

Table 5

Ovulation and reproductive organ weights at the terminal necropsy on postnatal week 32-33 (Exp. 1) or 22-23 (Exps. 2-4) in the animals treated with EE during the neonatal period.

Exp. 1 Single subcutaneous treatment on postnatal day 1

Dose of EE ($\mu\text{g}/\text{kg}$)	0	0.08	0.4	2
Animals examined	8	7	8	8
Animals on the day of estrus ^a	6	7	8	8
Animals found oocytes	5	1	0	1
Body weight (g)	377 \pm 21	409		453
Number of oocytes shed	13.2 \pm 1.2	1		13
Ovaries (mg)	94 \pm 6	113		137
Uterus (mg)	630 \pm 37	614		636
Animals found no oocytes	3	6	8	7
Body weight (g)	425 \pm 34	397 \pm 24	422 \pm 21	411 \pm 24
Ovaries (mg)	82 \pm 10	59 \pm 7	61 \pm 6	61 \pm 7
Uterus (mg)	606 \pm 77	780 \pm 54	664 \pm 47	700 \pm 54

^a Including animals showing persistent estrus

Exp. 2 Repeated oral administration from postnatal day 1 to 5

EE ($\mu\text{g}/\text{kg}$)	0 \times 5	0.4 \times 5	2 \times 5
N of animals examined	10	9	10
Animals found fresh oocytes	6	0	0
Ovaries (mg)	80.2 \pm 10.6		---
Uterus (mg)	621 \pm 35		---
N of oocytes	12.7 \pm 1.0		---
Ovaries (mg)	91.1 \pm 6.3	---	---
Uterus (mg)	463 \pm 33	---	---
Animals found no oocyte	4	9	10
Ovaries (mg)	74.8 \pm 0.7	59.2 \pm 0.7	45.0 \pm 0.3**
Uterus (mg)	617 \pm 28	584 \pm 31	465 \pm 38*

Table 5 (continued)

Exp. 3 Oral administration on postnatal day 1

EE ($\mu\text{g}/\text{kg}$)	0	10	20
N of animals examined	13	13	13

Animals found fresh oocytes	2	1	0
Ovaries (mg)	83.7	120	---
Uterus (mg)	664	322	---
N of oocytes	13.5	11	---
Animals found old oocytes	4	0	0
Ovaries (mg)	91.1 ± 6.3	---	---
Uterus (mg)	463 ± 33	---	---
Animals found no oocyte	7	12	13
Ovaries (mg)	69.0 ± 6.4	55.1 ± 1.5	61.1 ± 5.5
Uterus (mg)	464 ± 53	520 ± 15	503 ± 10

Values indicate mean ± SEM.

Table 5 (continued)

Exp. 4 Oral administration on postnatal day 1, 5 or 7

Administration of EE	Ovulated /N	Body weight (g)	Ovaries (mg)	Uterus (mg)	Oocytes in the ampullae
PND 1					
0 µg/kg	3/4	351 ± 10	83.7 ± 2.6	500 ± 73	13.0 ± 1.0
10 µg/kg	0/4	---	---	---	---
PND 5					
0 µg/kg	7/9	363 ± 13	81.6 ± 4.8	507 ± 15	13.4 ± 0.7
10 µg/kg	3/9	336 ± 16	72.7 ± 1.3	477 ± 51	13.3 ± 0.9
PND 7					
10 µg/kg	0/4	---	---	---	---
Administration of EE	Not ovulated /N	Body weight (g)	Ovaries (mg)	Uterus (mg)	
PND 1					
0 µg/kg	1/4	368	47	560	
10 µg/kg	4/4	368 ± 41	55.0 ± 6.9	497 ± 52	
PND 5					
0 µg/kg	2/9	351	74	512	
10 µg/kg	6/9	355 ± 10	54.3 ± 4.1	517 ± 19	
PND 7					
10 µg/kg	4/4	369 ± 18	85.0 ± 16.5	448 ± 44	

Table 6

Organ weights at the terminal necropsy on postnatal week 32-33 postnatal week or on postnatal week 22-23 in the animals treated with EE during the neonatal period

Exp. 1 Subcutaneous injection on postnatal day 1

Dose of EE ($\mu\text{g}/\text{kg}$)	0	0.08	0.4	2
Number of animals examined	8	7	8	8
BW at necropsy (g)	395 \pm 19	399 \pm 20	422 \pm 19	414 \pm 19
Pituitary	19 \pm 2	22 \pm 2	26 \pm 2	24 \pm 2
Thymus (mg)	148 \pm 13	145 \pm 14	133 \pm 13	161 \pm 13
Liver (g)	11.9 \pm 0.6	12.1 \pm 0.7	13.2 \pm 0.6	12.7 \pm 0.6
Kidneys (g)	2.2 \pm 0.1	2.2 \pm 0.1	2.3 \pm 0.1	2.4 \pm 0.1
Spleen (mg)	611 \pm 27	636 \pm 29	633 \pm 27	641 \pm 27
Adrenal glands (mg)	72 \pm 4	80 \pm 5	82 \pm 4	84 \pm 4

Exp. 2 Repeated oral administration from postnatal day 1 to 5

EE ($\mu\text{g}/\text{kg}$)	0 \times 5	0.4 \times 5	2 \times 5
N of animals	10	9	10
Body weight (g)	354 \pm 13	363 \pm 16	379 \pm 14
Pituitary (mg)	15.2 \pm 1.0	22.7 \pm 1.9*	22.2 \pm 2.7*
Liver (g)	11.1 \pm 0.3	12.4 \pm 0.5	13.6 \pm 0.5**
Kidneys (g)	2.1 \pm 0.1	2.2 \pm 0.1	2.4 \pm 0.1
Adrenal glands (mg)	75.3 \pm 2.6	79.0 \pm 4.6	82.7 \pm 4.7

Exp. 3 Repeated oral administration from postnatal day 1 to 5

EE ($\mu\text{g}/\text{kg}$)	0	10	20
N of animals	13	13	13
Body weight (g)	370 \pm 10	366 \pm 9	343 \pm 9
Pituitary (mg)	18.0 \pm 1.1	22.8 \pm 1.2*	22.0 \pm 1.3*
Liver (g)	11.4 \pm 0.5	12.5 \pm 0.4	11.6 \pm 0.3
Adrenal glands (mg)	71.9 \pm 3.2	85.6 \pm 3.5*	90.1 \pm 3.6**

Adrenal glands were weighed for 10, 11 and 11 in the 0, 10 or 20 $\mu\text{g}/\text{kg}$ of EE treated group, respectively.

Table 6 (continued)

Exp. 4 Oral administration on postnatal day 1, 5 or 7

Administration of EE	N	Body weight (g)	Pituitary (mg)	Liver (g)	Adrenal glands (mg)
PND 1					
0 µg/kg	4	355 ± 8	18.1 ± 3.7	4.4 ± 0.5	64.3 ± 7.1
10 µg/kg	4	368 ± 41	23.0 ± 4.4	3.2 ± 2.5	81.0 ± 4.4
PND 5					
0 µg/kg	9	360 ± 10	18.3 ± 4.8	4.1 ± 0.8	69.1 ± 2.7
10 µg/kg	9	348 ± 7	20.5 ± 4.1	3.9 ± 1.4	71.1 ± 3.9
PND 7					
10 µg/kg	4	369 ± 18	23.5 ± 13.4	5.0 ± 1.4	64.0 ± 3.4

Values indicate mean ± SEM.

* and ** Significantly different from control at $p < 0.05$ and $p < 0.01$, respectively.

Table 7

Histological findings of ovaries collected at the terminal necropsy on postnatal week 32-33 (Exp. 1) or 22-23 (Exps. 2, 3) in the animals treated with EE during the neonatal period.

Exp. 1 Single subcutaneous treatment on postnatal day 1

Dose of EE (µg/kg)	0	0.08	0.4	2
Animals examined	8	7	8	8
Absence of corpus luteum	1	3	5	4
Presence of cystic follicles	2	5	5	7

Exp. 2 Repeated oral administration from postnatal day 1 to 5

Dose of EE (µg/kg)	0×5	0.4×5	2×5
Animals examined	10	8	10
Absence of corpus luteum	2	7	10
Presence of cystic follicles	5	9	10

Exp. 3 Exp. 3 Repeated oral administration from postnatal day 1 to 5

EE (µg/kg)	0	10
N of animals examined	5	6
No corpus luteum	0	3
Formation of cystic follicles	0	6

Table 8

Cell densities of adrenal glands collected at the terminal necropsy on postnatal week 22-23 from the animals treated orally with 0, 10 or 20 µg/kg of EE on postnatal day 1.

Values indicate mean ± SEM.

EE ($\mu\text{g}/\text{kg}$)	0	10	20
N of animals examined	3	4	4
N of cells/50mm ² zona fasciculata	140 \pm 19	102 \pm 9	91 \pm 6*
N of cells/50mm ² zona fasciculata	315 \pm 22	290 \pm 31	197 \pm 22*

* Significantly different from control at $p < 0.05$.

Table 9

Incidences of macroscopic abnormality of the mammary gland at the terminal necropsy on postnatal week 32-33 (Exp. 1) or 22-23 (Exps. 2-4) in the animals treated with EE during the neonatal period

Exp. 1 Single subcutaneous treatment on postnatal day 1

Exp. 2 Exp. 2 Repeated oral administration from postnatal day 1 to 5

EE ($\mu\text{g}/\text{kg}$)	0 \times 5	0.4 \times 5	2 \times 5	
Dose of EE ($\mu\text{g}/\text{kg}$)	0	0.08	0.4	2
N of animals examined	8	8	8	8
N of animals with a white spot	0	3	4	3
N of animals examined	10	10	10	
N of animals with a white spot	0	2	6	
Exp. 3 Repeated oral administration from postnatal day 1 to 5				
EE ($\mu\text{g}/\text{kg}$)	0	10	20	
N of animals examined	13	13	13	
N of animals with a white spot	0	0	1	

Table 9 (continued)

Exp. 4 Oral administration on postnatal day 1, 5 or 7

Administration of EE	N	N of animals with a white spot
PND 1 0 $\mu\text{g}/\text{kg}$	4	0
10 $\mu\text{g}/\text{kg}$	4	0
PND 5 0 $\mu\text{g}/\text{kg}$	9	1
10 $\mu\text{g}/\text{kg}$	9	1
PND 7 10 $\mu\text{g}/\text{kg}$	4	1

Table 10

Ovulation and organ weights on the day of vaginal opening (VO) in the animals

treated orally with EE during the neonatal period

Exp. 2 Repeated oral administration from postnatal day 1 to 5

EE ($\mu\text{g}/\text{kg}$)	0	0.4	2
N of animals	12	8	11
Age at V0	33.2 \pm 0.3	30.4 \pm 0.3	32.2 \pm 0.5
Body weight at V0	137 \pm 3	122 \pm 3	129 \pm 4
N of animals ovulated	12	7	5
N of oocytes in the ovulated animals	12.8 \pm 0.4	12.0 \pm 1.0	8.8 \pm 2.3*
Ovarian weight (mg)	42 \pm 2.8	46 \pm 2.8	29 \pm 2.8**
Uterus weight (mg)	187 \pm 12	166 \pm 15	149 \pm 18
Pituitary weight (mg)	6.7 \pm 0.3	6.5 \pm 0.3	6.1 \pm 0.4
Adrenal weight (mg)	30.5 \pm 0.1	28.1 \pm 0.1	32.5 \pm 0.1

Table 10 (continued)

Exp. 3 Oral administration on postnatal day 1

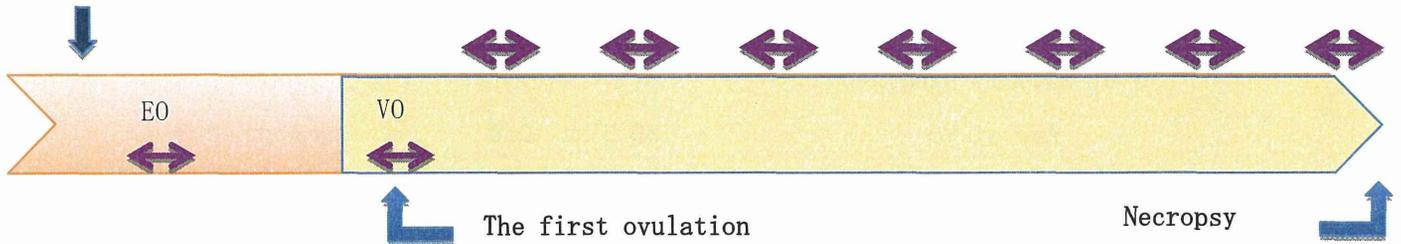
EE ($\mu\text{g}/\text{kg}$)	0	10
N of animals examined	6	7
Age at V0	30.3 \pm 0.4	29.1 \pm 0.3
Body weight at V0	114 \pm 14	106 \pm 8
N of animals ovulated	6	5
N of oocytes in the ovulated animals	12.2 \pm 1.9	12.2 \pm 2.2
Ovarian weight (mg)	35.1 \pm 2.8	31.5 \pm 2.8*
Uterus weight (mg)	180 \pm 35	143 \pm 19
Pituitary weight (mg)	5.7 \pm 0.7	5.6 \pm 0.5
Adrenal weight (mg)	28.8 \pm 2.5	25.7 \pm 2.4

Values indicate mean \pm SEM.

* and **, Significantly different from control at $p < 0.05$ and $p < 0.01$, respectively.

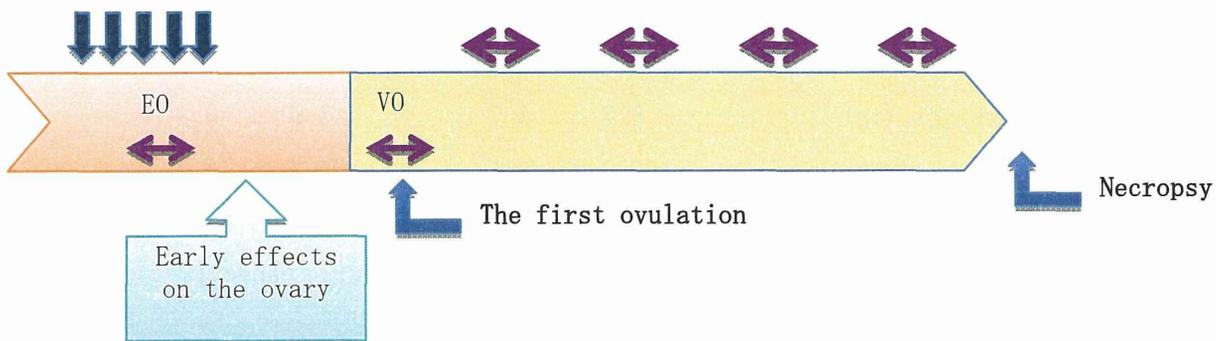
Exp. 1

Single subcutaneous treatment with 0, 0.08, 0.4 or 2.0 µg/kg



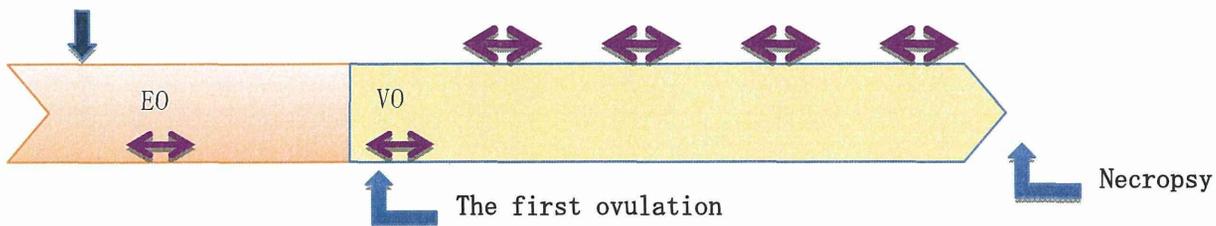
Exp. 2

Repeated oral treatment with 0, 0.4 or 2.0 µg/kg from PND 1 to 5



Exp. 3

Single oral treatment with 0, 10 or 20 µg/kg on PND 1



Exp. 4

Single oral treatment with 0 or 10 µg/kg on PND 1, 5 or 7

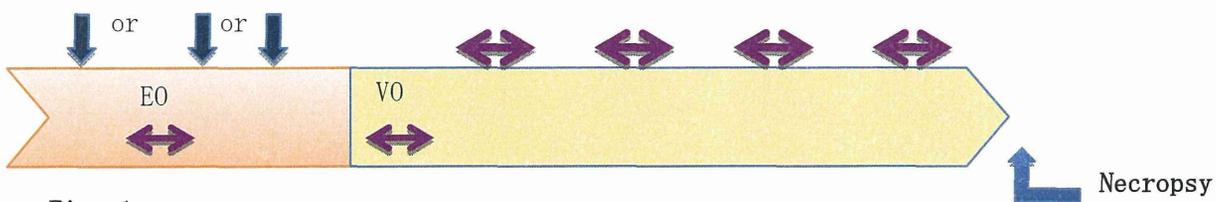


Fig. 1

Schematic representation of the experimental designs of the studies on the effects of neonatal exposure to EE

Treated animals were weaned on postnatal day (PND 21).

Every two weeks of estrous cycle monitoring from postnatal week (PNW) 8 to 33 in the Exp. 1 and to 21 in the Exps. 2-4

EO, eyelid opening; VO, vaginal opening

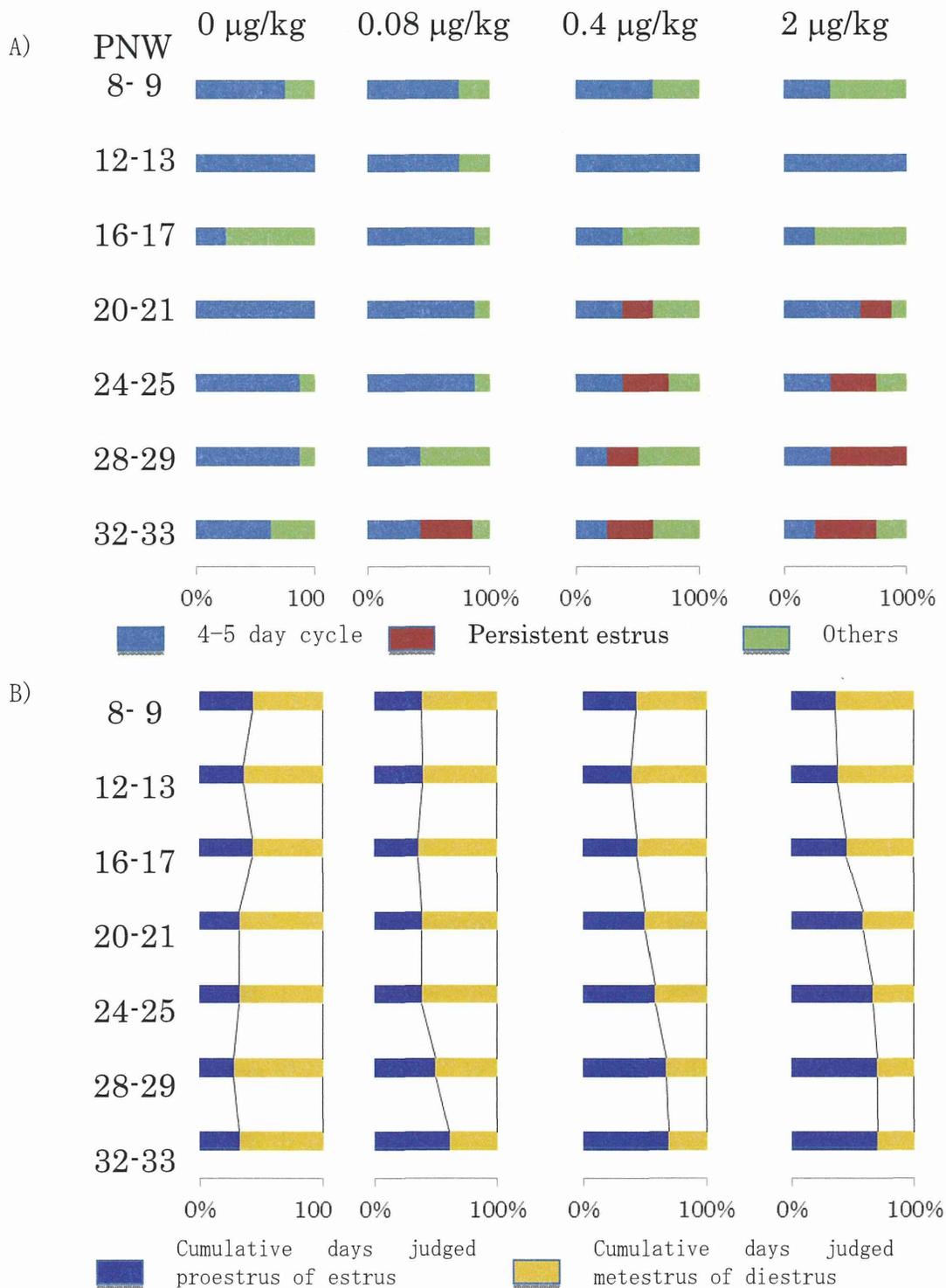
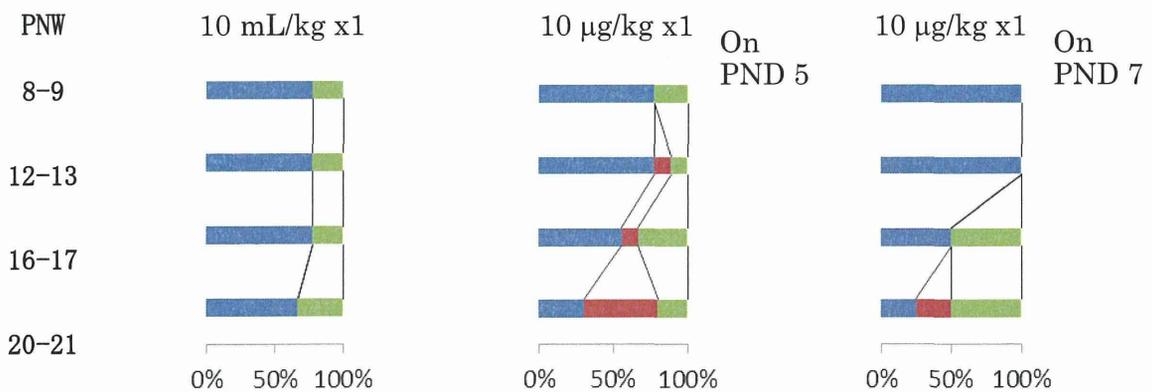
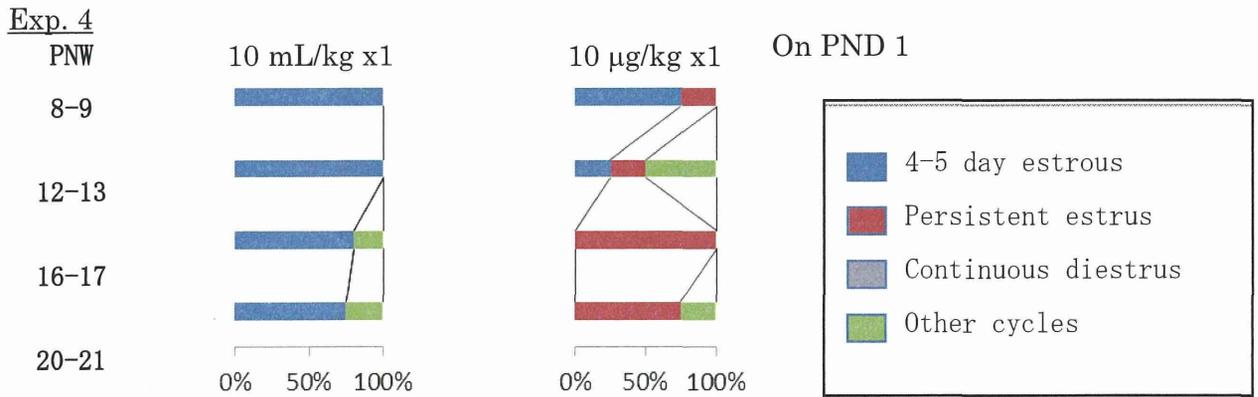
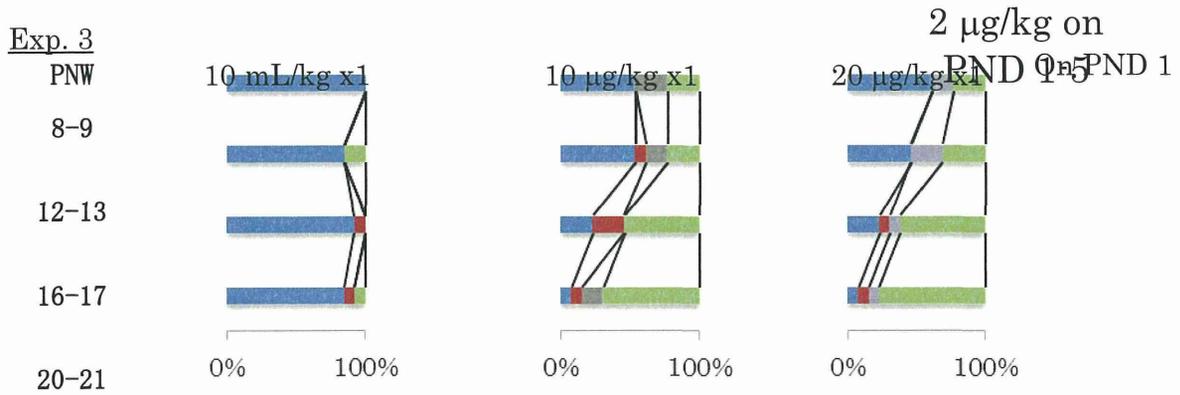
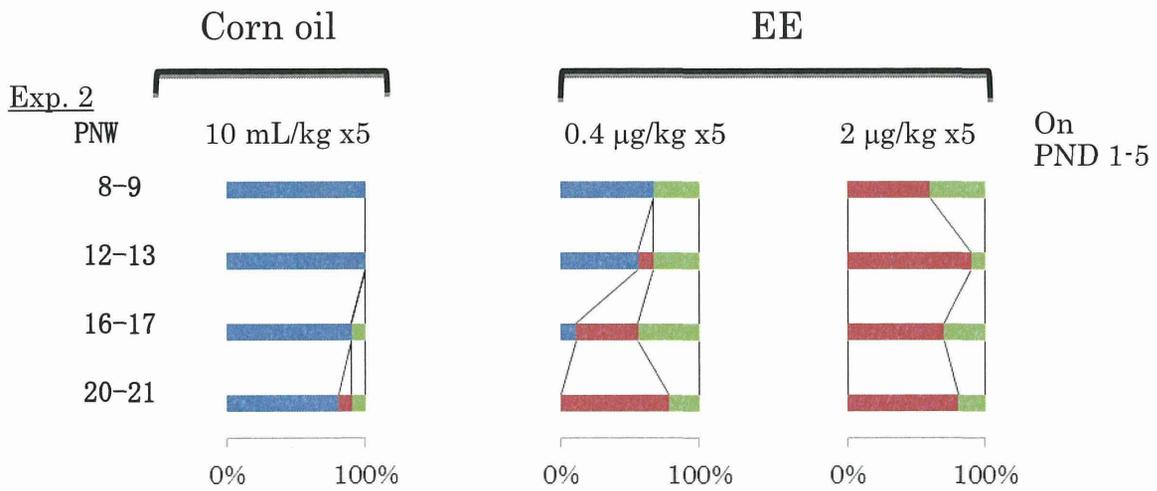


Fig. 2

Estrous cycle of female rats treated subcutaneously with 0 (corn oil), 0.08, 0.4 or 2 $\mu\text{g}/\text{kg}$ of EE on postnatal day 1. Each bar in panel A indicates ratio of animals showing 4-5 day cycle, persistent estrus, continuous diestrus or the other types. Each bar in panel B indicates ratio of cumulative days judged proestrus or estrus and those judged metestrus or diestrus in each observation period.



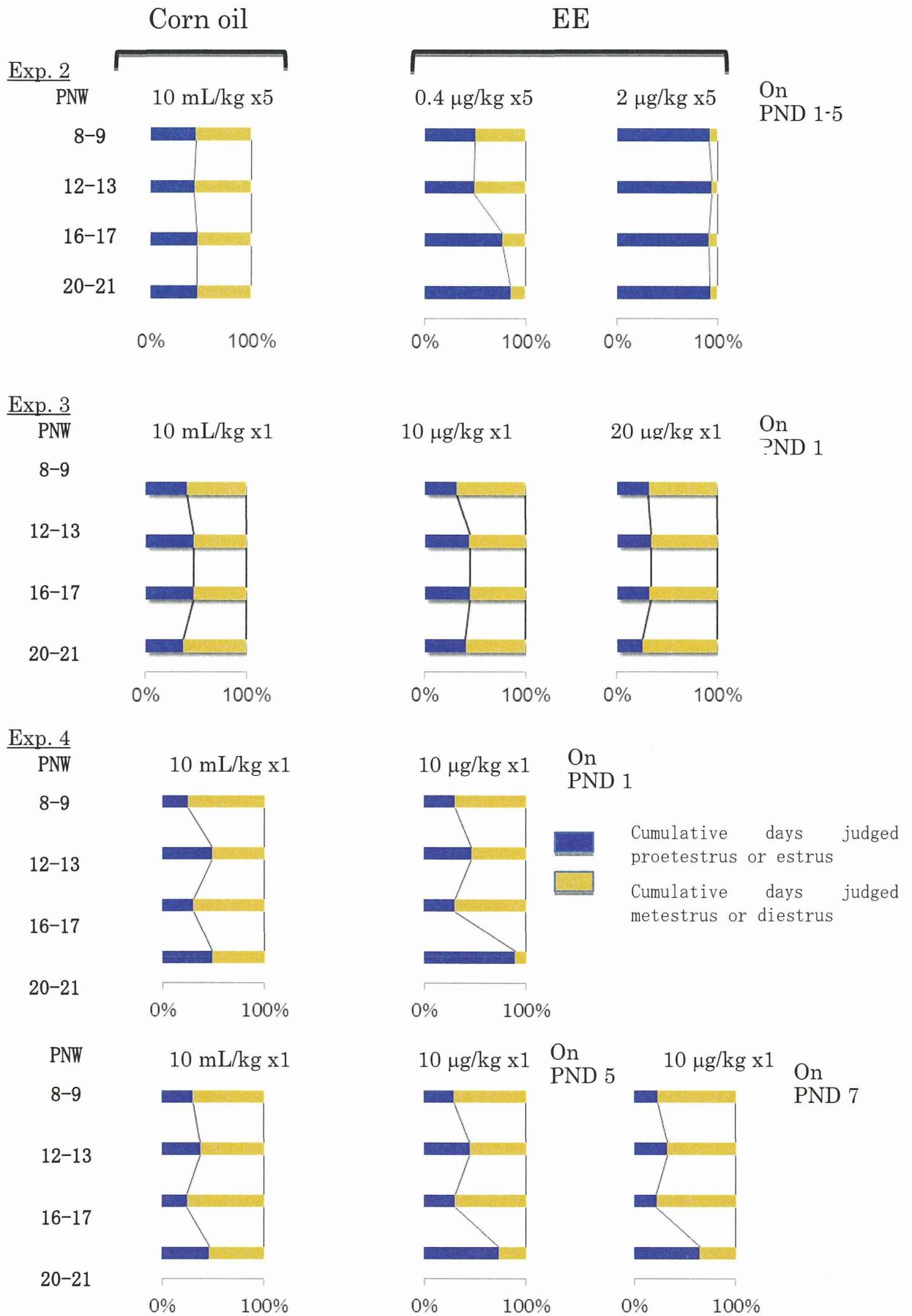


Fig. 4

Fig. 3

Estrous cycle of female rats treated orally with 0 (corn oil), 0.4, 2, 10 or 20 $\mu\text{g}/\text{kg}$ of EE at different timing during the neonatal period. In the Exp. 2, the animals treated for 5 days from postnatal day 1 with 0 (corn oil), 0.4 or 2 $\mu\text{g}/\text{kg}$ of EE. In the Exp. 3, the animals treated with 0 (corn oil), 10 or 20 $\mu\text{g}/\text{kg}$ of EE on postnatal day 1. In the Exp. 4, the animals treated with 0 (corn oil) or 10 $\mu\text{g}/\text{kg}$ of EE on postnatal day 1, 5 or 7. Each bar indicates ratio of animals showing 4-5 day cycle, persistent estrus, continuous diestrus or the other types. Each bar in panel B indicates ratio of cumulative days judged proestrus or estrus and those judged metestrus or diestrus in each observation period.

Fig. 4

Estrous cycle of female rats treated orally with 0 (corn oil), 0.4, 2, 10 or 20 $\mu\text{g}/\text{kg}$ of EE at different timing during the neonatal period. In the Exp. 2, the animals treated for 5 days from postnatal day 1 with 0 (corn oil), 0.4 or 2 $\mu\text{g}/\text{kg}$ of EE. In the Exp. 3, the animals treated with 0 (corn oil), 10 or 20 $\mu\text{g}/\text{kg}$ of EE on postnatal day 1. In the Exp. 4, the animals treated with 0 (corn oil) or 10 $\mu\text{g}/\text{kg}$ of EE on postnatal day 1, 5 or 7. Each bar indicates ratio of cumulative days judged proestrus or estrus and those judged metestrus or diestrus in each observation period.

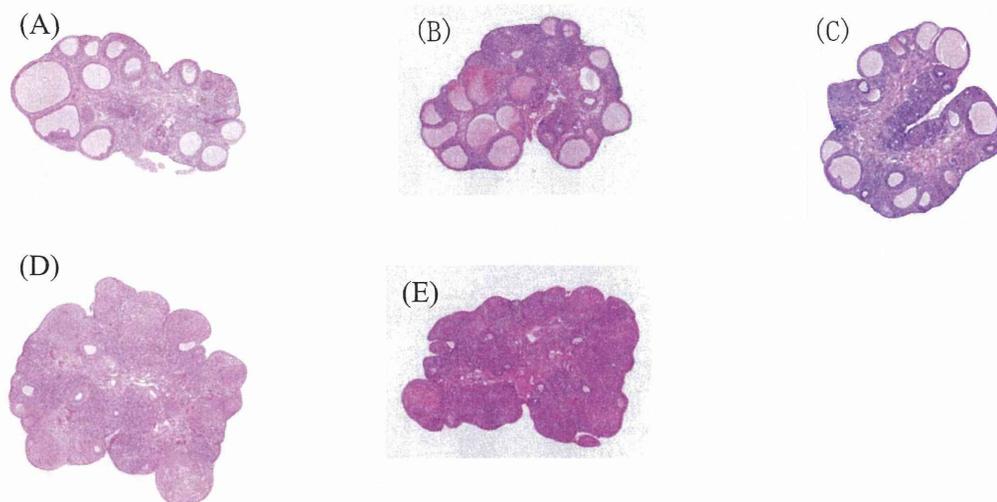


Fig. 5

Representative histology of ovaries collected at postnatal week 32-33 (A, D) or postnatal week 22-23 (B, D, E) from animals treated with EE (A, B, C) or corn oil (D, E) on postnatal day 1 subcutaneously (A, D), on postnatal day 1 to 5 orally (B, E) or on postnatal day 1 orally (C).

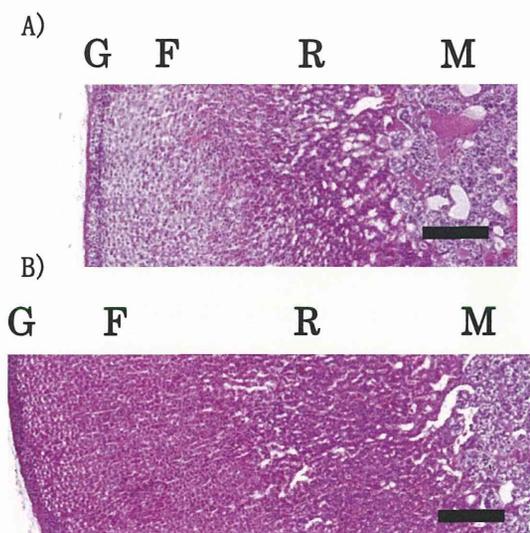


Fig. 6

Representative histology of adrenal glands collected at postnatal week 22-23 from animals treated orally with 0 (A) or 20 $\mu\text{g}/\text{kg}$ (B) of EE on postnatal day 1.

G, zona glomerulosa; F, zona fasciculata; R, zona reticularis; M, medulla. Hematoxyline and eosine staining; Bars indicate 200 μm .

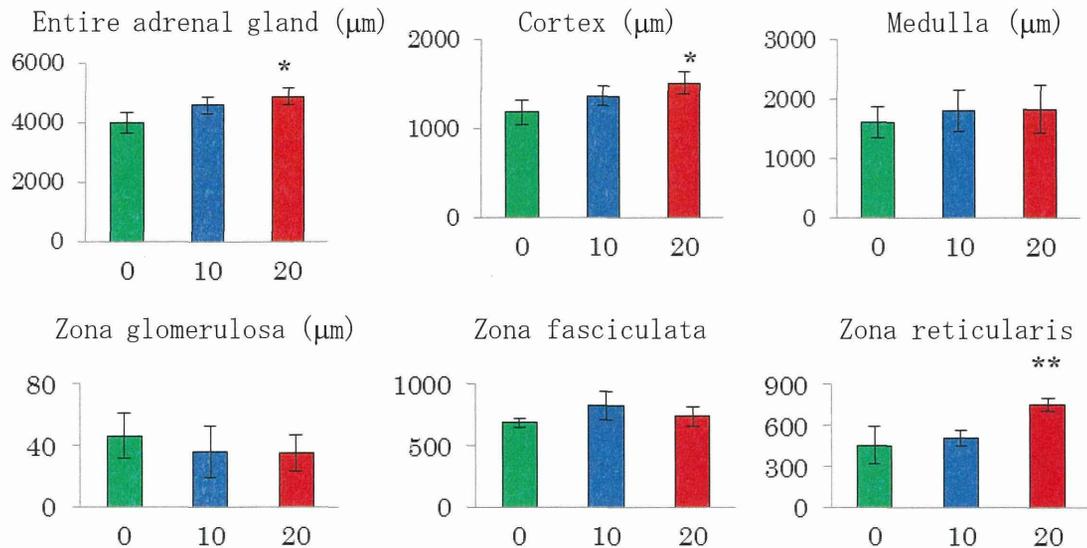


Fig. 7

Lengths of the entire and each part of adrenal glands collected at postnatal week 22-23 from the animals treated orally with 0, 10 or 20 µg/kg of EE on postnatal day 1. The lengths represent average for those of the longest and the shortest axes. Each column represents mean value for average length of the longest and the shortest axes from 3, 5 or 5 animals in the 0, 10 or 20 µg/kg treated group, respectively. Bars indicate standard deviation. * $p < 0.05$, ** $p < 0.01$

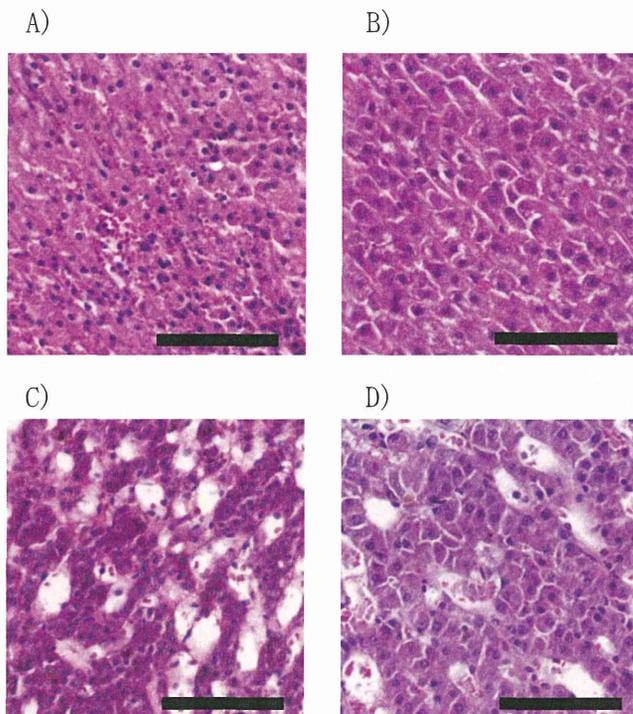


Fig. 8

Representative photographs of histology in zona fasciculata (A and C) and zona reticularis (B and D) of adrenal glands collected at postnatal week 22-23 from the animals treated orally with 0 (A and B) or 20 µg/kg (C and D) of EE on postnatal day 1. Bars indicate 100 µm.

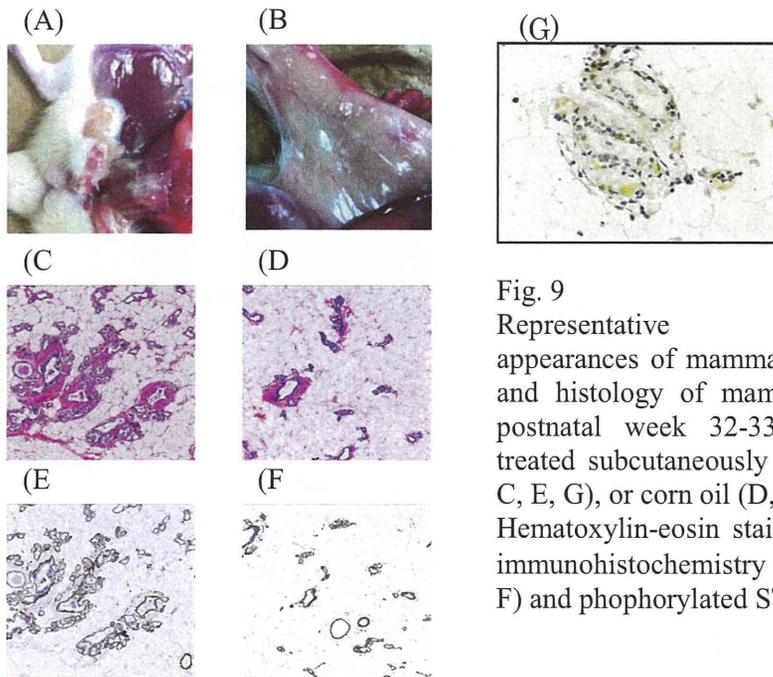


Fig. 9
 Representative macroscopic appearances of mammary gland (A, B) and histology of mammary glands at postnatal week 32-33 from animals treated subcutaneously with EE (A, B, C, E, G), or corn oil (D, F). Hematoxylin-eosin staining (C, D) and immunohistochemistry for α -SMA (E, F) and phosphorylated STAT-5 (G).

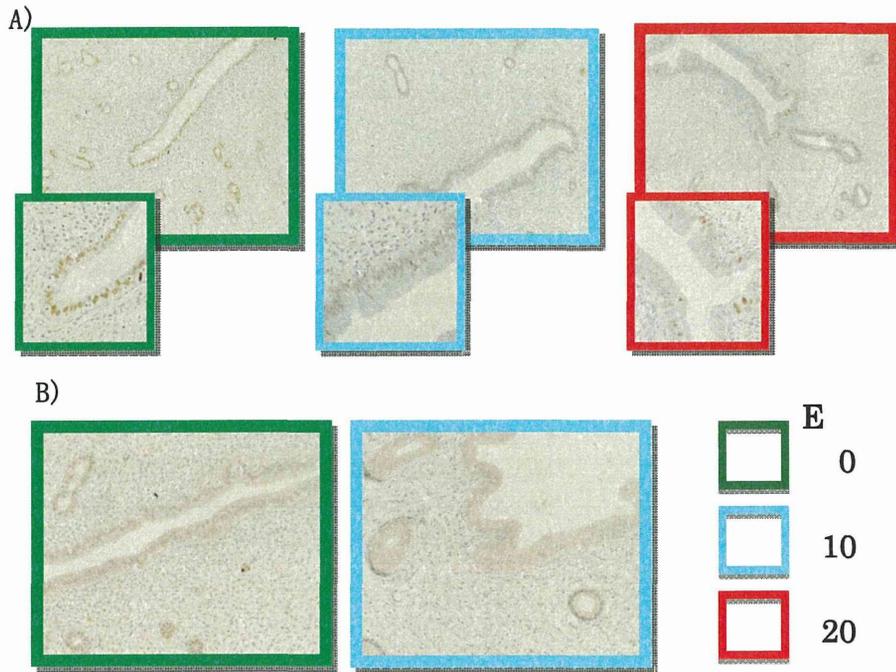


Fig. 10

Immunohistochemical localizations of PCNA (A) and estrogen α receptor (B) in the uterine tissue collected at postnatal week 22-23

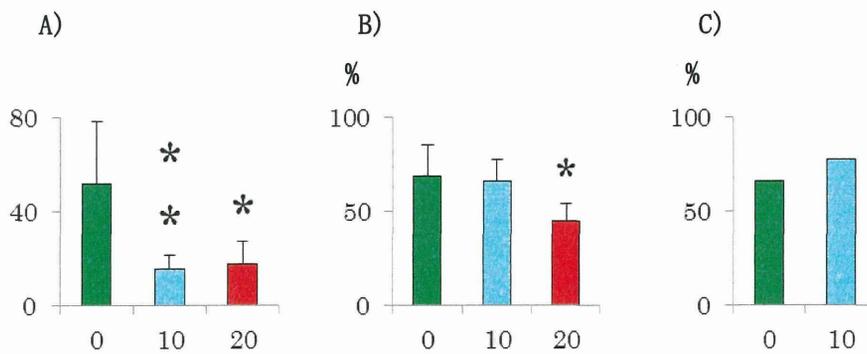


Fig. 11

Number uterine glands in a uterine section (A) and ratios of PCNA-positive (B) and estrogen α receptor-positive (C) cells to the luminal epithelial cells in the uterine tissue collected at postnatal week 22-23 from animals treated orally with 0, 10 or 20 $\mu\text{g/kg}$ of EE on postnatal day 1.

* $p < 0.05$, ** $p < 0.01$

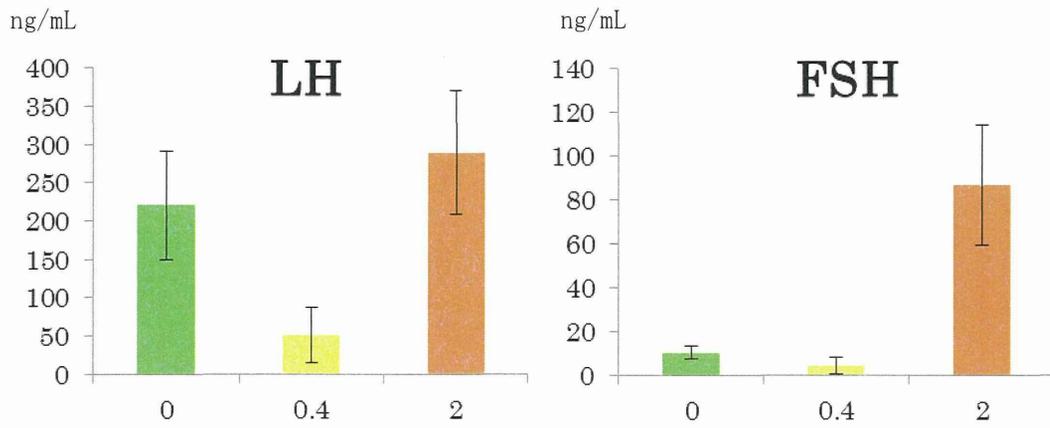


Fig. 12

Serum concentrations of LH and FSH at postnatal week 22-23 in the animals treated orally with 0, 0.4 or 2 µg/kg/day of EE from postnatal day 1 to 5.

Vertical bars represent S.E.M.

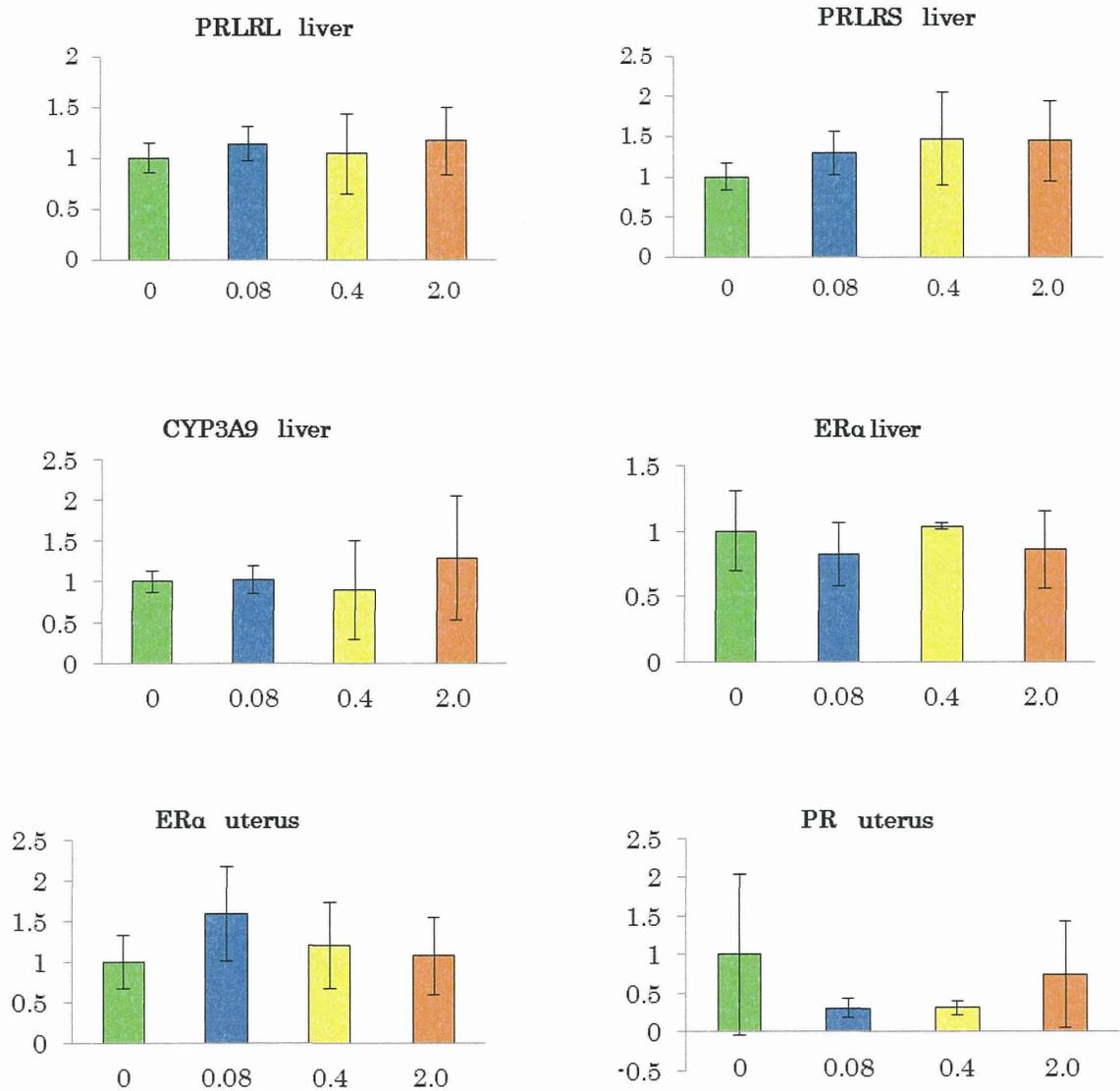


Fig. 13

Relative expressions of mRNAs encoding long form prolactin receptor (PRLRL), short form prolactin receptor (PRLRS), cytochrome P450 (CYP) 3A9, estrogen α receptor (ER α) and progesterone receptor (PR) in the liver or uterus at postnatal week 32-33 from the animals treated subcutaneously with 0, 0.08, 0.4 or 2 $\mu\text{g}/\text{kg}$ of EE on postnatal day 1.

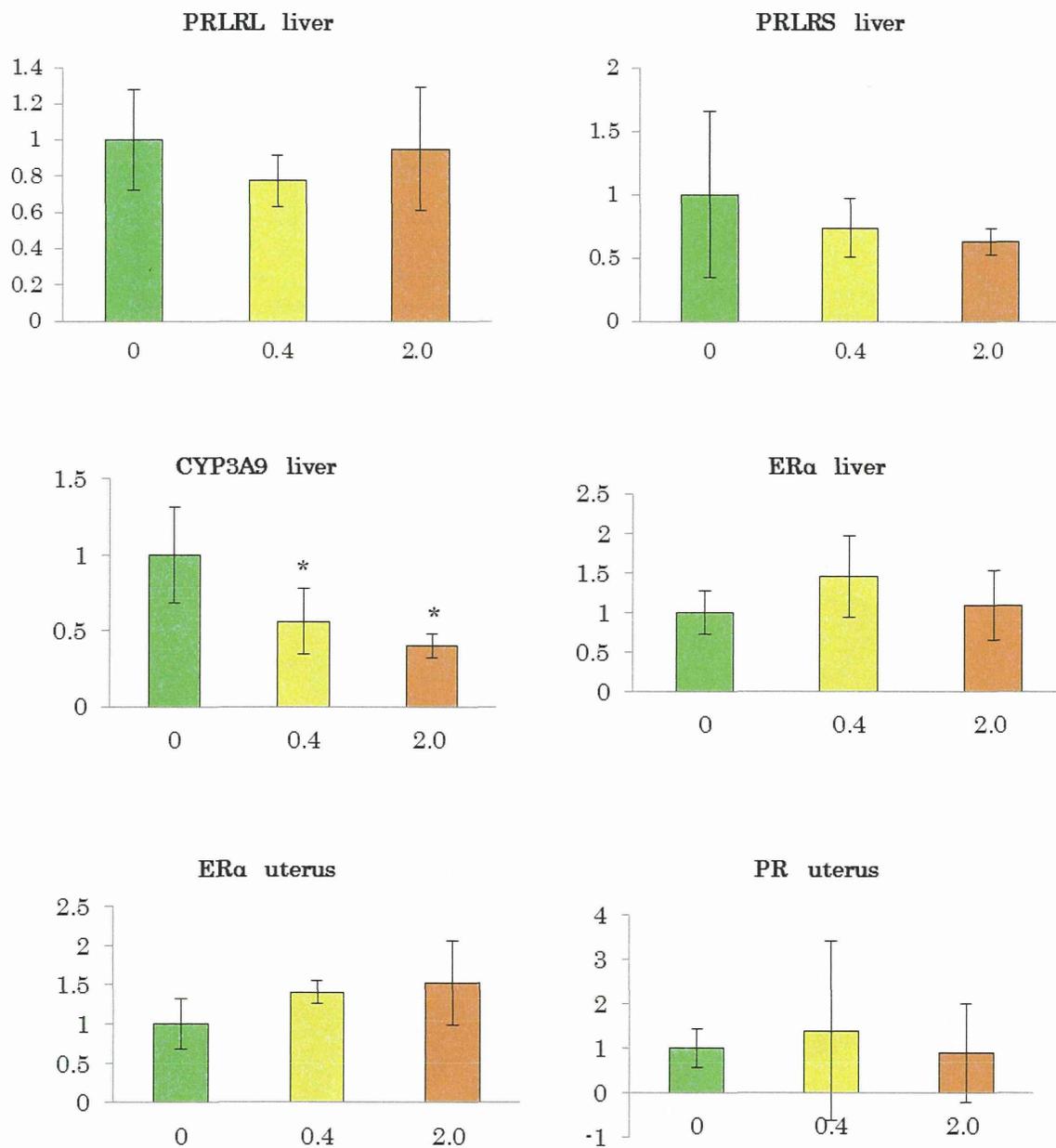


Fig. 14

Relative expressions of mRNAs encoding long form prolactin receptor (PRLRL), short form prolactin receptor (PRLRS), cytochrome P450 (CYP) 3A9, estrogen α receptor ($ER\alpha$) and progesterone receptor (PR) in the liver or uterus at postnatal week 22-23 from the animals treated orally with 0, 0.4 or 2 $\mu\text{g}/\text{kg}$ of EE from postnatal day 1 to 5.