhibit oocyte nest breakdown and primordial follicle assembly in the neonatal mouse ovary *in vitro* and *in vivo*. Endocrinology 2007;148:3580–90.
[31] Zhang FP, Poutanen M, Wilbertz J, Huhtaniemi I. Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. Mol Endocrinol 2001;15:172–83.
[32] Abel MH, Huhtaniemi I, Pakarinen P, Kumar TR, Charlton HM. Age-related uterine and ovarian hypertrophy in FSH receptor knockout and FSHβ subunit knockout mice. Reproduction 2003;125:165–73.
[33] Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen AP, et al. Anti-Müllerian hormone and anti-Müllerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. Endocrinology 1995;136:4951–62.
[34] Visser AJ, Themmen APN. Anti-Müllerian hormone and folliculogenesis. Mol Cell Endocrinol 2005;234:81–6.
[35] Nagai A, Ikeda Y, Aso T, Eto K, Ikeda M. Exposure of neonatal rats to diethylstilbestrol affects the expression of genes involved in ovarian differentiation. J Med Dent Sci 2003;50:35–40.
[36] Ikeda Y, Nagai A, Ikeda M, Hayashi S. Increased expression of Müllerian-inhibiting substance correlates which inhibition of follicular growth in the developing ovary rats treated with E2 benzoate. Endocrinology 2002;143:304–12.
[37] Knight PG, Glister C. TGF-beta superfamily members and ovarian follicle development. Reproduction 2006;132:191–206.
[38] Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk M. Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 1996;383:531–5.

[39] Lee WS, Yoon SJ, Yoon TK, Cha KY, Lee SH, Shimasaki S, et al. Effects of bone morphogenetic protein-7 (BMP-7) on primordial follicular growth in the mouse ovary. Mol Reprod Dev 2004;69:159–63.
[40] Nilsson EE, Skinner MK. Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. Biol Reprod 2003;69:1265–72.
[41] Liu L, Rajareddy S, Reddy P, Jagarlamudi K, Shen Y, Gunnarsson D, et al. Infertility caused by retardation of follicular development in mice with oocyte-specific expression of Foxo3a. Development 2007;134:199–209.
[42] Matzuk MM, Kumar TR, Shou W, Coerver KA, Lau AL, Behringer RR, et al. Transgenic models to study the roles of inhibins and activins in reproduction, oncogenesis, and development. Rec Progr Horm Res 1996;51:123–54.
[43] Kipp JL, Kilen SM, Bristol-Gould S, Woodruff TK, Mayo KE. Neonatal exposure to estrogens suppresses activin expression and signaling in the mouse ovary. Endocrinology 2007;148:1968–76.
[44] Couse JF, Yates MM, Deroo BJ, Korach KS. Estrogen receptor β is critical to granolosa cell differentiation and the ovulatory response to gonadotropins. Endocrinology 1999;146:3247–62.
[45] Shomberg DW, Couse JF, Mukerjee A, Lubahn DB, Sar A, Mayo KE, et al. Targeted disruption of estrogen receptor-α gene in female mice: characterization of ovarian responses and phenotype in adult. Endocrinology 1999;140:2733–44.
[46] Emmen JMA, Couse JF, Elmore SA, Yates MM, Kissling GE, Korach KS. *In vitro* growth and ovulation of follicles from ovaries of estrogen receptor (ER) α and ERβ null mice indicate a role for ERβ in follicular maturation. Endocrinology 2005;146:2817–26.
[47] Britt KL, Saunders PK, Mcpherson SJ, Misso ML, Simpson ER, Findlay JK. Estrogen actions on follicle formation and early follicle development. Biol Reprod 2004;71:1712–23.
[48] Hegele-Hartung C, Siebel P, Peters O, Kosemund D, Müller G, Hillisch A, et al. Impact of isotype-selective estrogen receptor agonists on ovarian function. Proc Natl Acad Sci USA 2004;101:5129–34.
[49] Couse JF, Hewitt SC, Bunch DO, Sar M, Walker VR, Davis BJ, Korach. Postnatal sex reversal of the ovaries in mice lacking estrogen receptors α and β. Science 1999;286:2328–31.
[50] Marino M, Galluzzo P, Ascenzi P. Estrogen signaling multiple pathways to impact gene transcription. Curr Genomics 2006;7:497–508.
[51] Iguchi T, Ohta Y, Fukuzawa Y, Takasugi N. Strain differences in the induction of polyovular follicles by neonatal treatment with diethylstilbestrol in mice. Med Sci Res 1987;15:1407–8.
[52] Imamov O, Morani A, Shim GJ, Omoto Y, Andersson CT, Warner M, et al. Estrogen receptor β regulates epitherial cellular differentiation in the mouse ventral prostate. Proc Natl Acad Sci USA 2004;101:9375–80.
[53] Hiraike OW, Hiraike H, Okinaga H, Imamov O, Barros RPA, Morani A, et al. Role of estrogen receptor β in uterine stroma and epithelium: insights from estrogen receptor $\beta^{-/-}$ mice. Proc Natl Acad Sci USA 2006;103:18350–5.
[54] Jefferson WN, Couse JF, Padilla-Banks E, Korach KS, Newbold RR. Neonatal exposure to genistein induces estrogen receptor (ER)α expression and multioocyte follicles in the maturing mouse ovary: evidence for ERβ-mediated and nonestrogenic actions. Biol Reprod 2002;67:1285–96.

Environ. Sci. Technol. **2009**, *43*, 1219–1225

Sexual Reprogramming and Estrogenic Sensitization in Wild Fish Exposed to Ethinylestradiol

ANKE LANGE,*,† GREGORY C. PAULL,† TOBIAS S. COE,† YOSHINAO KATSU,‡ HIROSHI URUSHITANI,‡ TAISEN IGUCHI,‡ AND CHARLES R. TYLER†

School of Biosciences, University of Exeter, Exeter EX4 4PS, United Kingdom, and Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, National Institutes of Natural Sciences, 5-1 Higashiyama, Myodaiji, Okazaki, 444-8787, Japan

Received September 18, 2008. Revised manuscript received December 10, 2008. Accepted December 16, 2008.

Globally, feminization responses in wild male freshwater fish are caused by exposure to estrogenic chemicals, including natural and synthetic estrogens, contained in effluents from wastewater treatment works. In U.K. rivers, feminization responses, including intersex, are widespread in wild roach (*Rutilus rutilus*) populations, and severely affected fish have a reduced reproductive success. We exposed roach to environmentally relevant concentrations of the contraceptive estrogen 17α-ethinylestradiol (EE_2) for up to 2 years, including intermittent and repeated exposures, to determine effects on sexual development and subsequent responsiveness to estrogen. Exposure of roach to EE_2 (at 4 ng/L) for 2 years resulted in sex reversal in males, leading to an all-female population with two cohorts in terms of their stages of ovarian development, one paralleling the control females and one at a significantly less advanced stage, which we propose were sex-reversed males. Differing developmental and maturing rates of the putative sex-reversed males compared with control females would question their functional capability as females in the wild. Early-life exposure to environmentally relevant concentrations of EE_2 sensitized females to estrogen, as determined by the measurement of the responses of estrogen-sensitive genes in a further EE_2 challenge 398 days after the original exposure. In the wild, exposure to environmentally relevant concentrations of EE_2 during early life has significantly wider implications for the sexual physiology in fish than has thus far been determined.

Introduction

Endocrine-disrupting chemicals (EDCs) including natural and synthetic steroid estrogens, alkylphenol ethoxylates, various pesticides, phthalates, and bisphenols are widespread in the environment, and many have been shown to disrupt endocrine function in wildlife and humans (reviewed in ref *1*). Wildlife living in and/or closely associated with freshwater ecosystems is especially at risk of EDC exposure because many freshwaters receive discharges, principally via wastewater treatment works (WwTWs), and in the United Kingdom, it is not uncommon for half of the flow of a river to be comprised of treated WwTW effluent (*2*). Globally, exposure to WwTW effluents has been associated with a range of deleterious effects on reproduction in fish and fish populations (e.g., in the United Kingdom (*3, 4*), France (*5*), Germany (*6*) or the United States (*7*)). There are other studies, however, that have found no adverse effects of WwTW effluents on fish (*8, 9*).

Extensive studies carried out on wild roach (*Rutilus rutilus*) living downstream of WwTWs have shown a range of feminization responses, including elevated concentrations of blood vitellogenin (VTG; an estrogen-dependent yolk precursor) in males and immature females, the presence of a female-like ovarian cavity in the testis of males, and a high incidence of intersex (*3*). We would emphasize that the prevalence of intersex reported in wild roach is considerably lower in rivers in other European countries compared with the United Kingdom (*10, 11*). Although some species of fish are hermaphrodites, containing both male and female sex cells in their gonads, or undergo changes in sex as part of their normal sexual development, roach are normally gonochorists (single sexed). Disruption in sexual development in roach has been shown to impact negatively the reproductive success of affected fish (*12, 13*) with the potential for population-level ramifications. All of the feminized phenotypes seen in wild fish can be induced experimentally by controlled exposure to WwTW effluents (*2, 14, 15*).

In fish, sex determination and sexual differentiation are controlled by a delicate balance of genetic and environmental factors, and any alteration created by other exogenous influences, including EDCs, can ultimately impact sex assignment, even in gonochoristic species (e.g., ref *16*). Estrogens play key roles in sexual differentiation and gametogenesis, and exposure to estrogen-mimicking chemicals during critical periods of differentiation can affect sexual development and have consequences for subsequent reproductive capabilities. It has been hypothesized that steroidal estrogens, both natural and synthetic, present in effluents play a major role in the disruption of sexual function in wild roach in U.K. rivers. Of these estrogens, the pharmaceutical 17α-ethinylestradiol (EE_2) used in the contraceptive pill is the most potent (*17*), with high persistence and a tendency to bioconcentrate in organisms (*18*). EE_2 thus has the potential to disrupt reproductive processes in fish at relatively low (ng/L) concentrations that occur in the aquatic environment. In Europe, EE_2 has been measured at concentrations from below the detection limit up to 15 ng/L in effluents and up to 5 ng/L in surface waters (*14, 18–26*). Laboratory exposures of fish to EE_2 have been shown to disrupt normal sexual development and differentiation (e.g., ref *27*), alter reproductive behaviors (e.g., ref *28*), and reduce reproductive success via effects on fecundity (e.g., ref *29*) or fertilization success/embryo viability (e.g., refs *30* and *31*), but often at exposure concentrations exceeding those measured in the aquatic environment. In mammals, neonatal exposure to estrogens and their mimics can alter regulation of gene transcription, producing long-term changes in a number of signaling pathways including those regulating cell proliferation, differentiation, and survival, and some of these changes in gene regulation, or imprinting, have been implicated in the susceptibility to environmentally related diseases, including cancer (*32, 33*). Whether environmental estrogens produce permanent changes in gene function or "imprint" on the endocrine system in nonmammalian species

* Corresponding author phone: +44.1392.269298; fax: +44 0.1392.263700; e-mail: A.Lange@exeter.ac.uk.
† University of Exeter.
‡ National Institutes of Natural Sciences.

10.1021/es802661p CCC: $40.75
Published on Web 01/21/2009

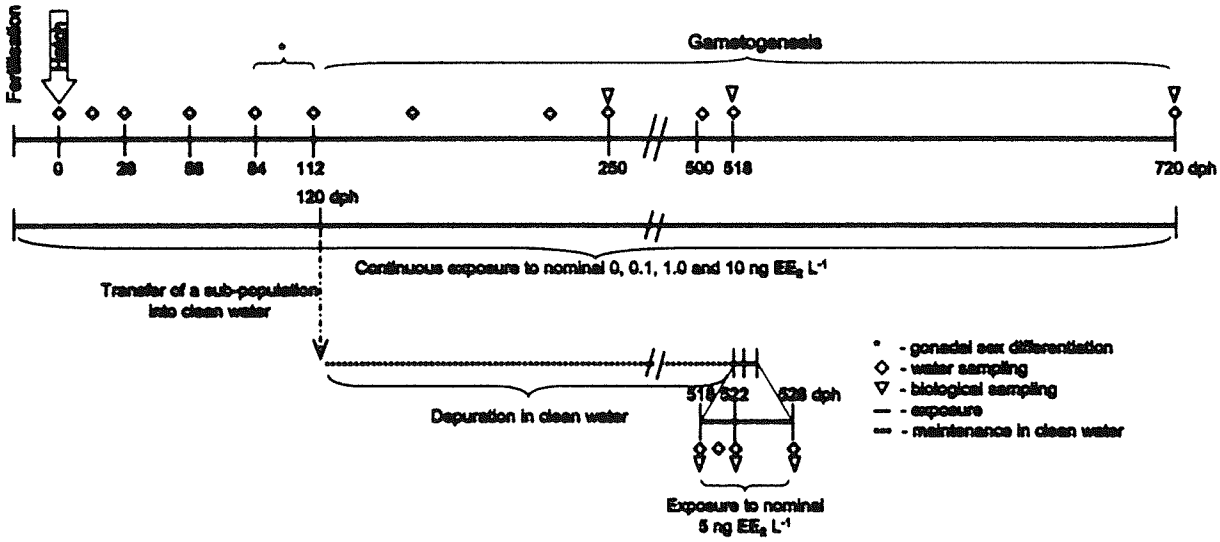


FIGURE 1. Experimental design of the long-term exposures of roach to EE_2. Roach were exposed from fertilization to 720 dph to environmental concentrations of EE_2 (nominal 0.1, 1.0, and 10 ng/L). Some fish from each treatment were transferred to clean water after gonadal sex differentiation at 120 dph and allowed to depurate for 398 days, when a subpopulation was sampled. The remaining depurated fish were then re-exposed to one concentration of EE_2 (nominal 5 ng/L) and sampled at days 4 and 10 of the rechallenging experiment.

is not known, but this could have serious implications for wildlife populations.

In this study we conducted exposures of roach to environmentally relevant concentrations of EE_2 for up to 2 years and showed complete sex reversal and delayed gonadal maturation in roach. Furthermore, a particularly novel finding was that roach exposed during early life to EE_2 become more sensitive and responsive when rechallenged with EE_2, more than 1 year after the original exposure.

Materials and Methods

Fish Source and Husbandry. Prespawning, sexually mature male and female roach were obtained from the Environment Agency's National Coarse Fish Farm (Calverton, Nottinghamshire, U.K.) and brought into the aquarium facility where they were induced artificially to spawn using established procedures with carp pituitary extract (*12*). The fertilized eggs were deployed into glass aquaria under flow-through conditions. Embryos hatched 7−10 days postfertilization and the resulting fish were provisioned with dietary requirements according to their age (*34*). In brief, fish were fed with Cyprico Crumble EX dry food (Coppens International bv, Helmond, The Netherlands), and at all life stages, the diet was supplemented with live food.

Fish were maintained at ambient water temperature (18 ± 1 °C) with a 16 h light/8 h dark photoperiod in their first year. Between December and February of their second year, the temperature of the incoming water gradually decreased to 12 ± 2 °C before progressively increasing again to 18 ± 2 °C in April. With decreasing temperature, the photoperiod regime was also reduced in a stepwise manner to 12 h/12 h light/dark and then in a stepwise manner back to 16 h/8 h light/dark by May.

Exposure Systems and Experimental Design. Roach were exposed continuously to one of three concentrations of EE_2 (Sigma-Aldrich, Poole, U.K.; nominal concentrations of 0.1, 1.0, and 10 ng/L) from fertilization up to 720 days post-hatching (dph) in flow-through conditions, in duplicate tanks (Figure 1). EE_2 was made up in ethanol, and the solvent dosing to all the tanks was less than 0.0001%, v/v. Control roach were maintained in dilution water tanks. Dilution water and the EE_2 dosing stock solution were both delivered to the tanks using peristaltic pumps. Water flows and EE_2 dosing rates were monitored regularly, and the EE_2 dosing stock solution was renewed every 4−7 days. Fish were sampled at regular intervals for assessments on sexual development.

In a further experiment roach from this EE_2 exposure were transferred into clean water at 120 dph, subsequently depurated for 398 days (when the status of their sexual development was assessed). Fish were then re-exposed to one concentration of EE_2 (nominal 5 ng/L EE_2) for 10 days (Figure 1) and effects on the rate and level of plasma VTG induction and transcription of a suite of estrogen-responsive genes determined.

Water samples were collected periodically from each tank throughout the experiment to determine the actual exposure concentrations of EE_2. The extraction method and the applied radioimmunoassay were as described by Katsu et al. (*35*).

Fish Sampling and Biological Analyses. All fish were sacrificed humanely by terminal anesthesia with benzocaine followed by cervical dislocation as approved by the U.K. Home Office (Animals (Scientific Procedures) Act 1986). In the long-term exposure to EE_2, fish (n = 12−30) were sampled at random from the exposure populations and analyzed for gonadal development at 250, 518, and 720 dph and VTG induction (250 and 518 dph only). Fish exposed to EE_2 during early life and then maintained in clean water were analyzed for gonadal development and for VTG induction at 518 dph and also subsequently for plasma VTG induction and the transcription of gonadal aromatase (*cyp19a1a*), estrogen receptor α (*esr1*), and estrogen receptor β2 (*esr2b*) using qRT-PCR, at 522 and 528 dph, after 4 and 10 days of re-exposure to a single concentration of EE_2. These genes were chosen for study because of their fundamental role within estrogen-responsive pathways.

At all sampling points, the total wet body weight and standard length were determined for each fish. The condition factor was calculated by expressing the cube of the fish length as a percentage of the body weight. At the final sampling point for EE_2 exposure (720 dph), the gonadosomatic index (GSI) was calculated as a measure of gonadal growth by expressing the dissected gonad weight as a percentage of the total body weight.

At all time points, gonads excised were divided in half, and one of the halves was used for histological analysis and, in the re-exposure study, the other half for molecular analysis.

For histopathology, gonads were first preserved in Bouin's fixative for 4–24 h, depending on the size of the gonad, processed as described previously (*35*), and analyzed for the presence of sex cells and their developmental stages. Excised gonadal tissue for molecular analyses was immediately snap frozen in liquid nitrogen and stored at −80 °C until analysis. Total RNA was extracted from the gonadal tissue and reverse transcribed as described previously (*35*). Subsequently, the mRNA expression of the target genes was established by qRT-PCR and normalized to the endogenous reference gene ribosomal protein L8 (*rpl8*) as described previously (*27, 35*).

For VTG analysis, blood samples were collected from the caudal sinus into heparinized hematocrit tubes or heparinized syringes, transferred into microfuge tubes containing aprotinin (Sigma-Aldrich), and centrifuged. The resulting plasma was stored at −20 °C until analysis. VTG was determined using an ELISA originally established for common carp VTG which has been validated for measuring VTG in roach (*36*).

Statistical Analysis. Unless stated otherwise, the data are presented as means ± SEM and a probability level of $p < 0.05$ was considered to be statistically significant. Data were examined for conformity with the assumptions of normality. If these were not met, the data were transformed, as appropriate.

For the continuous EE_2 exposure, statistical analyses were carried out using SigmaStat 3.1 (Systat Software, Inc.), and the effects on each end point were analyzed by one-way ANOVA if the data met the assumptions of normality and homogeneity of variance, followed by Tukey's all pairwise multiple comparison procedures. Data not meeting the assumptions of normality after transformation were analyzed by Kruskal–Wallis ANOVA on Ranks followed by Dunnett's or Dunn's all pairwise multiple comparison procedures.

The data of the EE_2 rechallenge experiment were analyzed using two-way ANOVAs (via the software R 2.5.1) to assess the effect of exposure concentration during early life and duration of estrogen rechallenge on the chosen end points and the interaction between both factors. Between-treatment comparisons within each time point were carried out using Tukey's honest significant differences test (Tukey's HSD test).

Results

Effects of Long-Term Exposure to EE_2. EE_2 was not detected in water in the control tank, and EE_2 in the nominal 0.1 ng/L treatment was below the detection limit of the radioimmunoassay (the detection limit for the assay in this study was 40 pg/L). The mean measured exposure concentrations of EE_2 in the nominal 1.0 and 10 ng/L test tanks were 0.3 ± 0.1 and 4.0 ± 0.3 ng/L, respectively (as reported previously in refs *27* and *35*). No significant mortalities occurred during the course of the 2 year exposure experiment, and no concentration-dependent mortality rates were observed. There were no concentration-related effects of EE_2 on the length, weight, or condition factor in male or female roach exposed to EE_2 (Figure S1, Supporting Information). After 720 days of exposure, no effect was observed on the GSI in male or female roach exposed to nominal 0.1 and measured 0.3 ng/L EE_2. The GSI of fish exposed to 4 ng/L EE_2 was highly variable (Figure S2, Supporting Information), and all fish in this treatment group had an ovarian morphology (see below). It was not possible to assign fish as females or (putative sex-reversed) males, and consequently, these fish were excluded from the statistical analyses for effects of EE_2 on growth.

In control females, ovaries at 250 dph contained all sex cells up to primary oocytes at the Balbiani body stage, and in males, testes had well-defined lobules with all stages of sex cells up to and including spermatogonia A and spermatogonia B (Figure S3, Supporting Information). At 518 dph, the ovaries in females included early vitellogenic oocytes and in males the testes contained spermatocytes. At 720 dph, all females contained vitellogenic oocytes and all males contained spermatocytes, with one male containing spermatozoa. There was a male bias in the population throughout the study: at 250 dph, there were 60% males versus 40% females ($n = 53$), at 518 dph, 59% males versus 41% females ($n = 17$), and, at 720 dph, 69% males versus 31% females ($n = 26$).

There were no histological differences in the gonadal status of fish exposed to nominal 0.1 ng/L EE_2 compared with control fish throughout the 720 day exposure. In fish exposed to measured 0.3 ng/L EE_2 at 250 dph the sex ratio was equally balanced (49% males and 51% females, $n = 35$), but at 720 dph there was a female sex bias (42% males and 58% females, $\chi^2(1, n = 24) = 8.05$, $p < 0.01$) compared to the control population at 720 dph. At each time point, gonads of fish exposed to 0.3 ng/L EE_2 contained sex cells at the same developmental stage as gonads of control fish throughout, with the exception of a single male fish in this treatment group at 720 dph that was intersex (Figure S4, Supporting Information).

Exposure to 4 ng/L EE_2 feminized the population completely (Figure S3, Supporting Information) for all life stages sampled. At 250 dph ($n = 22$), most ovaries contained sex cells up to and including primary oocytes at the Balbiani body stage, comparable to the control fish at this time, but few fish in this treatment group were less developed. At 518 dph ($n = 12$), there was a far greater degree of variation in gonadal development between individuals in this treatment group compared with control females, and this persisted in these fish at 720 dph ($n = 24$), where ovaries from 58% of the fish analyzed were comparable to those of the control females, containing all stages of sex cells, including vitellogenic oocytes, and 42% had predominantly primary oocytes with just a few sex cells at a more advanced stage of development.

Concentrations of VTG in controls at 250 dph were similar in males (142.8 ± 36.6 ng/mL) and females (147 ± 46.6 ng/mL), but at 518 dph they were widely divergent and in accordance with sex (20.3 ± 6.0 ng/mL in males compared with 5416 ± 4875 ng/mL in females). Significantly higher concentrations of VTG occurred in fish exposed to 4 ng/L EE_2 compared with controls. Exposure to 4 ng/L EE_2 resulted in VTG inductions of 472- and 489-fold after 250 dph (data not shown) and 103581-fold and 388-fold after 518 dph compared with the values in control female and male roach, respectively (Figure S5, Supporting Information).

Gonadal Impacts of Early-Life Exposure to EE_2. There were no significant differences in the length or weight of fish exposed to EE_2 during early life until 120 dph and maintained in clean water thereafter to 518 dph compared with fish at 518 dph that had been exposed continuously to EE_2 or maintained in clean water throughout (Figure S6, Supporting Information). There were also no effects on gonad development in roach exposed during early life (encompassing the period of gonadal sex differentiation) to a nominal concentration of 0.1 ng/L EE_2 and a measured concentration of 0.3 ng/L EE_2. Some fish exposed to 4 ng/L EE_2 during early life did not differ from control fish (unexposed throughout) at 518 dph in terms of the stages of germ cells present (Figure 2A,B). In this treatment group, however, there were some intersex fish (4 out of 11, 36%) at 518 dph (Figure 2C). The gonads of intersex roach were characterized by the presence of a few primary oocytes scattered throughout gonad sections of the testicular tissue in a multifocal arrangement. The primary oocytes occurred singly or in clusters. The gonads of these fish were dissected out prior to histological analysis, and thus, it was not possible to observe any malformations of the reproductive ducts.

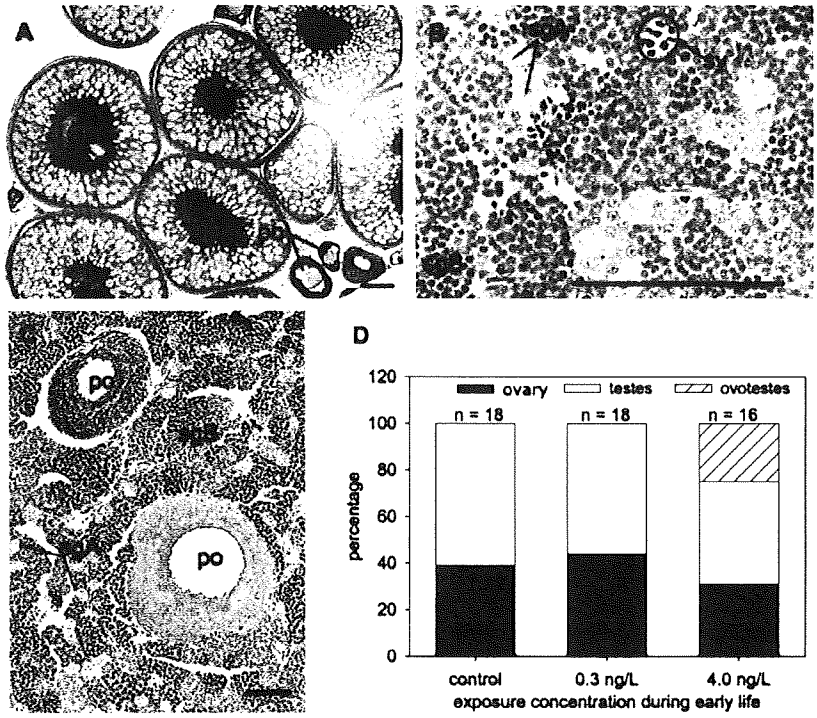


FIGURE 2. Gonad histopathology and sex ratio of roach at 518 dph. Sections of ovary (A), testis (B), and intersex testis (C) of roach exposed to measured 4 ng/L EE_2 until 120 dph and then kept in clean water: po, primary oocyte; so, secondary oocyte; vo, vitellogenic oocyte; sgA, spermatogonia A; sgB, spermatogonia B; sy, spermatocytes. Bars indicate 50 μm. (D) Sex ratios of roach exposed to EE_2 until 120 dph and then kept in clean water for 398 days.

At 518 dph, there was a male bias in the depurated fish population, as occurred for the unexposed controls, and the percentages of males ranged between 56% (0.3 ng/L EE_2 during early life) and 69% (4 ng/L EE_2) (Figure 2D).

Exposure of roach to 4 ng/L EE_2 during early life to 120 dph significantly induced plasma VTG in exposed fish (2116 ± 726 ng/g) compared to 40 ± 6 ng/g in controls, but after 398 days in clean water, VTG concentrations did not differ from those of the control fish (unexposed throughout; Figure S5, Supporting Information).

Estrogenic Sensitization. During the course of the re-challenge experiment, the EE_2 exposure concentration was 2.3 ± 0.2 ng/L EE_2. Overall, and as expected, re-exposure to EE_2 induced VTG synthesis and the duration of the EE_2 rechallenge had a significant effect on the level of the VTG induction in both females and males (females, $F_{1,47} = 46.45$, $p < 0.0001$; males, $F_{1,43} = 78.55$, $p < 0.0001$). In females, the EE_2 exposure concentration during early life appeared to have an effect on the level of induction of VTG in the estrogen rechallenge (plasma VTG levels in females exposed to 4 ng/L EE_2 during sexual differentiation were almost twice those in fish exposed to 0.3 ng/L EE_2—5.0- and 9.9-fold differences on day 4 and 1.7- and 3.3-fold on day 10 compared to re-exposed control fish); however, there was no statistically significant difference ($F_{2,48} = 1.39$, $p = 0.74$) due to the high variability in the responses between individuals. In males, there was no effect of EE_2 treatment during early life on the VTG response on rechallenge to estrogen ($F_{2,44} = 0.76$, $p = 0.47$), and males appeared to be less responsive generally to estrogen at this time compared with females (Figure 3A,B).

The expression of *esr1*, *esr2b*, and *cyp19a1a*, genes key in estrogen signaling, appeared to show an enhanced responsiveness to estrogen in fish that had been exposed to estrogen during early life when compared with control fish previously unexposed; i.e., there was a sensitization in the responses of these genes as a consequence of prior exposure to EE_2 during early life. This apparent trend was more pronounced in females compared with males (Figure 3). Overall, the effect of exposure concentration during early life was significant for the expression of *esr1*, *esr2b*, and *cyp19a1a* in ovaries and the expression of *esr2b* in testes (Table S1, Supporting Information). In females, the gonadal responses of *esr2b* and *cyp19a1a* in the EE_2 rechallenge experiment were dependent on the EE_2 exposure concentration during early life; e.g., the expression of *cyp19a1a* in the ovary differed significantly from that of the controls (fish not previously exposed to estrogen) with 5.1-fold and 10.5-fold elevations in females exposed previously (during early life) to 0.3 and 4.0 ng/L EE_2, respectively, compared to fish that received no estrogen exposure during early life (and were exposed during the estrogen rechallenge experiment).

Discussion

This study shows that life-long exposure of roach to concentrations of EE_2 found in some of the more polluted aquatic environments resulted in complete feminization of the population, and exposure during early life encompassing the phase of sex determination and sex differentiation induced the development of testis−ova. For the first time, we also show that exposure to estrogen during early life sensitizes fish to subsequent (and environmentally relevant) estrogen challenge, as shown by the responses in estrogen-responsive genes.

In most reported exposures of fish to environmental estrogens that have feminized gonadal development and/or caused complete sex reversal, pharmacological concentrations of estrogens have been used that bear no environmental relevance (*37*). Here we show that long-term exposure to a concentration of only 4 ng/L EE_2 (a concentration found in some of the more polluted WwTW effluents) feminizes roach completely, resulting in an all-female population, an effect that would result in population failure in the wild. Populations of wild roach living in U.K. rivers heavily polluted with

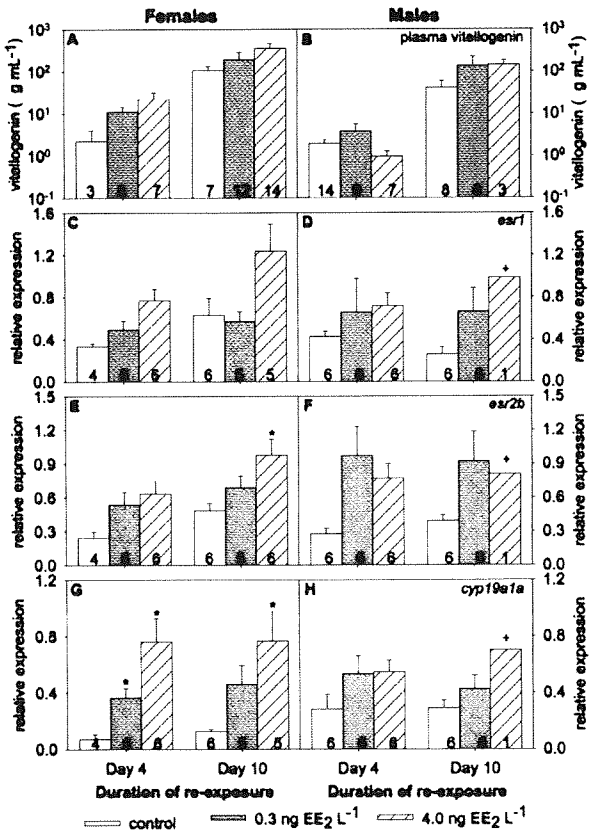


FIGURE 3. Effects of an estrogen rechallenge on plasma vitellogenin and expression of genes key in estrogenic signaling. Concentrations of plasma vitellogenin (A, B) and relative mRNA expression of *esr1* (C, D), *esr2b* (E, F), and *cyp19a1a* (G, H) in female (left) and male (right) roach exposed to measured 2.3 ng/L EE_2 for 10 days following early-life exposure to different concentrations of EE_2 until 120 dph and a subsequent depuration period of 398 days. Each column represents the mean ± SEM, and numbers in the columns indicate the number of samples analyzed. Asterisks indicate a significant difference from the control at that specific time point. Note in graphs for gene expression in males that at day 10 only one male fish was analyzed from the 4 ng/L EE_2 early-life treatment group (bar marked with a plus sign) and was therefore excluded from any analysis.

estrogenic WwTW effluents have been found with a sex ratio that is skewed toward females (*38*), and in some cases they are all-female (S. Jobling, personal communication). The lack of a genetic sex marker for roach, however, has excluded a definitive answer at to whether this bias in females is due to the presence of sex-reversed males. If sex-reversed roach occur in U.K. rivers and even if they were able to produce functional gametes, a retarded maturation rate, as occurred in this study, would likely mean the gametes were released at an inappropriate time in the wild; timing of reproduction and gamete release is critical in this seasonally spawning fish.

Generally, concentrations of EE_2 in U.K. WwTW effluents are measured between 0.15 and 2.85 ng/L (*39*), lower than that required to cause complete sex reversal in roach (here 4 ng/L); however, these effluents also contain biologically active concentrations of natural steroidal estrogen and other estrogenic EDCs that are additive in their feminizing effects (*40*), and therefore, roach populations may be at risk, given the overall estrogenic loadings entering U.K. rivers.

In mammals, (xeno)estrogens can induce effects via both genomic and nongenomic mechanisms (*41*). Genomic mechanisms include direct effects on the expression of genes involved with estrogen signaling that define the pathways of development for the two sexes and/or effects on coregulators of estrogen receptor (ER) function. Imprinting of genes by estrogens during critical periods of development has been suggested as an important mechanism for functional defects later in life (*42, 43*). Epigenetic (nongenomic) mechanisms include effects on DNA methylation controlling selective transcription or silencing tissue-specific genes. The mechanism by which sexual reprogramming occurs in roach as a consequence of exposure to steroidal estrogens is not known. In fish, however, exposures to 17β-estradiol (E_2) and EE_2 have both been shown to lead to global genomic hypermethylation in the testis and to induce tissue- and sex-specific changes in the methylation pattern of ER and aromatase genes (*44, 45*). It is therefore possible that exposure of roach to EE_2 during early life led to persistent changes in the DNA methylation pattern of these and/or other crucial sex-determining genes, resulting in alterations in the regulation of their gene expression. This might explain the induction of intersex (and even possibly the differences in the subsequent responsiveness of estrogen-controlled genes to stimulation in later life), but the fact that exposure of roach to 4 ng/L EE_2 during the window of sexual differentiation did not induce complete sex reversal, which occurred only for the continuous exposure to EE_2, suggests another operational mechanism(s) for this effect. Our findings in this study showing that sex reversal occurs only for prolonged exposures to estrogen support other studies showing longevity of exposure affects the magnitude (and severity) of the effects seen for sexual disruption, including for the vitellogenic response (*15*) and the intersex condition (*46*), and emphasize the need to consider the exposure period when the possible hazards posed by steroidal estrogens are assessed. Our study also shows that detrimental effects of long-term exposure to EE_2 (at 4 ng/L) were not limited to the males, and they also occurred for females, where the ovaries (at 720 dph) were relatively smaller compared with those of "normal" females.

Early-life-stage windows are especially sensitive to estrogenic (and other EDC) effects, and this is especially so for many fish species (e.g. ref *47*). In some cases, effects induced by exposure during early life do not become manifest until puberty or sexual maturation (*47, 48*). In our studies on the roach too, we showed the induction of intersex (ovotestis) occurs as a consequence of exposure to 4 ng/L EE_2 during early life. Our most novel finding, however, was that exposure to EE_2 during early life altered the subsequent responsiveness of roach to estrogen in later life. Females in particular showed enhanced responses on rechallenge to EE_2, and this occurred in a concentration-dependent manner for the exposures during early life. These findings strongly indicate that multiple exposures to estrogen, even with considerable time intervals between the successive exposures, can markedly affect the dynamics of the response to an environmental estrogen. If this response also occurs for endogenous estrogen (as is likely), then the reproductive dynamics could be altered, given the importance of steroidal estrogens such as E_2 in both fecundity and timing of maturation (*49, 50*). These findings have further implications for assessing the hazards and health risks associated with exposure to this synthetic steroidal estrogen as, for instance, thresholds for biomarker responses may be altered. Again, the mechanism(s) in operation that enhance estrogen responsiveness in later life in roach is (are) not known, but potentially they might include a molecular priming effect, where the sensitivity of, e.g., ERs and aromatases is enhanced. These molecular markers studied are known for their functional role as part of estrogen-responsive pathways: ER expression has implications for receptor availability and regulation, whereas aromatase expression has implications for the regulation of the ratios

between estrogens and androgens (see refs *27* and *35* and references within).

This study demonstrates that life-long exposure of roach to environmental concentrations of EE_2 (4 ng/L) induced sex reversal of males, resulting in an all-female population, and further that the induction of ovotestis is a consequence of (re)programming during early life. These data strongly support the hypothesis that EE_2 plays a major role in the induction of feminization responses in wild male fish living in rivers contaminated with estrogenic WwTW effluents. A further significant finding is that exposure to EE_2 during early life at concentrations as low as 0.3 ng/L induced more subtle disruptions in normal sexual programming, altering the subsequent responsiveness to estrogens in later life and with wider potential health implications.

Acknowledgments

We thank members of the Environmental and Molecular Fish Biology group at the University of Exeter and staff at Calverton Fish Farm (U.K. Environment Agency) who supported this project and Alexander Scott (CEFAS Laboratory Weymouth) for access to the EE_2 radioimmunoassay. The work was funded by the U.K. Natural Environmental Research Council (Grants NE/D002818/1 and NER/T/S/2002/00182) and the U.K. Environment Agency (C.R.T.) and the Ministry of Environment and the Ministry of Education, Culture, Sports, Science and Technology, Japan (T.I.) and (Y.K.). Defra (United Kingdom) supported research visits for A.L. to Japan.

Supporting Information Available

Statistical results of two-way ANOVAs, histopathology of continuously EE_2-exposed roach, and growth data and vitellogenin results for the continuous exposure and depuration experiment. This information is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Hotchkiss, A. K.; Rider, C. V.; Blystone, C. R.; Wilson, V. S.; Hartig, P. C.; Ankley, G. T.; Foster, P. M.; Gray, C. L.; Gray, L. E. Fifteen years after "Wingspread"—environmental endocrine disrupters and human and wildlife health: Where we are today and where we need to go. *Toxicol. Sci.* **2008**, *105*, 235–259.

(2) Harries, J. E.; Janbakhsh, A.; Jobling, S.; Matthiessen, P.; Sumpter, J. P.; Tyler, C. R. Estrogenic potency of effluent from two sewage treatment works in the United Kingdom. *Environ. Toxicol. Chem.* **1999**, *18*, 932–937.

(3) Jobling, S.; Nolan, M.; Tyler, C. R.; Brighty, G.; Sumpter, J. P. Widespread sexual disruption in wild fish. *Environ. Sci. Technol.* **1998**, *32*, 2498–2506.

(4) van Aerle, R.; Nolan, M.; Jobling, S.; Christiansen, L. B.; Sumpter, J. P.; Tyler, C. R. Sexual disruption in a second species of wild cyprinid fish (the gudgeon, *Gobio gobio*) in United Kingdom freshwaters. *Environ. Toxicol. Chem.* **2001**, *20*, 2841–2847.

(5) Flammarion, P.; Brion, F.; Babut, M.; Garric, J.; Migeon, B.; Noury, P.; Thybaud, E.; Tyler, C. R.; Palazzi, X. Induction of fish vitellogenin and alterations in testicular structure: Preliminary results of estrogenic effects in chub (*Leuciscus cephalus*). *Ecotoxicology* **2000**, *9*, 127–135.

(6) Hecker, M.; Tyler, C. R.; Hoffmann, M.; Maddix, S.; Karbe, L. Plasma biomarkers in fish provide evidence for endocrine modulation in the Elbe River, Germany. *Environ. Sci. Technol.* **2002**, *36*, 2311–2321.

(7) Vajda, A. M.; Barber, L. B.; Gray, J. L.; Lopez, E. M.; Woodling, J. D.; Norris, D. O. Reproductive disruption in fish downstream from an estrogenic wastewater effluent. *Environ. Sci. Technol.* **2008**, *42*, 3407–3414.

(8) Aerni, H. R.; Kobler, B.; Rutishauser, B. V.; Wettstein, F. E.; Fischer, R.; Giger, W.; Hungerbuhler, A.; Marazuela, M. D.; Peter, A.; Schonenberger, R.; Vogeli, A. C.; Suter, M. J. F.; Eggen, R. I. L. Combined biological and chemical assessment of estrogenic activities in wastewater treatment plant effluents. *Anal. Bioanal. Chem.* **2004**, *378*, 688–696.

(9) Nichols, K. M.; Miles-Richardson, S. R.; Snyder, E. M.; Giesy, J. P. Effects of exposure to municipal wastewater in situ on the reproductive physiology of the fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* **1999**, *18*, 2001–2012.

(10) Bjerregaard, L. B.; Korsgaard, B.; Bjerregaard, P. Intersex in wild roach (*Rutilus rutilus*) from Danish sewage effluent-receiving streams. *Ecotoxicol. Environ. Saf.* **2006**, *64*, 321–328.

(11) Minier, C.; Caltot, G.; Leboulanger, F.; Hill, E. M. An investigation of the incidence of intersex fish in Seine-Maritime and Sussex regions. *Analusis* **2000**, *28*, 801–806.

(12) Jobling, S.; Coey, S.; Whitmore, J. G.; Kime, D. E.; Van Look, K. J. W.; McAllister, B. G.; Beresford, N.; Henshaw, A. C.; Brighty, G.; Tyler, C. R.; Sumpter, J. P. Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biol. Reprod.* **2002**, *67*, 515–524.

(13) Jobling, S.; Beresford, N.; Nolan, M.; Rodgers-Gray, T.; Brighty, G. C.; Sumpter, J. P.; Tyler, C. R. Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biol. Reprod.* **2002**, *66*, 272–281.

(14) Rodgers-Gray, T. P.; Jobling, S.; Morris, S.; Kelly, C.; Kirby, S.; Janbakhsh, A.; Harries, J. E.; Waldock, M. J.; Sumpter, J. P.; Tyler, C. R. Long-term temporal changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. *Environ. Sci. Technol.* **2000**, *34*, 1521–1528.

(15) Liney, K. E.; Jobling, S.; Shears, J. A.; Simpson, P.; Tyler, C. R. Assessing the sensitivity of different life stages for sexual disruption in roach (*Rutilus rutilus*) exposed to effluents from wastewater treatment works. *Environ. Health Perspect.* **2005**, *113*, 1299–1307.

(16) Strüssmann, C. A.; Nakamura, M. Morphology, endocrinology, and environmental modulation of gonadal sex differentiation in teleost fishes. *Fish Physiol. Biochem.* **2002**, *26*, 13–29.

(17) Segner, H.; Navas, J. M.; Schäfers, C.; Wenzel, A. Potencies of estrogenic compounds in in vitro screening assays and in life cycle tests with zebrafish in vivo. *Ecotoxicol. Environ. Saf.* **2003**, *54*, 315–322.

(18) Larsson, D. G. J.; Adolfsson-Erici, M.; Parkkonen, J.; Pettersson, M.; Berg, A. H.; Olsson, P. E.; Forlin, L. Ethinyloestradiol—an undesired fish contraceptive. *Aquat. Toxicol.* **1999**, *45*, 91–97.

(19) Baronti, C.; Curini, R.; D'Ascenzo, G.; Di Corcia, A.; Gentili, A.; Samperi, R. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. *Environ. Sci. Technol.* **2000**, *34*, 5059–5066.

(20) Belfroid, A. C.; Van der Horst, A.; Vethaak, A. D.; Schafer, A. J.; Rijs, G. B. J.; Wegener, J.; Cofino, W. P. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. *Sci. Total Environ.* **1999**, *225*, 101–108.

(21) Cargouët, M.; Perdiz, D.; Mouatassim-Souali, A.; Tamisier-Karolak, S.; Levi, Y. Assessment of river contamination by estrogenic compounds in Paris area (France). *Sci. Total Environ.* **2004**, *324*, 55–66.

(22) Desbrow, C.; Routledge, E. J.; Brighty, G. C.; Sumpter, J. P.; Waldock, M. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environ. Sci. Technol.* **1998**, *32*, 1549–1558.

(23) Kuch, H. M.; Ballschmiter, K. Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NCI)-MS in the picogram per liter range. *Environ. Sci. Technol.* **2001**, *35*, 3201–3206.

(24) Rutishauser, B. V.; Pesonen, M.; Escher, B. I.; Ackermann, G. E.; Aerni, H. R.; Suter, M. J. F.; Eggen, R. I. L. Comparative analysis of estrogenic activity in sewage treatment plant effluents involving three in vitro assays and chemical analysis of steroids. *Environ. Toxicol. Chem.* **2004**, *23*, 857–864.

(25) Ternes, T. A.; Stumpf, M.; Mueller, J.; Haberer, K.; Wilken, R. D.; Servos, M. Behavior and occurrence of estrogens in municipal sewage treatment plants—I. Investigations in Germany, Canada and Brazil. *Sci. Total Environ.* **1999**, *225*, 81–90.

(26) Williams, R. J.; Johnson, A. C.; Smith, J. J. L.; Kanda, R. Steroid estrogens profiles along river stretches arising from sewage treatment works discharges. *Environ. Sci. Technol.* **2003**, *37*, 1744–1750.

(27) Lange, A.; Katsu, Y.; Ichikawa, R.; Paull, G. C.; Chidgey, L. L.; Coe, T. S.; Iguchi, T.; Tyler, C. R. Altered sexual development in roach (*Rutilus rutilus*) exposed to environmental concentrations of the pharmaceutical 17α-ethinylestradiol and associated expression dynamics of aromatases and estrogen receptors. *Toxicol. Sci.* **2008**, *106*, 113–123.

(28) Larsen, M. G.; Hansen, K. B.; Henriksen, P. G.; Baatrup, E. Male zebrafish (*Danio rerio*) courtship behaviour resists the feminising effects of 17α-ethinyloestradiol—morphological sexual characteristics do not. *Aquat. Toxicol.* **2008**, *87*, 234–244.
(29) Nash, J. P.; Kime, D. E.; Van der Ven, L. T. M.; Wester, P. W.; Brion, F.; Maack, G.; Stahlschmidt-Allner, P.; Tyler, C. R. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environ. Health Perspect.* **2004**, *112*, 1725–1733.
(30) Länge, R.; Hutchinson, T. H.; Croudace, C. P.; Siegmund, F.; Schweinfurth, H.; Hampe, P.; Panter, G. H.; Sumpter, J. P. Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* **2001**, *20*, 1216–1227.
(31) Schultz, I. R.; Skillman, A.; Nicolas, J. M.; Cyr, D. G.; Nagler, J. J. Short-term exposure to 17 alpha-ethynylestradiol decreases the fertility of sexually maturing male rainbow trout (*Oncorhynchus mykiss*). *Environ. Toxicol. Chem.* **2003**, *22*, 1272–1280.
(32) Jirtle, R. L.; Sander, M.; Barrett, J. C. Genomic imprinting and environmental disease susceptibility. *Environ. Health Perspect.* **2000**, *108*, 271–278.
(33) Miyagawa, S.; Katsu, Y.; Watanabe, H.; Iguchi, T. Estrogen-independent activation of erbBs signaling and estrogen receptor α in the mouse vagina exposed neonatally to diethylstilbestrol. *Oncogene* **2004**, *23*, 340–349.
(34) Paull, G. C.; Lange, A.; Henshaw, A. C.; Tyler, C. R. Ontogeny of sexual development in the roach (*Rutilus rutilus*) and its interrelationships with growth and age. *J. Morphol.* **2008**, *269*, 884–895.
(35) Katsu, Y.; Lange, A.; Ichikawa, R.; Urushitani, H.; Paull, G. C.; Cahill, L. L.; Jobling, S.; Tyler, C. R.; Iguchi, T. Functional associations between two estrogen receptors, environmental estrogens and sexual disruption in the roach (*Rutilus rutilus*). *Environ. Sci. Technol.* **2007**, *41*, 3368–3374.
(36) Tyler, C. R.; van Aerle, R.; Hutchinson, T. H.; Maddix, S.; Trip, H. An in vivo testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. *Environ. Toxicol. Chem.* **1999**, *18*, 337–347.
(37) Devlin, R. H.; Nagahama, Y. Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture* **2002**, *208*, 191–364.
(38) Environment Agency. *The Identification of Oestrogenic Effects in Wild Fish—Phase II*; R&D Technical Report W2-014/TR; Bristol, U.K., 2002; available at http://publications.environment-agency.gov.uk/epages/eapublications.storefront (accessed June 18, 2008).
(39) Thorpe, K. L.; Gross-Sorokin, M.; Johnson, I.; Brighty, G.; Tyler, C. R. An assessment of the model of concentration addition for predicting the estrogenic activity of chemical mixtures in wastewater treatment works effluents. *Environ. Health Perspect.* **2006**, *114*, 90–97.
(40) Thorpe, K. L.; Cummings, R. I.; Hutchinson, T. H.; Scholze, M.; Brighty, G.; Sumpter, J. P.; Tyler, C. R. Relative potencies and combination effects of steroidal estrogens in fish. *Environ. Sci. Technol.* **2003**, *37*, 1142–1149.
(41) Moggs, J. G. Molecular responses to xenoestrogens: Mechanistic insights from toxicogenomics. *Toxicology* **2005**, *213*, 177–193.
(42) Jirtle, R. L.; Skinner, M. K. Environmental epigenomics and disease susceptibility. *Nat. Rev. Genet.* **2007**, *8*, 253–262.
(43) McLachlan, J. A.; Burow, M.; Chiang, T. C.; Li, S. F. Gene imprinting in developmental toxicology: A possible interface between physiology and pathology. *Toxicol. Lett.* **2001**, *120*, 161–164.
(44) Aniagu, S. O.; Williams, T. D.; Allen, Y.; Katsiadaki, I.; Chipman, J. K. Global genomic methylation levels in the liver and gonads of the three-spine stickleback (*Gasterosteus aculeatus*) after exposure to hexabromocyclododecane and 17β-oestradiol. *Environ. Int.* **2008**, *34*, 310–317.
(45) Contractor, R. G.; Foran, C. M.; Li, S. F.; Willett, K. L. Evidence of gender- and tissue-specific promoter methylation and the potential for ethinylestradiol-induced changes in Japanese medaka (*Oryzias latipes*) estrogen receptor and aromatase genes. *J. Toxicol. Environ. Health, A* **2004**, *67*, 1–22.
(46) Jobling, S.; Williams, R.; Johnson, A.; Taylor, A.; Gross-Sorokin, M.; Nolan, M.; Tyler, C. R.; van Aerle, R.; Santos, E.; Brighty, G. Predicted exposures to steroid estrogens in U.K. rivers correlate with widespread sexual disruption in wild fish populations. *Environ. Health Perspect.* **2006**, *114*, 32–39.
(47) Fenske, M.; Maack, G.; Schäfers, C.; Segner, H. An environmentally relevant concentration of estrogen induces arrest of male gonad development in zebrafish *Danio rerio*. *Environ. Toxicol. Chem.* **2005**, *24*, 1088–1098.
(48) Maunder, R. J.; Matthiessen, P.; Sumpter, J. P.; Pottinger, T. G. Impaired reproduction in three-spined sticklebacks exposed to ethinyl estradiol as juveniles. *Biol. Reprod.* **2007**, *77*, 999–1006.
(49) Ankley, G. T.; Miller, D. H.; Jensen, K. M.; Villeneuve, D. L.; Martinovic, D. Relationship of plasma sex steroid concentrations in female fathead minnows to reproductive success and population status. *Aquat. Toxicol.* **2008**, *88*, 69–74.
(50) Tyler, C. R.; Sumpter, J. P. Oocyte growth and development in teleosts. *Rev. Fish Biol. Fish.* **1996**, *6*, 287–318.

ES802661P

Environ. Sci. Technol. **2009,** *43,* 3897–3904

Environmental Health Impacts of Equine Estrogens Derived from Hormone Replacement Therapy

CHARLES R. TYLER,*,▽,† AMY L. FILBY,*,▽,† LISA K. BICKLEY,† ROB I. CUMMING,∥ RICHARD GIBSON,‡,⊥ PIERRE LABADIE,‡ YOSHINAO KATSU,§ KATHERINE E. LINEY,†,# JANICE A. SHEARS,† VANESSA SILVA-CASTRO,⊥ HIROSHI URUSHITANI,§ ANKE LANGE,† MATTHEW J. WINTER,∥ TAISEN IGUCHI,§ AND ELIZABETH M. HILL‡

School of Biosciences, University of Exeter, Exeter, United Kingdom, School of Life Sciences, University of Sussex, Falmer, United Kingdom, Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Okazaki, Japan, Brixham Environmental Laboratory, AstraZeneca UK Limited, Brixham, United Kingdom, and Instituto de Ingeniería, Universidad Nacional Autónoma de México, Ciudad Universitaria, México

Received December 15, 2008. Revised manuscript received January 30, 2009. Accepted February 3, 2009.

Many factors have been considered in evaluations of the risk–benefit balance of hormone replacement therapy (HRT), used for treating menopausal symptoms in women, but not its potential risks for the environment. We investigated the possible environmental health implications of conjugated equine estrogens (CEEs), the most common components of HRT, including their discharge into the environment, their uptake, potency, and ability to induce biological effects in wildlife. Influents and effluents from four UK sewage treatment works (STWs), and bile of effluent-exposed fish, were screened for six equine estrogens. In vitro estrogen receptor (ER) activation assays were applied in humans and fish to compare their potencies, followed by in vivo exposures of fish to equine estrogens and evaluation of bioaccumulation, estrogenic responses, and ER gene expression. The equine estrogen equilenin (Eqn), and its metabolite 17β-dihydroequilenin (17β-Eqn), were detected by tandem GC-MSMS in all STW influent samples and 83% of STW effluent samples analyzed, respectively, at low concentrations (0.07–2.6 ng/L) and were taken-up into effluent-exposed fish. As occurs in humans, these estrogens bound to and activated the fish ERs, with potencies at ERα 2.4–3490% of that for 17β-estradiol. Exposure of fish for 21 days to Eqn and 17β-Eqn induced estrogenic responses including hepatic growth and vitellogenin production at concentrations as low as 0.6–4.2 ng/L. Associated with these effects were inductions of hepatic ERα and ERβ1 gene expression, suggesting ER-mediated mechanism(s) of action. These data provide evidence for the discharge of equine estrogens from HRT into the aquatic environment and highlight a strong likelihood that these compounds contribute to feminization in exposed wildlife.

* Corresponding author phone: +44 (0)1392 264450; fax: +44 (0)1392 263700; e-mail: c.r.tyler@exeter.ac.uk.
▽ C.R.T. and A.L.F. are joint first authors of this manuscript.
† University of Exeter.
‡ University of Sussex.
§ Okazaki Institute for Integrative Bioscience.
∥ AstraZeneca UK Limited.
⊥ Universidad Nacional Autónoma de México.
Current affiliation: Cascade Consulting, Manchester, United Kingdom.

Introduction

Estrogen has been used since the 1940s as a hormone replacement therapy (HRT) in women both during and following menopause, a physiological process characterized by the cessation of the menstrual cycle (amenorrhea) and typically occurring when women reach their late 40s/early 50s (*1*). Different HRT preparations use different estrogen compounds, the most common being the conjugated equine estrogens (CEEs; brand names Premarin and Prempro, Wyeth-Ayerst (*2*)), an extract from the urine of pregnant mares that consists of a mixture of estrogens, including estrone sulfate (which naturally occurs in women) and metabolites of the B-ring unsaturated estrogens, equilin (Eq) and equilenin (Eqn) (which are specific to horses).

Although the use of HRT has declined since 2002, when a much-publicized study identified associated risks (*3*), it is generally considered that the overall risk–benefit ratio favors its use (reviewed in *4, 5*). Indeed, the most recent estimates available indicate that approximately 17% of women aged 50+ (and up to 25% of women aged 50–59) remain on HRT in the United States (*6*). Moreover, with human population projections in the developed world predicting increases in both the number of women and their life expectancy (*7*), the actual volumes of HRT usage (kilograms/year) will likely rise in future years.

What has not been addressed in the risk–benefit balance of HRT, however, are its potential implications for the environment. It is well established that estrogens from women enter the aquatic environment via excretion in the urine and that sewage treatment processes vary in their removal efficacy (*8*). The widespread environmental presence of the estrogens estrone (E_1) and 17β-estradiol (17β-E_2), naturally produced by women, and the synthetic estrogen 17α-ethinylestradiol (EE_2), pharmaceutically administered via the contraceptive pill, has been clearly linked to endocrine disruption and reproductive abnormalities in aquatic wildlife, most notably fish (reviewed in (*9*)). However, there have been no thorough assessments on equine estrogens used in HRT in the environment or indeed any assessments of their ability to induce effects in exposed wildlife, which is surprising given that this was highlighted as a priority in environmental health more than a decade ago (*10*).

In a previous study, we identified for the first time the bioconcentration of the equine estrogen metabolite 17β-dihydroequilenin (17β-Eqn) in fish exposed to final (treated) effluent from a sewage treatment works (STW) in the UK (*11*). In this study, we set out to clarify and extend this finding by screening different UK STW effluents for the presence of six equine estrogens common in CEE-based HRT preparations, and further determined the uptake of these estrogens into exposed fish, and their ability to bind to and activate the fish estrogen receptor (ER). Through subsequent controlled in vivo exposures to equine estrogens in two fish species, we evaluated bioconcentration, estrogenic responses and potencies, and ER-mediated pathways of effect.

Materials and Methods

Chemicals. Equine estrogens were purchased from Steraloids, Inc. (Newport, RI). E_1, E_2, and EE_2, bis(trimethylsilyl)-tri-

10.1021/es803135q CCC: $40.75
Published on Web 04/01/2009

fluoroacetamide containing 1% trimethylchlorosilane and pyridine were obtained from Sigma-Aldrich (Poole, UK). Deuterated standards (2, 4, 16, 16-[$^2H_4$], E_1-[$^2H_4$], >98% [^2H] atom, E_2-[$^2H_4$], 95–97% [^2H] atom, and EE_2-[$^2H_4$], >96% [^2H] atom) were obtained from Cambridge Isotope Laboratories (Andover, MA). All solvents were HPLC-grade purchased from Rathburn Chemicals (Walkerburn, UK) or Fisher Scientific (Loughborough, UK).

STW Influents and Effluents. Influents and effluents from four STWs in the UK were studied (Table 1). STWs A and B were selected because they had been shown in previous studies to have a reasonably strong estrogenic potency (11.1–38.2 and 1.5–8.9 ng 17β-E_2 equivalent/L, respectively) and to induce marked feminized responses in fish (e.g., *12–14*). On the basis of an analysis of population data obtained from the UK Office for National Statistics (ONS)'s most recent census (*15*), two further STWs (STWs C and D) were selected because their coverage areas contained the highest proportions of women of menopausal/postmenopausal age (defined as 45+) of all regions of the UK (see Table 1).

Detection and Quantification of Equine Estrogens in STW Influents and Effluents. Our analyses of STW influents and effluents focused on six equine estrogens present in CEE-based HRT formulations. These were Eq, Eqn, and their metabolites 17α-dihydroequilin (17α-Eq), 17β-dihydroequilin (17β-Eq), 17α-dihydroequilenin (17α-Eqn), and 17β-dihydroequilenin (17β-Eqn), and these equine estrogens were quantified together with other steroidal estrogens commonly found in STW effluents (E_1, 17α-E_2, 17β-E_2 and EE_2). Samples of influent and effluent from each STW (100–500 mL) were collected in triplicate and the estrogens were isolated as reported elsewhere (*16*). Briefly, samples were prefiltered through glass wool, acidified with glacial acetic acid, and extracted onto OASIS HLB solid-phase extraction (SPE) cartridges (Waters Ltd., Elstree, UK). Profiles of estrogenic activity were obtained for STW A effluent samples; estrogens were eluted from SPE with methanol, and the extracts were fractionated by HPLC. Fractions were tested for estrogenic activity using a recombinant yeast estrogen screen (YES (*17*)) and active fractions, together with fractions eluting at the same time as standard equine estrogens, were analyzed by GC-MS (see (*11*)). Estrogens were also quantified in all STW samples using SPE methodology described by (*16*), silylation, and tandem GC-MSMS (see Supplemental Table S1).

Detection of Equine Estrogens in STW Effluent-Exposed Fish. We also determined whether the equine estrogens concentrated in the bile (the major route of excretion of estrogenic compounds in fish) of fish exposed to STW effluent (effluent A) (see (*11*)). Briefly, 25 juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed for 10 days to effluent or dechlorinated tap water under continuous flow-through conditions, after which fish were killed, their bile was removed, and the estrogenic compounds in the bile were deconjugated by incubation with β-glucuronidase, sulfatase, and β-glucosidase in 0.1 M phosphate buffer at pH 6.0 for 16 h at 37 °C. Composite bile extracts from 20 fish were acidified and extracted onto OASIS cartridges and, after elution, extracts were fractionated by HPLC and tested for estrogenic activity using YES. Fractions containing estrogenic activity or eluting at the same retention time as equine estrogens were silylated, and analyzed by GC-MS. During the exposure, the overall estrogenic activity of the effluent ranged from 24.3–104.1 ng 17β-E_2 equivalent/L and increases in the plasma concentrations of the estrogen-inducible protein vitellogenin (VTG) were between 240- and 700-fold in the exposed fish (see (*18*)).

In Vitro Analyses of the Estrogenic Potency of Equine Estrogens in Fish Compared with Humans. The estrogenic potency of equine estrogens in fish compared with humans was assessed by comparing the ability of the six target equine estrogens to activate the ER (using in vitro assays) in relation to that of the natural steroidal estrogen 17β-E_2. Responses at the ERα were evaluated in human using the YES, and in fish (roach, *Rutilus rutilus*) using an ERα reporter gene assay (*19*). The roach is a fish species for which widespread feminization has been demonstrated in UK rivers contaminated with treated sewage effluent (reviewed in (*9*)). For roach ERα and human ERα, dose–response curves were established with triplicate measurements and the potencies of the equine estrogens relative to 17β-E_2 were compared using EC_{50} values. Additionally, all equine estrogens were assayed in a reporter assay containing the roach ERβ ((*19*), here, at a single concentration of 10^{-5}M) to compare, approximately, their responses with that determined at the roach ERα.

In Vivo Exposures of Fish to Individual Equine Estrogens. *Chemical Dosing.* To assess the estrogenic potency of the equine estrogens detected in the effluents (17β-Eqn and Eqn) in fish, we conducted laboratory exposures with two species: the rainbow trout, as a representative salmonid, and common carp (*Cyprinus carpio*), as a representative cyprinid (the dominant family of UK freshwater fish). Fish were obtained from local fish farms and maintained in dechlorinated (trout) or reconstituted reverse osmosis (carp) water under flow-through conditions at 10–12 °C (trout) or 14–16 °C (carp) with a 12 h:12 h light/dark photoperiod and a commercial pelleted food diet.

For the in vivo exposures, stock solutions of 17β-Eqn, Eqn, 17β-E_2, and EE_2 were prepared in ethanol and dilutions (at 400× final exposure concentrations) used to dose the exposure tanks with a replacement of approximately 4 tank volumes per day. Groups of all-female immature rainbow trout (n = 10; 1 year old) or juvenile mixed sex common carp (n = 12; 20 months old) were exposed in duplicate in 150 L tanks for a period of 21 days to 4–5 concentrations of the equine estrogens. In experiments 1 and 2, trout were exposed to 17β-Eqn (1–320 ng/L) and Eqn (1–100 ng/L), respectively. In experiment 3, carp were exposed to 17β-Eqn (3.2–320 ng/L). In each exposure, groups of the same numbers of fish were exposed to dilution water (DWC) or dilution water containing solvent (SC; final concentration 0.0000125%) as negative controls, and to a concentration of an estrogen known to elicit biological effects (17β-E_2 at 100 ng/L in experiment 1, 17β-Eqn at 100 ng/L in experiment 2, and EE_2 at 10 ng/L in experiment 3) as positive controls. The actual concentrations of equine estrogens present in the tanks in all experiments were measured weekly by GC-MSMS.

Sampling and Morphometric Analyses. At the end of the exposures, fish were sacrificed by a lethal dose of anesthesia (benzocaine; Sigma), blood was sampled, and body weights and lengths were recorded. Gonads and livers were removed and weighed for determination of gonadosomatic and hepatosomatic index (GSI and HSI; gonad or liver weight as a percentage of total body weight) and, in the 17β-Eqn exposures, samples of liver were snap-frozen in liquid nitrogen for assessment of ER mRNA expression. In the trout exposures bile was also collected for assessment of estrogen bioconcentration, and in the carp exposure, gonads were fixed in 10% neutral-buffered formalin for histological determination of sex. Blood samples were centrifuged at 13,000 rpm for 5 min and the plasma was removed and stored at −20 °C for quantification of VTG via ELISA (as in *20, 21*). Bile and liver samples were stored at −80 °C until use.

Determination of Equine Estrogen Bioconcentration. Bile samples (0.1 mL) were deconjugated with β-glucuronidase and sulfatase for 16 h at 37 °C, then purified on Strata WAX (Phenomenex Ltd., Cheshire, UK) and aminopropyl (Supelco, Sigma-Aldrich, Dorset, UK) SPE cartridges. The final extracts were silylated and analyzed by GC-MSMS with limits of

TABLE 1. Concentrations (ng/L ± 1 SD) of the Equine Estrogens 17β-Eqn and Eqn[a] Relative to Other Steroidal Estrogens in UK STWs

STW	population equivalent	% of female population aged 45+[b]	treatment processes employed	sample	month of year	time of day (h)	equine estrogens[a]		other steroidal estrogens			
							17β-Eqn	Eqn	E_1	17α-E_2	17β-E_2	17α-EE_2
A	138,000	42.47	primary treatment followed by bubble-diffused air-activated sludge and percolating filter secondary treatments	influent	November	09.00	0.37 ± 0.10[d]	1.96 ± 0.24	45.2 ± 13.0	2.30 ± 0.67	27.6 ± 5.1	N/Q
				influent	November	12.00	0.23[c,d]	2.32 ± 0.47	45.4 ± 25.2	1.77 ± 0.57	13.4 ± 2.8	N/Q
				influent	November	15.00	N/Q	N/Q	34.0 ± 5.3	0.81 ± 0.72	13.5 ± 5.6	N/Q
						mean ± SD	**0.30 ± 0.10**	**2.14 ± 0.26**	**42.1 ± 4.0**	**1.63 ± 0.76**	**18.2 ± 8.2**	**N/Q**
				effluent	September	09.00	0.09 ± 0.05	0.32 ± 0.09	36.9 ± 4.4	0.11 ± 0.01	1.97 ± 0.11	0.32 ± 0.07
				effluent	September	12.00	0.07 ± 0.03	0.33 ± 0.04	40.9 ± 0.4	0.11 ± 0.02	2.09 ± 0.18	0.23 ± 0.05
				effluent	September	15.00	0.07 ± 0.01	0.34 ± 0.07	39.1 ± 3.0	0.12 ± 0.02	2.21 ± 0.10	0.25 ± 0.05
				effluent	November	09.00	0.14 ± 0.02	0.52 ± 0.13	47.2 ± 3.3	0.20 ± 0.01	2.99 ± 0.29	0.87 ± 0.09
				effluent	November	12.00	0.12 ± 0.04	0.47 ± 0.07	42.0 ± 5.5	0.23 ± 0.03	2.74 ± 0.34	0.85 ± 0.28
				effluent	November	15.00	0.13 ± 0.04	0.59 ± 0.11	46.2 ± 13.7	0.22 ± 0.05	2.93 ± 0.58	0.87 ± 0.16
						mean ± SD	**0.10 ± 0.03**	**0.43 ± 0.11**	**42.14.0**	**0.17 ± 0.06**	**2.49 ± 0.45**	**0.57 ± 0.33**
B	107,250	46.28	primary treatment followed by biological (percolating) filters and humus tanks	effluent	September	12.00	0.08 ± 0.07	1.02 ± 0.15	6.85 ± 0.34	N/A	5.54 ± 0.33	1.22 ± 0.18
C	20,270	58.82	primary treatment followed by secondary activated sludge and final UV treatment	influent	October	11.00	<LOD	2.59 ± 2.31	25.1 ± 3.6	4.47 ± 3.50	13.7 ± 2.3	1.05 ± 076
				effluent	October	11.00	<LOD	0.37 ± 0.07	1.54. ± 0.15	0.08 ± 0.03	0.67 ± 0.11	0.11 ± 0.01
D	15,472	59.14	preliminary screening treatment, followed by primary treatment, submerged biological contactor (SBC), then final settlement and UV treatment	influent	October	14.00	N/Q	N/Q	50.8 ± 35.6	N/Q	66.7 ± 2.7	0.53 ± 0.04
				effluent	October	14.00	0.18 ± 0.09	1.32 ± 0.76	18.0 ± 5.6	1.62 ± 0.53	10.5 ± 4.2	0.39 ± 013

[a] Data are a mean of 3 replicate samples. Concentrations of other equine estrogens were below the limit of detection in all samples (see Supplemental table S1 for LOD and LOQ values). N/A: Not analyzed. N/Q: Not quantified due to interference from influent matrix. [b] Data calculated based on 2005 population census data from districts of the UK (*15*). [c] Detected in 1 of 3 replicate samples. [d] Below LOQ value.

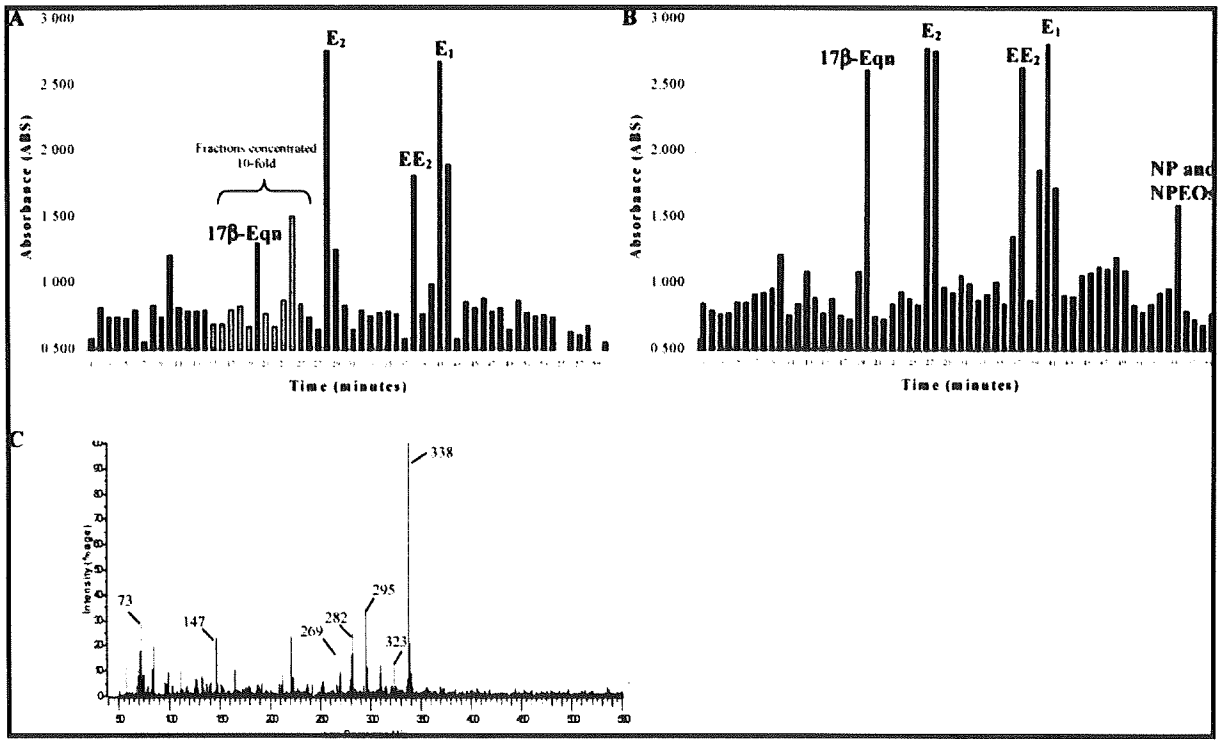


FIGURE 1. Identification of equine estrogens in STW final effluents and effluent-exposed fish. Profiles of estrogens in (a) effluent and (b) bile of effluent-exposed trout, and mass spectrum identity of (c) Eqn in bile of effluent-exposed trout. Extracts of effluent and hydrolyzed bile were fractionated by reversed-phase HPLC and fractions were analyzed for estrogenic activity by measuring the response (absorbance) in the YES assay. The identities of the estrogenic compounds in the active fractions were determined by GC-MS. NP, nonylphenol; NPEO, nonylphenol polyethoxylates.

detection (LOD) of 60 and 180 pg/0.1 mL bile for 17β-Eqn and Eqn, respectively.

ER Gene Expression Analyses. Real-time PCR was conducted to determine the effect of exposure to 17β-Eqn on the hepatic expression of the different ER subtypes in fish (ERα, ERβ1, ERβ2) and their isoforms (ERα1 "short" and "long" isoforms in trout). Total RNA was extracted from the liver samples, DNase-treated, and reverse transcribed as previously described (*22*). Real-time quantitative PCR was performed in triplicate for each sample with the iCycler iQ Real-time Detection System (Bio-Rad Laboratories Inc., Hercules, CA) with gene-specific primers (see Table S2 in the Supporting Information) using the methods previously described (*22*). Ribosomal protein L8 (RPL8) was used for efficiency-corrected relative quantitation because its expression in liver did not change following the exposures.

Data Analyses. Unless otherwise stated, all data are presented as mean ± SEM. Statistical differences ($P < 0.05$, or $P < 0.01$ for the roach ER assay) between groups were assessed by Student's *t*-test or one-way ANOVA followed by Dunnets's post hoc test, or nonparametric alternatives when required (SigmaStat 2.03; Jandel Scientific Software; or Graph Pad Prism3 DEMO version software; Graph Pad Software, Inc.). In the in vivo fish exposures, there was no effect of the solvent on any of the end points, so experimental groups were compared to the DWC. For the carp in vivo exposure, there were no differences in any of the responses of males compared to females so the data for the two sexes were pooled.

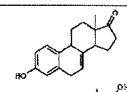
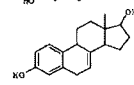
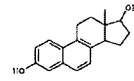
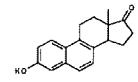
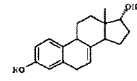
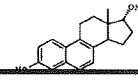
Results

Detection of Equine Estrogens in STW Influents and Effluents and Effluent-Exposed Fish. Profiles of estrogenic activity in effluent A revealed fractions containing E_2, EE_2, and E_1 (for a representative profile, see Figure 1A). The equine estrogen 17β-Eqn was also identified in an estrogenic fraction (after a 10-fold concentration) eluting at 20 min. Characteristic fragments of the mass spectra comprised *m/z* 412, M^+; *m/z* 322, loss of trimethylsilanol, fragments *m/z* 397 and 307 formed by methyl loss from these two species; and *m/z* 281, loss of the D ring (see (*11*)). Analysis of other fractions with either minor estrogenic activity or eluting at the same retention time as standard equine estrogens did not reveal the presence of any other steroidal estrogens. Quantitative analysis of the STW influents/effluents revealed that, of the six equine estrogens screened, only 17β-Eqn and Eqn were detected. Concentrations of Eqn ranged between 1.32−2.59 ng/L (influent) and 0.32−1.32 ng/L (effluent) and for 17β-Eqn between <0.2 (LOD)−0.37 ng/L (influent) and 0.07−0.18 ng/L (effluent); data presented in Table 1. Other steroidal estrogens (E_1, 17α-E_2, 17β-E_2 and EE_2) were detected (Table 1) at concentrations typically found in STW influents and effluents (*8*).

Analysis of bile of trout exposed to effluent A confirmed the presence of 17β-Eqn in an estrogenic fraction (identified previously by (*11*); Figure 1B). Eqn was also detected from analysis of other HPLC fractions that corresponded to the same retention times as standard equine estrogens. The fraction containing Eqn (which eluted at 32 min) revealed a mass spectrum identical to that of the silylether of standard Eqn and contained ions at *m/z* 338 (M^+), 323 (loss of methyl) 295 (loss of ethyl) and *m/z* 281 (loss of the D ring; Figure 1C). There was no evidence of other equine estrogens in bile of effluent-exposed fish. Bile of fish exposed to tap water contained only one peak of estrogenic activity, which was confirmed to be 17β-E_2 (data not shown).

In Vitro Analyses of the Estrogenic Potency of Equine Estrogens in Fish Compared with Humans. In the in vitro assays, estrogenic responses were induced in a concentration-dependent manner by all six equine estrogens both at the

TABLE 2. EC_{50}s and Estrogenic Potencies of Six Equine Estrogens at the Human and Fish ERα

Name	Molecular weight and formula	Structure	EC_{50} (M)[a]		Estrogenic potency relative to 17β-E_2 (%)[d]	
			Human ERα[b]	Roach ERα[c]	Human ERα[b]	Roach ERα[c]
Equilin (Eq)	268 $C_{18}H_{20}O_2$		5.5×10^{-10}	1.2×10^{-9}	15	25.6
17β-dihydroequilin (17β-Eq)	270 $C_{18}H_{22}O_2$		1.1×10^{-10}	8.7×10^{-12}	77	3490
17β-dihydroequilenin (17β-Eqn)	268 $C_{18}H_{20}O_2$		3.3×10^{-10}	3.7×10^{-9}	25	8.24
Equilenin (Eqn)	266 $C_{18}H_{18}O_2$		1.2×10^{-9}	1.3×10^{-8}	7	2.35
17α-dihydroequilin (17α-Eq)	270 $C_{18}H_{22}O_2$		1.2×10^{-9}	1.1×10^{-8}	7	2.88
17α-dihydroequilenin (17α-Eqn)	268 $C_{18}H_{20}O_2$		4.2×10^{-9}	4.7×10^{-9}	2	6.45

[a] Data based on triplicate measurements. [b] Data generated using YES assay. [c] Data generated using roach (*Rutilus rutilus*). [d] Relative potencies were calculated using EC_{50} values (The EC_{50} for 17β-E_2in the YES assay was 8.3×10^{-11}M and in the roach ERα transactivation assay was 3.0×10^{-10} M).

human ERα and roach ERα (Table 2, also see Figure S1). At the human ERα, all equine estrogens were found to be of a lower potency than 17β-E_2 (relative potencies of 2−77%; Table 2), with the rank order of potency as follows: 17β-Eq > 17β-Eqn > Eq > 17α-Eq = Eqn > 17α-Eqn. At the roach ERα, one equine estrogen, 17β-Eq, was found to be 35-fold more potent than 17β-E_2, while the other equine estrogens had relative potencies compared to 17β-E_2 of 2.35−25.6% (Table 2). The order of potency was 17β-Eq > Eq > 17β-Eqn > 17α-Eqn > 17α-Eq > Eqn and showed strong similarities to that observed for the same estrogens at the human ERα. For example, 17β-Eq in both cases had the highest potency of the six equine estrogens and 17α-Eq and Eqn had the least. All six equine estrogens were additionally able to activate transcriptional activity of roach ERβ at the concentration tested (10^{-5} M) (see Figure S2).

In Vivo Exposures of Fish to Individual Equine Estrogens. Measured concentrations of the test equine estrogens in the exposure water were 60−93% and 87−132% of nominal for the trout and carp exposures to 17β-Eqn, respectively, and 42−100% of nominal for the trout exposure to Eqn (see Table S3). There were no effects of any of the treatments on body size, condition factor, or GSI in either species. Exposure to the highest concentration of 17β-Eqn (299 ng/L) resulted in a significantly increased HSI in the trout ($P = 0.007$; by 1.2-fold from 0.0086 ± 0.0003% to 0.0104 ± 0.0003%; which was the same fold increase as observed for the fish exposed to 100 ng of 17β-E_2/L; $P = 0.009$) but Eqn did not affect this end point.

Exposure to both 17β-Eqn and Eqn led to increases in the concentrations of VTG in the plasma of trout and carp in a concentration-dependent manner (all tests $P < 0.001$; Figure 2A) as occurred for the estrogenic positive controls. In trout, exposure to 17β-Eqn resulted in significant induction in VTG at all concentrations tested (from 0.6 ng/L), with a maximal induction (400-fold compared to the water controls) in the fish exposed to 299 ng 17β-Eqn/L (Figure 2A (i)). In contrast, significant induction of plasma VTG by 17β-Eqn in the carp occurred after exposure to the highest concentration tested (421 ng/L) only, where the VTG concentration was 830-fold higher than in the DWCs (Figure 2A (ii)) (and this was a significantly ($P < 0.001$) smaller induction than that observed for trout exposed to 299 ng of 17β-Eqn/L). The LOEC for induction of VTG in trout exposed to Eqn was 4.2 ng/L (no induction occurred at 1 ng/L) and the maximal level of induction was 28-fold in the 50 ng/L treatment group (Figure 2A (i)).

Neither 17β-Eqn nor Eqn were detected in the bile of the trout from the DWC group. However, 17β-Eqn was present in the bile of trout exposed to 17β-Eqn, and both Eqn and 17β-Eqn were present in the bile of trout exposed to Eqn. Bioconcentration factors (based on measured water concentrations) for the equine estrogens in bile of exposed trout were 1.5×10^6 in trout exposed to 17β-Eqn and 2.2×10^6 in trout exposed to Eqn (taking into account the measured concentrations of both Eqn and 17β-Eqn).

Exposure to 17β-Eqn altered ER gene expression in the liver of both fish species, although this varied according to ER subtype/isoform (Figure 2B). In trout (Figure 2B (i)), there was a significant induction (2.1−4.1-fold) of both isoforms of ERα at the highest concentration of 17β-Eqn (299 ng/L), but the large isoform appeared more sensitive, and was additionally induced (by 2.1-fold) at 75 ng 17β-Eqn/L ($P = 0.003$ and $P = 0.006$, respectively). Similarly, hepatic expression of ERα was also induced in carp exposed to the highest concentration of 17β-Eqn ($P < 0.001$; although the 15.6-fold induction was greater than that observed in trout (Figure 2B (ii)). The most notable species difference was the induction of ERβ1 in trout exposed to 17β-Eqn (at both 75 and 299 ng/L; 1.6- and 1.7-fold, respectively; $P = 0.011$) which did not occur in carp.

Discussion

Our screening detected only two equine estrogens, Eqn and its metabolite 17β-Eqn, in the STWs surveyed but their presence in influents demonstrated that they were not solely products of steroid metabolism during STW treatment processes, suggesting that they very likely result from the pharmaceutical use of CEEs in HRT formulations (*11*). We cannot, however, confirm the absence of the other equine estrogens as these may be present at concentrations below

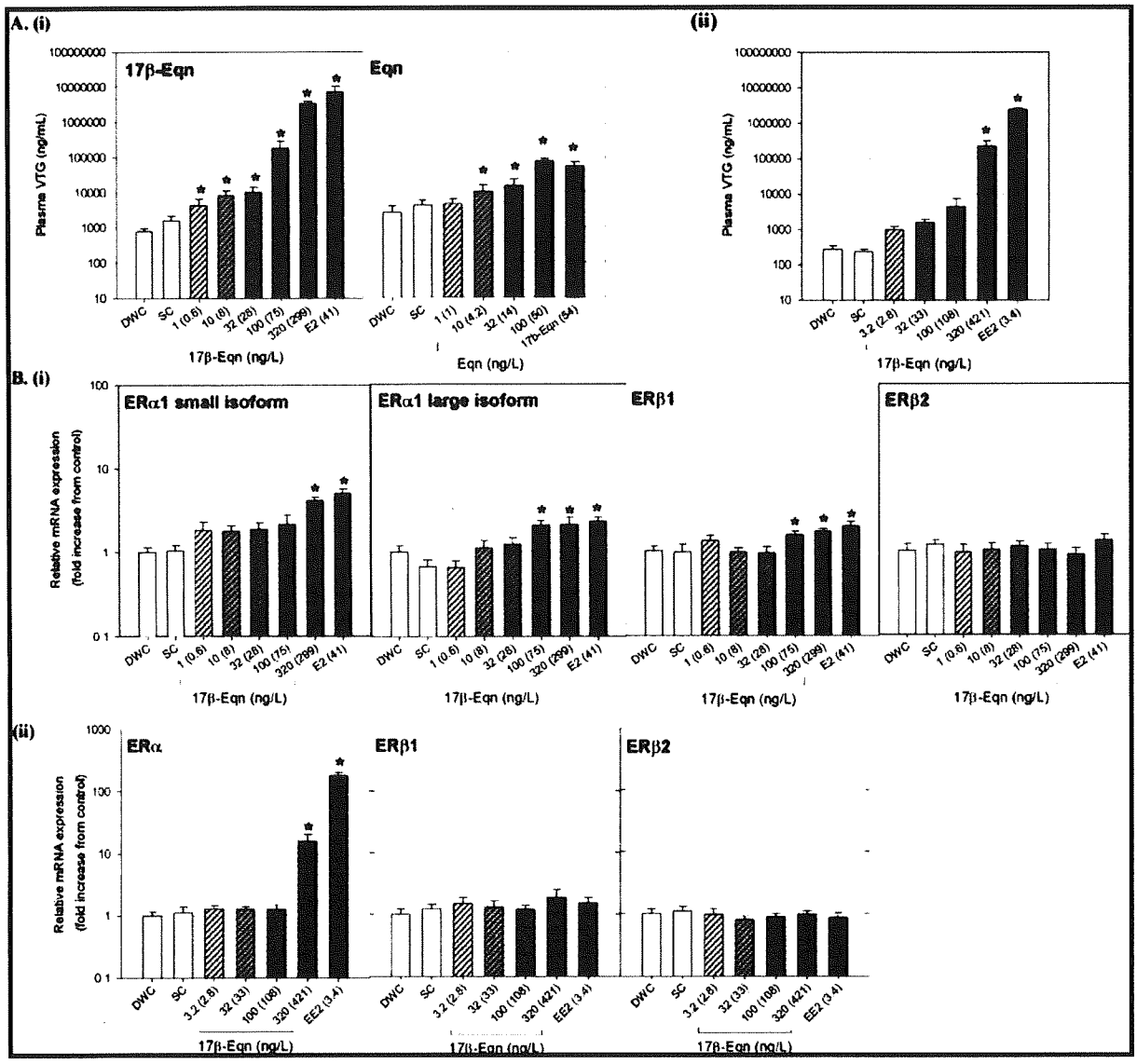


FIGURE 2. Estrogenic responses in fish following in vivo exposure (21 days) to the equine estrogens. (A) Induction of the estrogen-inducible protein VTG in the plasma of rainbow trout (i) and common carp (ii) following exposure to the equine estrogens 17β-Eqn (both species) and Eqn (trout only). (B) Induction of hepatic expression of ERα, β1, and β2 subtypes and their isoforms in rainbow trout (i) and common carp (ii) exposed to 17β-Eqn. Rainbow trout were all female and carp were mixed sex. Measured chemical concentrations are shown in parentheses. Statistically significant differences compared to the dilution water controls are indicated by an asterisk ($P < 0.05$). DWC, dilution water control; SC, solvent control.

the LOD of our analytical methodology. The equine estrogens detected were present at concentrations up to 1.32 ng/L in effluents, and at concentrations similar to the pharmaceutical EE_2. These low levels of equine estrogens might suggest they are of limited environmental concern, but they do have the capacity to bioconcentrate to very high levels (see later discussion) and there is now a strong body of evidence demonstrating that steroidal estrogens can act additively in their biological effects (e.g., (*23*)).

No other studies have examined for the presence of equine estrogens in STW effluents or surface waters in Europe. However, in a recent study in the United States the 17α-reduced form of Eq (which represents 25% of the estrogen content of Premarin), was detected in a municipal STW in Pennsylvania (*24*). In that study, 17α-Eq was measured at 24 ng/L and only a 1% removal was observed during sewage treatment (a form of activated sludge), contrasting with our study which indicated a far higher removal/degradation within the STWs studied (at between 66−86%, when making a simple comparison of measured content in the influent compared with in the effluent).

Nevertheless, these studies combined demonstrate that equine estrogens can survive current sewage treatment procedures and suggest that the source of CEEs in the aquatic environment is from pharmaceutical use (rather than industry or agricultural runoff).

Our analyses of bile of trout exposed to effluent containing Eqn and 17β-Eqn (effluent A) confirmed our previous finding with this effluent for 17β-Eqn (*11*) and also discovered the presence of Eqn. Here our application of GC-MS analysis of selected bile fractions enabled the detection of the sub-nanogram concentrations of equine estrogens that would not have been possible previously using YES detection of estrogenic HPLC fractions of effluent alone. These results show that equine estrogens present in effluent are taken up into exposed fish and become highly concentrated within the bile.

It is well established that the biological effects of estrogens are principally elicited via their binding to and activation of nuclear ERs (*25*) and we used in vitro ER assays to compare the potency of equine estrogens in human with roach, a widespread fish species common in UK watercourses into which these effluents are discharged. Our finding showed that all six equine estrogens tested were able to bind to and activate both ER subtypes in roach with relatively high potencies as occurred in humans (this study and others *26–29*), demonstrating similar sensitivities of humans and fish to these compounds, and their potential for exerting biological effects in vertebrates generally. Although there were some differences in the exact rank order of potency of the equine estrogens across phyla, several clear commonalities were observed. These included the greater potency of 17β-Eq compared to all other equine estrogens tested. Furthermore, the 17β-stereoisomers were more potent compared with the corresponding 17α- or -keto isomers, the latter of which fits with the belief that the 17β-reduced products of Eq and Eqn are the active metabolites in menopausal women (*27*). One notable difference, however, was the observation that the potency of 17β-Eq greatly exceeded that of 17β-E_2 in roach. While this was not observed for the human ERα in this study, a greater binding affinity of 17β-Eq compared to 17β-E_2 at the ER of human and rat has been observed in previous studies (*26, 27, 29*), differences perhaps resulting from differences in the methodologies used.

Our in vivo exposures of fish to the equine estrogens detected in STW effluents (17β-Eqn and Eqn) demonstrated that CEEs induce estrogenic responses in intact animals, with effects including hepatic growth and induction of the yolk precursor protein VTG, both common biomarkers of estrogen-exposure in oviparous species (reviewed in (*21*)). The fact that a vitellogenic response was observed in trout at all tested concentrations of 17β-Eqn (and as low as 0.6 ng/L), and at all except the lowest concentration of Eqn (i.e., from 4.2 ng/L), illustrates the high potency of these equine estrogens in salmonid fish, even after relatively short-term exposure. The effect concentrations were, however, considerably higher in carp (with a LOEC of 421 ng/L for 17β-Eqn). Other studies have also shown that trout are more sensitive to estrogens compared with fish of the carp family (*18, 30*) and these species-differences in responses of fish to environmental contaminants may result from differential chemical uptake, bioconcentration, receptor interaction, processing or turnover (e.g., (*18*)). A further finding from these exposures was the lower potency of Eqn compared to its metabolite 17β-Eqn in fish, again consistent with ER binding studies in mammals (*26–29*). Nevertheless, relative binding affinities of equine estrogens (including Eqn) for the ER have not always been found to be correlated with their biological effects (*27, 29*).

Finally, the results from the hepatic ER gene expression assays in these exposed fish demonstrated that an induction of the hepatic ER is a plausible mechanism for the estrogenic effects of CEEs in fish. Estrogen induction of ERα here was a response common to both trout and carp, and has been observed consistently in studies in fish (e.g., (*31*)). The lack of a significant induction of ERα gene expression on exposure to 17β-Eqn in trout at concentrations at which VTG protein induction occurred indicated that VTG induction was more sensitive to this equine estrogen compared with ER gene expression. VTG however undergoes a far higher magnitude of induction (potentially up to a million-fold) compared with ER mRNA induction and is thus more easily detected at threshold effect concentrations. The parallel increase in the expression of the ERβ1 subtype in exposed trout is especially intriguing. In mammals the majority of (if not all) equine estrogens possess a higher binding affinity for ERβ, unlike for any other natural steroid estrogens (*27–29*). As our investigations on the activation of fish ERβ by equine estrogens used only a single concentration, we do not know their relative potency at the two ERs in fish and this would be a very interesting focus for future work.

In summary, these data provide clear evidence that the discharge into the aquatic environment of equine estrogens used pharmaceutically in HRT can induce estrogenic effects in exposed wildlife and may contribute to the feminization observed in wild fish populations in the UK, and elsewhere. Furthermore, given the evidence from mammalian research for wider adverse health effects of equine estrogens (including DNA damage, cytotoxicity, carcinogenesis, and immune modulation *32, 33*), it is possible that the effects of these compounds in exposed wildlife may extend beyond endocrine disruption alone. These implications on environmental health should perhaps be considered when weighing up the risks and benefits of HRT for women.

Acknowledgments

This work was supported by the UK Natural Environmental Research Council (refs: NE/D002818/1, NER/T/S/2002/00182, NE/F007787/1), British Biotechnology and Biological Sciences Research Council, and AstraZeneca on grants to C.R.T., by the Ministry of Environment, Japan, and Grants-in-Aid for Scientific Research B from the Ministry of Education, Culture, Sports, Science and Technology, Japan, on grants to T.I., and by the EU Interreg IIIA programme on a grant to E.M.H. We thank Richard Williams of CEH Wallingford, Gregory Paull and Tessa Scown, of the University of Exeter, and participating water companies for their assistance.

Supporting Information Available

Ions used in quantification of equine estrogens by GC-MSMS and the recoveries and limit of detection values from spiked samples (Table S1), details of real-time PCR assays (Table S2) and water chemistry results from in vivo exposures (Table S3), results for equine estrogen activation of roach ERα (Figure S1) and ERβ (Figure S2) in the in vitro assays. This information is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Nelson, H. D. Menopause. *The Lancet* **2008**, *371*, 760–770.
(2) Hersch, A. L.; Stefanick, M. L.; Stafford, R. S. National use of postmenopausal hormone therapy - annual; trends and response to recent evidence. *JAMA* **2004**, *291*, 47–53.
(3) Rossouw, J. E.; Anderson, G. L.; Prentice, R. L.; LaCroix, A. Z.; Kooperberg, C.; Stefanick, M. L. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. *JAMA* **2002**, *288*, 321–33.
(4) Nelson, H. D.; Humphrey, L. L.; Nygren, P.; Teutsch, S. M.; Allan, J. D. Postmenopausal hormone replacement therapy: scientific review. *JAMA* **2002**, *288*, 872–881.
(5) Harman, S. M. Estrogen replacement in menopausal women: recent and current prospective studies, the WHI and the KEEPS. *Gend. Med.* **2006**, *3*, 254–269.
(6) Kim, J. K.; Alley, D.; Hu, P.; Karlamangla, A.; Seeman, T.; Crimmins, E. M. Changes in postmenopausal hormone therapy use since 1988. *Womens Health Issues* **2007**, *17*, 338–341.
(7) Diczfalusy, E.; Bengiano, G. Women and the third and fourth age. *Int. J. Gynecol. Obstet.* **1997**, *58*, 177–188.
(8) Johnson, A. C.; Aerni, H. R.; Gerritsen, A.; Gibert, M.; Giger, W.; Hylland, K.; Jurgens, M.; Nakari, T.; Pickering, A.; Suter, M. J. F.; Svenson, A.; Wettstein, F. E. Comparing steroid estrogen, and nonylphenol content across a range of European sewage plants with different treatment and management practices. *Water Res.* **2005**, *39*, 47–58.
(9) Tyler, C. R.; Jobling, S.; Sumpter, J. P. Endocrine disruption in wildlife: a critical review of the evidence. *Crit. Rev. Toxicol.* **1998**, *28*, 319–361.
(10) Arcand-Hoy, L. D.; Nimrod, A. C.; Benson, W. H. Endocrine-modulating substances in the environment: estrogenic effects of pharmaceutical products. *Int. J. Toxicol.* **1998**, *17*, 139–158.

(11) Gibson, R.; Smith, M. D.; Spary, C. J.; Tyler, C. R.; Hill, E. M. Mixtures of estrogenic contaminants in bile of fish exposed to wastewater treatment works effluents. *Environ. Sci. Technol.* **2005**, *39*, 2461–2471.
(12) Rodgers-Gray, T. P.; Jobling, S.; Kelly, C.; Morris, S.; Brighty, G.; Waldock, M. J.; Sumpter, J. P.; Tyler, C. R. Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption of gonadal duct development. *Environ. Sci. Technol.* **2001**, *35*, 462–470.
(13) Environment Agency. *Assessment of (anti-) oestrogenic and (anti-) androgenic activities of final effluents from sewage treatment works*; Science Report SC020118/SR; Environment Agency: Bristol, UK, 2007; http://publications.environment-agency.gov.uk/epages/eapublications.storefront.
(14) Filby, A. L.; Neuparth, T.; Thorpe, K. L.; Owen, R.; Galloway, T. S.; Tyler, C. R. Health impacts of estrogens in the environment, considering complex mixture effects. *Environ. Health Perspect.* **2007**, *115*, 1704–1710.
(15) Office for National Statistics (ONS). *Key population and vital statistics - local and health authority areas*; Report 2005 vs no. 32 PP1 no. 28, ISSN: 14692732; 2005; http://www.statistics.gov.uk/statbase/product.asp?vlnk=539.
(16) Gibson, R.; Becerril-Bravo, E.; Silva-Castro, V.; Jimenez, B. Determination of acidic pharmaceuticals and potential endocrine disrupting compounds in wastewaters and spring waters by selective elution and analysis by gas chromatography-mass spectrometry. *J. Chromatgr. A* **2007**, *1169*, 31–39.
(17) Routledge, E. J.; Sumpter, J. P. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ. Toxicol. Chem.* **1996**, *15*, 241–248.
(18) Tyler, C. R.; Spary, C.; Gibson, R.; Santos, E. M.; Shears, J.; Hill, E. M. Accounting for differences in estrogenic responses in rainbow trout (*Oncorhynchus mykiss*: Salmonidae) and roach (*Rutilus rutilus*: Cyprinidae) exposed to effluents from wastewater treatment works. *Environ. Sci. Technol.* **2005**, *39*, 2599–2607.
(19) Katsu, Y.; Lange, A.; Urushitani, H.; Ichikawa, R.; Paull, G. C.; Cahill, L. L.; Jobling, S.; Tyler, C. R.; Iguchi, T. Functional associations between two estrogen receptors, environmental estrogens, and sexual disruption in the roach (*Rutilus rutilus*). *Environ. Sci. Technol.* **2007**, *41*, 3368–3374.
(20) Tyler, C. R.; van Aerle, R.; Hutchinson, T. H.; Maddix, S.; Trip, H. An in vivo testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. *Environ. Toxicol. Chem.* **1999**, *18*, 337–347.
(21) Tyler, C. R.; van Aerle, R.; Nilsen, M. V.; Blackwell, R.; Maddix, S.; Nilsen, B. M.; Berg, K.; Hutchinson, T. H.; Goksoyr, A. Monoclonal antibody enzyme-linked immunosorbent assay to quantify vitellogenin for studies on environmental estrogens in the rainbow trout (*Oncorhynchus mykiss*). *Environ. Toxicol. Chem.* **2002**, *21*, 47–54.
(22) Filby, A. L.; Tyler, C. R. Molecular characterization of estrogen receptors 1, 2a, and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). *Biol. Reprod.* **2005**, *73*, 648–662.
(23) Thorpe, K. L.; Cummings, R. I.; Hutchinson, T. H.; Scholze, M.; Brighty, G.; Sumpter, J. P.; Tyler, C. R. Relative potencies and combination effects of steroid estrogens in fish. *Environ. Sci. Technol.* **2003**, *37*, 1142–1149.
(24) Chimchirian, R. F.; Suri, R. P. S.; Fu, H. Free synthetic and natural estrogen hormones in influent and effluent of three municipal wastewater treatment plants. *Water Environ. Res.* **2007**, *79*, 969–974.
(25) Cheskis, B. J.; Greger, J. G.; Nagpal, S.; Freedman, L. P. Signaling by estrogens. *J. Cell Physiol.* **2007**, *213*, 610–617.
(26) Bhavnani, B. R.; Woolever, C. A. Interaction of ring B unsaturated estrogens with estrogen receptors of human endometrium and rat uterus. *Steroids* **1991**, *56*, 201–209.
(27) Bhavnani, B. R. Estrogens and menopause: pharmacology of conjugated equine estrogens and their potential role in the prevention of neurodegenerative diseases such as Alzheimer's. *J. Steroid Biochem. Mol. Biol.* **2003**, *85*, 473–482.
(28) Sun, Y.; Gu, C.; Liu, X.; Liang, W.; Yao, P.; Bolton, J. L.; van Breemen, R. B. Ultrafiltration tandem mass spectrometry of estrogens for characterization of structure and affinity for human estrogen receptors. *J. Am. Soc. Mass Spectrom.* **2004**, *16*, 271–279.
(29) Bhavnani, B. R.; Tam, S. P.; Lu, X. F. Structure activity relationships and differential interactions and functional activity of various equine estrogens mediated via estrogen receptors ERα and ER*β*. *Endocrinology* **2008**, *149*, 4857–4870.
(30) Jobling, S.; Casey, D.; Rodgers-Gray, T.; Oehlmann, J.; Schulte-Oehlmann, U.; Pawlowski, S.; Baunbeck, T.; Turner, A. P.; Tyler, C. R. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. *Aquat. Toxicol.* **2004**, *66*, 207–222.
(31) MacKay, M. E.; Raelson, J.; Lazier, C. B. Upregulation of estrogen receptor mRNA and estrogen receptor activity by estradiol in liver of rainbow trout and other teleostean fish. *Comp. Biochem. Physiol. C* **1996**, *115*, 201–209.
(32) Chen, Y.; Shen, L.; Zhang, F.; Lau, S. S.; van Breemen, R. B.; Nikolic, D.; Bolton, J. L. The equine estrogen metabolite 4-hydroxyequilenin causes DNA single-strand breaks and oxidation of DNA bases in vitro. *Chem. Res. Toxicol.* **1998**, *11*, 1105–1111.
(33) Yilmazer, M.; Fenkci, V.; Fenkci, S.; Aktepe, O.; Sonmezer, M.; Kurtay, G. Association of serum complement (C3, C4) and immunoglobulin (IgG, IgM) levels with hormone replacement therapy in healthy postmenopausal women. *Hum. Reprod.* **2003**, *18*, 1531–1535.

ES803135Q

Genetic Interactions of the Androgen and Wnt/β-Catenin Pathways for the Masculinization of External Genitalia

Shinichi Miyagawa, Yoshihiko Satoh, Ryuma Haraguchi, Kentaro Suzuki, Taisen Iguchi, Makoto M. Taketo, Naomi Nakagata, Takahiro Matsumoto, Ken-ichi Takeyama, Shigeaki Kato, and Gen Yamada

Institute of Molecular Embryology and Genetics (S.M., K.S., G.Y.), Center for Animal Resources and Development (S.M., Y.S., R.H., K.S., N.N., G.Y.), and Global Center of Excellence Cell Fate Regulation Research and Education Unit, Kumamoto University, Kumamoto 860-0811, Japan; Okazaki Institute for Integrative Bioscience (I.T.), National Institutes of Natural Sciences, Okazaki 444-8787, Japan; Graduate School of Medicine (M.M.T), Kyoto University, Kyoto 606-8501, Japan; and Institute of Molecular and Cellular Biosciences (T.M., K.T., S.K), University of Tokyo and Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Tokyo 113-0032, Japan

In most mammals, the sexually dimorphic development of embryos is typically achieved by the differentiation of the external genitalia. Hence, the sexual distinction of mammalian newborns is based on the external genital structure. Although it was shown in the 1940s and 1950s that androgen from the testes establishes the male sexual characteristics, the involvement of nongonadal and locally produced masculine effectors remains totally unknown. It is noteworthy that the disorders of fetal masculinization, including hypospadias, one of the most frequent birth defects, occur at a high frequency. Furthermore, their causative factors remain unclear. In this study, the involvement of the coordinated actions of androgen and the growth factor systems was genetically analyzed for the first time on mammalian reproductive organ formation. The results demonstrated that the Wnt/β-catenin pathway is indispensable masculine factor for the external genital development. The bilateral mesenchymal region adjacent to the urethral plate epithelium displayed a sexually dimorphic activity of Wnt/β-catenin signaling. Loss- and gain-of-function β-catenin mutants displayed altered sexual development of the external genitalia. These results indicate the novel functions of the Wnt/β-catenin pathway as a locally expressed masculine effector. This could be the first genetic study analyzing the roles of the genetic interactions between androgen and locally expressed growth factor signaling during the development of reproductive organs. These results also shed new insight on the reproductive genetics and the causative factors of genital disorders. (***Molecular Endocrinology*** **23: 871–880, 2009)**

The male sexual characteristics of embryos are generally established by androgens, which depend on testis development. Such hormonal control of sexual development has been shown since the beginning of the 1900s (1). However, the involvement of nongonadal and locally produced masculine factors in the sexually developing organs has not yet been elucidated. Sexual differentiation is a remarkably complex process, which depends on the orchestration of the signaling network. The presence of putative effectors that can potentially interact with hormonal signaling or can function parallel with the hormonal pathway remains completely unknown.

Disorders of sexual development are among the most common human birth defects. Of those, hypospadias is the most frequent malformation in which the urethral meatus is located at the ventral (lower) side of the penis. Males with severe hypospadias are born with ambiguous genitalia. The prevalence of hypospadias is increasing, occurring in 0.4–1% of boys at birth (2–4). Although any defects in the pathways along with androgen signaling can cause feminization and urogenital defects (5–9), the etiology of such disorders is obscure in most cases. Therefore, a better understanding of the principles of sexually dimorphic development will shed light on the causative mechanisms of the genital malformations.

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in U.S.A.

doi: 10.1210/me.2008-0478 Received December 24, 2008. Accepted March 3, 2009.
First Published Online March 12, 2009

Abbreviations: AR, Androgen receptor; E, embryonic day; GT, genital tubercle; KO, knockout; TM, tamoxifen; UPE, urethral plate epithelium.

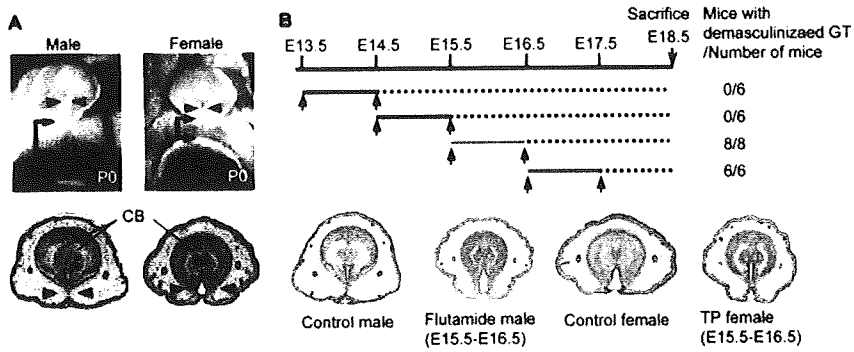


FIG. 1. The time window for external genital masculinization. A, Sexual dimorphisms of the external genitalia in newborn mice (postnatal d 0). In males, the fusion of the urethral folds in the ventral midline results in canalization of the urethral epithelium (*arrowheads*) and bilateral fusion of the prepuce. The prospective corporal body (CB) condenses and bilaterally separates in the male but not in the female GTs. In the male, the anogenital distance, the distance from the external genitalia to the anus, is longer than that in the female (*arrows*). *, Urethral meatus. B, The timeline for flutamide treatment (*upper panel*). The GTs of all male embryos treated with flutamide on E15.5–16.5 and E16.5–17.5 (*red lines*) are demasculinized, displaying a morphology similar to those of the female GTs. The representative demasculinized and masculinized GTs and the GT of control mice with the vehicles are shown.

The development of the fetal external genitalia is divided into two processes. The first phase corresponds to the initial outgrowth and early patterning of the genital primordia. The external genitalia of both sexes are derived from a common undifferentiated anlage, the genital tubercle (GT). In mice, it develops from the cloacal region starting at embryonic day (E) 10.5. The after GT outgrowth is a consequence of mesenchymal swelling around the cloaca, which is accompanied by the formation of the urethral plate epithelium (UPE), the future embryonic urethral epithelium, in the ventral midline. Androgen pathway-independent severe hypospadias with groove-like defects can therefore be affected at this phase of development (10, 11).

The subsequent second phase is a hormonally regulated process. As an embryo develops, the prepuce elevates from the proximal to the distal region of the GT, which eventually engulfs the GT. The preputial elevation is accompanied by the thickening of the preputial mesenchyme and the enlarged future glans region. After the development of the prepuce, the urethral folds fuse in the midline of the GT, which eventually develops the tubular urethra (12). The mesenchyme in the glans gradually condenses and develops the various mesenchymal derivatives, such as corporal tissues and penile bones. On the other hand, the female external genitalia display few such characteristic processes. Hence, morphological sexual dimorphism is apparent at birth with larger external genitalia, a well-developed prepuce, tubular urethra, and the condensation of a bilaterally segmented prospective corporal body in males (Fig. 1A).

Sexual differentiation of the male external genitalia is under the influence of androgen. Fetal androgen production begins shortly after Leydig cell differentiation at around E13 in the mouse and reaches a peak shortly before birth (13). However, the timing of sexually dimorphic development has not yet been fully characterized. In mice, the obvious morphological sexual difference of the GTs appears at E16.5 (14). On the other hand, a recent study indicated that the androgen action commences several days earlier than the timing of the morphological differentiation in rat embryos (15). Not only the timing but also the spatially regulated androgen signaling for the masculinization remain poorly understood. Previous tissue-recombination experiments indicated that the GT develops through epithelial-mesenchymal interactions (16, 17). However, the contribution of mesenchymally or epithelially expressed androgen receptor (AR) in the masculinization processes of the external genitalia is still not known.

Various mutant studies have shown that the initial GT outgrowth and patterning requires the coordinated output of several growth factors, including Hedgehog (Hh), Wnt, bone morphogenetic protein (Bmp), and fibroblast growth factor (Fgf) (18–24). However, virtually no studies have focused on these growth factor cascades during masculinization. This is partly because the mutants for such genes exhibit drastic defects often with lethal phenotypes before the masculinization processes. Recently, loss-of-function mutants for β-catenin display very early developmental arrest of the GTs (25). However, the role of canonical Wnt/β-catenin, with regard to the GT masculinization processes remains totally unknown. Wnt/β-catenin signaling also regulates the growth and development of the prostate around the perinatal stage, implying a possibility that they can affect urogenital organ development in later sexually dimorphic organogenesis (26). Wnt/β-catenin signaling is therefore an attractive candidate growth factor system to be involved in sexually dimorphic GT development.

Androgen has been thought to play a central role in such a network, but it is not even clear whether androgen is necessary or sufficient for male external genital development. Furthermore, the downstream pathway of androgen signaling has not been elucidated at all. The current study examined the contribution of growth factor signaling in the external genital development, especially focusing on the Wnt/β-catenin signaling. To this end, molecular genetic analyses using the spatially and temporally regulated inducible expression of Cre recombinase and several mice with the loxP-flanked gene alleles were applied. First, the critical time window of external genital masculinization was identified, and the indispensable function of mesenchymally expressed AR was clarified. Intriguingly, the degree of Wnt/β-catenin signaling activity was higher in the male GTs than that of the female GTs. Loss- and gain-of-function mutant studies revealed the function of Wnt/β-catenin signaling as a masculine effector in the GTs. In fact, the current study showed that excessive Wnt/β-catenin activity could yield adult male-type external genitalia in the female background. This study therefore indicates that Wnt/β-catenin signal is an essential masculine effector for the external genitalia. Because external genital development is a typical and sensitive organogenesis displaying embryonic sexual dimorphisms, this study could be the first