Transfer of [^3H]estradiol-17 β and [^3H]progesterone from conspecifics to cohabiting female mice

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Abstract

Estradiol-17 β (E_2) and progesterone (P_4) play critical roles in female reproductive physiology and behavior. Given the sensitivity of females to exogenous sources of these steroids, we examined the presence of E_2 and P_4 in conspecifics' excretions and the transfer of excreted steroids between conspecifics. We paired individual adult female mice with a stimulus male or female conspecific given daily injections of [^3H] E_2 or [^3H] P_4 . Following 48 h of direct interaction with the stimulus animal, we measured radioactivity in the uterus, ovaries, muscle, olfactory bulbs, mesencephalon and diencephalon (MC+DC), and cerebral cortex of the untreated female cohabitant. Radioactivity was significantly present in all tissues of female subjects after individual exposure to a stimulus male or female given [^3H] E_2 . In females exposed to males given [^3H] P_4 , radioactivity was significantly present in the uterus, ovaries, and muscle, but not in other tissues. In females exposed to stimulus females given [^3H] P_4 , radioactivity was significantly present in all tissues except the MC+DC. In mice directly administered [^3H]steroids, greater radioactivity was found in the urine of females than of males. Among females directly administered [^3H]steroids, greater radioactivity was found in urine of those given [^3H] P_4 than of those given [^3H] E_2 . When females were administered unlabeled E_2 before exposure to [^3H] E_2 -treated females, less radioactivity was detected in most tissues than was detected in the tissues of untreated females exposed to [^3H] E_2 -treated females. We suggest that steroid transfer among individuals has implications for the understanding of various forms of pheromonal activity.

Key Words

- ▶ estradiol
- ▶ progesterone
- ▶ [^3H]steroids
- ▶ pheromones
- ▶ steroid transfer

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Introduction

Conspecifics' excretions can impact mammalian females' reproductive state in many ways. Male excretions can accelerate female sexual development (Vandenbergh 1967, Harder & Jackson 2003), terminate pregnancies sired by other males (Bruce 1960, Rohrbach 1982, Marashi & Rüllicke 2012, Thorpe & deCatanzaro 2012), and alter estrous cycling (Whitten 1956, Dodge *et al.* 2002, Rivas-Muñoz *et al.* 2007). In laboratory mice, males

develop polyuria and polydipsia in the presence of females and progressively target their urine toward these females, while females in this context have nasal and dermal contact with male urine droplets (deCatanzaro *et al.* 2006, 2009). Female excretions can also have effects on the reproductive physiology of other females, prolonging or even completely disrupting estrus (Lee & Boot 1955, 1956, Ma *et al.* 1998). Female mice generally excrete less urine

than do males, but their urinary output is greater while they are in estrus than in diestrus, which coincides with a greater ability during estrus to affect the development and physiology of cohabiting females (Drickamer 1982, 1986, 1995). Although evidence indicates that some pheromonal effects could be mediated by females' responses to conspecifics' urinary proteins (Ma *et al.* 1999, Marchlewska-Koj *et al.* 2000, Leinders-Zufall *et al.* 2004), growing evidence also implicates steroids, especially estrogens, in conspecifics' excretions (Beaton *et al.* 2006, deCatanzaro *et al.* 2006, Khan *et al.* 2009, Thorpe & deCatanzaro 2012). Indeed, recent data suggest that small lipophilic steroids such as estradiol-17 β (E_2) could transfer directly between cohabiting individuals (Guzzo *et al.* 2010, 2012).

The steroids that regulate many aspects of the female reproductive state, E_2 and progesterone (P_4), are abundant in unconjugated form in mouse urine from both sexes (deCatanzaro *et al.* 2004, 2006, Khan *et al.* 2009, Guzzo *et al.* 2012, Thorpe & deCatanzaro 2012). By contrast, urinary estrone conjugates in female mice were measured at about 1% of the level of urinary unconjugated E_2 , whereas urinary estrone conjugates in male mice were below the threshold for detection (Muir *et al.* 2001). Both E_2 and P_4 are lipophilic and have low molecular masses (272.4 and 314.5 Da respectively) and high chemical stability, allowing for cutaneous (Scheuplein *et al.* 1969, Schaefer *et al.* 1982, Hueber *et al.* 1994) and nasal (Bawarshi-Nassar *et al.* 1989) entry into circulation. When E_2 or P_4 is administered nasally to rats, the majority of the administered quantity is bioavailable as unconjugated E_2 in circulation (Bawarshi-Nassar *et al.* 1989). Following cutaneous application of [3H] E_2 and [^{14}C] P_4 , as much as 44% of the applied quantity of each appears in urine within 72 h (Goldzieher & Baker 1960).

Accordingly, we reasoned that small lipophilic steroids excreted by individuals could enter into and journey through the circulatory system of cohabiting conspecifics, where they could then bind to any available receptors throughout the body. We tested this for both E_2 and P_4 using tritium-labeled versions ([3H] E_2 and [3H] P_4) injected into either male or female mice, and then measured the level of radioactivity in various tissues of untreated female mice after a few days of cohabitation. Previously (Guzzo *et al.* 2010), we showed that males injected i.p. with [3H] E_2 excrete it in their urine, and that females nasally administered [3H] E_2 had radioactivity in brain and reproductive tissues. Radioactivity was particularly evident in the uterus and was significantly reduced by pre-administration of unlabeled E_2 to females. We subsequently (Guzzo *et al.* 2012) demonstrated that [3H] E_2 injected into male mice

is detectable in the brain and reproductive tissues of inseminated female and juvenile female cohabitants. Here we examined reproductive, brain, and other tissues of freely cycling females. We showed both male-to-female and female-to-female transfer of [3H] P_4 and [3H] E_2 , and showed greater absorption of [3H] E_2 than [3H] P_4 when equivalent quantities were given in terms of radioactivity. We also demonstrated differential excretion over time of administered [3H] E_2 and [3H] P_4 between the sexes. Finally, we showed that directly administering unlabeled E_2 to female subjects significantly reduced radioactivity in their tissues when they were exposed to [3H] E_2 -treated stimulus females.

Materials and methods

Animals

Mice (*Mus musculus*) were housed in standard polypropylene cages measuring 28 $\times$ 16 $\times$ 11 (height) cm with wire tops allowing access to food and water, except where otherwise stated. Sexually experienced males aged 3–8 months and sexually inexperienced females aged 2–6 months were of CF1 strain and were either bred from stock or obtained from Charles River (Kingston, NY, USA). Age was counterbalanced across conditions. The colony was maintained at 21 °C with a reversed 14 h light:10 h darkness cycle. All research was approved by the Animal Research Ethics Board of McMaster University, conforming to standards of the Canadian Council on Animal Care.

Measurement of natural urinary E_2 and P_4

Individual males and females (Ten per sex) were each placed in a Plexiglas apparatus measuring 15 $\times$ 21 $\times$ 13 (height) cm with a wire-mesh grid floor with squares measuring 0.5 cm². The floor consisted of wire-mesh raised ~1 cm above a Teflon stainless-steel surface covered with wax paper, which permitted non-invasive collection of urine. The animal was introduced to the chamber at the onset of darkness and left undisturbed for 2 days to acclimate to the apparatus. Following the onset of darkness on the subsequent 6 days, urine was collected from each animal, placed into a labeled vial, and then frozen at –20 °C.

Validations and full procedures for enzyme immunoassays for mouse urine were previously reported (Muir *et al.* 2001, deCatanzaro *et al.* 2003, 2004). Steroids and creatinine standards were obtained from Sigma. Steroid antibodies and corresponding HRP conjugates were obtained from the Department of Population Health and Reproduction at the University of California, Davis.

Cross-reactivities for anti-E₂ are: E₂ 100%, estrone 3.3%, P₄ 0.8%, testosterone 1.0%, androstenedione 1.0%, and all other measured steroids <0.1%. Cross-reactivities for anti-P₄ are: P₄ 100.0%, 11 α -hydroxyprogesterone 45.2%, 5 α -pregnen-3,20-dione 18.6%, 17 α -hydroxyprogesterone 0.38%, 20 α -hydroxyprogesterone 0.13%, 2, pregnanediol <0.001%, pregnenolone 0.12%, E₂ <0.001%, and estrone <0.04%. Following convention, urinary steroid levels were considered with and without adjustment for urinary creatinine (deCatanzaro *et al.* 2009).

Direct exposure of cycling females to [³H]steroid-injected males

Stimulus males were gradually tolerated to ethanol as described previously (Guzzo *et al.* 2012). Briefly, the ethanol concentration in the water given to these males was increased by 1% v/v per week, from 1% to 4%, and then maintained at 4% until the time of the experiment. At 7 h following the onset of darkness on the first day of the experiment (day 1), each stimulus male was given an i.p. injection of 10 μ l 95% ethanol (control), 10 μ Ci [2,4,6,7-³H](N)-E₂ (in ethanol, 1.0 μ Ci/ μ l, 89.2 Ci/mM, PerkinElmer, Waltham, MA, USA), or 10 μ Ci [1,2,6,7-³H](N)-P₄ (in ethanol, 1.0 μ Ci/ μ l, 101.3 Ci/mM, PerkinElmer). Injections were performed using a 50 μ l syringe (705RN, Hamilton, Reno, NV, USA) with a 26 GA needle (19131U Type 2-S, Hamilton). For animals given [³H]E₂, this is equivalent to 30.5 ng per dose, for a total of 91.5 ng per animal. For animals given [³H]P₄, this is equivalent to 31 ng per dose or 93 ng per animal. The injection site was swabbed with cotton doused in 95% ethanol to prevent external contamination.

At the onset of darkness on day 2, randomly selected females with regular estrous cycles were each paired in a clean cage with a stimulus male. There were five male-exposed females prepared in each treatment. Stimulus males were each given additional i.p. injections identical to their first at 7 h following the onset of darkness on days 2 and 3 of the experiment, and were temporarily housed in a separate cage for 1 h following each additional injection in order to prevent direct contamination of the paired female or the shared bedding. On day 4 of the experiment, at 4 h following the onset of darkness, each female was heavily anesthetized with sodium pentobarbital and then perfused with 30 ml saline. Whole uterus, ovaries, mesencephalon and diencephalon (MC+DC), olfactory bulbs, cerebral cortex, and a sample of muscle tissue were extracted as described previously (Guzzo *et al.* 2012) and placed in pre-weighed scintillation vials. The

vials were re-weighed to determine tissue wet mass and then processed for measurement of radioactivity as described below.

Following previously published procedures (Guzzo *et al.* 2012), female tissue samples were homogenized by adding 1.0 (all tissues except cerebral cortex) or 1.5 (cerebral cortex) ml SOLVABLE (PerkinElmer) to each vial after re-weighing. Vials were then placed in a 60 °C water bath, swirled after 1 h, and then left in the bath for an additional 1–3 h until no visible pieces of tissue remained. After removal from the bath and at least 10 min of cooling, 10 ml Ultima Gold scintillation cocktail (PerkinElmer) was added to each vial. Vials were stored in darkness in a TriCarb 2910 TR Liquid Scintillation Analyser (PerkinElmer) for at least 12 h to eliminate background noise in the form of heat and luminescence. Following this interval, radioactivity was measured for 5 min per sample, and counting efficiencies and final adjusted estimates for the amount of radioactivity per sample (in d.p.m.) were automatically calculated by the accompanying QuantaSmart software package (PerkinElmer).

Direct exposure of cycling females to [³H]steroid-injected females

The procedures for this experiment replicated the procedures for the previous experiment, except that the stimulus animals were non-sibling females instead of males. There were five female subjects prepared in each of the control and [³H]E₂ treatments, and four subjects prepared in the [³H]P₄ treatment. All other details, including ethanol tolerance, timing of the injections, solutions used for the injections, timing of separation, timing of the surgeries, tissues analyzed in the female paired with the stimulus animal, tissue processing, and scintillation counting were identical to those of the previous experiment.

Measurement of urinary radioactivity in [³H]steroid-injected males and females

This experiment was conducted in order to determine the rates and time profiles of urinary excretion of [³H]E₂ and [³H]P₄ in injected males and females. At the onset of the darkness phase of the light:darkness cycle, seven males and four females aged 4–5 months were each given an i.p. injection of 10 μ Ci [2,4,6,7-³H](N)-E₂ (in ethanol, 1.0 μ Ci/ μ l, 72.1 Ci/mM, PerkinElmer), and ten males and four females received 10 μ Ci [1,2,6,7-³H](N)-P₄ (in ethanol, 1.0 μ Ci/ μ l, 101.3 Ci/mM, PerkinElmer).

These doses provide a total of 37.7 ng [^3H]E₂ and 31 ng [^3H]P₄. Larger numbers of males were prepared than females as male urination patterns differ from those of females, rendering sample collection much more difficult (deCatanzaro *et al.* 2006, 2009). Immediately following injection, animals were individually placed in a Plexiglas apparatus measuring 15×21×13 (height) cm with a wire-mesh grid floor raised ~1 cm above a surface covered with wax paper, which permitted non-invasive collection of urine. Urine was first collected 1 h after injection at about the commencement of the darkness phase of the animals' reversed lighting cycle, and subsequently every hour for a total of 10 h. Animals were left undisturbed through the light phase of the light:darkness cycle and a final urine sample was taken 24 h following injection. Urine was aspirated using 1 ml syringes with 26 GA needles and placed into a 1.5 ml microtube. 10 µl of each urine sample was subsequently transferred to vials containing 5 ml Ultima Gold scintillation cocktail (PerkinElmer). Vials were shaken for 10 min and then placed in the scintillation counter. Radioactivity was measured for 5 min per sample, and counting efficiencies and final adjusted estimates for the amount of radioactivity per sample (in d.p.m.) were calculated as described earlier.

[^3H]E₂ transfer to females administered E₂

We then tested whether or not administering unlabeled E₂ to female subjects would alter the uptake of radioactivity in tissues resulting from exposure to a [^3H]E₂-treated conspecific. At the onset of the darkness phase of the light:darkness cycle, ten stimulus females were each given an i.p. injection of 50 µCi [2,4,6,7- ^3H](N)-E₂ (stock solution in ethanol, 1.0 µCi/µl, 72.1 Ci/mM, PerkinElmer) diluted in 0.15 ml saline to yield a 1:3 ethanol:saline solution. This dose provides a total of 188.6 ng [^3H]E₂. Five other stimulus females for the control treatment were simply given 0.2 ml ethanol:saline solution. Within 15 min, ten female subjects were given 0.05 ml peanut oil s.c., while five others were injected s.c. with 0.05 ml oil containing 5 µg E₂ (Sigma). After 1 h, female subjects were each paired with a stimulus female in a clean cage, such that there were five oil-treated subjects matched with five ethanol-treated stimulus females in the control treatment, five oil-treated female subjects paired with five [^3H]E₂-treated stimulus females, and five E₂-treated female subjects paired with five [^3H]E₂-treated stimulus females. After another 24 h, female subjects were anaesthetized as described earlier and perfused with 20 ml heparinized PBS

(1000 units heparin/ml). Uterus, ovaries, olfactory bulbs, and cerebral cortex were collected as in previous experiments. Additional tissues were also taken, including lateral slices of the cerebellum, a section of the hypothalamic region (bilateral sections posterior to the optic chiasm on the ventral surface of the brain above the pituitary stalk), a sample of the heart, a sample of one lung, a sample of the liver, and a sample of abdominal adipose. Tissues were processed and measured for radioactivity on the scintillation counter as described earlier.

Statistical analyses

Data are presented as means ± S.E.M. The threshold for statistical significance (α level) was set at $P < 0.05$, unless otherwise stated. Where possible, data were analyzed by ANOVA, and significant effects were followed by Newman-Keuls multiple comparisons on all pairs of treatment combinations. In experiments where female subjects were exposed to stimulus conspecifics given [^3H]steroids or the ethanol vehicle (controls), there were highly unequal variances among treatments by Bartlett's test due to very low variance in the control treatments. Therefore, such data were analyzed using a conservative nonparametric statistic, the Wilcoxon rank-sum test. Bonferroni adjustments were applied to the probabilities in order to correct for the fact that there were multiple tissues measured in each experiment. We focused statistical comparisons on differences between treatments within each tissue, omitting comparisons across tissues due to potentially differential impacts of perfusion upon these tissues.

Results

Natural urinary E₂ and P₄

E₂ and P₄ were present in every urine sample from both males and females, consistently over 6 successive days of measurement. An average value over days was taken for each subject, and the means (± S.E.M.) of those values for each subject are presented (Table 1). The ranges among individual samples for females were 0.8–66.2 ng E₂/ml and 6.1–138.4 ng P₄/ml, whereas those for males were 0.6–5.6 ng E₂/ml and 4.0–66.7 ng P₄/ml.

Direct exposure of cycling females to [^3H]steroid-injected males

Following ~48 h of free interaction with stimulus males given ethanol (control), [^3H]E₂, or [^3H]P₄, female subjects

Table 1 Urinary P_4 , E_2 , creatinine-adjusted P_4 , creatinine-adjusted E_2 , and creatinine levels of female and male adult CF1 mice. Each subject was measured for 6 successive days and assigned the average value for all available samples over those days, and the mean $\pm$ s.e.m. of those values across subjects is presented

	Females	Males
ng P_4 /ml urine	28.54 $\pm$ 5.78	13.54 $\pm$ 2.75
ng P_4 /mg creatinine	29.33 $\pm$ 6.19	17.14 $\pm$ 2.93
ng E_2 /ml urine	13.23 $\pm$ 1.74	4.14 $\pm$ 0.50
ng E_2 /mg creatinine	13.57 $\pm$ 1.76	6.37 $\pm$ 0.99
mg creatinine/ml urine	0.97 $\pm$ 0.07	0.78 $\pm$ 0.06

were killed and their tissues were processed for liquid scintillation counting to assess radioactivity. Planned orthogonal comparisons were made for each tissue between controls and subjects exposed to $[^3H]$ steroid-treated males. Considering that six tissues were measured, the Bonferroni-corrected threshold value for significance is $P < 0.0083$. Radioactivity (d.p.m./mg) among females paired with males injected with $[^3H]E_2$ was in a completely non-overlapping range from that of control females (Fig. 1); this comparison was significant for all tissues, $W_s = 15$, $P < 0.001$. Females paired with males injected with $[^3H]P_4$ also had significantly more radioactivity (in a non-overlapping range) compared with controls for uterus, ovaries, and muscle, $W_s = 15$, $P < 0.001$. Radioactivity in other tissues showed some overlap in range between the $[^3H]P_4$ and control treatments and did not reach significance. In comparing only the animals exposed to $[^3H]$ steroid-treated males through ANOVA, there was a main effect of condition, $F(1,8) = 10.62$, $P = 0.0115$, indicating more radioactivity in females exposed to $[^3H]E_2$ -injected males than in those exposed to $[^3H]P_4$ -injected males.

Direct exposure of cycling females to $[^3H]$ steroid-injected females

Following 48 h of pairing and free interaction with stimulus females injected with ethanol (control), $[^3H]E_2$, or $[^3H]P_4$, radioactivity was measured in tissues of female subjects. Planned orthogonal comparisons were made for each tissue between controls and subjects exposed to $[^3H]$ steroid-treated males. Considering that six tissues were measured, the Bonferroni-corrected threshold value for significance is $P < 0.0083$. Radioactivity among females paired with stimulus females injected with $[^3H]E_2$ was in a completely non-overlapping range from that of control females (Fig. 2); this comparison was significant for all

tissues, $W_s = 15$, $P < 0.001$. Females paired with stimulus females injected with $[^3H]P_4$ also had significantly more radioactivity (in a non-overlapping range) for uterus, ovaries, muscle, olfactory bulb, and cortex, $W_s = 10$, $P < 0.005$; however, the comparison for the MC+DC was not significant due to overlap in range between the treatments. In comparing only the animals exposed to $[^3H]$ steroid-treated females, there was a main effect of condition, $F(1,7) = 13.89$, $P = 0.0074$, indicating more radioactivity in females exposed to $[^3H]E_2$ -injected females than in those exposed to $[^3H]P_4$ -injected females.

Patterns of urinary excretion after $[^3H]$ steroid injection

When adult males or females were given a single injection of $[^3H]E_2$ or $[^3H]P_4$, radioactivity was measured in all samples taken within the next 24 h. Mean values are given for all obtained samples at each collection time (Fig. 3). As expected, urine collections were more readily obtained for females than for males, such that there were substantially more missing values for males. Radioactivity was generally more abundant in female samples than in male samples. In female samples, radioactivity was more evident in earlier samples than later ones for those given $[^3H]E_2$, whereas there was a clear peak in radioactivity 4–6 h after administration in females given $[^3H]P_4$. For males, excreted radioactivity appeared less variable across measurement times. Because of missing values,

Figure 1 Mean ($\pm$ s.e.m.) radioactivity in the uterus, ovaries, muscle, olfactory bulbs (OB), MC+DC, and cerebral cortex of females following exposure to sexually experienced stimulus males for 2 days. Stimulus males were injected with either 10 μ l ethanol (control), 10 μ Ci $[^3H]P_4$, or 10 μ Ci $[^3H]E_2$ once a day for 3 days. Females were each paired with a stimulus male at the onset of darkness following the male's first injection.

Figure 2

Mean ($\pm$ S.E.M.) radioactivity in the uterus, ovaries, muscle, olfactory bulbs (OB), MC+DC, and cerebral cortex of females following exposure to stimulus females for 2 days. Stimulus females were injected with either 10 μ l ethanol (control), 10 μ Ci [³H]P₄, or 10 μ Ci [³H]E₂ once a day for 3 days. The females represented by the data were each paired with a stimulus female at the onset of darkness following the stimulus female's first injection.

statistics were conducted only on a single average sample value for each mouse, collapsing all available samples. Factorial (2 $\times$ 2) ANOVA indicated significant effects of sex, $F(1,21)=111.27$, $P<0.0001$; of [³H]E₂ vs [³H]P₄, $F(1,21)=4.22$, $P=0.0500$; and of the interaction, $F(1,21)=4.26$, $P=0.0490$. Multiple comparisons indicated that urinary radioactivity for females given [³H]P₄ exceeded that from all other treatments and that urinary radioactivity for females given [³H]E₂ exceeded that for both treatments of males.

[³H]E₂ transfer to females administered E₂

Following exposure to stimulus females given [³H]E₂, females pre-treated with unlabeled E₂ had lower uptake of radioactivity from steroid transfer than did females pre-treated with oil vehicle (Fig. 4). Ranges of the data for each tissue were completely non-overlapping between the control subjects and subjects exposed to [³H]E₂-treated stimulus females and given oil. There was some overlap in range between controls and subjects given E₂ and exposed to [³H]E₂-treated stimulus females for the olfactory bulbs, heart, liver, and adipose, but not for the other tissues. Statistical analysis focused on planned comparisons between the two groups exposed to [³H]E₂-treated stimulus females. In comparison with a Bonferroni-corrected threshold of $P<0.005$ for ten tissues, Wilcoxon tests on this contrast in each tissue showed non-overlapping ranges and significant effects in the uterus, lungs, and adipose ($W_s=15$, $P<0.001$), and small overlap

with nevertheless significant effects in the heart and ovaries ($W_s=16$, $P<0.005$). Other tissues did not show significance by the corrected threshold.

Discussion

These data show that [³H]E₂ and [³H]P₄ excreted by a mouse of either sex can be readily absorbed by an unmanipulated adult female cohabitant, with radioactivity observed in her reproductive, brain, and other tissues. Although identical amounts of radioactivity were injected in the [³H]E₂ and [³H]P₄ treatments, radioactivity was more evident in females exposed to [³H]E₂-treated conspecifics than among those exposed to [³H]P₄-treated conspecifics. Our results corroborate those of previous studies with regard to interindividual steroid transfer in this species (Guzzo *et al.* 2010, 2012). That previous work was conducted in the context of the Bruce effect (disruption of blastocyst implantation by non-sire males) and the Vandenberg effect (promotion of reproductive maturation of juvenile females by males). Those studies involved a distinct strain of out-bred males (HS) that show substantially higher levels of urinary E₂ than those observed here for CF1 males (deCatanzaro *et al.* 2006, 2009, Thorpe & deCatanzaro 2012). The previous E₂ measures from HS males were proven to be especially elevated after a few days of exposure to inseminated or juvenile females, which also causes males to show polydipsia, polyuria, and direction of urine toward the

Figure 3

Mean radioactivity in urine of females and males given a single injection of either 10 μ Ci [³H]P₄ or 10 μ Ci [³H]E₂, measured in samples taken hourly for 10 h during the animals' active period (dark phase of the light cycle) and also 24 h after the injection. S.E.M. is given above the mean for females and below the mean for males.

Figure 4

Mean ($\pm$ S.E.M.) radioactivity in tissues of females exposed to ethanol-injected stimulus females (control) or [³H]E₂-treated stimulus females. Females exposed to [³H]E₂-treated stimulus females also directly received either an E₂ or an oil injection before exposure to the stimulus females.

stimulus females (deCatanzaro *et al.* 2006, 2009). Here, we measured natural urinary E₂ and P₄ in isolated CF1 males and females. We also demonstrated for the first time that E₂ and P₄ transfer can occur from male to female and from female to female simply through 2 days of cohabitation.

In samples of urine taken from animals injected with [³H]P₄ or [³H]E₂, radioactivity was greater in urine of females than that of males. Nevertheless, comparable levels of radioactivity were evident in the tissues of non-injected females exposed to [³H]steroid-injected males and females. This is consistent with the fact that males actively deliver their urine to females in their presence (deCatanzaro *et al.* 2009). Male mice tend to urinate more than do females (Drickamer 1995). Males also tend to disperse their urine in small droplets whereas females urinate in puddles (Desjardins *et al.* 1973, Maruniak *et al.* 1974, deCatanzaro *et al.* 2006, 2009), which makes male urine much more difficult to collect. Polyuria and polydipsia can make male urine more dilute (deCatanzaro *et al.* 2009), which could help to account for differences between males and females seen in Fig. 3. The data presented here also indicate that females exposed to [³H]E₂-treated males or females show much greater radioactivity in their tissues than do those exposed to [³H]P₄-treated males or females. This is clearly not due to differences in excretory rates in injected animals, as radioactivity in urine is equal after injection of the two [³H]steroids in males and somewhat greater for females given [³H]P₄ than for those given [³H]E₂. The superior

levels of radioactivity in tissues of recipient females exposed to conspecifics given [³H]E₂ are accordingly due to parameters of absorption and metabolism in these recipients, as was also previously observed when [³H]P₄ and [³H]E₂ were directly given to females intranasally (Guzzo *et al.* 2012).

The quantities of [³H]E₂ and [³H]P₄ that were injected into the stimulus animals were miniscule relative to the endogenous levels of these steroids. In the experiments showing transfer to untreated females, the total quantity injected into each stimulus animal was 91.5 ng [³H]E₂ or 93 ng [³H]P₄ per dose. By contrast, a dose of 60 µg E₂ every 2 days was necessary to restore normal urinary levels of E₂ in castrated males (Thorpe & deCatanzaro 2012), which is almost one thousand times the dose of [³H]E₂ given in these experiments. E₂ and P₄ from endogenous sources each pass into urine in both sexes, where it is detectable by specific antibodies in enzyme immunoassay (see Table 1, also Muir *et al.* (2001) and deCatanzaro *et al.* (2004, 2009)). E₂ from exogenous sources is similarly detectable in urine (Thorpe & deCatanzaro 2012), and the current data show this for both E₂ and P₄ by radioactive tracing. Small, lipophilic sex steroids can readily enter the circulation following nasal or dermal exposure (Scheuplein *et al.* 1969, Waddell & O'Leary 2002, Guzzo *et al.* 2012), and we here show that females exposed to conspecific-excreted [³H]E₂ or [³H]P₄ have radioactivity dispersed throughout the females' bodies. Following nasal entry into the system in rats, the bioavailability of E₂ has been shown to be at least 50% of an equivalent i.v. dose, with the remainder possibly being oxidized to the less active estrogen estrone (Bawarshi-Nassar *et al.* 1989). For P₄, the bioavailability from nasal absorption has been shown to be equal to that from i.v. administration (Bawarshi-Nassar *et al.* 1989). Another indication that nasally administered E₂ remains biologically active is the fact that it can disrupt blastocyst implantation in inseminated females, as does s.c. administration of E₂ (deCatanzaro *et al.* 2001, 2006). Moreover, i.m. injection of E₂ into castrated male mice restores not only their urinary E₂ but also their abilities to disrupt blastocyst implantation in inseminated females and accelerate uterine growth in juvenile females (Thorpe & deCatanzaro 2012), suggesting that a meaningful proportion of males' systemic E₂ arrives in bioactive form in the reproductive tract of nearby females (*cf.* Guzzo *et al.* 2012).

We also demonstrated here that pre-administration of E₂ to females exposed to [³H]E₂-treated conspecifics significantly lessens the uptake of radioactivity in at least some tissues of these females. This suggests that the

injected E₂ displaced [³H]E₂ at receptors and other binding factors (Terenius 1969, Sasson & Notides 1983). While some of the [³H]E₂ administered to the stimulus animals could have been metabolized, this provides further evidence that [³H]E₂ remains bioactive despite potential metabolism in both the stimulus animal and the recipient. We have not made direct comparisons among tissues given the likelihood that perfusion techniques differentially remove blood from these tissues. It is not technically feasible to remove all blood from tissues of mice, even when heparinized saline is used in perfusion as was the case in this last experiment. While many tissues were blanched after perfusion, muscle tissue for example remained relatively red, which, in addition to the involvement of E₂ in glucose homeostasis in skeletal muscle and adipose (Barros *et al.* 2009), could account for the high radioactivity readings observed in this tissue. Estrogen receptors are also involved in blood vessel nitric oxide regulation (Rubanyi *et al.* 1997), and so E₂ bound in capillary walls may factor into the total levels of radioactivity detected in various tissues.

Estrogen and P₄ receptors are heavily concentrated in reproductive tissues of females (e.g. Hiroi *et al.* 1999, Uotinen *et al.* 1999) and are also found in hypothalamus and limbic structures of the brain (e.g. Pfaff 1980, Simerly *et al.* 1990, Mani *et al.* 1997). Increasingly, evidence indicates that estrogen receptors are diversely located throughout the body. Studies using reverse transcription PCR for estrogen receptors α and β showed heavy expression of both receptors in ovary and uterus, detectable expression of estrogen receptor α in liver and heart, and expression of estrogen receptor β in bladder, lung, cerebellum, and other areas of the brain (Kuiper *et al.* 1997). The expression of estrogen receptors imperfectly corresponds with the general distribution of [³H]E₂ following i.v. administration (Eisenfeld & Axelrod 1966). Following nasal or cutaneous administration of [³H]E₂ to non-cycling females, radioactivity can be detected in every tissue examined, with generally greater levels in the uterus and ovaries (Guzzo *et al.* 2010, 2012). An exogenous steroid's route of entry into the body can greatly affect its subsequent distribution (Anand Kumar *et al.* 1974, Guzzo *et al.* 2012). Data from this study showed that steroids transferred from another individual were detectable in diverse tissues of the recipient, but some of the highest concentrations were in the uterus as would be predicted from receptor density.

These data could have implications for the common laboratory practice of group housing of animals. Moreover, the data potentially have implications for a broad range of

phenomena where males and other females influence a female's reproductive state. E₂ and P₄ play critical roles in control of the estrous cycle (Butcher *et al.* 1974, Hsueh *et al.* 1976, Ryan & Schwartz 1980). Exogenous E₂ followed by exogenous P₄ is sufficient to induce behavioral estrus in ovariectomized rats, and supraphysiological levels of P₄ can inhibit female sexual receptivity (Blaustein & Wade 1977). Across species, the estrous cycle is known to be sensitive to housing with conspecifics and exposure to their urine. Mice of some strains can exhibit pseudopregnancy when housed in groups of four per cage (Lee & Boot 1955, 1956), and exposure to males can induce regular estrous cycling in females (Whitten 1956). Similarly, exposure to males will induce estrous behavior in grouped anestrous female goats (Rivas-Muñoz *et al.* 2007), and female Siberian hamsters exposed to the excretions of intact males enter pro-estrus 3 days following the onset of exposure (Dodge *et al.* 2002). In mice, urine from females that are in estrus and under long photoperiods can accelerate the onset of first estrus in other females (Drickamer 1982, 1986). Steroid transfer could also be relevant to some human endocrine phenomena. Axillary glands have some enzymatic capacity to modify steroids and even synthesize them *de novo* (Rothardt & Beier 2001, Zouboulis *et al.* 2007) and very high levels of unconjugated E₂ and P₄ are found in axillary perspiration of young men (Muir *et al.* 2008). Women repeatedly exposed to the axillary extracts of men show more stable menstrual cycle lengths, with fewer aberrant cycles (Cutler *et al.* 1986). Axillary extracts from one woman may influence the recipient woman's menstrual cycle (Preti *et al.* 1986, Preti 1987, *cf.* Stern & McClintock 1998). Given the importance of steroids such as E₂ and P₄ in regulating the female reproductive cycle, we suggest that the possibility of interindividual steroid transfer should not be overlooked in the design and analysis of endocrine research.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Anand Kumar TC, David GFX, Umberkoman B & Saini KD 1974 Uptake of radioactivity by body fluids and tissues in rhesus monkeys after intravenous injection or intranasal spray of tritium-labelled oestradiol and progesterone. *Current Science* **43** 435–439.
- Barros RPA, Gabbi C, Morani A, Warner M & Gustafsson J-Å 2009 Participation of ER α and ER β in glucose homeostasis in skeletal muscle and white adipose tissue. *American Journal of Physiology. Endocrinology and Metabolism* **297** E124–E133. (doi:10.1152/ajpendo.00189.2009)
- Bawarshi-Nassar RN, Hussain AA & Crooks PA 1989 Nasal absorption and metabolism of progesterone and 17 β -estradiol in the rat. *Drug Metabolism and Disposition* **17** 248–254.
- Beaton EA, Khan A & deCatanzaro D 2006 Urinary sex steroids during sexual development in female mice and in proximate novel males. *Hormone and Metabolic Research* **38** 501–506. (doi:10.1055/s-2006-949521)
- Blaustein JD & Wade GN 1977 Concurrent inhibition of sexual behavior, but not brain [3 H]estradiol uptake, by progesterone in female rats. *Journal of Comparative and Physiological Psychology* **91** 742–751. (doi:10.1037/h0077366)
- Bruce HM 1960 A block to pregnancy in the mouse caused by proximity of strange males. *Journal of Reproduction and Fertility* **1** 96–103. (doi:10.1530/jrf.0.0010096)
- Butcher RL, Collins WE & Fugo NW 1974 Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17 β throughout the 4-day estrous cycle of the rat. *Endocrinology* **94** 1704–1708. (doi:10.1210/endo-94-6-1704)
- Cutler WB, Preti G, Krieger A, Huggins GR, Garcia CR & Lawley HJ 1986 Human axillary secretions influence women's menstrual cycles: the role of donor extract from men. *Hormones and Behavior* **20** 463–473. (doi:10.1016/0018-506X(86)90008-5)
- deCatanzaro D, Baptista MAS & Vella ES 2001 Administration of minute quantities of 17 β -estradiol on the nasal area terminates early pregnancy in inseminated female mice. *Pharmacology, Biochemistry, and Behavior* **69** 503–509. (doi:10.1016/S0091-3057(01)00554-8)
- deCatanzaro D, Muir C, Beaton E, Jetha M & Nadella K 2003 Enzymeimmunoassay of oestradiol, testosterone and progesterone in urine samples from female mice before and after insemination. *Reproduction* **126** 407–414. (doi:10.1530/rep.0.1260407)
- deCatanzaro D, Muir C, Beaton EA & Jetha M 2004 Non-invasive repeated measurement of urinary progesterone, 17 β -estradiol, and testosterone in developing, cycling, pregnant, and postpartum female mice. *Steroids* **69** 687–696. (doi:10.1016/j.steroids.2004.07.002)
- deCatanzaro D, Beaton EA, Khan A & Vella E 2006 Urinary oestradiol and testosterone levels from novel male mice approach values sufficient to disrupt early pregnancy in nearby inseminated females. *Reproduction* **132** 309–317. (doi:10.1530/rep.1.00965)
- deCatanzaro D, Khan A, Berger RG & Lewis E 2009 Exposure to developing females induces polyuria, polydipsia, and altered urinary levels of creatinine, 17 β -estradiol, and testosterone in adult male mice (*Mus musculus*). *Hormones and Behavior* **55** 240–247. (doi:10.1016/j.yhbeh.2008.10.013)
- Desjardins C, Maruniak JA & Bronson FH 1973 Social rank in house mice: differentiation revealed by ultraviolet visualization of urinary marking patterns. *Science* **182** 939–941. (doi:10.1126/science.182.4115.939)
- Dodge JC, Kristal MB & Badura LL 2002 Male-induced estrus synchronization in the female Siberian hamster (*Phodopus sungorus sungorus*). *Physiology & Behavior* **77** 227–231. (doi:10.1016/S0031-9384(02)00851-X)
- Drickamer LC 1982 Delay and acceleration of puberty in female mice by urinary chemosignals from other females. *Developmental Psychobiology* **15** 433–445. (doi:10.1002/dev.420150505)
- Drickamer LC 1986 Effects of urine from females in oestrus on puberty in female mice. *Journal of Reproduction and Fertility* **77** 613–622. (doi:10.1530/jrf.0.0770613)
- Drickamer LC 1995 Rates of urine excretion by house mouse (*Mus domesticus*): differences by age, sex, social status, and reproductive condition. *Journal of Chemical Ecology* **21** 1481–1493. (doi:10.1007/BF02035147)
- Eisenfeld AJ & Axelrod J 1966 Effect of steroid hormones, ovariectomy, estrogen pretreatment, sex and immaturity on the distribution of 3 H-estradiol. *Endocrinology* **79** 38–42. (doi:10.1210/endo-79-1-38)
- Goldzieher JW & Baker RE 1960 The percutaneous absorption of estradiol-17 β and progesterone. *Journal of Investigative Dermatology* **35** 215–218. (doi:10.1038/jid.1960.106)
- Guzzo AC, Berger RG & deCatanzaro D 2010 Excretion and binding of tritium-labelled oestradiol in mice (*Mus musculus*): implications for the Bruce effect. *Reproduction* **139** 255–263. (doi:10.1530/REP-09-0382)
- Guzzo AC, Jheon J, Imtiaz F & deCatanzaro D 2012 Oestradiol transmission from males to females in the context of the Bruce and Vandenbergh effects in mice (*Mus musculus*). *Reproduction* **143** 539–548. (doi:10.1530/REP-11-0375)
- Harder JD & Jackson LM 2003 Male pheromone stimulates ovarian follicular development and body growth in juvenile female opossums (*Monodelphis domestica*). *Reproductive Biology and Endocrinology* **1** 21. (doi:10.1186/1477-7827-1-21)
- Hiroi H, Inoue S, Watanabe T, Goto W, Orimo A, Momoeda M, Tsutsumi O, Taketani Y & Muramatsu M 1999 Differential immunolocalization of estrogen receptor α and β in rat ovary and uterus. *Journal of Molecular Endocrinology* **22** 37–44. (doi:10.1677/jme.0.0220037)
- Hsueh AJW, Peck EJ & Clark JH 1976 Control of uterine estrogen receptor levels by progesterone. *Endocrinology* **98** 438–444. (doi:10.1210/endo-98-2-438)
- Hueber F, Besnard M, Schaefer H & Wepierre J 1994 Percutaneous absorption of estradiol and progesterone in normal and appendage-free skin of the hairless rat: lack of importance of nutritional blood flow. *Skin Pharmacology* **7** 245–256. (doi:10.1159/000211301)
- Khan A, Berger RG & deCatanzaro D 2009 Preputialectomised and intact adult male mice exhibit an elevated urinary ratio of oestradiol to creatinine in the presence of developing females, whilst promoting uterine and ovarian growth of these females. *Reproduction, Fertility, and Development* **21** 860–868. (doi:10.1071/RD08299)
- Kuiper GG, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S & Gustafsson J-Å 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β . *Endocrinology* **138** 863–870. (doi:10.1210/en.138.3.863)
- Lee S van der & Boot LM 1955 Spontaneous pseudopregnancy in mice. *Acta Physiologica et Pharmacologica Neerlandica* **4** 442–444.
- Lee S van der & Boot LM 1956 Spontaneous pseudopregnancy in mice. II. *Acta Physiologica et Pharmacologica Neerlandica* **5** 213–215.
- Leinders-Zufall T, Brennan P, Widmayer P, Chandramani SP, Maul-Pavicic A, Jäger M, Li X-H, Breer H & Zufall F 2004 MHC class I peptides as chemosensory signals in the vomeronasal organ. *Science* **306** 1022–1037. (doi:10.1126/science.1102818)
- Ma W, Miao Z & Novotny MV 1998 Role of the adrenal gland and adrenal-mediated chemosignals in suppression of estrus in the house mouse: the Lee-Boot effect revisited. *Biology of Reproduction* **59** 1317–1320. (doi:10.1095/biolreprod59.6.1317)
- Ma W, Miao Z & Novotny MV 1999 Induction of estrus in grouped female mice (*Mus domesticus*) by synthetic analogues of preputial gland constituents. *Chemical Senses* **24** 289–293. (doi:10.1093/chemse/24.3.289)
- Mani SK, Blaustein JG & O'Malley BW 1997 Progesterone receptor function from a behavioral perspective. *Hormones and Behavior* **31** 244–255. (doi:10.1006/hbeh.1997.1393)
- Marashi V & Rülcke T 2012 The Bruce effect in Norway rats. *Biology of Reproduction* **86** 1–5. (doi:10.1095/biolreprod.111.093104)

- Marchlewska-Koj A, Cavaggioni A, Mucignat-Caretta C & Olejniczak P 2000 Stimulation of estrus in female mice by male urinary proteins. *Journal of Chemical Ecology* **26** 2355–2366. (doi:10.1023/A:1005578911652)
- Maruniak JA, Owen K, Bronson FH & Desjardins C 1974 Urinary marking in male house mice: responses to novel environmental and social stimuli. *Physiology & Behavior* **12** 1035–1039. (doi:10.1016/0031-9384(74)90151-6)
- Muir C, Vella ES, Pisani N & deCatanzaro D 2001 Enzyme immunoassay of 17 β -estradiol, estrone conjugates, and testosterone in urinary and fecal samples from male and female mice. *Hormone and Metabolic Research* **33** 653–658. (doi:10.1055/s-2001-18692)
- Muir CC, Treasurywala K, McAllister S, Sutherland J, Dukas L, Berger RG, Khan A & deCatanzaro D 2008 Enzyme immunoassay of testosterone, 17 β -estradiol, and progesterone in perspiration and urine of preadolescents and young adults: exceptional levels in men's axillary perspiration. *Hormone and Metabolic Research* **40** 819–826. (doi:10.1055/s-0028-1082042)
- Pfaff DW 1980 Estrogens and brain function: neural analysis of a hormone-controlled mammalian reproductive behavior. New York: Springer-Verlag.
- Preti G 1987 Reply to Wilson. *Hormones and Behavior* **21** 547–550. (doi:10.1016/0018-506X(87)90013-4)
- Preti G, Cutler WB, Garcia CR, Huggins GR & Lawley HJ 1986 Human axillary secretions influence women's menstrual cycles: the role of donor extract of females. *Hormones and Behavior* **20** 474–482. (doi:10.1016/0018-506X(86)90009-7)
- Rivas-Muñoz R, Fitz-Rodríguez G, Poindron P, Malpoux B & Delgadillo JA 2007 Stimulation of estrous behavior in grazing female goats by continuous or discontinuous exposure to males. *Journal of Animal Science* **85** 1257–1263. (doi:10.2527/jas.2006-115)
- Rohrbach C 1982 Investigation of the Bruce effect in the Mongolian gerbil (*Meriones unguiculatus*). *Journal of Reproduction and Fertility* **65** 411–417. (doi:10.1530/jrf.0.0650411)
- Rothardt G & Beier K 2001 Peroxisomes in the apocrine sweat glands of the human axilla and their putative role in pheromone production. *Cellular and Molecular Life Sciences* **58** 1344–1349. (doi:10.1007/PL00000946)
- Rubanyi GM, Freay AD, Kauser K, Sukovich D, Burton G, Lubahn DB, Couse JF, Curtis SW & Korach KS 1997 Vascular estrogen receptors and endothelium-derived nitric oxide production in the mouse aorta. Gender difference and effect of estrogen receptor gene disruption. *Journal of Clinical Investigation* **99** 2429–2437. (doi:10.1172/JCI119426)
- Ryan KD & Schwartz NB 1980 Changes in serum hormone levels associated with male-induced ovulation in group-housed adult female mice. *Endocrinology* **106** 959–966. (doi:10.1210/endo-106-3-959)
- Sasson S & Notides AC 1983 Estriol and estrone interaction with the estrogen receptor: II. Estriol and estrone-induced inhibition of the cooperative binding of [³H]estradiol to the estrogen receptor. *Journal of Biological Chemistry* **258** 8118–8122.
- Schaefer H, Zesch A & Stüttgen G 1982 Skin permeability. Berlin: Springer Verlag.
- Scheuplein RJ, Blank I, Brauner G & MacFarlane D 1969 Percutaneous absorption of steroids. *Journal of Investigative Dermatology* **52** 63–70. (doi:10.1038/jid.1969.9)
- Simerly RB, Chang C, Muramatsu M & Swanson LW 1990 Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an *in situ* hybridization study. *Journal of Comparative Neurology* **294** 76–95. (doi:10.1002/cne.902940107)
- Stern K & McClintock MK 1998 Regulation of ovulation by human pheromones. *Nature* **392** 177–179. (doi:10.1038/32408)
- Terenius L 1969 The effect of steroidal hormones on the binding of 17 β -estradiol by the mouse uterus and vagina. *Steroids* **13** 311–325. (doi:10.1016/0039-128X(69)90041-5)
- Thorpe JB & deCatanzaro D 2012 Oestradiol treatment restores the capacity of castrated males to induce both the Vandenberg and the Bruce effects in mice (*Mus musculus*). *Reproduction* **143** 123–132. (doi:10.1530/REP-11-0251)
- Uotinen N, Puustinen R, Pasanen S, Manninen T, Kivineva M, Syväla H, Tuohimaa P & Ylikomi T 1999 Distribution of progesterone receptor in female mouse tissues. *General and Comparative Endocrinology* **115** 429–441. (doi:10.1006/gcen.1999.7333)
- Vandenberg JG 1967 Effect of the presence of a male on the sexual maturation of female mice. *Endocrinology* **81** 345–349. (doi:10.1210/endo-81-2-345)
- Waddell B & O'Leary P 2002 Distribution and metabolism of topically applied progesterone in a rat model. *Journal of Steroid Biochemistry* **80** 449–455. (doi:10.1016/S0960-0760(02)00032-8)
- Whitten WK 1956 Modification of the oestrous cycle of the mouse by external stimuli associated with the male. *Journal of Endocrinology* **13** 399–404. (doi:10.1677/joe.0.0130399)
- Zouboulis CC, Chen WC, Thornton MJ, Qin K & Rosenfield R 2007 Sexual hormones in human skin. *Hormone and Metabolic Research* **39** 85–95. (doi:10.1055/s-2007-961807)

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