The vonomeronal organ in rodents is an important social and sexual signaling pathway. We have investigated whether the housing of intact immature females in close proximity to mature males would interfere with the sensitivity of the immature rodent uterotrophic assay as the result of vonomeronal signals transmitted by male urinary proteins. The hypothesis was that the proximity of males might induce early puberty, thereby increasing mean uterine weight and reducing the responsiveness of the assay. The hypothesis was tested in both rats and mice by housing mature males above immature females, separated only by a wire screen, for 3 days and determining possible changes in uterine weight. The results were negative. Neither the mean uterine weight nor the group mean standard deviation of the uterine weights were changed in the uterotrophic assay. Given that the timing of sexual maturation may vary with the strain of mouse used, we also evaluated the sensitivity of the immature mouse uterotrophic assay to diethylstilbestrol (DES) using four strains of mice. Similar sensitivity was observed for the CD-1, C57Bl6, and Alpk strains, but B6CBF1 mice were marginally less sensitive to DES than were the other strains. These findings add to earlier data indicating the robustness of the rodent uterotrophic assay protocol. Key words: puberty, sexual development, strain differences, uterine weight, uterotrophic assay, vonomeronal.

Materials and Methods

Chematics. DES (> 99% pure) and arachis oil were obtained from Sigma Chemicals (Poole, Dorset, UK).

Animals. Alpk (Alpk:ApfSD, Wistar-derived) rats and Alpk (Alpk:AP, CD-1, Swiss-derived) and B6CBF1 (C57BL/6J-Alpk X CBA/CA-Alpk) mice were obtained from the AstraZeneca breeding unit (Alderley Park, Macclesfield, Cheshire, UK). CD-1 (CD-1 CrI:CD-1 (ICR) BR) mice were obtained from Charles River UK (Margate, Kent, UK). C57Bl6/J (C57BL/6JolaHsd) mice were obtained from Harlan UK (Bicester, Oxford, UK). Male rats were 18–19 days of age on arrival (body weights ≤ 45 g; male rats were 10–12 weeks of age. Female mice were 19–20 or 20–21 days of age on arrival, and male mice were 10–12 weeks of age. Immature animals were selected randomly from a large number of litters by the suppliers and were
already weaned at delivery. Animals were allowed 24 hr acclimatization before the start of the uterotrophic assays. Female animals were housed (up to five per cage) in metal cages with wire mesh bases and were supplied with shredded paper bedding. Males were not supplied with bedding to allow the females maximum contact with the male urine. Rat and Mouse No. 1 diet (Special Diet Services Ltd., Witham, Essex, UK) and water were available ad libitum. Animal care and procedures were conducted according to in-house standards as described previously (Odum et al. 1999).

**Uterotrophic assays.** Immature rat and mouse uterotrophic assays were conducted in an identical manner.

The effect of the close proximity of mature males on female uterine growth was tested in Alpk rats and Alpk mice that were 19–20 days of age (pnd 19–20) and 20–21 days of age (pnd 20–21), respectively, at the start of treatment. Untreated females were housed for 3 days in groups of three in a cage placed directly under a cage containing a mature (untreated) male. The base of the male’s cage was open wire mesh, thus allowing direct contact with the male’s urine. Control untreated animals were housed for 3 days in a separate room containing no male animals. DES (5 µg/kg) or vehicle (arachis oil) were administered by subcutaneous (sc) injection (dosing volumes for rats and mice were 2.5 and 5 mL/kg, respectively) daily for 3 days. DES groups were limited to three animals. For power, the groups housed in proximity to mature males and with males absent were enlarged to 9 or 10 animals. Blotted and oven-dried uterine weights were recorded, as well as body weight at termination.

In both studies, the DES-treated group had significantly increased uterine weights and the vehicle control group had uterine weights consistent with previous results in each species (Odum et al. 2002; Tinwell et al. 2000). As shown in Table 1, the mean blotted and dry uterine weights of females in close proximity to males were similar to those of untreated females in the absence of males and those of the vehicle controls. Further, the standard deviations of females in close proximity to males were also similar to the controls. Therefore, we detected no impact of mature males in close proximity to immature females in the uterotrophic bioassay using either rats or mice.

The second series of experiments was designed to investigate the response of different strains of mice to DES. All strains showed a highly significant uterotrophic response to DES at 10 µg/kg/day, although the uterine weight increase varied from 64 mg in the Alpk to 35 mg in the B6CBF1 strain (Table 2).

### Table 1. The effect of males on uterine weight of immature females (mean ± SD) in Alpk rats and Alpk mice.

| Treatment/condition | No. | Uterine blotted weight (mg) | Uterine dry weight (mg) | Body weight at termination (g) |
|---------------------|-----|-----------------------------|------------------------|-----------------------------|
| **Rats**            |     |                             |                        |                             |
| Untreated females, close proximity to males | 9 | 21.3 ± 2.3 | 4.1 ± 0.5 | 16.3 ± 2.8 |
| Untreated females (males absent) | 10 | 22.8 ± 1.7 | 4.5 ± 0.4 | 16.3 ± 2.8 |
| Arachis oil, 5 mL/kg/day (males absent) | 3 | 23.6 ± 5.8 | 4.4 ± 1.1 | 16.3 ± 2.8 |
| DES, 5 µg/kg/day (males absent) | 3 | 107.2 ± 7.6* | 18.6 ± 0.9* | 16.3 ± 2.8 |
| **Mice**            |     |                             |                        |                             |
| Untreated females, close proximity to males | 9 | 11.7 ± 2.9 | 2.5 ± 0.6 | 16.3 ± 2.8 |
| Untreated females (males absent) | 10 | 10.8 ± 3.6 | 2.4 ± 0.6 | 16.3 ± 2.8 |
| Arachis oil, 5 mL/kg/day (males absent) | 3 | 12.7 ± 3.7 | 2.8 ± 0.6 | 16.3 ± 2.8 |
| DES, 5 µg/kg/day (males absent) | 3 | 70.8 ± 11.5* | 11.3 ± 1.8* | 16.3 ± 2.8 |

*Significantly different from the appropriate vehicle control group (p < 0.01).

### Table 2. Uterine weight (mean ± SD) of different strains of immature female mice exposed to DES.

| Experiment/strain/compound | Age (days) at start of dosing | Uterine blotted weight (mg) | Body weight at termination (g)*a |
|---------------------------|------------------------------|-----------------------------|---------------------------------|
| **Experiment 1**          |                              |                             |                                 |
| Alpk                      |                             |                             |                                 |
| Arachis oil, 5 mL/kg/day  | 20–21                        | 11.0 ± 2.8                  | 16.0 ± 1.7                      |
| DES, 1 µg/kg/day          |                             | 25.7 ± 8.6*                 | 16.0 ± 1.5                      |
| DES, 10 µg/kg/day         |                             | 64.2 ± 8.1*                 | 16.0 ± 1.5                      |
| C57BL6J                   |                             |                             |                                 |
| Arachis oil, 5 mL/kg/day  | 20–21                        | 8.8 ± 1.3                   | 11.3 ± 1.1                      |
| DES, 1 µg/kg/day          |                             | 20.3 ± 8.0*                 | 11.2 ± 1.0                      |
| DES, 10 µg/kg/day         |                             | 45.7 ± 6.2*                 | 11.7 ± 1.6                      |
| **Experiment 2**          |                              |                             |                                 |
| Alpk                      |                             |                             |                                 |
| Arachis oil, 5 mL/kg/day  | 20–21                        | 9.6 ± 3.2                   | 17.4 ± 0.7                      |
| DES, 1 µg/kg/day          |                             | 16.3 ± 3.1*                 | 17.4 ± 0.7                      |
| DES, 10 µg/kg/day         |                             | 51.7 ± 7.4*                 | 18.5 ± 1.5                      |
| B6CBF1                    |                             |                             |                                 |
| Arachis oil, 5 mL/kg/day  | 20–21                        | 8.8 ± 1.6                   | 10.3 ± 0.9                      |
| DES, 1 µg/kg/day          |                             | 11.7 ± 2.6                  | 10.6 ± 1.6                      |
| DES, 10 µg/kg/day         |                             | 34.6 ± 5.0*                 | 10.7 ± 1.0                      |
| **Experiment 3**          |                              |                             |                                 |
| Alpk                      |                             |                             |                                 |
| Arachis oil, 5 mL/kg/day  | 21–22                        | 10.9 ± 1.9                  | 15.4 ± 1.2                      |
| DES, 1 µg/kg/day          |                             | 26.8 ± 5.9*                 | 16.0 ± 1.1                      |
| DES, 10 µg/kg/day         |                             | 45.1 ± 8.6*                 | 15.6 ± 2.2                      |
| CD-1                      |                             |                             |                                 |
| Arachis oil, 5 mL/kg/day  | 21–22                        | 21.0 ± 6.8                  | 15.4 ± 1.2                      |
| DES, 1 µg/kg/day          |                             | 29.6 ± 7.3                  | 14.9 ± 1.0                      |
| DES, 10 µg/kg/day         |                             | 66.9 ± 9.0*                 | 15.1 ± 1.5                      |

*Significantly different from the appropriate vehicle control group (p < 0.01).
effects on testes and sperm were evident have been observed with male mice exposed to five weak estrogen agonists (Kanno et al. 2001, 2003a, 2003b). Thegravimetric weight of the uterus in response to chemical administration can be assayed in sexually immature or ovariectomized rodents, and either rats or mice can be used. Recently, several experiments have been conducted on the possible influence of several variables on the responsiveness of the bioassay, including the age of immature animals (Yamasaki et al. 2001), diet (Ashby et al. 2000, 2001; Degen et al. 2002; Owens et al. 2003; Yamaki et al. 2002), and vehicle (Yamasaki et al. 2001). One interest here was to define whether certain animal husbandry conditions may lead to early puberty and decreased assay responsiveness. A second interest was to determine possible strain differences in the response to estrogen agonists such as DES.

Both the group mean of blotted and dry uterine weights and the standard deviations of these group means were unaffected by proximity of males for either rats or mice. These data, therefore, provide no evidence that the presence of mature males will alter the results of the uterotrophic bioassay or lead to conditions that will interfere with the responsiveness of the bioassay. The absence of an effect in these studies is probably due to the young age of the animals (24 days of age at termination). Colby and Vandenberg (1974) showed accelerated first estrus in mice 24–29 days of age at the time of exposure to male urine, an effect that is absent in younger animals.

Strain differences were not a major variable in the OECD validation of the rat uterotrophic assay. No differences were observed between Sprague-Dawley and Wistar rat strains among several laboratories using ethinal estradiol as a potent reference and also five weak estrogen agonists (Kanno et al. 2001, 2003a, 2003b). However, strain differences in response to estrogen receptor agonists have been observed with male mice exposed to estradiol, where marked differences in the effects on testes and sperm were evident (Spearow et al. 1999). Strand differences in the response of rats to bisphenol A (Long et al. 2000) have also been reported, with F344 rats more sensitive than Sprague-Dawley rats, although we found no difference in the magnitude of the uterotrophic effect of nonylphenol in Sprague-Dawley and Alpk rats (Odum et al. 1999). The work of Thigpen et al. (1987) and Schlumpf et al. (2001) clearly demonstrate the impact of the early events of prepuberty in mice and rats, respectively. In both species, an increase in mean uterine weight and a rapid increase in the standard deviations of a group occurs. Thus, the timing of puberty must be taken into account with each species and each strain. In this investigation, B6C3F1 mice were less sensitive to the action of DES, and control CD-1 mice had relatively high uterine weights. However, in each of the strains, DES was clearly detected as an estrogen. Although the possibility remains that the response to nonylphenol signals may differ with strain, the age of the immature animals appears to be the primary determinant of sensitivity, in agreement with others (Thigpen et al. 1987; Schlumpf et al. 2001). Our chosen strain was unaffected.

These findings add to earlier data (Kanno et al. 2001, 2003a, 2003b) indicating the robustness of the rodent uterotrophic assay protocol, and they raise the possibility that some strains of animal or the conditions under which they are used may lead to differences in sensitivity to the action of estrogens. This supports the efforts of Kanno et al. (2001, 2003a, 2003b) to standardize the age of their animals and of Owens et al. (2003) to investigate the influence of dietary phytoestrogens.

References

Ashby J, Tinwell H, Odum J. 2000. Uterotrophic activity of a “phytoestrogen-free” rat diet [Letter]. Environ Health Perspect 108:251–254.

———. 2001. DNA adducts, estrogenicity and rodent diets. Mutat Res 483:105–106.

Brennan PA, Schellinck HM, Keverne EB. 1999. Patterns of expression of the immediate early gene egr-1 in the accessory olfactory bulb of female mice exposed to pheromonal constituents of male urine. Neuroscience 90:1463–1470.

Christian MS, Böckmann A, Bachmann S, Hellwig J. 1998. Variability in the uterotrophic response assay (in vivo estrogenic response assay) in untreated control and positive control (DES-SP, 2.5 µg/kg, bid) Wistar and Sprague-Dawley rats. Drug Chem Toxicol 21(suppl 1):51–100.

Cisloord PM, Bishop JD. 1982. Variation in the mouse major urinary protein (MUP) genes isolated from a single inbred line. Gene 18:221–230.

Colby DR, Vandenberg JG. 1974. Regulatory effects of urinary pheromones on puberty in the mouse. Biol Reprod 11:268–279.

Degen GH, Janning P, Diel P, Bolt HM. 2002. Estrogenic compounds with ICI 182,780 or antide. Arch Toxicol 76:613–620.

———. 2003b. The OECD program to validate the rat uterotrophic bioassay. Phase 2: dose–response studies. Environ Health Perspect 111:1530–1548.

Degen J, Onyon L, Peddada S, Ashby J, Jacob E, Owens W. 2001. The OECD program to validate the rat uterotrophic bioassay to screen compounds for in vivo estrogenic responses: phase 1. Environ Health Perspect 109:785–794.

Degen J, Onyon L, Peddada S, Ashby J, Jacob E, Owens W. 2003a. The OECD program to validate the rat uterotrophic bioassay. Phase 2: coded single-dose studies. Environ Health Perspect 111:1550–1558.

———. 2003b. The OECD program to validate the rat uterotrophic bioassay. Phase 2: dose–response studies. Environ Health Perspect 111:1550–1548.

Long K, Steinmetz R, Bejon-Donnan N, Capeller-Grant A, Young PCM, Nephew KP, et al. 2000. Strain differences in vaginal responses to the xenoestrogen bisphenol A. Environ Health Perspect 108:237–248.

Mucignat-Caretta C,aretta A, Cavaggioni A. 1995. Acceleration of puberty onset in female mice by male urinary proteins. J Physiol 486:517–522.

Nielson JF, Karelus K, Felicio LA, Johnson TE. 1990. Genetic influences on the timing of puberty in mice. Biol Reprod 44:649–655.

Odum J, Lefeva PA, Tinwell H, Van Miller JP, Joiner RL, Chapman RE, et al. 2003. Comparison of the developmental and reproductive toxicity of diethylstilbestrol administered to rats in utero, lactationally, pre-weaning or post weaning Toxicol Sci 86:147–163.

Odum J, Lefeva PA, Tittens S, Patton D, Roud Jeffrey E, Sumpter JP, et al. 1997. The rodent uterotrophic assay: critical protocol features, studies with nonylphenols, and comparison with a yeast estrogenicity assay. Regul Toxicol Pharmacol 25:176–189.

Odum J, Pihlaj ITG, Soames AR, Foster JR, Van Miller JP, Joiner RL, et al. 1999. Effects of nonylphenol (NP) and diethylystilbestrol (DES) on the Alderley Park (Alpk) rat: comparison of mammary gland and uterus sensitivity following oral gavage or implanted minipumps. J Appl Toxicol 19:367–378.

Owens W, Ashby J, Odum J, Onyon L. 2003. The OECD program to validate the rat uterotrophic bioassay. Phase 2: dietary phytoestrogen analyses. Environ Health Perspect 111:1559–1567.

Price MA, Vandenbergh JG. 1982. Analysis of puberty-accelerating pheromones. J Exp Zool 241:42–45.

Sanson CE, North ACT, Sawyer LH. 1994. Structural analysis and classification of lipocalins and related proteins using a profile-search method. Biochem Biophys Acta 1208:247–255.

Schlumpf M, Berger L, Cotton B, Conscience-Engel M, Durrer S, Fleischmann I, et al. 2001. Estrogen active UV screens. Seifen-Öle-Fette-Wachse 127:10–18.

Spearow JL, Doemeny P, Sera R, Leffler R, Barkley M. 1999. Genetic variation in susceptibility to estrogen and xenoestrogen disruption by estrogen in mice. Science 285:1259–1261.

Thigpen JE, Li LA, Richter CB, Lebekin EH, Jameson CW. 1987. The mouse bioassay for the detection of estrogenic activity in rodent diets. A standardized method for conducting the mouse bioassay. Lab Anim Sci 37:596–601.

Tinwell H, Joiner R, Pate I, Ashby J. 2000. Uterotrophic activity of bisphenol A (BPA) in the immature mouse. Regul Toxicol Pharmacol 32:118–128.

Vandenberg JG. 1996. Male odor accelerates female sexual maturation in mice. Endocrinology 148:858–860.

Vandenberg JG, Whitsett J, Lombardi JR. 1975. Partial isolation of a pheromone accelerating puberty in female mice. J Reprod Fertil 43:515–523.

van der Lee S, Boot LM. 1995. Spontaneous pseudopregnancy in mice. Acta Physiol Pharmacol Neurog 4:442–443.

Whitten WK. 1956. Modification of estrous cycle of the mouse by external stimuli associated with the male. J Endocrinol 13:399–404.

Yamasaki K, Sawaki M, Noda S, Takatsuki M. 2001. Effects of age and weaning on the immature rat uterotrophic assay using ethynylestradiol. Exp Anim 50:77–89.

Yamasaki K, Sawaki M, Noda S, Wada T, Hara T, Takatsuki M. 2002. Immature uterotrophic assay of estrogenic compounds in rats given diets of different phytoestrogen content and the ovarian changes in the immature rat uterotrophic of estrogenic compounds with ICI 182,780 and antiand. Arch Toxicol 76:613–620.