MALES, DIET, PRENATAL ANDROGENS AND FEMALE SEXUAL MATURITY

INFLUENCE OF ADULT MALES, DIETARY PHYTOESTROGENS, AND AN INDEX OF *IN UTERO* ANDROGEN EXPOSURE ON SEXUAL DEVELOPMENT IN THE FEMALE MOUSE (*MUS MUSCULUS*)

By

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Abstract

The age at which a juvenile female reaches sexual maturity can be modulated by a variety of environmental and social factors. Experiments described in this thesis were designed to enhance the current understanding of the relationships among three variables that influence the onset of sexual maturation in female mice (*Mus musculus*), including: [1] exposure to dietary phytoestrogens during development, [2] variations in prenatal androgens, and [3] the presence or absence of genetically-unrelated males after weaning. For the first time, age at onset of male-induced female puberty was investigated using non-invasive behavioural and fertility measures. Through enzyme immunoassay procedures, daily output of urinary creatinine, 17β-estradiol, and progesterone was profiled in developing females that were either isolated or exposed to adult males. Uterine and ovarian tissue was also measured in such females, and male exposure was observed to increase reproductive tissue mass and was influenced by prior androgen exposure in interaction with diet and male presence. Male-exposed females fed a diet containing phytoestrogens immediately became sexually receptive when housed directly with males, and they conceived earlier than females in other conditions. Females with longer anogenital distance, which reflects higher *in utero* androgen exposure, displayed more escape attempts and aggressive posturing in the direct presence of males, especially when they had been housed near males and fed the phytoestrogen-containing diet. Urinary 17β-estradiol was substantially reduced in females raised on the phytoestrogen-free diet. Urinary output of progesterone was not strongly influenced by diet. Male-exposed females' output of progesterone and 17β-estradiol was more dynamic in

comparison to that of isolated females. The size of this effect depended on diet, prior androgen exposure, and whether urinary steroid measures were adjusted by urinary creatinine. Urinary creatinine was elevated by the low phytoestrogen diet and reduced by male exposure. These data suggest that dietary phytoestrogens and *in utero* androgen exposure interact with presence or absence of males in determining the age at onset of sexual maturity in developing females.

A final experiment was designed to examine two components of adult male urine, preputial gland emissions and unconjugated estrogens, that have been posited to act on females to advance reproductive maturation. Intact and preputialectomized males were compared in their output of urinary creatinine, 17β-estradiol, and testosterone, and in their influence on reproductive tissue in juvenile females. Lack of preputial glands did not hinder the capacity of males to induce uterine and ovarian growth in females. Male urinary creatinine was reduced by exposure to juvenile females. Creatinine-adjusted 17β-estradiol and testosterone were greater in female-exposed males, regardless of whether the preputial glands were present. Based on these findings and those reported elsewhere, it is probable that male excreted urinary steroids are important in regulating reproductive changes in developing females exposed to males.

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LIST OF ABBREVIATION

Enzyme-linked immunosorbant assay	ELISA
Hypothalamic-pituitary-gonadal	HPG
Gonadotropin-releasing hormone	GnRH
Luteinizing hormone	LH
Follicle-stimulating hormone	FSH
Estrogen receptor	ER
Intrauterine position	IUP
Female fetus gestating between male siblings	2M
Female fetus gestating between female siblings	0M
Low molecular weight	LMW
2-sec-butyl-4, 5-dihydrothiazole	SBT
3,4-dehydro-exo-breviocomin	DHB
6-hydroxy-6-methyl-3-heptanone	Heptanone
Major urinary proteins	MUPs
Insulin-like growth factor type 1	IGF-1
Ano-genital distance index	AGDI
Metabolizable energy	ME

Chapter 1

General Introduction

The reproductive biology of a developing female is influenced by a series of physiological, environmental, and behavioural events that generally act to maximize reproduction (Bronson & Stetson; 1973; Ebling, 2005; Ryan & Vandenbergh, 2002). The age at which a juvenile female reaches sexual maturity may be modulated by a number of factors including genetics (Drickamer, 1981), hormonal environment during gestation (McDermott *et al.*, 1978; vom Saal, 1989), diet throughout development (Odum *et al.*, 2001), and external conditions such as temperature and photoperiods (Barnett & Coleman, 1959; Drickamer, 1975a; 1975b). Chemosignals from other individuals can also greatly accelerate or retard development in juvenile females (Colby & Vandenbergh, 1974; Drickamer, 1982, 1983, 1984a, 1984b, 1984c, 1986; Drickamer & Murphy, 1978; McIntosh & Drickamer, 1977) and influence ovarian cycling once adulthood ensues (Bronson & Whitten, 1968).

The acceleration of sexual maturity due to male stimulation is an established phenomenon in a variety of rodent species (meadow vole in Baddaloo & Clulow, 1981; hopping mouse in Breed, 1976; opossum in Harder & Jackson, 2003; lemming in Hasler & Banks, 1975; prairie vole in Hasler & Nalbandov, 1974; pine vole in Lepri & Vandenbergh, 1986; prairie deermouse in Lombardo & Terman, 1980; vole in Spears & Clarke, 1986; house mouse in Vandenbergh, 1969; rat in Vandenbergh, 1976). It has also been observed in some larger mammals (boar in Brooks & Cole, 1970; tamarin in Epple & Katz, 1980; cattle in Izard & Vandenbergh, 1982). It is well accepted that male urine serves as a key vector for the transmission of biologically active signals (Colby & Vandenbergh, 1974; Drickamer, 1984a, 1988; Drickamer & Mikesic, 1990; Jemiolo *et*

al., 1985; Lombardi *et al.*, 1976; Novotny *et al.*, 1999a, 1999c; Vandenbergh *et al.*, 1976). Adult male urine contains a rich repository of chemicals that are important in regulating endocrine and reproductive changes in recipient females (Bronson & Whitten, 1968; Drickamer, 1983, 1984b; Drickamer & Murphy, 1978; Jemiolo *et al.*, 1985; Morè, 2006; Mucignat-Caretta *et al.*, 1995a, 1995b). Topical nasal application of male urine alone can elicit major developmental changes in exposed females (Colby & Vandenbergh, 1974; Drickamer, 1982; 1983). However, the precise urinary components that accelerate sexual maturity in immature females have not been entirely identified. As well, sources in the male that are essential for the production of these chemosignals, such as which organs or glands, have yet to be fully characterized.

The hormonal environment of a female fetus may also influence developmental events occurring later in life. In litter-bearing mammals, androgens released into the uterus by neighbouring male littermates can have masculinizing effects on female siblings (vom Saal, 1979). The timing of puberty is complex, being regulated by environmental and genetic factors, with age at puberty in males often occurring later than in females (Glucksmann, 1974). Females gestating between neighbouring males show delays in onset of sexual maturity in comparison to their counterparts with less androgen exposure (McDermott *et al.*, 1978; vom Saal, 1989). Therefore, in studies investigating female developmental trajectories, it becomes important to account for *in utero* androgen exposure in experimental subjects. One commonly used index of this has been the female's anogenital distance (McDermott *et al.*, 1978; vom Saal, 1989; vom Saal & Bronson, 1978; Zehr *et al.*, 2001).

Dietary phytoestrogen content may also influence timing to puberty. Recently there have been some concerns that laboratory rodent diets formulated with soybean as a primary protein ingredient result in high and steady-state serum concentrations of phytoestrogens (Brown & Setchell, 2001). These physiologically active compounds are non-steroidal but similar in structure to 17β-estradiol, the most potent mammalian estrogen (Kurzer & Xu, 1997). Phytoestrogens are capable of binding to estrogen receptors (Kuiper *et al.*, 1998) and are also able to cross the placental barrier (Brown & Setchell, 2001; Ikegami *et al.*, 2006), potentially having an effect on prenatal hormonal exposure.

The experiments presented in this thesis were designed to examine how proximity to adult males, dietary phytoestrogens, and prior *in utero* androgen exposure interact to influence timing to sexual maturity in female mice. One major goal was to assess the onset of female sexual maturity using non-invasive measures that minimized human handling. Traditional laboratory methods used to assess female development in small laboratory rodents often involve invasive procedures such as blood sampling or repeated collection of cells from the vaginal epithelium (Bronson & Desjardins, 1974; Bronson & Stetson, 1973; Vandenbergh, 1967; 1969; 1976). These methods can introduce artifacts such as cessation of cycling in receptive females (Diamond, 1970) or induction of abnormal cellular changes in the vaginal canal (Emery & Bchwabe, 1936). Human handling itself can be a stressor (deCatanzaro & MacNiven, 1992), which could influence hormonal dynamics and obscure results. I have assessed onset of female sexual maturity using non-invasive behavioural measures of sexual receptivity and fertility. I have also

used enzyme-linked immunosorbant assay (ELISA) to profile output of urinary progesterone and 17β-estradiol from developing females for 2 weeks, starting at the time of weaning (at 28 days of age) and during housing alone or near two unrelated adult-males. Non-invasiveness of techniques used to collect urine samples from subjects allowed examination of ongoing hormonal levels within the same individual, which is preferable to techniques previously employed to monitor hormonal levels in blood.

A final study focused on the capacity of adult males to induce female sexual maturity following removal of the preputial glands, considered as important contributors to puberty-accelerating chemosignals in male urine (Ma *et al.*, 1999; Novotny *et al.*, 1999a, 1999c). Since female sexual maturity is also sensitive to exogenous estrogens (Bronson, 1975), and males excrete higher levels of urinary estradiol in the presence of developing females than in isolation (Beaton *et al.*, 2006; deCatanzaro *et al.*, 2009), I also profiled urinary output of testosterone and17β-estradiol in preputialectomized and intact males while in the presence or absence of juvenile females.

Three major experiments are presented in this thesis. Broadly, these studies were designed to investigate whether exposure to exogenous estrogens in the environment of a developing female will affect the rate of her sexual maturation. It was hypothesized that female mice exposed to high quantities of dietary phytoestrogens and/or estradiol from the excretions of unrelated males would reach sexual maturity earlier than unexposed counterparts. The three main objectives of this thesis were to: [1] examine the influence of dietary and male factors that influence timing to female sexual maturity, [2] explore

the impact of *in utero* androgen exposure on female development, and [3] assess female sexual maturity using non-invasive experimental procedures.

The remainder of this chapter will first discuss endocrine changes that accompany female development, starting from gestation and until the time of sexual maturity. Next, the impact of unrelated adult males on the physiology of pre-pubertal females will be addressed, followed by a discussion of how sexual maturity is assessed in laboratory rodents and some potential confounds that might arise due to excessive handling of experimental subjects. Subsequently, a general introduction on dietary phytoestrogens will be presented, including concerns that have been raised about their ability to influence development and reproduction in females. A final section will discuss literature on various pheromonal factors that might influence sexual development in females. These include the impact of steroid exposure from neighbouring male littermates during gestation, preputial secretions from non-related males, and the influences of male urinary constituents such as proteins and steroids on sexual maturity in exposed females.

Female Sexual Development

Studies of the rat and mouse have contributed substantially to our understanding of female sexual development. Laboratory rodents are convenient animals since they grow rapidly, reproduce quickly, and are able to mate throughout the year. There is a general assumption that mechanisms underlying the processes of sexual development are conserved across mammalian species (Ojeda & Skinner, 2006). Accordingly, many of

the results obtained from experiments using rodent subjects might be extrapolated to other mammals, including humans.

Sexual maturity in female mammals can be defined by the attainment of gonadal cycling and fertility, a process that encompasses major changes in the morphology, physiology, and behaviour of an individual (Bronson & Desjardins, 1974; Bronson & Stetson, 1973; Ojeda & Skinner, 2006; Safranski *et al.*, 1993; Takahashi, 1990; Zehr *et al.*, 2005). These changes can largely be attributed to adjustments in functioning of the hypothalamic-pituitary-gonadal (HPG) system (Ebling, 2005; Ojeda *et al.*, 2003; Ojeda & Skinner, 2006). Around the time of puberty, there are modifications in the pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, important in the attainment of adult-like activity within the pituitary-gonadal axis (Ebling, 2005; Watanabe & Terasawa, 1989). This in turn results in alterations in output of pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which are important in initiating follicular development, steroid production, and ovulation (Ojeda & Skinner, 2006; Ubranski & Ojeda, 1985a). The fundamental role of GnRH in reproduction is best seen in the hypogonadal mouse in which the GnRH gene is mutated; affected animals have underdeveloped gonads and exhibit low concentrations of pituitary gonadotropins (Ward & Charlton, 1981). GnRH injections can stimulate LH and FSH production (Yoshimoto *et al.*, 1975) and with appropriate pulsations can even induce onset of pubertal maturation (Wildt *et al.*, 1980).

GnRH neurons in the adult rat are located predominantly in the medial preoptic area of the hypothalamus (King & Anthony, 1984; Silverman *et al.*, 1979). They are

embryonically derived from cells in the olfactory placode (Schwanzel-Fukuda *et al.*, 1985) and are found diffusely throughout the brain in regions such as the septum and organum vasculosum of the lamina terminalis (Jennes & Schwanzel-Fukuda, 1992). GnRH is a decapeptide that enters the pituitary portal vasculature in a pulsatile manner to signal the synthesis and secretion of pituitary gonadotropins. These gonadotropins act on target cells in the ovaries to direct production of ova as well as secretion of other hormones such as estrogens and progestins. Within the brain, these steroids influence GnRH secretion through a neuroendocrine feedback loop and facilitate ovarian cycling and sexual behaviour (Reiter & Grumbach, 1982; Becú-Villalobos *et al.*, 1997).

Although gonadotropin presence can be detected as early as gestational day 17 in the neonatal rat, the fetal and neonatal ovary fail to respond to FSH or LH stimulation (Chowdhury & Steinberger, 1976; Funkenstein *et al.*, 1980; Ojeda *et al.*, 1986; Salisbury *et al.*, 1982). It is likely that ovarian unresponsiveness to gonadotropins in the neonate is due to lack of gonadotropin receptors (Peters *et al.*, 1973; Siebers *et al.*, 1977). Consequently, it is suspected that early ovarian development might be independent of gonadotropin control (Kraiem *et al.*, 1976; Lamprecht *et al.*, 1973). The mechanisms driving ovarian development this early in life are not well understood. It is not until the second week of postnatal life that ovarian follicles become subject to gonadotropin influence, in particular FSH, which facilitates development of follicles (Ojeda & Skinner, 2006; Oktay *et al.*, 1997).

In the infantile animal, sources other than those residing in the immature HPG axis might be playing a role in regulating ovarian activity. The presence of plasma α-

fetoprotein, which readily binds to estrogens, might hinder the operation of the HPG feedback system very early in development (Raynaud *et al.*, 1971). In addition, maternal milk contains a GnRH-like substance indistinguishable from hypothalamic GnRH, which also has the ability to stimulate gonadotropin release *in vitro* (Amarant *et al.* 1982; Smith & Ojeda, 1984). Chronic exposure to exogenous GnRH has been shown to suppress ovarian function (Hsueh & Jones, 1981), and thus GnRH may play an important role in regulating performance of the ovary in a maturing female.

Around the time a developing individual becomes less reliant on mother's milk for nutrition, the ovary begins to grow under the influence of low serum levels of LH and FSH (Ojeda *et al.*, 1986). Although the juvenile ovary undergoes waves of follicular atresia and development, the follicles do not reach the ovulatory stage (Hage *et al.*, 1978; Ingram, 1962; Schwartz, 1974). It is probable that this is due to the relatively low levels of circulating steroids, since the ovary during this phase of development can ovulate if challenged with sufficiently large amounts of exogenous progesterone (McCormack *et al.*, 1964).

The key factors involved in initiation of female sexual maturity have yet to be completely understood. One theory ("gonadostat hypothesis") suggests that as the individual matures, there is a decrease in sensitivity of the hypothalamus and pituitary to steroid-negative feedback (Ramirez & McCann, 1963). In negative feedback, estrogens and progestins from the ovaries influence the hypothalamus to decrease gonadotropin output, which in turn inhibits production of ovarian steroids. Prior to sexual maturity, the underdeveloped gonads secrete relatively small amount of steroids. Circulating levels of

these hormones are enough to inhibit gonadotropin secretion. At about the time of puberty, the hypothalamic-pituitary unit loses its sensitivity to estrogens and progestins, an increase in LH and FSH occurs and results in the onset of sexual maturity (Ramirez & McCann, 1963). Andrews *et al.* (1981) have called the gonadostat hypothesis into question by examining the effect of estradiol on LH secretion before and after the first ovulation in the female rat. They demonstrated that the decrease in responsiveness to estradiol in the HPG system occurs after the first ovulation. A more recent theory suggests involvement of gonad-independent influences on the hypothalamic-pituitary unit, and is commonly referred to as the "central drive" hypothesis (Ojeda & Bilger, 1999). According to this theory, GnRH secretion is thought to be low during early development due to the presence of a central restraint mechanism and the lack of excitatory inputs to hypothalamic neurons (Ojeda & Bilger, 1999). Onset of sexual maturity is now thought to be initiated by a combination of decreases in GABAergic inhibition and increases in glutamatergic stimulation to GnRH neurons (Mitsushima *et al.*, 1994; Terasawa, 1999; Ubranski & Ojeda, 1990)

The complex series of events that culminate in a female's first ovulation, an important hallmark of sexual maturity, has yet to be fully characterized. The initial hormonal manifestation that sexual maturity is underway is a daily change in the manner of LH release. Ubranski and Ojeda (1985b) examined alterations in the secretory pattern of LH between the juvenile and peripubertal period in rats maintained under a photoperiod of 14 hours of light per day (lights on from 0500 – 1900 hours). There was a 1.8 fold increase in mean LH levels in peripubertal animals with some evidence of large

amplitude pulses accompanied by a daily midafternoon minisurge that lasted up to 2 hours. These LH pulses elicit significantly more estrogen and progesterone release by the ovary than other (e.g., morning) LH pulses, suggesting that these mini-surges may be important for the peripubertal activation of ovarian function (Ubranski & Ojeda, 1985a).

There is little debate that the diurnal changes in the pulsatile LH release pattern result from the activation of GnRH secretion (Ebling, 2005; Mitsushima *et al.*, 1994; Ojeda & Bilger, 1999; Terasawa, 1999; Ubranski & Ojeda, 1990), although the underlying mechanisms are less clear. The resulting changes in LH secretion are important for the continuation of development since they stimulate the ovary to produce more estrogens, which can induce further ovarian activation (Ubranski & Ojeda, 1985a). It is suspected that the first preovulatory surge of LH occurs when the hypothalamic-pituitary unit is fully mature and the ovary becomes capable of producing estrogen levels of sufficient magnitude and for a significantly long period of time (Ojeda & Skinner, 2006). The influence of estradiol on LH release can be suppressed if estradiol antibodies are administered up to 15 hours prior to the expected time of ovulation (Ferin *et al.*, 1969). A direct stimulatory effect of progesterone on GnRH discharge has also been demonstrated (Kim & Ramirez, 1982). In this report, *in vitro* treatment with progesterone activated GnRH in hypothalamic fragments from ovariectomized, estradiol-primed immature rats (Kim & Ramirez, 1980).

Morphological and Histological Characteristics of the Ovarian Cycle

The ovarian cycle in rats and mice ranges between 4 – 6 days in length (Asdell, 1964) and is generally divided into four major phases, mainly based on morphological and histological criteria. Cellular changes in the vaginal epithelium follow a regular course throughout the estrous cycle. Three basic types of cells can be obtained by flushing the vagina via a saline solution by method of vaginal lavage. These include leukocytes, cornified epithelial cells (named due to their large, irregular shape and granulated cytoplasm), and nucleated epithelial cells (named due to presence of a clearly visible nucleus). Much like the vagina, the uterus and ovaries also undergo changes during different phases of the estrous cycle. In the late juvenile phase (*anestrus*), the uterus is small and no intrauterine fluid can be detected. The vagina is also closed. Animals in the phase of *early proestrus* have larger uteri with intraluminal fluid with their vagina closed. The phase of *late proestrus* corresponds to a uterus filled with intraluminal fluid and the ovaries show the appearance of large follicles. There is also predominance of nucleated epithelial cells. The phase of *estrus* is the day of ovulation when the uterine fluid is no longer present, fresh corpora lutea can be readily observed, the vagina opens, and vaginal cytology shows cornified cells. The phase of *diestrus* is characterized by a vaginal cytology showing a predominance of leukocytes and by the presence of mature corpora lutea within the ovaries. The corpus luteum forms from a mature ovarian follicle following ovulation and persists if pregnancy occurs (Asdell, 1964; Green, 1966; Ojeda & Skinner, 2006; Westwood, 2008).

Hormonal Control of the Ovarian Cycle

Serum levels of LH are lowest from early morning of estrus, shortly after ovulation, and until mid-day of proestrus. On the afternoon of proestrus, circulating levels of LH begin to increase rapidly in a pulsatile manner (Fox & Smith, 1985; Gallo, 1981) and reach peak levels by the evening. This rapid surge in LH induces follicular rupture and ovulation (Freeman, 2006). The pattern of FSH secretion throughout the cycle is similar to that of LH (Butcher *et al.*, 1974; Gay *et al.*, 1970). Basal FSH levels are secreted from late estrus through mid-day proestrus. From mid-afternoon and onward, proestrous FSH secretion increases along with LH, reaching peak levels by the same evening. LH levels begin to decrease and reach baseline by the early morning of estrus. Although both FSH and LH decline during the early part of estrus, a second rise of FSH occurs during the early morning hours of estrus (Pupkin *et al.*, 1966).

The cyclical release of FSH and LH results in stimulatory (positive) and inhibitory (negative) feedback control by ovarian steroids (estrogens and progestins) acting both on the hypothalamus and pituitary (Freeman, 2006). The secretion of LH and FSH is low from late estrus until early proestrus. Baseline levels of FSH cause the follicles to develop and produce estradiol and progesterone, which in turn participate in a negative feedback loop to maintain low gonadotropin levels (Freeman, 2006). The most potent ovarian steroids for inhibiting gonadotropin release are estrogens (Schwartz & McCormack, 1972). Paradoxically, increases in the secretion of estradiol at about the time of diestrus participate in the positive feedback important in the ovulation-inducing surge of LH (Neil *et al.*, 1971). A large estrogen surge around proestrus participates in a

positive feedback mechanism producing further secretion of GnRH and ultimately resulting in ovulation due to a corresponding LH surge. Estrogen levels decrease to baseline rapidly but progesterone remains elevated for several hours, stimulating mating behaviour (Zucker, 1966).

The Vandenbergh Effect

Influence of Unrelated Adult Males on Female Sexual Development

The finding that genetically-unrelated adult males could decrease time to onset of sexual maturity in females was simultaneously reported by Vandenbergh (1967) and Castro (1967). However this phenomenon has often been called the "Vandenbergh Effect." Vandenbergh reported that when immature female mice (21 days of age) were reared in the presence of an unrelated adult male, they displayed first estrus roughly 20 days earlier than isolated controls. This effect was most prominent when adult males were introduced to females at an early age. For instance, females that were exposed to males at 21 days of age reached sexual maturity at about 37 days of age. Females that were exposed to males starting at 30 or 38 days of age reached sexual maturity at 41.9 and 45.6 days of age respectively. Interestingly, when females were exposed to unrelated males very early in development (*e.g.*, starting at day 2 after birth), their sexual maturity was not hastened. Females exposed to a male after weaning reached first estrus at an earlier age than did females exposed to a male prior to weaning, and both treatments resulted in an earlier appearance of first estrus than seen in females that were denied access to a male (Vandenbergh, 1967).

This effect does not require direct contact with the stimulus male. When pre-pubertal female mice were reared across a wire-mesh screen from adult males, their vaginal openings and first estrus lagged behind those of females housed directly with males by about 1 day. For example, females each housed in the direct company of a male exhibited first estrus at about 39.6 days of age, whereas those indirectly exposed to a male showed first estrus at about 40.6 days of age (Vandenbergh, 1969). Moreover, exposure to bedding soiled with male excretions or treatment with male urine also reduced time to female sexual maturity (Drickamer, 1983). In comparison to urine of dominant males, urine from subordinate males was not as effective in accelerating development (Lombardi & Vandenbergh, 1977), suggesting that certain male characteristics are important in fully inducing this effect.

Nevertheless, the Vandenbergh effect is most robust when juvenile females are housed in the direct presence of males (Vandenbergh *et al.*, 1972), which indicates that chemosignals in male urine synergize with male behaviour. Direct contact with the male and in particular the sexual nature of the contact may help to account for a portion of this effect. For example, Drickamer (1974) housed adult androgen-treated females with juvenile females and observed that the latter matured earlier than did those housed in isolation, but later than did those housed with males. In an attempt to classify behavioural interactions among individuals, juvenile females were paired with normal females, adult neonatally-androgenized females, or adult males. Contact stimulation with the young females by androgenized females was equal in quantity and similar in quality (*e.g.*, body contact, mounts, and thrusts) to the contact observed between such females

and normal adult males. In an additional experiment, when developing females were continuously exposed to a neonatally-androgenized female along with a daily sample of soiled male bedding, they matured at the same age as did females placed with intact adult males.

Hormonal Changes in Juvenile Females in Response to Male Presence

Dramatic hormonal changes begin to take place in juvenile females within one hour of male exposure. The first observable effect consists of a rapid elevation in LH concentration that is maintained for about 4 hours. Serum estradiol levels also begin to rise and are significantly greater at 3 hours from the time of male introduction and increase to about 15 – 20 fold by 12 hours, and a second peak occurs after about 36 hours of male exposure. This second peak is followed by a rapid rise in LH, FSH, and progesterone by about the third day, which is similar to periovulatory changes observed in the adult estrous cycle (Bronson & Desjardins, 1974). These changes could possibly reflect the final maturation of the HPG system (Bronson & Desjardins, 1974). Uterine mass in male-exposed females showed increases as early as 24 hours and a marked twofold increase by about 36 hours after the start of male exposure (Bronson & Stetson, 1973). The initial response to males in juvenile females was noticeable in the uterus well before changes in gonadotropin levels (Bronson & Stetson, 1973). Increasing levels of estrogens can induce changes in FSH and LH similar to those observed in proestrus (Ying *et al.*, 1971). The uterine response to the preovulatory rise in plasma estradiol is required to prepare the tissue for an upcoming increase in progesterone that accompanies

ovulation and is also important for embryo implantation if pregnancy occurs (Freeman, 2006; Huet-Hudson *et al.*, 1989; Martin *et al.*, 1973).

Assessment of Sexual Maturity in Developing Females

Traditional methods used in assessing female pubertal development have been invasive, often requiring repeated human manipulation of the animal (*e.g.*, Bingel, 1972; Bronson & Whitten, 1968; Colby & Vandenberg, 1974; Drickamer, 1974, 1975a, 1975b, 1982, 1983, 1990; Drickamer & Murphy, 1978; Gangrade & Dominic, 1983; Hasler & Banks, 1975; Ma *et al.*, 1999; Massey & Vandenbergh, 1981; Morè, 2006; Pandey & Pandey, 1988; Price & Vandenbergh, 1992; Vandenbergh, 1967, 1969, 1976). In a typical experimental paradigm, an adult male is introduced to either an individually-housed female or a group of developing females. A variety of measures are then conducted in succession, starting with daily inspection to detect occurrence of vaginal opening. Once vaginal introitus is apparent, daily collection of cells from the vaginal epithelium commences in order to determine reproductive states corresponding with shifts in the changing hormonal profile of the female. Determination of the estrous cycle is made by microscopically examining epithelial cell types. For example, proestrus is defined by the presence of entirely-nucleated epithelial cells, estrus is denoted by the prevalence of cornified cells with leukocytes absent or low in number, and the start of diestrus is indicated by the recurrence of leukocytes (Gorbman & Bern, 1962; Stiff *et al.*, 1974).

Multiple examination of vaginal cytology might be impractical for small laboratory rodents since constant physical examination itself could influence experimental outcome. In one instance (Stiff *et al.*, 1974), epithelial cells were reported to be out of phase with expected corresponding changes in uterine mass. Proestrus in these females also lasted up to 12 days. Oviducts of females achieving their first estrus were free of ova, suggesting that a vaginal smear indicative of cycling might not always accompany the full onset of sexual maturity (Stiff *et al.*, 1974). Moreover, frequent smearing may result in persistent estrus (Wade & Doisy, 1935) and disrupt cycling (Emery & Bchwabe, 1936; Stiff *et al.*, 1974). Determination of cell counts from vaginal smears has also been argued to be labour intensive and somewhat subjective (Safranski *et al.*, 1993). Others have suggested that the first appearance of cornified cells is less reliable than is the occurrence of estrus-like epithelia at regular intervals, which might better reflect physiological maturity (Nelson *et al.*, 1990).

Age at vaginal opening, onset of first estrus, and occurrence of normal cycling are also not well correlated (Nelson *et al.*, 1990). Safranski *et al.* (1993) conducted a series of investigations to determine the best measure for assessing the onset of sexual maturity in female mice. Age at vaginal opening occurred at 27.2 days, age of first vaginal estrus occurred at 29.1 days, and age of first mating as evident by presence of a copulatory plug (a protein based mucous produced by male ejaculation, Whittingham & Wood, 1983) occurred at 32.3 days. Mean concentrations of estradiol did not differ among days of vaginal opening, estrus, or copulatory-plug detection. Estradiol levels did peak on the day of vaginal estrus, which was approximately two days following the detection of

vaginal opening and one day prior to the detection of a copulatory plug. The highest proportion of females ovulating was in individuals examined at detection of a copulatory plug. Absence of ovulation at vaginal opening demonstrated that vaginal opening is inaccurate as a measure for attainment of puberty in female mice. The fact that 91% of the females ovulated at first copulatory plug indicated that this might be the most appropriate measure (Safranski *et al.*, 1993). If puberty is defined as the first fertile estrus, the presence of a mating plug in a females housed with a male could be the best indicator of sexual maturity.

An additional parameter used to assess sexual maturity is weight of reproductive tissue (Beaton *et al.*, 2006; Bronson & Desjardins, 1974; Bronson & Stetson, 1973; Hasler & Banks, 1975; Lepri & Vandenbergh, 1986; Mucignat-Caretta *et al.*, 1995a, 1995b; Spears & Clarke, 1986; Stiff *et al.*, 1974; Teague & Bradley, 1978). Around sexual maturity, estrogenic stimulation causes uterine and ovarian cells to proliferate, allowing tissue to grow and increase in mass (Alonso & Rosenfield, 2002; Cooke *et al.*, 1997; Fitzpatrick *et al.*, 1999; Kirkland *et al.*, 1979; Quarmby *et al.*, 1984). Whole body mass may also be measured due to the suspected relationship between puberty and the attainment of a critical body mass (Frisch *et al.*, 1975; Kennedy & Mitra, 1963). However, some reports have not replicated this within the context of male-induced female puberty (Teague & Bradley, 1978). Other methods that monitor occurrence of first estrus, through sectioning of ovaries for mature follicles or detection of the corpora lutea as an indication of successful ovulation, have now become rare (Cheung *et al.*, 1997; Takashima-Sasaki *et al.*, 2006). Researchers investigating sexual development in

female rodents have typically preferred age at vaginal opening, cell counts via vaginal smears and/or weighing of reproductive tissue as their primary method of assessment.

Phytoestrogens

Background

Steroid hormones exert a powerful effect on female sexual maturity, and chemicals that mimic these hormones may alter endogenous hormonal levels and disrupt normal developmental trajectories (Hilakivi-Clarke *et al.*, 1998; Jefferson *et al.*, 2002; Kouki *et al.*, 2003; Levy *et al.*, 1995; Lund *et al.*, 2001; Nagao *et al.*, 2001; Nikaido *et al.*, 2004; Takashima-Sasaki *et al.*, 2006; Thigpen *et al.*, 2003). Phytoestrogens are naturally-occurring compounds found most commonly in plants and are among diphenolic compounds with structural similarities to natural (*e.g.*, 17β-estradiol) and synthetic estrogens (*e.g.*, diethylstilbestrol) and antiestrogens (*e.g.*, tamoxifen). This phenolic ring is found naturally in the vertebrate endocrine system as part of the chemical structure common to estrogens. The presence of the phenolic ring is an important prerequisite for binding to the estrogen receptor (ER; Kuiper *et al.*, 1998; Kurzer & Xu, 1997). Accordingly, phytoestrogens have the ability to exert estrogenic effects on the central nervous system (Lund *et al.*, 2001), to induce delays or hasten the onset of female sexual maturity (Casanova *et al.*, 1999; Hilakivi-Clarke *et al.*, 1998; Kouki *et al.*, 2003; Levy *et al.*, 1995; Lewis *et al.*, 2003; Nikaido *et al.*, 2004, 2005; Takashima-Sasaki *et al.*, 2006; Thigpen *et al.*, 2002, 2003; Whitten & Naftolin, 1992), and to stimulate abnormal changes in the developing female genital tract (Nikaido *et al.*, 2004). They also have the

capability to bind to ERs (Morito *et al.*, 2001) and induce specific estrogen-responsive gene products (Patisaul *et al.*, 2007).

Defined broadly, there are three major groups of biologically-active phytoestrogens, including isoflavones (a subclass of a larger and more ubiquitous group of nutraceuticals called isoflavonoids), lignans, and coumestans. Almost every vegetable, fruit, grain, egg, milk, alcoholic beverage, fish, and meat product contains some combination of these categories (Kuhnle *et al.*, 2008; Thompson *et al.*, 2006). Lignans are commonly found in high quantities in flaxseed and dried fruits such as apricots, dates, and prunes (Thompson *et al.*, 2006). Coumestans (coumestrol, 4'-methoxycoumestrol) are very similar in structure to isoflavones but less frequently found in human diets. They are present in limited quantities in a variety of fruits (*e.g.*, blueberries, dried apricots, grapes, oranges), vegetables (*e.g.*, collards) and teas (black and green) (Thompson *et al.*, 2006). The key sources of isoflavones in human and animal diets are soybean and related products (*e.g.*, soy nuts, soy yogurt, soy bean sprouts, soy protein powder, tofu) along with alfalfa sprouts and beans (Thompson *et al.*, 2006). Principal isoflavones identified thus far are daidzein (4',7-dihydroxy-isoflavone) and genistein (4',5,7-trihydroxyisoflavone). Daidzein and genistein occur in four different forms, including the aglycones or the unconjugated form (daidzein and genistein), and the 7-*O*-β-glucosides (daidzin and genistin), acetylglucosides (6"-*O*-Acetydaidzin and 6"-*O*-Acetylgenistin) and malonylglucosides (6"-*O*-Malonyldaidzin and 6"-*O*-Malonylgenistin) (Kudou *et al.*, 1991). A minor component of isoflavones is glycitein in

the unconjugated form and 6"-*O*-Malonyglycitin in the glucoside form and acetylglycitin (6"-*O*-Acetylglycitin) and malonyglycitin (6"-*O*-Malonylglycitin) (Kudou *et al.*, 1991).

Phytoestrogens and Reproductive Abnormalities in Female Mammals

Major interest in isoflavone research began in the 1940s with the report of breeding problems in female sheep of Western Australia (Bennetts *et al.*, 1946). Infertility resulted from the development of cystic endometrium, also known as clover disease, attributed to increased consumption of *Trifolium subterraneum*, a type of clover rich in isoflavones (Lundh *et al.*, 1990). Similar reproductive abnormalities were observed in New Zealand rabbits fed a diet comprised 50% soybean hay (Kendall *et al.*, 1950). When the effects of genistin were tested on reproductive capacities in female mice, animals fed diets enriched with 0.2% genistin exhibited decreases in the number and size of litters (Carter *et al.*, 1955).

Setchell and colleagues (1984, 1987) have advocated caution against repeated consumption of soy related products, particularly in the laboratory animal population (Brown & Setchell, 2001). This group suggested that major reproductive disorders could occur due to excessive consumption of soy (Setchell *et al.*, 1984). In another report, they attributed the inability of the captive cheetah to reproduce to the presence of soy in its diet (Setchell *et al.*, 1987). Brown and Setchell (2001) reported that isoflavones found in commercial rodent diets lead to high steady-state serum and urine isoflavone concentrations in adult rats and mice, exceeding these animals' normal endogenous estrogen levels by almost 30,000 fold (Barkley *et al.*, 1985; Butcher *et al.*, 1974; Nequin

et al., 1979). In one experiment, groups of adult female mice were fed different commercial diets containing isoflavone content ranging from 0 – 829 μg/g (Brown & Setchell, 2001). Animals that were fed diets that contained 394 μg/g of isoflavones or more showed serum isoflavone levels of up to 2,338 ng/mL (Brown & Setchell, 2001). The bacterially-derived estrogenic metabolite equol (Muthyala *et al.*, 2004; Setchell *et al.*, 1984) was the major isoflavone identified in the urine and serum of animals fed diets high in phytoestrogens (Brown & Setchell, 2001).

Phytoestrogens and Female Reproductive Maturity

Based on the work of Setchell and colleagues, developmental exposure to phytoestrogens has become a particular concern for those using animal models to assess sexual development. However there have been some contradictory findings concerning the influence of phytoestrogens on the timing of female puberty. In some reports, developmental exposure to phytoestrogens has been shown to accelerate time to maturity (Casanova *et al.*, 1999; Nikaido *et al.*, 2004, 2005; Takashima-Sasaki *et al.*, 2006; Thigpen *et al.*, 2003, 2007; Whitten & Naftolin, 1992). However others have shown a delay (Levy *et al.*, 1995) or no change (Goldman *et al.*, 2000).

Differences in the direction of effects of phytoestrogens on sexual maturity could be explained by variations in dose and perhaps timing of exposure. For instance, Kouki *et al.* (2003) exposed rats, starting from the day of birth until postnatal day 10, to once daily subcutaneous injections of 1 mg genistein, 1 mg daidzein, 100 μg of 17β-estradiol, or sesame oil. Vaginal opening was advanced in the genistein- and estradiol-treated

animals, occurring at 28 days of age in the estradiol and genistein groups in comparison to 35 days of age in the oil and diadzein group. Vaginal smears indicated that oil-treated and diadzein-treated females showed a constant 4- or 5-day estrous cycle, whereas genistein- and estradiol-treated animals showed persistent estrus. On the other hand, Levy *et al.* (1995) administered genistein by subcutaneous injection to pregnant dams on gestation days 16-20 at two doses (5 mg and 25 mg), and found a delay in vaginal opening at the lower dose. Accordingly, phytoestrogen-induced developmental effects in females require further investigation.

Pheromonal Communication in Rodents

Relatively little is known about intra-species communication among rodents in the wild. Much of the work on the social structure of mice has been conducted using caged enclosures with either laboratory or wild stocks (DeFries & McClearn, 1972; Nikitina et al., 1976; Reimer & Petras, 1967). Generally, each territory contains a dominant male, several females, and some unrelated subordinate males (Reimer & Petras, 1967). Migration between territories has been most commonly observed in females (Reimer & Petras, 1967). Socially dominant males appear to have a reproductive advantage as they sire most of the young within a territory (DeFries & McClearn, 1972). Dominant males are usually replaced by their offspring (Reimer & Petras, 1967) and most individuals stay within one territory throughout their lifetime (Newsome, 1969).

Social cues among members of an established territory or migrants to a new habitat are important in regulating reproductive behaviour (Bronson, 1979). The action

of pheromonal signals that serve to identify the sex, status, and reproductive state of an individual has been extensively explored (*cf.* Bronson, 1971, 1979). The term "pheromone" is commonly used for externally-emitted substances that convey information between members of the same species. These substances are typically believed to exert their effects via olfactory reception. Two general types of pheromones have been recognized: [1] "signaling" pheromones from the emitting animal produce immediate changes in motor activity on the recipient, and [2] "priming" pheromones trigger neuroendocrine and endocrine activity (Bronson, 1971). Pheromonal factors can be found in saliva, urine, sweat, feces, and other glandular secretions (Birch, 1974; Bronson, 1971; Nishimura *et al.*, 1989; Novotny *et al.*, 1990, 1999a; Teague & Bradley, 1978).

Intrauterine Position

The effects exerted by males on female development are not simply confined to male presence around the time of sexual maturity. *In utero* exposure to testosterone originating from male siblings serves as a priming pheromone since it has important physiological and developmental consequences on recipient females (vom Saal, 1983, 1989; vom Saal & Bronson, 1980; vom Saal & Moyer, 1985; vom Saal *et al.*, 1981). Around the 2nd week of gestation the testes in males differentiate and begin secreting testosterone (Feldman & Bloch, 1978), which steers the subsequent development of the body, central nervous system, and behaviour in a masculine direction (vom Saal, 1979). In litter-bearing mammals, multiple male and female fetuses develop *in utero* and as a

result are exposed to differing amounts of androgens based upon the sex of neighbouring siblings. Intrauterine position (IUP) results in differential transport of androgens between adjacent fetuses. A female fetus gestating between male siblings (2M) will exhibit higher titers of testosterone in her serum and amniotic fluid than does a female located between two female siblings (0M; vom Saal & Bronson, 1980). As a result, the uterine position of a female fetus relative to males will determine the extent to which she will become morphologically (Gandelman *et al.*, 1977; Kinsley *et al.*, 1986), physiologically (Faber & Hughes, 1992), and behaviourally masculinized (Gandelman *et al.*, 1977). This variability in the uterine environment is important for normal development, since females without littermates mature abnormally and reproduce poorly (Clark *et al.*, 1997).

Clemens (1974) originally proposed that the potential mechanism of intrauterine steroid transfer might be due to the inter-amniotic diffusion of androgens from contiguous male fetuses (the "Continguity Hypothesis"). However Meisel and Ward (1981) suggested an alternative hypothesis based on the vascular organization of the uterine horns and capacity of the venous system to drain into the arterial supply. They proposed that venous drainage from male fetuses introduces androgens into the circulation of the uterus. The proximity of the uterine vein and artery may allow hormones to pass into the arterial flow, thus supplying blood to fetuses located downstream from androgen producing males (the "Transvasculature Hypothesis"). In contrast to the Contiguity Hypothesis, which labels respective fetuses simply on the basis of their location relative to adjacent male fetuses, the Transvasculature Hypothesis

suggests that location of animals with regard to the direction of blood flow within the uterine vasculature is a better predictor of masculinization.

Although there has been some support for the Transvasculature Hypothesis (Meisel & Ward, 1981), the majority of investigations have successfully predicted timing to sexual maturity, receptivity, and reproductive capacity based on the classification proposed by the Contiguity Hypothesis (McDermott *et al.*, 1978; Rines & vom Saal, 1984; vom Saal & Moyer, 1985; vom Saal, 1989; vom Saal *et al.*, 1981; vom Saal *et al.*, 1990). The female anogenital distance has been used as a successful bioassay of natural androgen variation due to IUP (Hotchkiss & Vandenbergh, 2005; Vandenbergh & Huggett, 1995). The anogenital region is located between the anus and genital papilla. It elongates in response to androgen exposure during gestation, resulting in 0M female exhibiting shorter anogenital distances than 2M females (Hotchkiss & Vandenbergh, 2005; Vandenbergh & Huggett, 1995). 0M females show an earlier age at vaginal opening and at first estrus than do 2M females (McDermott *et al.*, 1978; vom Saal 1989). Length of the estrous cycle is shorter in 0M females than in 2M females (vom Saal, 1989; vom Saal *et al.*, 1981; vom Saal *et al.*, 1990). When sexually experienced males are given the opportunity to mate with either 0M or 2M females, they show marked preference for 0M females (Rines & vom Saal, 1984; vom Saal & Bronson, 1978; 1980). In return, 0M females are more sexually receptive to males (Rines & vom Saal, 1984) and over repeated intromissions with the same male become inseminated more rapidly than do 2M females (Rines & vom Saal, 1984). 0M females also produce more viable litters and cease giving birth at later ages than do 2M females (vom Saal & Moyer, 1985).

Male Preputial Glands

It is now well accepted that males' capacity to decrease time to sexual maturity in proximate females is largely mediated through bioactive constituents in urine (Bronson & Whitten, 1968; Colby & Vandenbergh, 1974; Drickamer, 1982, 1983, 1984a, 1984b; Drickamer & Mikesic, 1990; Drickamer & Murphy, 1978; Lombardi *et al.*, 1976; Novotny *et al.*, 1999a, 1999c; Schellinck *et al.*, 1993). In male mice the major source of pheromones is urine, containing chemicals that indicate the identity, health, reproductive status, and social status of the emitter (Keverne, 1983; Penn & Potts, 1998). Male mice routinely mark their territory with urine and use it in the presence of both male and female conspecifics (Bronson & Desjardins, 1974; deCatanzaro *et al.*, 2009; Maruniak *et al.*, 1974; Reynolds, 1971). The classical view of the chemical composition of male urine has linked pheromones to low molecular weight (LMW) volatile compounds that transmit information by virtue of their volatility (Whitten *et al.*, 1968).

Novotny and colleagues (1999a, 1999b, 1999c) suggested that an assortment of small, LMW volatiles originating from the bladder, such as 2-sec-butyl-4, 5-dihydrothiazole (SBT), 3,4-dehydro-exo-breviocomin (DHB) and 6-hydroxy-6-methyl-3-heptanone (heptanone), could be serving as male pheromones. The preputial glands, part of the male accessory gland system located on both sides of the urethral meatus (Beaver, 1960), have also been suggested to secrete putative pheromones α- and β-farnesenes into male urine. The assumption is that under the control of testosterone, males produce lipophilic farnesenes via the preputial glands to be excreted into urine, while more polar molecules such as SBT, DHB, and heptanone are contained in the bladder (Harvey *et al.*,

1989). Thus far, artificially-applied synthetic analogues of each of these have been shown to stimulate uterine growth (Novotny, 1999a, 1999b).

Major Urinary Proteins

It has also been suggested that high molecular weight molecules in urine, particularly proteins, might be aiding in the mediation of pheromonal communication among individuals (Beynon & Hurst, 2004; Moncho-Bogani *et al.*, 2002; Timm *et al.*, 2001). The putative function of these proteins is to carry volatile pheromones through an aqueous environment and offer protection from decomposition (Beynon & Hurst, 2004). Major urinary proteins (MUPs) are barrel-shaped lipocalin proteins containing a central cavity that binds small lipophilic and hydrophobic molecules (Bacchini *et al.*, 1992; Zidek *et al.*, 1999). MUP mRNA has been detected in the nasal mucosa and in other mucous and saliva producing glands (Shahan *et al.*, 1987). Generally, MUPs are produced in the liver, secreted in plasma, and excreted in urine via the kidneys (Finlayson *et al.*, 1965). The typical concentration of MUPs in mouse urine is about 1 – 5 mg/mL (Ferrari *et al.*, 1997). Males excrete three to four times more MUPs in their urine than do females (Beynon & Hurst, 2004) with the urine of prepubertal or castrated males exhibiting low MUP concentration (Marchlewska-Koj *et al.*, 2000).

Some reports have recommended that MUPs are not simply carriers of signaling molecules but are themselves priming pheromones (Morè, 2006; Mucignat-Caretta *et al.*, 1995a; Nevison *et al.*, 2003). Mucignat-Caretta and co-workers (1995a) delivered purified MUPs without their associated ligands to the nose of developing female mice,

and induced uterine mass increases. These females were exposed to MUPs dissolved in the urine of prepubertal males. Control groups received only prepubertal male urine. In an additional experiment, this group used volatile ligands separated from their MUP complex and suspended in the urine of prepubertal males and found no effect on uterine mass. Elsewhere it has been demonstrated that nasal exposure to MUPs dissolved in the urine of either prepubertal or castrated males or ovariectomized females induced estrus in adult females (Marchlewska-Koj *et al.*, 2000; Morè, 2006).

Contrary to the findings reported by Mucignat-Caretta *et al.* (1995a), others have shown that synthetic analogues of farnesenes and other volatile ligands can increase uterine weights following nasal administration to juvenile females (Novotny *et al.*, 1999b). The main ligands bound to MUPs are SBT, DHB, heptanone, and α- and β-farnesenes (Bacchini *et al.*, 1992; Robertson *et al.*, 1993). Ma *et al.* (1999) showed that synthetic farnesenes also have the capacity to induce estrus in sexually-experienced females. Nevertheless this work requires replication with solutions containing natural ligands extracted from the urine of adult males.

Steroids

Steroids have been quantified in bodily excretions of several mammals (Carroll *et al.*, 1990; Graham *et al.*, 1995; Shideler *et al.*, 1993; Wasser *et al.*, 1996). Data from larger mammals in the field show strong correlations between measures of blood steroids and their conjugates in urine and feces (Shideler *et al.*, 1993; Wasser *et al.*, 1996). Our laboratory has developed ELISA methods for measuring unconjugated steroids in

noninvasively-collected urine and other excretions of mice (Muir *et al.*, 2001; Vella & deCatanzaro, 2001). Methods have been validated for 17β-estradiol, testosterone, and estrone conjugates (Muir *et al.*, 2001) and for progesterone (deCatanzaro *et al.*, 2003). Many of the general urinary trends are similar to those established for systemic levels of these same steroids (deCatanzaro *et al.*, 2003, 2004; Vella & deCatanzaro, 2001).

Exposure to both endogenous and exogenous estrogens has been implicated in female puberty (Alonso & Rosenfield, 2002; Bronson, 1975; Cook *et al.*, 1953). Bronson (1975) showed that two injections of roughly 0.2 μg/animal 17β-estradiol benzoate could induce ovulation in pre-pubertal females, resembling the effects of male exposure. Estrogens bind to GnRH neurons with high affinity (Ng *et al.*, 2001) and are thus important in reproductive cycling. Uterine cells also proliferate in response to estrogens (Ogasawara *et al.*, 1983), and estradiol stimulates activity of growth hormone and insulin-like growth factor type 1 (IGF-1) that mediate development of the uterus (Kahlert *et al.*, 2000; Leung *et al.*, 2004; Sato *et al.*, 2002).

When in the company of developing females, males exhibit higher output of creatinine-adjusted estradiol and testosterone in their urine (Beaton *et al.*, 2006; deCatanzaro *et al.*, 2009). Proximity to juvenile females also causes adult males to increase water consumption and urination, thereby enhancing females' exposure to urinary steroids. Female-exposed males also actively direct their urine toward females (deCatanzaro *et al.*, 2009). Accordingly, exposure to males' excreted estrogens may help to account for male-induced female sexual maturation (Beaton *et al.*, 2006; deCatanzaro *et al.*, 2009). Male urinary testosterone could also contribute, possibly through

aromatization to estradiol. Polydipsia (excessive drinking) and polyuria (excessive urine secretion) in adult males during housing near developing females would serve to increase the transmission of urinary steroidal constituents and thus alter physiology of exposed females (deCatanzaro *et al.*, 2009).

Rationale and Overview of Experiments

The purpose of experiments provided in this thesis was to shed light on the role of three major influences on the timing to sexual maturity in developing females. These included the presence of genetically-unrelated adult males after weaning, phytoestrogen content in diet, and prior *in utero* androgen exposure of female subjects. An additional aim was to assess the onset of female sexual maturity through the use of procedures that minimized human handling. Attainment of female puberty was investigated using non-invasive behavioural and fertility measures. Enzyme immunoassay procedures were used to profile output of urinary progesterone and 17β-estradiol from juvenile females during exposure to adult males. Noninvasively-collected urinary measures allowed ongoing hormonal monitoring within the same individual, which is preferable to techniques previously employed to monitor hormonal levels in blood. In order to assess the influence of pheromonal contributions from the male preputial glands, an additional experiment was designed to investigate the capacity of preputialectomized males to induce puberty in females. A secondary purpose for this experiment was to examine whether these males would show changes in the urinary profile of excreted 17β-estradiol

and testosterone, implicated in accelerating sexual maturation in proximate females (Beaton *et al.*, 2006; deCatanzaro *et al.*, 2009).

Chapter 2: Khan, A., Bellefontaine, N., & deCatanzaro, D. (2008). Onset of sexual maturation in female mice as measured in behavior and fertility: Interactions of exposure to males, phytoestrogen content of diet, and ano-genital distance. *Physiology & Behavior*, *3*, 588-594.

Abstract: Age of puberty was examined in female mice through non-invasive behavioral and fertility measures, in relationship to ano-genital distance, phytoestrogen content of diet, and exposure to males post weaning. Throughout gestation and postnatal development, females were exposed to a regular diet or one that was nutritionally similar but deficient in phytoestrogens. After segregation at weaning on the basis of a short or long ano-genital distance index (AGDI), an indirect measure of *in utero* androgen exposure, females were housed alone or underneath two outbred adult males for two weeks. Subsequently, an outbred male was placed in the cage of each developing female, and mating behavior, escape attempts, biting gestures, and boxing postures were recorded. Next, females were monitored for the occurrence of a copulatory plug and allowed to bear young, with pregnancy and litters monitored up to weaning. Male-exposed females fed a regular diet were immediately sexually receptive when housed directly with males, and their conceptions occurred earlier than did those of other females. Subjects fed a diet deficient in phytoestrogens were least likely to show sexual receptivity. Male-exposed females with longer AGDI displayed more escape attempts in

the presence of males, regardless of diet. Once inseminated, most females carried to term and the majority of pups survived until weaning. These data suggest that phytoestrogens and AGDI interact with exposure to males in determining age at onset of puberty.

Chapter 3: Khan, A., Berger, R.G., & deCatanzaro, D. (2008). The onset of puberty in female mice as reflected in urinary steroids and uterine/ovarian mass: interactions of exposure to males, phyto-oestrogen content of diet, and ano-genital distance. *Reproduction*, *135*, 99-106.

Abstract: Development of puberty in female mice was examined in relationship to the ano-genital distance index (AGDI), phytoestrogen content of diet, and exposure to males post weaning. Throughout gestation and postnatal development, females were exposed to a regular diet or to a nutritionally similar diet deficient in phytoestrogens. After segregation at weaning on the basis of short or long AGDI, an indirect measure of *in utero* androgen exposure, females were housed alone or underneath two outbred adult males for two weeks. Female urinary samples were collected non-invasively throughout this exposure, then assayed for estradiol, progesterone, and creatinine. Females were then sacrificed and uterine and ovarian mass was determined. Urinary estradiol was substantially reduced in females raised on the phytoestrogen free diet. Estradiol levels were more dynamic over days in urine of male-exposed females, especially among those on the regular diet. Urinary progesterone was not strongly influenced by diet. Progesterone was more dynamic in urine of male-exposed females, and was generally elevated compared to levels in isolated females, with the size of this effect dependent on

AGDI, diet, and whether the measure was adjusted for creatinine. Urinary creatinine was elevated by the phytoestrogen free diet and reduced by male exposure, tending to decline over days in females exposed to males. Male exposure increased uterine and ovarian mass and was influenced by AGDI in interaction with diet and male exposure.

Chapter 4: Khan, A., Berger, R.G., & 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.

Abstract: Exposure to novel adult males and their urine can hasten the onset of sexual maturity in female mice. Some evidence implicates chemosignals from males' preputial glands, while other evidence suggests that male urinary steroids, especially 17β-oestradiol, contribute to this effect. The current experiment was designed to determine whether preputial gland removal would influence males' capacity to accelerate female sexual development, and to measure male urinary oestradiol and testosterone in the presence or absence of these glands. Juvenile females aged 28 days were housed for two weeks in isolation or underneath two outbred males that had undergone preputialectomy or sham surgery. Urine samples were collected non-invasively from males that were isolated or exposed to females, then assayed for oestradiol, testosterone, and creatinine. Combined uterine and ovarian mass from females sacrificed at day 43 of age was increased by exposure to males, regardless of whether these males had been preputialectomised. Male urinary creatinine was reduced by exposure to developing

females. Creatinine-adjusted oestradiol and testosterone were significantly greater in female-exposed than in isolated males, in both preputialectomised and intact males. These data suggest that the preputials are not necessary for males' capacity to hasten female uterine and ovarian growth. As exogenous oestrogens can promote uterine growth and other parameters of female reproductive maturation, oestradiol in males' urine may contribute to earlier sexual maturity in male-exposed females.

Chapter 2

Onset of Sexual Maturation in Female Mice as Measured in Behavior and Fertility: Interactions of Exposure to Males, Phytoestrogen Content of Diet, and Ano-Genital Distance

Khan, A., Bellefontaine, N., & deCatanzaro, D. (2008).

Physiology & Behavior, 3, 588-594.

Authors' Contributions

Ayesha Khan: Concept, formation of experimental design, data collection, literature review, data analysis and manuscript writing.

Nicole Bellefontaine: Data collection and manuscript editing.

Denys deCatanzaro: Assistance with concept development and experimental design, data analysis and manuscript editing.

Additional Support

Undergraduate Students: Kirk Wong, Yewande Akinfemiwa, Trina Hancock, Elaine Lewis and Jordan Shaw provided assistance with experimental procedures.

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Introduction

The timing of sexual maturation in female mammals can be strongly influenced by extrinsic factors during the pre-pubertal period, such as the presence of adult males and exposure to exogenous estrogenic substances [1-5]. The current study was designed to examine the potential interactions of three factors in determining age of puberty; the phytoestrogen content of diet during the females' development, variation in prenatal androgen exposure as reflected in the ano-genital distance index (AGDI) at weaning, and the presence or absence of novel adult males during post-weaning development. Non-invasive behavioral measures, including those of sexual receptivity and of aggressive and evasive actions toward males, were used to assess puberty. These were followed by measures of the age at insemination and fertility after mating.

The presence of novel adult males can induce early onset of sexual maturation in juvenile females of several species [1,5-14]. When sexually immature female mice are reared with novel adult males, first estrus can be accelerated by as much as twenty days [1]. Traditional methods used in assessing sexual maturity in small laboratory rodents often involve invasive procedures such as sacrificing large cohorts of animals to obtain blood samples for steroid analysis [15,16], measurement of reproductive tissue mass [5] or performance of repeated vaginal flushing in order to detect cyclical changes in vaginal cytology [1,6,7,17,18]. Noninvasive measures such as visual inspection of vaginal opening or detection of mating by presence of a copulatory plug are often conducted in conjunction with vaginal smears [1] which require swabbing cells from the vaginal lumen. Such artificial stimulation can induce artifacts such as pseudopregnancy [19] or

an abnormally high occurrence of estrus-like smears [20]. Human handling in itself can produce stress [21] which could further obscure results.

Prenatal conditions also produce variability among female subjects in morphological, physiological, and behavioral markers of puberty. In litter-bearing mammals, intrauterine position produces differential transport of androgens between adjacent fetuses [22]. A female fetus located between male siblings (2M) will have higher *in utero* testosterone and lower estradiol exposure than a female located between female siblings (0M) [22]. Ano-genital distance can be used as a bioassay for this, as *in utero* exposure to androgens promotes growth of the perineal region such that it is greater in males than in females [23] and longer in 2M females than 0M females [24]. 2M females are also more likely to mount other females [25], display higher levels of aggression [25,26], generate less interest by males [27] and less sexual arousal [26], have lengthier estrous cycles [28], and show decreased lordosis response [29] and fertility [30].

Dietary phytoestrogen content may also influence age of puberty. Commercial rodent diets formulated with soybean as a primary protein ingredient result in high steady-state serum concentrations of isoflavones, a major class of phytoestrogens [31]. These are non-steroidal but similar in structure to 17β-estradiol [32]. They also have the ability to bind with both estrogen receptor (ER) isoforms, ERα and ERβ with a higher affinity for ERβ [33,34]. In the female mouse, both receptors can be found in the brain and reproductive organs [35]. Adult rodents consuming commercial soy-containing diets *ad libitum* show serum isoflavone levels which can exceed estradiol levels by 30,000 to 60,000 fold [31]. Since isoflavones cross the placental barrier [36], it is possible that this

exposure can produce biological effects on estrogen sensitive tissues vital in sexual development. Investigations into the developmental effects of phytoestrogens have yielded inconclusive results. Some studies found that female mice exposed to phytoestrogens or soy derivatives during prenatal or neonatal development reached puberty substantially earlier than did untreated controls [37-40], while others have failed to replicate these findings or found delays in onset of puberty in phytoestrogen treated females [41,42]; different routes of administration, timing of phytoestrogen exposure, and invasiveness of procedures may be responsible for these discrepancies.

The current experiment was designed to investigate the influence of dietary phytoestrogens and adult male presence on sexual development in female mice using non-invasive markers of puberty. Starting from day one of gestation, females were exposed to a commercially available diet rich in phytoestrogens or one that was nutritionally similar but deficient in phytoestrogen content. At the time of weaning, females were segregated into those with short and those with long AGDI. Females were then either housed alone or with sexually experienced adult males for two weeks via an indirect exposure paradigm which allowed for full olfactory but limited tactile contact. Subsequently, a male was placed directly with each female, and behavioral measures of sexual receptivity and aggressiveness were recorded during the initial two hours. Each male-female pair remained together during the subsequent days and fertility was compared among conditions through measures of the latency of copulatory plugs, number of pups born, and survivorship of mother and/or pups to the point of weaning.

Methods

Subjects

CF1 strain mice (*Mus musculus*) were bred from laboratory stock originally obtained from Charles River Breeding Farms (Québec, Canada). Heterogeneous strain (HS) mice were produced by interbreeding C57-B6, Swiss Webster, CF1, and DBA-2 strains, also obtained from Charles River Breeding Farms. HS male were aged 5-10 months when used in this experiment and had each previously had at least two fertile matings. Housing conditions consisted of standard polypropylene cages measuring 28x16x11 (height) cm connected to wire grid tops allowing continuous access to food and water. Except as otherwise indicated, animals were placed on 8640 Teklad Certified Rodent Chow in pellet form obtained from Harlan Teklad® (Madison, Wisconsin). The ingredients of this "regular diet" are listed by the manufacturer as dehulled soybean meal, ground corn, wheat middlings, flaked corn, fish meal, cane molasses, soybean oil, ground wheat, dried whey, brewers dried yeast, plus vitamins and minerals. All animals were maintained under a reversed 14:10 hour light:dark 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.

Breeding and Nutrition

Female subjects were selected from offspring of 44 CF-1 females fed regular laboratory chow and 40 others fed a nutritionally similar phytoestrogen-free diet, Advanced Protocol™ Verified Casein Diet 1 IF from Purina Mills Inc. LabDiet® (Ren's

Feed & Supply Ltd., Ontario, Canada), commencing one week before pairing with a CF-1 breeder male. The ingredients of this "phytoestrogen-free" diet are listed by the manufacturer as ground wheat, ground corn, wheat middlings, ground oats, fish meal, casein, corn gluten meal, corn oil, dicalcium phosphate, brewers dried yeast, plus vitamins and minerals. This diet is specified as consistently containing less then 1.0 ppm total isoflavones (aglycone equivalents of genistein, daidzein and glycitein).

Litters from regular diet dams had an average of 11.8 pups, and were 48.8% female with mean body weight of 19.7 g and ano-genital distance of 9.66 mm at 28 days among female pups. Litters from phytoestrogen-free diet dams had an average of 11.4 pups, and were 44.3% female with mean body weight of 20.1 g and ano-genital distance of 9.49 mm at 28 days among female pups. Subjects were selected from among female pups with care to distribute representatives from all litters randomly among conditions; remaining pups used in other experiments in this laboratory.

Ano-Genital Distance

At 28 days following birth, pups were weaned. An ano-genital distance index (AGDI) was generated for each female, using a Mastercraft® digital caliper by measuring the distance (mm) between base of the genital papilla and proximal end of the anal opening, then dividing this distance by body mass (g) and multiplying the resultant value by 100 [24]. Care was taken to ensure that the ano-genital region was neither stretched nor compressed during measurements. We first determined an AGDI range (between 37.15 and 66.07), then females with AGDI of 46 or lower were labeled as short AGDI

while those of 53 or higher were labeled as long AGDI. Remaining pups were excluded from subsequent experimentation.

Experimental Treatment

Immediately following AGDI measurements, weanling females were each assigned to one of eight conditions in a 2x2x2 factorial design involving diet (regular vs. phytoestrogen-free), social situation (isolated vs. male-exposed), and AGDI (short vs. long). Among those reared on the regular diet, there were 15 isolated short AGDI, 11 isolated long AGDI, 15 male-exposed short AGDI, and 11 male-exposed long AGDI females. Among those reared on the phytoestrogen-free diet, there were 15 isolated short AGDI, 10 isolated long AGDI, 14 male-exposed short AGDI, and 11 male-exposed long AGDI females. Females raised on each specific diet continued on it for the remainder of the experiment.

Each female was housed in the lower compartment of a double-decker cage system described previously [43]. Briefly, this apparatus was constructed from clear Plexiglas, measuring 30 x 21 x 27 cm, divided into upper and lower compartments (each measuring 30 x 21 x 13) by a stainless steel wire-mesh grid (squares of $0.5cm^2$). Isolated females were in the apparatus alone, whereas male-exposed females were housed directly below two sexually-experienced adult HS males in the upper compartment. The grid floor between the upper and lower compartment allowed excretions from the males to pass into the female's compartment. An opaque Plexiglas partition separated the two males in two equal sub-compartments to prevent aggression. The female had full

olfactory and auditory contact with each male, but tactile interaction was limited such that mating was not possible while nasal-nasal and nasal-genital contact could occur. Each compartment provided continuous independent access to food and water, with food in the female's compartment protected from excretions of the males above. Females remained in this apparatus from 28 to 43 days of age.

At age 43 days, a single stimulus male from the upper compartment of male-exposed females was placed into the lower compartment with the female. The other male was placed in the compartment of a female that had been kept in isolation. Each stimulus male therefore had had two weeks of continuous exposure to developing female through a wire-mesh grid.

Behavioral Observations and Pregnancy Outcome Measures

Behavioral observations were conducted for each female in a 2-h session under dim illumination commencing 3-7 h after the start of the dark phase of the lighting cycle, with time of measurement counterbalanced across conditions. Each session commenced with the introduction of the stimulus male directly into the female's compartment. An observer who was blind with respect to the specific condition from which each female derived took counts taken of discrete instances of specific sexual and defensive postures, modeled after measures in previous studies of this species [*e.g.* 44,45]. These included mounts by the male (male climbing onto the female's back), lordosis responses (female immobile in response to a mount, with hindquarters raised potentially permitting intromission), and actual intromissions (discrete male insertion of penis). Female non-

sexual behavior that was recorded included boxing postures (standing on rear limbs with forelimbs raised), biting gestures (gestures with the teeth directed toward the male, not necessarily pinching the male's skin), escape attempts (locomotion away from the male), and freezing responses (immobility without raising hindquarters) in response to male mounting, exploration of female genitals, and grooming.

Following behavioral observations, each pair was placed into a standard polypropylene cage and generally left undisturbed, except that the hindquarters of females were inspected for copulatory plugs on three occasions daily, under dim illumination at approximately 1, 6, and 10 h after the start of the light phase of the cycle. All copulatory plugs were detected visually and without the use of metal probes. On the day of copulatory plug detection, the male was removed from the female's cage and the female was left undisturbed for the remainder of gestation. Females were visually monitored daily for health and parturition. Counts were taken of the number of pups born, and litters were visually inspected on a daily basis until each litter was ready to be weaned at 28 days following birth.

Results

During the 2-h session of behavioral observation following direct introduction of the male into the female's compartment, males mounted in all conditions, but female sexual response was most common among male-exposed females on the regular diet. For each measure, factorial analysis of variance (2x2x2 design) was used to analyze the data

with male presence (isolated vs. male-exposed), diet (regular vs. phytoestrogen-free) and AGDI (short vs. long) as between-subjects factors. Male mounting was observed in all conditions; there were no significant main effects or interactions for this measure despite a relatively low number exhibited toward short-AGDI females on the phytoestrogen-free diet (see Table 1). Among the regular diet females, there was clearly more receptivity (lordosis) among male-exposed females than among isolated ones; this trend was absent in females on the phytoestrogen-free diet, with some lordosis responses seen in isolated short-AGDI females. Analysis of variance on lordosis responses per mount (see Figure 1) indicated a significant 2-way interaction of male presence and diet, $F(1,94) = 4.77$, $p = 0.029$, but no main effect and no other interaction reached significance at the conventional ($p < 0.05$) level. The same statistical profile was seen for unadjusted lordosis responses, with only the interaction of male presence and diet reaching significance, $F(1,54) = 4.47$, $p = 0.035$. The number of intromissions showed a similar trend (see Figure 2), however variance within conditions meant that no significant effects occurred.

Table 1. Mean (±S.E.) number of mounts, unadjusted lordosis responses, biting gestures, and freezing responses observed during the 2 h immediately after sexually experienced adult males were introduced into females' compartments when females were aged 43 days.

	Regular Diet				Phytoestrogen-Free Diet			
Behavior	Isolated Short AGD	Isolated Long AGD	Male Exposed Short AGD	Male Exposed Long AGD	Isolated Short AGD	Isolated Long AGD	Male Exposed Short AGD	Male Exposed Long AGD
Mounts	18.9±5.5	25.3±7.3	25.5±12.2	53.4±16.6	24.5±8.4	18.3±9.0	3.2±1.2	29.9±19.5
Lordosis Postures	0.07±0.07	0.36±0.36	2.93±1.86	2.73±1.71	0.93±0.52	0.00±0.00	0.00±0.00	0.27±0.27
Biting Gestures	361±63	582±65	517±111	543±64	567±67	522±68	414±72	615±85
Freezing Responses	6.20±2.68	5.00±1.34	4.47±2.02	3.54±2.15	9.53±3.46	2.90±1.64	1.57±0.72	0.55±0.25

Figure 1. Mean (±S.E.) number of lordosis postures by females divided by the number of mounts by stimulus males during 2 hours of observations starting from time of placement of a stimulus male in the female's compartment at 43 days of age.

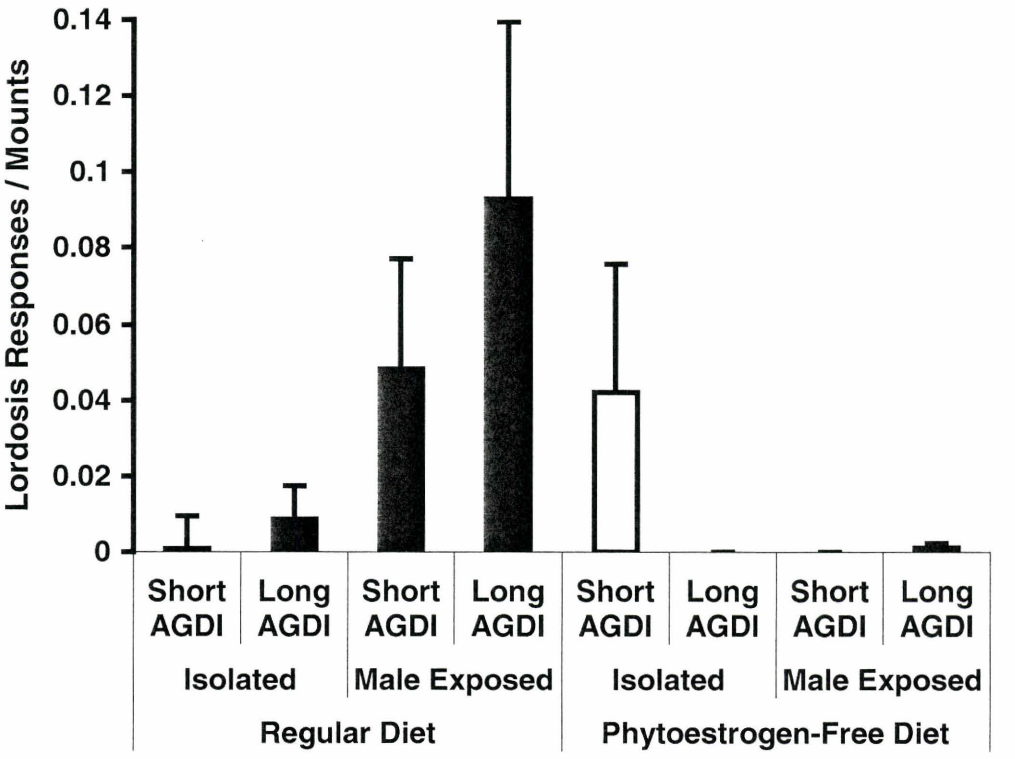
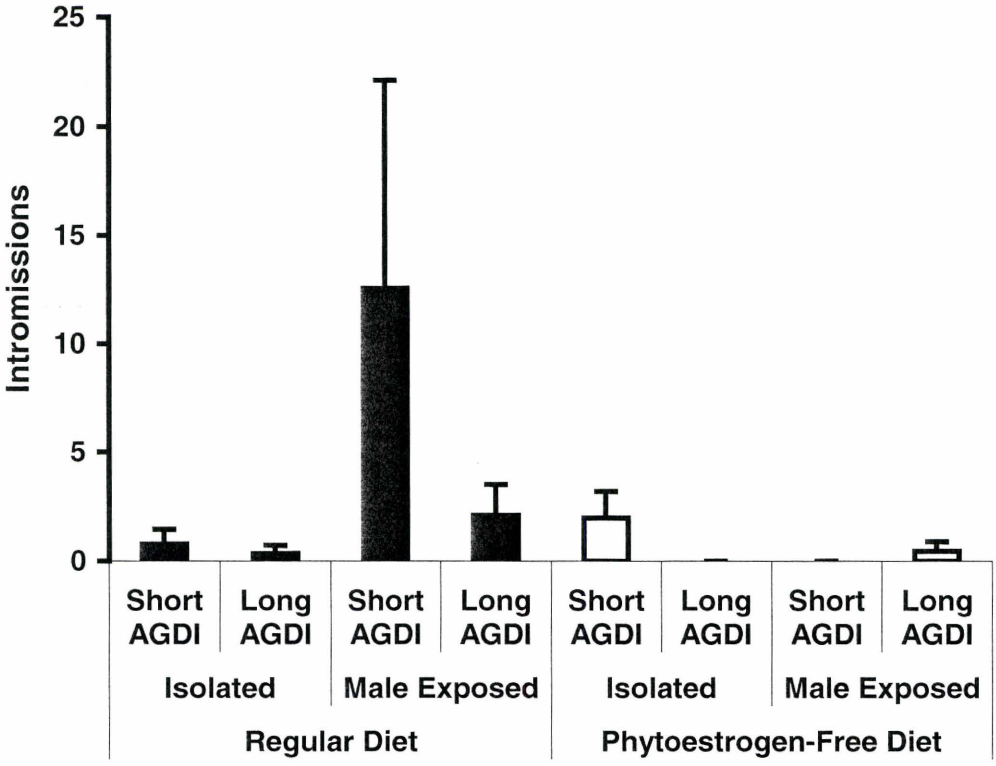


Figure 2. Mean (±S.E.) number of intromissions observed during 2 hours of observations starting from time of placement of a stimulus male in the female's compartment at 43 days of age.



Female defensive posturing was also observed in all conditions in the 2 h following introduction of the male to the female's compartment, often in the same females that responded sexually to males. Females placed on the regular diet displayed highest number of boxing postures (see Figure 3), especially those that were male-exposed with long AGDI. For boxing postures, the main effect of diet was significant, $F(1,94) = 10.90$, $p = 0.002$, while main effects of male presence, $F(1,94) = 3.656$, $p = 0.056$, and AGDI, $F(1,94) = 3.210$, $p = 0.073$, approached significance, while none of the interactions were at or near significance. Active attempts to escape from the male in response to his mounting, exploration of genitals or grooming (see Figure 4) showed a significant main effect of AGDI $F(1,94) = 7.75$, $p = 0.007$ and a significant interaction of AGDI and male presence, $F(1,94) = 4.13$, $p = 0.042$. There were numerous biting gestures displayed toward males in all conditions (see Table 1), but these did not generally produce overt wounds on the males; the 3-way interaction, $F(1,94) = 3.80$, $p = 0.051$, and the main effect of AGDI, $F(1,94) = 3.17$, $p = 0.075$, approached significance. Freezing responses (see Table 1) were more frequent in isolated than male-exposed females, with a significant effect of male presence, $F(1,94) = 4.49$, $p = 0.034$ but no other significant main effects or interactions.

Figure 3. Mean (±S.E.) number of boxing postures exhibited by females during 2 hours of observations starting from time of placement of a stimulus male in the female's compartment at 43 days of age.

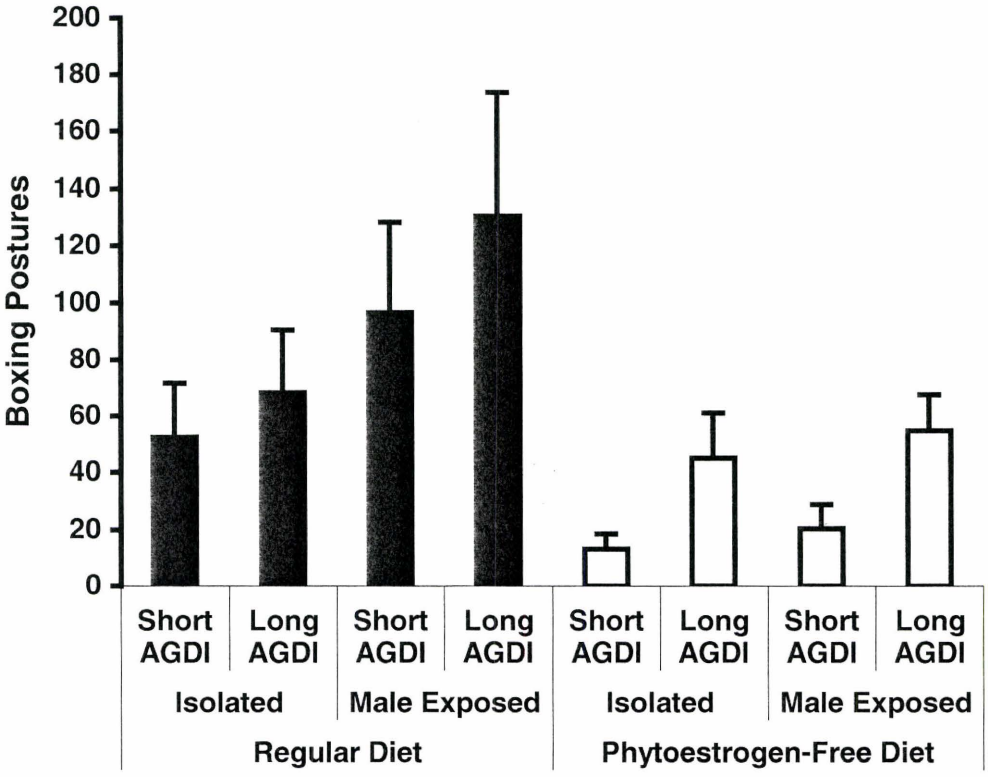
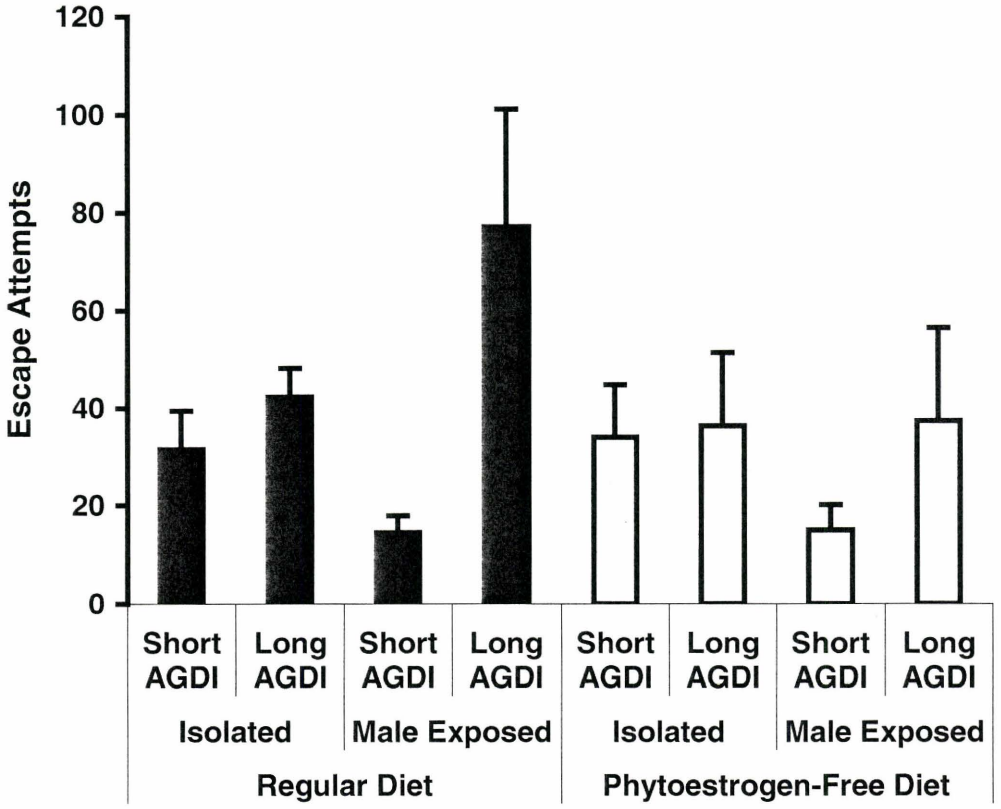


Figure 4. Mean (±S.E.) number of escape attempts by females in response to male mounting, exploration of female genitals, and grooming during 2 hours of observations starting from time of placement of a stimulus male in each female's compartment at 43 days of age.



During the subsequent days, copulatory plugs were observed earlier in male-exposed females on the regular diet than in females from the other conditions (see Figure 5). There was a significant main effect of diet, $F(1,94) = 6.35$, $p = 0.013$, and a significant interaction of male presence during development and diet, $F(1,94) = 4.61$, $p = 0.032$, but no other significant effects were found. Gestation length was comparable in all conditions, such that age of the dam at birth showed trends that corresponded to those for latency of copulatory plugs. Although in a number of cases females and/or pups died after birth, most inseminated females were parturient and produced litters where both dams and pups survived until weaning (see Table 2). Analysis via chi-squared tests of association showed no differences among conditions for proportion parturient, $\chi^2(7) = 7.95$, $p < 0.50$, or proportion producing viable litters, $\chi^2(7) = 5.57$, $p < 0.75$.

Figure 5. Mean (±S.E.) number of days between the placement of a stimulus male in the female's compartment at 43 days of age and the detection of a copulatory plug in the female's hindquarters.

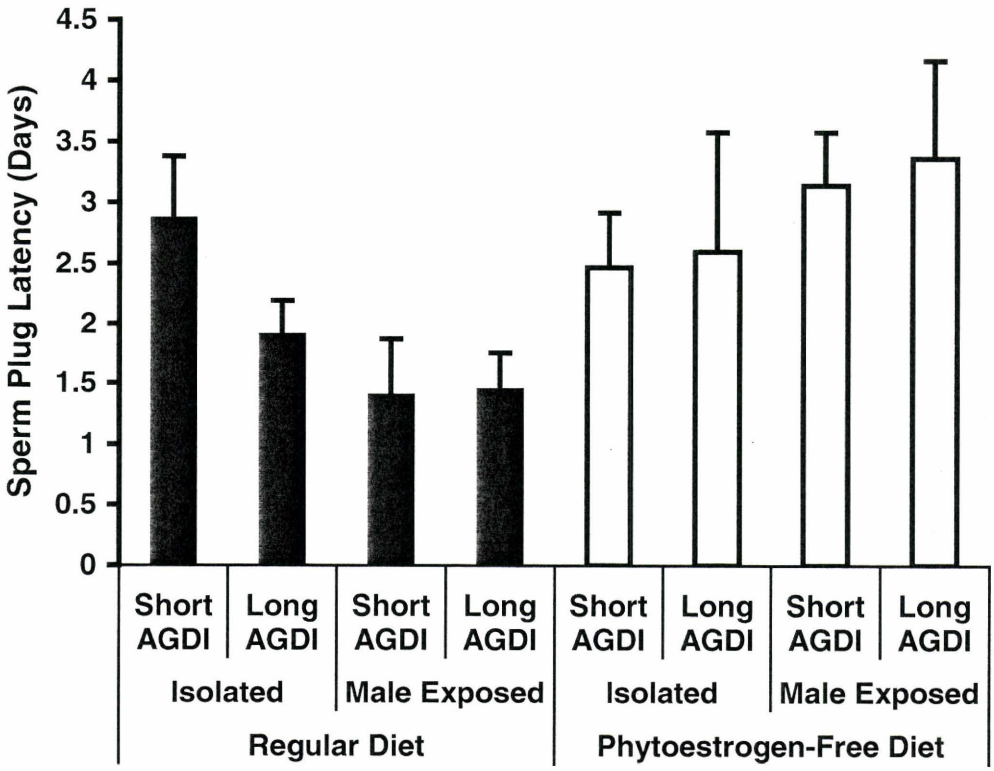


Table 2. Percentage of females that were parturient and the percentage of females that delivered litters where the dam and pups survived until weaning (viable litters).

	Regular Diet				Phytoestrogen-Free Diet			
	Isolated Short AGDI	Isolated Long AGDI	Male Exposed Short AGDI	Male Exposed Long AGDI	Isolated Short AGDI	Isolated Long AGDI	Male Exposed Short AGDI	Male Exposed Long AGDI
% Parturient	**93.3**	**72.7**	**86.7**	**90.9**	**100.0**	**90.0**	**100.0**	**90.9**
% Viable Litters	**86.7**	**54.5**	**60.0**	**81.8**	**80.0**	**60.0**	**78.6**	**72.7**

Discussion

We believe that this is the first demonstration via noninvasive behavioral measures of the influence of novel males on the onset of female puberty. In females on the regular diet, this was evident in the frequency of lordosis responses and in the age at which females first received copulatory plugs. However, this influence of novel males was absent among females on a phytoestrogen-free diet. The data from regular-diet females reinforce the ecological validity of previous demonstrations of that exposure to adult males advances the age of maturation of vaginal and uterine morphology [1,2,5-11], indicating that exposure to males also advances the age of sexual receptivity and fertility.

These data suggest an important role of dietary phytoestrogens in the rate of sexual maturation. Females raised on the phytoestrogen-free diet showed substantially less sexual receptivity and a later date of insemination than did females raised on the regular diet, and they were resistant to the influence of novel males on the age of puberty. Nevertheless, it is noteworthy that females on the phytoestrogen-free diet were not deficient in sexual response and fertility, but rather simply showed these milestones later than did females on the regular diet. Females from both dietary conditions were eventually able to mate, and once inseminated, most females were able to carry to term and the majority of pups survived until weaning. The present data are consistent with previous data indicating that female mice exposed to phytoestrogens during prenatal or neonatal development reached puberty substantially earlier than did untreated controls [37-40]. Much evidence indicates that estrogens are involved in maturation of the female reproductive tract [2,5,46] as well as in preparation of female sexual receptivity [47,48].

Natural unconjugated estrogens are also abundant in male urine [5,49], which may help to explain how novel males and their urine can advance puberty in nearby females [5].

We found that female escape behavior was greater in long AGDI females than in short AGDI ones. Elsewhere [26] it has been found that females with high *in utero* androgen exposure exhibit an increased tendency for aggressive behavior. In that study, such aggression was usually directed at female conspecifics with aggression toward males only in the form of *post partum* aggression to a male intruder [26]. Females with high prenatal androgen exposure are also less likely to assume sexually receptive postures in the presence of adult males [29]. Female mice have been found to exhibit the highest levels of male-directed aggression during days preceding ovulation [50]. Since long AGDI females placed on the regular diet had delayed copulatory plug latency in comparison to their short AGDI counterparts, and there were increased escape attempts in response to male attention (e.g., mounting, exploration of genitals or grooming), it is possible that some of these females were displaying pre-ovulatory behaviors during the time we conducted the ethogram.

We found that the age of receiving a first copulatory plug was advanced in male-exposed females on the regular diet, and these females were accordingly parturient a few days younger than females in other conditions. One report [51] suggests that a non-invasive measure such as presence of a copulatory plug is the best indicator for ovulation, superior to estrus smears and vaginal opening. Since the set of genes that become activated at time of vaginal opening differ from those coding for vaginal cornification and cyclicity, a nonconcordance has been shown for vaginal opening and first

cornification to the estrous cycle [52]. Although vaginal opening and first cornification occur due to increases in plasma estradiol, the onset of estrous also requires that the hypothalamus and pituitary respond to plasma estradiol resulting in a pre-ovulatory surge of luteinizing hormone, important in ovulation induction [53]. We suggest that the age of first insemination and pregnancy could be the clearest indicators of sexual maturation.

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Chapter 3

The onset of puberty in female mice as refelcted in urinary steroids and uterine/ovarian mass: interactions of exposure to males, phyto-oestrogen content of diet, and ano-genital distance.

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Authors' Contributions

Ayesha Khan: Concept, experimental design, data collection, ELISA procedures, literature review, data analysis and manuscript writing.

Robert G. Berger: Assistance with ELISA procedures and manuscript editing.

Denys deCatanzaro: Assistance with concept development, data analysis and manuscript editing.

Additional Assistance

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deCatanzaro lab graduate students: Jordan Shaw and Adam Guzzo provided assistance with experimental procedures.

Introduction

The onset of sexual maturation in female mammals can be influenced by environmental and social factors (Drickamer 1974; 1975; Hasler & Banks 1975; Teague & Bradley 1978; Spears & Clarke 1986; vom Saal 1989). The presence of adult conspecifics (Vandenbergh 1967; 1976), hormonal exposure during gestation (Zehr *et al.* 2001) and diet (Whitten & Naftolin 1992; Thigpen *et al.* 2003; Takashima-Sasaki *et al.* 2006) have all been found to influence the timing of sexual maturity. The present study was designed to explore how the combination of these factors affects reproductive tissue maturation and steroid production in developing female mice through the examination of uterine and ovarian growth and profiles of urinary oestradiol and progesterone.

Exposure to adult males can induce early onset of sexual maturation in juvenile females of several mammals including mice (Vandenbergh 1967), rats (Vandenbergh 1976), lemmings (Hasler & Banks 1975), deer mice (Teague & Bradley 1978), voles (Spears & Clarke 1986), opossums (Harder & Jackson 2003), and cattle (Roberson *et al.*, 1991), a phenomenon often called the *Vandenbergh effect*. Within 24 hours of male presence, juvenile females begin to show changes in uterine growth followed by surges in serum LH and FSH by the third day (Bronson & Stetson 1973). Plasma oestradiol spikes after six hours of male exposure, while progesterone levels show an influence after about sixty hours (Bronson & Desjardin 1974). Following a few weeks of male exposure, cyclical changes in vaginal cytology are evident in samples from repeated vaginal flushing of male-exposed females (Vandenbergh 1967; 1976; Bingel 1972), and uterine mass is substantially higher than in isolated females (Beaton *et al.* 2006). These

traditional techniques of assessing precocious puberty are invasive, as blood sampling and human handling will influence endogenous steroid levels (deCatanzaro & MacNiven 1992) and repeated vaginal stimulation can introduce artifacts including pseudopregnancy (Diamond 1970) and several hormonal changes (Komisaruk & Steinman 1986). In the present study, we undertook to determine steroid profiles over time in urine collected non-invasively from developing female mice, following recently developed methods for this species (Muir *et al.* 2001; deCatanzaro *et al.* 2003; 2004; 2006; Beaton *et al.* 2006).

Phyto-oestrogens, commonly found in commercial rodent diets, can also significantly influence the onset of puberty (Thigpen *et al.* 1987; Takashima-Sasaki *et al.* 2006). These physiologically active compounds are similar in structure to 17β-oestradiol (Kurzer & Xu 1997) and are capable of binding to oestrogen receptors (Kuiper *et al.* 1998). Given the rich distribution of oestrogen receptors in female reproductive tissue (Cooke *et al.* 1997; Couse *et al.* 1997; Fitzpatrick *et al.* 1999; Jefferson *et al.* 2002), exposure to phyto-oestrogens can influence oestrogen-related action at the pre-pubertal uterus (Wang *et al.* 2005; Takashima-Sasaki *et al.* 2006) and ovaries (Jefferson *et al.* 2002; Takashima-Sasaki *et al.* 2006). These compounds are also able to cross the placental barrier (Brown & Setchell 2001; Ikegami *et al.* 2006), potentially having a significant effect on prenatal hormonal exposure.

The onset of sexual maturation in females can also be influenced by the hormonal milieu *in utero* (Clark & Galef 1988; Zehr *et al.* 2001). Testosterone produced by male foetuses in the third week of gestation (Pointis *et al.* 1980) can be passively transferred to

adjacent foetuses (Even *et al.* 1992; vom Saal & Dhar 1992). A female foetus located between two male foetuses will be exposed to higher levels of androgens than will a female surrounded by two females. Differences in intrauterine position have significant implications for developing females, as those located in between two males reach sexual maturity later (Clark & Galef 1988; Zehr *et al.* 2001) and have later occurrence of vaginal opening (Zielinski & Vandenbergh 1991) and lengthier cycles once oestrus ensues (vom Saal *et al.* 1981). Vandenbergh and Huggett (1995) have suggested the importance of segregating female subjects based on their prior *in utero* steroid hormone exposure to reduce variation found in many experimental designs. The ano-genital distance index (AGDI) is a noninvasive bioassay that can be used to assign females accordingly, as it correlates closely with prenatal exposure to androgens (Vandenbergh & Huggett 1995).

The present study was designed to investigate how proximity to adult male conspecifics, dietary phyto-oestrogens, and AGDI interact in sexual maturation of female mice. Repeated noninvasive urinary collections, as previously developed in this laboratory (Muir *et al.* 2001; deCatanzaro *et al.* 2003), were conducted throughout a two-week exposure period. An enzyme-linked immunosorbent assay was used to measure oestradiol and progesterone levels in female urine samples. Following this exposure, females were sacrificed and wet and dry uterine and ovarian mass was recorded.

Materials and Methods

Subjects

CF1 strain mice (*Mus musculus*) were bred from stock originally obtained from Charles River Breeding Farms of Canada (Québec, Canada). Heterogeneous strain (HS) mice were produced by interbreeding C57-B6, Swiss Webster, CF1, and DBA-2 strains, also obtained from Charles River Breeding Farms. Housing conditions consisted of standard polypropylene cages measuring 28 x 16 x 11 (height) cm connected to wire grid tops allowing continuous access to food and water. Animals normally had continuous access to 8640 Teklad Certified Rodent chow in pellet form obtained from Harlan Teklad® (Madison, Wisconsin). The ingredients of this "regular diet" are listed by the manufacturer as dehulled soybean meal, ground corn, wheat middlings, flaked corn, fish meal, cane molasses, soybean oil, ground wheat, dried whey, brewers dried yeast, plus vitamins and minerals. All animals were maintained under a reversed 14:10 hour light:dark 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.

Breeding and Nutrition

Female subjects were selected from offspring of 44 CF-1 females fed regular laboratory chow and 40 others fed a nutritionally similar diet designed for very low levels of phyto-oestrogens (Advanced Protocol™ Verified Casein Diet 1 IF from Purina Mills Inc. LabDiet® obtained from Ren's Feed & Supply Ltd., Ontario, Canada), commencing

one week before pairing with a CF-1 breeder male. The ingredients of this "phyto-oestrogen free" diet are listed by the manufacturer as ground wheat, ground corn, wheat middlings, ground oats, fish meal, casein, corn gluten meal, corn oil, dicalcium phosphate, brewers dried yeast, plus vitamins and minerals, and is specified as consistently containing less then 1.0 ppm total isoflavones (aglycone equivalents of genistein, daidzein and glycitein).

Litters from regular diet dams had an average of 11.8 pups, and were 48.8% female with mean body weight of 19.7 g and ano-genital distance of 9.66 mm at 28 days among female pups. Litters from phyto-oestrogen-free diet dams had an average of 11.4 pups, and were 44.3% female with mean body weight of 20.1 g and ano-genital distance of 9.49 mm at 28 days among female pups. Subjects were selected from among female pups with care to distribute representatives from specific litters in a counterbalanced fashion among all conditions, such that no condition contained more than two females from a specific litter. Remaining pups were used in other experiments in this laboratory.

Ano-Genital Distance

Twenty-eight days following birth of litters, an ano-genital distance index (AGDI) was generated for each female. Individual ano-genital distance (mm) was determined using a Mastercraft® digital caliper to measure the distance between base of the genital papilla and proximal end of the anal opening. An AGDI was calculated by dividing the ano-genital distance (mm) by body mass (g) and multiplying the resultant value by 100 (Vandenbergh & Huggett 1995). Care was taken to ensure that the ano-genital region

was neither stretched nor compressed during measurements. All indices were calculated by a single experimenter well trained in animal handling. Since there have been no previous reports on AGDI in females of this age, we first calculated an index that ranged between 37.15 and 66.07. Females with AGDIs of 46 or lower were considered as having short AGDI while those with an AGDI of 53 or higher were considered as having long AGDI. Remaining pups were excluded from subsequent experimentation.

Experimental Treatment

Immediately following calculation of the AGDI, weanling females were each assigned to one of eight conditions in a 2 x 2 x 2 factorial design involving diet (regular vs. phyto-oestrogen-free), social situation (isolated vs. male-exposed), and AGDI (short vs. long). Among those reared on the regular diet, there were 12 isolated short AGDI, 14 isolated long AGDI, 12 male-exposed short AGDI, and 12 male-exposed long AGDI females. Among those reared on the phyto-oestrogen-free diet, there were 12 isolated short AGDI, 12 isolated long AGDI, 11 male-exposed short AGDI, and 11 male-exposed long AGDI females. Females raised on each specific diet continued on it for the remainder of the experiment.

Apparatus

Each female was placed in the lower compartment of a double-decker cage system described in deCatanzaro *et al.* (1996). Briefly, this apparatus was constructed from clear Plexiglas, measuring 30 x 21 x 27 cm, divided into upper and lower

compartments (each measuring 30 x 21 x 13) by a stainless steel wire-mesh grid (squares of 0.5 cm^2) which allowed for excretions from the males to pass into the females compartment. An opaque Plexiglas partition separated the males to prevent aggression. The female had full olfactory contact with each male but limited behavioural interaction. Each compartment provided continuous independent access to food and water, with food in the female's compartment protected from excretions of the males above. After a 48 hour acclimation period to control for urinary hormone dynamics attributable to placement in the novel environment (deCatanzaro *et al.* 2004), daily urine collection began at day 30 and continued until day 43 of age. On each day of collection, the upper compartment containing the HS males was separated from the lower compartment containing the female for a period of 4 ± 2 hours. Each day, a clean tray was placed underneath each subject at the start of the dark phase of the light cycle. Pooled urine was then aspirated via 1-cc syringes with 26-gauge needles. A fresh syringe and needle were used for each animal on each day. Collection was extended up to 6 hours where necessary to obtain a sufficient urine sample (approximately 0.5 ml). Care was taken to ensure that urine samples were not contaminated with food residue or feces, by only taking urinary samples clearly free of such deposits. All samples were stored in labeled 1.5 ml Nalgene® cryo-tubes and frozen immediately after collection at -20 °C until they were assayed simultaneously for the whole experiment.

Uterine and Ovarian Mass Measurement

After completion of urine collections on day 43 of age, females were euthanized using a lethal dose of CO_2 gas. Ovaries and uterus were extracted through a dorsolateral incision made near the abdominal cavity. Excess fat and mesentery were removed as per standard criteria (Wang *et al.* 2005). All surgeries were performed by a single experimenter blind to each female's experimental condition. Dry weights were calculated after tissue samples were stored in calcium sulfate crystals for a period of thirty days at approximately 3 °C.

Urinary Steroid and CreatinineDetermination

Validations of enzyme immunoassay procedures for this laboratory for adult male and female mice have been reported elsewhere for oestradiol (Muir *et al.* 2001) and for progesterone (deCatanzaro *et al.* 2003). Creatinine, 17β-oestradiol, and progesterone were obtained from Sigma Chemical Co. (St Louis, MO). Antibodies to 17β-oestradiol and progesterone and corresponding horseradish peroxidase conjugates were obtained from the Department of Population Health and Reproduction at the University of California (Davis). Cross-reactivities for anti-oestradiol and anti-progesterone have been previously reported (deCatanzaro *et al.* 2004). The assay were carried out on Nunc® Maxisorb plates which were first coated with 50 µl of antibody stock diluted at 1:10 000 in a coating buffer (50 mmol bicarbonate buffer l^{-1}, pH 9.6) and stored for 12–14 h at 4 °C. Wash solution (0.15 mol NaCl l^{-1} containing 0.5 ml of Tween 20 l^{-1}) was added five times to each well using an automated strip (Bio-Tek Instruments Inc. model ELx50) to

rinse away any unbound antibody and then 50 µl phosphate buffer per well was added. The plates were incubated at room temperature (21 °C) for 2 h for oestradiol determination and 1 h for progesterone determination before adding standards, samples, or controls.

For oestradiol and progesterone, urine samples were diluted 1:9 in phosphate buffer before they were added to the plate. Standard curves were derived by serial dilution from a known stock solution. Oestradiol stock was 2000 pg ml^{-1} yielding values of 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 1.95, 0.98, 0.46 and 0.23 pg ml^{-1}. 5000 pg ml^{-1} progesterone standard stock was serially diluted to 2500, 1250, 650, 325, 162.5, 81.25, 40.63 20.31, 10.16, 5.08, 2.54, 1.27, and 0.63. For all assays, 50 µl oestradiol or progesterone horseradish peroxidase was added to each well, with 20 µl of standard, sample, or control for oestradiol or 50 µl of standard, sample, or control for progesterone. The plates were incubated for 2 h at room temperature. Subsequently, the plates were washed and 100 µl of a substrate solution of citrate buffer, H_2O_2 and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) were added to each well and the plates were covered and incubated while shaking at room temperature for approximately 30 to 60 min. The plates were then read with a single filter at 405 nm on the microplate reader (Bio-Tek Instruments Inc., model ELX 808).

It is common practice with urinary steroid analyses to compensate for variations in fluid intake and output by adjusting sample values for creatinine (Munro *et al.* 1991; Muir *et al.* 2001; deCatanzaro *et al.* 2003). Standard creatinine values of 100.0, 50.0, 25.0, 12.5, 6.25, 3.12, 1.56, and 0.78 µg ml^{-1} were used. All urine samples were diluted

1:41 urine: phosphate buffer (0.1 mol l^{-1} sodium phosphate buffer, pH 7.0 containing 8.7 g of NaCl and 1 g of BSA per litre). Dynatech Immulon flat bottom plates were used and 50 µl per well of standard was added together with a solution of 50 µl distilled water, 50 µl 0.75 mol l^{-1} NaOH and 50 µl 0.4 mol l^{-1} picric acid. The plates were then shaken and incubated at room temperature for 30 min. The plate was measured for absorbance on a plate reader with a single filter at 490 nm. Standard curves were generated, regression lines were fit, and the regression equation was applied to the optical density for each sample. Steroid measurements were adjusted for creatinine by dividing the obtained value by the measure of creatinine per ml or urine. Both creatinine-adjusted and unadjusted steroid measures were analysed statistically.

Results

Figures 1 gives values for urinary creatinine, unadjusted and creatinine-adjusted urinary oestradiol, and unadjusted and creatinine-adjusted urinary progesterone, with each female receiving a single average value of the 14 daily samples for each measure. Urinary oestradiol among phyto-oestrogen-free diet females was in a non-overlapping range below that of females raised on the regular diet. A 2 (diet) x 2 (male exposure) x 2 (AGDI) factorial analysis of variance indicated a significant effect of diet, $F(1,88) = 76.93$, $p < 0.0001$ and an effect of male exposure approaching the conventional ($p < 0.05$) level of significance, $F(1,88) = 3.41$, $p = 0.0648$, but no other significant main effect or interaction. Urinary progesterone showed a significant 3-way interaction among the

variables, $F(1,88) = 5.64$, $p = 0.0187$, but no other significant main effect or interaction. Multiple comparisons (Duncan's New Multiple Range Test, $p < 0.05$) could not identify the source of this interaction. Creatinine levels also varied among conditions, showing significant main effects of diet, $F(1,88) = 19.08$, $p = 0.0001$, and of male exposure, $F(1,88) = 15.12$, $p = 0.0004$. As with unadjusted oestradiol, creatinine-adjusted oestradiol showed a significant main effect of diet, $F(1,88) = 135.83$, $p < 0.0001$. Creatinine-adjusted progesterone showed significant main effects of diet, $F(1,88) = 3.90$, $p = 0.0486$ and of male exposure, $F(1,88) = 6.60$, $p = 0.0115$, and a significant 3-way interaction, $F(1,88) = 6.92$, $p = 0.0098$. Multiple comparisons for adjusted progesterone indicated that regular-diet male-exposed long AGDI females exceeded their isolated counterparts and all phyto-oestrogen-free conditions except male-exposed short-AGDI females, while among phyto-oestrogen-free females, short-AGDI male-exposed females exceeded their isolated counterparts.

Figure 1. Mean (±S.E.M.) of the average value for each individual over 14 days for urinary creatinine, oestradiol, progesterone, creatinine-adjusted oestradiol and creatinine-adjusted progesterone. Females were of short or long ano-genital distance index (AGDI), raised on either a regular or phyto-oestrogen-free diet, and either housed alone or exposed through a grid to two adult males.

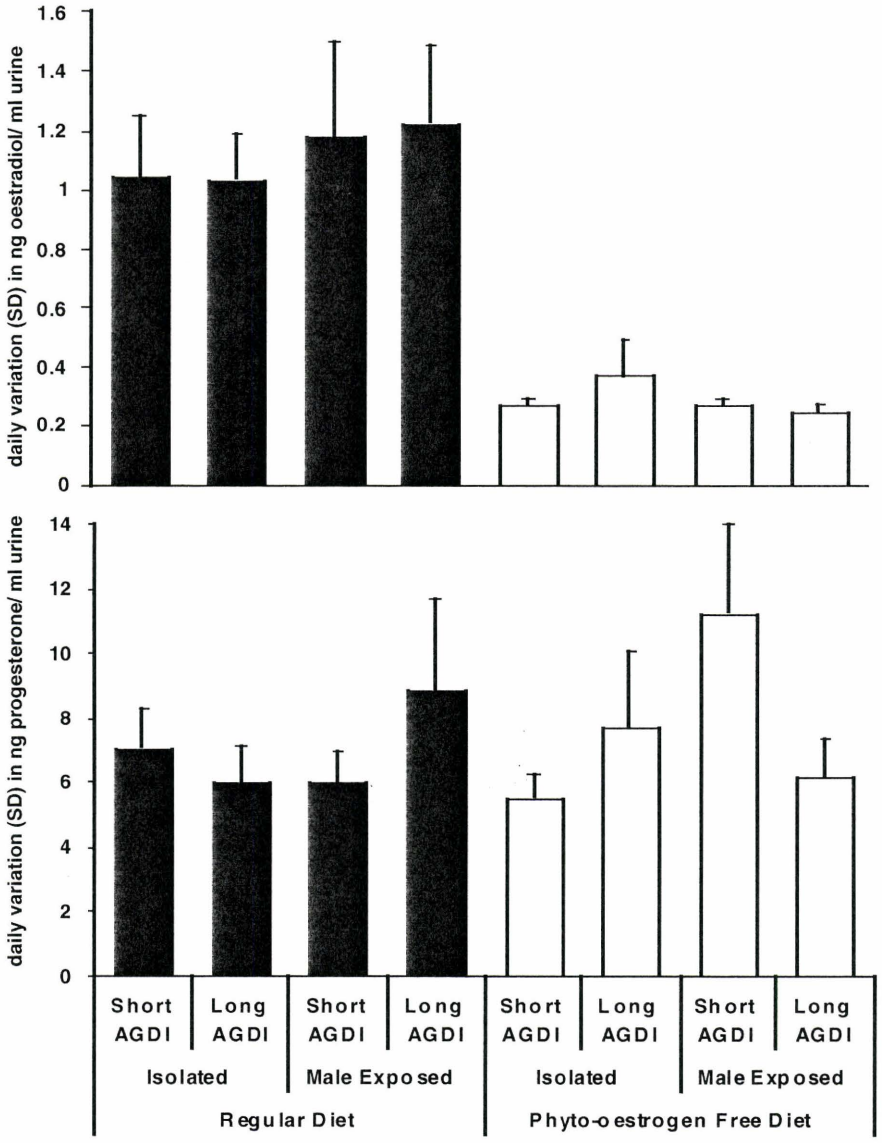

daily variation (SD) in ng oestradiol/ ml urine
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
daily variation (SD) in ng progesterone/ ml urine
14
12
10
8
6
4
2
0
Short AGDI
Long AGDI
Short AGDI
Long AGDI
Short AGDI
Long AGDI
Short AGDI
Long AGDI
Isolated
Male Exposed
Isolated
Male Exposed
Regular Diet
Phyto-oestrogen Free Diet

Figure 2 shows the mean daily urinary oestradiol, progesterone, and creatinine levels for females raised on either a regular or phyto-oestrogen-free diet, among those of short or long ano-genital distance (AGDI) either housed alone or exposed through a grid to two adult males. For each measure, a factorial analysis of variance was conducted separately for each diet, with male exposure and AGDI treated as between subjects and day treated as within subjects. Among females on the regular diet, urinary oestradiol showed a significant effect of day, $F(13,598) = 3.05$, $p = 0.0004$, and a significant interaction of day and male exposure, $F(13,598) = 7.11$, $p < 0.0001$. Among females on the phyto-oestrogen-free diet, there were main significant effects of male exposure, $F(1,42) = 12.95$, $p = 0.0012$, and of day, $F(13,546) = 3.35$, $p = 0.0002$. Among females on the regular diet, urinary progesterone showed only a significant effect of day, $F(13,598) = 2.06$, $p = 0.0146$. Among those on the phyto-oestrogen-free diet, there was a significant main effect of day, $F(13,546) = 8.25$, $p < 0.0001$, and a significant interaction of male exposure and AGDI, $F(1,42) = 4.96$, $p = 0.0296$. Urinary creatinine declined over days in females that were exposed to males. For females on the regular diet, creatinine levels showed significant main effects of male exposure, $F(1,46) = 4.11$, $p = 0.0456$, and day, $F(13,598) = 4.19$, $p < 0.0001$, and a significant interaction of male exposure and day, $F(13,598) = 3.92$, $p < 0.0001$. For females on the phyto-oestrogen-free diet, creatinine levels showed significant main effects of male exposure, $F(1,42) = 14.11$, $p = 0.0008$, and day, $F(13,546) = 8.82$, $p < 0.0001$, and a significant 3-way interaction, $F(13,546) = 1.90$, $p = 0.0276$.

Figure 2. Mean daily urinary oestradiol, progesterone, and creatinine levels for females raised on either a regular or phyto-oestrogen-free diet, among those of short or long ano-genital distance index (AGDI) either housed alone or exposed through a grid to two adult males.

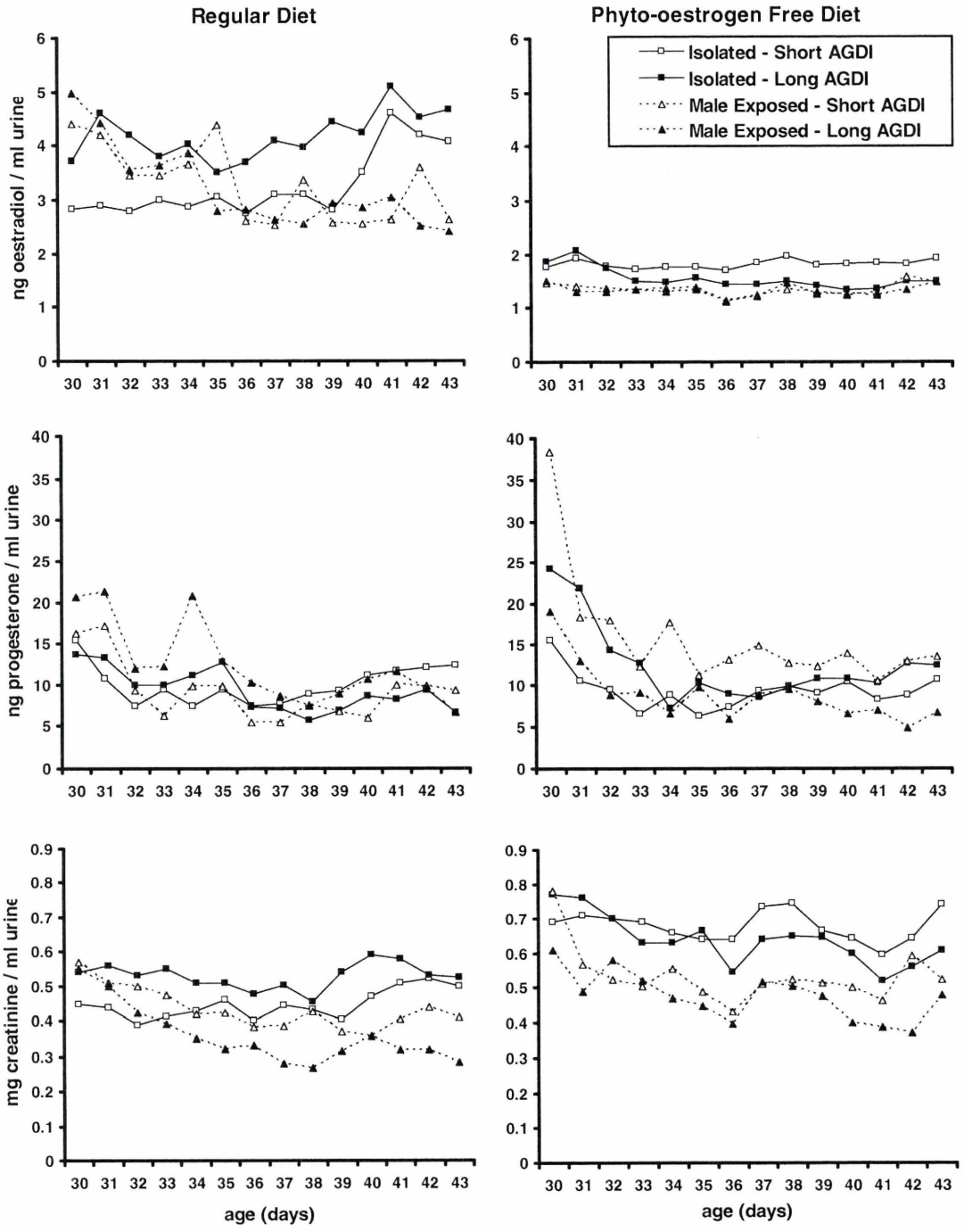

Regular Diet
Phyto-oestrogen Free Diet
Isolated - Short AGDI
Isolated - Long AGDI
Male Exposed - Short AGDI
Male Exposed - Long AGDI
ng oestradiol / ml urine
ng progesterone / ml urine
mg creatinine / ml urine
age (days)
age (days)

Figure 3 shows a measure of variation over days in urinary oestradiol and progesterone levels, the standard deviation of the daily measure for each individual over days 30-43 of age. A 2 (diet) x 2 (male exposure) x 2 (AGDI) factorial analysis of variance for oestradiol indicated a significant main effect of diet on variation over days, $F(1, 88) = 42.97$, $p < 0.0001$ with no other significant main effects or interaction. Variation over days in progesterone levels showed a significant 3-way interaction, $F(1,88) = 4.89$, $p = 0.0279$, and no other significance main effect or interaction. Multiple comparisons on this measure did not identify the nature of this interaction. Variation over days was also analysed for urinary creatinine, and this showed no significant effects.

Figure 3. Mean (±S.E.M.) of the standard deviation for each individual over daily measures of urinary oestradiol and progesterone, for females of short or long ano-genital distance index (AGDI), raised on either a regular or phyto-oestrogen-free diet, and either housed alone or exposed through a grid to two adult males.

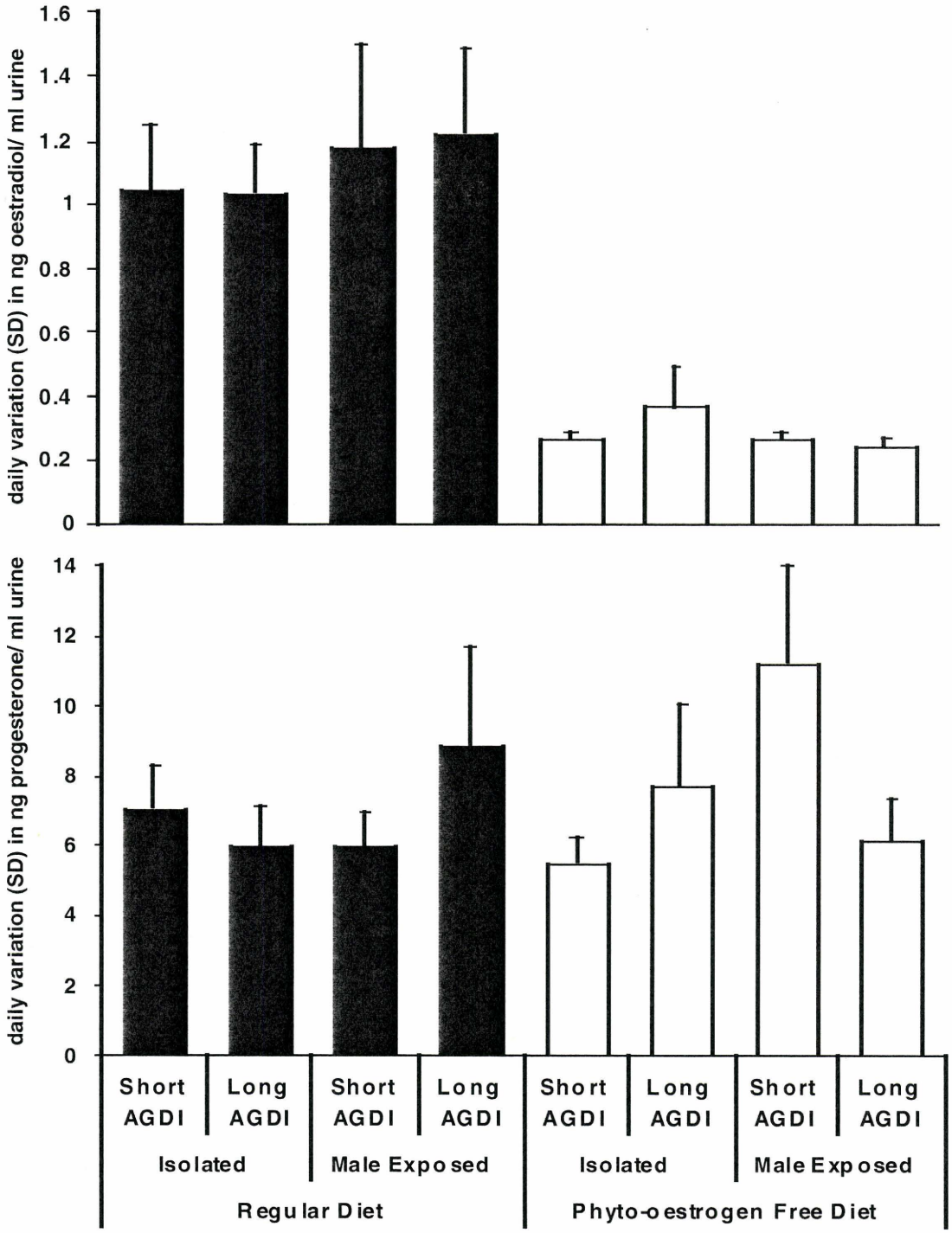
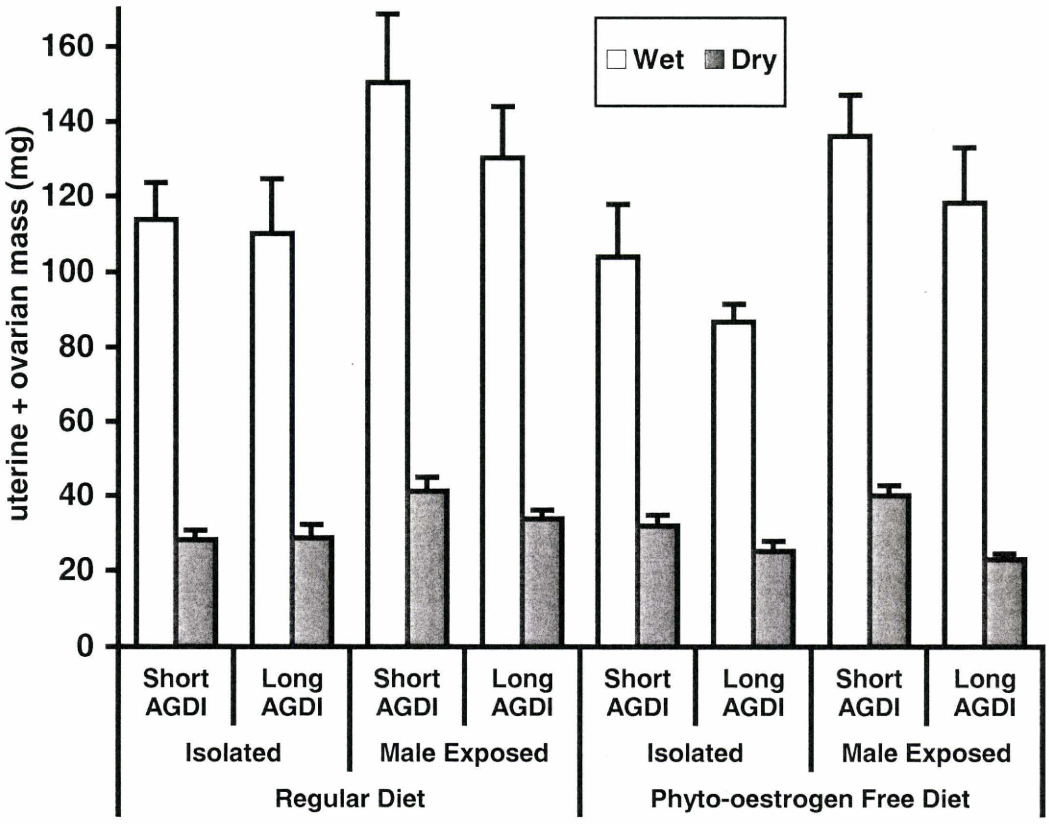

daily variation (SD) in ng oestradiol/ ml urine
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
daily variation (SD) in ng progesterone/ ml urine
14
12
10
8
6
4
2
0
Short AGDI
Long AGDI
Short AGDI
Long AGDI
Short AGDI
Long AGDI
Short AGDI
Long AGDI
Isolated
Male Exposed
Isolated
Male Exposed
Regular Diet
Phyto-oestrogen Free Diet

Figure 4 provides wet and dry combined uterine and ovarian weights on day 43 of development for females by conditions. A 2 (diet) X 2 (male exposure) X 2 (AGDI) factorial analysis of variance for wet weights indicated a significant main effect of male exposure and no other significant main effect or interaction, $F(1,88) = 10.10$, $p = 0.0024$. Analysis of dry tissue weight showed significant main effects of male exposure, $F(1,88) = 8.90$, $p = 0.0040$, and AGDI $F(1,88) = 14.64$, $p = 0.0005$, and interactions between AGDI and diet, $F(1,88) = 4.64$, $p = 0.0319$ and between AGDI and male exposure, $F(1,88) = 5.33$, $p = 0.0222$. Multiple comparisons indicated that male-exposed females on either diet with short-AGDI had heavier reproductive tissue than those of all other groups except each other and the regular-diet, male-exposed, long-AGDI females.

Figure 4. Mean (±S.E.M.) of wet and dry combined uterine and ovarian weight on day 43 of development, for females of short or long ano-genital distance index (AGDI), raised on either a regular or phyto-oestrogen-free diet, and either housed alone or exposed through a grid to two adult males.



Discussion

These data indicate that the influence of male exposure on female sexual maturation is modulated by phyto-oestrogen content of diet and to a lesser extent by prenatal hormonal milieu as reflected in AGDI. They also suggest that the onset of female sexual maturity can be assessed by urinary steroid measures, but more in the variability of these measures over days than in their absolute levels. Urinary oestradiol and progesterone were more dynamic over days in urine of male-exposed females, especially among females on the regular diet. Urinary progesterone was elevated in urine of male-exposed females compared to isolated females, with the size of this effect dependent on AGDI, diet, and whether the measure was adjusted for creatinine. Urinary creatinine itself was elevated by the phyto-oestrogen-free diet and reduced by male exposure, tending to decline over days in females exposed to males. Male exposure increased uterine and ovarian mass and the size of this effect was influenced by AGDI in interaction with diet.

Urinary steroid measures have the advantages of being noninvasive as they are not altered by human handling and blood sampling. This permits repeated measures and profiling of individuals over time and across conditions (Muir *et al.* 2001; Vella & deCatanzaro 2001; deCatanzaro *et al.* 2003; 2004; 2006; Beaton *et al.* 2006). Unconjugated ovarian and testicular steroids are reliably measurable in the urine of this species, and in general, patterns of urinary excretion have reflected known systemic steroid trends. Levels of testicular and ovarian steroids are substantially reduced by castration (Vella & deCatanzaro 2001; deCatanzaro *et al.* 2003). Oestradiol and

progesterone levels in urine are dynamic over days of the oestrous cycle and rise substantially during mid-pregnancy then decline in later pregnancy (deCatanzaro *et al.* 2003; 2004). Nevertheless, it remains possible that some variation in excreted levels is distinct from that of systemic levels, given dynamics of receptor binding and excretory mechanisms.

It is usual practice to adjust urinary steroid output for creatinine in order to compensate for variation in hydration (Munro *et al.* 1991; Muir *et al.* 2001; deCatanzaro *et al.* 2003). We found that urinary creatinine was itself dynamic among conditions, tending to be lower and to decline progressively in male-exposed as opposed to isolated females, and to be somewhat higher in females raised on the phyto-oestrogen-free diet. An influence of proximity to males was also reported elsewhere (Beaton *et al.* 2006). One possibility is that movements of male-exposed females were impeded due to presence of males in adjacent compartments. Isolated and group-housed mice have been found to have a variety of differences in hormone levels in previous experimentation (Brain 1975). As well, fluid intake can also be influenced by social housing conditions, and urinary output in mice is also influenced by reproductive status (Drickamer 1995). Our major findings with urinary oestradiol and progesterone were observed both with and without creatinine adjustment. We suggest that it may not always be appropriate to adjust urinary steroid levels for creatinine for at least this species, especially given that laboratory animals have constant access to water.

Urinary oestradiol was substantially reduced in females raised on the phyto-oestrogen-free diet. The amount of phyto-oestrogen content in diet has been shown to

directly correlate with phyto-oestrogen concentrations in plasma and urine. Rodents fed diets high in phyto-oestrogen content also display high levels of isoflavone concentrations in their urine (Brown & Setchell 2001). The source of the difference in urinary oestradiol observed here is unknown. The antibodies used in our enzyme assay were polyclonal, and could possibly show some binding with oestrogenic components excreted in urine of females that were fed the regular diet. Nevertheless, the increased intra-individual variability in oestradiol with male exposure was observed despite any such possible direct influence of phyto-oestrogens on urinary measures.

The increase observed here in combined mass of uterus and ovaries among male-exposed females at age 43 is consistent with previous findings (e.g. Beaton *et al.* 2006). The current data also indicate that AGDI has a significant impact upon dry uterine and ovarian mass, with long AGDI females having lesser reproductive tract mass. There was also some interaction of diet with this influence of AGDI upon dry uterine and ovarian mass, with the effect being most apparent in females raised on the phyto-oestrogen-free diet. Uterine growth over development and within oestrous cycles is highly influenced by oestrogens, with specific roles depending on timing and species (Gray *et al.* 2001). In mice, uterine cells proliferate in response to exogenous oestradiol (Ogasawara *et al.* 1983). Oestrogens regulate growth hormone and IGF-1 action (Kahlert *et al.* 2000; Leung *et al.* 2004), and local IGF-1 activity mediates uterine growth in response to oestradiol (Sato *et al.* 2002). Male urine also contains significant quantities of unconjugated oestradiol (Vella & deCatanzaro 2001; deCatanzaro *et al.* 2006; Beaton *et al.* 2006), and male mice actively direct urine droplets at proximate females (Reynolds

1971; deCatanzaro *et al.* 2006). Since oestrogens are established to play a critical role in male-induced female precocious puberty (Bronson 1975), oestrogens in male excretions could contribute to pubertal development in proximate females.

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Chapter 4

Preputialectomised and intact adult male mice exhibit an elevated urinary ratio of oestradiol to creatinine in the presence of developing females, whilst promoting these females' uterine and ovarian growth

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Authors' Contributions

Ayesha Khan: Concept, experimental design, data collection, ELISA procedures, literature review, data analysis and manuscript writing.

Robert G. Berger: Assistance with ELISA procedures and manuscript editing.

Denys deCatanzaro: Assistance with concept development, data analysis and manuscript editing.

Additional Assistance

Undergraduate Students: Elizabeth Ackloo, Shaun Fernandes, and Erin Lillie provided assistance with experimental procedures.

Introduction

In mammals, adult males can accelerate sexual maturity in nearby juvenile females of several species including mice (Vandenbergh 1967, 1969, 1975; Drickamer 1975), rats (Vandenbergh 1976), deer mice (Teague and Bradley 1978), lemmings (Hasler and Banks 1975) voles (Spears and Clarke 1986), opossums (Harder and Jackson 2003), and cattle (Roberson *et al.* 1991). Chemosignals emitted by male conspecifics are known to modify the reproductive status of developing females (Colby and Vandenbergh 1974; Lombardi and Vandenbergh 1977; Drickamer and Murphy 1978; Massey and Vandenbergh 1981; Izard and Vandenbergh 1982; Pandey and Pandey 1988; Price and Vandenbergh 1992). Male preputial glands have been implicated in a variety of pheromonally-mediated phenomena, including sexual attraction (Caroom and Bronson 1971; Orsulak and Gawienowski 1972; Gawienowski *et al.* 1975; Ninomiya and Kimura 1988), intermale aggression (McKinney and Christian 1970; Jones and Nowell 1973; Hayashi 1987; Thompson *et al.* 2007), intermale dominance (Bronson and Marsden 1973; Barnett *et al.* 1980; Novotny *et al.* 1990), and induction of oestrus in females (Marchlewska-Koj *et al.* 1990; Ma *et al.* 1999).

The male preputials are bilateral leaf-like subcutaneous organs that lie on each side of the midline genitalia (Brown and Williams 1972) with excretory ducts that run along the lateral surface of the penis and empty through an opening on the surface of the urethral meatus (Beaver 1960). Maturation of the preputial glands is androgen dependent; they are more developed in males than in females and are enhanced by exposure to testosterone (Beaver 1960) or its esters (Nickerson *et al.* 1976). In male

mice, the average combined mass is 15 mg at three weeks of age, then increases to ~60-75 mg at sexual maturity and remains constant until a decrease in old age (Rudali *et al.* 1974).

Some evidence indicates that male preputial emissions influence oestrous behaviour and contribute to the onset of female sexual maturity in mice. Exposure to preputial gland homogenates (Marchlewska-Koj *et al.* 1990) or extracts (Ma *et al.* 1999) causes oestrous cycling to resume in group-housed adult females. Preputialectomised males' urine is less effective than intact males' urine in stimulating oestrus in adult females, although both are more effective than castrated males' urine (Chipman and Albrecht 1974). Exposure to preputial fluid alone can elicit oestrus in adult females, but less than does intact males' urine (Chipman and Albrecht 1974). Among constituents of preputial fluid are the sesquiterpenic compounds E,E-α-farnesene and E-β-farnesene (Novotny *et al.* 1990). The concentration of farnesenes can be contingent upon endocrine and social status, with elevated levels in emissions of dominant males (Harvey *et al.* 1989; Novotny *et al.* 1990). Synthetic mixtures of farnesenes can induce onset of oestrus in adult females with suspended cycles (Ma *et al.* 1999) and decrease time to sexual maturity in developing females (Novotny *et al.* 1999a). Nevertheless, incubated preputial fluids are more effective than fresh fluids in attracting females while farnesene concentration remains unchanged (Ninomiya and Kimura 1988; Ninomiya *et al.* 1993).

Other evidence suggests that the preputials are not required for some parameters of male-induced puberty acceleration. Urine collected directly from the bladder is as effective as excreted urine in accelerating time to first oestrus in developing females

(Bronson and Whitten 1968). Bladder urine does not contain preputial secretions since these glands are located below the connection of the bladder with the urethra (Green 1966). Ages of vaginal opening and first oestrus are comparable in female mice exposed to urine of preputialectomised males or intact males (Colby and Vandenbergh 1974). One possibility is that male urinary steroids, especially oestrogens, also promote sexual development in proximate females. Male mouse urine contains substantial quantities of unconjugated 17β-oestradiol and other steroids (Muir *et al.* 2001; Beaton *et al.* 2006; deCatanzaro *et al.* 2006, 2009). Exogenous oestrogens can promote female sexual maturation, mimicking influences of male exposure (Bronson 1975). Oestrogens play critical roles in development of the female reproductive tract (Ogasawara *et al.* 1983; Sato *et al.* 2002) and preparation of oestrous behaviour (Pfaff 1980; deCatanzaro 1987).

The current study was designed to investigate whether the preputials are necessary for males' capacity to accelerate sexual maturation in post-weaning females, and to examine whether male urinary steroid content depends on these glands. Uterine and ovarian mass, which is highly sensitive to male exposure (Beaton *et al.* 2006; Khan *et al.* 2008), was compared in isolated females and those exposed for two weeks to preputialectomised or sham-operated males. We also profiled urinary 17β-oestradiol, testosterone, and creatinine collected non-invasively throughout a two-week period while preputialectomised or sham-operated males were housed in isolation or exposed to developing females.

Materials and Methods

Subjects and experimental treatment

Female CF1 strain mice (*Mus musculus*) were bred from stock originally obtained from Charles River Breeding Farms of Canada (LaPrairie, Québec). Male mice were of heterogeneous strain (HS), produced by interbreeding across C57-B6, Swiss Webster, CF1, and DBA-2 strains. Housing conditions consisted of standard polypropylene cages measuring 28 x 16 x 11 (height) cm connected to wire grid tops allowing continuous access to food and water. The colony was maintained under a reversed 10:14 h dark:light cycle at 21°C. This research was approved by the McMaster University Animal Research Ethics Board, conforming to the standards of the Canadian Council on Animal Care.

Weanling female CF1 pups aged 28 days were randomly assigned to one of three conditions: exposed to two preputialectomised HS males (n=12), two sham-operated HS males (n=12), or housed in isolation (n=10). Proven HS males aged 7-10 months were pre-selected for sexual vigour and isolated for one week prior to surgery. Half (n=34) were preputialectomised and the remainder (n=34) underwent a sham surgical procedure. All animals successfully recovered from surgery and were in good health throughout the experiment. Experimental procedures began 3 weeks following surgery.

Preputialectomy

Animals were anaesthetised via inhalation of isoflurane in conjunction with pure oxygen. A single incision measuring approximately 5 mm was made on the right side of the external genitalia. The glands were found immediately under the skin and subsequently cut off at the stem. In some individuals, a thin membranous tissue was

present either over or between the two glands; this was removed as necessary. Once the glands were extracted, the skin incision was closed using silk suture thread. Animals were kept on a heating pad until normal activity resumed. Sham animals received identical treatment as those receiving full surgery, but the preputial glands were left undisturbed.

Apparatus

Two HS males were placed in the upper compartment of a two-tiered cage system containing either no female or a single juvenile CF1 female in the lower compartment as described in deCatanzaro *et al.* (1996). This apparatus was constructed from clear Plexiglas, measuring 30 x 21 x 27 cm, divided into upper and lower compartments (each measuring 30 x 21 x 13) by a stainless steel wire-mesh grid (squares of 0.5 cm^2) that allowed for excretions from the males to pass directly into the female's compartment below. An opaque Plexiglas partition prevented contact between the two males in the upper compartment. The female had full olfactory exposure to each male but the grid limited behavioural interaction. Each compartment provided continuous independent access to food and water, with food in the female's compartment protected in an outset closet from excretions of the males above.

After a 48-hour acclimation period to control for urinary hormone dynamics attributable to placement in a novel environment (deCatanzaro *et al.* 2004), daily urine collection began and continued for 14 days. On each day at the start of the dark phase of the lighting cycle, the upper compartments containing HS males were separated from the lower compartment for a period of 4 ± 2 hours. Male cages were gently lifted and placed

on a collection pan lined with wax paper in order to facilitate urine droplet identification. Urine was aspirated via 1-cc syringes with 26-gauge needles. A fresh syringe and needle were used for each animal on each day. Care was taken to ensure that urine samples were not contaminated with food residue or faeces. All samples were stored in labeled 1.5 ml Nalgene cryo-tubes and frozen immediately after collection at -20°C until assayed. Urine was collected from 16 of 24 female-exposed preputialectomised males, 16 of 24 female-exposed sham males and from all 10 isolated males in both sham and preputialectomised conditions.

Body, Uterine, and Ovarian Mass Measurement

Following 16 days of male exposure, females were weighed and then euthanised at 43 days of age using a lethal dose of CO_2 gas. Ovaries and uteri were extracted through a dorsolateral incision made near the abdominal cavity. Excess fat and mesentery were removed via standard criteria (Wang *et al.* 2005). All surgeries were performed by a single experimenter blind to each female's experimental condition. Wet tissue was measured immediately following surgery, whereas dry tissue mass was measured after tissue samples were stored in calcium sulfate crystals for thirty days at ~3°C before weighing.

Urinary Steroid and Creatinine Determination

Validations of enzyme immunoassay procedures have been previously reported (Muir *et al.*, 2001). Creatinine, 17β-oestradiol, and testosterone were obtained from Sigma Chemical Co. (St Louis, MO, USA). Antibodies to 17β-oestradiol and testosterone and corresponding horseradish peroxidase conjugates were obtained from the

Department of Population Health and Reproduction at the University of California (Davis). Cross reactivities for anti-17β-oestradiol are: 17β-oestradiol 100%, oestrone 3.3%, progesterone 0.8%, testosterone 1.0%, androstenedione 1.0% and all other measured steroids <0.1%. Cross reactivities for anti-testosterone are: testosterone 100%, 5α-dihydrotestosterone 57.4%, androstenedione 0.27%, androsterone, and DHEA, cholesterol, 17β-oestradiol, progesterone, and pregnenolone <0.05%. Nunc Maxisorb plates were first coated with 50 µl of antibody stock diluted at 1:10,000 in a coating buffer (50 mmol bicarbonate buffer l^{-1}, pH 9.6) and stored for 12–14 h at 4 °C. Wash solution (0.15 mol NaCl l^{-1} containing 0.5 ml of Tween 20 l^{-1}) was then added five times to each well using an automated strip washer (Bio-Tek Instruments Inc. model ELx50) to rinse away any unbound antibody and then 50 µl phosphate buffer (0.1 mol l^{-1} sodium phosphate buffer, pH 7.0 containing 8.7 g of NaCl and 1 g of BSA per litre) per well was added. Plates were incubated at room temperature (21 °C) for 2 h for oestradiol determination and 30 min for testosterone determination before adding standards, samples, or controls.

For oestradiol and testosterone, urine samples were diluted 1:8 in phosphate buffer before they were added to the plate. Standard curves were derived by serial dilution from a known stock solution. Oestradiol stock was 2000 pg ml^{-1} diluted to 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 1.95, 0.98, 0.46 and 0.23 pg ml^{-1}. Testosterone stock was 50,000 pg ml^{-1} diluted to 1250, 650, 325, 162.5, 81.25, 40.63 20.31, 10.16, 5.08, 2.54, 1.27, and 0.63 pg ml^{-1}. For all assays, 50 µl oestradiol or testosterone horseradish peroxidase was added to each well, with 20 µl of standard, sample, or control for

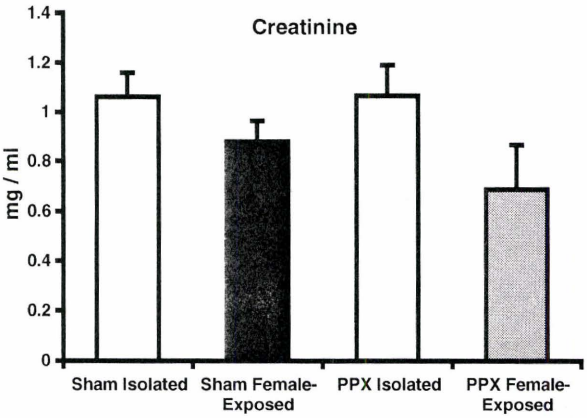
oestradiol or 50 µl of standard, sample, or control for testosterone. The plates were incubated for 2 h for oestradiol and 30 min for testosterone at room temperature. Subsequently, the plates were washed and 100 µl of a substrate solution of citrate buffer, H_2O_2 and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) were added to each well and the plates were covered and incubated while on an orbital shaker at room temperature for approximately 30 to 60 min. The plates were then read with a single filter at 405 nm on the microplate reader (Bio-Tek Instruments Inc., model ELX 808).

It is convention with urinary steroid analyses to compensate for variations in fluid intake and output by adjusting sample values for creatinine (Munro *et al.* 1991; Muir *et al.* 2001; deCatanzaro *et al.* 2003). Standard creatinine values of 100.0, 50.0, 25.0, 12.5, 6.25, 3.12, 1.56, and 0.78 µg ml^{-1} were used. All urine samples were diluted 1:41 in phosphate buffer. Dynatech Immulon flat bottom plates were used and 50 µl per well of standard was added together with a solution of 50 µl distilled water, 50 µl 0.75 mol l^{-1} NaOH and 50 µl 0.4 mol l^{-1} picric acid. The plates were then incubated at room temperature for 30 min. Plates were measured for absorbance on a microplate reader with a single filter at 490 nm. Standard curves were generated, regression lines were fit, and the regression equation was applied to the optical density for each sample. Steroid measurements were adjusted for creatinine by dividing the obtained value by the measure of creatinine per ml of urine. Both creatinine-adjusted and unadjusted steroid measures were analysed statistically.

Results

Figure 1 gives values for urinary creatinine, and unadjusted and creatinine-adjusted urinary oestradiol and testosterone, with each male receiving a single average value of the 14 daily samples. One isolated preputialectomised animal was removed due to insufficient urine samples. Urinary creatinine was reduced in female-exposed as compared to isolated males. A 2 (female-exposed vs. isolated) by 2 (preputialectomised vs. sham-operated) factorial analysis of variance indicated a significant effect of female exposure on creatinine, $F(1,47) = 13.47$, $p = 0.001$, but no main effect of surgery and no interaction. Unadjusted urinary oestradiol was greater in isolated males than in those exposed to females, with this main effect reaching significance, $F(1,47) = 4.72$, $p = 0.033$, while the main effect of surgery and the interaction were not significant. There were no significant effects for unadjusted urinary testosterone. Creatinine-adjusted oestradiol and testosterone were elevated in female-exposed as opposed to isolated males. For creatinine-adjusted oestradiol, the main effect of female exposure was significant, $F(1,47) = 5.85$, $p = 0.018$, but there was no effect of surgery and no interaction. Creatinine-adjusted testosterone showed significant main effects of female exposure, $F(1,47) = 9.94$, $p = 0.003$, and surgery, $F(1,47) = 5.34$, $p = 0.024$, but no interaction.

Figure 1. Mean (±S.E.) urinary creatinine, 17β-oestradiol, testosterone, creatinine-adjusted 17β-oestradiol, and creatinine-adjusted testosterone, giving the average value for each individual male during 14 days of exposure to developing females or isolation, for preputialectomised (PPX) or intact sham-operated (sham) males. In 2 x 2 factorial analyses of variance, the main effect of female exposure versus isolation was significant ($p < 0.05$) for creatinine, oestradiol, creatinine-adjusted oestradiol, and creatinine-adjusted testosterone.


Creatinine
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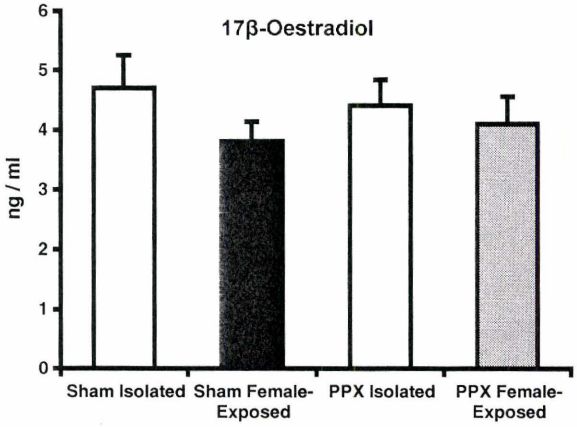
PPX Female-Exposed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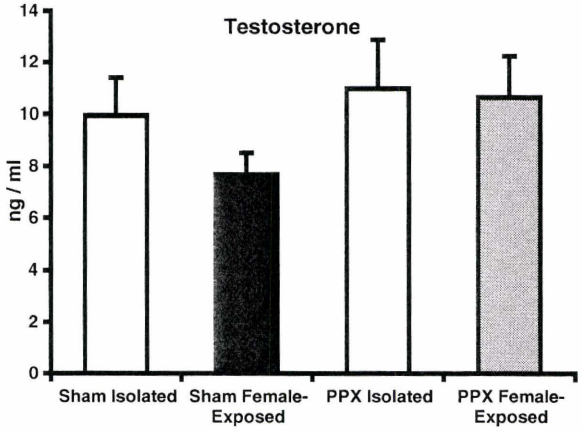
Testosterone
ng / ml
14
12
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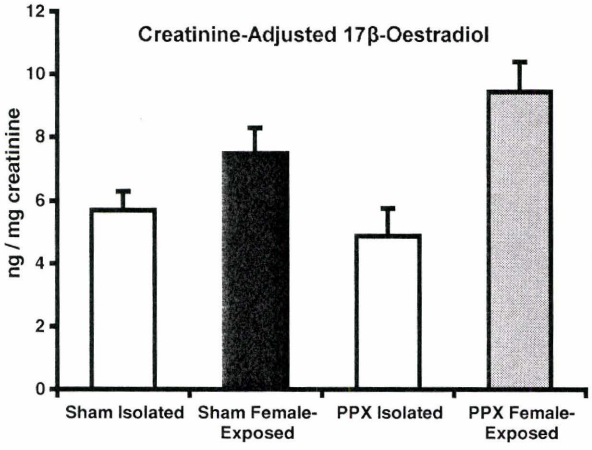
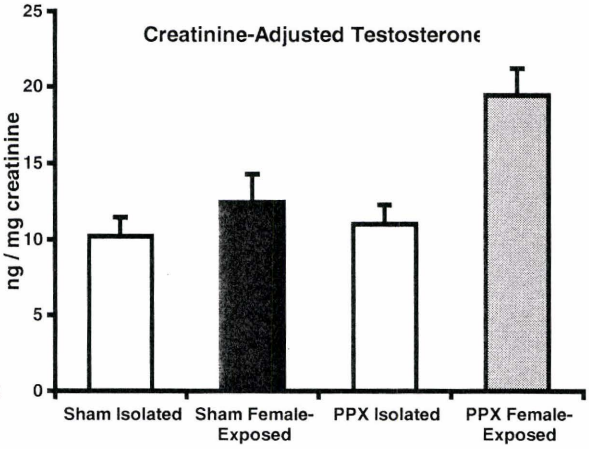
Sham Isolated
Sham Female-Exposed
PPX Isolated
PPX Female-Exposed


Creatinine-Adjusted 17β-Oestradiol
ng / mg creatinine
12
10
8
6
4
2
0
Sham Isolated
Sham Female-Exposed
PPX Isolated
PPX Female-Exposed


Creatinine-Adjusted Testosterone
ng / mg creatinine
25
20
15
10
5
0
Sham Isolated
Sham Female-Exposed
PPX Isolated
PPX Female-Exposed

As some males did not produce sufficient urine samples on all days, measures were averaged into seven 2-day measures to eliminate missing values. Figure 2 provides mean values in the four conditions for urinary creatinine, oestradiol, testosterone, creatinine-adjusted oestradiol, and creatinine-adjusted testosterone. A 2 (female-exposed vs. isolated) by 2 (preputialectomised vs. sham-operated) by 7 (repeated measure) factorial analysis of variance was conducted for each measure. For creatinine, there was a significant main effect of female exposure, $F(1,47) = 13.33$, $p < 0.001$, and a significant interaction of surgery and day, $F(6,282) = 2.43$, $p = 0.026$, but no other main effects or interaction. Analysis of unadjusted oestradiol revealed main effects of female exposure, $F(1,47) = 5.02$, $p = 0.028$, and day, $F(6,282) = 2.24$, $p = 0.038$, and a significant three-way interaction, $F(6,282) = 3.01$, $p = 0.007$. Unadjusted urinary testosterone showed a significant two-way interaction of surgery and day, $F(6,282) = 2.48$, $p = 0.023$, but no other effects. For creatinine-adjusted oestradiol, there was a main effect of female exposure, $F(1,47) = 5.30$, $p = 0.024$, but no other main effects or interactions. Creatinine-adjusted testosterone showed a significant effect of female exposure, $F(1.46) = 6.44$, $p = 0.014$, but no other main effects or interactions.

Figure 2. Mean urinary creatinine, 17β-oestradiol, testosterone, creatinine-adjusted 17β-oestradiol and creatinine-adjusted testosterone during seven 2-day intervals over 14 days of exposure to developing females or isolation for preputialectomised (PPX) or intact sham-operated (sham) males. In 2 x 2 x 7 factorial analyses of variance, the main effect of female exposure versus isolation was significant ($p < 0.05$) for creatinine, oestradiol, creatinine-adjusted oestradiol, and creatinine-adjusted testosterone.

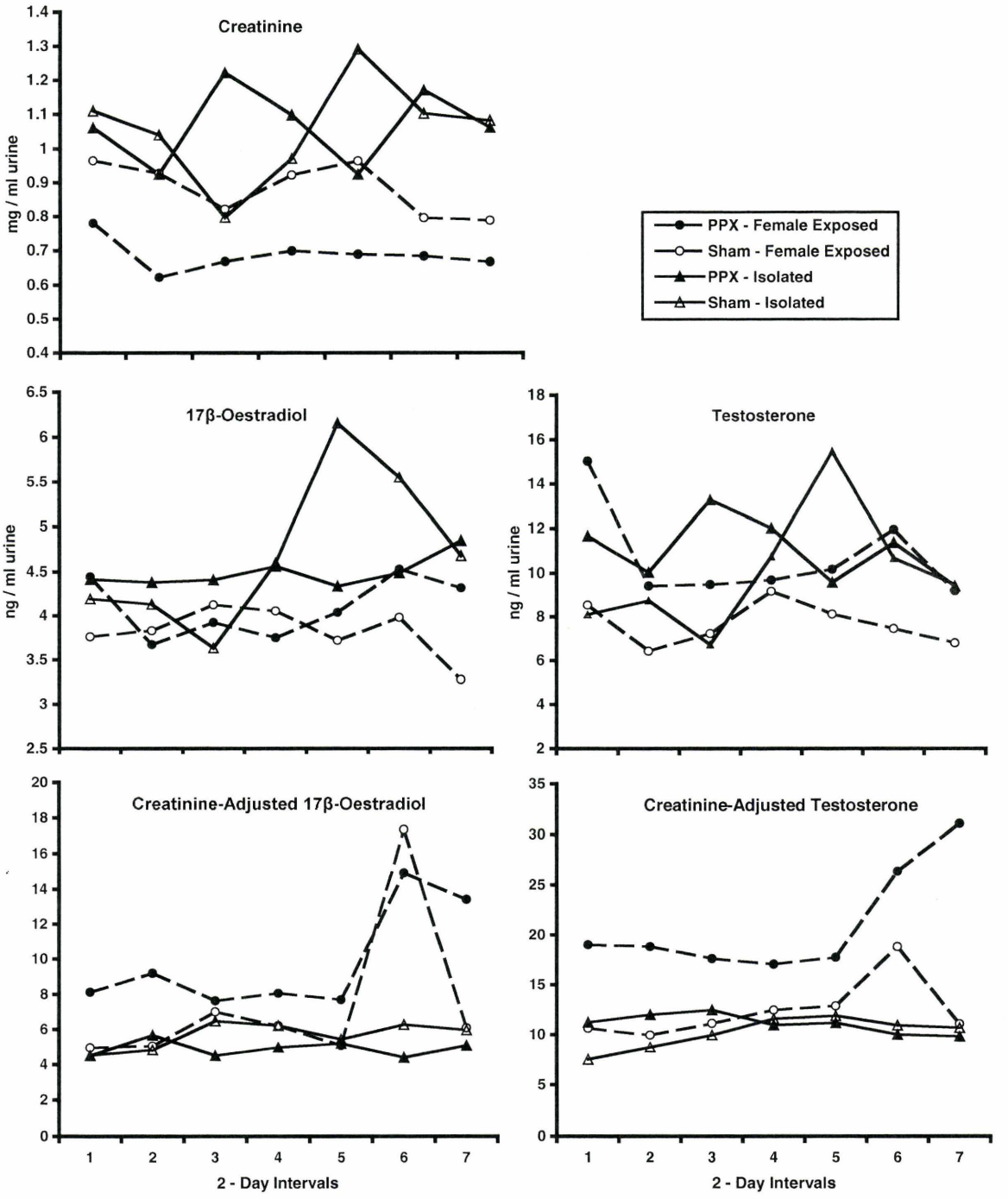
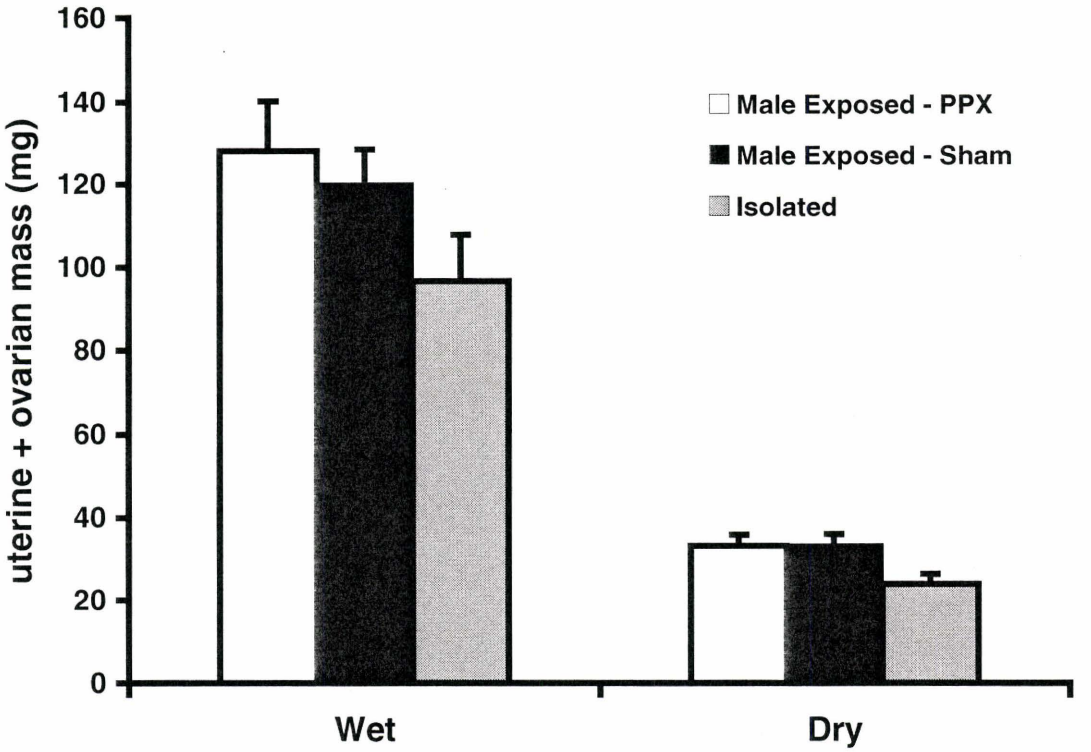

Creatinine
mg / ml urine
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
PPX - Female Exposed
Sham - Female Exposed
PPX - Isolated
Sham - Isolated
17β-Oestradiol
ng / ml urine
6.5
6
5.5
5
4.5
4
3.5
3
2.5
Testosterone
ng / ml urine
18
16
14
12
10
8
6
4
2
Creatinine-Adjusted 17β-Oestradiol
20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
2 - Day Intervals
Creatinine-Adjusted Testosterone
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
2 - Day Intervals

Figure 3 provides wet and dry combined uterine and ovarian mass on day 43 of development for females by condition. Analysis of variance on dry tissue mass revealed a significant effect of conditions, $F(2,31) = 4.21$, $p = 0.024$. Newman-Keuls multiple comparisons ($p < 0.05$) indicated that mass for the isolated females was significantly lower than that of both other groups. Mean body mass was 27.10±0.72 for isolated females, 25.83±0.49 for females exposed to sham-operated males, and 25.33±0.63 for females exposed to preputialectomised males; this measure did not show statistical significance.

Figure 3. Mean (±S.E.) of wet and dry uterine plus ovarian mass for 43-day old females following two weeks of housing in isolation, below two preputialectomised (PPX) males, or below two intact sham-operated (sham) males. One-way analysis of variance on dry mass was significant; multiple comparisons ($p < 0.05$) identified differences between isolated females and those exposed to preputialectomised males, and between isolated females and those exposed to sham-operated males.



Discussion

These data demonstrate that female uterine and ovarian growth can be hastened by exposure to adult males despite removal of the males' preputial glands. They also indicate that the chemistry of males' urine is altered by exposure to developing females. Levels of creatinine were reduced in males' urine by exposure to females, and similar trends were evident in unadjusted oestradiol and testosterone. However, creatinine-adjusted oestradiol and testosterone were both significantly elevated in female-exposed males. Each change in urine chemistry was observed in both intact and preputialectomised males.

These data indicate that the preputial glands are not necessary for males' capacity to accelerate growth of reproductive tissue in juvenile female mice. This is consistent with an earlier report (Colby and Vandenbergh 1974) that females show similar ages of first vaginal opening and oestrus when exposed to urine of preputialectomised or intact males. Nevertheless the preputials contribute to other measures of female sexual receptivity. Previous investigations implicate male preputials in stimulation of oestrus in reproductively-mature female mice (Chipman and Albrecht 1974; Marchlewska-Koj *et al.* 1990; Ma *et al.* 1999). However urine extracted directly from the bladder induces oestrus in adult females, suggesting factors already present prior to contact with accessory gland secretions (Bronson and Whitten 1967); these findings have been replicated in developing females (Drickamer and Murphy 1978). Others have suggested that male pheromones derive from both the bladder and preputials. Along with preputial-originating E,E-α-farnesene and E-β-farnesene, bladder-originating male urinary

constituents such as 2-*sec*-butyl-4,5-dihydrothiazole (SBT), 3,4-dehydro-*exo*-brevicomin (DHB) and 6-hydroxy-6-methy-3-heptanone have been suggested as putative puberty-inducing pheromones in females (Novotny *et al.* 1999b). All of these readily bind with major urinary proteins, which may release volatile chemosignals into the environment (Novotny *et al.* 1999a; Beynon and Hurst 2004). Pharmacological nasal application of synthetic analogues of SBT and DHB, 6-hydroxy-6-methy-3-heptanone, and farnesenes, mixed with castrated male urine in various combinations and alone, can increase uterine mass in exposed females (Novotny *et al.* 1999a).

As demonstrated here and elsewhere (Vella and deCatanzaro 2001; deCatanzaro *et al.* 2006; Beaton *et al.* 2006), male mouse urine contains abundant quantities of unconjugated 17β-oestradiol and testosterone. These quantities diminish gradually during the two months after castration (Vella and deCatanzaro 2001). Given that male mice actively direct urine at nearby females (Reynolds 1971; Maruniak *et* al., 1974; Hurst, 1990; deCatanzaro *et al.* 2006, 2009), oestrogens in males' excretions could impinge upon proximate females' physiology, with absorption facilitated by the small molecular size and lipophilic nature of oestrogens (*cf.* deCatanzaro *et al.* 2006). Females' circulating oestradiol is dynamic within the initial days of exposure to novel males, showing spikes relative to measures in isolated females (Bronson and Desjardins, 1974). Two injections of less than 0.2 μg/animal of 17β-oestradiol benzoate can induce ovulation in pre-pubertal females, resembling the effects of three full days of male exposure (Bronson 1975). Moreover, as observed here and elsewhere (Beaton *et al.* 2006; deCatanzaro *et al.* 2006, 2009), the ratio of urinary oestradiol to creatinine rises in

males when females are proximate, suggesting that males have evolved to emit greater quantities of oestrogens while in the presence of females. There is a rich distribution of oestrogen receptors in female reproductive tissue (Cooke *et al.* 1997; Fitzpatrick *et al.* 1999; Jefferson *et al.* 2002). Both the uterus (Das 1972; Cooke *et al.* 1997) and ovaries (Yang *et al.* 2002) are responsive to exogenous oestrogens. Oestradiol causes uterine cells to proliferate and drives the growth of the uterus over development in interaction with local growth hormone and IGF-1 activity (Ogasawara *et al.* 1983; Ghahary and Murphy 1989; Kahlert *et al.* 2000; Sato *et al.* 2002; Leung *et al.* 2004). The influence of oestrogens on uterine growth in laboratory rodents is so reliable that uterine growth is widely used in toxicological assessment of oestrogenicity of diverse exogenous substances (*e.g.* Evans *et al.* 1941; Shaw and deCatanzaro 2009).

In the present study we did not observe a similar effect of male exposure on body mass, despite the general correlation of sexual maturity in females with increasing body mass (Frisch *et al.* 1980; Chehab *et al.* 1997; Lin-Su *et al.* 2002; Zeinoaldini *et al.* 2006). Individual rats that are slow in somatic growth reach sexual maturity at the same body mass but at a later age than do those growing rapidly (Kennedy and Mitra 1963). However in one report, attainment of vaginal opening occurred at roughly the same age despite differences in body mass (Davis and Lamberson 1991). Our observations are concordant with previous findings showing either no influence or just a small increase in body mass in females exposed to males, despite major growth of the reproductive tract (*e.g.* Lombardi and Vandenbergh 1977; Beaton *et al.* 2006).

Urinary creatinine is an index of metabolic activity and is routinely used in urinary steroid analyses to compensate for variations in fluid intake and excretion within and among individuals (*e.g.* Erb *et al.* 1970; Munro *et al.* 1991; Muir *et al.* 2001; deCatanzaro *et al.* 2003). This is based on the assumption that the rate of creatinine excretion is fairly constant (*e.g.* Erb *et al.* 1970; Munro *et al.* 1991; Boeniger *et al.* 1993; Muir *et al.* 2001). The present data indicate that urinary output of creatinine itself varies as a function of social condition. Elsewhere, proximity to developing females has been shown to increase males' rates of fluid intake and urination, consistent with lower creatinine levels indicating greater dilution of urine (deCatanzaro *et al.* 2009). Urine output may also be influenced by age, sex, endocrine and social status (Drickamer 1989, 1995). Accordingly, analysis of creatinine and both unadjusted and adjusted steroid levels would be appropriate in interpreting urinary steroid dynamics in future studies.

We have demonstrated here that, regardless of the presence of the preputial glands, adult males excrete substantial quantities of unconjugated oestradiol and testosterone in their urine. Via absorption by proximate females and in combination with other pheromonal factors, males' excreted steroids could contribute to growth of reproductive tissue and the onset of female sexual maturity.

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Chapter 5

General Discussion

Rationale

The experiments in this thesis were designed to investigate the role of three major factors that influence the timing to sexual maturity in female mice. These were the presence or absence of adult males during the two weeks following weaning, phytoestrogen content of diet during development, and varied prenatal exposure to siblings' androgens as inferred from the females' AGDI.

Some evidence indicates that preputial emissions from proximate adult males contribute to developmental changes in juvenile females (Chipman & Albrecht, 1974; Ma *et al.*, 1999; Marchlewska-Koj *et al.*, 1990; Novotny *et al.*, 1999a, b, c). Accordingly, a final experiment was designed to examine the capacity of preputialectomized males to induce sexual maturity in nearby females. An additional aim for this study was to examine whether these males would show changes in the urinary profile of excreted 17β-estradiol and testosterone, implicated in accelerating sexual maturation in exposed females (Beaton *et al.*, 2006; deCatanzaro *et al.*, 2009).

One common limitation among previous experiments that have investigated the influence of adult males on timing to female sexual maturity has been the invasiveness of procedures used to assess onset of estrous cycling (Bingel, 1972; Bronson & Whitten, 1968; Colby & Vandenberg, 1974; Drickamer, 1975a, 1975b, 1982, 1983; Drickamer & Murphy, 1978; Gangrade & Dominic, 1983; Hasler & Banks, 1975; Ma *et al.*, 1999; Massey & Vandenbergh, 1981; Morè, 2006; Pandey & Pandey, 1988; Price & Vandenbergh, 1992; Vandenbergh, 1967, 1969, 1976). When laboratory animals are subjected to actions such as repeated handling, weighing and vaginal lavage, they display

elevated heart rate and mean arterial blood pressure providing evidence of stress due to experimental procedures (Sharp *et al.*, 2003). This could be problematic as chronic stress can influence hormonal dynamics (deCatanzaro & MacNiven, 1992) and possibly obscure results. Therefore, a key goal in designing the studies presented in this thesis was to minimize the amount of handling done by the investigator on experimental subjects.

Overview of Results

In Experiments 1 and 2 (Chapter 2 and Chapter 3), development of puberty was examined in relationship to AGDI, phytoestrogen content of diet, and exposure to males post weaning. To the best of my knowledge, data obtained from Experiment 1 are the first demonstration that onset of male-facilitated sexual maturity in developing females can be assessed exclusively via non-invasive behavioural markers (Chapters 2). These results also indicate that dietary phytoestrogens are an important component of sexual development in juvenile females. This supports previous findings that female mice exposed to phytoestrogens during development reach puberty earlier than do untreated controls (Casanova *et al.*, 1999; Nikaido *et al.*, 2004, 2005; Takashima-Sasaki *et al.*, 2006; Thigpen *et al.*, 2003; Whitten & Naftolin, 1992). Among the regular-diet subjects there were more displays of sexual receptivity (lordosis) in male-exposed females than in isolates. This trend was absent in animals given the phytoestrogen-free diet. Copulatory plugs, which correlate with the time of ovulation (Safranski *et al.*, 1993), were also observed earlier in male-exposed females on the regular diet than in females from all

other conditions (Chapter 2). The influence of adult males was absent among females on the phytoestrogen-free diet, as these females showed less sexual receptivity and a later date of insemination than did females raised on the regular diet (Chapter 2). However, females raised on the phytoestrogen-free diet were not impaired in their sexual response or fertility, but rather exhibited these events later in life. Male-exposed females raised on the regular diet displayed copulatory plugs roughly 2 days earlier (*ca.* 44.8 days of age) than did male-exposed females on the phytoestrogen-free diet (*ca.* 46.5 days of age). All females were eventually able to mate, gestation length was comparable in all conditions, and the majority of pups survived until weaning. These data suggest that although removal of phytoestrogens from the females' diet delayed sexual maturity, this did not produce gross abnormalities in the ability of such females to mate and produce viable litters (Chapter 2).

In Experiment 2 (Chapter 3), development of sexual maturity was examined using female urinary samples that were collected non-invasively throughout a 2-week period of adult-male exposure, then assayed for 17β-estradiol, progesterone, and creatinine. Following housing either alone or in proximity to adult males, uterine and ovarian tissue was extracted and weighed. This experiment is the first to profile output of urinary steroids in developing females segregated on the basis of their AGDI, under different dietary conditions and during exposure to adult males. The data indicate that the influence of male exposure on female sexual maturation is modulated by the phytoestrogen content of diet and to a lesser extent by AGDI. Male exposure increased uterine and ovarian mass, and the size of this effect was influenced by AGDI in

interaction with diet. Male-exposed females on either diet with short AGDI had heavier tissue than did those of all other groups, except females with long AGDI that were on the regular diet and exposed to males. Urinary estradiol among phytoestrogen-free diet females was well below that of females raised on a regular diet. Male presence had a significant impact on creatinine-adjusted progesterone and its output interacted with diet and female AGDI. Male-exposed females on the regular diet exceeded all other conditions in their output of creatinine-adjusted progesterone, except male-exposed short-AGDI females on the phytoestrogen-free diet. Urinary estradiol was more dynamic in urine of male-exposed females, especially those of the regular diet, however this effect was subtle. Urinary progesterone was more dynamic in male-exposed females, and was generally elevated compared with levels in isolated females. However the size of this effect depended on AGDI, diet, and whether the measure was adjusted for creatinine. Urinary creatinine was elevated by the phytoestrogen-free diet and reduced by male exposure, and declined over days in females exposed to males. This suggests that urinary creatinine itself can vary as a function of diet and social environment.

Experiment 3 (Chapter 4) was designed to investigate whether the preputial glands are necessary for males' capacity to accelerate sexual maturation in juvenile females, and to provide the first account of whether male urinary steroid content depended on these glands. Females' uterine and ovarian growth was hastened by exposure to adult males despite removal of the preputials. The chemistry of males' urine was altered in both intact and preputialectomized males by exposure to developing females, since creatinine-adjusted estradiol and testosterone were both significantly

elevated in female-exposed males. As seen in Experiment 2 (Chapter 3), output of urinary creatinine varied based on the social environment of experimental subjects. Levels of creatinine were reduced in the urine of males exposed to developing females. Exogenous estrogens can promote uterine growth (Galand *et al.*, 1971; Ogasawara *et al.*, 1983; Padilla-Banks *et al.*, 2001; Sato *et al.*, 2002; Yamashita *et al.*, 1990) and other parameters of female reproduction (Drouva *et al.*, 1984; Herbison, 1998; Legan *et al.*, 1975; Legan & Karsch, 1975). These data provide further support that steroids in males' urine contribute to earlier sexual maturity in male-exposed females (Beaton *et al.*, 2006; deCatanzaro *et al.*, 2009).

The Female Anogenital Distance Index

The female AGDI has been used as a successful biomarker of natural variations in prenatal androgenization (Hotchkiss & Vandenbergh, 2005; Vandenbergh & Huggett, 1995). One long-held assumption has been that females that have gestated between two male siblings display more "male-like" behavioural characteristics, such as higher rates of urine marking and more aggressiveness, than do females that have gestated between female siblings (Quadagno *et al.*, 1987; vom Saal & Bronson, 1978). Data obtained from Experiment 1 (Chapter 2) showed that in the direct presence of males, females on the regular diet with long AGDI displayed increased escape attempts and defensive posturing. These results support previous work demonstrating that long-AGDI females exhibit high levels of aggression in some settings (Quadagno *et al.*, 1987).

Long-AGDI females have also been shown to reach sexual maturity substantially later than do short-AGDI females (Clark & Galef, 1988; McDermott *et al.*, 1978; vom Saal, 1989; Zehr *et al.*, 2001). In Experiment 1 (Chapter 2), a significant main effect of AGDI on latency to copulatory plug was not observed. However under both diet categories, male-exposed females with long-AGDI lagged behind their short-AGDI counterparts by about 2-5 hrs in the appearance of a copulatory plug. In Experiment 2 (Chapter 3), AGDI was significantly related to dry uterine and ovarian mass, with long-AGDI females exhibiting lesser reproductive tissue mass, particularly in the phytoestrogen-free animals. However in the presence of males, long-AGDI females on the regular diet displayed similar tissue mass to that of short-AGDI females from both diets. These results suggest that dietary phytoestrogens and *in utero* androgen exposure exhibit some interaction with presence or absence of males in determining the age at onset of sexual maturity in developing females.

Phytoestrogens and Female Sexual Maturity

Data concerning female behaviour in Experiment 1 (Chapter 2) are consistent with findings that phytoestrogens advance sexual maturation in female mice (Casanova *et al.*, 1999; Nikaido *et al.*, 2004, 2005; Takashima-Sasaki *et al.*, 2006; Thigpen *et al.*, 2003; Whitten & Naftolin, 1992). Nevertheless, the influences of phytoestrogens on the age of female sexual maturity may still require further clarification. While some studies have found that developing females exposed to phytoestrogens or soy derivatives reach sexual maturity much earlier than do untreated counterparts (Casanova *et al.*, 1999;

Nikaido *et al.*, 2004, 2005; Takashima-Sasaki *et al.*, 2006; Thigpen *et al.*, 2003; Whitten *et al.*, 1992), others have failed to replicate these findings (Thigpen *et al.*, 2002). There have even been reports of delays in pubertal onset due to phytoestrogen exposure (Levy *et al.*, 1995; Lund *et al.*, 2001). For example, Lund *et al.* (2001) found a delay in sexual maturity of female rats maintained on a phytoestrogen-rich diet in comparison to those fed a phytoestrogen-free diet. Conversely, Casanova *et al.* (1999) observed that a diet enriched with genistein (100 mg/ 100 g of feed) markedly increased the ratio of uterine mass to body mass in such females. Although offspring from both studies were exposed to high levels of phytoestrogens starting from the day of conception, their attainment of sexual maturity was assessed at different time points. Whereas Casanova *et al.* (1999) weaned female subjects at 21 days of age, Lund *et al.* (2001) weaned females at 30 days of age. Sexual maturity in these females was measured through the occurrence of vaginal opening, which occurred at approximately 39.3 days of age, four days later than animals maintained on the phytoestrogen-free diet (Lund *et al.*, 2001). The lack of information about the amount of genistein provided to animals in the Lund *et al.* (2001) experiment makes it difficult to compare experimental outcomes between studies. Variations in phytoestrogen content, different routes of administration (dietary vs. subcutaneous injection), timing of phytoestrogen exposure (during gestation vs. postnatal), age at developmental assessment and type of measure used to evaluate female maturity (*e.g.*, appearance of vaginal opening vs. uterine/ovarian mass or vaginal smear) may be responsible for inconsistencies reported in the literature.

There has been concern about the ability of dietary phytoestrogens to decrease the sensitivity of biological endpoints assessing estrogenic activity in toxicological studies, such as the uterotrophic assay (Boettger-Tong *et al.*, 1998). Typically, this assay involves measurement of the mass of the immature uterus following oral or subcutaneous exposure to exogenous substances (Kim *et al.*, 2002; Odum *et al.*, 1997). The basic assumption is that since the uterus contains numerous cells that are sensitive to estrogens, and uterine growth is driven by endogenous estrogens, the estrogenicity of exogenous substances can be assessed by their impact on uterine growth and function. Owens *et al.* (2003) evaluated the immature rat uterotrophic assay as part of the Organization for Economic Cooperation and Development initiative to develop guidelines for testing endocrine disruptors. This group suggested that investigators may use diets with less than 325-350 μg/g total phytoestrogens without impairing responsiveness of the uterotrophic assay.

Data presented in Experiment 2 (Chapter 3) showed no significant impact of diet on the mass of uterine and ovarian tissue. Odum *et al.* (2001) reported that female rat pups exposed to a phytoestrogen-free diet with less than 5 μg/g isoflavone starting from conception displayed equivalent uterine weights to those reared on phytoestrogen-rich diets (containing up to 300 μg/g isoflavone). Phytoestrogens may not be the only dietary factors influencing uterine mass (Odum *et al.*, 2004; Thigpen *et al.*, 2002, 2003). Thigpen *et al.* (2002) investigated the influence of percent crude fiber, protein, fat, and carbohydrate content as well as total metabolizable energy (ME) on uterine mass in immature mice during a seven-day exposure period. Total ME was significantly

predictive of changes in uterine mass. The ME reported by manufacturers of diets used in this thesis were 3.11 Kcal/g for the regular diet and 3.15 Kcal/g for the phytoestrogen-free diet. It is possible that increased reproductive tissue mass in male-exposed females on the phytoestrogen-free diet was due to estrogenic stimulation from male urine.

Phytoestrogens are ubiquitous in the plant kingdom, and many rodents are regularly exposed to them whether reared in the laboratory or the wild. From an evolutionary perspective, some plants have evolved the capacity through phytoestrogens to induce hormonal disruption in vertebrates that graze upon them (Wynne-Edwards, 2001). Animals require nutrients from plant tissue for growth and survival, and evolutionary counterstrategies to lessen the damage are likely. Vertebrate strategies might involve steroid receptors in the oral cavity or vomeronasal organ to adjust food intake, increases in endogenous hormones, and/or alterations in binding affinities that decrease activity of exogenous compounds (Wynne-Edwards, 2001). It is not known for certain whether mice in the wild are herbivores, eating mainly plants or seeds, or omnivores, eating a mix of plants and animals (Berry & Jakobson, 1974; Fitzgerald *et al.*, 1996; Moro & Bradshaw, 2002). The choice may simply vary in response to available foods, season, and habitat (Bomford, 1987).

Influence of Diet and Social Environment on Urinary Creatinine

Creatinine is a breakdown product of creatine phosphate in muscles, and is produced at a fairly constant rate in healthy individuals. It is mainly filtered out of the blood by the kidneys, with a small amount being actively excreted in urine (Wyss &

Kaddurah-Daouk, 2000). Urinary creatinine can thus be considered as an index of metabolic activity, and the rate of its excretion is assumed to be fairly constant (Boeniger *et al.*, 1993; Erb *et al.*, 1970; Muir *et al.*, 2001; Munro *et al.*, 1991). Investigators routinely adjust urinary steroid values for creatinine to compensate for variations in fluid intake and excretion within and among individuals (Beaton *et al.*, 2006; deCatanzaro *et al.*, 2003; Erb *et al.*, 1970; Muir *et al.*, 2001; Munro *et al.*, 1991). However, some reports indicate that urinary hormonal profiles are similar with and without creatinine adjustment (Denari *et al.*, 1981; Munro *et al.*, 1991). A number of researchers have also criticized this exercise as unwarranted and perhaps even misleading (Boeniger *et al.*, 1993; deCatanzaro *et al.*, 2009; Hakim *et al.*, 1994; Hall Moran *et al.*, 2001; Miro *et al.*, 2004).

Data presented in Experiments 2 (Chapter 3) and 3 (Chapter 4) demonstrate that creatinine levels in urine can vary based on an animal's diet and social environment. Urinary creatinine was somewhat higher in females raised on the phytoestrogen-free diet and lower in females housed near males (Chapter 3). Likewise, levels of creatinine were reduced in males' urine by exposure to females (Chapter 4). Elsewhere, it has been shown that water consumption increases among female-exposed male mice, in conjunction with decreased urinary creatinine levels indicating dilution of urine (deCatanzaro *et al.*, 2009). Accordingly, I suggest that urinary steroid analyses should always accompany evaluation of urinary creatinine output in experimental subjects (Chapter 3 and Chapter 4).

Male Emission of Urinary Estradiol and Its Influence on Female Sexual Maturation

Chemical communication between unrelated males and developing females is clearly implicated in the Vandenbergh effect. However the precise nature of male pheromones involved in this effect has yet to be fully elucidated. As reported in Experiment 3 (Chapter 4), excretions from the male preputials are not necessary for the onset of male-facilitated sexual maturity in juvenile females. As shown here (Chapter 4) and presented elsewhere (Beaton *et al.*, 2006; deCatanzaro *et al.*, 2006, 2009), adult males excrete significant amounts of 17β-estradiol and testosterone in their urine. When males are housed near developing females, their urinary estradiol levels rise relative to creatinine (deCatanzaro *et al.*, 2009). Female exposure to males' excreted estrogens might help to account for the Vandenbergh effect.

It is well established that urination in male mice is a social response (Arakawa *et al.*, 2007; Drickamer, 2001; Hurst, 1987, 1990). Males actively direct urine toward juvenile females (deCatanzaro *et al.*, 2009), whereas in the presence of other males they tend to direct urination around their own housing area (Arakawa *et al.*, 2007). It has been widely accepted that males' capacity to advance sexual maturity in nearby females is mediated through pheromonal components in urine (Bronson & Whitten, 1968; Colby & Vandenbergh, 1974; Drickamer, 1982, 1983, 1984a, 1984b; Drickamer & Mikesic, 1990; Drickamer & Murphy, 1978; Lombardi *et al.*, 1976; Novotny *et al.*, 1999c; Schellinck *et al.*, 1993). Similarly, another pheromonally-mediated phenomenon, the capacity of unrelated males' to disrupt implantation in previously inseminated females (the "Bruce effect"), has been linked with steroids excreted through male urine (Beaton &

deCatanzaro, 2005; deCatanzaro *et al.*, 2006). This phenomenon can be mimicked by nasal or subcutaneous administration of minute quantities of exogenous estrogens (deCatanzaro *et al.*, 2001, 2006).

The female reproductive tract and the pituitary and hypothalamus are important targets for estrogen action (Anderson & Greenwald, 1969; Caraty *et al.*, 1998; Wang *et al.*, 2000). Estradiol plays an important role in ovulation, uterine receptivity, and implantation (Kapur *et al.*, 1992; Labhsetwar, 1970; Yoshinaga, 1988). Cyclical fluctuations in the amplitude and release of GnRH are determined largely by estrogenic activity (Herbison, 1998). Recent evidence has shown the existence of ERβ in GnRH neurons of the hypothalamus (Hrabovszky *et al.*, 2000). Accordingly, estrogens in males' excretions could impinge upon proximate females' physiology at the reproductive tract, hypothalamus, and pituitary, with absorption facilitated by the small molecular size and lipophilic nature of estrogens (*cf.* deCatanzaro *et al.*, 2006). Urinary testosterone may also contribute through aromatization to estradiol in the female's system.

One outcome of estrogen action at the immature uterus is the proliferation of multiple cell types (Quarmby & Korach, 1984). Activation of a number of growth factors is likely to be involved in the actions of estrogens on the uterus (Cullinan-Bove & Koos, 1993; Nelson *et al.*, 1992). Administration of estrogens to ovariectomized rats results in an increase in IGF-I messenger RNA (Norstedt *et al.*, 1989). In *in vitro* cultures, IGF-I stimulates DNA synthesis in uterine tissue from immature rats, thereby inducing cell proliferation (Murphy & Ghahary, 1990). Tissue measurements presented in Experiment 2 (Chapter 3) showed that combined mass of uterus and ovaries was increased due to

male exposure for females on both diets. As stated earlier, increased excretion of urinary estradiol and testosterone has been documented when males are housed near developing females (Beaton *et al.*, 2006; deCatanzaro *et al.*, 2009). This increase in males' urinary estradiol occurs concurrently to accelerations in growth of both the uterus and ovaries in male-exposed females (Beaton *et al.*, 2006). It is possible that the change in reproductive tissue mass observed in females in Experiment 2 (Chapter 3) was due to direct exposure from steroid excretions of males.

Methodological Limitations

Urinary steroid measures presented in this thesis have the advantage of being non-invasive. Collection of urine was conducted without disturbing the experimental subjects and permitted the study of ongoing hormonal levels within the same animal. However the collection of adequate amounts of urine from mice can be challenging. Although females typically urinate in puddles, males in social situations disperse their urine across the experimental apparatus, making it difficult to collect sufficient samples for analysis. The removal of data points due to missing samples can reduce statistical power. One possible solution is to average steroid values across two-day measures and eliminate missing values (Chapter 4).

The antibodies used in ELISA procedures described in this thesis were polyclonal and could possibly exhibit binding with multiple urinary components apart from 17β-estradiol, testosterone, or progesterone. Although ELISA data on urinary steroidal output from females derived thus far largely reflect established systemic trends of the

unconjugated hormones (deCatanzaro *et al.*, 2003, 2004), and cross-reactivities of the antibodies to other examined steroids including estrone conjugates are quite low, it is not possible to rule out some reactivity to other urinary constituents such as steroid metabolites or phytoestrogens.

A general limitation associated with both Experiments 1 (Chapter 2) and 2 (Chapter 3) is the lack of information on the amounts and types of phytoestrogens found in the regular diet. For experiments using diets that contain estrogenic components such as soy, chemical analyses should be conducted in order to detect the amounts of isoflavones such as genistein, daidzein, and glycitein as well as their respective glucosides. Coward *et al.* (1993) have described methods using high-pressure liquid chromatography, mass spectrometry, and proton nuclear magnetic resonance spectroscopy for the extraction and measurement of isoflavone constituents in soy-containing products.

Future Directions

Evidence presented in this thesis (Chapter 4) and reported elsewhere (Beaton *et al.*, 2006; deCatanzaro *et al.*, 2009) suggests an important role of male urinary steroids in the Vandenbergh effect. Males' urinary testosterone and estradiol diminishes gradually during the six weeks following castration. Castrated males also gradually lose their ability to disrupt pregnancy in previously inseminated females (Vella & deCatanzaro, 2001). A similar experiment could be designed during which castrated males would be exposed to juvenile females roughly six weeks post castration. Urinary output of

estradiol and testosterone during female exposure could be measured. Male fluid intake and urination patterns should also be recorded. Additional conditions could include castrated males with administrations of testosterone or 17β-estradiol. Measuring the capacity of these males to induce sexual maturity in females might provide further insight into the role of male-excreted testosterone and estradiol in the Vandenbergh effect.

The reduction of 17β-estradiol in the urine of male mice through chronic administration of an aromatase inhibitor, anastrozole, in combination with a low-phytoestrogen diet has been shown to decrease the capacity of such males to induce the Bruce effect (Beaton & deCatanzaro, 2005). Similarly, an experiment could be designed to investigate the ability of such males to produce the Vandenbergh effect. In the Beaton & deCatanzaro (2005) study, only males in the aromatase-inhibitor condition were given a diet low in phytoestrogens. The vehicle-treated males received a standard rodent chow. Dietary phytoestrogens can have a significant impact on male reproductive function (Robertson *et al.*, 2002). In one experiment (Robertson *et al.*, 2002), when male aromatase-knockout mice with spermatogenic abnormalities were given a diet enriched with soy, there was a beneficial effect on sperm production. The experiment suggested above should also include control males maintained only on a phytoestrogen-free diet in order to examine their output of urinary estradiol and testosterone as well as their ability to bring about reproductive changes in developing females. Since Experiment 2 (Chapter 3) showed that urinary creatinine can also vary based on diet, an examination of creatinine output in these males would be required.

Female behaviour is an important component in the successful delivery of excretions emitted by males. Although it has been shown that adult males target their urinations towards developing females (deCatanzaro *et al.*, 2009), responses of females to male emissions have yet to be thoroughly quantified. Do females behave differently in the presence of fathers and brothers versus unrelated males? Discrete counts could be taken of female behavioural patterns including females' nose touching the grid (containing males in an adjacent compartment), climbing on the males' grid, nasal contact with the males' genitals and nasal area, and grooming of the females own nasal area. Observation of male behaviour could include number and direction of urinations, location of urine droplets, sniffing of the grid adjacent to females, and nasal contact with females' nasal, body, and genital areas. Male urine samples during exposure to females could also be collected and analyzed for potential differences in steroid output.

Contributions to the Literature

These experiments are the first to show interactions of diet, male-exposure, and an index of prenatal androgen exposure (AGDI) in determining the time of sexual maturity in female mice. I have provided the first demonstration that onset of male-facilitated sexual maturity in developing females can be assessed exclusively via non-invasive behavioural markers. I am also the first to measure output of creatinine, urinary steroids, and mass of uterine and ovarian tissue in developing females of varying AGDI under different dietary conditions and in relation to exposure to males. I have provided empirical support to a small number of studies which suggest that urinary creatinine is

dynamic in relation to social condition. I have further shown that urinary creatinine can vary based on an animal's nutrition. Data in this thesis also provide evidence supporting an important role of males' excreted steroids in the Vandenbergh effect, while indicating that male preputial emissions are not necessary for this effect.

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