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Exposure to developing females induces polyuria, polydipsia, and altered urinary levels of creatinine, 17β-estradiol, and testosterone in adult male mice (*Mus musculus*)

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ABSTRACT

Novel male mice can accelerate reproductive maturation in proximal developing females, an effect mediated by the chemistry of the males' urine. Exogenous estrogens can similarly accelerate female sexual development. In Experiment 1, adult male mice were housed across wire grid from either empty compartments or those containing post-weanling females. Proximity of females caused males to urinate more, progressively over days of exposure, with most urination directed towards females' compartments. Male urine collected after 5 days in these conditions was analyzed by enzyme immunoassay for 17β-estradiol, testosterone, and creatinine. Urinary creatinine of isolated males significantly exceeded that of female-exposed males. Unadjusted urinary steroids also trended toward higher levels in isolates, but creatinine-adjusted estradiol and testosterone of female-exposed males significantly exceeded that of isolated males. In Experiment 2, measurement of water consumption indicated significantly greater drinking by female-exposed as opposed to isolated males. In Experiment 3, males were housed in isolation or beside post-weanling intact (sham-operated) females, ovariectomized females, or intact (sham-operated) males. Male water consumption was elevated in all conditions involving social contact. Urinary creatinine was significantly lower in female-exposed males compared to isolated controls, while unadjusted testosterone was significantly lower in males in all social conditions. Again, creatinine-adjusted estradiol in female-exposed males significantly exceeded that of isolates. These data indicate that adult males drink and urinate more, have more dilute urine, and have a higher ratio of estradiol to creatinine when they are near developing females. These dynamics increase females' exposure to urinary steroids and other urinary constituents that can hasten sexual maturity.

Introduction

Proximity to novel adult males can accelerate sexual maturation in juvenile females of several mammalian species, including mice (Vandenbergh, 1967), rats (Vandenbergh, 1976), deer mice (Teague and Bradley, 1978), voles (Spears and Clarke, 1986), lemmings (Hasler and Banks, 1975), opossums (Harder and Jackson, 2003), and cattle (Roberson et al., 1991). In post-weanling female mice, exposure to adult males can advance sexual maturity by as much as twenty days (Vandenbergh, 1967). This is reflected in increased vaginal opening (Vandenbergh, 1967), cyclical changes in vaginal cytology (Bingel 1972; Vandenbergh 1967, 1976), increased reproductive tissue mass (Beaton et al., 2006; Khan et al., 2008b), and earlier display of sexual receptivity and more rapid insemination during direct exposure to adult males (Khan et al., 2008a).

Sexual maturation in female mice and other mammals is dependent to a large degree upon ovarian steroid secretion. Growth of the uterus during development and within estrous cycles is highly dependent upon estrogens, with some species-specific variations in timing (Gray et al., 2001). In mice, exogenous estradiol causes uterine cells to proliferate (Ogasawara et al., 1983). Estrogens regulate growth hormone and IGF-1 activity (Kahlert et al., 2000; Leung et al., 2004), and local IGF-1 activity mediates uterine growth in response to estradiol (Sato et al., 2002). Estrogens also play critical roles in the preparation of female sexual receptivity (*e.g.* deCatanzaro, 1987; Pfaff, 1980). Given that male mouse urine and other excretions contain abundant quantities of unconjugated estradiol (Beaton et al., 2006; deCatanzaro et al., 2006; Vella and deCatanzaro 2001), and male mice actively direct urine droplets at proximate females (deCatanzaro et al., 2006; Hurst, 1990b; Reynolds 1971), estrogens in males' excretions could contribute to male-induced pubertal acceleration in proximal females.

Previous investigations have indicated that males' excretions may contain higher quantities of creatinine-adjusted estradiol and testosterone after males have been exposed for 3 or more days to post-weanling, pre-pubertal females (Beaton et al., 2006) or females inseminated by other males around the time of intrauterine implantation (deCatanzaro et al., 2006). It is common practice in conducting urinary steroid analyses to compensate for variations in hydration by adjusting sample values for creatinine, an index of metabolic activity, due to variation within and among animals in fluid

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intake and excretion (*e.g.* deCatanzaro et al., 2003; Erb et al., 1970; Muir et al., 2001; Munro et al., 1991). However, creatinine itself can vary, at least in laboratory mice, depending on environmental, background, and social variables (Beaton et al., 2006; Khan et al., 2008b). It has also been observed that adult males that are proximal to females can be extraordinarily aroused, active, and aggressive (deCatanzaro et al., 1996, 2000), which could impact their delivery of urine and its bioactive constituents, such as estradiol, to nearby females. On this basis, we hypothesized that there might be differences between isolated males and males housed in proximity to developing females in fluid intake, urination, and urinary content of creatinine and sex steroids.

The current experiments were designed to shed further light on the causation of male-induced pubertal acceleration in mice, specifically the possibility that this is mediated by delivery of exogenous estradiol and other steroids by males to females through urine. As it is already established that exogenous estrogens can accelerate female sexual maturation (*cf.* Bronson, 1975; Khan et al., 2008b; Ogasawara et al., 1983), that male urine contains unconjugated estradiol and other steroids (Beaton et al., 2006; deCatanzaro et al., 2006; Muir et al., 2001), and that males deliver their urine to proximal females (deCatanzaro et al., 2006; Reynolds, 1971), the validity of this hypothesis depends upon quantitative issues. Thus it is important to establish the dynamics of male urination during exposure to developing females and the concentrations of unconjugated estrogens in male urine. Fluid intake is also quite relevant given its likely impact upon urinary quantity, while creatinine levels in male urine should also reflect fluid intake and excretion. Accordingly, we compared adult males that were housed alone to those housed by developing females in frequency of urination, location of urination, water intake, and urinary concentrations of 17β-estradiol, testosterone, and creatinine. We also compared water intake, creatinine, and steroid dynamics in males' urine in the presence of developing intact females, ovariectomized females, and males.

Methods

Subjects

Heterogeneous strain (HS) mice (*Mus musculus*) had previously been produced by interbreeding C57-B6, Swiss Webster, CF1, and DBA-2 strains obtained from Charles River Breeding Farms (Québec, Canada). HS male subjects, aged 7–9 months with mean (±SE) weight of 46.7 ± 1.5 g at the commencement of procedures, had been housed individually since weaning in standard polypropylene cages measuring 28×16×11 (height) cm and had no previous sexual experience. CF-1 strain stimulus female mice were of stock from Charles River Breeding Farms. Stimulus females were weaned from full litters at 28 days, with no more than two weanling females chosen per litter, then randomly placed in compartments of an experimental apparatus as described below. Prior to and throughout experimental procedures, animals had continuous access to water and 8640 Teklad Certified Rodent Chow. The animal colony was maintained under a reversed 10:14 hour dark:light cycle at 21 °C. This research was approved by the McMaster University Animal Research Ethics Board, conforming to standards of the Canadian Council on Animal Care.

Experimental apparatus

A urinary collection apparatus, constructed of Plexiglas and stainless-steel wire-mesh grid, was divided into three compartments. The male's rectangular compartment measured 30×9×15(height) cm. Two adjacent square compartments for developing stimulus animals measured 15×15×15 cm such that each compartment had a 225 cm^2 interface through vertical grid with each other compartment. Vertical wire mesh between the two square compartments had grid of 0.25 cm^2, while that between each square (stimulus animal) compartment and the rectangular (male) compartment had a grid of 1 cm^2, allowing limited interactions between the male and each developing stimulus animal. Each compartment had an outset closet away from the grid walls that provided continuous access to food and water. All compartments had wire-grid floor with squares of 0.5 cm^2 raised 2 cm above a Teflon-coated collection tray.

Experiment 1

This experiment was designed to compare urination patterns and urinary creatinine, estradiol, and testosterone in isolated males and those exposed to developing females. On the first day of experimental procedures (day 1), at 1 h after commencement of the dark phase of the lighting cycle, an HS male subject was placed in the rectangular compartment of the apparatus. For 12 randomly-selected isolated control males, the two adjacent square compartments remained empty. For 12 other female-exposed males, a 28-day old female was placed in each of the two square compartments of the apparatus. Two developing females were used for each male in order to ensure robust effects and to reduce the potential influence of inter-female variance.

Behavioral observations were conducted for each animal under dim illumination on each of days 1–4 of experimental procedures. Each session lasted 30 min and commenced approximately 1 to 2.5 h after the start of the dark phase of the lighting cycle, with time of sessions counterbalanced across conditions and within conditions over days, such that each animal was tested at each of four possible half-hour intervals. A trained observer recorded each instance of male urination, and the location of urination in terms of three categories: toward the floor of the male's compartment, toward a Plexiglas wall, or toward an adjacent compartment through grid. Instances in which the male made nasal contact with the grid interface on one or the other adjacent compartments were also recorded for each male. Each session was videotaped via a Sony model TVR33 digital video camera equipped with an infrared camera mounted spotlight. Videotapes were reviewed as necessary to confirm counts.

On day 5 of the experiment, at the commencement of the dark phase of the lighting cycle, each cage was gently lifted from the existing collection tray and placed on a clean tray which was covered with wax paper to facilitate urine droplet identification and collection. Males were monitored in dim illumination until urination was observed. Urine samples that were uncontaminated by feces, water, or food residue were aspirated using a 1 ml syringe with a 26-gauge needle and stored in labeled 1 ml microtubes. If necessary, males were observed and samples collected up until 5 h after commencement of the dark phase of the lighting cycle. On day 6 of the experiment, this whole procedure was repeated in order to gain a second urine sample for each male. All samples remained frozen at −20 °C until they were assayed for 17β-estradiol, testosterone, and creatinine as described below. Assays were conducted on all samples, simultaneously for each substance.

Experiment 2

This experiment was designed to assess fluid intake in isolated males and those exposed to developing females. Sixteen HS males were exposed to 28-day-old females, while 16 others remained isolated in the experimental apparatus. Each male was given a graduated water bottle with a measured quantity of 200 ml water on the initial day of the experiment at the point of introduction to the apparatus, approximately 1 h after commencement of the dark phase of the light cycle. After 5 days (120 h) of undisturbed habitation in these conditions, the volume remaining in the water bottle was measured for each male, and subtracted from the initial quantity to calculate water consumption for each animal.