REPRODUCTIVE PLASTICITY DURING MALE EXPOSURE AND STRESS

AN EXPLORATION OF FEMALE REPRODUCTIVE PLASTICITY IN THE CONTEXT OF STRANGE MALE EXPOSURE AND STRESS IN MICE (*MUS MUSCULUS*)

By JOELLE B. THORPE, B.SC.H, M.SC.

A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Doctorate of Philosophy

McMaster University DOCTORATE OF PHILOSOPHY (2013)

Hamilton, Ontario (Psychology, Neuroscience & Behaviour)

TITLE: An exploration of female reproductive plasticity in the context of strange male exposure and stress in mice (*Mus musculus*)

AUTHOR: Joelle B. Thorpe, B.Sc.H, M.Sc. (Queen's University)

SUPERVISOR: Professor Denys deCatanzaro

NUMBER OF PAGES: xiii, 219

Abstract

Females experience reproductive plasticity in several situations. Under severe stress, females can experience ova-implantation failure. In the presence of unfamiliar males, females can also experience implantation failure (the Bruce effect), or hastened sexual maturation (the Vandenbergh effect). These physiological events (sexual maturation and implantation failure) are also induced by 17β-estradiol (E_2). Male mice excrete E_2 in their urine and direct it toward females; furthermore, stress can increase endogenous E_2 in females. Accordingly, I explored the role of male urinary E_2 in the Bruce and Vandenbergh effects, and examined endogenous E_2 and progesterone (P_4) during stress-induced implantation failure in mice. I showed that a male mouse's ability to induce the Bruce and Vandenbergh effects depends on his urinary E_2, as castration removed this ability, and injections of E_2 restored it and urinary E_2 levels. I also developed urinary measurements of the stress hormone, corticosterone, for use in subsequent stress studies. I showed that serum and urinary corticosterone follow a similar circadian rhythm, and that urinary corticosterone responds appropriately to acute stress in male mice. Also, urinary and serum corticosterone, P_4, and E_2 in female mice show similar responses to stress, with slightly different time courses. Lastly, I showed that rat-exposure is stressful and causes implantation failure in female mice. Furthermore, P_4 is elevated in females maintaining their pregnancies, and suppressed in females losing their pregnancies, during stress. E_2 is elevated in stress, particularly in females losing their pregnancies. Exogenous P_4 did not mitigate stress-induced pregnancy failure, unless it was combined with a low dose of E_2. Taken together with previous research, I suggest that exogenous

and endogenous E_2 contribute to reproductive plasticity in female mice, particularly their ability to abandon early pregnancy. Additionally, I suggest that serum and urinary steroid measures in response to stress have value under various experimental circumstances.

Acknowledgements

I am immensely grateful to my supervisor Dr. Denys deCatanzaro for the past four years. Sometimes I think of the graduate student experience like a highly compressed period of development from academic infancy to young adulthood. In the beginning, a graduate student is heavily dependent on her supervisor for guidance, support, and advice. Through this, she gradually develops as an independent scientist with the skills to ask and answer important and interesting questions. Unfortunately, before this can happen, a period of academic adolescence is inevitable, during which the graduate student may become less than patient with this process, and somewhat irritable at times! Thank you, Denys, for your patience, understanding, guidance, and support, even during my "academic adolescence". I can't thank you enough for all you've taught me, and for the freedom you've given me to explore my (very) varied interests.

Thank you also to my committee members, Dr. Sigal Balshine and Dr. Joanna Wilson. Sigal, you've been a wonderful person to get to know. Thank you so much for your encouragement, enthusiasm, thoughtful questions, and for reminding me of why I love evolutionary biology. Joanna, you've been an invaluable source of challenging and thoughtful questions. Thank you for your attention, even during your sabbatical!

Thank you to my labmates, both past and present. Dr. Robert Berger, you were in your last year when I started in the deCat lab. I now understand and appreciate just what it took to help me in my first year, as you were finishing up! I am grateful for all of our conversations about research – they were stimulating, and reminded me of why I love it. Dr. Ayesha Khan, although our time in the deCat lab didn't overlap for more than a

couple of weeks, you've always treated me like a labmate and colleague. Thank you so much for the opportunities you've given me to flex my teaching muscles. You are a wonderful teacher and friend. Naz and Brent, you two were experts at keeping things fun and lighthearted in the lab – I always knew when you two were in the office just by listening for laughter! Naz, you were energetic even at 3am while we were doing blood collections. How could that *not* rub off on me? Tyler and Evan, you have both helped me survive my last year. Thank you for putting up with my curmudgeonly self, and for reminding me that I'm not quite *that* old (yet). Dr. Adam Guzzo, I've had many labmates over the past several years, but none for as long as you. Believe it or not, you've kept me sane for the past four years. You were always there when I was panicking about one thing or another, and were a master at talking me off ledges, so to speak! Thank you so much for all of our talks (research-related and otherwise!), for your neverending tech support, for collecting urine at all hours of the night for me (I still owe you for that big time), and for all that television! I was lucky to have you as a labmate and am lucky to be able to call you a friend.

Thank you to the undergraduate students who have helped me along the way. Kaitlin, you were full of inquisitive questions, and became a pro at running from mouse room to surgery suite in less than 30 seconds. Your dedication was admirable! Paige and Marta, you two had amazing work ethic. You never grumbled at having to be in the lab every weekend for a solid 3 months. You were both careful and thoughtful researchers, and I couldn't have asked for better helpers in my last year!

Thank you to the staff of the Psychology animal facility for making it a safe and caring environment. Heather and Wendy, you were always willing to teach me various animal techniques, and were the best at taking care of my animals in the most thoughtful and least disruptive way. Tamara and Dawn, you have been so understanding of the difficulties of doing stress research, and have bent over backwards to change your schedule in order to fit my animals' and my needs. To the ladies in the main office – Milica, Wendy, Sally, and Nancy – thank you all so much for putting up with my questions, and for having all the answers!

Finally, to my friends and family who have formed a great support system for me over the years. Karen, you have been a wonderful friend and officemate. Our coffee dates were often a saving grace for me during the most stressful of times! It was always comforting to know I wasn't alone in the things (academic and otherwise) that kept me up at night with worry! To my sisters, thank you for providing me with distractions from school, and showing me that there is life off campus. To my mom, thank you for listening to me vent about the tiniest of things, and for your excellent editorial skills. To my dad, thank you for passing on your love of research to me, and for encouraging me to see the value of, and find huge satisfaction in, hard work.

And last, but most certainly not least, thank you to Mark. I could not have asked for a more understanding partner over the years. Somehow you've survived through writing your own thesis, and through me writing *two*. I honestly don't know how you've done it (I know I can be quite a handful!). Countless times, and without complaint, you've driven me to school on early weekend mornings. You haven't said a thing when I

return home smelling like mouse urine! You, above all others, best understand the lifestyle of a graduate student – having to put research above all else, even if it means missing family functions and putting off vacations. And yet you constantly remind me to enjoy the experience while it lasts, because nothing is ever as good, flexible, fun, and rewarding as being a graduate student! And although I now have to enter the "real world", I know it'll be great since I get to live in it with you!

Table of Contents

List of Figures and Tables

List of Abbreviations

Estradiol (17β-estradiol, oestradiol)	E_2, E2
Testosterone	T_5, T
Progesterone	P_4, P4
Ovariectomized	OVX
Gestation day	GD
Glucocorticoid(s)	GC(s)
Dehydroepiandrostenedione	DHEA
Hypothalamic-pituitary-gonadal	HPG
Gonadotropin-releasing hormone	GnRH
Luteinizing hormone	LH
Follicle-stimulating hormone	FSH
Hypothalamic-pituitary-adrenal	HPA
Corticotropin-releasing hormone	CRH
Adrenocorticotropic hormone	ACTH
G-protein coupled receptor 54	GPR54
Estrogen receptor (alpha)	ER(α)
Insulin-like growth factor 1	IGF-1
Epithelial-cadherin	e-cadherin
Major urinary protein(s)	MUP(s)
Major histocompatibility complex	MHC
Standard error of the mean	S.E.M., S.E.

Intramuscular	i.m.
Horse radish peroxidase	HRP
Analysis of variance	ANOVA
Intraperitoneal	i.p.
Subcutaneous	s.c.

Chapter 1

General Introduction

Overview

A common concept in evolutionary biology is that of the choosy female and the eager male. While this pattern is not followed in all species, it is prevalent in those with internal fertilization and female-dominant parental care. These two characteristics are present in the vast majority of mammalian species (Kleiman 1977). Most mammalian males need only provide relatively inexpensive sperm in order to increase their fitness. Mammalian females, on the other hand, are saddled with the energetic burdens of creating large, nutrient-rich ova, gestating, lactating, and providing other maternal care necessary to ensure offspring survival to reproductive age (Kleiman 1977). Consequently, a male can abandon a female after insemination, because the female will provide all of the resources and care required for his offspring to survive and pass his genes on to future generations. Males in most mammalian species where there is no pair-bonding or paternal care may increase their lifetime fitness best by following a "quantity over quality" strategy, inseminating as many females as possible (Trivers 1972). Most females increase their lifetime fitness best by following the opposite strategy, mating with only the highest quality of available males, thereby increasing the probability that their energetic investment is not wasted on unhealthy or subpar offspring that may not even survive to reproduce themselves (Trivers 1972).

The unequal investment in parental care between the sexes, with females bearing most of the burden, means that it might be beneficial for females to be reproductively plastic. Certainly, males can be reproductively plastic as well, particularly in their mating strategies (Gross 1996). However, throughout this thesis, I define reproductive plasticity

in females as their ability to alter their reproductive strategies post-fertilization. It could be adaptive for females to be flexible in their parental commitment depending on various environmental variables such as the identity of surrounding conspecifics, the presence of predatory heterospecifics, climate, and resource availability, to name a few. One way in which females are often reproductively plastic is in their ability to abandon early pregnancy when non-sire males are present (perhaps to avoid infanticide that may occur if pregnancy was carried to term, e.g., Hrdy 1979, Labov 1981, Roberts *et al.* 2012), or when stress is experienced (perhaps a signal of reduced probability of maternal or offspring survival under current poor environmental conditions, e.g., Wingfield *et al.* 1998). Males may have evolved to take advantage of this female plasticity under some circumstances (Hrdy 1979, Schwagmeyer 1979). For instance, in some species including several rodents, a male has the capacity to disrupt pregnancies that he did not sire in surrounding females (possibly to bring females back into estrus speedily so he can inseminate them himself, e.g., Mallory & Brooks 1978). A male may also hasten the process of sexual maturation in juvenile females (possibly to increase the availability of females for insemination, e.g., Vandenbergh 1971). This sets the stage for several phenomena seen in mice (*Mus*) and some other species, including pheromonal effects such as disruption of blastocyst implantation and acceleration of female puberty, and the endocrine effect of stress-induced pregnancy failure. Broadly, in this thesis I examine female mouse reproductive plasticity in 2 situations: 1) in the presence of unfamiliar males, and 2) in the presence of a predator, and I examine the physiological

underpinnings of such plasticity, with a focus on adrenal and gonadal steroids, particularly estrogens.

Steroid Hormones

Steroids are a class of hormone synthesized and released into the bloodstream by the major endocrine glands: the gonads (the ovaries and testes) and the adrenal cortex (the outer shell of the adrenal gland). Steroids are divided into 5 classes: the mineralocorticoids (including aldosterone), the glucocorticoids (GCs, including cortisol and corticosterone), the progestins (including progesterone [P_4]), the androgens (including testosterone [T_5]), and the estrogens (including 17β-estradiol [E_2]). Each steroid hormone in each class is structurally distinct except for its core, the steroid backbone common to all steroids, which consists of three 6-carbon rings and one 5-carbon ring. Since steroids are small and lipophilic, they can easily pass through the cell phospholipid bilayer. As a result, steroids produce most of their effects via intracellular receptors (Walters 1985). Once bound by a steroid in the cytosol, the steroid/receptor unit moves to the nucleus and acts as a transcription factor, binding to hormone response elements on DNA and upregulating the transcription of specific genes (Walters 1985, Beato *et al.* 1995, Mangelsforf *et al.* 1995).

The physiological and behavioural effects of steroid hormones vary widely. Steroids are involved in the regulation of parental (Wynne-Edwards & Reburn 2000, Saltzman & Maestripieri 2011), sexual (Feder 1984), and aggressive (Lonstein & Gammie 2002) behaviours, the maintenance of pregnancy (Schindler 2005), the initiation

of parturition (Nathanielsz 1998), and the upregulation or suppression of immune function (Sapolsky *et al.* 2000), among many other processes. Despite the variety of physiological and behavioural effects of steroid hormones, all steroids arise from the modification of the same molecule, cholesterol, through several enzymatic steps (Payne & Hales 2004). In fact, the steroid biosynthetic pathway is essentially a series of enzymatic reactions leading from one steroid to another by cleaving the appropriate molecules from the steroid backbone.

Depending on the presence or absence of specific enzymes, various endocrine glands synthesize different steroid hormones. Classically, the steroids are grouped into two categories: the sex steroids (e.g., T_5, P_4, and E_2) released from the gonads, and the adrenal steroids (e.g., aldosterone and the GCs cortisol and corticosterone) released from the adrenal glands. The gonads do not possess the enzymes necessary to synthesize adrenal steroids, but the adrenal glands can synthesize sex steroids including P_4, and the androgens androstenedione and dehydroepiandrostenedione (DHEA) (Payne & Hales 2004). Estrogens, being the smallest of the steroid hormones, are located at the end of the steroid biosynthetic pathway. Their immediate precursors are the androgens (Payne & Hales 2004). One important enzymatic step is the aromatization of T_5 to E_2 by the enzyme aromatase (Simpson *et al.* 1994). The direct conversion of T_5 to E_2 has led to the discovery that some traditionally male characteristics regulated by T_5 are actually only indirectly so regulated, with direct effects being attributed to E_2 (e.g., masculinization of the brain: McCarthy 2008). This regulation of the synthesis and release of steroids

including P_4, E_2, and the GCs is controlled by signals from the hypothalamus and pituitary.

The Hypothalamic-Pituitary-Gonadal Axis

Reproduction in both male and female mammals is ultimately controlled by the hypothalamic-pituitary-gonadal (HPG) axis. Communication between the hypothalamus, pituitary, and ovaries is essential for all major reproductive milestones in females: sexual maturation (Ojeda & Urbanski 1988), estrous/menstrual cycling leading to ovulation (Freeman 1988, Knobil & Hotchkiss 1988), and pregnancy (Niswender & Nett 1988). The major effectors of the HPG axis are kisspeptin and gonadotropin-releasing hormone (GnRH) from the hypothalamus, the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the gonadotrope cells of the anterior pituitary, and the steroid hormones P_4 and E_2 from the ovaries.

Generally, kisspeptin stimulates the pulsatile release of GnRH (Dungan *et al.* 2006), which in turn acts at the anterior pituitary gonadotropes to prompt the secretion of LH and FSH (Schally *et al.* 1973, Conn & Crowley 1990). These gonadotropins stimulate the cells of the follicles of the ovaries to release the sex steroids P_4 and E_2 (Jamnongjit & Hammes 2006). Over most of the rodent estrous cycle, the HPG axis functions in a classic negative feedback fashion, with E_2 feeding back to inhibit kisspeptin (and therefore indirectly inhibiting GnRH and the gonadotropins) in the arcuate nucleus of the hypothalamus (Dungan *et al.* 2006). However, during the afternoon of proestrus (the day before ovulation and sexual receptivity in rodents), E_2 feeds back to the

anteroventral periventricular nucleus of the hypothalamus in a positive feedback fashion to stimulate kisspeptin, all of which ultimately results in a surge of LH and culminates in ovulation (Kauffman *et al.* 2007, Smith 2008). If mating does not occur at this time, E_2 and P_4 levels fall back to baseline levels and the cycle continues anew. However, if the ova shed are fertilized, P_4 remains elevated to support pregnancy (Freeman 1988).

The Hypothalamic-Pituitary-Adrenal Axis

Like sex steroid release from the ovaries, the release of GCs from the adrenal glands is controlled by signals from the hypothalamus and pituitary. Corticotropin-releasing hormone (CRH) released from the hypothalamus stimulates pituitary corticotropes to release adrenocorticotropic hormone (ACTH), which in turn acts at the adrenal cortex to initiate GC synthesis and secretion. The synthesis and secretion of these hormones, particularly the GCs, display a distinct circadian pattern, with levels increasing in conjunction with the start of the activity cycle (corresponding to the morning for diurnal species like humans, and the evening for nocturnal species like mice and rats), and decreasing continuously until reaching a trough at the beginning of the sleep cycle (the evening for diurnal species, and the morning for nocturnal species) (Dickmeis 2009). As well as displaying a distinct circadian pattern, the hypothalamic-pituitary-adrenal (HPA) axis displays a distinct response to stress.

When faced with a stressor (any perceived or real challenge to homeostasis), an individual mounts a highly stereotyped stress response, mediated partly by the HPA axis. Almost immediately after the perception of stress, CRH is released from the

hypothalamus, leading to increased ACTH from the anterior pituitary, and ultimately to increased GCs (cortisol or corticosterone) from the adrenal cortex (Sapolsky *et al.* 2000). The stress response is generally considered adaptive, allowing an individual to mobilize resources, escape threat, and return to homeostasis (Wingfield *et al.* 1998, but see Breuner *et al.* 2008). Indeed, without the ability to mount an appropriate GC response to stress, the individual is in an extremely fragile state (e.g., Addison's disease, Oelkers 1996). However, the actions of GCs themselves, when remaining high (for instance, in situations of chronic stress), can be detrimental (Sapolsky *et al.* 1986, 2000). As such, in the young, healthy animal, GC levels in response to a stressor are tightly regulated by negative feedback to the hypothalamus (decreasing CRH) and pituitary (decreasing ACTH). This is not so in the aged animal: after a lifetime of exposure to stressors, the mechanisms of GC negative feedback break down, leading to a prolonged increase in GCs during stress and even hastening the aging process in a positive feedback loop (Sapolsky *et al.* 1986).

Female Sexual Maturation

The process through which juvenile females gain the capacity to reproduce (puberty, or sexual maturation) is highly complex, being influenced by hormonal (Ojeda & Urbanski 1988), genetic (Drickamer 1981), and environmental (Ellis & Garber 2000, Nebesio & Pescovitz 2005) factors. Sexual maturation in females is characterized by an awakening of the HPG axis. The first sign of this is a change in the pattern of LH secretion, with increased pulse amplitude and mean concentrations of LH occurring in the

afternoon in female rats (diurnal pattern; Urbanski & Ojeda 1985a) or overnight in human girls (nocturnal pattern; Goji 1993). The ovary matures, and LH receptor content and sensitivity to gonadotropins increases (Peluso *et al.* 1976, Ojeda & Urbanski 1988). This may then allow the diurnal pulses of LH to stimulate ovarian steroidogenesis of E_2 and P_4 in sufficient amounts to lead to the first preovulatory gonadotropin surge via E_2 positive feedback to the hypothalamus (Urbanski & Ojeda 1985b).

It is largely assumed that these LH pulses are driven by GnRH release from the hypothalamus, since GnRH is a potent stimulator of LH, and increases in GnRH during puberty have been noted (Terasawa *et al.* 1999, Ebling 2005). Indeed, administration of GnRH in pulses that produce a profile of LH similar to that seen during the pubertal transition causes the onset of precocious puberty in female rats (Urbanski & Ojeda 1987). Moreover, excitatory activation of GnRH neurons itself initiates puberty (Urbanski & Ojeda 1987), leading to the hypothesis that some factor upstream of GnRH may be involved in the onset of sexual maturation. This factor remained unknown until recently.

It is now generally well accepted that a driving force initiating the awakening of the HPG axis in puberty is kisspeptin and its receptor, G protein-coupled receptor 54 (GPR54) (Dungan *et al.* 2006, Smith 2008). Humans and mice lacking functional GPR54 fail to mature sexually, with females having abnormally low gonadotropin and E_2 levels leading to acyclicity and infertility (de Roux *et al.* 2003, Seminara *et al.* 2003). On the other hand, increased kisspeptin activity can result in precocious puberty: in one case study, a mutation in GPR54 leading to prolonged activation by kisspeptin was hypothesized as the cause of a young girl's precocious puberty (Teles *et al.* 2008). This

early sexual maturation can be attained experimentally in immature female rats by central administration of kisspeptin (Navarro *et al.* 2004a).

During normal maturation, there is a distinct pattern in the development of the kisspeptin/GPR54 system, with an apparent increase in the sensitivity of hypothalamic neuronal circuitry to kisspeptin. For instance, the percentage of GnRH neurons firing in response to kisspeptin increases from 27% in juvenile rats to 44% in pubertal rats to 90% in adult rats, and maximal circulating LH levels can be reached with lower doses of kisspeptin in adult animals compared to juveniles (Han *et al.* 2005). There is also a distinct pattern in the expression of GPR54 gene and kisspeptin gene and peptide expression in the hypothalamus during sexual maturation, with increases seen in rats (Navarro *et al.* 2004b), mice (Mayer *et al.* 2010), and rhesus macaques (Shahab *et al.* 2005) as they mature. The pubertal upregulation of kisspeptin in female mice may in part be under the control of E_2, since mice lacking estrogen receptor alpha (ERα, a major estrogen receptor) specifically in kisspeptin-immunoreactive neurons do not experience a normal increase in kisspeptin during maturation. Moreover, these mice have abnormal sexual development and are acyclic (Mayer *et al.* 2010), indicating the importance of E_2 in female sexual maturation.

E_2 plays an essential role in female puberty, including the sexual maturation of the reproductive tract (Alonso & Rosenfield 2002). Exposure to E_2 or estrogen-mimicking substances (xenoestrogens) can cause precocious puberty in humans and rodents (Feuillan *et al.* 1999, Partsch & Sippell 2001, Rasier *et al.* 2006). There are several measures used as biomarkers of sexual maturity in rodents, including onset of sexual behaviour and

vaginal opening, uterine weight, and circulating LH and E_2 levels (e.g., Navarro *et al.* 2004a, Khan *et al.* 2008a, 2008b), many of which are biomarkers of estrogenic activity. Sexual behaviour in female rodents occurs only around the time of ovulation (estrus), and is due to high levels of E_2 against a backdrop of increasing P_4 (Beach 1976). In the mature ovariectomized (OVX) rat, administration of both E_2 and P_4 is necessary to stimulate sexual behaviour similar to that seen during estrus (Powers 1970), although sufficient levels of E_2 alone can stimulate sexual behaviour (Davidson *et al.* 1968). Vaginal opening occurs at the attainment of estrus due to rises in E_2, and is therefore used as a sign of sexual maturity and estrogenic activity (Reel *et al.* 1996, Rasier *et al.* 2006). Uterine changes occur during sexual maturation as well, including increased endometrial thickness and change in shape in girls (Bridges *et al.* 1996) and increased weight in female mice (Ogasawara *et al.* 1983). This increased uterine weight during sexual maturation is thought to be due to increasing levels of E_2 seen during puberty, since it does not occur in the absence of the ovaries (Ogasawara *et al.* 1983). Indeed, it is well known that E_2 promotes growth of the uterus, since administration of the steroid increases uterine weight (Davidson *et al.* 1968, Ogasawara *et al.* 1983). E_2 stimulates uterine growth in puberty by reducing cell cycle length (Quarmby & Korach 1984) thereby increasing mitotic cell division (Ogasawara *et al.* 1983), and also by signaling through the uterotrophic insulin-like growth factor-1 (IGF-1)/IGF-1 receptor system (Murphy & Ghahary 1990, Zhu & Pollard 2007).

Pregnancy and Implantation

After a successful mating during estrus coinciding with ovulation and resulting in the fertilization of ova in the female reproductive tract, several events must occur for pregnancy to progress. The corpus luteum (an endocrine gland in the ovaries formed from cells of the ovulated follicle) must remain functional in the mated female and secrete sufficient quantities of P_4 necessary for the maintenance of pregnancy (Niswender & Nett 1988). Early studies of the importance of the corpus luteum for pregnancy in mice showed that ovariectomy in mid- to late-gestation leads to pregnancy failure (Harris 1927, Parkes 1928) unless P_4 is replaced (Rubenstein & Forbes 1963, Milligan & Finn 1997). The fertilized ova must undergo mitotic cell division while travelling through the oviduct to the uterus, and develop into blastocysts that are capable of implanting into the uterine wall to establish physical maternal-fetal connection (Wang & Dey 2006). Concurrently, the uterine wall must undergo decidualization, a complex process dependent upon E_2 and P_4 (Ramathal *et al.* 2010), involving transformation of the uterine cells to produce a thickened endometrium (Cross *et al.* 1994), increased vascular permeability (Weitlauf 1988) and to facilitate the upregulation of immune cells (Croy *et al*. 2002). The product of decidualization, the decidua, is thought to support early pregnancy and embryonic as well as placental development (Croy *et al.* 2002, Ramathal *et al*. 2010). Upon implantation and decidualization, the outer layer of the blastocyst, the trophectoderm, begins to develop into the placenta, which is essential for the transport of nutrients between the mother and fetus, and therefore the healthy growth and development of the fetus to term (Cross *et al.* 1994).

There is particular interest in the process of blastocyst implantation into the uterine wall, perhaps because it is the initiation of the physical maternal-fetal connection that ultimately leads to the development of a new individual. Implantation itself is extremely complex, and involves multiple hormonal, immune and genetic components (Dey *et al.* 2004). Except for a brief period of time, the uterus is a hostile environment for the blastocyst, preventing attachment from occurring (Paria *et al.* 2002, Wang & Dey 2006). It is during this brief window of uterine receptivity that the blastocyst and uterine wall come into contact and implantation can begin (Dey *et al.* 2004). If the blastocyst does not arrive in the uterus during the window of receptivity, implantation may fail since the uterus quickly shifts into a refractory state that is damaging to the blastocyst and incompatible with implantation (Wang & Dey 2006). Thus, the development of the uterus, and the development of the fertilized ova into blastocysts as they travel through the reproductive tract, must be exquisitely timed so that the blastocysts arrive at the uterine wall during the window of receptivity in order for successful implantation to occur (Wang & Dey 2006).

Implantation is dependent on E_2 and P_4 in the mouse (Dey *et al.* 2004), failing in the absence of P_4 (Huet-Hudson & Dey 1990) or in the absence of E_2 signaling (Curtis Hewitt *et al.* 2002). It can occur normally in OVX females only when both P_4 and E_2 are administered (Humphrey 1967, Moore Smith & Biggers 1968). In the mouse, implantation begins on day 3 of pregnancy, when the day of mating is considered day 0 (Dey *et al.* 2004). When the natural rise of E_2 seen in females on day 3 of pregnancy is prevented, implantation is inhibited (McCormack & Greenwald 1974), indicating the

importance of E_2 in this process. In particular, E_2 is necessary to bring the P_4-primed uterus into its receptive state (Dey *et al.* 2004, Paria *et al.* 2002).

Although E_2 is necessary for implantation in mice, it can also, paradoxically, disrupt implantation (deCatanzaro *et al.* 1991) through several mechanisms. Low doses of E_2 can extend the window of receptivity to blastocyst implantation in P_4-primed uteri; however, doses of E_2 slightly above this can prematurely close the window, bringing the uterus into a refractory state early and preventing implantation (Ma *et al.* 2003). This E_2-induced refractory state is associated with, and possibly caused by, an abnormal or a complete lack of expression of several genes in the uterus involved in implantation (Ma *et al.* 2003).

Implantation failure caused by supra-optimal levels of E_2 can also be a result of altered blastocyst transport through the reproductive tract. Given the importance of synchronous development of the blastocyst and uterine wall to the establishment of implantation, any alteration of the transport of blastocysts may result in their untimely arrival into the uterus and therefore in implantation failure. Depending on the dose and species examined, E_2 can increase (Burdick & Whitney 1937, Banik & Pincus 1964, Greenwald 1967) or decrease (Greenwald 1967, Humphrey & Martin 1968) the rate of transport through the reproductive tract. Increased transport often results in expulsion of fertilized ova from the female (Banik & Pincus 1964), whereas decreased transport results in retention of ova in the oviducts (Greenwald 1967).

Finally, elevated levels of E_2 may also disrupt implantation by preventing adhesion of the blastocyst to the uterine wall, and of the uterine walls to each other

around the blastocyst, resulting in failure of the uterine lumen (the space in the uterus) to close. Adhesion molecules belonging to the cadherin family, including epithelial-cadherin (e-cadherin) have several functions, including promoting and maintaining adhesion between cells (Rowlands *et al.* 2000). During normal implantation, e-cadherin is increased in the apical surface of uterine epithelial cells (Slater *et al.* 2002), specifically at the points of implantation (Jha *et al*. 2006). Increased e-cadherin promotes enhanced endometrial receptivity for blastocyst attachment (Rahnama *et al.* 2009), and may be involved in the adhesion of opposing uterine walls to each other, resulting in luminal closure around implanting blastocysts (Hyland *et al.* 1998). E_2 can decrease the expression of e-cadherin in reproductive tissues (Potter *et al.* 1996, Oesterreich *et al.* 2003, Jha *et al.* 2006), and can also cause an influx of fluid into the uterine lumen (Salleh *et al.* 2005). Both of these estrogenic processes together may prevent uterine luminal closure and blastocyst attachment, leading to implantation failure.

Male-Induced Changes in Female Reproductive Development and Pregnancy

Communication between conspecifics can occur through many means including visual, auditory, and olfactory. Pheromones are chemicals released from one individual and received by another, and can be classified as either signaling or priming (Vandenbergh 1988). Signaling pheromones result in an immediate behavioural change in the recipient individual (e.g., male attraction to a female in estrus), whereas priming pheromones result in a long-term physiological change in the recipient individual (e.g.,

sexual maturation in juvenile females) (Vandenbergh 1988). Two of the most studied priming pheromonal effects in mice are the Vandenbergh and the Bruce effects.

The Vandenbergh Effect: Acceleration of Female Sexual Maturation

The Vandenbergh effect was first described in the mouse by John Vandenbergh in the 1960s, when he noted that juvenile females housed with an adult male became sexually mature earlier than juveniles housed in the absence of a male (Vandenbergh 1967). Although he first quantified sexual maturation by the age at which the females attained vaginal opening, first estrus, and first mating, other typical measures of the Vandenbergh effect include increased uterine weight (Lombardi & Vandenbergh 1977, Khan *et al.* 2008b) and sexual behaviour (Khan *et al.* 2008a). Shortly thereafter, Vandenbergh discovered that direct contact between the juvenile female and adult male was not necessary, as females became sexually mature when housed with a male behind a wire-mesh grid preventing physical contact, and even when housed only with a male's soiled bedding (Vandenbergh 1969). It is now known that the signal inducing sexual maturation in juvenile females is in adult male urine, since exposure of juvenile females to adult male urine alone is sufficient to induce the Vandenbergh effect (Colby & Vandenbergh 1974). Moreover, this urinary pheromone is androgen-dependent, as urine from castrated males does not accelerate sexual maturation in females (Colby & Vandenbergh 1974), but urine from castrated males given exogenous T_5 does (Lombardi *et al.* 1976). Since its initial description in mice, the Vandenbergh effect has been observed in several other species, including rats (Vandenbergh 1976), prairie voles (Carter *et al.* 1980), field voles (Spears & Clarke 1986), Djungarian hamsters (Reasner &

Johnston 1987), lemmings (Hasler & Banks 1975), opossums (Harder & Jackson 2005), cows (Izard & Vandenbergh 1982), and pigs (Brooks & Cole 1970).

There are several proposed urinary constituents that contribute to a male's capacity to induce sexual maturation in juvenile females (Petrulis 2013). Most of these are ligands in the urine found bound to major urinary proteins (MUPs) (Novotny *et al.* 1999). Two such ligands, α farnesene and β farnesene, are chemicals produced by the male preputial glands (Novotny *et al*. 1990). That there are multiple components of male urine that can induce the Vandenbergh effect is evidenced by the fact that preputialectomized male mice are still capable of hastening sexual maturation in nearby juvenile females (Khan *et al.* 2009). Since E_2 can stimulate precocious puberty in females, just as the urinary pheromone from adult males can, it is possible that male urinary E_2 also contributes to male-induced sexual maturation of juvenile females.

Radiolabelled E_2 injected into adult male mice appears in their urine within minutes (Guzzo *et al.* 2010), and can be seen in a cohabiting juvenile female's brain and reproductive tract, likely gaining entry through her nose and skin (Guzzo *et al.* 2012). Pre-treatment of adult females with unlabeled E_2 reduces radioactivity in the uterus, suggesting that the radiolabelled E_2 in male urine is binding to estrogen receptors and is therefore biologically active (Guzzo *et al*. 2010, 2013). Urination is a social response in mice, with male urination increasing particularly in the presence of females (Reynolds 1971). Male mice experience increased circulating T_5 in the presence of unfamiliar females (Macrides *et al.* 1975). This could lead to increases in circulating E_2 (aromatized from T_5) and subsequently increased urinary E_2. Male mice housed with juvenile females

experience increased urinary E_2, and aim their urine toward the female (Beaton *et al.* 2006, deCatanzaro *et al.* 2009). Interestingly, female voles experience increased serum E_2 when in the presence of males, which is hypothesized to lead to sexual maturation (Cohen-Parsons & Carter 1986). It is not known whether this E_2 is endogenous, from the male, or both. Since steroids easily pass through the skin (Guzzo *et al.* 2012), it is possible that E_2 in male urine is absorbed by juvenile females, thus contributing to precocious puberty in the Vandenbergh effect.

The Bruce Effect: Disruption of Early Pregnancy

Hilda Bruce first described the phenomenon of pregnancy disruption in inseminated female mice caused by housing them with a non-sire male (Bruce 1959). Several indicators of pregnancy disruption in females induced by strange (non-sire) males have been used, including a return to estrous cycling (Bruce 1960, Dominic 1966), the absence of parturition (Beaton & deCatanzaro 2005), and a decrease in uterine implantation sites (Keverne & de la Riva 1982). Much like the Vandenbergh effect, the Bruce effect is induced by a chemical in male urine (Dominic 1966) that disappears after castration (Bruce 1965), and reappears if the male is treated with T_5 (deCatanzaro & Storey 1989). While it is most widely known in the house mouse, the Bruce effect has also been reported in deer mice (Bronson & Eleftheriou 1963), meadow voles (Clulow & Langford 1971), Mongolian gerbils (Rohrbach 1982) and the wild gelada baboon (Roberts *et al.* 2012).

There is much interest in identifying the mechanism by which male urine disrupts pregnancy. A prominent hypothesis involves the formation of an olfactory memory of the

sire by the mated female (Brennan 2004). The ability of an inseminated female to "recognize" the sire prevents him from blocking her pregnancy, whereas unfamiliar males produce a Bruce effect (Brennan 2009). Individual recognition in mice may be mediated through the major histocompatibility complex (MHC) or MUPs in the urine, both of which can vary between unrelated individuals (Brennan & Peele 2003). Chemosignals bound to either the MHC or MUPs in unfamiliar male urine are suggested to enter the female through her vomeronasal organ, cause a surge of dopamine in the arcuate nucleus which then decreases prolactin release from the pituitary, leading to the degradation of the corpora lutea and a subsequent drop in P_4, resulting in pregnancy loss (Brennan 2004). It is also possible that, in conjunction with a female's behaviour based on the identity of the male (sire or novel), E_2 in the male's urine enters the inseminated female's system and contributes to pregnancy failure in the Bruce effect.

Recently inseminated females approach and investigate a strange male much more than their own sire when given the choice (deCatanzaro & Murji 2004). Additionally, when present, the sire will prevent a female from investigating the other male, leading to reduced incidence of pregnancy block (deCatanzaro & Murji 2004). It is possible that females investigate unfamiliar males with MHC or MUP complexes different from their sire. By investigating unfamiliar males, inseminated females may then absorb E_2 found in their urine, leading to disruption of implantation (deCatanzaro 2011). Like juvenile females, inseminated females housed with unfamiliar males injected with radiolabelled E_2 absorb it into their brains and reproductive tracts (Guzzo *et al.* 2012). Furthermore, manipulating male urinary E_2 can change his capacity to block pregnancy in inseminated

females. Castration (Vella & deCatanzaro 2001) or aromatase inhibition plus a phytoestrogen-free diet (Beaton & deCatanzaro 2005) reduces males' urinary E_2 and their ability to produce a Bruce effect. Furthermore, like those in the presence of juvenile females, males housed in close proximity to inseminated females experience increased urinary E_2 (deCatanzaro *et al.* 2006). Interestingly, sires themselves do not experience the same increase in urinary E_2 when housed with their mates, and novel males only experience an increase in E_2 in the presence of an inseminated female when her sire is absent (deCatanzaro *et al.* 2006). This lack of an E_2 rise in strange males when the sire is present could contribute to the sire protection effect (Murji & deCatanzaro 2004). Several mechanisms by which E_2 disrupts implantation have been discussed, and it is hypothesized that E_2 present in novel male urine may contribute to the Bruce effect through these mechanisms.

Stress-Induced Reproductive Suppression

The stress response facilitates escape from immediate danger, thereby potentially increasing survival. GCs released in stress mobilize energetic resources by increasing circulating glucose, and also increase blood pressure and cardiac output, all of which presumably facilitate escape from the stressor (Sapolsky *et al.* 2000). Another characteristic of the stress response is the inhibition of physiological systems unnecessary for immediate survival, such as digestion, growth, and reproduction (Wingfield & Sapolsky 2003, Sapolsky 2004). These long-term processes are energetically costly and therefore are generally considered maladaptive in the short-term during stress (Sapolsky

2004). Indeed, the energetic demands of gestation and lactation may not allow a female to use her energy stores maximally to escape a threat. Moreover, rearing young under stressful conditions may not be conducive to either maternal or offspring survival. Thus, forestalling reproduction until more favourable conditions arise is considered to be adaptive (Wingfield & Sapolsky 2003).

There are several ways in which stress suppresses reproduction. Stress causes decreased gonadotropin levels (Rivier *et al.* 1986, Sapolsky & Krey 1988, Norman & Smith 1992, Breen *et al.* 2007), indicating a suppression of the HPG axis, which could originate at the hypothalamic or pituitary level. CRH released in stress can suppress the HPG axis by acting at the level of the hypothalamus. CRH suppresses both kisspeptin gene and GPR54 expression (Kinsey-Jones *et al.* 2008), which would presumably lead to decreased GnRH release. Interestingly, CRH infusion into immature female rats delays sexual maturation (Kinsey-Jones *et al.* 2010), perhaps by suppressing the kisspeptin system. CRH may also directly suppress GnRH: GnRH neurons express CRH receptors (Kinsey-Jones *et al.* 2006), and CRH administration reduces GnRH levels (Rivest *et al.* 1993).

GCs released in stress suppress the HPG axis at the hypothalamic, pituitary and gonadal levels. GCs reduce gonadotropin release from the pituitary partly by reducing gonadotrope sensitivity to GnRH stimulation (Breen & Karsch 2004). GCs may also inhibit the HPG axis by stimulating the release of gonadotropin-inhibitory hormone, a recently discovered hypothalamic peptide that is involved in suppression of GnRH and gonadotropins (Kirby *et al.* 2009). Finally, GCs can render the gonads hyposensitive to

the stimulatory effects of gonadotropins, thereby reducing sex steroid production and release (Charpenet *et al.* 1982, Sapolsky 1985, Michael *et al.* 1993). Given the importance of the HPG axis hormones to reproduction, suppression at any level by stress can result in reduced spermatogenesis in males, impaired sexual behaviour in males and females, and reduced cyclicity leading to anovulation in females (McGrady 1984, Negro-Vilar 1993, Wingfield & Sapolsky 2003).

The above mechanisms of stress-induced reproductive suppression result in disruptions of hormone cycling and sexual behaviour. However, reproductive suppression can also occur in female mammals after successful mating has already occurred. A severe stressor encountered after insemination likely indicates that the current environment is not conducive to maternal or offspring survival. Termination of pregnancy in the peri-implantation period due to stress has been reported (deCatanzaro & MacNiven 1992, deCatanzaro 2011). Failure of early pregnancy to proceed to term can be caused by several physical and psychological stressors, including overcrowding (Helmreich 1960), restraint (Euker & Riegle 1973, Wiebold *et al.* 1986), social subordination (Huck *et al.* 1988), loud noise (Joachim *et al.* 2003), heat (Garcia-Ispierto *et al.* 2006), and predator exposure (deCatanzaro 1988). While stress is closely associated with increased GCs, it is not the direct action of these steroids that causes pregnancy to fail (deCatanzaro *et al.* 1991). Instead, it appears to be a modulation of the sex steroids E_2 and P_4 in stress that disrupts early pregnancy.

Implantation is sensitive to the ratio of circulating E_2 and P_4: elevated E_2 relative to P_4 is associated with implantation failure in humans and mice (Gidley-Baird *et al.*

1986, Safro *et al.* 1990). In pregnant females, P_4 is decreased in stress and this decrease is associated with loss of pregnancy (Parker & Douglas 2009). Administration of P_4 or progestin analogues, or blocking the conversion of P_4 to corticosterone can protect against stress-induced pregnancy loss (MacNiven & deCatanzaro 1990, Joachim *et al.* 2003). There are several reports of E_2 rising in response to stress (MacNiven *et al.* 1992, Shors *et al.* 1999, Misdrahi *et al.* 2005, Agrawal *et al.* 2011). Although the source of this E_2 is unknown (adrenal, ovarian, or other), it appears to contribute to stress-induced pregnancy loss, since antibodies to E_2 can mitigate pregnancy failure in stressed mice (deCatanzaro *et al.* 1994). Given the known detrimental effects of E_2 on implantation, it is possible that rises in E_2 caused by stress experienced shortly after insemination, along with decreases in P_4, contribute to stress-induced reproductive suppression in pregnant females.

Objectives and Overview

The objectives of this thesis are fourfold: 1) to determine whether male estrogens are involved in the Bruce and Vandenbergh effects; 2) to develop urinary and serum immunoassays that allow documentation of adrenal and gonadal steroid responses to stress in male and female mice; 3) to assess the relative merit of urinary and serum steroid immunoassays in studies of stress; and 4) to determine whether indirect rat exposure constitutes a robust stressor in inseminated female mice capable of disrupting implantation, and to examine E_2 and P_4 dynamics surrounding such stress-induced pregnancy loss.

In **Chapter 2**, I examine the role of male E_2 in implantation failure (the Bruce effect) and sexual maturation (the Vandenbergh effect) induced in nearby female mice. The following two chapters are methodological descriptions of urinary and serum immunoassays. In **Chapter 3**, I document the circadian rhythm and acute stress response of corticosterone in urine and serum of male mice. Here, I also document the urinary circadian rhythm of T_5, and its response to acute physical stress. In **Chapter 4**, I document the time course of adrenocortical corticosterone, P_4, and E_2 response to acute, mild, stress in female mouse urine and serum. In **Chapter 5**, I examine several responses of inseminated female mice to predator exposure during the first 5 days of pregnancy: corticosterone, P_4, and E_2 levels, behavioural avoidance, and the number of blastocyst implantation sites in the uterus. Here, I also examine the implantation response to various doses of E_2 and P_4 administered in the presence or absence of predator stress. In **Chapter 6**, I summarize and discuss the results of Chapters 2-5, and suggest areas of future research.

Chapter 2: Thorpe JB, deCatanzaro D 2012 Oestradiol treatment restores the capacity of castrated males to induce both the Vandenbergh and the Bruce effects in mice (*Mus musculus*). *Reproduction* **143** 123-132.

Abstract: Androgen-dependent urinary constituents from males hasten reproductive maturation (the Vandenbergh effect) and disrupt peri-implantation pregnancy (the Bruce effect) in nearby females. Each of these effects can be mimicked in socially isolated females by direct administration of exogenous oestrogens. The current experiments were

designed to determine the role of males' urinary 17β-oestradiol (E_2) in their capacities to induce these effects. A preliminary experiment showed that both males on a phyto-oestrogen-rich soy-based diet and those on a phyto-oestrogen-free diet could induce both effects. For subsequent experiments, males were castrated and treated with either oil vehicle or E_2. Enzyme immunoassay was conducted on non-invasively collected urine samples from these males. Concentrations of urinary testosterone were subnormal in both conditions, but urinary E_2 was restored to the normal range for intact males in castrates given E_2. Urinary creatinine was also quantified as a measure of hydration and was significantly reduced in males treated with E_2. Castration diminished the capacity of males to promote growth of the immature uterus and also their capacity to disrupt blastocyst implantation in inseminated females. Injections of E_2 to castrated males restored both capacities. These data converge with other studies indicating that E_2 is the main constituent of male urine responsible for induction of both the Vandenbergh and the Bruce effects.

Chapter 3: Thorpe JB, Rajabi N, deCatanzaro D 2012 Circadian rhythm and response to an acute stressor of urinary corticosterone, testosterone, and creatinine in adult male mice. *Hormone and Metabolic Research* **44** 429-435.

Abstract: In small laboratory species, steroid measures can be obtained more frequently and less invasively from urine than blood. Insofar as urinary levels reflect systemic levels, they could provide advantages particularly for measurement of glucocorticoids, whose blood levels react rapidly to handling and stress. In Experiment 1, urinary samples were

collected from male mice every second hour over a 14:10 h light:dark cycle. Samples were analyzed via enzyme immunoassay for corticosterone, testosterone, and creatinine. Corticosterone had peak concentrations 1 h after light offset and a trough 1 h after light onset. Testosterone showed peak concentrations 5-7 h after light onset and lowest concentrations during the dark phase of the cycle. Creatinine showed some variation over the light-dark cycle, but steroid measures showed similar trends with and without adjustment for creatinine. In Experiment 2, mice were stressed via an injection at times close to the determined peak and trough levels of corticosterone. In urinary samples taken 90 min after injection, corticosterone was significantly higher in injected animals at both times relative to levels in control animals, but testosterone was unaffected by injection stress. In Experiment 3, serum and urine samples were collected from mice every sixth hour across the diurnal cycle. Corticosterone peaked in urine and serum immediately after light offset, and urinary measures predicted those in serum. These data indicate that urinary corticosterone reflects systemic levels in mice, document circadian variation in urinary testosterone, and indicate that circadian variation in creatinine is minimal, but potentially relevant in stressed animals.

Chapter 4: Thorpe JB, Gould KE, deCatanzaro D (submitted) Circulating and urinary adrenal steroids in response to acute, mild stress in female mice (*Mus musculus*).

Abstract: During acute stress, glucocorticoids and progesterone from the adrenal gland are upregulated. Estradiol may also rise, although its origin is unknown. In studies of stress, it can be difficult to obtain blood rapidly enough to avoid confounding steroid

measures. In such cases, non-invasive urinary steroid measures may be more appropriate insofar as they reflect systemic steroid levels. The current study was designed to examine the urinary steroid response to an acute, mild stressor in female mice, and to compare serum and urinary steroid responses to this mild stressor. In Experiment 1, we profiled urinary corticosterone, progesterone, and estradiol in ovariectomized female mice at 11 time points following a one-hour stressor (placement on an elevated platform). This increased urinary corticosterone for 3 hours and urinary progesterone for 4 hours after stressor offset. In Experiment 2, terminal blood and urine samples were obtained at 0, 2, 4, and 6 hours after the stressor offset. Stressed females showed increased serum corticosterone and progesterone immediately after the stressor offset. Urinary levels of corticosterone were increased at both 0 and 2 hours post-stress, while those of progesterone trended toward increases 2-6 hours after stress offset. Neither serum nor urinary estradiol was significantly influenced by stress. Urinary measures generally reflected systemic measures, however with a different time course resulting in a longer return to baseline. We suggest that the relative value of serum or urinary steroid measures in mice depends upon the experimental design.

Chapter 5: Thorpe JB, Burgess PS, Sadkowski M, deCatanzaro D (In Press) Estrogen-progesterone balance in the context of blastocyst implantation failure induced by predator stress. *Psychoneuroendocrinology.*

Abstract: Diverse stressors can disrupt blastocyst implantation in inseminated female mammals. Stress-induced implantation failure can be mimicked by minute doses of

exogenous estradiol, and some evidence indicates that it may be mitigated by exogenous progesterone. In Experiment 1, we showed that acute exposure to a rat across a wire-mesh grid caused elevation of corticosterone and progesterone. In Experiment 2, we showed that exposure of inseminated mice to rats across a grid during gestation days 1-5 was associated with avoidance of proximity to the grid and a significantly reduced number of implantation sites on gestation day 6. Rat-exposure also resulted in elevated progesterone levels in females that maintained their pregnancies, and elevated estradiol levels in females that lost their pregnancies. In Experiment 3, we investigated whether exogenous progesterone, estradiol, or a combination of both could influence implantation failure induced by rat-exposure stress. Treatment with 100ng estradiol per day on gestation days 1-5 induced a complete absence of implantation sites on gestation day 6, regardless of the presence or absence of the stressor. Administration of 500μg progesterone per day was insufficient to prevent the stress-induced pregnancy loss. However, 500μg progesterone plus 10ng estradiol per day did prevent implantation failure in rat-exposed females. These findings are consistent with the hypothesis that estradiol elevations contribute to stress-induced pregnancy loss, but show paradoxically that low doses of estradiol can act together with progesterone to mitigate stress-induced pregnancy loss.

Chapter 2

Oestradiol treatment restores the capacity of castrated males to induce both the Vandenbergh and the Bruce effects in mice (*Mus musculus*)

Joelle B. Thorpe and Denys deCatanzaro (2012)

Reproduction, 143, 123-132

Authors' Contributions

Joelle B. Thorpe: Experimental design, data collection, data analysis, manuscript writing, editing, and preparation.

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

Abstract

Androgen-dependent urinary constituents from males hasten reproductive maturation (the Vandenbergh effect) and disrupt peri-implantation pregnancy (the Bruce effect) in nearby females. Each of these effects can be mimicked in socially isolated females by direct administration of exogenous oestrogens. The current experiments were designed to determine the role of males' urinary 17β-oestradiol (E_2) in their capacities to induce these effects. A preliminary experiment showed that both males on a phyto-oestrogen-rich soy-based diet and those on a phyto-oestrogen-free diet could induce both effects. For subsequent experiments, males were castrated and treated with either oil vehicle or E_2. Enzyme immunoassay was conducted on non-invasively collected urine samples from these males. Concentrations of urinary testosterone were subnormal in both conditions, but urinary E_2 was restored to the normal range for intact males in castrates given E_2. Urinary creatinine was also quantified as a measure of hydration and was significantly reduced in males treated with E_2. Castration diminished the capacity of males to promote growth of the immature uterus and also their capacity to disrupt blastocyst implantation in inseminated females. Injections of E_2 to castrated males restored both capacities. These data converge with other studies indicating that E_2 is the main constituent of male urine responsible for induction of both the Vandenbergh and the Bruce effects.

Introduction

A female's reproductive physiology can be profoundly impacted by nearby males. Of particular interest are the abilities of adult males to hasten reproductive maturation in developing females (Vandenbergh 1967) and to disrupt peri-implantation pregnancy (Bruce 1960). These phenomena, termed the Vandenbergh and Bruce effects, respectively, have been studied extensively and are best known in house mice (e.g. Bruce 1960, Vandenbergh 1967, Drickamer 1983). The Vandenbergh effect has also been observed in deer mice (Teague & Bradley 1978), voles (Lepri & Vandenbergh 1986), hamsters (Reasner & Johnston 1988), lemmings (Hasler & Banks 1975), opossums (Harder & Jackson 2003), pigs (Brooks & Cole 1970) and cows (Izard & Vandenbergh 1982). The Bruce effect has also been reported in deer mice (Bronson & Eleftheriou 1963), voles (Clulow & Langford 1971) and gerbils (Rohrbach 1982). The current study was designed to explore the hypothesis that males' urinary oestradiol (E_2) is critical for their capacities to accelerate female reproductive maturation and to disrupt blastocyst implantation.

It is well established that the Vandenbergh and Bruce effects are attributable to urinary constituents passed from males to nearby females. These constituents are androgen dependent, since castrated males lose their abilities to hasten reproductive maturation and disrupt implantation in nearby females (Bruce 1965, Colby & Vandenbergh 1974, Vella & deCatanzaro 2001), and androgen replacement restores these abilities (Lombardi *et al.* 1976, deCatanzaro & Storey 1989). Along with testosterone, E_2 is decreased after castration (Vella & deCatanzaro 2001), and since testosterone is readily

converted to E_2 by aromatase (Simpson *et al.* 2002), androgen replacement in previous studies could have its influences through E_2. Injections of E_2 benzoate can promote reproductive maturity in juvenile females (Bronson 1975), and E_2 given to inseminated females can disrupt blastocyst implantation (deCatanzaro *et al.* 2001, 2006), in both cases mimicking the effects of male exposure. Moreover, when inseminated females are nasally administered urine from males given radiolabelled E_2, radioactivity can be detected in the females' uteri and brains (Guzzo *et al.* 2010). Both females' reproductive maturation and blastocyst implantation are highly sensitive to oestrogens. Oestrogens drive growth of the uterus in juveniles (Ogasawara *et al.* 1983, Quarmby & Korach 1984), and although oestrogens are critical for preparing the uterus for implantation, small elevations above optimal levels can completely disrupt implantation (deCatanzaro *et al.* 2001, Ma *et al.* 2003). Collectively, these data suggest that E_2 could be the urinary "pheromone" responsible for both the Vandenbergh and Bruce effects.

Biologically active unconjugated sex steroids have been quantified in urine of mice and largely reflect systemic steroid fluctuations (Muir *et al.* 2001, Vella & deCatanzaro 2001, deCatanzaro *et al*. 2003, 2004). An increase in urinary E_2 concentration is seen in adult male mice after they have been housed for a few days in proximity to either juvenile or recently inseminated females (Beaton *et al.* 2006, deCatanzaro *et al.* 2006, 2009). The presence of females causes males to consume more water, urinate more and direct their urine towards females (deCatanzaro *et al.* 2009). Sex steroids are lipophilic and have low molecular weight (e.g. E_2, 272.4 Da) and therefore easily pass from the skin or nasal area into circulation where they can bind to receptors in

the uterus, ovaries and brain (Waddell & O'Leary 2002, Guzzo *et al.* 2010). Thus, early reproductive maturation and disruption of peri-implantation gestation in male-exposed females may be due in part to absorption by females of male-excreted oestrogens.

The purpose of this study was to determine whether the Vandenbergh effect is mediated by oestrogens in male urine and to confirm that the same is true for the Bruce effect. A preliminary experiment was designed to compare the capacities of males to induce the Vandenbergh and Bruce effects when they were fed either a common laboratory soy-based diet or a phyto-oestrogen-free diet. This was based on evidence that dietary phyto-oestrogens can influence measures of urinary E_2 and possibly the Bruce effect (Beaton & deCatanzaro 2005) and evidence that they can also influence female reproductive maturation (Whitten & Naftolin 1992, Thigpen *et al.* 2003, Khan *et al.* 2008*a,b*). Subsequent experiments were conducted with all males on a phyto-oestrogen-free diet, used several weeks after castration in order to diminish baseline urinary testosterone and E_2 (Vella & deCatanzaro 2001). Some of these castrated males were given replacement injections of E_2, while control males were given oil vehicle injections, and their urinary E_2 and testosterone levels were then compared. We exposed juvenile females to males in the two conditions, and then measured these females' uterine and ovarian mass as an indication of the Vandenbergh effect. We also exposed recently inseminated females to males in the two conditions and counted the number of intrauterine implantation sites on day 6 of gestation. The number of implantation sites is one traditional measure of the Bruce effect (e.g. Keverne & de la Riva 1982), and it is an

indicator of gestational failure during the pre-implantation period that is not confounded by post-implantation influences (Berger *et al.* 2008).

Results

Initially, we compared the influences of males maintained on diets that were either soy based (rich in phyto-oestrogens) or phyto-oestrogen free. Phyto-oestrogen content of males' diet did not strongly influence their capacity to hasten reproductive maturity in juvenile females exposed to them and their excretions through a wire-mesh grid (Table 1). Combined uterine and ovarian wet mass, $F(2,47) = 3.63$ $P = 0.033$, and dry mass, $F(2,47) = 4.58$, $P = 0.015$, differed among conditions. Multiple comparisons indicated that dry mass in both male-exposed groups was increased relative to that of isolated controls; for wet mass, only females exposed to males on a soy-based diet exceeded controls. For uterine mass alone, there was overall significance for both wet tissue, $F(2,47) = 3.23$, $P = 0.047$, and dry tissue, $F(2,47) = 4.24$, $P = 0.020$, with females exposed to males on the soy-based diet having significantly increased uterine mass compared with isolated females. Ovarian wet mass was altered by experimental condition, $F(2,47) = 8.25$, $P = 0.001$, with this mass significantly increased in females exposed to males on either diet. Dry ovarian mass approached the conventional level of significance, $F(2,47) = 3.05$, $P = 0.055$.

Table 1 Mean (± S.E.M.) wet and dry uterine and ovarian mass of 28-day-old female mice after 7 days of isolation (isolated) or 7 days of exposure to adult males that were on either a soy-based diet or a phyto-oestrogen-free diet. Multiple comparisons indicated that females exposed to either group of males had significantly higher combined uterine and ovarian dry mass compared with isolated control females (see Results section for details).

		Uterine mass (mg)		**Ovarian mass (mg)**	
Condition	***n***	**Wet**	**Dry**	**Wet**	**Dry**
Isolated	17	56.45 ± 8.92	10.32 ± 1.28	12.65 ± 0.49	3.45 ± 0.19
Exposed to males on a soy-based diet	17	94.78 ± 13.14	15.58 ± 1.41	15.32 ± 0.47	3.72 ± 0.13
Exposed to males on a phytoestrogen-free diet	16	80.60 ± 10.02	14.09 ± 1.27	15.17 ± 0.61	4.06 ± 0.19

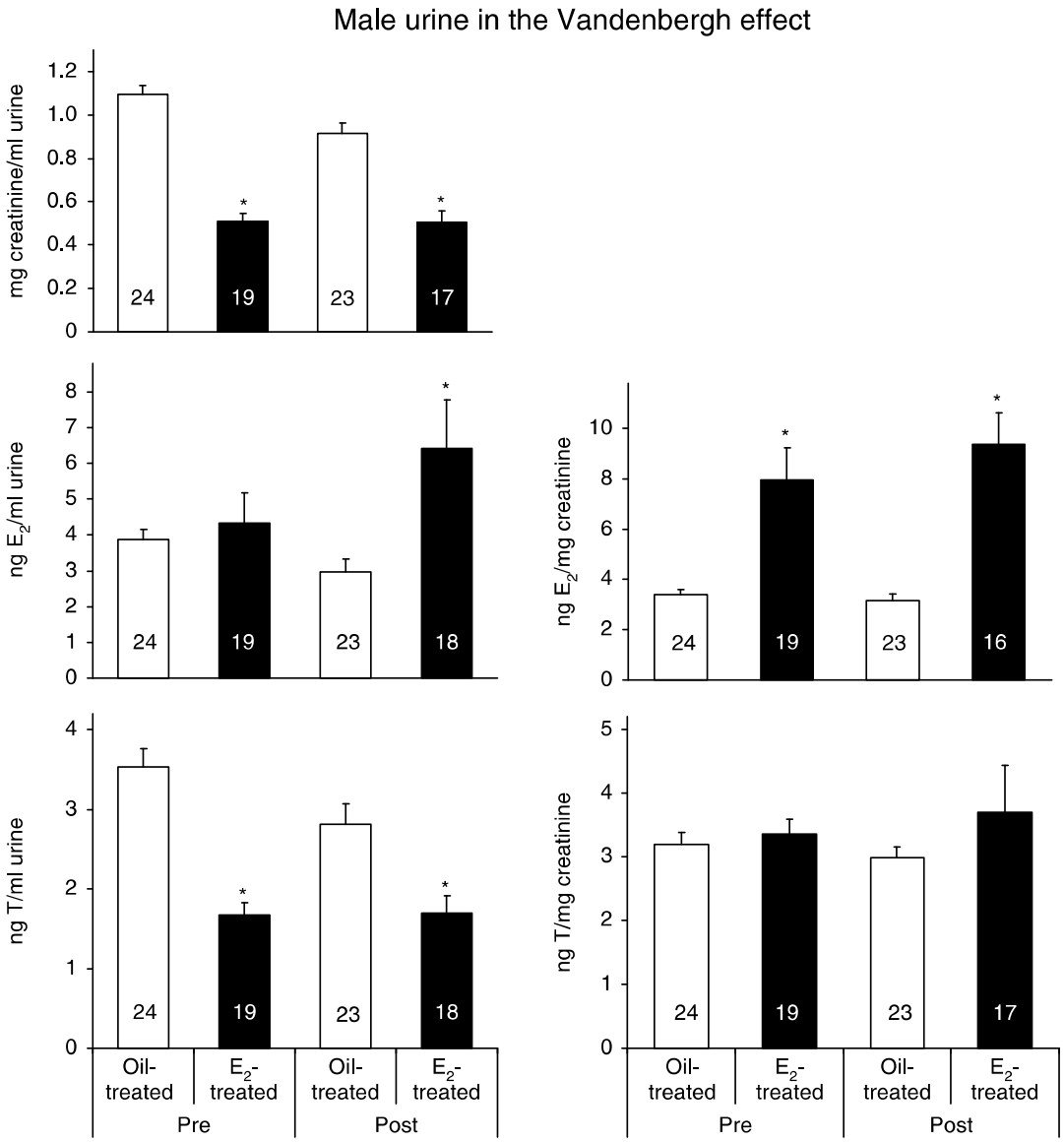
The phyto-oestrogen content of diet did not strongly impact males' capacity to disrupt implantation in recently inseminated females exposed to these males and their excretions through a wire-mesh grid (Table 2). Overall, experimental condition impacted the proportion of females that were pregnant, $\chi^2(2) = 14.50$, $P < 0.001$. Specific tests for each pair of conditions showed that isolated females were more likely to be pregnant than those exposed to males on the soy-based diet, $\chi^2(1) = 8.18$, $P = 0.004$, and those exposed to males on the phyto-oestrogen-free diet, $\chi^2(1) = 14.70$, $P < 0.001$. Experimental condition also altered the number of implantation sites found on gestation day 6, $F(2,64) = 8.53$, $P < 0.001$, with females exposed to males on either diet having fewer implantation sites than isolated females.

Table 2 Number of females showing any implantation sites (*n* pregnant) and mean (± S.E.M.) number of implantation sites on day 6 of gestation, after 5 days of isolation (isolated) or exposure to adult males that were on either a soy-based diet or a phyto-oestrogen-free diet. Multiple comparisons indicated that females exposed to either group of males had significantly fewer implantation sites compared with isolated control females.

Condition	***n***	***n* pregnant**	**Implantation sites**
Isolated	23	22	12.82 ± 0.76
Exposed to males on a soy-based diet	23	14	8.57 ±1.50
Exposed to males on a phytoestrogen-free diet	21	9	5.33 ±1.47

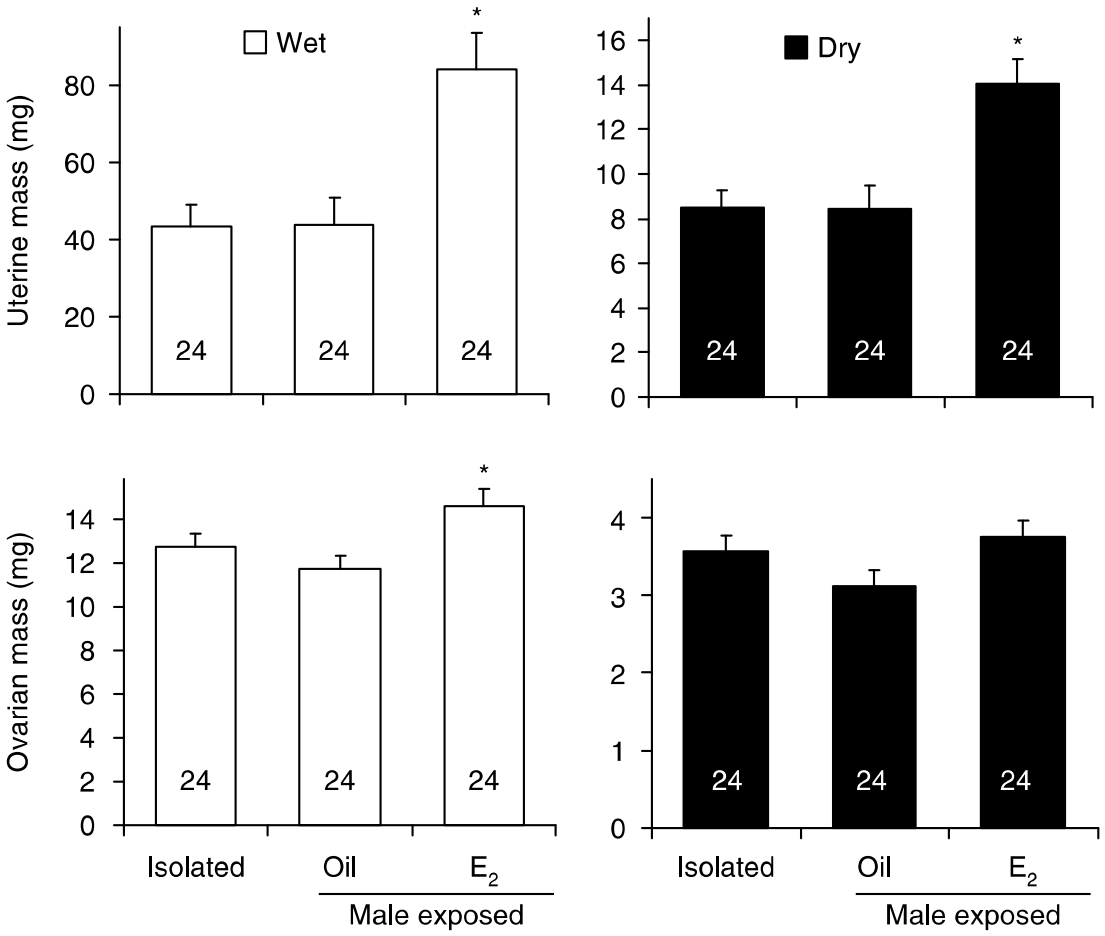
The subsequent experiments compared influences on females of castrated males given either oil (control) or E_2 injections. There were clear influences of E_2 treatment on male urinary creatinine and steroid concentrations before and after 6 days of exposure to juvenile females (Fig. 1). E_2-treated males had significantly reduced urinary creatinine compared with oil-treated males both before exposure to females, $t(41) = 10.62$, $P < 0.0001$, and after, $t(38) = 5.62$, $P < 0.0001$. Although unadjusted urinary E_2 was not significantly different between experimental and control males before exposure to females, $t(41) = 0.58$, $P = 0.286$, it was significantly increased in E_2-treated castrates after such exposure, $t(39) = 2.75$, $P = 0.004$. Creatinine-adjusted urinary E_2 was significantly increased by E_2 administration both before exposure, $t(41) = 3.89$, $P < 0.001$, and after, $t(37) = 5.73$, $P < 0.0001$. Unadjusted testosterone was significantly decreased in E_2-treated castrates both before exposure, $t(41) = 6.40$, $P < 0.0001$, and after, $t(39) = 3.20$, $P = 0.003$, but these trends were absent when urinary testosterone was adjusted for creatinine.

Figure 1 Mean (± S.E.M.) urinary creatinine, oestradiol (E_2), testosterone (T), creatinine-adjusted E_2 and creatinine-adjusted testosterone concentration, immediately before (pre) and after 6 days (post) of exposure to a juvenile female, in castrated male mice given oil or E_2 injections. Sample sizes are indicated on the bars. *Significant difference from controls within measures at the same time.


Male urine in the Vandenbergh effect
mg creatinine/ml urine
ng E2/ml urine
ng E2/mg creatinine
ng T/ml urine
ng T/mg creatinine
24
19
23
17
18
16
Oil-treated
E2-treated
Pre
Post

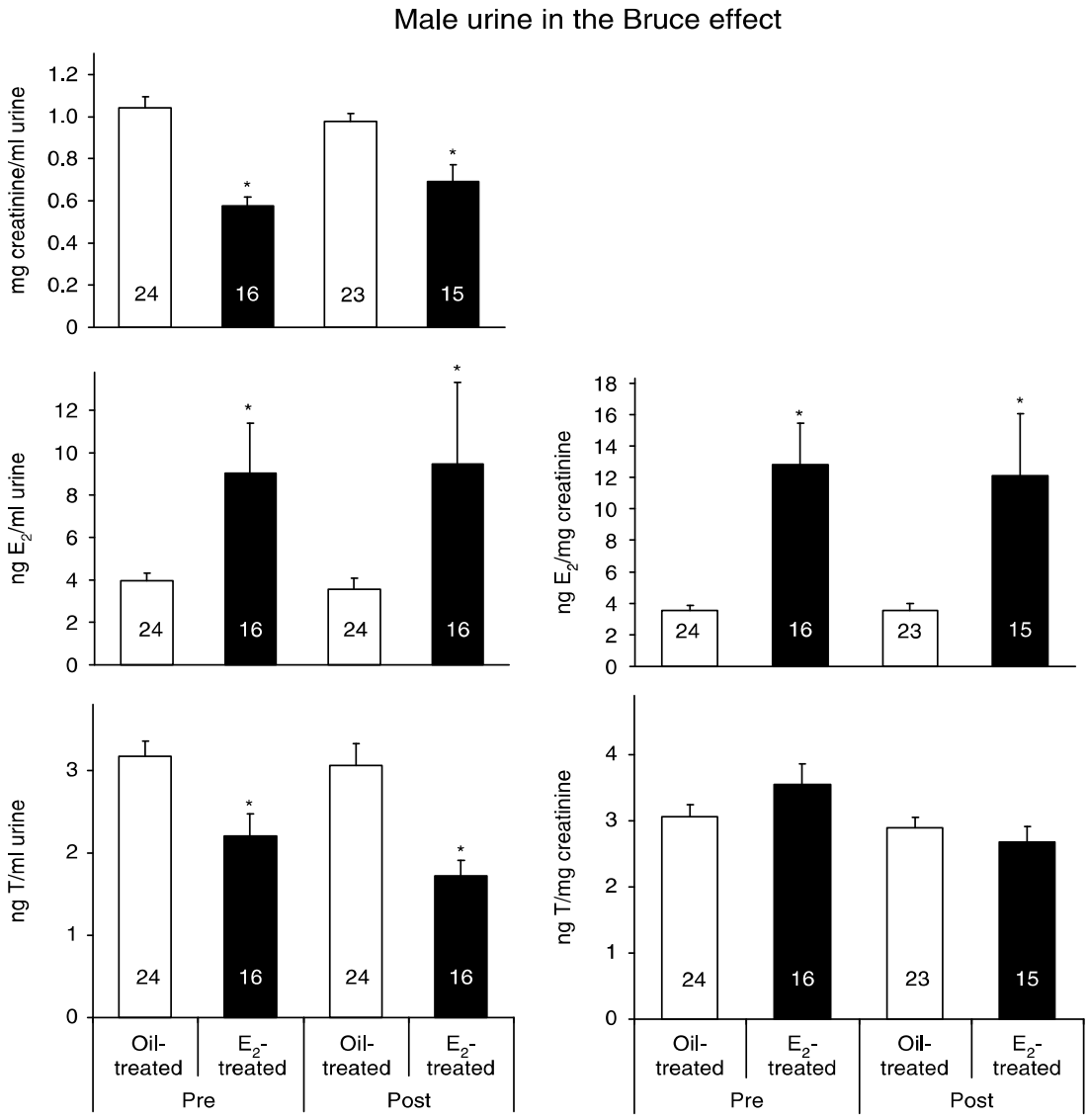
E_2 treatment of castrated males restored their capacity to hasten reproductive maturity in nearby juvenile females, as reflected in uterine and ovarian mass (Fig. 2). Combined uterine and ovarian mass was clearly impacted by experimental condition both when tissue was wet, $F(2,69) = 9.92$, $P < 0.001$, and dry, $F(2,69) = 10.47$, $P < 0.001$. When the uterus was considered separately, experimental condition significantly impacted both wet mass, $F(2,69) = 9.58$, $P < 0.001$, and dry mass, $F(2,69) = 10.37$, $P < 0.001$. Ovarian mass was significantly impacted by experimental treatment for only wet tissue, $F(2,69) = 5.12$, $P = 0.009$. Multiple comparisons indicated that for all of these measures, mass was higher in females exposed to E_2-treated castrates than in both isolated females and those exposed to oil-treated castrates.

Figure 2 Wet and dry mean (± S.E.M.) uterine and ovarian masses of 28-day-old female mice after 7 days of isolation (isolated) or 7 days of exposure to castrated adult males on a phyto-oestrogen-free diet receiving either oil or oestradiol (E_2) injections. Sample sizes are indicated on the bars. *Significant difference from the other two groups.



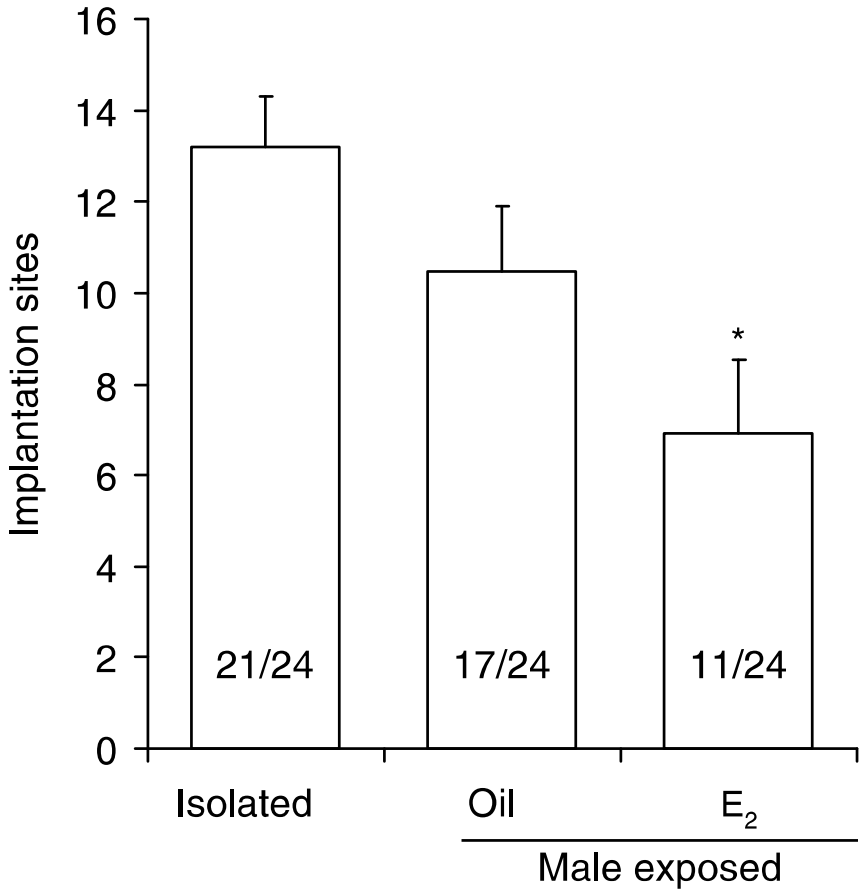
For castrated males exposed to inseminated females, there were clear influences of E_2 treatment on male urinary creatinine and steroid concentrations (Fig. 3). Urinary creatinine was significantly reduced in castrates treated with E_2 compared with oil-treated castrates both before female exposure, $t(38) = 6.51$, $P < 0.0001$, and after, $t(36) = 3.63$, $P = 0.001$. Urinary E_2 was significantly increased in castrated males treated with E_2 both before exposure, $t(38) = 2.55$, $P = 0.007$, and after, $t(38) = 1.85$, $P = 0.034$. When adjusted for urinary creatinine, urinary E_2 was increased by E_2 treatment both before exposure, $t(38) = 4.19$, $P < 0.001$, and after, $t(36) = 2.68$, $P = 0.011$. Urinary testosterone was decreased by E_2 treatment before exposure, $t(38) = 3.07$, $P = 0.004$, and after, $t(38) = 3.64$, $P = 0.001$, but this was not the case when testosterone was adjusted for urinary creatinine.

Figure 3 Mean (± S.E.M.) urinary creatinine, oestradiol (E_2), testosterone (T), creatinine-adjusted E_2 and creatinine-adjusted testosterone concentration, immediately before (pre) and after 4 days (post) of exposure to a recently inseminated adult female, in castrated male mice given oil or E_2 injections. Sample sizes are indicated on the bars. *Significant difference from controls within measures at the same time.


Male urine in the Bruce effect
mg creatinine/ml urine
ng E2/ml urine
ng E2/mg creatinine
ng T/ml urine
ng T/mg creatinine
24
16
23
15
Oil-treated
E2-treated
Pre
Post

On day 6 after insemination, isolated females were most often pregnant and had the most implantation sites (Fig. 4). Experimental treatment impacted the number of females that were pregnant (> 0 implantation sites), $\chi^2(2) = 9.71$, $P = 0.008$. Specific tests of pairs of conditions indicated that there were fewer pregnancies among females exposed to E_2-treated males than among isolated females, $\chi^2(1) = 9.38$, $P = 0.002$. Similarly, experimental treatment affected the number of implantation sites on gestation day 6, $F(2,69) = 5.07$, $P = 0.009$, with females exposed to E_2-treated males having significantly fewer implantation sites than isolated females.

Figure 4 Total number of implantation sites (± S.E.M.) in female mice on gestation day 6, after 5 days of isolation (isolated) or 5 days of exposure to castrated adult males receiving either oil or oestradiol (E_2) injections. Number of females pregnant (numerator) and total number of females (denominator) are indicated on the bars for each condition. *Significant difference from controls.



Discussion

These data indicate that males' urinary oestrogens contribute to their abilities to hasten reproductive maturation in juvenile females and to disrupt peri-implantation pregnancy in inseminated females. These effects are not attributable to the males' dietary phyto-oestrogens, as males on diets that are either rich in or free of phyto-oestrogens induced both the Vandenbergh and Bruce effects. Castration has long been known to eliminate males' capacity to induce both effects (Bruce 1965, Colby & Vandenbergh 1974), and testosterone replacement restores it (Lombardi *et al.* 1976, deCatanzaro & Storey 1989). We found that E_2 replacement was similarly effective, suggesting that this metabolite of testosterone is responsible for the testes' role in both effects. More specifically, our results indicate that the presence of E_2 in male urine is critical. Castrated males with low urinary testosterone and E_2 were incapable of inducing either the Vandenbergh effect or the Bruce effect. Replacement injections of E_2 resulted in castrated males with a normal range of urinary E_2 (cf. deCatanzaro *et al.* (2006, 2009) and Khan *et al.* (2009)) but low urinary testosterone concentrations. These males were capable of both hastening reproductive maturation and disrupting peri-implantation gestation in nearby females.

The current data show for the first time that E_2 treatment restores the capacity of castrated males to hasten female reproductive maturation in the Vandenbergh effect. They also refine understanding of the role of male urinary E_2 in the Bruce effect, replicating previous data where a similar dose of E_2 restored the capacity of castrated males to induce the effect and a higher dose disrupted even more pregnancies (deCatanzaro *et al.* 1995).

The design here was improved in that implantation sites were examined rather than litter size at birth, excluding potential post-implantation effects. The current data are also complemented by quantification of male urinary E_2 levels, showing them to be at a physiological level. For both effects, our data indicate a relationship to male urinary E_2 *per se*.

Maturation and growth of the female mammalian reproductive tract is driven by endogenous oestrogens (Ogasawara *et al.* 1983, Quarmby & Korach 1984). Both the uterus and the ovaries express oestrogen receptors and are responsive to E_2 (Clark & Gorski 1970, Medlock *et al.* 1981, Cooke *et al.* 1997, Yang *et al.* 2002). E_2 stimulates uterine growth by increasing DNA synthesis and cell proliferation (Mukku *et al.* 1982, Ogasawara *et al.* 1983, Quarmby & Korach 1984, Cooke *et al.* 1997). It can act through oestrogen receptor α to upregulate the expression of insulin-like growth factor 1 (IGF1) and its receptor (Murphy *et al.* 1988, Ghahary & Murphy 1989, Kahlert *et al.* 2000). The interaction of E_2 and IGF1 has mitogenic effects on the uterus (Sato *et al.* 2002, Zhu & Pollard 2007). The involvement of oestrogen in uterine growth is so well established that such growth is frequently used as a measure of oestrogenicity (the uterotrophic assay) of chemicals in toxicological research (e.g. Ashby & Tinwell 1998, Shaw & deCatanzaro 2009). Thus, exposure to exogenous E_2 via absorption from male urine could account for growth of the uterus in the Vandenbergh effect.

Similarly, absorption of E_2 from male urine could account for implantation loss in the Bruce effect, given the fact that peri-implantation gestation is highly sensitive to oestrogens. Diverse stressors can also disrupt blastocyst implantation, and this may be

mediated by small rises in endogenous oestrogens above optimal levels (reviewed by deCatanzaro (2011)). Systemically administered E_2 in daily doses as low as 37 ng during the peri-implantation period results in gestation failure (deCatanzaro *et al.* 1991, 2001), and nasal administration also does so at somewhat higher doses (deCatanzaro *et al.* 2001, 2006). Uteri that are primed to be receptive to implantation are rendered unreceptive by a single 10 ng dose of E_2 (Ma *et al.* 2003). Supraoptimal levels of E_2 can prevent implantation by accelerating or delaying ovum transport through the reproductive tract, depending on the dose of E_2 and the species examined (Greenwald 1967, Ortiz *et al.* 1979). They can also render the uterine epithelium entirely refractory to blastocyst implantation (Ma *et al.* 2003) and be deleterious to survival of blastocysts themselves (Valbuena *et al.* 2001).

Male mice are a source of exogenous oestrogens for female conspecifics. Over the initial 4 days of female exposure through a wire-mesh grid, males develop polydipsia and polyuria, and increasingly direct droplets of their urine towards females' compartments (deCatanzaro *et al*. 2009). Males excrete unconjugated E_2 in their urine (Muir *et al.* 2001) and show increased urinary E_2 relative to creatinine when they are exposed to juvenile or inseminated females (deCatanzaro *et al.* 2006, 2009). Lipophilic steroids of small molecular weight can easily enter circulation after nasal or dermal exposure (Waddell & O'Leary 2002, Guzzo *et al.* 2010). Radiolabelled E_2 that is injected into adult males arrives in their urine, and radioactivity is especially present in the uterus after this urine is nasally administered to an inseminated female (Guzzo *et al.* 2010). Thus, it is clear that

unconjugated E_2 in male urine can enter the circulation of nearby females and bind where it could have impacts on implantation and uterine growth.

Historically, work on the Vandenbergh effect showed that exogenous E_2 could mimic this effect (Bronson 1975) and that endogenous E_2 in developing females changed in response to male exposure (Bronson & Desjardins 1974). Subsequently, evidence has indicated roles for preputial chemicals, such as farnesenes, in the induction of oestrus (Ma *et al.* 1999) and growth of the uterus in developing females (Novotny *et al.* 1999). However, other evidence has shown that a male's preputial glands are not necessary for his ability to induce growth of the female's uterus and ovaries (Khan *et al.* 2009). It is likely that there are convergent mechanisms that may each be sufficient and that multiple triggers are involved in various physiological and behavioural measures of female reproductive maturation (Vandenbergh 1967, Bronson & Desjardins 1974, Khan *et al.* 2008*a,b*). Our data implicating male urinary E_2 in the Vandenbergh effect are specific to measures of uterine growth, and further research would be necessary to generalize our hypothesized mechanism to other measures of reproductive maturity.

Historically, research on the Bruce effect has focused on the female's olfactory memory of the sire and a cascade of neural and endocrine events set off by impingement of novel male odours upon the female's vomeronasal organ (reviewed by Brennan (2004)). Some recent evidence indicates that short MHC-related peptides in novel male urine, different from those of the sire, help to trigger the female's pregnancy loss in the Bruce effect (Leinders-Zufall *et al.* 2004). Notably, however, these peptides can only disrupt pregnancy when placed in male urine rather than just water (Kelliher *et al.* 2006).

Potential reconciliation with the role of the male's urinary E_2 suggested by our data has yet to be developed. One possibility is that distinct odours of novel males draw the female's attention and stimulate her vomeronasal vascular pump (cf. Meredith (1994)), facilitating absorption of male urinary E_2 into her circulation (see review by deCatanzaro (2011)).

It is common practice to adjust urinary hormone concentrations by creatinine to control for differences in hydration among individuals, based on the assumption that rate of creatinine excretion is fairly constant (Erb *et al.* 1970, Muir *et al.* 2001, deCatanzaro *et al.* 2003). However, this practice has been criticised by some as unnecessary or misleading (e.g. Alessio *et al.* 1985, Hakim *et al.* 1994, Miro *et al.* 2004). Moreover, urinary creatinine can be dynamic in social situations in mice, decreasing substantially in conjunction with polyuria and polydipsia in males housed nearby females (deCatanzaro *et al.* 2009). Nevertheless, we found value here in creatinine adjustment of testosterone, as raw testosterone was unexpectedly reduced in castrated males by E_2 injections, while this effect disappeared with adjustment for creatinine. This is because creatinine itself differed across experimental conditions, being significantly reduced by E_2 treatment. Urination in male mice is reduced after castration (Drickamer 1995), which could have implications for creatinine. E_2 in the current study could have restored the profile of polydipsia and polyuria seen in intact males housed near females (deCatanzaro *et al.* 2006, 2009), thereby reducing urinary creatinine. We accordingly suggest that it is important to consider both raw and creatinine-adjusted urinary steroid measures.

These data confirm a strong relationship between the presence of E_2 in male urine and males' capacity to induce the Vandenbergh and Bruce effects. They also highlight the similarity of these two pheromonal effects, at least with respect to the necessary physiological state of the novel males, their behaviour of transmitting urine to females and the presence of E_2 in their urine. Once E_2-laden urine from the male reaches the female, the low molecular weight and lipophilic nature of E_2 permit its absorption into her system, enabling transport to the uterus. Once in the uterus, mechanisms are well established whereby small elevations of E_2 can promote uterine growth and disrupt implantation.

Materials and Methods

Animals

Stimulus males were heterogeneous strain (HS) mice derived from interbreeding C57-B6, Swiss Webster, CF-1 and DBA-2 strains originally obtained from Charles River Breeding Farms (St. Constant, QC, Canada). HS stimulus males were 4-8 months of age, with age counterbalanced across conditions within particular experiments. Females were CF-1 strain mice from stock obtained from Charles River Breeding Farms. For experiments concerning implantation failure, females were 3-5 months of age (33.6 ± 0.6 g) on day 1 of gestation, with age and weight counterbalanced across conditions within particular experiments. Females in experiments concerning reproductive maturation were 21 days old (12 ± 1.7 g) at the start of male exposure. Inseminating males were CF-1 adults of proven fertility. All mice were housed in standard polypropylene cages

(28x16x11 cm height) with access to water and rodent chow made available *ad libitum* (8640 Teklad Certified Rodent Chow, Harlan/Teklad, Madison, WI, USA) unless otherwise stated. All animals were maintained on a reversed 14 h light:10 h darkness 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.

Exposure apparatus

To achieve male exposure, each female was placed in the lower chamber of a double-decker apparatus adapted from that previously described (deCatanzaro *et al.* 1996). Briefly, this was a 30x21x27 cm (height) transparent Plexiglas cage, with the lower and upper chambers each ~13 cm in height and separated by stainless steel wire-mesh grid with openings of 0.5 cm^2. The upper chamber was divided into two compartments for two stimulus males from the same experimental treatment (see below), with the males separated by an opaque plastic divider in order to prevent aggression. The grid floor of the upper chamber let males' excretions fall through to the female below, allowing olfactory and limited tactile contact but preventing sexual interaction. The lower chamber of the apparatus, containing the female, had standard chip bedding and independent access to water and rodent chow made available *ad libitum* in an outset closet. Each male in the top chamber also had access to his own water and food *ad libitum* in an outset closet. Females that were isolated from male exposure were housed in a similar manner, with the top chamber left empty.

Comparison of males on soy-based and phyto-oestrogen-free diets

In a preliminary experiment, the potential effect of the phyto-oestrogen content of a male's diet on his capacity to induce both the Vandenbergh and the Bruce effects were examined. HS stimulus males were either kept on regular rodent chow ("soy-based diet"), or fed a nutritionally similar phyto-oestrogen-free diet ("phyto-oestrogen-free diet", Advanced Protocol Verified Casein Diet 1 IF from Purina Mills, Inc., Richmond, IN, USA; LabDiet, Ren's Feed & Supply Ltd, Oakville, ON, Canada). This phyto-oestrogen-free diet contains < 10.0 ppm total isoflavones (aglycone equivalents of genistein, daidzein and glycitein). Ingredients of the two diets are previously described (Khan *et al.* 2008*a*). After at least 2 months on their respective diets, males were exposed to juvenile females and recently inseminated females in the exposure apparatus using protocols described below.

Comparison of castrated males given oil or E_2

For subsequent experiments, HS stimulus males aged 2 months were bilaterally castrated under sodium pentobarbital anesthesia and then individually housed. Injections began 1.5 months following surgery. One week prior to female exposure, all males were placed on the phyto-oestrogen-free diet described above and maintained on this diet for the duration of the experiment. Five days before female exposure, each male commenced an i.m. injection regime of either 0.06 mg E_2 in 0.05 ml cottonseed oil or 0.05 ml cottonseed oil (control) under light isoflurane anaesthesia. Injections were repeated every other day throughout exposure to juvenile females and recently inseminated females

(described below). After each injection, the injection site was swabbed with 95% ethanol and males remained away from females for 30-60 min.

In order to collect an initial urine sample before female exposure, males were placed in the top chamber of the exposure apparatus (without the lower chamber) positioned 1 cm above a metal surface covered with wax paper. After 48 h of acclimation to this chamber, across the first 4 h of the dark phase, urine samples from each male were collected from the wax paper using 1 ml syringes and 25 gauge needles. Immediately after urine collection, the males' chamber was placed above the lower chamber containing a female subject. Urine was also collected after 6 days of exposure to a juvenile female or after 4 days of exposure to an inseminated female, by temporarily removing the upper chamber of the exposure apparatus and placing it above the collection surface. The day of urine sampling in all cases was a day on which males were not injected. Urine samples were stored immediately after collection in 1.5 ml Diamed microtubes at -20°C, until hormone assays were conducted concurrently for all samples from the experiment.

Vandenbergh effect protocol

CF-1 females were weaned from their dam at 21 days of age and placed in the exposure apparatus either alone or below pairs of males in the same experimental condition. Uterine and ovarian mass was used as an indicator of sexual maturation in females. At 28 days of age, after 7 days of male exposure or isolation, females were killed by cervical dislocation under isoflurane anaesthesia, and the uterus and ovaries were removed via a single abdominal incision, stripped of fat and mesentery, and each placed

into a pre-weighed 1.5 ml microtube. Uteri and ovaries were weighed immediately after harvesting (wet mass), stored in calcium sulphate crystals at 4°C for 30 days, and then reweighed to obtain dry tissue mass.

Bruce effect protocol

Adult CF-1 males and virgin females from our breeding colony were paired. Females' hindquarters were checked three times daily for the presence of a sperm plug beginning 2 h after pairing and continuing for 5 days. The day of sperm plug detection was designated day 0 of gestation. Around the commencement of the dark phase of the lighting cycle on gestation day 1, each female was removed from the sire and placed in the lower chamber of the exposure apparatus either alone or below pairs of males in the same experimental condition. At the beginning of the dark phase on day 6 of gestation (after 120 h in the apparatus), each female was killed by cervical dislocation under isoflurane anaesthesia. Uteri were removed through a single abdominal incision and the number of implantation sites in each uterine horn was counted. An implantation site was defined as a round protuberance in either uterine horn (Berger *et al.* 2008).

Urinary steroid and creatinine quantification

Enzyme immunoassay methods were previously validated (Muir *et al.* 2001) and described in full (deCatanzaro *et al.* 2006). Creatinine, E_2 and testosterone were obtained from Sigma Chemical Co. Antibodies to E_2, testosterone and corresponding HRP conjugates were obtained from the Department of Population Health and Reproduction at the University of California, Davis.

Briefly, steroid assays were conducted for each sample in duplicate and using the average. NUNC (Roskilde, Denmark) Maxisorb places were first coated with 50 µl antibody stock diluted 1:10,000 in a coating buffer consisting of 50 mmol/l bicarbonate buffer (pH 9.6), then stored for 12-14 h at 4°C. Wash solution (0.15 mol/l NaCl solution containing 0.5 ml Tween 20/l) was added to each well to rinse away unbound antibody, then 50 µl phosphate buffer was added to each well. The plates were incubated at room temperature for 2 h for E_2 and 30 min for testosterone before adding standards and samples. Before being added to the plate, urine samples were diluted 1:8 in phosphate buffer (0.1 mol/l sodium phosphate buffer, pH 7.0 containing 8.7 g NaCl and 1 g BSA/l). Standard curves were derived by serial dilution from a known stock solution. For all assays, 50 µl E_2 or testosterone HRP was added to each well, with 20 µl standard, sample or control for E_2 or 50 µl standard, sample or control for testosterone. Plates were incubated for 2 h at room temperature, then washed and then 100 µl substrate solution of citrate buffer, H_2O_2 and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid), was added to each well. The plates were then covered and incubated on a plate shaker at room temperature for 30-60 min. The plates were then read with a single filter at 405 nm on a microplate reader (Bio-Tek Instruments, Inc., Winnooski, VT, USA, model ELx 808). In all assays, optical densities were obtained, standard curves were generated and a regression line fit, with samples interpolated into the equation to get a value in pg/well.

Creatinine measures were also taken in duplicate. Urine samples were diluted 1:40 urine:phosphate buffer. Using Dynatech Immulon (VWR International, Mississauga, ON, Canada) flat bottom plates, 50 µl/well standard was added with 50 µl distilled water, 50 µl

0.75 M NaOH and 50 µl 0.4 M picric acid. The plate was then shaken and incubated at room temperature for 30 min. Optical density was measured on the 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. Where steroid measures were adjusted for creatinine, this was achieved by dividing the obtained value by the measure of creatinine/µl of urine for the particular sample. Both creatinine-adjusted and –unadjusted steroid measures are presented.

Statistical analyses

Data are presented as mean ± S.E.M. For female uterine/ovarian tissue and implantation site data, one-way ANOVA was conducted comparing conditions. Where there was significance, pairwise multiple comparisons were conducted using the Newman-Keuls method. For implantation site data, each female was also categorized as pregnant (at least one implantation site) or not, and a χ^2 test of association compared this to experimental conditions. For the male hormone and creatinine data, each measure was analysed separately before and after female exposure via a *t*-test for independent samples. As there was a predicted direction for the urinary E_2 measures, one-tailed *t*-tests were applied; all other probabilities are two tailed. All sample sizes are given in the figures and tables. The threshold for statistical significance (α level) was set at $P < 0.05$.

Declaration of interest

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

Funding

This research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada awarded to D deCatanzaro (RGPIN/1199-2010).

Acknowledgements

We greatly appreciate the assistance of Brent Crawford, Kaitlin Gould, and Adam Guzzo.

References

Alessio L, Berlin A, Dell'Orto A, Toffoletto F & Ghezzi I 1985 Reliability of urinary creatinine as a parameter used to adjust values of urinary biological indicators. *International Archives of Occupational and Environmental Health* **55** 99-106. (doi:10.1007/BF00378371)

Ashby J & Tinwell H 1998 Uterotrophic assay of bisphenol A in the immature rat. *Environmental Health Perspectives* **106** 719-720. (doi: 10.1289/ehp.98106719)

Beaton EA & deCatanzaro D 2005 Novel males' capacity to disrupt early pregnancy in mice (*Mus musculus*) is attenuated via a chronic reduction of males' urinary 17β-estradiol. *Psychoneuroendocrinology* **30** 688-697. (doi:10.1016/j.psyneuen.2005.03.001)

Beaton EA, Khan A & deCatanzaro D 2006 Urinary sex steroids during sexual development in female mice and in proximate novel males. *Hormones and Metabolic Research* **38** 501-506. (doi:10.1055/s-2006- 949521)

Berger RG, Shaw J & deCatanzaro D 2008 Impact of acute bisphenol-A exposure upon intrauterine implantation of fertilized ova and urinary levels of progesterone and 17β-estradiol. *Reproductive Toxicology* **26** 94-99. (doi:10.1016/j.reprotox.2008.06.007)

Brennan PA 2004 The nose knows who's who: chemosensory individuality and mate recognition in mice. *Hormones and Behavior* **46** 231-240. (doi:10.1016/j.yhbeh.2004.01.010)

Bronson FH 1975 Male-induced precocial puberty in female mice: confirmation of the role of estrogen. *Endocrinology* **96** 511-514. (doi:10.1210/endo-96-2-511)

Bronson FH & Eleftheriou BE 1963 Influence of strange males on implantation in the deermouse. *General and Comparative Endocrinology* **3** 515-518. (doi:10.1016/0016-6480(63)90084-4)

Bronson FH & Desjardins C 1974 Circulating concentrations of FSH, LH, estradiol, and progesterone associated with acute, male-induced puberty in female mice. *Endocrinology* **94** 1658-1668. (doi:10.1210/endo-94-6- 1658)

Brooks PH & Cole DJA 1970 The effect of the presence of a boar on the attainment of puberty in gilts. *Journal of Reproduction and Fertility* **23** 435-440. (doi:10.1530/jrf.0.0230435)

Bruce HM 1960 A block to pregnancy in the mouse caused by proximity of strange males. *Journal of Reproduction and Fertility* **1** 96-103. (doi:10.1530/jrf.0.0010096)

Bruce HM 1965 Effect of castration on the reproductive pheromones of male mice. *Journal of Reproduction and Fertility* **10** 141-143. (doi:10.1530/jrf.0.0100141)

deCatanzaro D 2011 Blastocyst implantation is vulnerable to stress-induced rises in endogenous estrogens and also to excretions of estrogens by proximate males. *Journal of Reproductive Immunology* **90** 14-20. (doi:10.1016/j.jri.2011.04.005)

deCatanzaro D & Storey AE 1989 Partial mediation of strange-male-induced pregnancy blocks by sexual activity in mice (*Mus musculus*). *Journal of Comparative Psychology* **103** 381-388. (doi:10.1037/0735- 7036.103.4.381)

deCatanzaro D, MacNiven E & Rucciuti F 1991 Comparison of the adverse effects of adrenal and ovarian steroids on early pregnancy in mice. *Psychoneuroendocrinology* **16** 525-536. (doi:10.1016/0306- 4530(91)90036-S)

deCatanzaro D, Smith M & Muir C 1995 Strange-male-induced pregnancy disruption in mice: potentiation by administration of 17β-estradiol to castrated males. *Physiology & Behavior* **58** 405-407. (doi:10.1016/0031- 9384(95)00064-P)

deCatanzaro D, Zacharias R & Muir C 1996 Disruption of early pregnancy by direct and indirect exposure to novel males in mice: comparison of influences of preputialectomized and intact males. *Journal of Reproduction and Fertility* **106** 269-274. (doi:10.1530/jrf.0.1060269)

deCatanzaro D, Baptista MAS & Vella ES 2001 Administration of minute quantities of 17β-estradiol on the nasal area terminates early pregnancy in inseminated female mice. *Pharmacology Biochemistry and Behavior* **69** 503-509. (doi:10.1016/S0091-3057(01)00554-8)

deCatanzaro D, Muir C, Beaton E, Jetha M & Nadella K 2003 Enzymeimmunoassay of oestradiol, testosterone and progesterone in urine samples from female mice before and after insemination. *Reproduction* **126** 407-414. (doi:10.1530/rep.0.1260407)

deCatanzaro D, Muir C, Beaton EA & Jetha M 2004 Non-invasive repeated measurement of urinary progesterone, 17β-estradiol, and testosterone in developing, cycling, pregnant and postpartum female mice. *Steroids* **69** 687-696. (doi:10.1016/j.steroids.2004.07.002)

deCatanzaro D, Beaton EA, Khan A & Vella E 2006 Urinary oestradiol and testosterone levels from novel male mice approach values sufficient to disrupt early pregnancy in nearby inseminated females. *Reproduction* **132** 309-317. (doi:10.1530/rep.1.00965)

deCatanzaro D, Khan A, Berger RG & Lewis E 2009 Exposure to developing females induces polyuria, polydipsia, and altered urinary levels of creatinine, 17β-estradiol, and testosterone in adult male mice (*Mus musculus*). *Hormones and Behavior* **55** 240-247. (doi:10.1016/j.yhbeh. 2008.10.013)

Clark JH & Gorski JB 1970 Ontogeny of the estrogen receptor during early uterine development. *Science* **169** 76-78. (doi:10.1126/science.169. 3940.76)

Clulow FV & Langford PE 1971 Pregnancy-block in the meadow vole, *Microtus pennsylvanicus*. *Journal of Reproduction and Fertility* **24** 275-277. (doi:10.1530/jrf.0.0240275)

Colby DR & Vandenbergh JG 1974 Regulatory effects of urinary pheromones on puberty in the mouse. *Biology of Reproduction* **11** 268-279. (doi:10.1095/biolreprod11.3.268)

Cooke PS, Buchanan DL, Young P, Setiawan T, Brody J, Korach KS, Taylor J, Lubahn DB & Cunha GR 1997 Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. *PNAS* **94** 6535-6540. (doi:10.1073/pnas.94.12.6535)

Drickamer LC 1983 Male acceleration of puberty in female mice (*Mus musculus*). *Journal of Comparative Psychology* **97** 191-200. (doi:10. 1037/0735-

7036.97.3.191)

Drickamer LC 1995 Rates of urine excretion by house mouse (*Mus domesticus*): differences by age, sex, social status, and reproductive condition. *Journal of Chemical Ecology* **21** 1481-1493. (doi:10.1007/ BF02035147)

Erb RE, Tillson, SA, Hodgen GD & Plotka ED 1970 Urinary creatinine as an index compound for estimating rate of excretion of steroids in the domestic sow. *Journal of Animal Science* **30** 79-85.

Ghahary A & Murphy LJ 1989 Uterine insulin-like growth factor-1 receptors: regulation by estrogen and variation throughout the estrous cycle. *Endocrinology* **125** 597-604. (doi:10.1210/endo-125-2-597)

Greenwald GS 1967 Species differences in egg transport in response to exogenous estrogen. *Anatomical Record* **157** 163-172. (doi:10.1002/ ar.1091570207)

Guzzo AC, Berger RG & deCatanzaro D 2010 Excretion and binding of tritium-labelled oestradiol in mice (*Mus musculus*): implications for the Bruce effect. *Reproduction* **139** 255-263. (doi:10.1530/REP-09-0382)

Hakim R, Gray RH & Zakur HA 1994 Is there a need for creatinine adjustment of urinary steroid hormone levels in studies of early fetal loss? *Clin. Chem. Acta* **230** 209-214. (doi:10.1016/0009-8981(94)90273-9)

Harder JD & Jackson LM 2003 Male pheromone stimulates ovarian follicular development and body growth in juvenile female opossums (*Monodelphis domestica*). *Reproductive Biology and Endocrinology* **1** 21. (doi:10.1186/1477-7827-1-21)

Hasler JF & Banks EM 1975 The influence of mature males on sexual maturation in female collared lemmings (*Dicrostonyx groenlandicus*). *Journal of Reproduction and Fertility* **42** 583-586. (doi:10.1530/jrf.0. 0420583)

Izard MK & Vandenbergh JG 1982 The effects of bull urine on puberty and calving date in crossbred beef heifers. *Journal of Animal Science* **55** 1160-1168.

Kahlert S, Nuedling S, van Eickels M, Vetter H, Meyer R & Grohe C 2000 Estrogen receptor α rapidly activates the IGF-1 receptor pathway. *Journal of Biological Chemistry* **275** 18447-18453. (doi:10.1074/jbc. M910345199)

Kelliher KR, Spehr M, Li X-H, Zufall F & Leinders-Zufall T 2006 Pheromonal recognition memory induced by TRPC2-independent vomeronasal sensing. *European Journal of Neuroscience* **23** 3385-3390. (doi:10.1111/ j.1460-9568.2006.04866.x)

Keverne EB & de la Riva C 1982 Pheromones in mice: Reciprocal interaction between the nose and brain. *Nature* **296** 148-150. (doi:10.1038/ 296148a0)

Khan A, Berger RG & deCatanzaro D 2008*a* 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. (doi:10.1530/REP-07- 0314)

Khan A, Bellefontaine N & deCatanzaro D 2008*b* Onset of sexual maturation in female mice as measured in behaviour and fertility: interactions of exposure to males, phytoestrogen content of diet, and ano-genital distance. *Physiology & Behavior* **93** 588-594. (doi:10.1016/ j.physbeh.2007.10.019)

Khan A, Berger RG & deCatanzaro D 2009 Preputialectomised and intact adult mice exhibit an elevated urinary ratio of oestradiol to creatinine in the presence of developing females, whilst promoting uterine and ovarian growth of these females. *Reproduction, Fertility and Development* **21** 860-868. (doi:10.1071/RD08299)

Leinders-Zufall T, Brennan P, Widmayer P, Chandramani SP, Maul-Pavicic A, Jäger M, Li X-H, Breer H & Zufall F 2004 MHC class I peptides as chemosensory signals in the vomeronasal organ. *Science* **306** 1022-1037. (doi:10.1126/science.1102818)

Lepri JJ & Vandenbergh JG 1986. Puberty in pine voles, *Microtus pinetorum*, and the influence of chemosignals on female reproduction. *Biology of Reproduction* **34** 370-377. (doi:10.1095/biolreprod34.2.370)

Lombardi JR, Vandenbergh JG & Whitsett JM 1976 Androgen control of the sexual maturation pheromone in house mouse urine. *Biology of Reproduction* **15** 179-186. (doi:10.1095/biolreprod15.2.179)

Ma W, Miao Z & Novotny MV 1999 Induction of estrus in grouped female mice (*Mus domesticus*) by synthetic analogues of preputial gland constituents. *Chemical Senses* **24**, 289-293. (doi:10.1093/chemse/ 24.3.289)

Ma W, Song H, Das SK, Paria BC & Dey SK 2003 Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. *PNAS* **100** 2963-2968. (doi:10.1073/pnas. 0530162100)

Medlock KL, Sheehan DM & Branham WS 1981 The postnatal ontogeny of the rat uterine estrogen receptor. *Journal of Steroid Biochemistry* **15** 285-288. (doi:10.1016/0022-4731(81)90285-5)

Meredith M 1994 Chronic recording of vomeronasal pump activation in awake behaving hamsters. *Physiology & Behavior* **56** 345-354. (doi:10. 1016/0031-9384(94)90205-4)

Miro F, Coley J, Gani MM, Perry PW, Talbot D & Aspinall LJ 2004 Comparison between creatinine and pregnanediol adjustments in the retrospective analysis of urinary hormone profiles during the human menstrual cycle. *Clinical Chemistry and Laboratory Medicine* **42** 1043-1050. (doi:10.1515/CCLM.2004.210)

Muir C, Vella ES, Pisani N & deCatanzaro D 2001 Enzyme immunoassay of 17β-estradiol, estrone conjugates, and testosterone in urinary and fecal samples from male and female mice. *Hormones and Metabolic Research* **33** 653-658. doi:10.1055/s-2001-18692)

Mukku VR, Kirkland JL, Hardy M & Stancel GM 1982 Hormonal control of uterine growth: temporal relationships between estrogen administration and deoxyribonucleic acid synthesis. *Endocrinology* **111** 480-487. (doi:10.1210/endo-111-2-480)

Murphy LJ, Murphy LC & Friesen HG 1988 Estrogen induces insulin-like growth factor-1 expression in the rat uterus. *Molecular Endocrinology* **1** 445-450. (doi:10.1210/mend-1-7-445)

Novotny MV, Ma W, Wiesler D & Zidek L 1999 Positive identification of the puberty-accelerating pheromone of the house mouse: The volatile ligands associating with the major urinary protein. *Proceedings of the Royal Society of London B* **266**, 2017-2022. (doi:10.1098/rspb.1999.0880)

Ogasawara Y, Okamoto S, Kitamura Y & Matsumoto K 1983 Proliferative pattern of uterine cells from birth to adulthood in intact, neonatally castrated, and/or adrenalectomized mice, assayed by incorporation of [^{125}I] Iododeoxyuridine. *Endocrinology* **113** 582-587. (doi:10.1210/ endo-113-2-582)

Ortiz ME, Villalon M & Croxatto HB 1979 Ovum transport and fertility following postovulatory treatment with estradiol in rats. *Biology of Reproduction* **21** 1163-1167. (doi:10.1095/biolreprod21.5.1163)

Quarmby VE & Korach KS 1984 The influence of 17β-estradiol on patterns of cell division in the uterus. *Endocrinology* **114** 694-702. (doi:10.1210/endo-114-3-694)

Reasner DS & Johnston RE 1988 Acceleration of reproductive development in female Djungarian hamsters by adult males. *Physiology & Behavior* **43** 57-64. (doi:10.1016/0031-9384(88)90098-4)

Rohrbach C 1982 Investigation of the Bruce effect in the Mongolian gerbil (*Meriones unguiculatus*). *Journal of Reproduction and Fertility* **65** 411-417. (doi:10.1530/jrf.0.0650411)

Sato T, Wang G, Hardy MP, Kurita T, Cunha GR & Cooke PS 2002 Role of systemic and local IGF-1 in the effects of estrogen on growth and epithelial proliferation of mouse uterus. *Endocrinology* **143** 2673-2679. (doi:10.1210/en.143.7.2673)

Shaw J & deCatanzaro D 2009 Estrogenicity of parabens revisited: impact of parabens on early pregnancy and an uterotrophic assay in mice. *Reproductive Toxicology* **28** 26-31. (doi:10.1016/j.reprotox.2009. 03.003)

Simpson ER, Clyne C, Rubin G, Boon WC, Robertson K, Britt K, Speed C & Jones M 2002 Aromatase - A brief overview. *Annual Review of Physiology* **64** 93-127. (doi:10.1146/annurev.physiol.64.081601.142703)

Teague LG & Bradley EL 1978 The existence of a puberty accelerating pheromone in the urine of the male prairie deermouse (*Peromyscus maniculatus bairdii*). *Biology of Reproduction* **19** 314-317. (doi:10. 1095/biolreprod19.2.314)

Thigpen JE, Haseman JK, Saunders HE, Setchell KDR, Grant MG & Forsythe DB 2003 Dietary phytoestrogens accelerate the time of vaginal opening in immature CD-1 mice. *Comparative Medicine* **53** 607-615.

Valbuena D, Martin J, de Pablo JL, Remoni J, Pellicer A & Simon C 2001 Increasing levels of estradiol are deleterious to embryonic implantation because they directly affect the embryo. *Fertility and Sterility* **76** 962-968. (doi:10.1016/S0015-0282(01)02018-0)

Vandenbergh JG 1967 Effect of the presence of a male on the sexual maturation of female mice. *Endocrinology* **81:** 354-349. doi:10.1210/endo-81-2-345)

Vella ES & deCatanzaro D 2001 Novel male mice show gradual decline in the capacity to disrupt early pregnancy and in urinary excretion of testosterone and 17β-estradiol during the weeks immediately following castration. *Hormones and Metabolic Research* **33** 681-686. (doi:10.1055/s-2001-18690)

Waddell BJ & O'Leary PC 2002 Distribution and metabolism of topically applied progesterone in a rat model. *Journal of Steroid Biochemistry and Molecular Biology* **80** 449-455. (doi:10.1016/S0960-0760(02)00032-8)

Whitten PL & Naftolin F 1992 Effects of a phytoestrogen diet on estrogen-dependent reproductive processes in immature female rats. *Steroids* **57** 56-61. (doi:10.1016/0039-128X(92)90029-9)

Yang P, Kriatchko A & Roy SK 2002 Expression of ER-α and ER-β in the hamster ovary: differential regulation by gonadotropins and ovarian steroid hormones. *Endocrinology* **143** 2385-2398. (doi:10.1210/en.143.6.2385)

Zhu L & Pollard JW 2007 Estradiol-17β regulates mouse uterine epithelial cell proliferation through insulin-like growth factor 1 signaling. *PNAS* **104** 15847-15851. (doi:10.1073/pnas.0705749104)

Received 11 July 2011

First decision 15 August 2011

Revised manuscript received 28 September 2011

Accepted 19 October 2011

Chapter 3

Circadian rhythm and response to an acute stressor of urinary corticosterone, testosterone, and creatinine in adult male mice

Joelle B. Thorpe, Nazanin Rajabi and Denys deCatanzaro

Hormone and Metabolic Research, 44(6), 429-435

Authors' Contributions

Joelle B. Thorpe: Experimental design, data collection, data analysis, manuscript writing, editing, and preparation.

Nazanin Rajabi: Data collection, and assistance with experimental design and manuscript editing.

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

Abstract

In small laboratory species, steroid measures can be obtained more frequently and less invasively from urine than blood. Insofar as urinary levels reflect systemic levels, they could provide advantages particularly for measurement of glucocorticoids, whose blood levels react rapidly to handling and stress. In Experiment 1, urinary samples were collected from male mice every second hour over a 14:10 h light:dark cycle. Samples were analyzed via enzyme immunoassay for corticosterone, testosterone, and creatinine. Corticosterone had peak concentrations 1 h after light offset and a trough 1 h after light onset. Testosterone showed peak concentrations 5-7 h after light onset and lowest concentrations during the dark phase of the cycle. Creatinine showed some variation over the light-dark cycle, but steroid measures showed similar trends with and without adjustment for creatinine. In Experiment 2, mice were stressed via an injection at times close to the determined peak and trough levels of corticosterone. In urinary samples taken 90 min after injection, corticosterone was significantly higher in injected animals at both times relative to levels in control animals, but testosterone was unaffected by injection stress. In Experiment 3, serum and urine samples were collected from mice every sixth hour across the diurnal cycle. Corticosterone peaked in urine and serum immediately after light offset, and urinary measures predicted those in serum. These data indicate that urinary corticosterone reflects systemic levels in mice, document circadian variation in urinary testosterone, and indicate that circadian variation in creatinine is minimal, but potentially relevant in stressed animals.

Key words: corticosterone, testosterone, creatinine, urine, circadian rhythm, stress

Introduction

Circulating glucocorticoids (cortisol and corticosterone) increase within minutes after the onset of a stressor [1-4]. Reactivity of glucocorticoids to acute and chronic events is overlaid upon an endogenous daily rhythm, entrained to the light cycle via retinal and pineal influences on the suprachiasmatic nucleus of the hypothalamus, with species variations related to nocturnal vs. diurnal activity [5-12]. Typically, glucocorticoids are measured in blood, but repeated measures over time and across circumstances can be precluded in smaller species, as blood sampling requires physical restraint and venipuncture, both of which can elevate glucocorticoids [13, 14]. Other simple laboratory practices including group housing, novel caging, and brief human handling can increase circulating corticosterone [2, 15-19]. As excretions can be collected with relatively little disturbance of the animal, excretory steroid measures could be advantageous insofar as they reflect systemic variations. Urinary measures of estradiol, progesterone, and testosterone in mice are more dynamic over time than are fecal measures, and generally reflect reported trends and patterns of these steroids in circulation [20-22]. In rats, the daily rhythm of urinary glucocorticoids has been found to mirror rhythms in circulation albeit with a time delay [23, 24], but such urinary rhythm has not previously been established for mice.

Here we validated an assay for urinary corticosterone and documented its daily rhythm. Urinary testosterone was quantified as well, given that this steroid shows a daily rhythm in a number of species albeit with some disagreement on the timing of peak and trough concentrations (e.g., [5, 11, 12, 25-31]). We also measured creatinine, which is

often used to adjust urinary steroid measures to control for differential hydration and activity [20, 22, 32]. Also, urinary creatinine itself can be dynamic across various experimental conditions in this species [33, 34], and could potentially show circadian variation related to activity and drinking. We were also interested in whether urinary steroid and creatinine measures would respond to an acute stressor. Therefore, in a second experiment we measured the reactivity of urinary corticosterone, testosterone, and creatinine to an acute stressor around the times of corticosterone peak and trough. The stressor was given 90 min prior to urine collection, based on previous evidence that acute stress increases urinary corticosterone within 1-2 h in female mice, with a return to baseline within 3 h [35]. Finally, in a third experiment we directly compared urinary and serum levels of corticosterone at 6-h intervals that included the times of peak and trough concentrations over the daily cycle.

Animals and Methods

Animals

All subjects were male CF1 strain mice bred from laboratory stock obtained originally from Charles River (Quebec, Canada). Mice were housed in standard polypropylene cages measuring 28cm x 16cm x 11cm (height) with continuous access to water and 8640 Teklad Certified Rodent Chow (Harlan Teklad, Madison, WI, USA). The colony was maintained at 21°C on a reversed 14:10 h light:dark cycle with the onset of the dark phase at 09:00 h. Measurement of light intensity during the light phase of the cycle averaged 64 ± 0.20 lux in this room. This research was approved by the McMaster

University Animal Research Ethics Board, conforming to the standards of the Canadian Council on Animal Care.

Experiment 1: Daily rhythm of urinary corticosterone, testosterone, and creatinine

Animals were housed individually for at least 1 week prior to the start of the experiment and had a mean age of 148 days (range of 133-159 days). They were randomly assigned to each time point (every 2 h from 00:00 h to 22:00 h) during which urine collection occurred. All experimental mice were housed in one room in the absence of other mice (Room A). Every 2 h, a subset of animals to be sampled was moved to an adjacent room (Room B), where urine collection occurred, in order to minimize disruption of animals to be sampled subsequently. A maximum of 15 min elapsed from the time Room A was entered to the time urine was collected from all subjects in Room B.

To obtain a urine sample, each animal was placed in a bucket measuring 24.8cm x 25.4cm x 16.5cm (height), the bottom of which was covered with clean wax paper. After a maximum of 3 min, each mouse was removed and urine was aspirated from the wax paper using a tuberculine syringe with a 25 ga needle. After sampling, all animals in Room B were taken to a third room. This process was repeated every 2 h with a new group of subjects from Room A for a 24-h period. During the dark cycle, lights in Room B were kept at 2% of maximum while urine sampling occurred. Urine was stored in 1.5 ml microtubes at -20°C until hormone analysis was conducted simultaneously for all samples.

Experiment 2: Urinary corticosterone, testosterone, and creatinine response to acute stress

Corticosterone, testosterone, and creatinine were measured in urine following an acute stressor applied 90 min before the established daily peak (10:00 h) and trough (20:00 h) of urinary corticosterone. Male mice (165 days of age) were prepared as in Experiment 1 and randomly assigned to testing at 10:00 h and 20:00 h (respectively 1 h after commencement of the dark and light phases of the cycle). Subjects were randomly assigned to the 2 times and to either a nonstressed control group or a stressed (i.p. injected) experimental group. All subjects were housed individually in Room A. Subjects in the experimental group were moved to Room B at either 08:30 h or 18:30 h. Each was captured by the base of the tail, held at the nape of the neck, and received an i.p. injection of 0.5 ml sterile saline. Each was then returned to its cage and remained undisturbed in Room B until urine sampling. Control males were then moved from Room A to Room B. After 90 min (either 10:00 h or 20:00 h), all males in Room B were placed in previously described [20] urine collection cages with a stainless steel wire grid floor, allowing males' excretions to fall through to a clean metal surface covered with wax paper. Urine was then collected from the wax paper and stored as described above.

Experiment 3: Comparison of urinary and serum corticosterone

Subjects were housed individually for at least 1 week prior to urine and blood collection. All males had a mean age of 143 days (range of 133-169 days). Males were randomly assigned to one of 4 sampling times (03:00 h, 09:00 h, 15:00 h, and 21:00 h), including peak and trough times evident from Experiment 1. At each sampling time, each

mouse was removed individually from the holding room, a blood sample was obtained using cardiac puncture under isoflurane anaesthesia, and a subsequent urine sample was obtained after cervical dislocation. Once completed, the next mouse was removed from the holding room for sampling. For each mouse, blood and urine samples were obtained within a maximum of 3 min of entering the holding room. Cohorts of up to 5 mice per time were tested on a single day, such that the last mouse was sampled no more than 15 min after the nominal sampling time. Urine was stored in 1.5 ml microtubes at -20°C until hormone assay. Blood samples were allowed to clot at room temperature for approximately 1 h, and were then centrifuged for 10 min at 4000 rpm. The serum supernatant was removed and stored in 1.5 ml microtubes at -20°C until hormone assay. All blood and urine samples were analyzed for corticosterone simultaneously, and urine samples were also analyzed for creatinine. Blood samples were thawed and extracted by adding 100 µl serum to 1 ml anhydrous ethanol, then vortexing for 15 s, centrifuging for 15 min at 3000 rpm, and removing 500 µl of the supernatant to a fresh 1.5 ml microtube. Samples remained under fumehood until the morning of the hormone assay, when the ethanol had evaporated.

Steroid and creatinine assays

Enzyme immunoassay methods for testosterone in mouse urine were previously validated [22]. Validations for corticosterone in mouse urine are reported below. Creatinine, corticosterone, and testosterone were obtained from Sigma Chemical Co. Antibodies to corticosterone, testosterone, and corresponding horseradish peroxidase (HRP) conjugates were obtained from the Department of Population Health and

Reproduction at the University of California, Davis. Cross reactivities for anti-corticosterone are: corticosterone 100 %, cortisol 0.23 %, deoxycorticosterone 14.25 %, tetrahydrocorticosterone 0.9 %, 11-deoxycortisol 0.03 %, progesterone 2.65 %, testosterone 0.64 %, prednisolone 0.07 %, prednisone, cortisone, 17α-estradiol; all <0.01%. Cross reactivities for anti-testosterone are: testosterone 100%, 5α-dihydrotestosterone 57.4 %, androstenedione 0.27 %, and each of androsterone, DHEA, cholesterol, 17α-estradiol, progesterone, and pregnenolone < 0.05 %.

Steroid assays were conducted with duplicate readings per sample; the average was used in statistical analysis. NUNC Maxisorb plates were first coated with 50 µl antibody stock diluted 1:10000 in a coating buffer (50mmol/l bicarbonate buffer, pH 9.6), then stored for 12–14 h at 4°C. Wash solution (0.15 mol/l NaCl solution containing 0.5 ml Tween 20 per liter) was added to each well to rinse away any unbound antibody, then 50 µl phosphate buffer/well was added. The plates were incubated at room temperature for 1 h for corticosterone and 30 min for testosterone before adding standards, samples, and controls. Urine samples were diluted 1:30 (corticosterone) or 1:8 (testosterone) in phosphate buffer (0.1mol/l sodium phosphate buffer, pH 7.0 containing 8.7 g NaCl and 1 g BSA/l) before being added to the plate. Extracted serum samples were reconstituted in 300 µl of phosphate buffer and vortexed before being added to the plate. Standard curves were derived by serial dilution from a known stock solution. For all assays, 50 µl corticosterone (1:140 000 in phosphate buffer) or testosterone (1:300 000 in phosphate buffer) HRP was added to each well, with 50 µl standard, sample, or control. The plates were incubated for 2 h at room temperature, then washed, and then 100 µl substrate

solution of citrate buffer, H2O2, and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), was added to each well. The plates were then covered and incubated, while shaking at room temperature for 45 min to 1.5 h. The plates were then read with a single filter at 405 nm on a microplate reader (Bio-Tek Instruments Inc, ELx 808). In all assays, optical densities were obtained, standard curves were generated, a regression line fit, and samples interpolated into the equation to get a value in pg per well.

Validations for the corticosterone assay included a test for parallelism to determine whether samples perform immunologically in a manner similar to steroid standards. Serial dilutions of samples were compared with a standard curve plotted against logarithmically transformed doses. This test indicates whether the steroid is present in samples in measurable quantities, and whether samples react with the antibodies in a predictable fashion.

Following convention for urinary steroid measures [20, 22, 32], urinary creatinine was also measured. All urine samples were diluted to 1:40 urine:phosphate buffer. Using Dynatech Immulon flat bottom plates, 50 µl/well of standard was added with 50 µl distilled water, 50 µl 0.75 M NaOH, and 50 µl 0.4 M picric acid. The plate was then shaken and incubated at room temperature for 30min. The plate was measured for optical density on the 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. The average of duplicates was taken for each sample. Where steroid measures were adjusted for creatinine, this was achieved by dividing the obtained value by the concentration of creatinine considered per ml of urine for the particular sample.

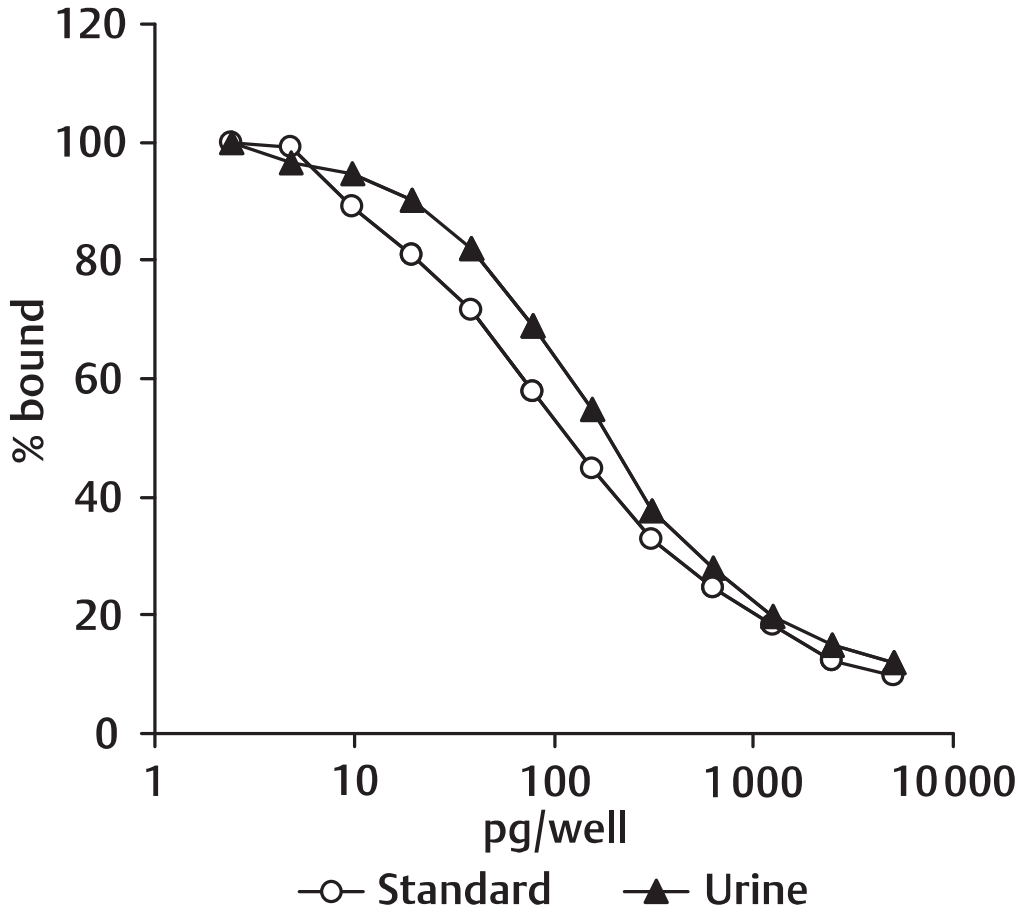
Both creatinine-adjusted and -unadjusted steroid measures are presented.

Results

Validation of the urinary corticosterone assay

Fig. 1 presents the results from a test of parallelism for serially diluted mouse urine samples pooled from 5 adult male mice and progressive dilutions of corticosterone. It is clear from these data that the plot of serially-diluted urine was parallel to that of serially-diluted corticosterone, indicating that the antibody is indeed measuring corticosterone in mouse urine.

Fig. 1 Serially diluted pooled mouse urine samples binding to antibody in parallel with serially diluted corticosterone samples.

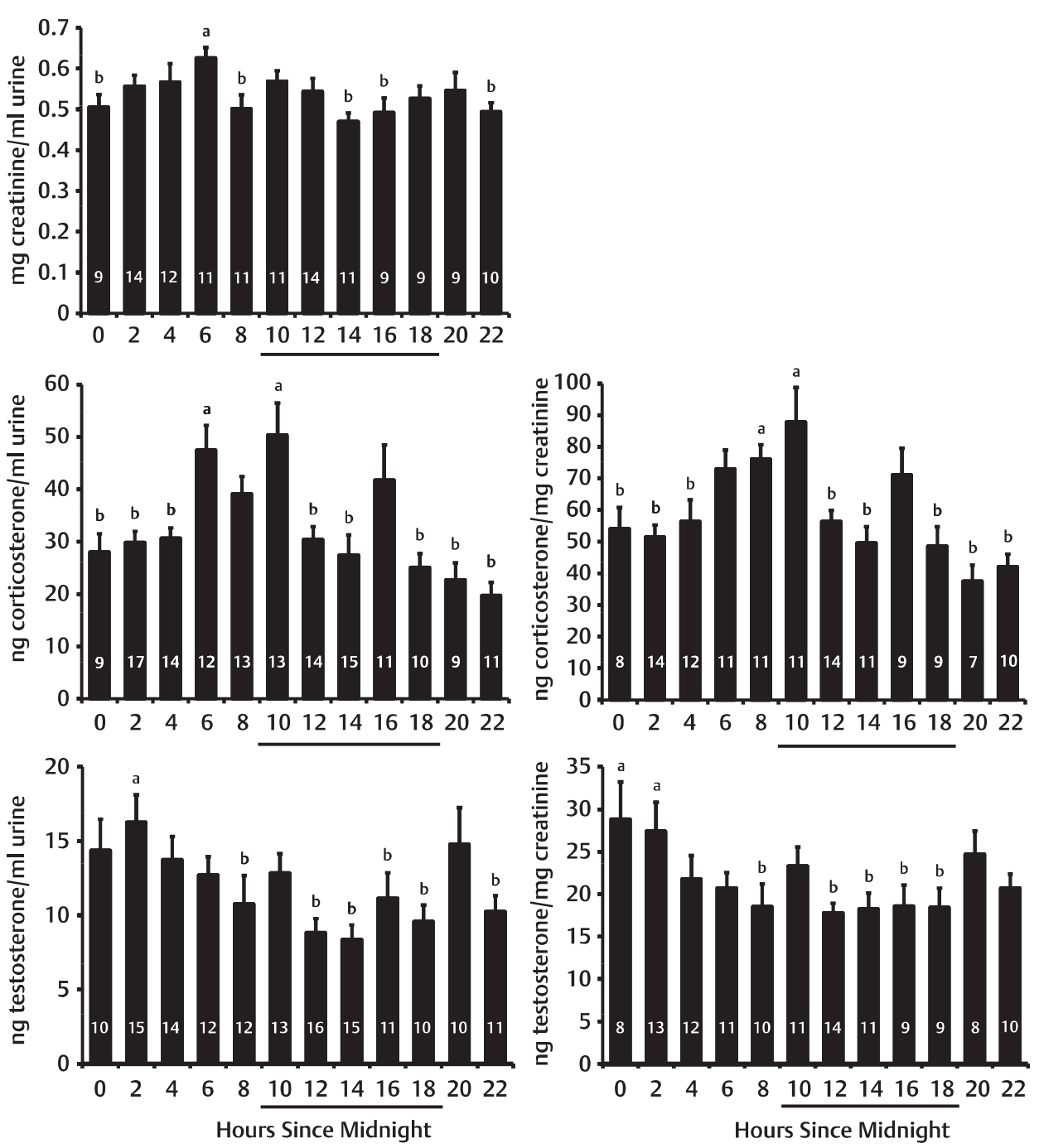


Experiment 1: Daily rhythm of urinary corticosterone, testosterone, and creatinine

Fig. 2 shows measures of creatinine, corticosterone, creatinine-adjusted corticosterone, testosterone, and creatinine-adjusted testosterone in male urine every 2 h across a 24-h period, with lights off from 09:00 h to 19:00 h. Both creatinine-adjusted and –unadjusted corticosterone showed a clear daily rhythm with peak concentrations just prior to and after commencement of the dark phase of the light cycle and trough concentrations in the immediate hours after commencement of the light phase of the cycle. Creatinine-adjusted and –unadjusted testosterone showed highest levels at 5-7 h after commencement of the light phase of the cycle and lowest levels during the dark phase of the cycle.

Each variable was analyzed via one-way analysis of variance comparing the 12 conditions of animals, followed by post hoc multiple comparisons (Duncan's test, $p < 0.05$). Analysis comparing time of day for creatinine approached but did not quite attain the conventional level ($p < 0.05$) of significance, $F(1,118) = 1.80$, $p = 0.060$. There was a clear effect of time of day on raw corticosterone, $F(11,136) = 5.98$, $p < 0.001$, and creatinine-adjusted corticosterone, $F(11,115) = 5.63$, $p < 0.001$. There was a significant effect of time of day for both unadjusted testosterone, $F(11,137) = 2.86$, $p = 0.002$, and creatinine-adjusted testosterone, $F(11,114) = 2.15$, $p = 0.022$. Results of multiple comparisons and sample sizes are indicated in Fig. 2

Fig. 2 The mean (± SE) measures of urinary creatinine, corticosterone, creatinine-adjusted corticosterone, testosterone, and creatinine-adjusted testosterone for adult male mice that were each sampled at one of 12 points in the diurnal cycle spaced at 2-h intervals. The times are identified related to midnight, with a reversed lighting cycle with lights off at 09:00 h and on at 19:00 h (the bar below the graph indicates the dark phase). Sample size is given at the base of each bar. The letters at the top of some bars indicate multiple comparisons, identifying conditions at the peak **a** that do not differ from one another, but that collectively differ significantly from those at the trough **b** which in turn do not differ from one another. Additional significant multiple comparisons occurred for corticosterone (16 vs. 0, 2, 14, 18, 20, 22; and 8 vs. 14, 18, 20, 22 h after midnight), creatinine-adjusted corticosterone (6, 16 vs. 2, 14, 18, 20, 22 h after midnight), and testosterone (12, 14 vs. 0, 4, 20 h after midnight).


mg creatinine/ml urine
ng corticosterone/ml urine
ng corticosterone/mg creatinine
ng testosterone/ml urine
ng testosterone/mg creatinine
Hours Since Midnight
Hours Since Midnight

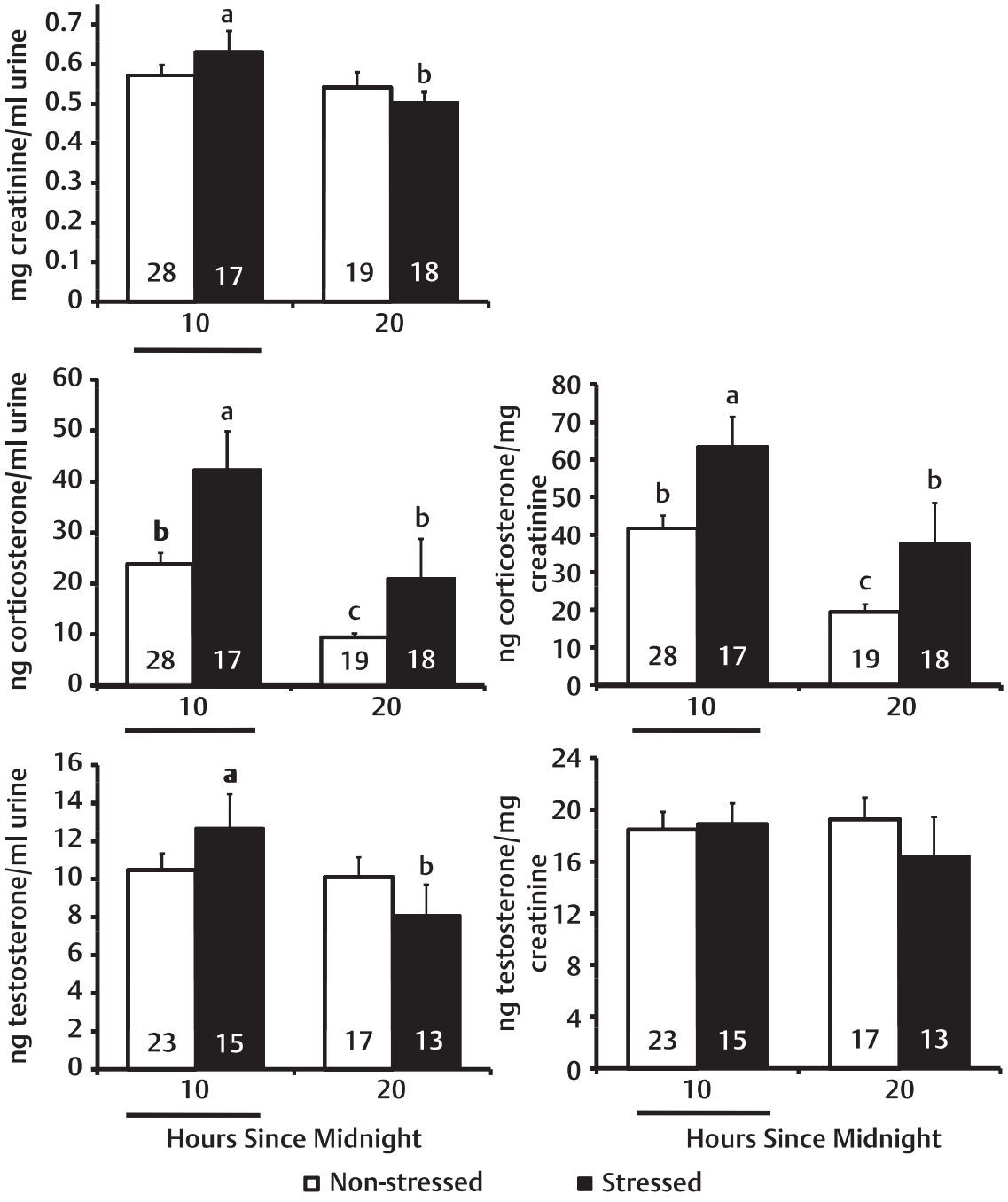
Experiment 2: Urinary corticosterone, testosterone, and creatinine response to acute stress

Fig. 3 shows measures of creatinine, corticosterone, creatinine-adjusted corticosterone, testosterone, and creatinine-adjusted testosterone in urine of males subjected to an acute stressor 90 min prior to sampling and nonstressed control males, sampled during the peak and trough of the corticosterone rhythm established in Experiment 1. The general influence of time of day upon levels of corticosterone was replicated, both considering only stressed animals and only controls. There was a clear effect of stress on adjusted and unadjusted corticosterone, with an elevation at both time points. Adjusted and unadjusted testosterone did not show influences of stress.

Two-factor analysis of variance (10 vs. 20 h after midnight, control vs. stressed animals) was conducted for each measure. For creatinine, there was a significant main effect of sampling time, $F(1,78) = 4.96$, $p = 0.027$, but neither the main effect of stress, $F(1,78) = 0.084$, $p = 0.765$, nor the interaction, $F(1,78) = 1.91$, $p = 0.168$, reached significance. For unadjusted corticosterone, there was an effect of both sampling time, $F(1,78) = 12.96$, $p = 0.001$, and stress, $F(1,78) = 9.15$, $p < 0.004$, but no interaction, $F(1,78) = 0.484$, $p = 0.596$. For creatinine-adjusted corticosterone, there was similarly an effect of sampling time, $F(1,78) = 14.04$, $p < 0.001$, and stress, $F(1,78) = 9.61$, $p = 0.003$, but no interaction, $F(1,78) = 0.083$, $p = 0.766$. For unadjusted testosterone, the effect of sampling time narrowly escaped the conventional level of significance, $F(1,64) = 3.63$, $p = 0.058$, and there was no effect of stress, $F(1,64) = 0.005$, $p = 0.899$, and no interaction, $F(1,64) = 2.67$, $p = 0.103$. For creatinine-adjusted testosterone, there was also no effect of

sampling time, $F(1,64) = 0.207$, $p = 0.655$, or stress, $F(1,64) = 0.409$, $p = 0.532$, and there was no interaction, $F(1,64) = 0.770$, $p = 0.387$. Results of multiple comparisons and sample sizes are indicated in Fig. 3.

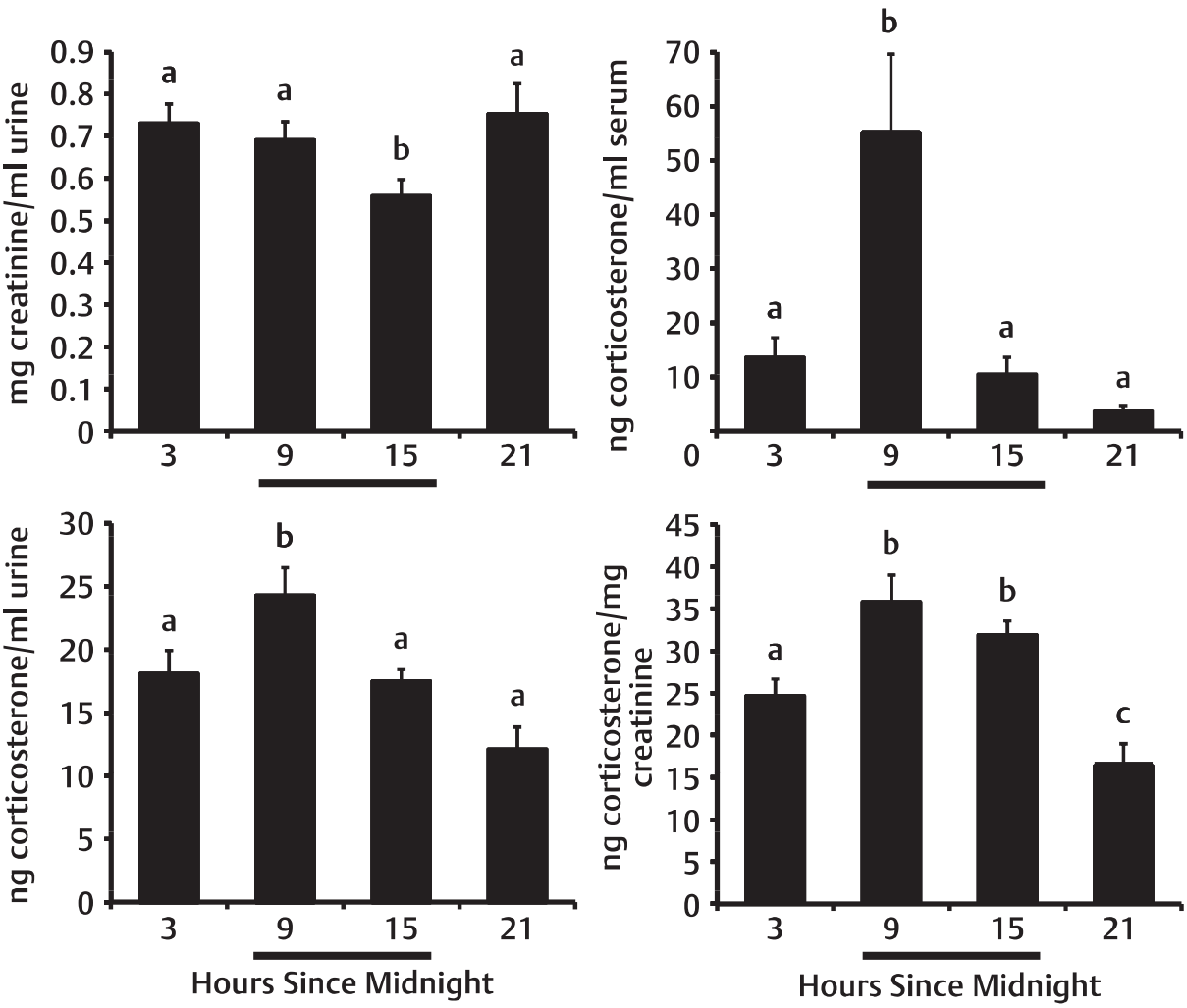
Fig. 3 The mean (± SE) measures of urinary creatinine, corticosterone, creatinine-adjusted corticosterone, testosterone, and creatinine-adjusted testosterone for adult male mice that were each sampled at 1 or 2 points in the diurnal cycle. These 2 points were 10 and 20 h after midnight, with a reversed lighting cycle with lights off at 09:00 h and on at 19:00 h, thus corresponding to 1 h after offset and 1 h after onset of lights. Sample size is given at the base of each bar. Multiple comparisons were conducted comparing all pairs of conditions within each measure. Significance is indicated by letters at the top of some bars; those that share the same letter do not differ from one another but collectively differ from those with a distinct letter. Those bars without a letter do not differ from any other condition.


mg creatinine/ml urine
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
28
17
19
18
10
20
ng corticosterone/ml urine
60
50
40
30
20
10
0
a
b
b
c
28
17
19
18
10
20
ng corticosterone/mg creatinine
80
70
60
50
40
30
20
10
0
a
b
b
c
28
17
19
18
10
20
ng testosterone/ml urine
16
14
12
10
8
6
4
2
0
a
b
23
15
17
13
10
20
Hours Since Midnight
ng testosterone/mg creatinine
24
20
16
12
8
4
0
23
15
17
13
10
20
Hours Since Midnight
Non-stressed
Stressed

Experiment 3: Comparison of urinary and serum corticosterone

Fig. 4 shows measures of urinary creatinine, serum corticosterone, unadjusted urinary corticosterone, and creatinine-adjusted urinary corticosterone in male mice sampled at 03:00 h, 09:00 h, 15:00 h, and 21:00 h. Measures of corticosterone in serum and urine (both unadjusted and creatinine-adjusted) all showed a clear daily rhythm, similar to that seen in Experiments 1 and 2, with peak concentrations at 09:00 h immediately after light offset. Each measure was analyzed using one-way analysis of variance comparing the 4 conditions of animals, followed by multiple comparisons. There was an effect of time of day on urinary creatinine, $F(3,30) = 3.34$, $p = 0.032$, serum corticosterone, $F(3,30) = 6.89$, $p = 0.01$, unadjusted urinary corticosterone, $F(3,30) = 6.41$, $p = 0.002$, and creatinine-adjusted urinary corticosterone, $F(3,30) = 9.41$, $p < 0.001$. Multiple comparisons and sample sizes are indicated in Fig. 4. Considering all subjects regardless of time of day, there were significant positive correlations between serum corticosterone and unadjusted urinary corticosterone, $r = 0.64$, $t(32) = 4.67$, $p < 0.001$, and between serum corticosterone and creatinine-adjusted urinary corticosterone, $r = 0.60$, $t(32) = 4.27$, $p < 0.001$. In addition, a multiple regression with time of day and unadjusted urinary corticosterone as predictor variables and serum corticosterone as the criterion was significant, $R^2 = 0.64$, $F(7,26) = 6.62$, $p < 0.001$.

Fig. 4 The mean (± SE) measures of urinary creatinine, serum corticosterone, unadjusted urinary corticosterone, and creatinine-adjusted urinary corticosterone for adult male mice that were each sampled at 1 of 4 times in the diurnal cycle spaced at 6-h intervals. The times are identified relative to midnight, with a reversed light cycle with lights off at 09:00 h and on at 19:00 h (the bar below the graph indicates the dark phase). Sample sizes are 10 at 03:00 h, 10 at 09:00 h, 9 at 15:00 h, and 5 at 21:00 h for all measures. Multiple comparisons within each measure are indicated by letters above the bar; those bars that share the same letter do not differ from one another, but collectively differ from those with a distinct letter.


mg creatinine/ml urine
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a
b
a
3
9
15
21
ng corticosterone/ml serum
70
60
50
40
30
20
10
0
b
a
a
a
3
9
15
21
ng corticosterone/ml urine
30
25
20
15
10
5
0
b
a
a
a
3
9
15
21
Hours Since Midnight
ng corticosterone/mg creatinine
45
40
35
30
25
20
15
10
5
0
b
a
b
c
3
9
15
21
Hours Since Midnight

Discussion

We believe that this is the first documentation of circadian urinary rhythms of corticosterone, testosterone, and creatinine in mice. In Experiment 1, urinary concentrations of corticosterone peaked around the onset of the dark phase of the diurnal cycle, and showed a trough around the onset of the light phase followed by gradual restoration. Urinary testosterone concentrations peaked at 5-7 h after light onset and showed a trough shortly before light offset and through most of the dark phase of the cycle. Urinary creatinine showed relatively small variations over the light-dark cycle. In Experiment 2, we documented responses of these urinary measures to an acute stressor applied at the peak and trough times of corticosterone concentrations observed in Experiment 1. Corticosterone was significantly higher 90 min after a stressor compared to that of control mice, both at the peak and the trough of the daily cycle. Testosterone was not significantly affected by the stressor, and creatinine was significantly lower around light onset (corticosterone trough) than around dark onset (corticosterone peak), but only in the stressed animals. In Experiment 3, we observed a distinct circadian rhythm in both urinary and serum corticosterone, and showed significant correlations between the 2 media. In both urine and serum, the circadian rhythm seen in Experiment 1 was clearly replicated.

Care was taken to ensure that animals were not subjected to any extraneous stimulation, handling, or light, and that procedures were standardized for all subjects. In order to achieve more precise timing than possible with spontaneous urination, we employed a method of urine collection that was mildly invasive, specifically brief

handling to place the animal in a novel environment. As plasma corticosterone takes over an hour to be excreted into urine in mice [35,36], and urine was collected no longer than 15 min after transport and handling, our procedures would not have influenced our measures.

Experiment 1 showed a clear daily rhythm in urinary corticosterone. Our observed urinary corticosterone rhythm is similar to that seen in blood measures of this hormone in nocturnal rodents. Rats and mice appear to have a stable cycle where peak plasma corticosterone occurs at the start of darkness and decreases until the onset of light [6, 8]. This contrasts with the pattern in diurnal mammals, such as humans and other primates, where cortisol is highest in the morning and decreases throughout the day [5, 11, 37]. Experiment 2 confirmed the finding that urinary corticosterone is greater at the onset of light, and indicated that an acute stressor elevated corticosterone at both times. Two studies using rats reported an increase in circulating corticosterone at both peak and trough times of cycle following an acute stressor [4, 17]. One of these studies [4] found similar increases in plasma corticosterone after stress during the dark and light phases of the daily cycle in female rats, whereas the other [17] showed a smaller increase in plasma corticosterone at the trough of the cycle. Another study in male rats [38] found that stress elevated plasma corticosterone around light onset (trough) but not around light offset (peak). It is likely that species, sex and stressor differences contribute to discrepancies among studies. Experiment 3 again replicated the pattern seen in diurnal variation of urinary corticosterone in Experiment 1 and 2, and furthermore showed that urinary corticosterone fluctuations across the diurnal cycle are very similar to those seen in serum

corticosterone.

In Experiment 1, we found that urinary testosterone peaked during the light phase of the cycle and was generally lower during the dark. Male rhythm in testosterone varies among species and shows a less consistent entrainment to light than does corticosterone. In men, testosterone rhythm coincides with that of corticosterone, with peaks around waking [5, 39, 40]; however, testosterone peaks at night in another diurnal primate, the rhesus monkey [11, 25]. Studies of circulating testosterone rhythm in nocturnal rodents show much less consensus than do those of corticosterone rhythm. Some have found trimodal peaks, whereas others have described a biphasic pattern [12, 41]. In rats, many studies have documented a circadian rhythm in circulating testosterone, however the reported timing of peaks and troughs varies greatly [27, 28, 42-44]. In laboratory mice, significant variance has been observed among strains [29]. Some evidence indicates that in men, mice and rats, testosterone rhythm may not be stable since this hormone's secretion is episodic and pulsatile [40, 45, 46]. It is difficult to compare our data on urinary rhythm to published rhythms in blood, due to variability in the rhythm of circulating testosterone that depends on species, strain, laboratory, and frequency of sampling. In Experiment 2, we found no change in urinary testosterone after acute injection stress, regardless of the time of day. Elsewhere, acute immobilization stress also did not alter testosterone in mice or rats, although more chronic immobilization stress did so [47-49]. Other studies have shown that multiple forms of acute stress decreased circulating testosterone in rats, however some found that the decrease did not occur until 2 h after stress onset [50, 51]. Another study [48] showed a decrease in circulating

testosterone after 1 h of continuous stress, which may be considered more chronic than that of the current study where stress duration was less than 30 s. Urinary measures of testosterone not only may lag those from blood, but also likely represent an integration of a longer time period than do more momentary measures from serum or plasma.

Creatinine is commonly used to correct urinary steroid concentrations for the animal's hydration, based on the assumption that its excretion remains generally constant [20, 21, 34, 52]. In all experiments reported here, trends in urinary corticosterone and testosterone were similar with and without adjustment for creatinine. In both Experiments 1 and 3, creatinine was relatively invariant compared to the steroid measures, but there was some evidence of small variation over time of day, likely related to changes in muscular activity, drinking, and urination. In Experiment 2, creatinine concentration was significantly higher when stress was applied around light offset (corticosterone peak) than around light onset (corticosterone trough). Elsewhere, creatinine has been observed to be dynamic across experimental conditions in mice, as social stimuli induce polydipsia and polyuria and reduce urinary creatinine concentration [33], while diet also has substantial influences [34]. Accordingly, we suggest that it is best to examine both raw and creatinine-adjusted value of urinary steroids.

Our observed circadian pattern of urinary corticosterone reflects patterns in blood reported elsewhere [6, 8]. Taken together with the positive correlation between urinary and serum corticosterone seen in Experiment 3, this suggests that urinary measures largely reflect systemic patterns. This paves the way for the use of noninvasive repeated urinary sampling and profiling of urinary corticosterone in individuals over time and

across circumstances. There is evidence that the liver in mice and rats is less efficient than in larger mammals at conjugating steroids through processes such a glucuronidation [22, 53]; this may increase confidence that unconjugated urinary steroid measures reflect systemic patterns in these steroids. Although inconsistencies among published studies of circulating testosterone rhythm prevent clear comparisons to urinary measures observed here, utility of urinary measures has been demonstrated with respect to other parameters such as castration and social stimulation (e.g., [21, 33, 52, 54]). Given constraints on repeated blood measures in small animals, noninvasive urinary measurement could lend itself well to investigations where steroids are examined over development and across circumstances [21, 34, 54].

Acknowledgements

This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) awarded to D. deCatanzaro. We greatly appreciate the assistance of Cameron Muir, Robert Berger, Adam Guzzo, Brent Crawford, and Jihwan Jheon.

References

1 *Fediuc S, Campbell JE, Riddell MC.* Effect of voluntary wheel running on circadian corticosterone release and on HPA axis responsiveness to restraint stress in Sprague-Dawley rats. J Appl Physiol 2006; 100: 1867–1875

2 *Gartner K, Buttner D, Dohler K, Friedel R, Lindena J, Trautschold I.* Stress response of rats to handling and experimental procedures. Lab Anim 1980; 14: 267–274

3 *Sapolsky RM, Romero LM, Munck AU.* How do glucocorticoids influence stress? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 2000; 21: 55–89

4 *Zimmermann E, Critchlow V.* Effects of diurnal variation in plasma corticosterone levels on adrenocortical response to stress. P Soc Exp Biol Med 1967; 125: 658–663

5 *Faiman C, Winter JSD.* Diurnal cycles in plasma FSH, testosterone and cortisol in men. J Clin Endocr Metab 1971; 33: 186–192

6 *Halberg F, Peterson RE, Silber RH.* Phase relations of 24-hour periodicities in blood corticosterone, mitoses in cortical adrenal parenchyma, and total body activity. Endocrinology 1959; 64: 222–230

7 *Kakihana R, Moore JA.* Circadian rhythm of corticosterone in mice: The effect of chronic consumption of alcohol. Psychopharmacologia 1976; 46: 301–305

8 *Leal AMO, Moreira AC.* Daily variation of plasma testosterone, androstenedione, and corticosterone in rats under food restriction. Horm Behav 1997; 31: 97–100

9 *MacAdam WR, Eberhart RJ.* Diurnal variation in plasma corticosteroid concentration in

dairy cattle. J Dairy Sci 1972; 55: 1792–1795

10 *Malisch JL, Breuner CW, Gomes FR, Chappell MA, Garland T Jr.* Circadian pattern of total and free corticosterone concentrations, corticosteroid-binding globulin, and physical activity in mice selectively bred for high voluntary wheel-running behavior. Gen Comp Endocr 2008; 156: 210–217

11 *Plant TM.* Time courses of concentrations of circulating gonadotropin, prolactin, testosterone, and cortisol in adult male rhesus monkeys (Macaca mulatta) throughout the 24h light-dark cycle. Biol Reprod 1981; 25: 244–252

12 *Waite E, Kershaw Y, Spiga F, Lightman SL.* A glucocorticoid sensitive biphasic rhythm of testosterone secretion. J Neuroendocrinol 2009; 21: 737–741

13 *Balcombe JP, Barnard ND, Sandusky C.* Laboratory routines cause animal stress. J Am Assoc Lab Anim Sci 2004; 43: 42–51

14 *Tuli JS, Smith JA, Morton DB.* Corticosterone, adrenal and spleen weight in mice after tail bleeding, and its effects on nearby animals. Lab Anim 1995; 29: 90–95

15 *Armario A, Montero JL, Balasch J.* Sensitivity of corticosterone and some metabolic variables to graded levels of low intensity stresses in adult male rats. Physiol Behav 1986; 37: 559–561

16 *Bronson FH.* Establishment of social rank among grouped male mice: relative effects on circulating FSH, LH, and corticosterone. Physiol Behav 1973; 10: 947–951

17 *Seggie JA, Brown GM.* Stress response patterns of plasma corticosterone, prolactin, and growth hormone in the rat, following handling or exposure to novel environment. Can J Physiol Pharm 1975; 53: 629–637

18 *van Herck H, Baumans V, de Boer SF, van der Gugten J, van Woerkom AB, Beynen AC.* Endocrine stress response in rats subjected to singular orbital puncture while under diethyl-ether anaesthesia. Lab Anim 1991; 25: 325–329

19 *Van Loo PLP, Mol JA, Koolhaas JM, Van Zutphen BFM, Baumans V.* Modulation of aggression in male mice: Influence of group size and cage size. Physiol Behav 2001; 72: 675–683

20 *deCatanzaro D, Muir C, Beaton E, Jetha M, Nadella K.* Enzymeimmunoassay of oestradiol, testosterone and progesterone in urine samples from female mice before and after insemination. Reproduction 2003; 126: 407–414

21 *deCatanzaro D, Muir C, Beaton EA, Jetha M.* Non-invasive repeated measurement of urinary progesterone, 17β-estradiol, and testosterone in developing, cycling, pregnant, and postpartum female mice. Steroids 2004; 69: 687–696

22 *Muir C, Vella ES, Pisani N, deCatanzaro D.* Enzyme immunoassay of 17β-estradiol, estrone conjugates, and testosterone in urinary and fecal samples from male and female mice. Horm Metab Res 2001; 33: 653–658

23 *Claustrat B, Valatx JL, Harthé C, Brun J.* Effect of constant light on prolactin and corticosterone rhythms evaluated using a noninvasive urine sampling protocol in the rat. Horm Metab Res 2008; 40: 398–403

24 *Hausler A, Hauser K, Meeker JB.* Effects of subchronic administration of psychoactive substances on the circadian rhythm of urinary corticosterone excretion in rats. Psychoneuroendocrinology 1985; 10: 421–429

25 *Goodman RL, Hotchkiss J, Karsch FJ, Knobil E.* Diurnal variations in serum

testosterone concentrations in the adult male rhesus monkey. Biol Reprod 1974; 11: 624–630

26 *Hoffman K, Nieschlag E.* Circadian rhythm of plasma testosterone in the male Djungarian hamster (Phodopus sungorus). Acta Endocrinol-Cop 1977; 86: 193–199

27 *Kalra PS, Kalra SP.* Circadian periodicities of serum androgens, progesterone, gonadotropins and luteinizing hormone-releasing hormone in male rats: the effects of hypothalamic deafferentation, castration and adrenalectomy. Endocrinology 1977; 101: 1821–1827

28 *Keating RJ, Tcholakian RK.* In vivo patterns of circulating steroids in adult male rats. I. Variations in testosterone during 24- and 48-hour standard and reverse light/dark cycles. Endocrinology 1979; 104: 184–188

29 *Lucas LA, Eleftheriou BE.* Circadian variation in concentrations of testosterone in the plasma of male mice: A difference between BALB/cBy and C57BL/6By inbred strains. J Endocrinol 1980; 87: 37–46

30 *Rowsemitt CN, Berger PJ.* Diel plasma testosterone rhythms in male Microtus montanus, the Montane vole, under long and short photoperiods. Gen Comp Endocr 1983; 50: 354–358

31 *Sharma OP.* Diurnal variations of plasma testosterone in stallions. Biol Reprod 1976; 15: 158–162

32 *Munro CJ, Stabenfeldt GH, Cragun JR, Addiego LA, Overstreet JW, Lasley BL.* Relationship of serum estradiol and progesterone concentrations to the excretion

profiles of their major urinary metabolites as measured by enzyme immunoassay and radioimmunoassay. Clin Chem 1991; 37: 838–844

33 *deCatanzaro D, Khan A, Berger RG, Lewis E.* Exposure to developing females induces polyuria, polydipsia, and altered urinary levels of creatinine, 17β-estradiol, and testosterone in adult male mice (Mus musculus). Horm Behav 2009; 55: 240–247

34 *Khan A, Berger RG, deCatanzaro D.* 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 2008; 135: 99–106

35 *Meijer MK, Lemmens AG, Van Zutphen BFM, Baumans V.* Urinary corticosterone levels in mice in response to intraperitoneal injections with saline. J Appl Anim Welf Sci 2005; 8: 279–283

36 *Touma C, Sachser N, Möstl E, Palme R.* Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. Gen Comp Endocr 2003; 130: 267–278

37 *Van Cauter E, Leproult R, Kupfer DJ.* Effects of gender and age on the levels and circadian rhythmicity of plasma cortisol. J Clin Endocr Metab 1996; 81: 2468–2473

38 *Brennan FX, Ottenweller JE, Seifu Y, Zhu G, Servatius RJ.* Persistent stress-induced elevations of urinary corticosterone in rats. Physiol Behav 2000; 71: 441–456

39 *Plymate SR, Tenover JS, Bremner WJ.* Circadian variation in testosterone, sex

hormone-binding globulin, and calculated non-sex hormone-binding globulin bound testosterone in healthy young and elderly men. J Androl 1989; 10: 366–370

40 *Veldhuis JD, King JC, Urban RJ, Rogol AD, Evans WS, Kolp LA, Johnson ML.* Operating characteristics of the male hypothalamo-pituitary-gonadal axis: Pulsatile release of testosterone and follicle-stimulating hormone and their temporal coupling with luteinizing hormone. J Clin Endocr Metab 1987; 65: 929–941

41 *Mock EJ, Norton HW, Frankel AI.* Daily rhythmicity of serum testosterone concentration in the male laboratory rat. Endocrinology 1978; 103: 1111–1121

42 *Kinson GA, Liu CC.* Diurnal variation in plasma testosterone of the male laboratory rat. Horm Metab Res 1973; 5: 233–234

43 *Wilson MJ, McMillin JM, Seal US, Ahmed K.* Circadian variation of serum testosterone in the adult male rat with a late morning acrophase. Cell Mol Life Sci 1976; 32: 944–945

44 *Hostetter MW, Piacsek BE.* Patterns of pituitary and gonadal hormone secretion during a 24h period in the male rat. Biol Reprod 1977; 16: 495–498

45 *Bartke A, Steele RE, Musto N, Caldwell BV.* Fluctuations in plasma testosterone levels in adult male rats and mice. Endocrinology 1973; 92: 1223–1228

46 *Ellis GB, Desjardins C.* Male rats secrete luteinizing hormone and testosterone episodically. Endocrinology 1982; 110: 1618–1627

47 *Armario A, Castellanos JM.* A comparison of corticoadrenal and gonadal responses to acute immobilization stress in rats and mice. Physiol Behav 1984; 32: 517–519

48 *Bliss EL, Frischat A, Samuels L.* Brain and testicular function. Life Sci 1972; 11: 231–238

49 *Demura R, Suzuki T, Nakamura S, Komatsu H, Odagiri E, Demura H.* Effect of immobilization stress on testosterone and inhibin in male rats. J Androl 1989; 10: 210–213

50 *Charpenet G, Tache Y, Bernier M, Ducharme JR, Collu R.* Stress-induced testicular hyposensitivity to gonadotropin in rats. Role of the pituitary gland. Biol Reprod 1982; 27: 616–623

51 *Marić D, Kostić T, Kovačević R.* Effects of acute and chronic immobilization stress on rat leydig cell steroidogenesis. J Steroid Biochem 1996; 58: 351–355

52 *Beaton EA, Khan A, deCatanzaro D.* Urinary sex steroids during sexual development in female mice and in proximate novel males. Horm Metab Res 2006; 38: 501-506

53 *Jäntti SE, Kiriazis A, Reinilä RR, Kostiainen RK, Ketola RA*. Enzyme-assisted synthesis and characterization of glucuronide conjugates of neuroactive steroids. Steroids 2007; 72: 287–296

54 *Vella ES, deCatanzaro D.* Novel male mice show gradual decline in the capacity to disrupt early pregnancy and in urinary excretion of tes- tosterone and 17β-estradiol during the weeks immediately following castration. Horm Metab Res 2001; 33: 681–686

Received 08.09.2011
Accepted 13.02.2012

Chapter 4

Circulating and urinary adrenal steroids in response to acute, mild stress in female mice (*Mus musculus*)

Joelle B. Thorpe, Kaitlin E. Gould & Denys deCatanzaro (2013)

Hormone and Metabolic Research, submitted July 19, 2013

Authors' Contributions

Joelle B. Thorpe: Experimental design, data collection, data analysis, manuscript writing, editing, and preparation.

Kaitlin E. Gould: Assistance with data collection.

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

Abstract

During acute stress, glucocorticoids and progesterone from the adrenal gland are upregulated. Estradiol may also rise, although its origin is unknown. In studies of stress, it can be difficult to obtain blood rapidly enough to avoid confounding steroid measures. In such cases, non-invasive urinary steroid measures may be more appropriate insofar as they reflect systemic steroid levels. The current study was designed to examine the urinary steroid response to an acute, mild stressor in female mice, and to compare serum and urinary steroid responses to this mild stressor. In Experiment 1, we profiled urinary corticosterone, progesterone, and estradiol in ovariectomized female mice at 11 time points following a one-hour stressor (placement on an elevated platform). This increased urinary corticosterone for 3 hours and urinary progesterone for 4 hours after stressor offset. In Experiment 2, terminal blood and urine samples were obtained at 0, 2, 4, and 6 hours after the stressor offset. Stressed females showed increased serum corticosterone and progesterone immediately after the stressor offset. Urinary levels of corticosterone were increased at both 0 and 2 hours post-stress, while those of progesterone trended toward increases 2-6 hours after stress offset. Neither serum nor urinary estradiol was significantly influenced by stress. Urinary measures generally reflected systemic measures, however with a different time course resulting in a longer return to baseline. We suggest that the relative value of serum or urinary steroid measures in mice depends upon the experimental design.

Key words: Stress, corticosterone, progesterone, estradiol, serum, urine

Introduction

The stress response allows individuals to cope with challenges or threats to homeostasis [1,2]. Upon exposure to a stressor (defined as an unpredictable or uncontrollable factor that challenges homeostasis), there is a distinct endocrine response that ultimately results in the secretion of cortisol or corticosterone (the glucocorticoids, GCs) from the adrenal cortex. The adrenal steroid response to stress is so well established that increases in circulating and excreted GCs are used as biomarkers of stress in field and laboratory studies in a variety of species including baboons [3], sheep [4], humans [5], meerkats [6], and mice [7,8].

During acute stress in female rats, progesterone (P_4) from the adrenal cortex is secreted [9]. Adrenocorticotropic hormone (ACTH) administration also has been found to increase P_4 in ovariectomized rats [10], cows [11], and fallow deer [12]. P_4 is situated early in the steroid biosynthetic pathway, and as such is a precursor to many steroids, including GCs. Estradiol (E_2) can also respond to stress in females; however, the physiological state of the female and the nature of the stressor may influence whether E_2 is increased or decreased. In cycling female rats, exposure to chronic stress (3 weeks of daily restraint) results in reduced E_2 [13], whereas more acute stress (20 minutes swimming) has been associated with increased E_2 [14]. In pregnant animals, chronic stress [15,16] or acute immune stress [17] can increase E_2. The source of this rise in circulating E_2 during stress is unknown. Some have suggested an ovarian origin [14,18], however it remains possible that the adrenal cortex secretes E_2 and/or its precursors in response to stress.

In small laboratory species like mice, obtaining a sufficient blood sample to quantify circulating steroids may require pooling of blood from several individuals or taking only terminal blood samples. Also, invasive handling and venipuncture are likely to alter subsequent steroid measures, so repeated measurement of the same individuals may be precluded. These issues are particularly troublesome when quantifying stress-reactive steroids like GCs, which can respond to even minor disturbances such as moving a cage in as little as 5 minutes [19]. As mentioned, in female mammals both P_4 and E_2 also respond to stress. Therefore, in situations where rapid blood sampling is inconvenient or impossible, it might benefit the researcher if steroids could be quantified in a different medium, in order to avoid the risk of blood collection procedures confounding stress measures.

In mice, excretions can be obtained in a relatively non-invasive and repeated manner that avoids human handling [8,20]. Our laboratory has previously demonstrated that urinary measures of unconjugated E_2, testosterone, and P_4 generally reflect expected trends in blood measures of these steroids [20-23]. In male mice, we have shown that urinary unconjugated corticosterone measures reflect those in serum across the circadian cycle [7], while others have shown the value of fecal corticosterone metabolites in assessing adrenocortical activity in mice [8,24]. As urinary and serum steroids have not been compared in female mice, we undertook to measure urinary corticosterone, P_4, and E_2 subsequent to an acute, mild stressor, and to compare urinary trends to those in serum. We used ovariectomized mice, since a preliminary experiment with intact females produced unclear results, likely because the estrus cycle influences E_2, P_4, and the steroid

response to stress [25]. In the first experiment, we measured steroids in repeated urine samples taken from female mice throughout the day following exposure to one hour on an elevated platform. In the second experiment, we compared the urinary and serum corticosterone, P_4, and E_2 response to this stressor in independent samples from female mice at different time points after offset of the stressor.

Animals and Methods

Animals

Female subjects were from an established colony of hybrid strain (HS) mice created by interbreeding CF-1, DBA-2, Swiss Webster, and C57-B6 parent strains originally obtained from Charles River Breeding Farms (St. Constant, QC, Canada). Bilateral ovariectomies were performed when females were 2-3 months old under sodium pentobarbital anaesthesia. They were given at least 2 weeks to recover from surgery. One week prior to each experiment, all females were placed on a phytoestrogen-free diet (Advanced Protocol Verified Casein Diet 1 IF from Purina Mills, Inc., Richmond, IN, USA; LabDiet, Ren's Feed & Supply Ltd., Oakville, ON, Canada) in order to reduce the potential impact of phytoestrogens on E_2 assays. This diet contains < 1 ppm total isoflavones (aglycone equivalents of genistein, daidzein and glycitein) and is nutritionally similar to standard laboratory rodent chow [26]. All animals were maintained at 21°C on a reversed 14 h light:10 h dark cycle with dark phase onset at 09:00 h, and housed in standard polypropylene cages measuring 28 cm x 16 cm x 11 cm (height) unless otherwise stated. This research was approved by the McMaster University Animal

Research Ethics Board, conforming to the standards of the Canadian Council on Animal Care.

Experiment 1: Repeated measures of urinary steroids after acute, mild stress

In the first experiment, we used a repeated measures design to verify the stressor protocol, and to document the urinary steroid response in female mice. Two days before sampling, all females (n = 15 experimental, n = 15 control) were placed in Plexiglas urine collection cages measuring 30 x 21 x 13 cm (height). The floors of these cages are wire-mesh grid, raised approximately 2 cm above a removable tray covered in wax paper, from which urine could be collected. Mice remained undisturbed in these cages in a holding room (Room A) for 48 h to allow for acclimation. At 09:30 h on the day of urine sampling, mice randomly assigned to the stress condition were removed from Room A and brought into the procedure room (Room B), whereas control mice remained undisturbed in Room A. Room B had 15 platforms elevated 1.22 m off the ground, each measuring 26.5 x 11 cm and tilted at an angle of approximately 45° relative to the plane of the floor. Each experimental mouse was placed individually on a platform. At 10:30 h, after 1 h on the platform, each mouse was returned to its cage and brought back to Room A. Beginning 1 h after stressor offset (11:30 h), urine samples were taken from each mouse every hour for 10 h, and one final sample was taken the following day, corresponding to 24 h after stressor offset. Urine was collected by sliding the removable tray out from under the urine collection cage and aspirating any pooled urine from the wax paper using 1 mL tuberculine syringes with 25 ga needles. After each sampling time, every tray was replaced with a fresh one covered in clean wax paper to ensure that each

sample was new from the previous hour. All urine was stored at -20°C in 1.5 mL Diamed microtubes until hormone assay (described below).

Immunoassay validation for serum P_4 and corticosterone

Immunoassays previously validated for female urinary P_4 [22] and male urinary and serum corticosterone [7] were modified and validated for P_4 and corticosterone in female mouse serum. Tests of parallelism were run to determine whether samples perform immunologically in a manner similar to steroid standards. Such tests indicate whether the steroid is present in measurable quantities in serum, and whether serum samples react with the antibodies in a predictable fashion. This also determines the dilution of serum that falls at 50% conjugate binding, which is an appropriate dilution when quantifying endogenous steroid concentration. While under isoflurane anaesthesia, a terminal blood sample was collected from females within 3 minutes into 1.5 mL Diamed microtubes by cardiac puncture with 1 mL tuberculine syringes and 25 ga needles. Blood was allowed to clot for 1.5-2 h at room temperature, and then centrifuged at 5000 rpm for 15 min. Serum supernatant from samples collected on the same day was pooled in a single microtube and stored at -20°C until hormone quantification (described below). Pooled serum used for the P_4 parallelisms was from 11 females and pooled serum used for the corticosterone parallelisms was from 6 females. For both, pooled serum was serially diluted in phosphate buffer and compared with a standard curve plotted against logarithmically transformed concentrations of steroid.

Experiment 2: Urinary and serum steroid response to an acute, mild stressor

A second experiment used the same acute, minor, psychological stressor in a between-subjects design, with different animals assigned to be measured at different times following the stressor or no treatment (controls). Prior to stressor procedures, experimental mice were removed from a common holding room containing both experimental and control females (Room A) and moved to Room B, where acute stress was applied from 09:30 h to 10:30 h. In Room B, each mouse was removed from its cage and placed individually on a raised platform (described for Experiment 1). Mice remained on the elevated platforms for 1 h before being returned to Room A at 10:30 h. All females remained in standard cages throughout the experiment. Mice were divided randomly into experimental and control groups, and these were subdivided into 4 sampling times chosen to capture the times in Experiment 1 at which urinary steroids differed between mice in the control and experimental treatments.

At the onset of the dark phase on the day of sampling, experimental females were moved from the common holding room (Room A) to Room B for stressor procedures (as described above). After 1 h, females were moved back to Room A. Samples were obtained from subsets of the females based on their assigned time slot. Times included immediately after stressor offset (10:30 h), and every 2 h after that for a total of 4 samples (last sample at 16:30 h). To obtain samples, each female was removed individually from Room A at the assigned sample time, and placed under isoflurane anaesthesia. While under anaesthesia, a blood sample was obtained by cardiac puncture with a 1 mL tuberculine syringe and 23 ga needle. After blood sampling, each female was euthanized

by cervical dislocation and expelled urine was collected from a piece of wax paper with a 1 mL tuberculine syringe. Blood and urine samples were obtained within a maximum of 3 minutes from when Room A was entered to remove the female. Only after the previous female had been sampled from was the next female removed from Room A for sampling. To ensure that sample times were as precise as possible between mice within groups, only 3 control and 3 experimental mice were sampled from at each time point on each day.

Urine samples were stored in a 1.5 mL Diamed microtube at -20°C immediately after collection until hormone analysis. Blood samples were allowed to clot at room temperature for approximately 1 h. After clotting, blood was centrifuged at 5000 rpm for 15 min, and serum was pipetted into individually labeled 1.5 mL Diamed microtubes and stored at -20°C until hormone analysis.

Steroid and creatinine assays

Serum E_2 was determined using a commercially available enzyme immunoassay kit (Calbiotech, product no. ES180S-100). Kit directions were followed exactly. Methods for all other steroid quantification, including urinary estradiol, are described below. Urinary E_2, P_4, and corticosterone immunoassay methods were previously validated and described [7,22,27]. Serum P_4 and corticosterone immunoassay methods are validated and reported here. All steroids were obtained from Sigma Chemical Co., and antibodies to corticosterone, P_4, and E_2, and corresponding horseradish peroxidase (HRP) conjugates were obtained from the Department of Population Health and Reproduction at the University of California, Davis.

All steroid assays were conducted with duplicate readings per sample and the average was used in statistical analysis. NUNC Maxisorb plates were first coated with 50 µl antibody stock diluted 1:10,000 for P_4, corticosterone, and E_2 in a coating buffer (50 mmol/l bicarbonate buffer, pH 9.6), then stored for 12-14 h at 4°C. Wash solution (0.15 mol/l NaCl solution containing 0.5 ml Tween 20 per liter) was added to each well to rinse away any unbound antibody, then 50 µl phosphate buffer/well was added. The plates were incubated at room temperature for 1 h for both P_4 and corticosterone, and 2 h for E_2, before adding standards, samples (pooled and serially diluted for parallelisms, or individual samples for steroid concentration determination), and controls. Urine samples were diluted 1:30 (corticosterone) or 1:8 (P_4 and E_2) in phosphate buffer (0.1 mol/l sodium phosphate buffer, pH 7.0 containing 8.7 g NaCl and 1 g BSA/l) before being added to the plate. Serum samples were diluted 1:15 (corticosterone) or 1:7 (P_4) in phosphate buffer before being added to the plate. These dilutions were chosen because tests of parallelism indicated they produced 50% conjugate binding. Standard curves were derived by serial dilution from a known stock solution. For all assays, 50 µl corticosterone (1:140,000 in phosphate buffer), P_4 (1:60,000 in phosphate buffer), or E_2 (1:350,000) HRP was added to each well, with 50 µl standard, sample, and control for both P_4 and corticosterone, or 20 µl of the same for E_2. The plates were incubated for 2 h at room temperature, then washed, and then 100 µl substrate solution of citrate buffer, H_2O_2 and 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid), was added to each well. The plates were then covered and incubated, while shaking at room temperature for 25 min to 1.5 h. The plates were then read with a single filter at 405 nm on a microplate

reader (Bio-Tek Instruments Inc, ELx 808). In all assays, optical densities were obtained, standard curves were generated, a regression line fit, and samples interpolated into the equation to get a value in pg per well.

Following convention for urinary steroid measures [22,27,28], urinary creatinine was also measured. All urine samples were diluted 1:40 in phosphate buffer. Using Dynatech Immulon flat bottom plates, 50 µl/well of standard was added with 50 µl distilled water, 50 µl 0.75 M NaOH and 50 µl 0.4 M picric acid. The plate was then shaken and incubated at room temperature for 30 min. The plate was measured for optical density on the 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. The average of duplicates was taken for each sample. Where steroid measures were adjusted for creatinine, this was achieved by dividing the obtained value by the concentration of creatinine considered per ml of urine for the particular sample.

Statistical analyses

In order to achieve a sufficient sample size despite a substantial number of missing values at each time point in the first (repeated-measures) experiment, the average value was taken for each subject within ranges of adjacent sampling times with similar trends, and analyzed through t-tests comparing the control and experimental conditions. For the second, between-subjects experiment, standard least squares analysis of variance with experimental group and sampling time included as predictor variables was conducted for each serum and urinary steroid and creatinine measure. Newman-Keuls

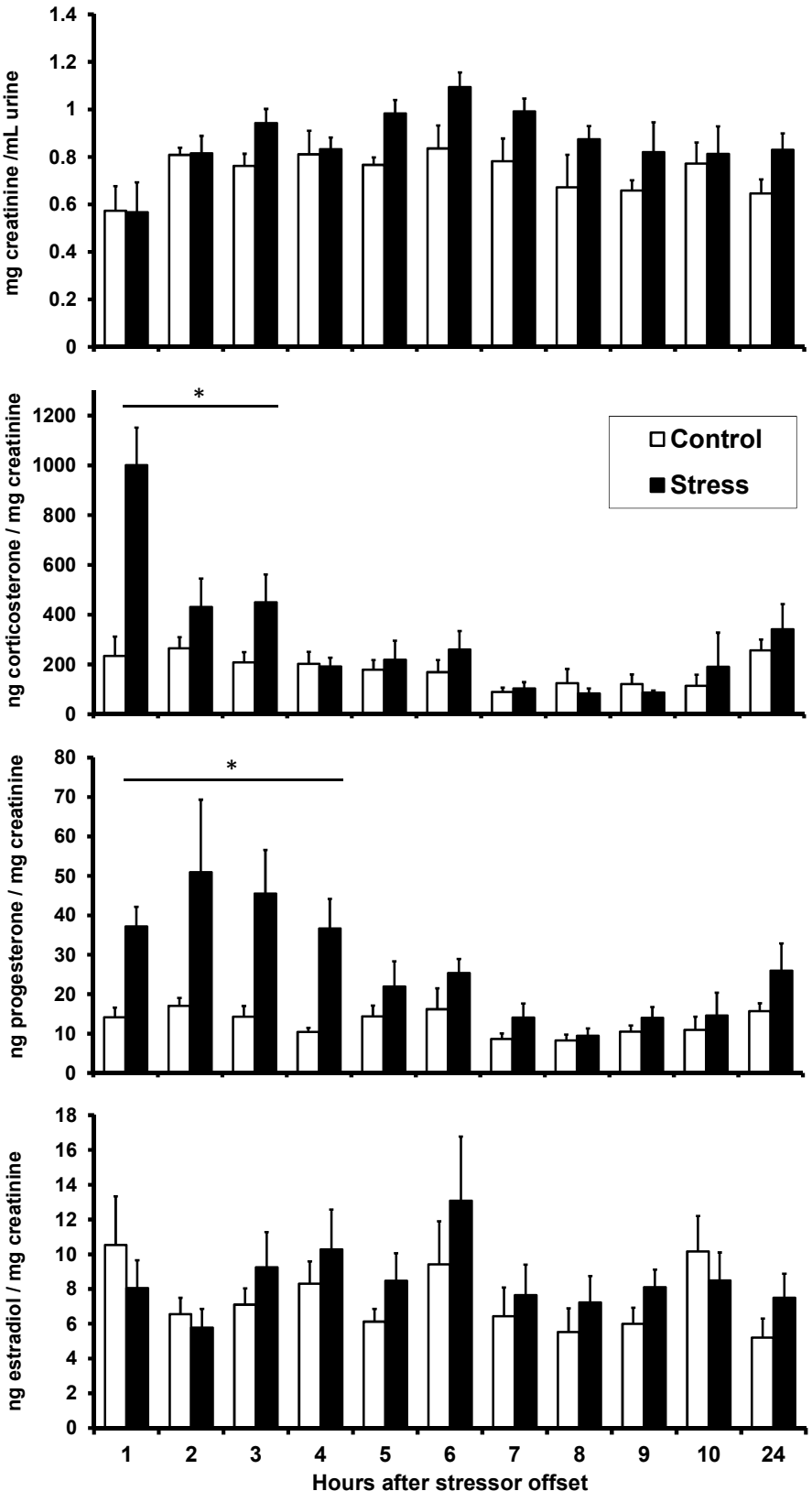
multiple comparisons ($p < 0.05$) were carried out where ANOVA models were significant ($p < 0.05$).

Results

Experiment 1

The height stressor clearly influenced urinary measures of corticosterone and P_4 (Fig. 1). Creatinine-adjusted urinary corticosterone was elevated during the first 3 h after stressor offset relative to controls; the average value from each subject in this range showed significant elevations in stressed mice compared to controls, $t(20) = 3.41$, $p = 0.003$. Creatinine-adjusted urinary P_4 was elevated during the first 4 h after stressor offset; the average value from each subject in this range showed a significant increase in stressed mice compared to controls, $t(22) = 4.89$, $p = 0.0002$. Creatinine-adjusted urinary E_2 was less influenced by stress than the other steroid measures; an apparent trend toward an elevation at 5-6 h after stressor offset was not significant considering the average value for each animal from these times, $t(23) = 1.28$, $p = 0.212$.

Fig. 1 Mean ± S.E. repeated measures of urinary creatinine, creatinine-adjusted corticosterone, creatinine-adjusted P_4, and creatinine-adjusted E_2 from ovariectomized mice after they either remained undisturbed (Control) or were placed on an elevated and angled platform for 1 h (Stress) in Experiment 1. Repeated urine samples were taken at 12 time points after offset of the stressor. Respective number of independent urine samples at each sample point were as follows: creatinine (control 5,9,7,7,12,7,7,3,6,5,6; stress 4,6,6,5,7,5,7,8,2,3,8); corticosterone (control 4,9,7,6,12,7,7,3,6,5,6; stress 4,6,6,5,7,5,7,8,2,3,8); P_4 (control 4,9,7,6,11,6,7,3,6,5,6; stress 4,6,6,5,7,5,7,8,2,3,8); E_2 (control 5,9,6,7,12,6,6,3,6,5,6; stress (4,6,6,5,7,5,7,8,2,3,8). Sample sizes varied as a result of mice not urinating every hour. * indicates range of sampling times that collectively show a significant difference between stressed and control values.


mg creatinine /mL urine
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ng corticosterone / mg creatinine
1200
1000
800
600
400
200
0
*
Control
Stress
ng progesterone / mg creatinine
80
70
60
50
40
30
20
10
0
*
ng estradiol / mg creatinine
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
24
Hours after stressor offset

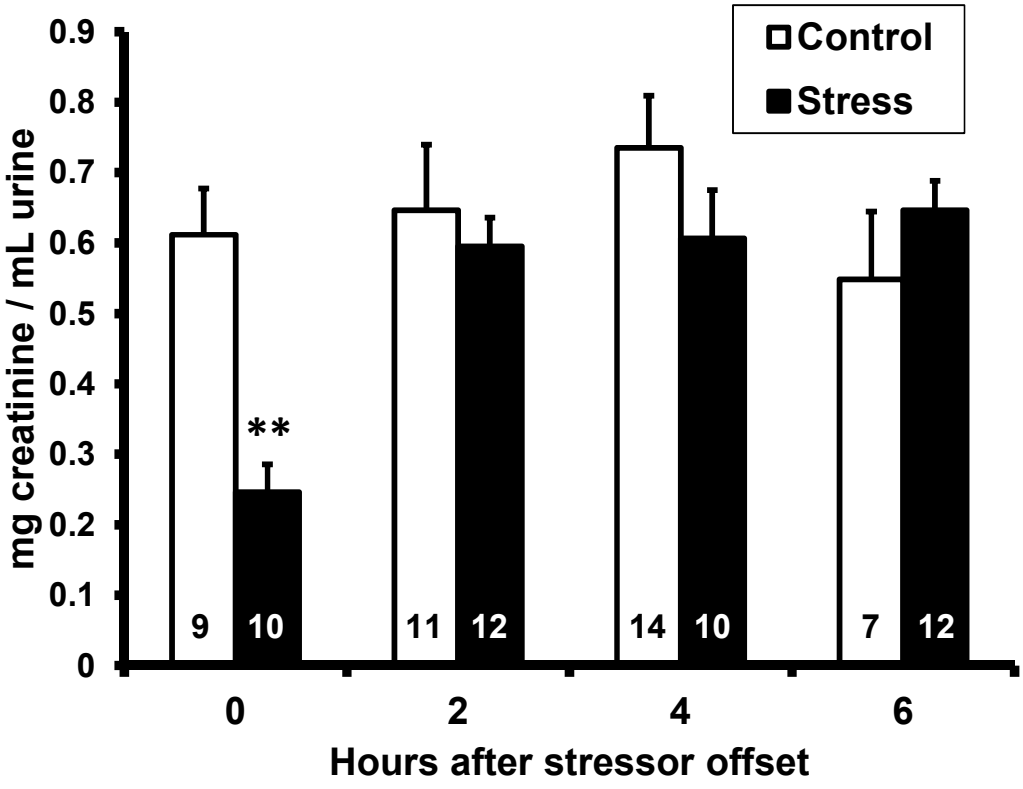
Validations of serum assays

Serial dilutions of pooled serum from adult cycling female mice were parallel to serial dilutions of corticosterone standard. This indicates that female mouse serum is binding to corticosterone antibody in a predictable fashion and in a manner similar to corticosterone itself. Serum diluted at 1:15 in phosphate buffer yielded 50% HRP binding. Serial dilutions of pooled serum from adult cycling female mice are also parallel to serial dilutions of P_4 standard. This again indicates that female mouse serum is binding to P_4 antibody in a predictable fashion and in a manner similar to P_4. Serum diluted at 1:7 in phosphate buffer yielded 50% HRP binding.

Experiment 2

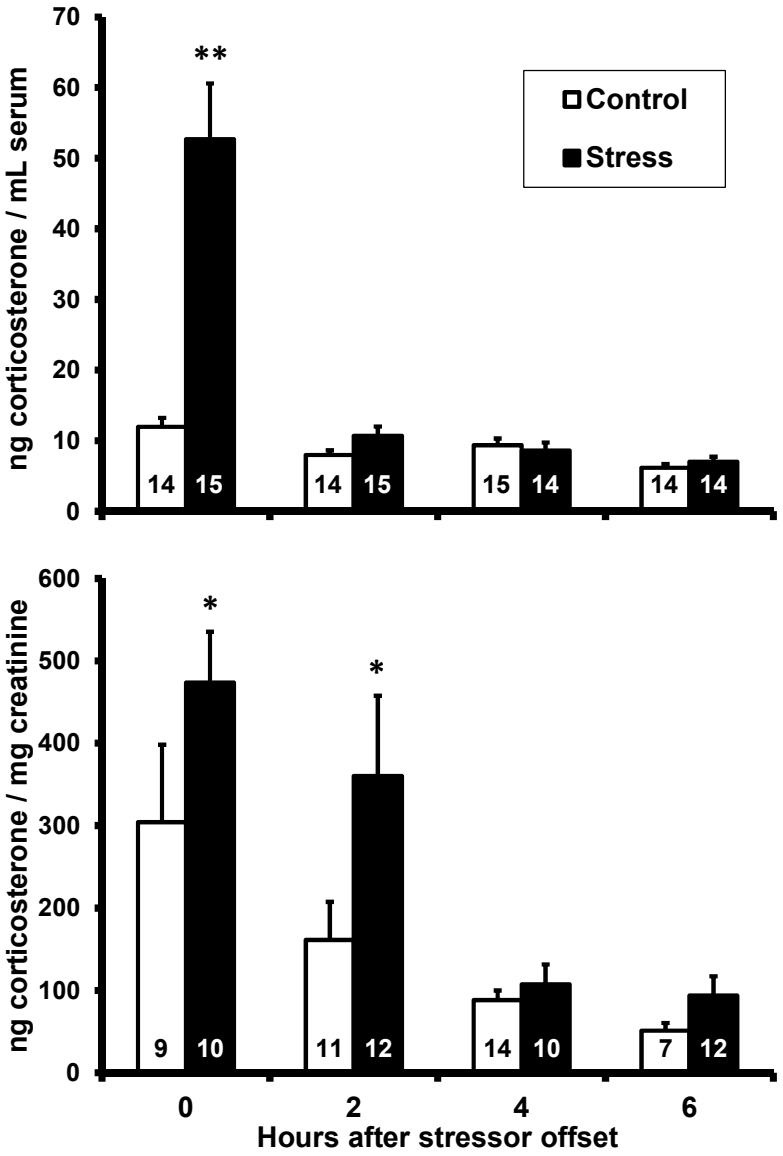
Urinary creatinine measures were altered by acute mild stress (Fig. 2). There were significant main effects of experimental treatment, $F(1,77) = 5.37$, $p = 0.020$, and of sampling time, $F(3,77) = 4.65$, $p = 0.005$, and a significant interaction between experimental treatment and sampling time, $F(3,77) = 3.65$, $p = 0.016$. Multiple comparisons indicated that creatinine measured immediately after 1 h of stress was significantly reduced compared to that of all other conditions.

Fig. 2 Mean ± S.E. urinary creatinine in samples collected from ovariectomized female mice at one of 4 times after they either remained undisturbed (Control) or were placed on an elevated and angled platform for 1 h (Stress) in Experiment 2. Sample sizes are given on the bars. ** indicates a significant difference from all other conditions.



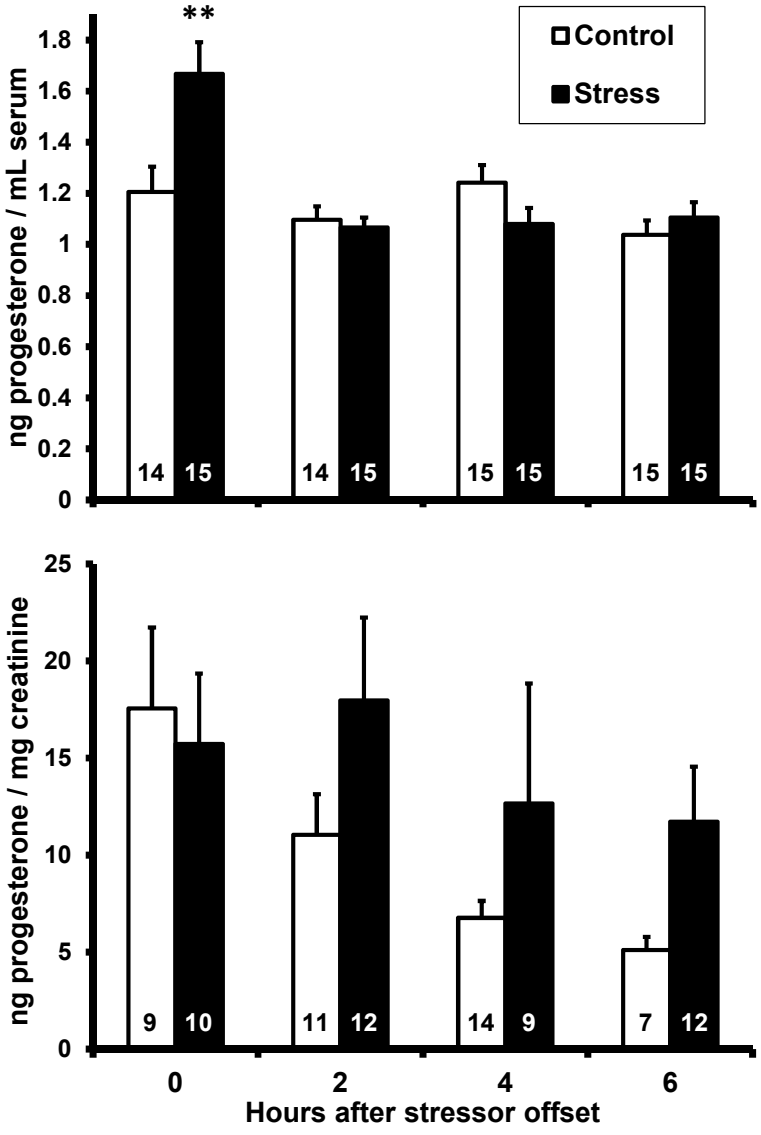
Both serum and urinary measures of corticosterone responded to the acute mild stressor (Fig. 3). In serum, there were main effects of experimental treatment, $F(1,107) = 25.17$, $p < 0.0001$, and sampling time, $F(3,107) = 31.09$, $p < 0.0001$, and a significant interaction between experimental treatment and sampling time, $F(3,107) = 21.26$, $p < 0.0001$. Multiple comparisons revealed that the condition in which mice were stressed for 1 h and sampled immediately after stress offset had significantly higher serum corticosterone than all other conditions. Urinary corticosterone was similarly altered by stress. For creatinine-adjusted urinary corticosterone, there were main effects of both experimental treatment, $F(1,77) = 6.91$, $p = 0.010$, and sampling time, $F(3,77) = 12.60$, $p < 0.0001$. Multiple comparisons revealed that stressed mice had significantly higher urinary corticosterone than controls at 0 and 2 h after stressor offset, and that stressed mice at 0 and 2 h after stressor offset had higher urinary corticosterone than stressed mice at 4 and 6 h after stressor offset.

Fig. 3 Mean ± S.E. serum corticosterone and creatinine-adjusted urinary corticosterone concentrations in ovariectomized female mice at one of 4 times after they were either placed on an elevated and angled platform for 1 h (Stress) or remaining undisturbed (Control) in Experiment 2. Sample sizes are given on the bars. * indicates a significant difference from the controls at the same sampling time. ** indicates a significant difference from all other conditions.



Serum P_4 was also increased by the stressor (Fig. 4). There was a significant main effect of sampling time on serum P_4, $F(3,110) = 10.31$, $p < 0.0001$, and a significant interaction between experimental treatment and sampling time, $F(3,110) = 6.42$, $p = 0.0007$. Multiple comparisons revealed that serum from stressed mice measured immediately after stressor offset had significantly elevated P_4 relative to mice from all other conditions. The response to stress was less clear in creatinine-adjusted urinary P_4. Although there were trends toward increased urinary P_4 in stressed animals at 2, 4, and 6 h after stressor offset, the main effects of experimental treatment, $F(1,76) = 3.19$, $p = 0.075$, and sampling time, $F(3,76) = 2.40$, $p = 0.074$, approached, but failed to reach, the conventional level of significance.

Fig. 4 Mean ± S.E. serum P_4 and creatinine-adjusted urinary P_4 concentrations in ovariectomized female mice at one of 4 times after they were either placed on an elevated and angled platform for 1 h (Stress), or remaining undisturbed (Control) in Experiment 2. Sample sizes are given on the bars. ** indicates a significant difference from all other conditions.



Serum E_2 was similar in all conditions, whereas urinary E_2 showed trends toward higher levels in stressed females than controls at 2 h after stressor offset (Table 1). However, neither serum nor urinary E_2 measures showed statistically significant main effects or interactions.

Table 1 Mean ± S.E. serum E_2 and creatinine-adjusted urinary E_2 concentrations in ovariectomized female mice at one of 4 times after they were either placed on an elevated and angled platform for 1 h (Stress), or remaining undisturbed (Control) in Experiment 2. Sample sizes are in brackets.

	pg estradiol / mL serum		ng estradiol / mg creatinine	
Hours after stressor offset	Control	Stress	Control	Stress
0	8.47 ± 0.31 (13)	8.93 ± 0.58 (14)	5.67 ± 0.91 (9)	5.14 ± 1.22 (6)
2	8.44 ± 0.34 (14)	8.40 ± 0.27 (14)	4.56 ± 0.43 (10)	6.23 ± 0.77 (12)
4	8.52 ± 0.28 (15)	8.63 ± 0.32 (15)	4.85 ± 0.32 (13)	4.97 ± 0.66 (10)
6	7.97 ± 0.25 (15)	8.86 ± 0.25 (15)	5.50 ± 0.67 (7)	5.42 ± 0.59 (12)

Discussion

Placement on a tilted, elevated platform for one hour is sufficiently stressful for ovariectomized female mice to cause elevations in serum corticosterone and P_4 within one hour. Urinary measures of corticosterone and P_4 were also both elevated by this stressor. In light of the many advantages of non-invasive urine sampling versus much more invasive blood sampling in mice, urinary measures of steroids may be used instead of serum measures with confidence that they reflect general systemic trends. We previously showed that urinary and serum measures of corticosterone correlate within individual male mice and across the daily cycle [7], confirming that urinary measures of steroids reflect serum measures in both male and female mice.

However, it is evident that the time course of the steroid response to stress in serum and urine is slightly different. Serum measures responded and returned to baseline control levels within 2 hours after stressor offset, whereas urinary measures appeared to take longer to return to baseline. This characteristic of urinary steroid response to stress may be useful in certain studies, particularly those where the effects of stress on steroids are of interest. For instance, if it is not possible to sample from all subjects in a study within 2 hours after stressor offset (before serum steroids return to baseline), urinary measures of GCs or P_4 may be superior, since they remain elevated after stressor offset for a longer period of time. On the contrary, it should be noted that urinary steroid measures have more inter-individual variation, and therefore may require larger sample sizes than serum measures to clarify stress-induced changes in steroids. This indicates that serum steroid measures may be superior when smaller sample sizes are used.

Previous studies have shown that adrenal P_4, like GCs, is upregulated in acute stress [9-12]. There are other studies indicating that stress can elevate subsequent circulating E_2 [14-17]. We did not find this effect in either serum or urine measures, and there are a number of possible reasons for this. First, stress-induced rises in E_2 may be of ovarian origin; if so, the effect will be absent in ovariectomized females as examined here. There is some indirect support to suggest that stress-induced rises in E_2 are from the ovaries [18] while there is some debate over the extent to which the rodent adrenal cortex is capable of synthesizing androgens and estrogens [29-32]. It is possible that stress-induced rises in E_2 require a more intense or chronic stressor, and that the relatively mild height stimulus used here was not sufficiently stressful. Other stressors that produce rises in E_2 are perhaps more traumatic, such as forced swimming [14], daily restraint for 5 hours [15], or immune infection [16]. Since stress-induced changes in E_2 and P_4 may have serious implications for female reproduction [33], it is important to explore further the relationship between stress and ovarian/adrenal sources of these steroids.

One issue with urinary measures is the fact that stress and social conditions can perturb patterns of drinking and urination. As is well known in laboratory rodents, acute stressors can induce immediate urination, which may be absent in controls, meaning that steroids in subsequent urinary measures can reflect a different interval of time in the two conditions. Also, stress and social exposure can induce polyuria and polydipsia in mice [34-36]. Increased urinary dilution can lead to reduced creatinine levels [36], as was observed in the urinary samples taken immediately after stressor offset in Experiment 2. As other constituents of urine such as unadjusted steroids are also reduced by greater

urinary dilution, we suggest that creatinine-adjusted steroid measures more accurately reflect steroid dynamics in the immediate aftermath of an acute stressor.

In female mice, both urinary and serum measures of corticosterone and P_4 respond to an acute, mild stressor in a similar manner, but urinary steroid measures show more inter-individual variability, and also remain elevated after stress longer compared to serum measures. Both serum and urinary steroid measures in response to stress have value. The prolonged return to pre-stress baseline levels in urinary corticosterone and P_4 may allow for the confirmation of stress-induced rises in these steroids in studies where rapid blood sampling within 2 hours of stressor offset is not possible. Moreover, in circumstances where repeated sampling from the same individuals is desired, urine collection allows for non-terminal sampling, is less invasive, and is less likely to confound results than is blood collection, therefore urinary steroid measures may be more convenient. On the other hand, the smaller variability in serum steroid measures between individuals may allow documentation of clear stress-induced changes in steroids even when small sample sizes must be used. Therefore, we suggest that urinary or serum steroid measures in response to stress be used depending on experimental design.

Acknowledgements

This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada to D. deCatanzaro.

References

1 *McEwen BS, Wingfield JC.* The concept of allostasis in biology and biomedicine. Horm Behav 2003; 43: 2-15.

2 *Korte SM, Koolhaus JM, Wingfield JC, McEwen BS*. The Darwinian concept of stress: Benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. Neurosci Biobehav R 2005; 29: 3-38.

3 *Sapolsky RM*. The endocrine stress-response and social status in the wild baboon. Horm Behav 1982; 16: 279-292.

4 *Hargreaves AL, Hutson GD.* The stress response in sheep during routine handling procedures. Appl Anim Behav Sci 1990; 26: 83-90.

5 *Nepomnaschy PA, Welch KB, McConnell DS, Low BS, Strassmann BI, England BG*. Cortisol levels and very early pregnancy loss in humans. PNAS 2006; 103: 3938-3942.

6 *Young AJ, Carlson AA, Monfort SL, Russell AF, Bennett NC, Clutton-Brock T*. Stress and the suppression of subordinate reproduction in cooperatively breeding meerkats. PNAS 2006; 103: 12005-12010.

7 *Thorpe JB, Rajabi N, deCatanzaro D*. Circadian rhythm and response to an acute stressor of urinary corticosterone, testosterone, and creatinine in adult male mice. Horm Metab Res 2012; 44: 429-435.

8 *Touma C, Palme R, Sachser N.* Analyzing corticosterone metabolite in fecal samples of mice: A noninvasive technique to monitor stress hormones. Horm Behav 2004; 45: 10-22.

9 *Plas-Roser S, Aron C.* Stress related effects in the control of sexual receptivity and the secretion of progesterone by the adrenals in cyclic female rats. Physiol Behav 1981; 27: 261-264.

10 *Resko JA.* Endocrine control of adrenal progesterone secretion in the ovariectomized rat. Science 1969; 164: 70-71.

11 *Watson ED, Munro CD*. Adrenal progesterone production in the cow. Brit Vet J 1984; 140: 300-306.

12 *Asher GW, Peterson AJ, Duganzich D*. Adrenal and ovarian sources of progesterone secretion in young female fallow deer, *Dama dama*. J Reprod Fertil 1989; 85: 667-675.

13 *Galea LM, McEwen BS, Tanapat P, Deak T, Spencer RL & Dhabhar FS*. Sex differences in dendritic atrophy of CA3 pyramidal neurons in response to chronic restraint stress. Neuroscience 1997; 81: 689-697.

14 *Shors TJ, Pickett J, Wood G, Paczynski M.* Acute stress persistently enhances estrogen levels in the female rat. Stress 1999; 3: 163-171.

15 *MacNiven E, deCatanzaro D, Younglai EV.* Chronic stress increases estrogen and other steroids in inseminated rats. Physiol Behav 1992; 52: 159-162.

16 *Misdrahi D, Pardon MC, Perez-Diaz F, Hanoun N, Cohen-Salmon C*. Prepartum chronic ultramild stress increases corticosterone and estradiol levels in gestating mice: Implications for postpartum depressive disorders. Psychiat Res 2005; 137: 123-130.

17 *Agrawal V, Jaiswal MK, Jaiswal YK*. Lipopolysaccharide induces alterations in ovaries and serum level of progesterone and 17β-estradiol in the mouse. Fertil Steril 2011; 95: 1471-1474.

18 *Wood GE, Shors TJ*. Stress facilitates classical conditioning in males, but impairs classical conditioning in females through activational effects of ovarian hormones. PNAS 1998; 95: 4066-4071.

19 *Gärtner K, Büttner D, Döhler K, Friedel R, Lindena J, Trautschold I*. Stress response of rats to handling and experimental procedures. Lab Anim 1980; 14: 267-274.

20 *deCatanzaro D, Muir C, Beaton EA, Jetha M*. Non-invasive repeated measurement of urinary progesterone, 17β-estradiol, and testosterone in developing, cycling, pregnant, and postpartum female mice. Steroids 2004; 69: 687-696.

21 *Vella ES, deCatanzaro D*. Novel male mice show gradual decline in the capacity to disrupt early pregnancy and in urinary excretion of testosterone and 17β-estradiol during the weeks immediately following castration. Horm Metab Res 2001; 33: 681-686.

22 *deCatanzaro D, Muir C, Beaton E, Jetha M, Nadella K*. Enzymeimmunoassay of oestradiol, testosterone and progesterone in urine samples from female mice before and after insemination. Reproduction 2003; 126: 407-414.

23 *Thorpe JB, deCatanzaro D.* Oestradiol treatment restores the capacity of castrated males to induce both the Vandenbergh and the Bruce effects in mice (*Mus musculus*). Reproduction 2012; 143: 123-132.

24 *Touma C, Sachser N, Möstl E, Palme R*. Effects of sex and time on metabolism and excretion of corticosterone in urine and feces of mice. Gen Comp Endocr 2003; 130: 267-278.

25 *Viau V, Meaney MJ*. Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. Endocrinology 1991; 129: 2503-2511.

26 *Khan A, Bellefontaine N, deCatanzaro D*. 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. Physiol Behav 2008; 93: 588-594.

27 *Muir C, Vella ES, Pisani N, deCatanzaro D*. Enzyme immunoassay of 17β-estradiol, estrone conjugates, and testosterone in urinary and fecal samples from male and female mice. Horm Metab Res 2001; 33: 653-658.

28 *Munro CJ, Stabenfeldt GH, Cragun JR, Addiego LA, Overstreet JW, Lasley BL.* Relationship of serum estradiol and progesterone concentrations to the excretion profiles of their major urinary metabolites as measured by enzyme immunoassay and radioimmunoassay. Clin Chem 1991; 37: 838-844.

29 *Perkins LM, Payne AH*. Quantification of $P450_{scc}$, $P450_{17\alpha}$, and iron sulfur protein reductase in leydig cells and adrenals of inbred strains of mice. Endocrinology 1988; 123: 2675-2682.

30 *van Weerden WM, Biering HG, van Steenbrugge GJ, de Jong FH, Schröder FH*. Adrenal glands of the mouse and rat do not synthesize androgens. Life Sci 1992; 50: 857-861.

31 *Tait AD, Hodge LC*. Adrenal androgen biosynthesis by a sesterpene pathway. J Steroid Biochem 1998; 30: 485-488.

32 *Touitou Y, Auzeby A, Bogdan A*. Cortisol and cortisone production in rat and mouse adrenal incubations. J Steroid Biochem 1990; 37: 279-284.

33 *deCatanzaro D.* Blastocyst implantation is vulnerable to stress-induced rises in endogenous estrogens and also to excretions of estrogens by proximate males. J Reprod Immunol 2011; 90: 14-20.

34 *Maruniak JA, Owen K, Bronson FH, Desjardins C.* Urinary marking in male house mice: responses to novel environmental and social stimuli. Physiol Behav 1974; 12: 1035-1039.

35 *Levine R, Levine S.* Role of the pituitary-adrenal hormones in the acquisition of schedule-induced polydipsia. Behav Neurosci 1989; 103: 621-637.

36 *deCatanzaro D, Khan A, Berger RG, Lewis E*. Exposure to developing females induces polyuria, polydipsia, and altered urinary levels of creatinine, 17β-estradiol, and testosterone in adult male mice (*Mus musculus*). Horm Behav 2009; 55: 240-247.

Chapter 5

Estrogen-progesterone balance in the context of blastocyst implantation failure induced by predator stress

Joelle B. Thorpe, Paige S. Burgess, Marta Sadkowski & Denys deCatanzaro

Psychoneuroendocrinology (Accepted for publication September 3, 2013)

Authors' Contributions

Joelle B. Thorpe: Experimental design, data collection, data analysis, manuscript writing, editing, and preparation.

Paige S. Burgess: Assistance with data collection and analysis.

Marta Sadkowski: Assistance with data collection and analysis.

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

Summary

Diverse stressors can disrupt blastocyst implantation in inseminated female mammals. Stress-induced implantation failure can be mimicked by minute doses of exogenous estradiol, and some evidence indicates that it may be mitigated by exogenous progesterone. In Experiment 1, we showed that acute exposure to a rat across a wire-mesh grid caused elevation of corticosterone and progesterone. In Experiment 2, we showed that exposure of inseminated mice to rats across a grid during gestation days 1-5 was associated with avoidance of proximity to the grid and a significantly reduced number of implantation sites on gestation day 6. Rat-exposure also resulted in elevated progesterone levels in females that maintained their pregnancies, and elevated estradiol levels in females that lost their pregnancies. In Experiment 3, we investigated whether exogenous progesterone, estradiol, or a combination of both could influence implantation failure induced by rat-exposure stress. Treatment with 100 ng estradiol per day on gestation days 1-5 induced a complete absence of implantation sites on gestation day 6, regardless of the presence or absence of the stressor. Administration of 500 μg progesterone per day was insufficient to prevent the stress-induced pregnancy loss. However, 500 μg progesterone plus 10 ng estradiol per day did prevent implantation failure in rat-exposed females. These findings are consistent with the hypothesis that estradiol elevations contribute to stress-induced pregnancy loss, but show paradoxically that low doses of estradiol can act together with progesterone to mitigate stress-induced pregnancy loss.

Introduction

Mammalian reproduction is sensitive to suppression by stress, especially in females (reviewed in deCatanzaro and MacNiven, 1992; Tilbrook et al., 2000). Blastocyst implantation in females, including women (Gallinelli et al., 2001), is particularly vulnerable to stress. Diverse stressors, such as social subordination (Huck et al., 1988), heat (Garcia-Ispierto et al., 2006), physical restraint (MacNiven and deCatanzaro, 1990), loud noise (Joachim et al., 2003), and predator exposure (deCatanzaro, 1988), can cause early pregnancy to fail. As implantation marks a transition early in pregnancy from relatively minimal to more energetically costly investment on the part of the female, stress-induced implantation failure is arguably adaptive, allowing females to abandon current reproductive efforts until more favorable conditions arise.

The process of implantation is highly complex, involving several endocrine and immune factors, and requiring the synchronized development of both the uterus and blastocyst (reviewed in Dey et al., 2004). For most of the reproductive cycle, the uterus is a hostile environment for blastocysts. During a narrow and defined window, the uterus becomes receptive to blastocyst attachment and implantation. In mice, this window is tightly regulated by 17β-estradiol (E_2), with low levels being required for implantation to occur in uteri primed with progesterone (P_4), but slight E_2 elevations above optimal levels rendering the uterus completely refractory to implantation (Ma et al., 2003). Administration of minute doses of E_2 during the peri-implantation period causes pregnancy failure in mice (deCatanzaro et al., 1991). Ovarian hyperstimulation causing

increases in endogenous E_2 can disrupt implantation in mice (Fossum et al., 1989) and is associated with higher instances of failed implantation in humans (Simon et al., 1995). Supra-optimal estrogen levels can prematurely close the window of uterine receptivity (Ma et al., 2003), accelerate or retard transport of blastocysts through the fallopian tubes (Greenwald, 1967), and damage the developing blastocyst itself (Valbuena et al., 2001). The ratio of E_2 to P_4 in maternal circulation appears to be particularly important, with high E_2:P_4 ratios being detrimental to implantation in both mice and humans (Gidley-Baird et al., 1986; Safro et al., 1990).

Circulating and excreted glucocorticoids are used as reliable biomarkers of stress in a variety of species including humans (Nepomnaschy et al., 2006) and mice (Thorpe et al., 2012). The ovarian steroid response to stress is less well established. Three weeks of chronic stress in female rats can decrease E_2 levels (Galea et al., 1997). Shorter durations of stress in non-pregnant females can increase E_2 (Shors et al., 1999) and P_4 (Plas-Roser and Aron, 1981; Parker et al., 2011). In pregnant females, stress can increase E_2 (e.g., MacNiven et al., 1992; Misdrahi et al., 2005; Agrawal et al., 2011), but contrary to its effects in their non-pregnant counterparts, it can decrease P_4 (Agrawal et al., 2011, Parker et al., 2011). The stress-induced rise of E_2 has been hypothesized to contribute to implantation failure, since the administration of E_2 antibodies mitigates the pregnancy disrupting effects of restraint stress in mice (deCatanzaro et al., 1994). In addition, stress-induced pregnancy failure can be prevented if progesterone (MacNiven and deCatanzaro, 1990; Pratt and Lisk, 1991) or a progesterone analogue (Joachim et al., 2003) is

administered in conjunction with stress, which may indicate a role of stress-induced decreases in P_4 in implantation failure.

The current study employed a paradigm involving peri-implantation exposure of inseminated mice to rats across a wire-mesh grid, which previously produced a robust reduction of the proportion of females that were parturient (deCatanzaro, 1988). Here we established that this exposure is stressful to mice, in that they showed elevated corticosterone titers and avoided proximity to the rats. We showed that it significantly diminished the number of implantation sites and the proportion of females that remained pregnant on gestation day (GD) 6 while altering both P_4 and E_2 concentrations. We subsequently administered doses of E_2 and P_4 to rat-exposed and control mice to observe whether this could enhance or diminish the impact of stress. The data support the notion that a high ratio of E_2:P_4 in stressed females results in implantation failure.

Methods

Animals

All mice were C57BL/6 strain and all rats were Long Evans strain, obtained from Charles River (Quebec, Canada). Mice and rats had *ad lib* access to food and water throughout the experiment. Mice were housed in standard polypropylene cages measuring 28 cm x 16 cm x 11 cm (height) and rats were housed in standard polypropylene cages measuring 44 cm x 23 cm x 20 cm (height), unless otherwise stated. One week before pairing for insemination, all female mice were placed on a phytoestrogen-free diet (Advanced Protocol Verified Casein Diet 1 IF from Purina Mills,

Inc., Richmond, IN, USA). This diet is nutritionally similar to standard laboratory rodent chow, but contains < 1 ppm total isoflavones (aglycone equivalents of genistein, daidzein and glycitein). Females were kept on this diet for the remainder of the experiment. Mice were maintained at 21°C on a reversed 14:10 h light:dark cycle with the onset of the dark phase at 0900h. This research was approved by the McMaster University Animal Research Ethics Board, conforming to the standards of the Canadian Council on Animal Care.

Experiment 1: Corticosterone and P_4 response to acute rat exposure on GD 2

One week after being placed on the phytoestrogen-free diet, each female was paired with a male (2-5 months of age). Females were examined 3 times daily for the presence of a vaginal sperm plug. The day of sperm plug detection was labeled gestation day (GD) 0. At the onset of darkness on GD 1, females (average age 114.70 ± 6.35 d, and average weight 22.75 ± 0.50 g) were separated from inseminating males, randomly assigned to either the rat-exposed or isolated control group, and placed into exposure cages. Exposure cages were modified 20cm high polypropylene rat cages with a wire-mesh grid (0.5 cm^2) separating the rat compartment (28 cm x 23 cm) from the mouse compartment (8.5 cm x 23 cm) to limit direct physical contact between the mouse and rat.

On GD 1, all females were moved into an empty room for 24 h of habituation to the exposure cages. On GD 2, females in the rat-exposed group were moved into a room housing a colony of Long Evans rats. One male rat (3 to 9 months old) was placed into the rat side of each exposure cage. Isolated control females were similarly moved, but into an empty room, and the rat side of the exposure cage remained empty. Mice

remained in their respective holding rooms for 1 h. Mice were then anesthetized with Isoflurane, and a blood sample was obtained via cardiac puncture with a 1 mL tuberculine syringe and 23 ga needle within 3 min of removal from the holding rooms. All blood samples were obtained 1.5 to 3 h after onset of the dark cycle. Blood was allowed to clot for 1 to 2 h then centrifuged at 4000 rpm for 15 min, and serum was removed into a 1.5 mL Diamed vial and stored at -20°C for later progesterone and corticosterone quantification as described below.

Experiment 2: Effects of rat exposure on steroid levels and implantation

Insemination and stressor procedures

Insemination and sperm plug detection procedures were as described for Experiment 1. On GD 1, females (average age 62.05 ± 0.51 d, and average weight 18.66 ± 0.16 g) were randomly assigned to either the rat-exposed or isolated control group. Females in the rat-exposed condition were moved to the room housing the rat colony as described above. Each rat-exposed female was placed in an exposure cage containing one male Long Evans rat. Females in the isolated control group were moved to a room containing only other control females. These females were each placed into an exposure cage with the rat compartment left empty. All females remained in the exposure cages for five days (until GD 6).

Proximity to the rat

Daily observations of all mice were conducted from GD 1-5 during the middle of the dark cycle. Two 10 min blocks of time were spent in each room, with observers alternating between rat-exposure and control rooms every 10 min. During these 10 min

blocks, each cage was scanned every 2 min, and the location of the mouse within the cage with respect to the grid separating the rat and mouse compartments was recorded (for a total of 10 daily observations per mouse). Location with respect to the wire grid was standardized using lines drawn on the outside of the cages demarcating the halfway point between the far wall and the wire grid within the mouse compartment. A mouse was considered to be "proximal" if it was located in between the wire grid and the middle of the compartment, "distal" if it was located in between the middle of the compartment and the far wall of the cage, and "middle" if any part of its body was located on the halfway line between the far wall and the wire grid. The proportion of scans during which each mouse was recorded as proximal, distal, or middle was calculated (e.g., number of scans rated as "proximal" divided by the total number of scans) for each day from GD 1-5.

Blood and urine collection, and implantation site counts

On GD 6, females were removed from the exposure apparatus and anesthetized with Isoflurane. For a subset of females from each condition, a blood sample was obtained by cardiac puncture within 2 min of induction of anesthesia as described for Experiment 1, then the mouse was euthanized by cervical dislocation and a urine sample was collected. The remaining mice from each condition were simply euthanized without blood and urine collection. The uterus was then excised through a single abdominal incision. Implantation sites (round protuberances along each uterine horn) were counted and recorded. All blood and urine samples were collected 1-2.5 h after the start of the dark cycle. Urine was stored at -20°C for later hormone quantification. Blood was

allowed to clot for 1-2 h, centrifuged at 4000 rpm for 15 min, serum was stored in 1.5 mL Diamed vials at -20°C for later hormone quantification as described below.

Experiment 3: Impact of stress and exogenous E_2 and P_4 on implantation

Pairing, sperm plug detection, and rat-exposure procedures were followed as described for Experiments 1 and 2. On GD 1, females (average age 74.05 ± 0.98 d, and average weight 19.66 ± 0.14 g) were randomly assigned to either the isolated control or rat-exposed condition, and were placed in exposure cages and housed in two separate rooms as described for Experiment 2. Within each of these two conditions, females were further assigned to one of six dosage groups. Approximately 1.5 h after onset of the dark cycle each day from GD 1 to GD 5, each female received a 0.05 mL sc injection of peanut oil containing no steroids (control), 10ng E_2, 100ng E_2, 500µg P_4, 10ng E_2 + 500µg P_4, or 100ng E_2 + 500µg P_4. All steroids were from Sigma-Aldrich. On GD 6, approximately 1.5 to 2.5 h after onset of the dark cycle, females were euthanized by cervical dislocation, and implantation sites were counted as described for Experiment 2.

Measurement of steroids in serum and urine

To quantify corticosterone and progesterone in female serum, assays previously validated for male urinary and serum corticosterone (Thorpe et al., 2012) and female urinary P_4 (deCatanzaro et al., 2003) were modified and validated for female serum corticosterone and P_4. Serially diluted pools of female serum were compared to a corticosterone or P_4 standard curve plotted against logarithmically-transformed concentrations of steroid. This test indicates whether the steroid is present in measurable quantities in serum, and whether serum samples react with the antibodies in a predictable

fashion. It also allows for the determination of the dilution of serum that falls at 50% conjugate binding. Serum androstenedione was measured using a commercial enzyme immunoassay kit (Neogen Corporation, Lexington, KY, USA). Kit directions were followed exactly. Urinary E_2 methods were previously validated (Muir *et al.,* 2001). Creatinine, corticosterone, E_2, and P_4 were obtained from Sigma-Aldrich. Antibodies to corticosterone, E_2, and P_4, and corresponding horseradish peroxidase (HRP) conjugates were obtained from the Department of Population Health and Reproduction at the University of California, Davis.

Steroid assays were conducted with duplicate readings per sample; the average was used in statistical analysis. NUNC Maxisorb plates were first coated with 50 µL corticosterone, P_4, or E_2 antibody stock diluted 1:10,000 in a coating buffer (50 mmol/L bicarbonate buffer, pH 9.6), then stored for 12-14 h at 4°C. Wash solution (0.15 mol/L NaCl solution containing 0.5 ml/L Tween 20) was added to each well to rinse away any unbound antibody, then 50 µl phosphate buffer/well was added. The plates were incubated at room temperature for 1 h for corticosterone and P_4, and 2 h for E_2, before adding standards, samples, and controls. Serum samples were diluted 1:18 (corticosterone) or 1:7 (P_4), and urine samples were diluted 1:8 (E_2) in phosphate buffer (0.1 mol/L sodium phosphate buffer, pH 7.0 containing 8.7 g NaCl and 1 g BSA/L) before being added to the plate. Standard curves were derived by serial dilution from a known stock solution. For all assays, 50 µL corticosterone (1:140,000 in phosphate buffer), P_4 (1:60,000 in phosphate buffer), or E_2 (1:350,000 in phosphate buffer) HRP was added to each well, with 50 µL standard, sample, or control (20 µL for E_2 plates).

The plates were incubated for 2 h at room temperature, washed, and then 100 μL substrate solution of citrate buffer, H_2O_2 and 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), was added to each well. The plates were then covered and incubated, while shaking at room temperature for 25 min to 1.5 h. The plates were read with a single filter at 405 nm on a microplate reader (Bio-Tek Instruments Inc, ELx 808). In all assays, optical densities were obtained, standard curves were generated, a regression line fit, and samples were interpolated into the equation to get a value in pg per well.

Following convention for urinary steroid measures (deCatanzaro et al., 2003; Muir et al., 2001), urinary creatinine was also measured. All urine samples were diluted 1:39 in phosphate buffer. Using Dynatech Immulon flat bottom plates, 50 μL/well of standard was added with 50 μL distilled water, 50 μL 0.75 M NaOH and 50 μl 0.4 M picric acid. The plate was then shaken and incubated at room temperature for 30 min. The plate was measured for optical density on the 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. The average of duplicates was taken for each sample.

Statistical analyses

Comparisons of continuous variables by experimental group, such as hormone concentration in Experiment 1 and implantation site counts in Experiment 2 were conducted using Student's t-tests. In Experiment 2, where experimental group and presence/absence of pregnancy were used as predictors of hormone concentration, standard least squares ANOVA models were used. Post hoc multiple comparisons used

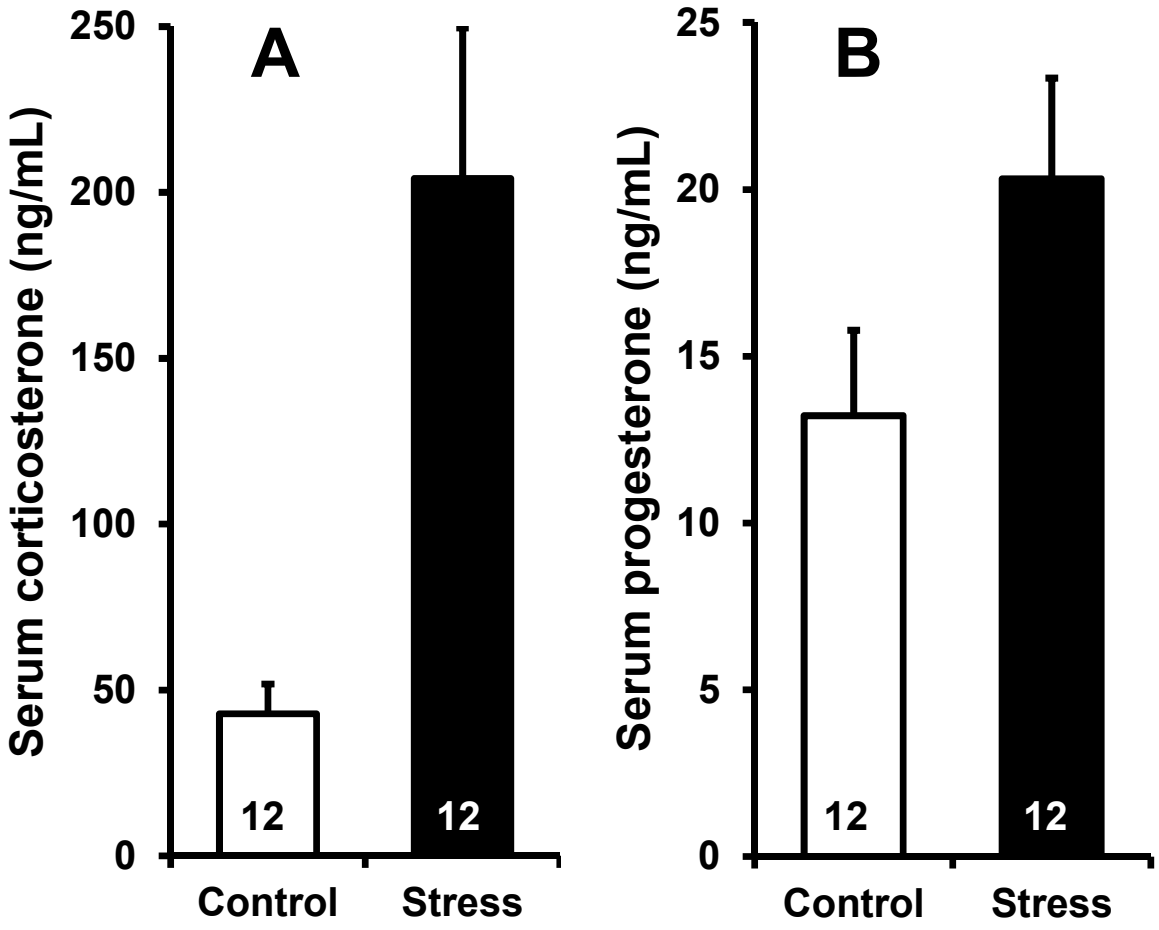
Duncan's test ($p < 0.05$). In Experiments 2 and 3, comparisons of presence or absence of pregnancy between experimental groups were done using the Pearson Chi Square test of association. Behavior during isolation or rat exposure in Experiment 2 was analyzed using Wilcoxon rank sum tests. All α levels were set at the conventional level of $p < 0.05$.

Results

Experiment 1: Corticosterone and P_4 response to acute rat exposure on GD 2

As expected, rat exposure on GD 2 increased serum corticosterone, $t(22) = 3.48$, $p = 0.001$, and P_4, $t(22) = 1.79$, $p = 0.042$ compared to isolated controls (Fig. 1A, B).

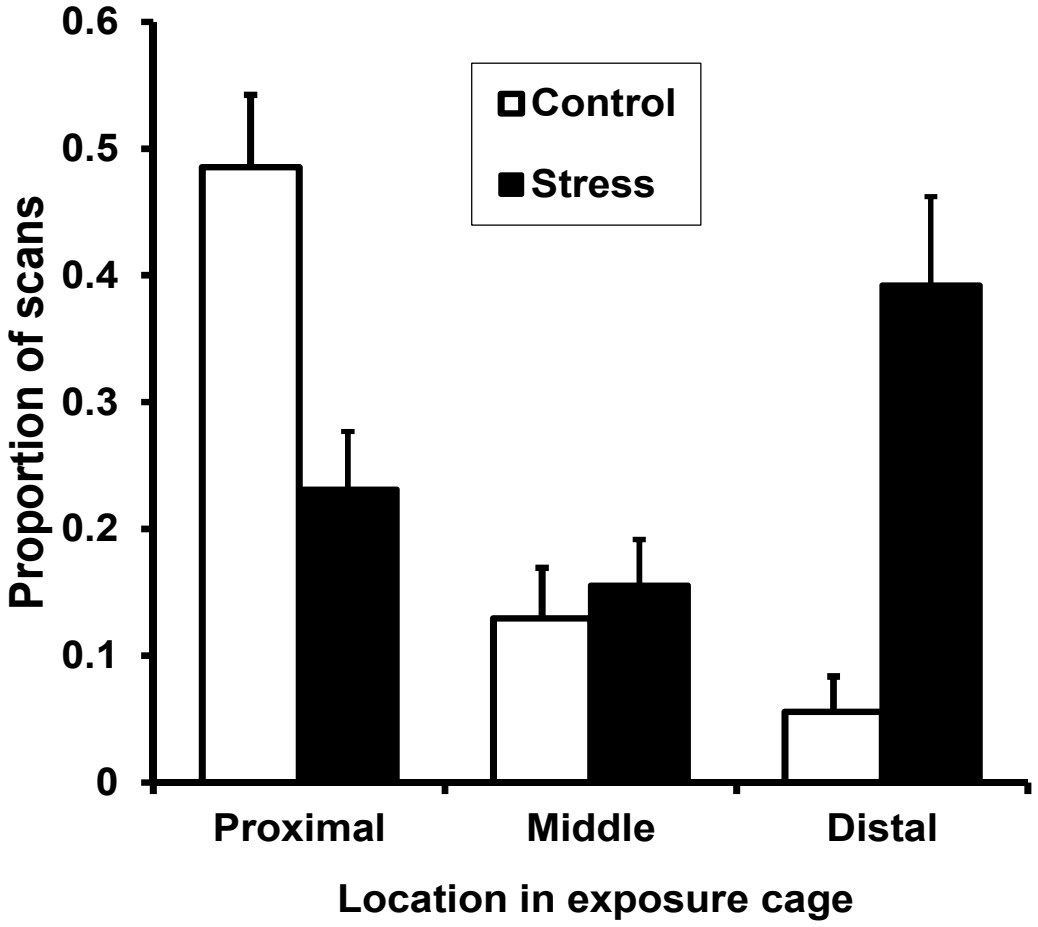
Figure 1 Mean serum A) corticosterone and B) progesterone concentration (± SEM) on GD 2 after 1 h of isolation (Control) or rat exposure (Stress). Sample size is denoted for each group on the bars.



Experiment 2: Effects of rat exposure on steroid levels and implantation

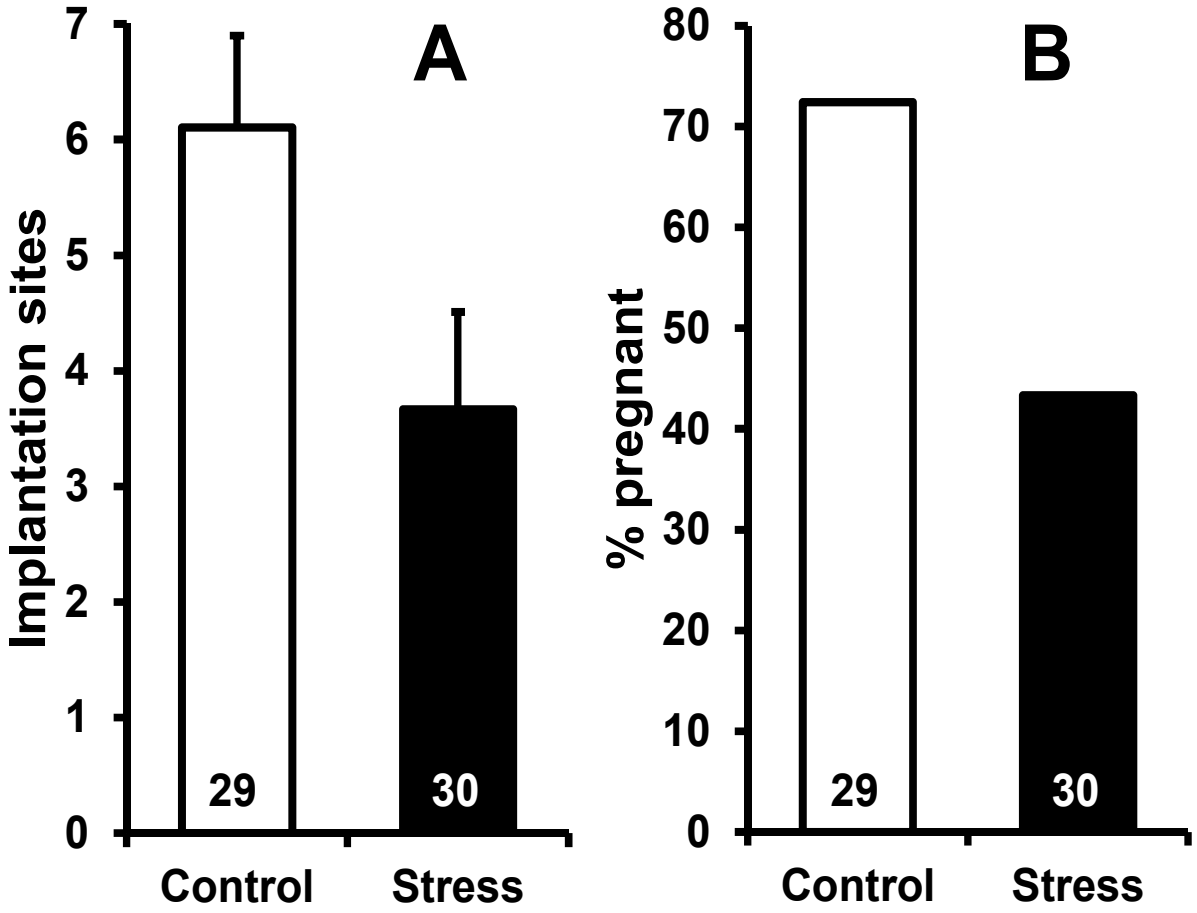
Observations of behavior indicated that the mice in the rat-exposed condition avoided areas of their compartment near the grid that separated them from the rat (Fig. 2). During observations on every day from GD 1 to GD 5, control females were more often found proximal to the grid (Wilcoxon, all $p < 0.05$), and rat-exposed females were more often found distal to the grid (Wilcoxon, all $p < 0.001$). There was no difference between experimental groups in the proportion of scans during which the females were found in the middle of the compartment. When proportions across all 5 days were averaged within experimental groups, the same trends were seen, with isolated control females spending a higher proportion of scans proximal to the grid compared to rat-exposed females ($W = 534.5$, $p < 0.0001$), rat-exposed females spending a higher proportion of scans distal to the grid compared to isolated control females ($W = 201$, $p < 0.0001$), and no difference between groups in proportion of scans spent in the middle of the compartment.

Figure 2 Mean proportion of scans (± SEM) isolated control (empty bars) and rat-exposed (black bars) females were located close to the rat compartment (Proximal), in the middle of the mouse compartment (Middle) or far from the rat compartment (Distal). Each mouse was scanned 10 times/day from GD 1 to GD 5. Proportions were calculated as described in the text, and were averaged over all 5 exposure days (GD 1 to GD 5) to produce one proportion for each location per mouse. There were 19 control and 20 stressed females.



The percentage of females that were pregnant (defined as those with at least one implantation site on GD 6) and the total number of implantation sites on GD 6 were decreased by rat exposure compared to isolated controls (Fig. 3A, B). Exposure to a rat from GD 1 to GD 5 significantly decreased the number of implantation sites, $t(57) = 2.10$, $p = 0.019$. A chi-square test of association indicated that rat-exposure significantly reduced the likelihood of pregnancy on GD 6, $\chi^2(1) = 5.11$, $p = 0.024$.

Figure 3 A) Mean number of implantation sites (± SEM) counted on GD 6 after 5 days of isolation (Control) or rat exposure (Stress). B) Percentage of isolated control (Control) and rat-exposed (Stress) females pregnant on GD 6 (defined as those females with at least one implantation site). Sample sizes are denoted for each group on the bars.



Serum P_4 on GD 6 after 5 days of isolation or rat-exposure varied depending on whether or not pregnancy was maintained (Fig. 4). There was a main effect of pregnancy on serum P_4 levels, $F(1, 34) = 18.05$, $p = 0.0003$, and an interaction between pregnancy and experimental group, $F(1, 34) = 13.72$, $p = 0.001$. Post hoc comparisons revealed that rat-exposed females that maintained their pregnancy had significantly higher P_4 levels than all other females, and that rat-exposed females that lost their pregnancies had significantly lower P_4 levels than all other females. Serum androstenedione was not substantially influenced by experimental treatment or pregnancy (nonpregnant controls, n = 6, 1.80±0.18 ng/mL; pregnant controls, n = 12, 1.91±0.06 ng/mL; nonpregnant rat-exposed, n = 12, 1.70±0.13 ng/mL; pregnant rat-exposed, n = 7, 1.98±0.18 ng/mL). Urinary creatinine was also not influenced by experimental group or whether the females were pregnant (nonpregnant controls, n = 4, 0.43±0.11 mg/mL; pregnant controls, n = 11, 0.49±0.05 mg/mL; nonpregnant rat-exposed, n = 5, 0.60±0.08 mg/mL; pregnant rat-exposed, n = 6, 0.53±0.03 mg/mL). Therefore, analyses were carried out on raw urinary E_2. Urinary E_2 was increased in rat-exposed mice, particularly those that lost their pregnancies (Fig. 5). There was a main effect of experimental group on urinary E_2 levels, $F(1, 22) = 5.36$, $p = 0.03$, but no effect of pregnancy or interaction between pregnancy and experimental group. Post hoc comparisons indicated that rat-exposed females that lost their pregnancies had significantly higher urinary E_2 levels than control females that were either pregnant or not.

Figure 4 Mean serum progesterone (± SEM) concentration on GD 6 after 5 days of isolation (empty bars) or rat exposure (black bars), in females with at least one implantation site counted on GD 6 (Pregnant) or no implantation sites counted on GD 6 (Not pregnant). Sample size is denoted for each group on the bars.

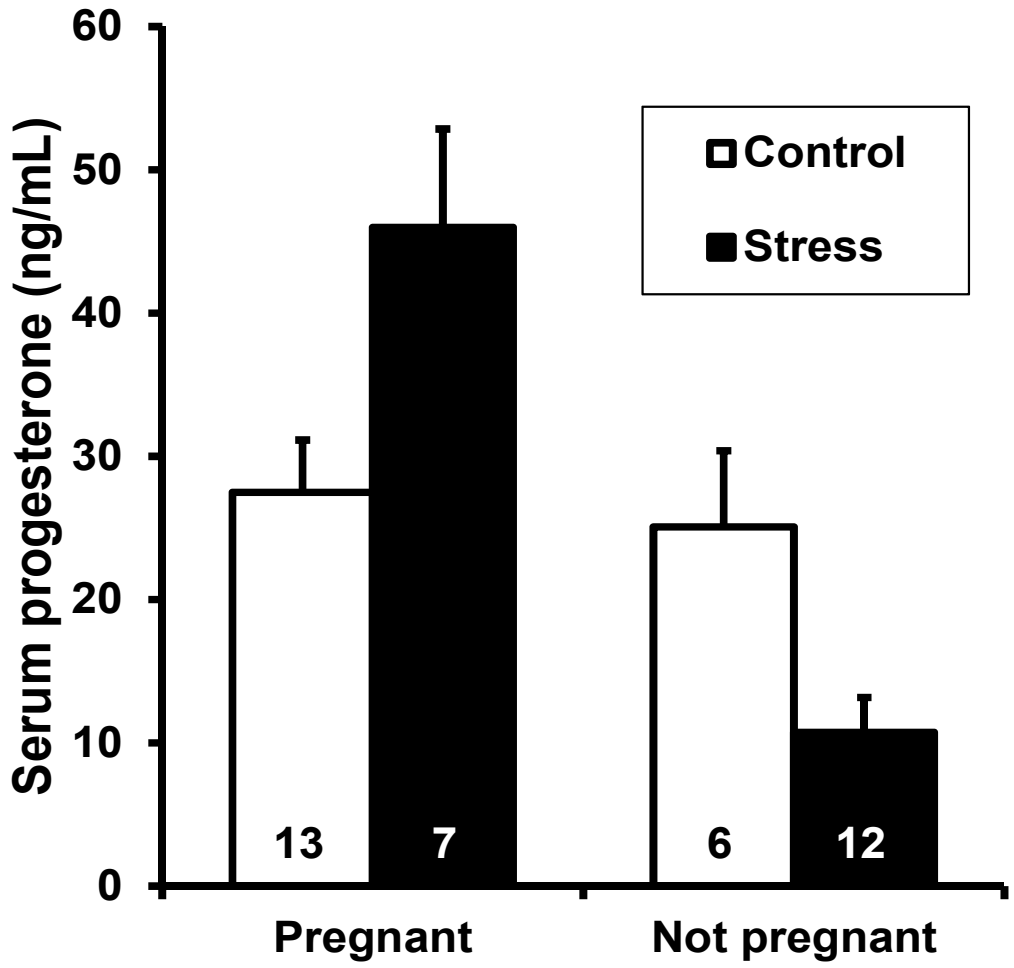
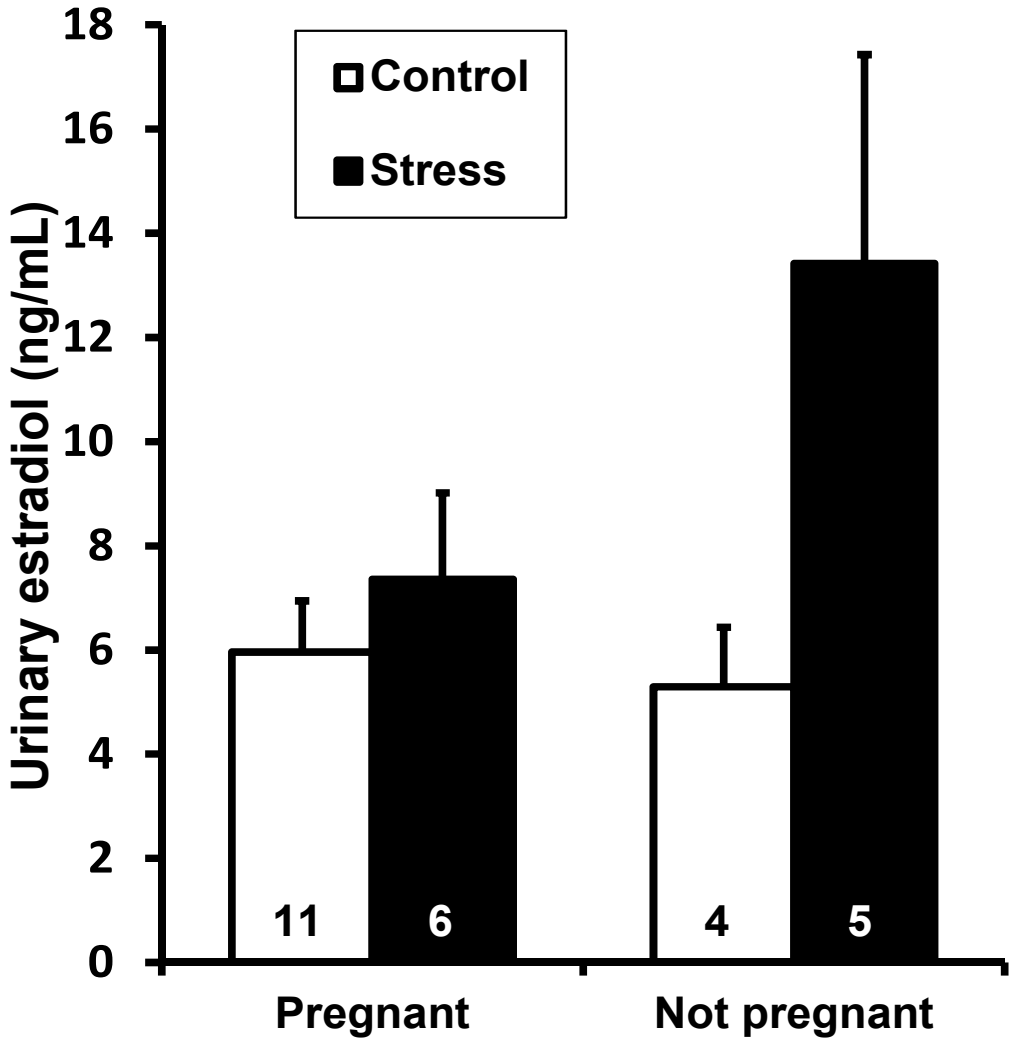


Figure 5 Mean urinary estradiol (± SEM) concentration on GD 6 after 5 days of isolation (empty bars) or rat exposure (black bars), in females with at least one implantation site counted on GD 6 (Pregnant) or no implantation sites counted on GD 6 (Not pregnant). Sample size is denoted for each group on the bars.



Experiment 3: Impact of stress and exogenous E_2 and P_4 on implantation

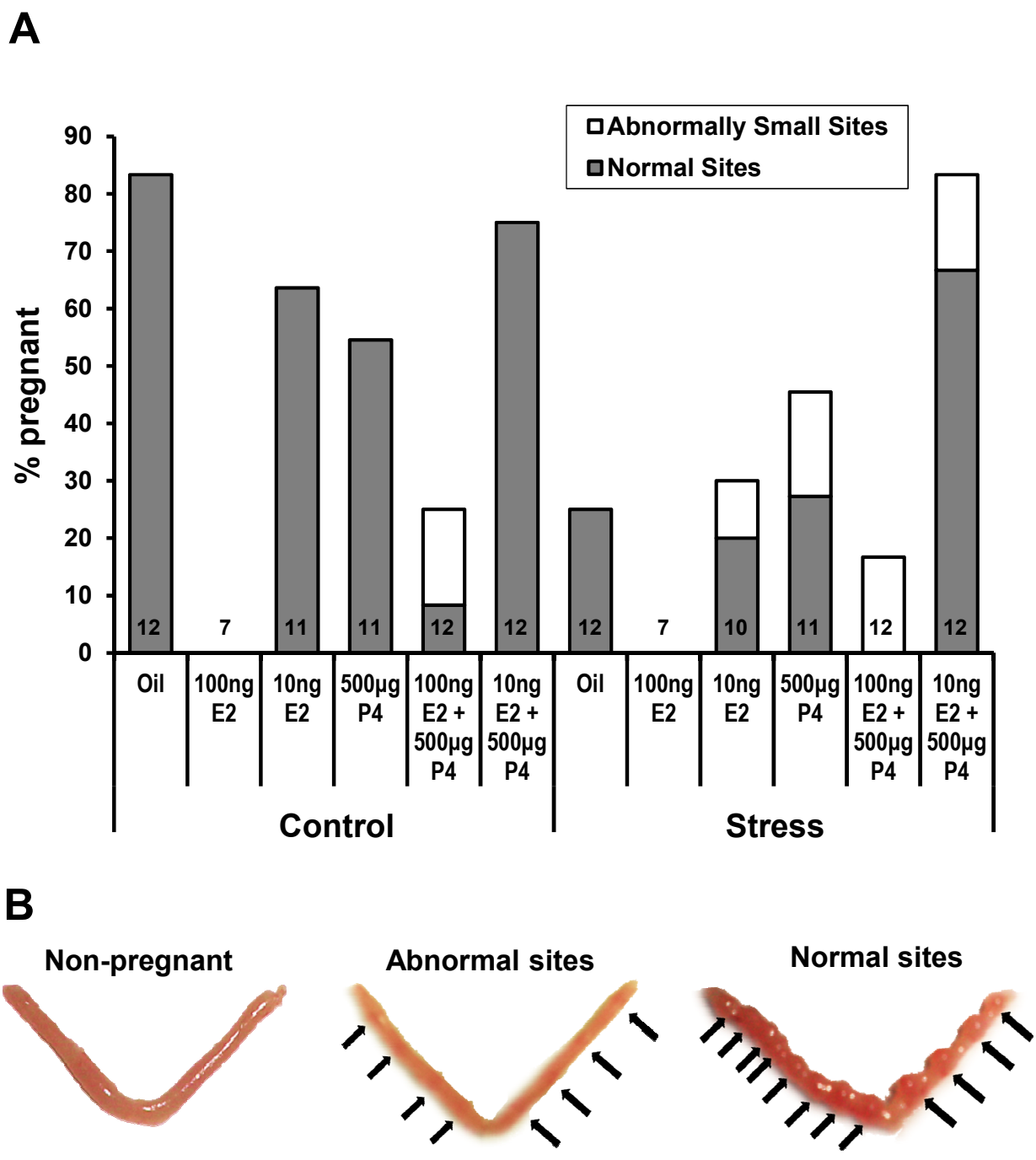
Measures of the number of implantation sites and the percentage of females pregnant on GD 6 showed a similar profile across conditions (Fig. 6A). There were significantly higher pregnancy rates on GD 6 in isolated control + oil females than in rat-exposed + oil females, $\chi^2(1) = 8.22$, $p = 0.004$. In some of the other conditions, it was noted that in a minority of females, the only observable implantation sites were exceptionally small and barely detectable (Fig. 6B). These results were analyzed in two ways, one considering all females but only counting those with healthy and normal sites as pregnant, and the second excluding females with abnormal sites from the statistics altogether.

Among the isolated controls, pregnancy rates did not differ between oil treated mice and each of 10ng E_2, 500µg P_4, and 10ng + 500µg P_4 injection groups, and all sites observed were normal. A dose of 100ng E_2 completely prevented implantation and significantly differed from the oil injection group, $\chi^2(1) = 12.32$, $p = 0.0004$. Three control females in the 100ng E_2 + 500µg P_4 dosage group had abnormal (very small) implantation sites; comparison of this condition to isolated oil-treated controls was significant both when these females were included, $\chi^2 (1) = 13.59$, $p = 0.0002$, and when they were excluded, $\chi^2 (1) = 10.76$, $p = 0.001$.

Among the females exposed to rats (stressed), pregnancy rates compared to oil injected females were not affected by 10ng E_2, 100ng E_2, 500µg P_4, or 100ng E_2 + 500µg P_4, regardless of whether the few cases involving abnormal sites were included. Notably, injections of 10ng E_2 + 500µg P_4 completely mitigated stress-induced pregnancy failure.

In comparison to the stressed oil-treated females, this trend held both when two females in the 10ng E_2 + 500µg P_4 condition with abnormally small sites were included, χ^2 (1) = 4.20, p = 0.041, and when they were excluded, χ^2 (1) = 6.60, p = 0.015. The comparison of stressed females that received 500 µg P_4, to those that received 10ng E_2 + 500µg P_4 approached the level of conventional significance when females with abnormal sites were included, χ^2 (1) = 3.57, P = 0.059, and reached significance when they were excluded, χ^2 (1) = 4.23, p = 0.040.

Figure 6 A) Percentage of females pregnant on GD 6 with normal implantation sites (grey bars) or abnormally small implantation sites (white bars) after 5 days of isolation (Control) or rat exposure (Stress) and 5 daily injections of oil, 100ng E_2, 10ng E_2, 500µg P_4, 100ng E_2 + 500µg P_4, or 10ng E_2 + 500µg P_4. Sample size is denoted for each group on the bars. B) Photographs of uteri containing no implantation sites (non-pregnant), abnormally small implantation sites, and normal implantation sites. Arrows indicate implantation sites.


A
Abnormally Small Sites
Normal Sites
% pregnant
90
80
70
60
50
40
30
20
10
0
12
7
11
11
12
12
12
7
10
11
12
12
Oil
100ng E2
10ng E2
500µg P4
100ng E2 + 500µg P4
10ng E2 + 500µg P4
Oil
100ng E2
10ng E2
500µg P4
100ng E2 + 500µg P4
10ng E2 + 500µg P4
Control
Stress
B
Non-pregnant
Abnormal sites
Normal sites

Discussion

Since wild and laboratory rats are known to kill mice in a predatory manner (Karli, 1956), it is not surprising that exposure to a rat is stressful for mice during early pregnancy. Increases in corticosterone in rat-exposed females and their avoidance of proximity to the rat validate the interpretation that such exposure constitutes a stressor. Since P_4 is a metabolic precursor to glucocorticoids, increased circulating P_4 after rat-exposure is likely of adrenal origin, due to upregulation of the adrenal cortex in stress (Plas-Roser and Aron, 1981). Previous work has shown that as little as 10 min of indirect rat exposure increases avoidance, freezing, and corticosterone in male mice (Amaral et al., 2010). The current study is the first to explore behavioral, corticosterone, and P_4 responses to rat exposure in early pregnant female mice.

We found that P_4 dynamics on GD 6 showed a strong interaction between the presence/absence of stress and pregnancy. Among control females, P_4 levels were similar regardless of whether or not they were pregnant. This could be due to pseudopregnancy in the non-pregnant controls, as all mice were proven by sperm plugs to have mated, and P_4 levels are similar in pseudopregnancy and true pregnancy (Garland et al., 1987). In contrast, we found that among stressed females, there was a stark difference in P_4 between those that maintained and those that lost their pregnancies. Females that maintained their pregnancies in the face of stress had significantly higher P_4 than all other females, which could suggest that high endogenous P_4 allows females to resist the negative impacts of stress on implantation. Females that lost their pregnancies while stressed showed significantly lower P_4 than all other animals in this experiment. This

may suggest that females with naturally lower P_4 in early pregnancy are more vulnerable to stress, or that the reduction in progesterone is a result of pregnancy loss due to stress, or that stress causes reductions in progesterone that in turn cause pregnancy to fail.

There is much support for the last of these explanations, that pregnancy is lost in response to lowered P_4 levels. In pregnant rodents, other stressors are associated with reduced P_4, including acute immune insults, fasting, noise, and social subordination (Huck et al., 1988; Joachim et al., 2003; Parker et al., 2011). Stress-induced early pregnancy loss can be mitigated by exogenous P_4 (MacNiven and deCatanzaro, 1990; Pratt and Lisk, 1991), progestin-mimicking drugs (Joachim et al., 2003), and drugs blocking the conversion of P_4 to corticosterone (MacNiven and deCatanzaro, 1990). Stress-induced reductions of P_4 lead to decreased progesterone-induced blocking factor (Joachim et al., 2003), a protein that maintains an appropriate balance of cytokines required for successful pregnancy (Szekeres-Bartho and Wegmann, 1996; Szekeres-Bartho et al., 1997). Decreases in P_4 may also prevent uterine closure around the implanting blastocyst. Luminal closure permits the uterine walls to come into close contact with the blastocyst and each other (Reinius, 1967), and is also associated with increased endocytotic activity of the uterine glands (Parr and Parr, 1977) resulting in reduced fluid in the uterine lumen. Exogenous P_4 increases such endocytotic activity, resulting in luminal closure (Parr, 1983; Salleh et al., 2005).

We measured E_2 in urine rather than serum since there is some debate over the ability of immunoassay methods to quantify circulating E_2 reliably in mice (Haisenleder et al., 2011). Urinary E_2 in mice is dynamic and largely reflects systemic trends (Muir et

al., 2001; deCatanzaro et al., 2004; Thorpe and deCatanzaro, 2012), and higher urinary E_2 levels on GD 2 correlate with smaller litter sizes at parturition (deCatanzaro et al., 2003). Here, the trends seen on GD 6 in urinary E_2 were opposite to those observed for P_4, with higher E_2 levels exhibited in stressed females than in controls, with the trend being especially strong in those that lost their pregnancies. This is consistent with the fact that elevated E_2 levels, slightly above those necessary for induction of uterine receptivity, are detrimental for implantation (Fossum et al., 1989; Simon et al., 1995; Ma et al., 2003).

E_2 may impede implantation by preventing luminal closure and decreasing expression of adhesion molecules such as e-cadherin. Contrary to P_4, E_2 increases fluid secretion into the uterine lumen, which physically should prevent closure (Parr, 1983; Salleh et al., 2005). E_2 may also prevent luminal closure by reducing adhesion of opposing uterine walls. During the peri-implantation period, adhesion molecules including e-cadherin (Jha et al., 2006) migrate towards the apical surface of the uterine epithelial cells as the blastocyst begins to implant and the lumen closes (Hyland et al., 1998). Adhesion molecules may also be important for initial blastocyst attachment to the uterine epithelium (Rahnama et al., 2009). E-cadherin is downregulated in various reproductive tissues by E_2 (Potter et al., 1996; Oesterreich et al., 2003), suggesting a mechanism through which stress-induced increases in E_2 may contribute to implantation failure. In contrast, P_4 upregulates e-cadherin (Jha et al., 2006), so it is also possible that reduced P_4 during stress also contributes to implantation failure by preventing expression of e-cadherin.

The sources of elevations of estradiol in pregnant females during stress are not established, in contrast to well-known suppressive effects of stress on the hypothalamic-pituitary-gonadal axis (Rivier and Rivest, 1991) and some evidence that glucocorticoids can inhibit estradiol action at the uterus (Rabin et al., 1990). Nevertheless, there are several observations that estradiol can be elevated by stressors during pregnancy (MacNiven et al., 1992; Misdrahi et al., 2005; Agrawal et al., 2011) and in some circumstances in cycling females (Shors et al., 1999). Moreover, much evidence implicates rises in estrogens in implantation failure (e.g. deCatanzaro et al., 1991, 2001; Ma et al., 2003; deCatanzaro, 2011), and exogenous estradiol antibodies can mitigate the negative influence of stress on implantation (deCatanzaro et al., 1994). While there is no evidence concerning potential upregulation in ovarian estrogens or androgen precursors during stress, adrenal sources would seem likely, and could include stress-induced increases in androstenedione (Wilke et al., 1982; Stahl et al., 1991). We did not see a substantial influence of stress on serum androstenedione levels on GD 6. Androgens such as testosterone or androstenedione require aromatization for conversion to estrogens (Payne and Hales, 2004), so conversion to estrogens could have obscured an upregulation of androstenedione in our data.

In Experiment 3, a high dose of 100ng E_2 injected daily from GD 1-5 resulted in complete disruption of implantation regardless of the presence or absence of stress. This replicates results seen elsewhere of detrimental effects of E_2 on early pregnancy (reviewed in deCatanzaro, 2011). Injections of 10ng E_2 did not significantly alter implantation in either control or stressed females. We predicted that this subthreshold

dose of E_2 might synergize with elevated endogenous E_2 in stress, and thus enhance implantation failure. However the strong pregnancy disruption by stress alone in Experiment 3 may have reduced our ability to distinguish between pregnancy rates in stressed females receiving oil versus those receiving 10ng E_2. A relatively large dose of 500μg P_4 was not sufficient to protect against stress-induced implantation failure, contrary to other reports (MacNiven and deCatanzaro, 1990; Pratt and Lisk, 1991; Joachim et al., 2003). In contrast, while neither 10ng E_2 nor 500μg P_4 alone influenced pregnancy rates, these doses in combination completely mitigated the disruptive effects of stress on implantation. To our knowledge, there are no previous reports of such a finding. We suspect that the 10ng E_2 dose upregulated uterine progesterone receptor (PR) expression, thus rendering the previously ineffective 500μg P_4 more potent. This is concordant with evidence that immune stress in mice is associated with downregulated PR expression in the pre-implantation uterus (Agrawal et al., 2012), and E_2 is known to upregulate the expression of uterine PR (Das et al., 1998).

The data reported here provide evidence for the involvement of E_2 and P_4 in stress-induced implantation failure. There is a complex interplay between E_2 and P_4 during implantation, with these steroids often having opposing roles. We suggest that stress-induced imbalances in these steroids favoring E_2 result in implantation failure through several mechanisms including prevention of uterine closure and reduced expression of uterine adhesion molecules. Pregnancy is energetically costly for females, and can fail in stress. Female mammals have apparently evolved the ability to abandon

reproductive efforts in very early pregnancy, avoiding the costly process of gestation where the environment is not conducive to offspring or maternal survival.

Acknowledgements

We appreciate the assistance of the department's animal care staff.

Role of the funding source

This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada to Denys deCatanzaro.

Conflict of interest

The authors declare that there are no conflicts of interest.

Contributors

JBT and DdeC designed the experiments together. JBT carried out the experiments together with PSB and MS. Data analysis was conducted by all authors. Initial versions of the manuscript were written by JBT and finalized together with DdeC.

References

Agrawal, V., Jaiswal, M.K., Jaiswal, Y.K., 2011. Lipopolysaccharide induces alterations in ovaries and serum level of progesterone in 17β-estradiol in the mouse. Fertil. Steril. 95, 1471-1474.

Agrawal, V., Jaiswal, M.K., Jaiswal, Y.K., 2012. Lipopolysaccharide-induced modulation in the expression of progesterone receptor and estradiol receptor leads to early pregnancy loss in mouse. Zygote e1-8.

Amaral, V.C.S., Gomes, K.S., Nunes-de-Souza, R.L., 2010. Increased corticosterone levels in mice subjected to the rat exposure test. Horm. Behav. 57, 128-133.

deCatanzaro, D., 1988. Effect of predator exposure upon early pregnancy in mice. Physiol. Behav. 43, 691-696.

deCatanzaro, D., MacNiven, E., Ricciuti, F., 1991. Comparison of the adverse effects of adrenal and ovarian steroids on early pregnancy in mice. Psychoneuroendocrinology 16, 525-536.

deCatanzaro, D., MacNiven, E., 1992. Psychogenic pregnancy disruptions in mammals. Neurosci. Biobehav. R. 16, 43-53.

deCatanzaro, D., MacNiven, E., Goodison, T., Richardon, D., 1994. Estrogen antibodies reduce vulnerability to stress-induced failure of intrauterine implantation in inseminated mice. Physiol. Behav. 55, 35-38.

deCatanzaro, D., Baptista, M.A.S., Vella, E.S., 2001. Administration of minute quantities of 17β-estradiol on the nasal area terminates early pregnancy in inseminated female mice. Pharmacol. Biochem. Behav. 69, 503-509.

deCatanzaro, D., Muir, C., Beaton, E., Jetha, M., Nadella, K., 2003. Enzymeimmunoassay of oestradiol, testosterone and progesterone in urine sample from female mice before and after insemination. Reproduction 126, 407-414.

deCatanzaro, D., Muir, C., Beaton, E.A., Jetha, M., 2004. Non-invasive repeated measurement of urinary progesterone, 17β-estradiol, and testosterone in developing, cycling, pregnant, and postpartum female mice. Steroids 69, 687-696.

deCatanzaro, D., 2011. Blastocyst implantation is vulnerable to stress-induced rises in endogenous estrogens and also to excretions of estrogens by proximate males. J. Reprod. Immunol. 90, 14-20.

Das, S.K., Tan, J., Johnson, D.C., Dey, S.K., 1998. Differential spatiotemporal regulation of lactoferrin and progesterone receptor genes in the mouse uterus by primary estrogen, catechol estrogen, and xenoestrogen. Endocrinology 139, 2905-2915.

Dey, S.K., Kim, H., Das, S.K., Reese, J., Paria, B.C., Daikoku, T., Wang, H., 2004. Molecular cues to implantation. Endocr. Rev. 25, 341-373.

Fossum, G.T., Davidson, A., Paulson, R.J., 1989. Ovarian hyperstimulation inhibits embryo implantation in the mouse. J. in Vitro. Fertil. Em. 6, 7-10.

Galea, L.M., McEwen, B.S., Tanapat, P., Deak, T., Spencer, R.L., Dhabhar, F.S., 1997. Sex differences in dendritic atrophy of CA3 pyramidal neurons in response to chronic restraint stress. Neuroscience 81, 689-697.

Gallinelli, A., Roncaglia, R., Matteo, M.L., Ciaccio, I., Volpe, A., Facchinetti, F., 2001. Immunological changes and stress are associated with different implantation rates

in patients undergoing in vitro fertilization-embryo transfer. Fertil. Steril. 76, 85-90.

Garcia-Ispierto, I., Lopez-Gatius, F., Santolaria, P., Yaniz, J.L., Nogareda, C., Lopez-Bejar, M. De Rensis, F., 2006. Relationship between heat stress during the peri-implantation period and early fetal loss in dairy cattle. Theriogenology 65, 799-807.

Garland, H.O., Atherton, J.C., Baylis, C., Morgan, M.R.A., Milne, C.M., 1987. Hormone profiles for progesterone, oestradiol, prolactin, plasma renin activity, aldosterone and corticosterone during pregnancy and pseudopregnancy in two strains of rat: Correlation with renal studies. J. Endocrinol. 113, 435-444.

Gidley-Baird, A.A., O'Neill, C., Sinosich, M.J., Porter, R.N., Pike, I.L., Saunders, D.M., 1986. Failure of implantation in human in vitro fertilization and embryo transfer patients: The effects of altered progesterone/estrogen ratios in humans and mice. Fertil. Steril. 45, 69-74.

Greenwald, G.S., 1967. Species differences in egg transport in response to exogenous estrogen. Anat. Rec. 157, 163-172.

Haisenleder, D.J., Schoenfelder, A.H., Marcinko, E.S., Geddis, L.M., Marshall, J.C., 2011. Estimation of estradiol in mouse serum samples: Evaluation of commercial estradiol immunoassays. Endocrinology 152 4443-4447.

Huck, U.W., Lisk, R.D., Miller, K.S., Bethel, A., 1988. Progesterone levels and socially-induced implantation failure and resorption in golden hamsters (*Mesocricetus auratus*). Physiol. Behav. 44, 321-326.

Hyland, R.A., Shaw, T.J., Png, F.Y., Murphy, C.R., 1998. Pan-cadherin concentrates apically in uterine epithelial cells during uterine closure in the rat. Acta Histochem. 100, 75-81.

Jha, R.K., Titus, S., Saxena, D., Kumar, P.G., Laloraya, M., 2006. Profiling of e-cadherin, β-catenin and Ca^{2+} in embryo-uterine interactions at implantation. FEBS Lett. 580, 5653-5660.

Joachim, R., Zenclussen, A.C., Polgar, B., Douglas, A.J., Fest, S., Knackstedt, M., Klapp, B.F., Arck, P.C., 2003. The progesterone derivative dydrogesterone abrogates murine stress-triggered abortion by inducing a Th2 biased local immune response. Steroids 68, 931-940.

Karli, P., 1956. The Norway rat's killing response to the white mouse: An experimental analysis. Behaviour 10, 81-103.

Ma, W., Song, H., Das, S.K., Paria, B.C., Dey, S.K., 2003. Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. P. Natl. Acad. Sci. USA. 100, 2963-2968.

MacNiven, E., deCatanzaro, D., 1990. Reversal of stress-induced pregnancy blocks in mice by progesterone and metyrapone. Physiol. Behav. 47, 443-448.

MacNiven, E., deCatanzaro, D., Younglai, E.V., 1992. Chronic stress increases estrogen and other steroids in inseminated rats. Physiol. Behav. 52, 159-162.

Misdrahi, D., Pardon, M.C., Pérez-Diaz, F., Hanoun, N., Cohen-Salmon, C., 2005. Prepartum chronic ultramild stress increases corticosterone and estradiol levels in

gestating mice: Implications for postpartum depressive disorders. Psychiatry Res. 137, 123-130.

Muir, C., Spironello-Vella, E., Pisani, N., deCatanzaro, D., 2001. Enzyme immunoassay of 17β-estradiol, estrone conjugates, and testosterone in urinary and fecal samples from male and female mice. Horm. Metab. Res. 33, 653-658.

Nepomnaschy, P.A., Welch, K.B., McConnell, D.S., Low, B.S., Strassmann, B.I., England, B.G., 2006. Cortisol levels and very early pregnancy loss in humans. P. Natl. Acad. Sci. USA. 103, 3938-3942.

Oesterreich, S., Deng, W., Jiang, S., Cui, X., Ivanova, M., Schiff, R., Kang, K., Hadsell, D.L., Behrens, J., Lee, A.V., 2003. Estrogen-mediated down-regulation of e-cadherin in breast cancer cells. Cancer Res. 63, 5203-5208.

Parker, V.J., Menzies, J.R.W., Douglas, A.J., 2011. Differential changes in the hypothalamic-pituitary-adrenal axis and prolactin responses to stress in early pregnant mice. J. Neuroendocrinol. 23, 1066-1078.

Parr, M.B., 1983. Relationship of uterine closure to ovarian hormones and endocytosis in the rat. J. Reprod. Fertil. 68, 185-188.

Parr, M.B., Parr, E.L., 1977. Endocytosis in the uterine epithelium of the mouse. J. Reprod. Fertil. 50, 151-153.

Payne, A.H., Hales, D.B., 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr. Rev. 25, 947-970.

Plas-Roser, S., Aron, C., 1981. Stress related effects in the control of sexual receptivity and in the secretion of progesterone by the adrenals in cyclic female rats. Physiol. Behav. 27, 261-264.

Potter, S.W., Gaza, G., Morris, J.E., 1996. Estradiol induces e-cadherin degradation in mouse uterine epithelium during the estrous cycle and early pregnancy. J. Cell. Physiol. 169, 1-14.

Pratt, N.C., Lisk, R.D., 1991. Role of progesterone in mediating stress-related litter deficits in the golden hamster (*Mesocricetus auratus*). J. Reprod. Fertil. 92, 139-146.

Rabin, D.S., Johnson, E.O., Brandon, D.D., Liapi, C., Chrousos, G.P., 1990. Glucocorticoids inhibit estradiol-mediated uterine growth: Possible role of the uterine estradiol receptor. Biol. Reprod. 42, 74-80.

Rahnama, F., Thompson, B., Steiner, M., Shafiei, F., Lobie, P.E., Mitchell, M.D., 2009. Epigenetic regulation of e-cadherin controls endometrial receptivity. Endocrinology 150, 1466-1472.

Reinius, S., 1967. Ultrastructure of blastocyst attachment in the mouse. Zeits. Zellforsch. 77, 257-266.

Rivier, C., Rivest, S., 1991. Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: Peripheral and central mechanisms. Biol. Reprod. 45, 523-532.

Safro, E., O'Neill, C., Saunders, D.M., 1990. Elevated luteal phase estradiol:progesterone ratio in mice causes implantation failure by creating a uterine environment that suppresses embryonic metabolism. Fertil. Steril. 54, 1150-1153.

Salleh, N., Baines, D.L., Naftalin, R.J., Milligan, S.R., 2005. The hormonal control of uterine luminal fluid secretion and absorption. J. Membrane Biol. 206, 17-28.

Shors, T.J., Pickett, J., Wood, G., Paczynski, M., 1999. Acute stress persistently enhances estrogen levels in the female rat. Stress 3, 163-171.

Simon, C., Cano, F., Valbuena, D., Remohi, J., Pellicer, A., 1995. Clinical evidence for a detrimental effect on uterine receptivity of high serum oestradiol concentrations in high and normal responder patients. Hum. Reprod. 10, 2432-2437.

Stahl, F., Götz, F., Dörner, G., 1991. The influence of fetal adrenals on the androgen levels during brain differentiation in human subjects and rats. Exp. Clin. Endocrinol. 98, 131-139.

Szekeres-Bartho, J., Wegmann, T.G., 1996. A progesterone-dependent immunomodulatory protein alters the Th1/Th2 balance. J. Reprod. Immunol. 31, 81-95.

Szekeres-Bartho, J., Par, G., Dombay, G., Smart, Y.C., Volgyi, Z., 1997. The antiabortive effect of progesterone-induced blocking factor in mice is manifested by modulating NK activity. Cell. Immunol.177, 194-199.

Thorpe, J.B., deCatanzaro, D., 2012. Oestradiol treatment restores the capacity of castrated males to induce both the Vandenbergh and the Bruce effects in mice (*Mus musculus*). Reproduction 143, 123-132.

Thorpe, J.B., Rajabi, N., deCatanzaro, D., 2012. Circadian rhythm and response to an acute stressor of urinary corticosterone, testosterone, and creatinine in adult male mice. Horm. Metab. Res. 44, 429-435.

Tilbrook, A.J., Turner, A.I., Clarke, I.J., 2000. Effects of stress on reproduction in non-rodent mammals: The role of glucocorticoids and sex differences. Rev. Reprod. 5, 105-113.

Valbuena, D., Martin, J., de Pablo, J.L., Remohi, J., Pellicer, A., Simon, C., 2001. Increasing levels of estradiol are deleterious to embryonic implantation because they directly affect the embryo. Fertil. Steril. 76, 962-968.

Wilke, D.L., Tseu, S.R., Rhees, R.W., Fleming, D.E., 1982. Effects of environmental stress or ACTH treatment during pregnancy on maternal and fetal plasma androstenedione in the rat. Horm. Behav. 16, 293-303.

Chapter 6

General Discussion

Summary of Results

In **Chapter 2**, I confirmed that castration reduces the capacity of male mice to induce both the Bruce and the Vandenbergh effects. Females exposed to castrated males did not differ from isolated control females in their probability of remaining pregnant (Bruce effect) or in the consistency of their investment in reproductive tissue (Vandenbergh effect). The capacity for castrated males to induce pregnancy disruption and sexual maturation in nearby females was restored when these castrated males were given injections of E_2. Moreover, while urinary T_5 levels in E_2-treated castrated males remained low and comparable to those levels seen in oil-treated castrated males, urinary E_2 levels were elevated. There are other reports showing that castrated males' abilities to induce the Bruce effect (deCatanzaro & Storey 1989) and the Vandenbergh effect (Lombardi *et al.* 1976) are restored with T_5 administration, leading to the conclusion that the pheromonal chemosignals responsible for both effects are androgen-dependent. However, castration reduces E_2 and T_5 in males (Vella & deCatanzaro 2001), and T_5 is converted to E_2 within the body (Simpson *et al.* 1994). My results showing that E_2 alone restores the capacity of castrated males to induce both pheromonal effects indicates that T_5 is important for the Bruce and Vandenbergh effects insofar as it provides a precursor for the production of E_2. Since E_2 plays important roles in implantation failure and female sexual maturation (Alonso & Rosenfield 2002, deCatanzaro 2011), and E_2 can transfer from male to female mice (Guzzo *et al.* 2010, 2012, 2013), it is possible that E_2 in male urine is absorbed by nearby females and contributes to the Bruce and Vandenbergh effects.

In **Chapter 3**, I evaluated the merit of a urinary corticosterone assay in male mice by documenting the circadian pattern of both urinary and serum corticosterone, and by examining the response of urinary corticosterone to an acute, physical stressor (an i.p. saline injection). Both urinary and serum measures of corticosterone followed a distinct circadian pattern similar to that in serum reported elsewhere (Halberg *et al.* 1959, Leal & Moreira 1997), with levels peaking around the start of the active cycle. Moreover, urinary and serum measures of corticosterone correlated positively over the daily cycle. Urinary corticosterone also responded as predicted to acute physical stress, with rises seen 1.5 hours after exposure to a 30 second stressor at both the peak and the trough of the circadian corticosterone rhythm. Therefore, urine provides an alternative medium to serum in which to study both basal and stress levels of corticosterone in male mice.

Given the connection between the adrenal and gonadal axes (Viau 2002), the circadian rhythm and response to acute physical stress of urinary T_5 were also analyzed in **Chapter 3**. The circadian pattern of urinary T_5 was much less distinct than that of corticosterone. T_5 was highest during the start of the light cycle, when corticosterone was lowest. The offset between the T_5 and corticosterone patterns in urine, in which the peak in corticosterone corresponded to the trough in T_5, is similar to, albeit weaker than, that reported in serum from rats (Waite *et al.* 2009). This could be due to the inhibitory action of T_5 on GCs (Kitay 1961, Handa *et al.* 1994, Viau 2002). Conversely, this offset may be due to the inhibitory action of GCs on T_5 (Waite *et al.* 2009). While other research has confirmed that GCs and stress can suppress T_5 (Kreuz *et al.* 1971, Bambino & Hsueh 1981, Sapolsky 1985), acute physical stress in **Chapter 3** associated with elevated urinary

corticosterone did not alter urinary T_5 levels 1.5 hours later. It is possible that a more severe or chronic stressor is necessary to suppress sex steroids, or that sampling 1.5 hours after stressor offset is too soon to observe any changes in urinary T_5.

In **Chapter 4**, I compared the urinary and serum adrenal steroid responses to acute psychological stress in ovariectomized female mice. First, I documented the time course of urinary corticosterone, P_4, and E_2 in response to 1 hour on an elevated platform. As predicted, both urinary corticosterone and P_4 were increased in response to this mild stressor. Compared to isolated controls, urinary corticosterone remained high for the first 3 hours, and urinary P_4 remained high for the first 4 hours, after stressor offset. Urinary E_2 did not appear to respond to acute psychological stress. Next, I compared serum and urinary steroid responses within individuals in response to the same acute psychological stressor. In ovariectomized female mice, urinary and serum corticosterone and P_4, but neither urinary nor serum E_2, increased in response to 1 hour on an elevated platform. The time taken by corticosterone and P_4 to return to baseline levels was different in serum compared to urinary measures. Serum corticosterone and P_4 both returned to baseline within 2 hours after stressor offset, whereas urinary corticosterone and P_4 took up to 4 hours to return to baseline, highlighting an important difference between urinary and serum steroids. Although both respond in a predictable way to acute stress, serum levels are more reflective of the immediate state of the animal, whereas urinary measures may reflect a more cumulative response over time. Both of these characteristics can have value depending on experimental design.

In **Chapter 5**, I explored the effects of a more chronic psychological stressor in inseminated female mice, and the associated reproductive and hormonal consequences. One hour of exposure to a male rat behind a wire grid on gestation day (GD) 2 increased serum corticosterone and P_4 compared to control females, suggesting that close proximity to a rat is stressful. In daily observations during continuous exposure to rats across a grid during GD 1 to 5, females were observed to avoid proximity to the grid, unlike control females where there was no rat across the grid. This confirmed that rat exposure was aversive, and that the mice did not habituate to rat exposure across GD 1 to 5. In the rat-exposed females, the probability of detecting pregnancy on GD 6 was significantly lower than it was in controls. Stress from predator exposure also resulted in interesting P_4 and E_2 dynamics on GD 6. In the stress condition, females that maintained their pregnancies had elevated P_4, and females that lost their pregnancies had reduced P_4. In addition, rat exposure resulted in elevated E_2 levels, particularly in females that lost their pregnancies. This is consistent with the known detrimental role upon implantation of high E_2 and the importance of P_4 in sustaining pregnancy. In a subsequent experiment, I examined the consequences of daily injections from GD 1 to 5 of E_2 and/or P_4 given to inseminated females that were also either isolated or rat-exposed. While a high dose (100 ng) of E_2 abolished all pregnancies by GD 6, a low dose (10 ng) of E_2 did not influence pregnancy rates in either control or stressed females. A standard dose of P_4 (500 μg) was not sufficient to mitigate stress-induced pregnancy failure, contrary to other reports (MacNiven & deCatanzaro 1990), nor could it counteract the negative impact of 100 ng E_2 on implantation. However, P_4 in combination with 10 ng of E_2 prevented stress-

induced pregnancy failure. This was contrary to expectation, and I hypothesize that the low dose of E_2 upregulated uterine progesterone receptor expression, enhancing the pregnancy-protecting effects of the co-administered P_4 dose.

The Role of Estrogens in Female Reproductive Plasticity

Exposure to exogenous E_2 can disrupt early pregnancy (deCatanzaro *et al.* 1991, **Chapter 5**) or induce female sexual maturation (Rasier *et al.* 2006). Although implantation, at least in mice and rats, requires some E_2 action (Dey *et al.* 2004), a small increase above the necessary level results in implantation failure through several mechanisms. For example, excessive E_2 can prematurely bring the uterus into a refractory stage (Ma *et al.* 2003), accelerate or retard blastocyst transport through the reproductive tract (Greenwald 1967), or reduce the expression of adhesion molecules important for uterine closure and implantation (Jha *et al.* 2006, Rahnama *et al.* 2009). E_2 also stimulates the growth and maturation of the reproductive tract in juvenile females by increasing mitotic activity of cells in the uterus (Ogasawara *et al.* 1983, Quarmby & Korach 1984). Moreover, E_2 causes the age of vaginal opening and first estrus (ovulation) to advance, and can also stimulate GnRH and LH release (Rasier *et al.* 2006), triggering the onset of sexual maturation.

A female mammal's susceptibility to these reproductive actions of E_2 is very likely to contribute to her reproductive plasticity. For decades, researchers have known that early pregnancy is disrupted in females in the presence of unfamiliar males (Bruce 1959) or severe stress (Euker & Riegle 1973), and that the age of sexual maturation in

juvenile females is advanced by exposure to adult males (Vandenbergh 1967). I found clear support for the involvement of E_2 in these various circumstances that influence female reproductive events.

Both the Bruce and Vandenbergh effects are mediated by one or more chemosignals in male urine that decrease in the absence of the testes (Dominic 1966, Colby & Vandenbergh 1974, **Chapter 2**) and are restored by T_5 treatment (Lombardi *et al.* 1976, deCatanzaro & Storey 1989). Since E_2 is an immediate metabolite of T_5, some effects originally attributed to T_5 are now known to be facilitated by E_2 (e.g., masculinization of the brain, McCarthy 2008). Like T_5 replacement, E_2 replacement restores castrated males' ability to induce the Bruce and Vandenbergh effects (**Chapter 2**). Based on the known negative impacts of E_2 on intrauterine blastocyst implantation and the stimulatory effects of E_2 on female sexual maturation, it is possible that E_2 present in male urine is absorbed by inseminated and juvenile females, thereby contributing to implantation failure and advanced sexual maturation. E_2 treatment of castrated males in **Chapter 2** not only restored their capacity to disrupt implantation in nearby inseminated females and to hasten sexual maturation in nearby juvenile females, but it also restored their urinary E_2 levels to levels similar to those in intact male mice (e.g., Khan *et al.* 2009). Moreover, T_5 levels in castrated males treated with E_2 remained low compared to intact males (e.g., **Chapter 3**), and were similar to T_5 levels in oil-treated castrated males that were unable to induce either the Bruce or the Vandenbergh effects (**Chapter 2**).

Although the data from **Chapter 2** do not on their own fully prove that E_2 excreted in male urine is absorbed by nearby inseminated and juvenile females, taken

together with previous research (described below) they do suggest that this is a plausible mechanism contributing to the Bruce and Vandenbergh effects. First, the ability of a male to induce the Bruce effect is reduced when E_2 is suppressed by aromatase inhibition plus a phytoestrogen-free diet (Beaton & deCatanzaro 2005), and is restored by administration of E_2 to castrated males (deCatanzaro *et al.* 1995). Secondly, the direct transmission of E_2 from male to female mice and its binding in the female brain and reproductive tract have been observed in both inseminated and juvenile females (Guzzo *et al.* 2010, 2012). Thirdly, male mice urinate more in the presence of females (Reynolds 1971), and their urinary E_2 is elevated in the presence of inseminated (Beaton *et al.* 2006) or juvenile (deCatanzaro *et al.* 2009) females. Finally, inseminated and juvenile females actively investigate unfamiliar males (deCatanzaro & Murji 2004, personal observation), thereby potentially facilitating E_2 transfer.

Like exposure to an unfamiliar male, exposure to stress can cause pregnancy failure (Helmreich 1960, Euker & Riegle 1973, Huck *et al.* 1988, Joachim *et al.* 2003, Garcia-Ispierto *et al.* 2006, **Chapter 5**). Stress can also increase endogenous E_2 in intact cycling (Shors *et al.* 1999) and pregnant (MacNiven & deCatanzaro 1992, Misdrahi *et al.* 2005, Agrawal *et al.* 2011, **Chapter 5**) females. The origin of this E_2 is unknown. Some have hypothesized that stress causes ovarian E_2 to rise (Wood & Shors 1998, Shors *et al.* 1999). In **Chapter 4**, a mild psychological stressor (one hour on an elevated platform) did not induce an increase in E_2 in ovariectomized female mice, which could indicate that stress-induced increases in E_2 are of gonadal origin. Still, it remains possible that a more severe stressor is required to induce an increase in E_2. A more straightforward, but

presently unsupported, hypothesis is that severe stress causes upregulation of steroidogenesis in the adrenal gland, resulting in increased E_2.

Regardless of its origin, E_2 is increased in females exposed to predator stress during the first 5 days of gestation (**Chapter 5**). Other researchers have found that P_4 is decreased in recently mated females in response to stress (Joachim *et al.* 2003, Parker *et al.* 2011). I did not find such a response: although P_4 was decreased in female mice that lost their pregnancies in the face of stress, it was actually increased in stressed females that maintained their pregnancies. On the other hand, although failing to reach significance, it appears that E_2 was elevated particularly in stressed females experiencing pregnancy failure. Taken together, females losing their pregnancies in stress had suppressed P_4 and trended towards elevated E_2. The ratio of E_2 to P_4 surrounding implantation appears to be more important than the absolute levels of each steroid alone. Elevations of E_2 relative to P_4 are associated with implantation failure both in humans undergoing in vitro fertilization (Gidley-Baird *et al.* 1986), and in mice (Safro *et al.* 1990). Immune stress in mice also causes elevations of E_2 against a backdrop of decreased P_4 in early pregnancy (Agrawal *et al.* 2011). Data from **Chapter 5** are consistent with the hypothesis that stress leads to implantation failure by increasing E_2 and suppressing P_4.

I did not find evidence that exogenous P_4 injections can counteract the negative impacts of exogenous E_2 injections on implantation in non-stress conditions (**Chapter 5**). This is contrary to other reports, which show that in non-stress conditions, injections of P_4 can prevent implantation failure induced by low E_2 doses, but not higher doses of E_2

(Gidley-Baird *et al.* 1986, Safro *et al.* 1990). The lack of such a finding here may be due to the E_2 doses used. The low dose (10 ng) did not influence implantation on its own, and the high dose (100 ng) completely disrupted pregnancy in all females. Had I included some intermediate E_2 doses between 10 ng and 100 ng, I may have seen some protective effects of P_4. Unlike other reports (MacNiven & deCatanzaro 1990), I did not find that exogenous P_4 mitigated stress-induced implantation failure. Since the levels of P_4 relative to E_2 are important, E_2 may have been so high from stress that the dose of P_4 used (500 μg) was not sufficient to counteract the effects of this endogenous E_2.

Surprisingly, two previous treatments that individually did not influence pregnancy rates (10 ng E_2 and 500 μg P_4) completely mitigated stress-induced implantation failure when given in combination (**Chapter 5**). This mitigation is most likely due to the protective actions of P_4. E_2 can upregulate uterine PR (Das *et al.* 1998), and immune stress in early pregnancy in mice is associated with decreased uterine PR (Agrawal *et al.* 2012). I suspect that 10 ng E_2 was sufficient to upregulate uterine PR, rendering the uterus more sensitive to the pregnancy-protective effects of the co-administered dose of P_4.

Urinary versus Serum Measures of Steroids

One of the challenges inherent to stress research is the rapidity with which steroids, particularly GCs, react to a stressor. Generally, to avoid an influence of stress of capture and restraint on GC levels, blood samples must be taken within 3 minutes of handling (Romero & Reed 2005). Although the rapidity with which GCs rise in response

to any disturbance is particularly challenging for field researchers, it should also be a concern for researchers working with laboratory animals. GCs are not the only steroids that respond to stress: adrenal P_4 is also increased in response to various stressors (Plas-Roser & Aron 1981, **Chapter 4**), and E_2 may respond as well (MacNiven *et al.* 1992, Shors *et al.* 1999, **Chapter 5**). Therefore, even in studies where stress is not the topic of interest, it is important to be aware that other steroids, sex steroids in particular, may respond to normal laboratory routines like moving a cage, which activates the HPA axis (Gärtner *et al.* 1980).

In studies requiring quantification of stress-induced increases in steroids, one challenge may be obtaining samples before blood levels of steroids return to baseline. In **Chapter 4**, serum measures of adrenal corticosterone and P_4 in ovariectomized females returned to control levels within 2 hours after stressor offset. Others have shown that corticosterone returns to baseline levels 1 to 2 hours after stressor offset in ovariectomized rats (Burgess & Handa 1992). One way to avoid some of the complications of blood measures of steroids is to quantify excreted steroids from non-invasively collected urine, which should reflect trends seen in blood, but with a slight time lag (Whitten *et al.* 1998). However, comparisons of blood and urinary measures of steroids are necessary to determine under what circumstances one measure may be superior to the other.

In **Chapter 3** it was found that male mice have a clear urinary corticosterone circadian rhythm similar to that seen in male rat urine (Claustrat *et al.* 2008) and blood (Waite *et al.* 2009). Additionally, urinary and blood corticosterone measured within the

same mice correlate across the day, which indicates that urinary corticosterone appropriately reflects basal levels of systemic corticosterone and gives confidence in the biological validity of the immunoassay procedure. Moreover, urinary corticosterone responds as predicted to acute physical stress in male mice (Meijer *et al.* 2005, **Chapter 3**) and acute psychological stress in female mice (**Chapter 4**). At least in female mice, this urinary corticosterone response to stress reflects a similar upregulation of systemic corticosterone (**Chapter 4**), which further supports the idea that urinary steroid measures accurately mirror circulating steroid measures.

Given the potential importance for reproductive outcomes of the P_4 and E_2 response to stress (**Chapter 5**), I not only examined corticosterone, but I also measured urinary and serum P_4 and E_2 in response to acute psychological stress in female mice. In a preliminary study of urinary corticosterone, P_4, and E_2 responses to stress in intact cycling females, data were highly variable with no clear trends. This unclear response to stress was most likely the result of the presence of the ovaries in the female subjects. The steroid response to stress in females, unlike in males, is complicated by the fact that the magnitude of the corticosterone response is affected by the estrous cycle due to the stimulatory effects of E_2 (Viau & Meaney 1991, Burgess & Handa 1992). To correct for this in **Chapter 4**, ovariectomized female mice were used because they no longer display an estrous cycle. Additionally, ovariectomy allowed me to specifically determine the adrenocortical response to stress in females. As expected, both urinary corticosterone and P_4 increased in response to stress, as did serum corticosterone and P_4 (**Chapter 4**). However, urinary measures of corticosterone and P_4 remained elevated after stressor

offset for 3 to 4 hours and were more variable between individuals, whereas serum measures of these steroids returned to control levels within 2 hours and had relatively low inter-individual variation (**Chapter 4**).

The delayed return to baseline after stress in urinary measures (**Chapter 4**) may allow detection of stress-induced rises in steroids in studies where sampling every individual before serum levels return to baseline is difficult. Moreover, the non-invasive nature of urine collection compared to blood collection permits repeated sampling in small species like mice, and also allows hormone quantification in non-laboratory animals in the field or in captivity (Whitten *et al.* 1998). However, as urinary measures after acute psychological stress have higher inter-individual variation compared to serum measures, they may require a larger sample size. In cases where small sample sizes are necessary, serum measures are probably superior for elucidating clear trends. Furthermore, the rapidity of the serum steroid response to stress has value for studies investigating the immediate response of an individual to a stressor. In field studies, urinary measures of stress hormones do not necessarily reflect the state of the animal in that moment; instead, they may reflect a systemic response to a stressor that occurred several hours prior to urination (Whitten *et al.* 1998). Therefore, in studies where the precise timing of the response to an acute stressor is desired, serum steroid measures are appropriate.

Future Directions

In addition to its impact on urinary E_2, treatment of castrated males with E_2 might restore a behavioural component important for the induction of the Bruce and Vandenbergh effects. Behaviour is likely an important component of both of these pheromonal effects, since females must be exposed to male urine specifically (Dominic 1966, Colby & Vandenbergh 1974). Intact, but not castrated, male mice urinate more than do females (Drickamer 1995), and intact males actively direct their urine towards nearby females (deCatanzaro *et al.* 2009). Both male-typical urine marking and sexual behaviour can be restored in castrated male mice by E_2 treatment (Wallis & Luttge 1975, Kimura & Hagiwara 1985). Therefore, it is possible that E_2 treatment of castrated male mice in **Chapter 2** restored behaviour in them that might have facilitated the transfer of pheromones to inseminated and juvenile females. More studies should be done to quantify castrated male behaviour in the presence of inseminated or juvenile females, and to compare the behaviour of oil- and E_2-treated castrates. Specifically, measures of drinking and urination behaviour, as well as investigatory behaviour directed towards the female, would be informative. However, it is very unlikely that the restoration of male behaviour by E_2 was solely responsible for the reinstatement of the capacity to induce the Bruce and Vandenbergh effects in **Chapter 2**, since the direct application of urine from castrated male mice does not disrupt implantation (Dominic 1966) or induce sexual maturation (Colby & Vandenbergh 1974) in females. Therefore, although male behaviour is important for directing urine towards the female, the androgen-dependent chemosignal itself must also be present in the urine in order for females to be affected.

Multiple measures have been used to determine whether a Bruce or Vandenbergh effect has occurred in male-exposed females. For the Bruce effect, these measures include the absence of parturition (Beaton & deCatanzaro 2005), decreased implantation site number in the uterus (Keverne & de la Riva 1982, **Chapter 2**), and a return to estrous cycling (Bruce 1960). Implantation site counts are fairly definitive, since by GD 6 healthy implantation sites are obvious (e.g., Figure 5.6B). For the Vandenbergh effect, several measures of female sexual maturation have been used, including the ages at which vaginal opening, first estrus, and first mating are attained (Vandenberh 1967), reproductive tissue weights (Lombardi & Vandenbergh 1977, Khan *et al.* 2008b, **Chapter 2**), the presence of sexual behaviour (Khan *et al.* 2008a), and hormone levels (Khan *et al.* 2008b), which reflect the multitude of changes that occur during female sexual maturation. Since I quantified only reproductive tissue weight in juvenile females in **Chapter 2**, I cannot extrapolate my results to other measures of sexual maturity in female mice. I would predict that these other commonly used measures would similarly be upregulated in juvenile females by exposure to E_2-treated castrated males, since they are all also dependent upon E_2. Therefore, it would be interesting to quantify the other measures of sexual maturity listed above in females exposed to E_2-treated castrated males. Male urine activates kisspeptin neurons in adult female mice (Bakker *et al.* 2010), and therefore the expression of hypothalamic kisspeptin in juvenile females exposed to adult males would also be of interest, especially in light of the involvement of kisspeptin in sexual maturation (Kauffman *et al.* 2007).

Given the similarities between implantation failure in the Bruce effect and in response to stress, it is possible that stress experienced by females due to the presence of unfamiliar males contributes to the loss of pregnancy. There is one report of the absence of a Bruce effect in adrenalectomized females (Snyder & Taggart 1967), which may implicate stress as a contributing factor. However, inseminated female mice investigate non-sire males (deCatanzaro & Murji 2004), and as shown in **Chapter 5**, mice tend to avoid aversive stimuli, which suggests that unfamiliar males may not be a stressor for inseminated females. Another common measure of the Bruce effect is a return to estrous cycling (Bruce 1960, Dominic 1966). If unfamiliar males are sufficiently stressful to impede implantation, then they would most likely be sufficiently stressful to also prevent cycling in females, which is a common consequence of stress (Wingfield & Sapolsky 2003). Preliminary data (Rajabi *et al.* 2013) indicate that systemic E_2 in females is not altered by male exposure, but P_4 is decreased, likely as a result of pregnancy loss. Corticosterone and other indicators of stress have not been quantified in inseminated females exposed to males, so it remains to be determined whether unfamiliar males constitute a stressor in inseminated female mice.

A closer look at the uterine mechanisms of stress-induced implantation failure in mice could be fruitful. Since E_2 and P_4 signal through their respective steroid receptors, the dynamics of the expression of these receptors in the uterus in response to the stress paradigm used in **Chapter 5** is of interest. Acute immune stress increases estrogen receptors (ER) while decreasing progesterone receptors (PR) in early pregnant mice (Agrawal *et al.* 2012). I suspect that upregulation of ER facilitates more estrogenic

action, and downregulation of PR prevents progestogenic action, thus contributing to implantation failure in stress. Whether the same trends occur in the uteri from mice exposed to 5 days of rat exposure is unknown. Moreover, given the involvement of e-cadherin in implantation (Rowlands *et al.* 2000), and the negative impact of E_2 on this adhesion molecule (Oesterreich *et al.* 2003), studying the expression of e-cadherin in the uteri of females losing or maintaining their pregnancies in stress would enable us to further elucidate the uterine mechanisms of stress-induced implantation failure.

To confirm without a doubt the involvement of E_2 in stress-induced implantation failure, experimentally blocking the actions of E_2 during stress and observing the outcome on implantation would be useful. Antibodies to E_2 can prevent stress-induced implantation failure in mice (deCatanzaro *et al.* 1994), however, corresponding endogenous E_2 levels in these mice are not known. An improved methodology involving administration of an aromatase inhibitor and measurement of endogenous E_2 would allow us to confirm the successful suppression of E_2 while also determining whether this suppression mitigates stress-induced implantation failure. The challenge with this method is that E_2 is required for implantation to occur in mice (Dey *et al.* 2004), so aromatase inhibition itself would probably block pregnancy. One way to circumvent this issue is to use a different model species that does not require E_2 for implantation. Hamsters and guinea pigs are common laboratory species that do not need E_2 for implantation (Wang & Dey 2006), and therefore could be appropriate models for aromatase inhibition studies.

In **Chapter 5**, I quantified E_2 and P_4 on GD 6, which is 2 to 3 days after the initiation of implantation. Measuring E_2 and P_4 trends in stress leading up to, and during the start of implantation would be informative. In a pilot study, I attempted to do this by taking daily urine samples from control and rat-exposed females from GD 1 to 5. However, the daily handling required to obtain urine from these females may have contributed to decreased control pregnancy rates, so this sampling schedule was discontinued. To observe dynamics in E_2 and P_4 leading up to implantation while reducing handling of the mice, a cross sectional approach should be taken in which each female is sampled from only once during isolation or rat exposure. Urinary measures would be superior over blood measures, since they can be obtained without euthanizing the mice. This way, a profile of E_2 and P_4 could be documented across all 5 days of rat exposure, and steroid levels could be compared to pregnancy outcome measured in all mice on GD 6.

Finally, there is a longstanding assumption in the literature that female reproductive plasticity seen in inseminated females exposed to stress or to unfamiliar males is adaptive. It has been proposed that females abandon early pregnancy in the presence of a non-sire male to avoid wasteful investment in gestation, since the new male may commit infanticide of unrelated infants upon their birth (Labov 1981). Additionally, there is a widely accepted hypothesis that suppression of energetically costly, long-term physiological processes, such as those involved in reproduction, allows for diversion of energetic resources to processes required for more immediate survival during a stressful

situation, and that this is adaptive[1] (Wingfield *et al.* 1998, Sapolsky 2004). Despite the wide acceptance of these hypotheses, it is important to note that few empirical studies exist showing the long-term fitness costs or benefits of reproductive suppression as a result of stress or the presence of an unfamiliar male. Previous calls have been made for the measurement of lifetime fitness and correlating it with HPA axis reactivity as a way of answering empirically whether or not the acute stress response is adaptive (Breuner *et al.* 2008). As a way of tackling the question of whether early pregnancy loss in particular can be adaptive, I suggest further study of the fitness consequences (e.g., age of senescence, number of lifetime litters, and litter sizes and sex ratios) of implantation failure or maintenance in the face of exposure to an unfamiliar male or to severe stress in female mice.

[1] However, there are other examples of situations and species in which *resistance* to stress-induced reproductive suppression occurs. This has been proposed to be adaptive under particular circumstances, such as very short breeding seasons (Wingfield & Sapolsky 2003).

General References (Chapters 1 & 6)

Agrawal V, Jaiswal MK & Jaiswal YK 2011 Lipopolysaccharide induces alterations in ovaries and serum level of progesterone and 17β-estradiol in the mouse. *Fertility and Sterility* **95** 1471-1474.

Agrawal V, Jaiswal MK & Jaiswal YK 2012 Lipopolysaccharide-induced modulation in the expression of progesterone receptor and estradiol receptor leads to early pregnancy loss in the mouse. *Zygote* **e** 1-8.

Alonso LC & Rosenfield RL 2002 Oestrogens and puberty. *Best Practice & Research Clinical Endocrinology and Metabolism* **16** 13-30.

Bakker J, Pierman S & González-Martínez D 2010 Effects of aromatase mutation (ArKO) on the sexual differentiation of kisspeptin neuronal numbers and their activation by same versus opposite sex urinary pheromones. *Hormones and Behavior* **57** 390-395.

Bambino TH & Hsueh AJW 1981 Direct inhibitory effect of glucocorticoids upon testicular luteinizing hormone receptor and steroidogenesis *in vivo* and *in vitro*. *Endocrinology* **108** 2142-2148.

Banik UK & Pincus G 1964 Estrogens and transport of ova in the rat. *Proceedings of the Society for Experimental Biology and Medicine* **116** 1032-1034.

Beach FA 1976 Sexual attractivity, proceptivity, and receptivity in female mammals. *Hormones and Behavior* **7** 105-138.

Beato M, Herrlich P, Schütz G 1995 Steroid hormone receptors: Many actors in search of a plot. *Cell* **83** 851-857.

Beaton EA & deCatanzaro D 2005 Novel males' capacity to disrupt early pregnancy in mice (*Mus musculus*) is attenuated via a chronic reduction of males' urinary 17β-estradiol. *Psychoneuroendocrinology* **30** 688-697.

Beaton EA, Khan A & deCatanzaro D 2006 Urinary sex steroids during sexual development in female mice and in proximate novel males. *Hormone and Metabolic Research* **38** 501-506.

Breen KM & Karsch FJ 2004 Does cortisol inhibit pulsatile luteinizing hormone secretion at the hypothalamic or pituitary level? *Endocrinology* **145** 692-698.

Breen KM, Oakley AE, Pytiak AV, Tilbrook AJ, Wagenmaker ER & Karsch FJ 2007 Does cortisol acting via the type II glucocorticoid receptor mediate suppression of pulsatile luteinizing hormone secretion in response to psychosocial stress? *Endocrinology* **148** 1882-1890.

Brennan PA 2004 The nose knows who's who: Chemosensory individuality and mate recognition in mice. *Hormones and Behavior* **46** 231-240.

Brennan PA 2009 Outstanding issues surrounding vomeronasal mechanisms of pregnancy block and individual recognition in mice. *Behavioural Brain Research* **200** 287-294.

Brennan PA & Peele P 2003 Towards an understanding of the pregnancy-blocking urinary chemosignals of mice. *Biochemical Society Transactions* **31** 152-155.

Breuner CW, Patterson SH & Hahn TP 2008 In search of relationships between the acute adrenocortical response and fitness. *General and Comparative Endocrinology* **157** 288-295.

Bridges NA, Cooke A, Healy MJR, Hindmarsh PC & Brook CGD 1996 Growth of the uterus. *Archives of Disease in Childhood* **75** 330-331.

Bronson FH & Eleftheriou BE 1963 Influence of strange males on implantation in the deermouse. *General and Comparative Endocrinology* **3** 515-518.

Brooks PH & Cole DJA 1970 The effect of the presence of a boar on the attainment of puberty in gilts. *Journal of Reproduction and Fertility* **23** 435-440.

Bruce HM 1959 An exteroreceptive block to pregnancy in the mouse. *Nature* **184** 105.

Bruce HM 1960 A block to pregnancy in the mouse caused by proximity of strange males. *Journal of Reproduction and Fertility* **1** 96-103.

Bruce HM 1965 Effect of castration on the reproductive pheromones of male mice. *Journal of Reproduction and Fertility* **10** 141-143.

Burdick HO & Whitney R 1937 Acceleration of the rate of passage of fertilized ova through the fallopian tubes of mice by massive injections of an estrogenic substance. *Endocrinology* **21** 637-643.

Burgess LH & Handa RJ 1992 Chronic estrogen-induced alterations in adrenocorticotropin and corticosterone secretion, and glucocorticoid receptor-mediated functions in female rats. *Endocrinology* **131** 1261-1269.

Carter CS, Getz LL, Gavish L, McDermott JL & Arnold P 1980. Male-related pheromones and the activation of female reproduction in the prairie vole (*Microtus ochrogaster*). *Biology of Reproduction* **23** 1038-1045.

Charpenet G, Taché Y, Bernier M, Ducharme JR & Collu R 1982 Stress-induced testicular hyposensitivity to gonadotropin in rats. Role of the pituitary gland. *Biology of Reproduction* **27** 616-623.

Claustrat B, Valatx J-L, Harthé C & Brun J 2008 Effect of constant light on prolactin and corticosterone rhythms evaluated using a noninvasive urine sampling protocol in the rat. *Hormone and Metabolic Research* **40** 398-403.

Clulow FV & Langford PE 1971 Pregnancy-block in the meadow vole, *Microtus pennsylvanicus*. *Journal of Reproduction and Fertility* **24** 275-277.

Cohen-Parsons M & Carter CS 1986 Males increase serum estrogen and estrogen receptor binding in brain of female voles. *Physiology & Behavior* **39** 309-314.

Colby DR & Vandenbergh JG 1974 Regulatory effects of urinary pheromones on puberty in the mouse. *Biology of Reproduction* **11** 268-279.

Conn PM & Crowley WF Jr 1990 Gonadotropin-releasing hormone and its analogues. *New England Journal of Medicine* **324** 93-103.

Cross JC, Werb Z & Fisher SJ 1994 Implantation and the placenta: Key pieces of the development puzzle. *Science* **266** 1508-1518.

Croy BA, Chantakru S, Esadeg S, Ashkar AA & Wei Q 2002 Decidual natural killer cells: Key regulators of placental development (a review). *Journal of Reproductive Immunology* **57** 151-168.

Curtis Hewitt S, Goulding EH, Eddy EM & Korach KS 2002 Studies using the estrogen receptor α knockout uterus demonstrate that implantation but not

decidualization-associated signaling is estrogen dependent. *Biology of Reproduction* **67** 1268-1277.

Das SK, Tan J, Johnson DC & Dey SK 1998 Differential spatiotemporal regulation of lactoferrin and progesterone receptor genes in the mouse uterus by primary estrogen, catechol estrogen, and xenoestrogen. *Endocrinology* **139** 2905-2915.

Davidson JM, Smith ER, Rodgers CH & Bloch GJ 1968 Relative thresholds of behavioral and somatic responses to estrogen. *Physiology & Behavior* **3** 227-229.

deCatanzaro D 1988 Effect of predator exposure upon early pregnancy in mice. *Physiology & Behavior* **43** 691-696.

deCatanzaro D & Storey AE 1989 Partial mediation of strange-male-induced pregnancy blocks by sexual activity in mice (*Mus musculus*). *Journal of Comparative Psychology* **103** 381-388.

deCatanzaro D, MacNiven E & Ricciuti F 1991 Comparison of the adverse effects of adrenal and ovarian steroids on early pregnancy in mice. *Psychoneuroendocrinology* **16** 525-536.

deCatanzaro D & MacNiven E 1992 Psychogenic pregnancy disruptions in mammals. *Neuroscience & Biobehavioral Reviews* **16** 43-53.

deCatanzaro D, MacNiven E, Goodison T & Richardson D 1994 Estrogen antibodies reduce vulnerability to stress-induced failure of intrauterine implantation in inseminated mice. *Physiology & Behavior* **55** 35-38.

deCatanzaro D, Smith M & Muir C 1995 Strange-male-induced pregnancy disruption in mice: Potentiation by administration of 17β-estradiol to castrated males. *Physiology & Behavior* **58** 405-407.

deCatanzaro D & Murji T 2004 Inseminated female mice (*Mus musculus*) investigate rather than avoid novel males that disrupt pregnancy, but sires protect pregnancy. *Journal of Comparative Psychology* **118** 251-257.

deCatanzaro D, Beaton EA, Khan A & Vella E 2006 Urinary oestradiol and testosterone levels from novel male mice approach values sufficient to disrupt early pregnancy in nearby inseminated females. *Reproduction* **132** 309-317.

deCatanzaro D, Khan A, Berger RG & Lewis E 2009 Exposure to developing females induces polyuria, polydipsia, and altered urinary levels of creatinine, 17β-estradiol, and testosterone in adult male mice (*Mus musculus*). *Hormones and Behavior* **55** 240-247.

deCatanzaro D 2011 Blastocyst implantation is vulnerable to stress-induced rises in endogenous estrogens and also to excretions of estrogens by proximate males. *Journal of Reproductive Immunology* **90** 14-20.

de Roux N, Genin E, Carel J-C, Matsuda F, Chaussain J-L & Milgrom E 2003 Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proceedings of the National Academy of Sciences* **100** 10972-10976.

Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T & Wang H 2004 Molecular cues to implantation. *Endocrine Reviews* **25** 341-373.

Dickmeis T 2009 Glucocorticoids and the circadian clock. *Journal of Endocrinology* **200** 3-22.

Drickamer LC 1981 Selection for age of sexual maturation in mice and the consequences for population regulation. *Behavioral and Neural Biology* **31** 82-89.

Drickamer LC 1995 Rates of urine excretion by house mouse (*Mus domesticus*): Differences by age, sex, social status, and reproductive condition. *Journal of Chemical Ecology* **21** 1481-1493.

Dominic CJ 1966 Observations on the reproductive pheromones of mice I. Source. *Journal of Reproduction and Fertility* **11** 407-414.

Dungan HM, Clifton DK & Steiner RA 2006 Minireview: Kisspeptin neurons as central processors in the regulation of gonadotropin-releasing hormone secretion. *Endocrinology* **147** 1154-1158.

Ebling FJ 2005 The neuroendocrine timing of puberty. *Reproduction* **129** 675-683.

Ellis BJ & Garber J 2000 Psychosocial antecedents of variation in girls' pubertal timing: Maternal depression, stepfather presence, and marital and family stress. *Child Development* **71** 485-501.

Euker JS & Riegle GD 1973 Effects of stress on pregnancy in the rat. *Journal of Reproduction and Fertility* **34** 343-346.

Feder HH 1984 Hormones and sexual behavior. *Annual Review of Psychology* **35** 165-200.

Feuillan P, Merke D, Leschek EW & Cutler GB Jr 1999 Use of aromatase inhibitors in precocious puberty. *Endocrine-Related Cancer* **6** 303-306.

Freeman ME 1988 The ovarian cycle of the rat. In *The Physiology of Reproduction*, edn 1, pp 1893-1928. Eds E Knobil and JD Neill. New York: Raven Press.

García-Ispierto I, Lopez-Gatius F, Santolaria P, Yaniz JL, Nogareda C, Lopez-Bejar M & De Rensis F 2006 Relationship between heat stress during the peri-implantation period and early fetal loss in dairy cattle. *Theriogenology* **65** 799-807.

Gärtner K, Büttner D, Döhler K, Friedel R, Lindena J & Trautschold I 1980 Stress response of rats to handling and experimental procedures. *Laboratory Animals* **14** 267-274.

Gidley-Baird A, O'Neill C, Sinosich MJ, Porter RN, Pike IL & Saunders DM 1986 Failure of implantation in human in vitro fertilization and embryo transfer patients: The effects of altered progesterone/estrogen ratios in humans and mice. *Fertility and Sterility* **45** 69-74.

Goji K 1993 Twenty-four-hour concentration profiles of gonadotropin and estradiol (E_2) in prepubertal and early pubertal girls: The diurnal rise of E_2 is opposite the nocturnal rise of gonadotropin. *Journal of Clinical Endocrinology and Metabolism* **77** 1629-1635.

Greenwald GS 1967 Species differences in egg transport in response to exogenous estrogen. *The Anatomical Record* **157** 163-172.

Gross MR 1996 Alternative reproductive strategies and tactics: Diversity within sexes. *Trends in Ecology and Evolution* **11** 92-97.

Guzzo AC, Berger RG & deCatanzaro D 2010 Excretion and binding of tritium-labelled oestradiol in mice (*Mus musculus*): Implications for the Bruce effect. *Reproduction* **139** 255-263.

Guzzo AC, Jheon J, Imtiaz F & deCatanzaro D 2012 Oestradiol transmission from males to females in the context of the Bruce and Vandenbergh effects in mice (*Mus musculus*). *Reproduction* **143** 539-548.

Guzzo AC, Pollock T & deCatanzaro D 2013 Transfer of [^{3}H]estradiol-17β and [^{3}H]progesterone from conspecifics to cohabiting female mice. *Journal of Endocrinology* **217** 1-10.

Halberg F, Peterson RE & Silber RH 1959 Phase relations of 24-hour periodicities in blood corticosterone, mitoses in cortical adrenal parenchyma, and total body activity. *Endocrinology* **64** 222-230.

Han S-K, Gottsch ML, Lee KJ, Popa SM, Smith JT, Jakawich SK, Clifton DK, Steiner RA & Herbison AE 2005 Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. *The Journal of Neuroscience* **25** 11349-11356.

Handa RJ, Nunley KM, Lorens SA, Louie JP, McGivern RF & Bollnow MR 1994 Androgen regulation of adrenocorticotropin and corticosterone secretion in the male rat following novelty and foot shock stressors. *Physiology & Behavior* **55** 117-124.

Harder JD & Jackson LM 2005 Male pheromone stimulates ovarian follicular development and body growth in juvenile opossums (*Monodelphis domestica*). *Reproductive Biology and Endocrinology.* **1** 21.

Harris RG 1927 Effect of bilateral ovariectomy upon the duration of pregnancy in mice. *Anatomical Record* **37** 83-93.

Hasler JF& Banks EM 1975 The influence of mature males on sexual maturation in female collared lemmings (*Dicrostonyx groenlandicus*). *Journal of Reproduction and Fertility* **42** 583-586.

Helmreich RL 1960 Regulation of reproductive rate by intra-uterine mortality in the deer mouse. *Science* **132** 417-418.

Hrdy SB 1979 Infanticide among animals: A review, classification, and examination of the implications for the reproductive strategies of females. *Ethology and Sociobiology* **1** 13-40.

Huck UW, Lisk RD, Miller KS & Bethel A 1988 Progesterone levels and socially-induced implantation failure and resorption in golden hamsters (*Mesocricetus auratus*). *Physiology & Behavior* **44** 321-326.

Huet-Hudson YM & Dey SK 1990 Requirement for progesterone priming and its long-term effects on implantation in the mouse. *Experimental Biology and Medicine* **193** 259-263.

Humphrey KW 1967 The induction of implantation in the mouse after ovariectomy. *Steroids* **10** 591-600.

Humphrey K & Martin L 1968 The effect of oestrogen and anti-oestrogens on ovum transport in mice. *Journal of Reproduction and Fertility* **15** 191-197.

Hyland RA, Shaw TJ, Png FY & Murphy CR 1998 Pan-cadherin concentrates apically in uterine epithelial cells during uterine closure in the rat. *Acta Histochemica* **100** 75-81.

Izard MK & Vandenbergh JG 1982 The effects of bull urine on puberty and calving date in crossbred beef heifers. *Journal of Animal Science* **55** 1160-1168.

Jamnongjit M & Hammes SR 2006 Ovarian steroids: The good, the bad, and the signals that raise them. *Cell Cycle* **5** 1178-1183.

Jha RK, Titus S, Saxena D, Kumar PG & Laloraya M 2006 Profiling of E-cadherin, β-catenin and Ca^{2+} in embryo-uterine interactions at implantation. *FEBS Letters* **580** 5653-5660.

Joachim R, Zenclussen AC, Polgar B, Douglas AJ, Fest S, Knackstedt M, Klapp BF & Arck PC 2003 The progesterone derivative dydrogesterone abrogates murine stress-triggered abortion by inducing a Th2 biased local immune response. *Steroids* **68** 931-940.

Kauffman AS, Clifton DK & Steiner RA 2007 Emerging ideas about kisspeptin-GPR54 signaling in the neuroendocrine regulation of reproduction. *Trends in Neurosciences* **30** 504-511.

Keverne EB & de la Riva C 1982 Pheromones in mice: Reciprocal interaction between the nose and brain. *Nature* **296** 148-150.

Khan A, Bellefontaine N & deCatanzaro D 2008a 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* **93** 588-594.

Khan A, Berger RG & deCatanzaro D 2008b 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.

Khan A, Berger RG & deCatanzaro D 2009 Preputialectomised and intact adult male mice exhibit an elevated urinary ratio of oestradiol to creatinine in the presence of developing females, whilst promoting uterine and ovarian growth of these females. *Reproduction, Fertility and Development* **21** 860-868.

Kimura T & Hagiwara Y 1985 Regulation of urine marking in male and female mice: Effects of sex steroids. *Hormones and Behavior* **19** 64-70.

Kinsey-Jones JS, Li XF, Bowe JE, Lightman SL & O'Byrne KT 2006 Corticotrophin-releasing factor type 2 receptor-mediated suppression of gonadotrophin-releasing hormone mRNA expression in GT1-7 cells. *Stress* **9** 215-222.

Kinsey-Jones JS, Li XF, Knox AMI, Wilkinson ES, Zhu XL, Chaudhary AA, Milligan SR, Lightman SL & O'Byrne KT 2008 Down-regulation of hypothalamic *Kisspeptin* and its receptor, *Kiss1r*, mRNA expression is associated with stress-induced suppression of luteinizing hormone secretion in the female rat. *Journal of Neuroendocrinology* **21** 20-29.

Kinsey-Jones JS, Li XF, Knox AMI, Lin YS, Milligan SR, Lightman SL & O'Byrne KT 2010 Corticotrophin-releasing factor alters the timing of puberty in the female rat. *Journal of Neuroendocrinology* **22** 102-109.

Kirby ED, Geraghty AC, Ubuka T, Bentley GE & Kaufer D 2009 Stress increases putative gonadotropin inhibitory hormone and decreases luteinizing hormone in male rats. *Proceedings of the National Academy of Sciences* **106** 11324-11329.

Kitay JI 1961 Sex differences in adrenal cortical secretion in the rat. *Endocrinology* **68** 818-824.

Kleiman DG 1977 Monogamy in mammals. *Quarterly Review of Biology* **52** 39-69.

Knobil E & Hotchkiss J 1988 The menstrual cycle and its neuroendocrine control. In *The Physiology of Reproduction*, edn 1, pp 1971-1994. Eds E Knobil and JD Neill. New York: Raven Press.

Kreuz LE, Rose RM & Jennings R 1971 Suppression of plasma testosterone levels and psychological stress: A longitudinal study of young men in officer candidate school. *Archives of General Psychiatry* **26** 479-482.

Labov JB 1981 Pregnancy blocking in rodents: Adaptive advantages for females. *The American Naturalist* **118** 361-371.

Leal AMO & Moreira AC 1997 Daily variation of plasma testosterone, androstenedione, and corticosterone in rats under food restriction. *Hormones and Behavior* **31** 97-100.

Lombardi JR, Vandenbergh JG & Whitsett JM 1976 Androgen control of the sexual maturation pheromone in house mouse urine. *Biology of Reproduction* **15** 179-186.

Lombardi JR & Vandenbergh JG 1977 Pheromonally induced sexual maturation in females: Regulation by the social environment of the male. *Science* **196** 545-546.

Lonstein JS & Gammie SC 2002 Sensory, hormonal, and neural control of maternal aggression in laboratory rodents. *Neuroscience and Biobehavioral Reviews* **26** 869-888.

Ma W, Song H, Das SK, Paria BC & Dey SK 2003 Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. *Proceedings of the National Academcy of Sciences* **100** 2963-2968.

MacNiven E & deCatanzaro D 1990 Reversal of stress-induced pregnancy blocks in mice by progesterone and metyrapone. *Physiology & Behavior* **47** 443-448.

MacNiven E, deCatanzaro D & Younglai EV 1992 Chronic stress increases estrogen and other steroids in inseminated rats. *Physiology & Behavior* **52** 443-448.

Macrides F, Bartke A & Dalterio S 1975 Strange females increase plasma testosterone levels in male mice. *Science* **189** 1104-1106.

Mallory FF & Brooks RJ 1978 Infanticide and other reproductive strategies in the collared lemming, *Dicrostonyx groenlandicus*. *Nature* **273** 144-146.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P & Evans RM 1995 The nuclear receptor superfamily: The second decade. *Cell* **83** 835-859.

Mayer C, Acosta-Martinez M, Dubois SL, Wolfe A, Radovick S, Boehm U & Levine JE 2010 Timing and completion of puberty in female mice depend on estrogen receptor α-signaling in kisspeptin neurons. *Proceedings of the National Academy of Sciences* **107** 22693-22698.

McCarthy MM 2008 Estradiol and the developing brain. *Physiological Reviews* **88** 91-134.

McCormack JT & Greenwald GS 1974 Evidence for a preimplantation rise in oestradiol-17β levels on day 4 of pregnancy in the mouse. *Journal of Reproduction and Fertility* **41** 297-301.

McGrady AV 1984 Effects of psychological stress on male reproduction: A review. *Archives of Andrology* **13** 1-7.

Meijer MK, Lemmens AG, Van Zutphen BFM & Baumans V 2005 Urinary corticosterone levels in mice in response to intraperitoneal injections with saline. *Journal of Applied Animal Welfare Science* **8** 279-283.

Michael AE, Pester LA, Curtis P, Shaw RW, Edwards CRW & Cooke BA 1993 Direct inhibition of ovarian steroidogenesis by cortisol and the modulatory role of 11β-hydroxysteroid dehydrogenase. *Clinical Endocrinology* **38** 641-644.

Milligan SR & Finn CA 1997 Minimal progesterone support required for the maintenance of pregnancy in mice. *Human Reproduction* **12** 602-607.

Misdrahi D, Pardon MC, Perez-Diaz F, Hanoun N & Cohen-Salmon C 2005 Prepartum chronic ultramild stress increases corticosterone and estradiol levels in

gestating mice: Implications for postpartum depressive disorders. *Psychiatry Research* **137** 123-130.

Moore Smith D & Biggers JD 1968 The oestrogen requirement for implantation and the effect of its dose on the implantation response in the mouse. *Journal of Endocrinology* **41** 1-9.

Murphy LJ & Ghahary A 1990 Uterine insulin-like growth factor-1: Regulation of expression and its role in estrogen-induced uterine proliferation. *Endocrine Reviews* **11** 443-453.

Nathanielsz PW 1998 Comparative studies on the initiation of labor. *European Journal of Obstetrics & Gynecology and Reproductive Biology* **78** 127-132.

Navarro VM, Fernández-Fernández R, Castellano JM, Roa J, Mayen A, Barreiro ML, Gaytan F, Aguilar E, Pinilla L, Dieguez C & Tena-Sempere M 2004a Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54. *Journal of Physiology* **561** 379-386.

Navarro VM, Castellano JM, Fernández-Fernández R, Barreiro ML, Roa J, Sanchez-Criado JE, Aguilar E, Dieguez C, Pinilla L & Tena-Sempere M 2004b Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. *Endocrinology* **145** 4565-4574.

Nebesio TD & Pescovitz OH 2005 Historical perspectives: Endocrine disruptors and the timing of puberty. *The Endocrinologist* **15** 44-48.

Negro-Vilar A 1993 Stress and other environmental factors affecting fertility in men and women: Overview. *Environmental Health Perspectives* **101** 59-64.

Niswender GD & Nett TM 1988 The corpus luteum and its control. In *The Physiology of Reproduction*, edn 1, pp 489-525. Eds E Knobil and JD Neill. New York: Raven Press.

Norman RL & Smith CJ 1992 Restraint inhibits luteinizing hormone and testosterone secretion in intact male rhesus macaques: Effects of concurrent naloxone administration. *Neuroendocrinology* **55** 405-415.

Novotny MV, Harvey S & Jemiolo B 1990 Chemistry of male dominance in the house mouse, *Mus domesticus*. *Experientia* **46** 109-113.

Novotny MV, Ma W, Wiesler D & Zidek L 1999 Positive identification of the puberty-accelerating pheromone of the house mouse: The volatile ligands associating with the major urinary protein. *Proceedings: Biological Sciences* **266** 2017-2022.

Oelkers W 1996 Adrenal insufficiency. *The New England Journal of Medicine* **335** 1206-1212.

Oesterreich S, Deng W, Jiang S, Cui X, Ivanova M, Schiff R, Kang K, Hadsell DL, Behrens J & Lee AV 2003 Estrogen-mediated down-regulation of E-cadherin in breast cancer cells. *Cancer Research* **63** 5203-5208.

Ogasawara Y, Okamoto S, Kitamura Y & Matsumoto K 1983 Proliferative pattern of uterine cells from birth to adulthood in intact, neonatally castrated, and/or

adrenalectomized mice, assays by incorporation of [^{125}I]Iododeoxyuridine. *Endocrinology* **113** 582-587.

Ojeda SR & Urbanski HF 1988 Puberty in the rat. In *The Physiology of Reproduction*, edn 1, pp 1699-1737. Eds E Knobil and JD Neill. New York: Raven Press.

Paria BC, Reese J, Das SK & Dey SK 2002 Deciphering the cross-talk of implantation: Advances and challenges. *Science* **296** 2185-2188.

Parker VJ & Douglas AJ 2009 Stress in early pregnancy: Maternal neuro-endocrine-immune responses and effects. *Journal of Reproductive Immunology* **85** 86-92.

Parker VJ, Menzies JRW & Douglas AJ 2011 Differential changes in the hypothalamic-pituitary-adrenal axis and prolactin responses to stress in early pregnant mice. *Journal of Neuroendocrinology* **23** 1066-1078.

Parkes AS 1928 The role of the corpus luteum in the maintenance of pregnancy. *Journal of Physiology* **65** 341-349.

Partsch C-J & Sippell WG 2001 Pathogenesis and epidemiology of precocious puberty. Effects of exogenous oestrogens. *Human Reproduction Update* **7** 292-302.

Payne AH & Hales DB 2004 Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocrine Reviews* **25** 947-970.

Peluso JJ, Steger RW & Hafez ESE 1976 Development of gonadotrophin-binding sites in the immature rat ovary. *Journal of Reproduction and Fertility* **47** 55-58.

Petrulis A 2013 Chemosignals, hormones and mammalian reproduction. *Hormones and Behavior* **63** 723-741.

Plas-Roser S, Aron C 1981 Stress related effects in the control of sexual receptivity and in the secretion of progesterone by the adrenals in cyclic female rats. *Physiology & Behavior* **27** 261-264.

Potter SW, Gaza G & Morris JE 1996 Estradiol induces e-cadherin degradation in mouse uterine epithelium during the estrous cycle and early pregnancy. *Journal of Cellular Physiology* **169** 1-14.

Powers JB 1970 Hormonal control of sexual receptivity during the estrous cycle of the rat. *Physiology & Behavior* **5** 831-835.

Quarmby VE & Korach KS 1984 The influence of 17β-estradiol on patterns of cell division in the uterus. *Endocrinology* **114** 694-701.

Rahnama F, Thompson B, Steiner M, Shafiei F, Lobie PE & Mitchell MD 2009 Epigenetic regulation of E-cadherin controls endometrial receptivity. *Endocrinology* **150** 1466-1472.

Rajabi N, Thorpe JB, Foster WG & deCatanzaro D 2013 (In Press) Novel male exposure reduces uterine e-cadherin, increases uterine luminal area, and diminishes progesterone levels while disrupting blastocyst implantation in inseminated mice. *Journal of Steroid Biochemistry and Molecular Biology* doi: http://dx.doi.org/10.1016/j.jsbmb.2013.08.005

Ramathal CY, Bagchi IC, Tayler RN & Bagchi MK 2010 Endometrial decidualization: Of mice and men. *Seminars in Reproductive Medicine* **28** 17-26.

Rasier G, Toppari J, Parent A-S & Bourguignon J-P 2006 Female sexual maturation and reproduction after prepubertal exposure to estrogens and endocrine disrupting

chemicals: A review of rodent and human data. *Molecular and Cellular Endocrinology* **254-255** 187-201.

Reasner DS & Johnston RE 1987 Acceleration of reproductive development in female Djungarian hamsters by adult males. *Physiology & Behavior* **43** 57-64.

Reel JR, Lamb JC & Neal BH 1996 Survey and assessment of mammalian estrogen biological assays for hazard characterization. *Fundamental and Applied Toxicology* **34** 288-305.

Reynolds E 1971 Urination as a social response in mice. *Nature* **234** 481-483.

Rivest S, Plotsky PM & Rivier C 1993 CRF alters the infundibular LHRH secretory system from the medial preoptic area of female rats: Possible involvement of opioid receptors. *Neuroendocrinology* **57** 236-246.

Rivier C, Rivier J & Vale W 1986 Stress-induced inhibition of reproductive functions: Role of endogenous corticotrophin-releasing factor. *Science* **231** 607-609.

Roberts EK, Lu A, Bergman TJ & Beehner JC 2012 A Bruce effect in wild geladas. *Science* **335** 1222-1225.

Rohrbach C 1982 Investigation of the Bruce effect in the Mongolian gerbil (*Meriones unguiculatus*). *Journal of Reproduction and Fertility* **65** 411-417.

Romero LM & Reed JM 2005 Collecting baseline corticosterone samples in the field: Is under 3 min good enough? *Comparative Biochemistry and Physiology, Part A* **140** 73-79.

Rowlands TM, Symonds JM, Farookhi R & Blaschuk OW 2000 Cadherins: Crucial regulators of structure and function in reproductive tissues. *Reviews of Reproduction* **5** 53-61.

Rubenstein L & Forbes TR 1963 Maintenance of pregnancy with subcutaneous pellets of progesterone in ovariectomized mice. *Experimental Biology and Medicine* **113** 1043-1046.

Safro E, O'Neill C & Saunders DM 1990 Elevated luteal phase estradiol:progesterone ratio in mice causes implantation failure by creating a uterine environment that suppresses embryonic metabolism. *Fertility and Sterility* **54** 1150-1153.

Salleh N, Baines DL, Naftalin RJ & Millian SR 2005 The hormonal control of uterine luminal fluid secretion and absorption. *Journal of Membrane Biology* **206** 17-28.

Saltzman W & Maestripieri D 2011 The neuroendocrinology of primate maternal behavior. *Progress in Neuro-Psychopharmacology & Biological Psychiatry* **35** 1192-1204.

Sapolsky RM 1985 Stress-induced suppression of testicular function in the wild baboon: Role of glucocorticoids. *Endocrinology* **116** 2273-2278.

Sapolsky RM, Krey LC & McEwen BS 1986 The neuroendocrinology of stress and aging: The glucocorticoid cascade hypothesis. *Endocrine Reviews* **7** 284-301.

Sapolsky RM & Krey LC 1988 Stress-induced suppression of luteinizing hormone concentrations in wild baboons: Role of opiates. *Journal of Clinical Endocrinology and Metabolism* **66** 722-726.

Sapolsky RM, Romero LM & Munck AU 2000 How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews* **21** 55-89.

Sapolsky RM 2004 Why zebras don't get ulcers: The acclaimed guide to stress, stress-related diseases, and coping. edn 3. New York: Henry Holt and Company, LLC.

Schally AV, Arimura A, Kastin AJ 1973 Hypothalamic regulatory hormones. *Science* **179** 341-350.

Schindler AE 2005 Endocrinology of pregnancy: Consequences for the diagnosis and treatment of pregnancy disorders. *Journal of Steroid Biochemistry & Molecular Biology* **97** 386-388.

Schwagmeyer PL 1979 The Bruce effect: An evaluation of male/female advantages. *The American Naturalist* **114** 932-938.

Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MBL, Crowley WF Jr, Aparicio SAJR & Colledge WH 2003 The *GPR54* gene as a regulator of puberty. *The New England Journal of Medicine* **349** 1614-1627.

Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR & Plant TM 2005 Increased hypothalamic GPR54 signaling: A potential mechanism for initiation of puberty in primates. *Proceedings of the National Academy of Sciences* **102** 2129-2134.

Shors TJ, Pickett J, Wood G & Paczynski M 1999 Acute stress persistently enhances estrogen levels in the female rat. *Stress* **3** 163-171.

Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD, Mendelson CR & Bulun SE 1994 Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrine Reviews* **15** 342-355.

Slater M, Murphy CR & Barden JA 2002 Tenascin, E-cadherin and P2X calcium channel receptor expression is increased during rat blastocyst implantation. *The Histochemical Journal* **34** 13-19.

Smith JT 2008 Kisspeptin signaling in the brain: Steroid regulation in the rodent and ewe. *Brain research Reviews* **57** 288-298.

Snyder RL & Taggart NE 1967 Effects of adrenalectomy on male-induced pregnancy block in mice. *Journal of Reproduction and Fertility* **14** 451-455.

Spears N & Clarke JR 1986 Effect of male presence and of photoperiod on the sexual maturation of the field vole (*Microtus agrestis*). *Journal of Reproduction and Fertility* **78** 231-238.

Teles MG, Bianco SDC, Brito VN, Trarbach EB, Kuohung W, Xu S, Seminara SB, Mendonca BB, Kaiser UB & Latronico AC 2008 A *GPR54*-activating mutation in a patient with central precocious puberty. *The New England Journal of Medicine* **358** 709-715.

Terasawa E, Luchansky LL, Kasuya E & Nyberg CL 1999 An increase in glutamate release follows a decrease in gamma aminobutyric acid and the pubertal increase

in luteinizing hormone releasing hormone release in female rhesus monkeys. *Journal of Neuroendocrinology* **11** 275-282.

Trivers RL 1972 Parental investment and sexual selection. In *Sexual Selection and the Descent of Man 1871-1971,* edn 1, pp 136-179. Ed B Campbell. Chicago: Aldine Publishing Company.

Urbanski HF & Ojeda SR 1985a The juvenile-peripubertal transition period in the female rat: Establishment of a diurnal pattern of pulsatile luteinizing hormone secretion. *Endocrinology* **117** 644-649.

Urbanski HF & Ojeda SR 1985b *In vitro* simulation of prepubertal changes in pulsatile luteinizing hormone release enhances progesterone and 17β-estradiol secretion from immature rat ovaries. *Endocrinology* **117** 638-643.

Urbanski HF & Ojeda SR 1987 Activation of luteinizing hormone-releasing hormone release advances the onset of female puberty. *Neuroendocrinology* **46** 273-276.

Vandenbergh JG 1967 Effect of the presence of a male on the sexual maturation of female mice. *Endocrinology* **81** 345-349.

Vandenbergh JG 1969 Male odor accelerates female sexual maturation in mice. *Endocrinology* **84** 658-660.

Vandenbergh JG 1971 The influence of the social environment on sexual maturation in male mice. *Journal of Reproduction and Fertility* **24** 383-390.

Vandenbergh JG 1976 Acceleration of sexual maturation in female rats by male stimulation. *Journal of Reproduction and Fertility* **46** 451-453.

Vandenbergh JG 1988 Pheromones and mammalian reproduction. In *The Physiology of Reproduction*, edn 1, pp 1699-1737. Eds E Knobil and JD Neill. New York: Raven Press.

Vella ES & deCatanzaro D 2001 Novel male mice show gradual decline in the capacity to disrupt early pregnancy and in urinary excretion of testosterone and 17β-estradiol during the weeks immediately following castration. *Hormone and Metabolic Research* **33** 681-686.

Viau V 2002 Functional cross-talk between the hypothalamic-pituitary-gonadal and -adrenal axes. *Journal of Neuroendocrinology* **14** 506-513.

Viau V & Meaney MJ 1991 Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. *Endocrinology* **129** 2503-2511.

Waite E, Kershaw Y, Spiga F & Lightman SL 2009 A glucocorticoid sensitive biphasic rhythm of testosterone secretion. *Journal of Neuroendocrinology* **21** 737-741.

Wallis CJ & Luttge WG 1975 Maintenance of male sexual behaviour by combined treatment with oestrogen and dihydrotestosterone in CD-1 mice. *Journal of Endocrinology* **66** 257-262.

Walters MR 1985 Steroid hormone receptors and the nucleus. *Endocrine Reviews* **6** 512-543.

Wang H & Dey SK 2006 Roadmap to embryo implantation: Clues from mouse models. *Nature Reviews* **7** 185-199.

Weitlauf HM 1988 Biology of implantation. In *The Physiology of Reproduction*, edn 1, pp 1699-1737. Eds E Knobil and JD Neill. New York: Raven Press.

Whitten PL, Brockman DK & Stavisky RC 1998 Recent advances in noninvasive techniques to monitor hormone-behavior interactions. *Yearbook of Physical Anthropology* **41** 1-23.

Wiebold JL, Stanfield PH, Becker WC & Hillers JK 1986 The effect of restraint stress in early pregnancy in mice. *Journal of Reproduction and Fertility* **78** 185-192.

Wingfield JC, Maney DL, Breuner CW, Jacobs JD, Lynn S, Ramenofsky M & Richardson RD 1998 Ecological bases of hormone-behavior interactions: The "Emergency Life History Stage". *American Zoologist* **38** 191-206.

Wingfield JC & Sapolsky RM 2003 Reproduction and resistance to stress: When and how. *Journal of Neuroendocrinology* **15** 711-724.

Wynne-Edwards KE & Reburn CJ 2000 Behavioral endocrinology of mammalian fatherhood. *Trends in Ecology and Evolution* **15** 464-468.

Zhu L & Pollard JW 2007 Estradiol-17β regulates mouse uterine epithelial cell proliferation through insulin-like growth factor 1 signaling. *Proceedings of the National Academy of Sciences* **104** 15847-15851.