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Estrogens, androgens, and the hormonal modulation of female primate sexual motivation in rhesus monkeys

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2016

Abstract

Estrogens, androgens, and the hormonal modulation of female primate sexual motivation in rhesus monkeys

By: Maurand M. Cappelletti

Ovarian steroids modulate female sexual motivation in human and nonhuman primates. Estrogens decrease following menopause, and many postmenopausal women experience decreased sexual desire. High-dose estrogen therapies increase sexual desire in postmenopausal women, but may increase women's risk of breast and uterine cancers. Estradiol para-quinol (DHED), a biologically inactive metabolite of estradiol, is converted to estradiol only in the brain, and thus may represent a brain-specific estrogen therapy. Because of cancer concerns, postmenopausal women are prescribed low-dose estrogen therapies, which do not increase sexual desire. Supraphysiological levels of testosterone improve the effectiveness of low-dose estrogen therapies; however, the mechanism by which testosterone increases women's sexual desire remains unknown. Testosterone may increase women's sexual desire via its aromatization to estradiol, or by binding sex hormone binding globulin (SHBG) and liberating SHBG-bound estradiol. Unlike testosterone, dihydrotestosterone (DHT) cannot be aromatized. The present dissertation investigated the roles of estrogens and androgens in the hormonal modulation of female primate sexual motivation. Study One investigated the effects of estradiol, DHED, and DHT on serum estradiol and female sexual initiation in group-housed ovariectomized rhesus monkeys. DHED treatment did not increase serum estradiol, suggesting that DHED is not converted to estradiol in the periphery. Neither DHED nor DHT treatment increased female sexual initiation; however, estradiol, the positive control, also did not increase female sexual initiation, suggesting that contextual factors inhibited female response to hormonal treatment. The social groups containing the study subjects were unstable, with high levels of conflict, throughout the study. The effects of estradiol on female sexual initiation were thus likely inhibited by group instability. Future studies should investigate the effects of DHED and DHT on female sexual initiation under stable social conditions. Study Two investigated changes in serum free estradiol following an acute DHT injection in estradiol-treated ovariectomized rhesus monkeys. Although preliminary, results indicated that free estradiol temporarily increased after an acute DHT injection, consistent with the hypothesis that DHT preferentially binds SHBG and liberates SHBG-bound estradiol. Collectively, the behavioral results of this dissertation indicate that the relationship between hormones and female primate sexual behavior is highly sensitive to social context.

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1. INTRODUCTION

1.1. Hormones and women's sexual desire

Ovarian steroids (estradiol, testosterone, and progesterone) maintain and modulate sexual desire in women. Women's self-reported levels of sexual desire, and rates of female-initiated sexual behavior, fluctuate across the menstrual cycle, and peak at midcycle just prior to ovulation (Dennerstein et al., 1994; Harvey, 1987; Stanislaw & Rice, 1988; Van Goozen et al., 1997), in accordance with the midcycle peak in estradiol and testosterone. The gradual and age-related cessation of ovarian function associated with natural menopause results in decreased levels of both estradiol and testosterone, and a substantial portion of postmenopausal women report experiencing decreased levels of sexual desire (Dennerstein et al., 2006; Jiroutek et al., 1998; Leiblum et al. 2006). Similarly, women who undergo bilateral ovariectomy (surgical menopause) report a post-operative decline in sexual desire following an abrupt drop in their circulating levels of ovarian steroids (Dennerstein et al., 2006; Korse et al., 2009; Leiblum et al. 2006; Sherwin et al., 1985). This menopause-related decrease in sexual desire can be extreme and even debilitating; the Women's International Study of Health and Sexuality (WISheS) found that roughly 9% of naturally and up to 26% of surgically postmenopausal women suffer from persistent and distressing lack of sexual desire (Dennerstein et al., 2006; Leiblum et al. 2006). While it is clear that women's sexual desire is subject to hormonal influence, the identity of the ovarian steroid(s) critical for the modulation of women's sexual desire remains a topic of debate.

Both estradiol and testosterone have been implicated as the steroid associated with increases in women's sexual desire. Circulating levels of estradiol and testosterone

peak at midcycle (Abraham, 1974; Korenman & Sherman, 1973; Moghissi et al., 1972), and either or both steroids therefore could theoretically be responsible for the midcycle peak in women's sexual desire. Estradiol, however, exhibits a much more pronounced, and briefer, midcycle peak than testosterone. Circulating estradiol levels increase by more than 800% over a 3-4 day period at midcycle, whereas circulating testosterone levels increase only by roughly 150% over a 6-8 day period (Abraham, 1974; Korenman & Sherman, 1973). Furthermore, in all other mammalian species that have been studied, the estrogens (primarily estradiol) have been shown to be critical for the manifestation of species-typical female sexual behavior (for review see Wallen, 1990; Wallen, 2013). If women's sexual desire was shown to be under androgenic rather than estrogenic modulation, it would seemingly discriminate humans as unique amongst mammals (Wallen, 2013).

Researchers have investigated whether women's self-reported levels of sexual desire are more closely associated with endogenous levels of estradiol or testosterone (Dennerstein et al., 2002; Roney & Simmons, 2013). Roney and Simmons (2013) examined the relationship between estradiol, testosterone, and progesterone, and women's sexual desire across the menstrual cycle. The authors asked 43 naturally cycling female participants (not using hormonal contraceptives) to fill out a daily questionnaire concerning sexual desire and activity across 1-2 menstrual cycles, and to provide a daily saliva sample for hormone analysis throughout the study. The authors examined the relationship between self-reported levels of sexual desire on a given day and steroid hormone levels on that same day, and on one and two days prior to that day. The authors reported that salivary estradiol was a significant positive predictor of sexual desire

measured two days later, while progesterone was a significant negative predictor of sexual desire at the time of sampling, and at a one or two day lag. By contrast, testosterone never predicted women's sexual desire. The results of Roney and Simmons (2013) suggest that circulating estradiol is more closely associated with the midcycle peak in women's sexual desire than is circulating testosterone.

Dennerstein et al. (2002) examined the relationship between waning levels of ovarian estradiol and testosterone across the menopausal transition and the postmenopausal decline in women's sexual desire. The authors followed a cohort of 226 perimenopausal women for eight years, as they transitioned from early to late menopause, and charted both hormonal condition and sexual functioning across that time span. They found that estradiol levels were significantly correlated with women's self-reported levels of sexual desire and sexual responsiveness across the menopausal transition, but that testosterone levels did not significantly correlate with any measure of female sexual functioning. The results of Dennerstein et al. (2002) suggest that the menopause-related decline in women's sexual desire is more closely associated with a loss of circulating estradiol rather than a loss of testosterone; nonetheless, both estradiol and testosterone are currently prescribed for the treatment of low libido in postmenopausal women.

1.2. Studies of hormone therapies for low libido in postmenopausal women

Researchers have invested a great deal of effort over the past three decades in assessing whether estrogen or androgen therapies are more effective at increasing sexual desire in postmenopausal women (for review see Alexander et al., 2004; Cappelletti & Wallen, 2016). Over the past 30 years, four double-blind randomized trials have examined the effectiveness of estrogen-only therapies at increasing sexual desire in

postmenopausal women (Dennerstein et al., 1980; Myers et al., 1990; Sherwin, 1991; Sherwin et al., 1985). These four studies produced conflicting results; two found that an estrogen-only therapy was effective at increasing sexual desire in postmenopausal women (Dennerstein et al., 1980; Sherwin, 1991), and two found that self-reported levels of sexual desire did not significantly differ between participants receiving an estrogen-only therapy and placebo controls (Myers et al., 1990; Sherwin et al., 1985). These contradictory results may reflect differences in the estrogen treatments administered in these four studies, which produced widely varying levels of circulating estradiol. For example, Myers et al. (1990) administered a conjugated equine estrogen (CEE) therapy alone or in combination with methyltestosterone to 40 naturally postmenopausal women, and found that neither treatment significantly increased self-reported levels of sexual desire. Their CEE therapy, however, produced circulating estradiol levels averaging <40 pg/mL throughout the study. In naturally cycling premenopausal women, estradiol levels normally range between 100 and 400 pg/mL during the periovulatory phase of menstrual cycle (Korenman & Sherman, 1973; Moghissi et al., 1972), the portion of the menstrual cycle associated with the midcycle peak in women's sexual desire. Thus, the estrogen therapy administered by Myers et al. (1990) would not have been expected to increase women's sexual desire, given the very low levels of estradiol it produced. Conversely, Sherwin (1991) administered a CEE therapy that produced periovulatory levels of circulating estradiol (>95 pg/mL) to 48 naturally postmenopausal women, and found that participants' self-reported levels of sexual desire were higher during weeks of the month when they were receiving CEE therapy as compared to hormone free weeks. However, Sherwin (1991) did not administer a placebo during the hormone free study weeks, and it

is not possible to confirm that their CEE therapy would have increased participants' sexual desire as compared to placebo.

Sherwin et al. (1985) conducted one of the most comprehensive investigations to date examining the effectiveness of estrogen and androgen therapies at improving sexual functioning in postmenopausal women. The authors administered an estrogen-only treatment (estradiol valerate), a testosterone-only treatment (testosterone enanthate), an estrogen in combination with testosterone (estradiol dienanthate, estradiol benzoate, testosterone enanthate benzilic acid hydrozone), or placebo to 53 surgically postmenopausal women immediately following oophorectomy. The authors found that self-reported levels of sexual desire did not differ between the testosterone-only and estrogen+testosterone treatment groups, and that both treatments increased sexual desire as compared to the estrogen-only treatment and placebo. Conversely, self-reported levels of sexual desire did not differ between the estrogen-only and placebo treatment groups. However, the dosage of testosterone administered in this study produced markedly supraphysiological levels of circulating testosterone in both the testosterone-only and estrogen+testosterone treatment groups (>100 ng/dL as compared to normal levels of 15-50 ng/dL). Unfortunately, the authors reported circulating estrogen levels produced by all four treatments as a combination of estradiol plus estrone (a relatively weak estrogen, typically present in high concentrations), making it impossible to determine whether their estrogen-only treatment produced periovulatory levels of circulating estradiol.

Apart from Sherwin et al. (1985), only one other double-blind randomized controlled trial has administered testosterone to postmenopausal women in the absence of a concurrent estrogen therapy (Davis et al., 2008). Davis et al. (2008) treated 814

postmenopausal women who were not taking an estrogen therapy with one of two different dosages of testosterone (150 ug/d and 300 ug/d) via transdermal patch, or with a placebo. The authors reported that the 300 ug/d transdermal testosterone patch (TTP) significantly increased sexual desire at both 12- and 24-weeks of treatment as compared to placebo; however, the authors also reported that the 300 ug/d TTP produced supraphysiological levels of circulating testosterone (>60 ng/dL). Furthermore, the increase in sexual desire reported for the women in the 300 ug/d TTP treatment group was of questionable clinical relevance. The authors used the Profile of Female Sexual Functioning (PFSF) to assess sexual desire, and reported that the mean sexual desire score for participants in the 300 ug/d TTP treatment group never exceeded 40, the clinical cutoff for low sexual desire (Dennerstein et al., 2006; McHorney et al., 2004). Thus, the women in the 300 ug/d TTP treatment group were still experiencing clinically low levels of sexual desire, while simultaneously experiencing supraphysiological levels of circulating testosterone. Additionally, the authors reported that the 150 ug/d TTP, which produced physiological levels of circulating testosterone (<50 ng/dL), did not increase sexual desire at 12 weeks of treatment, but *did* increase sexual desire at 24 weeks of treatment, as compared to placebo. Changes in sexual desire across the human menstrual cycle occur within a 14-day period, and it is unclear why 24 weeks of treatment would be required to find an effect of testosterone on women's sexual desire in this particular case. Unfortunately, the authors only reported the effect of the 150 ug/d TTP, but did not provide any data, so it is not possible to know how large an effect of testosterone was found. Today, Davis et al. (2008) remains the only study to administer physiological testosterone to postmenopausal women in the absence of a concurrent estrogen therapy,

and the effects of physiological testosterone on women's sexual desire remain unclear. The results of Sherwin et al. (1985) and Davis et al. (2008) suggest that testosterone, at supraphysiological levels, is capable of increasing sexual desire in postmenopausal women in the absence of a concurrent estrogen therapy.

Ten double-blind randomized controlled trials have compared the effectiveness of an estrogen therapy alone and in combination with testosterone at increasing sexual desire in postmenopausal women (Braunstein et al., 2005; Buster et al., 2005; Davis et al., 2006b; Flöter et al., 2002; Lobo et al., 2003; Panay et al., 2010; Sarrel et al., 1998; Shifren et al., 2000; 2006; Simon et al., 2005). Sarrel et al. (1998) administered an esterified estrogen (EE) therapy by itself or in combination with methyltestosterone to postmenopausal women, and reported that neither treatment increased sexual desire as compared to baseline. Their EE treatment, however, produced low levels of circulating estradiol (40-70 pg/mL), which may explain why neither treatment increased sexual desire. Lobo et al. (2003) administered methyltestosterone or placebo to 218 postmenopausal women currently taking, but dissatisfied with, an estrogen therapy, and reported that methyltestosterone increased sexual desire as compared to placebo; however, estradiol levels were again very low in these women (<40 pg/mL), which may explain why they were dissatisfied with their estrogen therapies to begin with. There is currently no assay for methyltestosterone, and thus the authors of these two studies were unable to determine whether their methyltestosterone treatments were physiological. Flöter et al. (2002) administered estradiol valerate alone or in combination with testosterone propionate to 50 surgically postmenopausal women. The authors reported that both treatments significantly increased sexual desire as compared to baseline, but

that the combined treatment increased sexual desire more than did the estradiol-only treatment. However, the authors also reported that their combined estradiol+testosterone treatment produced supraphysiological levels of circulating testosterone (>140 ng/dL). The results of Flöter et al. (2002) and Lobo et al. (2003) suggest that testosterone may enhance the effectiveness of a low-dose estrogen therapy at increasing sexual desire in postmenopausal women.

In the seven remaining estrogen versus estrogen+testosterone studies, researchers administered either testosterone (via TTP) or placebo to postmenopausal women currently taking, but dissatisfied with, an estrogen therapy (Braunstein et al., 2005; Buster et al., 2005; Davis et al., 2006b; Panay et al., 2010; Shifren et al., 2000; 2006; Simon et al., 2005). Six of these seven studies found that adding the 300 ug/d TTP to an existing estrogen regimen significantly increased women's sexual desire as compared to placebo (Braunstein et al., 2005; Buster et al., 2005; Davis et al., 2006b; Panay et al., 2010; Shifren et al., 2006; Simon et al., 2005). However, as mentioned above, the 300 ug/d TTP produces supraphysiological levels of circulating testosterone (>50ng/dl; Braunstein et al., 2005; Buster et al., 2005; Davis et al., 2006b; Panay et al., 2010; Shifren et al., 2000; 2006; Simon et al., 2005). By contrast, the 150 ug/d TTP, which produces physiological levels of circulating testosterone (<50 ng/dl), has proven ineffective at increasing sexual desire in postmenopausal women currently taking an estrogen therapy (Braunstein et al., 2005; Shifren et al., 2000). For example, Braunstein et al. (2005) administered the 150 ug/d, 300 ug/d, 450 ug/d TTP, or placebo to 318 surgically postmenopausal women currently taking an estrogen therapy. The authors reported that, consistent with findings from other TTP studies, the 300 ug/d TTP significantly increased participants' sexual

desire as compared to placebo, but neither the 150 ug/d nor the 450 ug/d TTP increased sexual desire. Why the 450 ug/d TTP, which produced supraphysiological levels of testosterone (>100 ng/dl) that were significantly higher than the levels produced by the 300 ug/d TTP, did not similarly increase sexual desire remains unresolved. However, and as with Davis et al. (2008), the improvements in sexual desire reported in these six TTP studies, while statistically significant, left participants with levels of sexual desire that would still clinically be considered dysfunctional. The results of these six TTP studies again suggest that supraphysiological levels of testosterone – with the exception of the 450 ug/d TTP dosage – enhance the effectiveness of an estrogen therapy at increasing sexual desire in postmenopausal women, but not to levels that would reverse the clinical condition of low sexual desire.

Three single-blind randomized controlled trials have also investigated the effects of estradiol alone or in combination with testosterone on sexual desire and functioning in postmenopausal women (Burger et al., 1987; Davis et al., 1995; Dow et al., 1983). Burger et al. (1987) reported that only the estradiol+testosterone treatment increased sexual desire as compared to baseline, but also reported that their estradiol+testosterone treatment produced supraphysiological levels of circulating testosterone (>100 ng/dl). Furthermore, the authors did not report levels of circulating estradiol produced by either treatment, leaving unresolved whether their estradiol-only treatment produced periovulatory levels of circulating estradiol. Dow et al. (1983) and Davis et al. (1995) both reported that estradiol alone and in combination with testosterone increased participants' levels of sexual desire as compared to baseline, and that self-reported levels of sexual desire did not differ between the estradiol-only and estradiol+testosterone

treatment groups. Importantly, these two studies administered the same estradiol-only treatment, and Davis et al. (1995) reported that this treatment produced periovulatory levels of circulating estradiol (90-250 pg/mL), which may explain why these two studies found that estradiol alone was as effective at increasing sexual desire in postmenopausal women as was estradiol in combination with supraphysiological amounts of testosterone.

In summary, four out of five studies found that estrogen-only therapies that produced periovulatory levels of circulating estradiol increased sexual desire in naturally and surgically postmenopausal women (Davis et al., 1995; Dennerstein et al., Dow et al., 1983; 1980; Sherwin, 1991; Sherwin & Gelfand, 1987); nonetheless, estrogen-only therapies for women have not been enthusiastically pursued, due in large part to safety concerns about exposure to elevated levels of estradiol (Grady et al., 1995, Kurman et al., 1985; Rossouw et al., 2002; Smith et al., 1975; Yager et al., 2006). Ten out of twelve studies found that the addition of supraphysiological testosterone enhanced the effectiveness of a low-dose estrogen therapy at increasing sexual desire in postmenopausal women (Burger et al., 1987; Flöter et al., 2002; Sherwin et al., 1985; Sherwin & Gelfand, 1987; Braunstein et al., 2005; Buster et al., 2005; Davis et al., 2006b; Panay et al., 2010; Shifren et al., 2006; Simon et al., 2005); however, the mechanism by which testosterone influences women's sexual desire in the presence of estradiol remains unknown.

1.3. DHED: a brain-specific estrogen

There is an unmet need for new and safer estrogen therapies for women to treat the cognitive and behavioral symptoms associated with hypoestrogenic conditions – such as natural and surgical menopause – including mood disorders, sleep disorders, memory

problems, hot flushes, and loss of sexual desire (Bachmann & Leiblum, 2004; Dennerstein et al., 2000; Ensrud et al., 2009; Freeman, 2010; Weber & Mapstone, 2009). High-dose estrogen therapies that produce periovulatory levels of circulating estradiol (>100 pg/mL) increase sexual desire in postmenopausal women (Cappelletti & Wallen, 2016); however; many women cannot, or do not want to, use high-dose estrogen therapies because of concerns about increased risk of breast and uterine cancers (Grady et al., 1995, Rossouw et al., 2002; Yager et al., 2006.) Estradiol stimulates the uterine endometrium, resulting in hyperplasia (excessive growth of the endometrium), which has been linked to the development of endometrial carcinomas (Kurman et al., 1984; Smith et al., 1975). Some estradiol metabolites can cause oxidative depurination of DNA, which may initiate tumor growth in breast tissue (Cavalieri et al., 1997; Yue et al., 2003). In consequence, postmenopausal women are currently prescribed low-dose estrogen therapies, which produce marginal levels of circulating estradiol (<40 pg/mL). Low-dose estrogen therapies are not associated with an increased risk of cancer (Writing group for the Women's Health Initiative, 2004), but are also not effective at increasing sexual desire in postmenopausal women (Cappelletti & Wallen, 2016). Due to the potential carcinogenic effects of high-dose estrogen therapies, a current tension exists between the goals of improving quality of life for postmenopausal women and reducing their risk of cancer.

The compound 10β,17β-dihydroxyestra-1,4-dien-3-one (estradiol paraquinol; DHED), a naturally occurring metabolite of estradiol, offers promise as an estrogen therapy. DHED has no receptor-mediated biological activity in its native form; DHED shows no intrinsic affinity for the estrogen receptors (Prokai-Tatrai et al., 2008) and

DHED's affinity for the androgen receptor is roughly 50-times weaker than that of estradiol (Prokai, et al., 2003). Furthermore, DHED shows no affinity for the progesterone receptor (Prokai, et al., 2003). However, data out of the lab of Dr. Laszlo Prokai suggest that DHED can be converted to active estradiol by a short-chain dehydrogenase/reductase enzyme expressed *only* in the brain (Prokai et al., 2015; Prokai-Tatrai et al., 2008).

Prokai et al. (2015) conducted a series of bioanalytical and imaging experiments in ovariectomized (OVX) rodents to test the brain-selectivity of DHED bioactivation. DHED was added to various OVX rat tissue homogenates, and was found to be preferentially converted to estradiol in brain tissue as compared to other estrogen-sensitive peripheral tissues, including uterine tissue. In agreement with these findings, the authors also reported that intravenous DHED treatment in OVX rats significantly increased estradiol concentrations in the brain, but had no effect on circulating levels of estradiol in the periphery. Furthermore, and unlike estradiol, DHED treatment did not increase mean wet uterine weight as compared to vehicle in OVX mice, suggesting that DHED treatment did not result in estrogenic stimulation of uterine tissue. Prokai et al. (2015) also examined the effects of DHED treatment on progesterone receptor (PR) expression in the preoptic area (POA) of the hypothalamus in OVX rats. In mammals, PR expression in the hypothalamus is sensitive to levels of estrogens, and treatment with exogenous estradiol reliably increases PR expression in the POA (Bethea et al., 1996; Romano et al., 1989; Roy et al., 1978). The authors reported that both DHED and estradiol treatment similarly increased PR expression in the POA as compared to vehicle. Furthermore, the effects of DHED treatment on PR expression in the POA were

attenuated by the co-administration of an estrogen receptor antagonist (ICI 182,780), indicating that the effects of DHED treatment on PR expression were likely the result of estradiol generated from DHED. Taken together, these findings support the view that DHED is converted to estradiol only in the brain, and that estradiol generated from DHED *in situ* remains confined to the central nervous system.

There is preliminary data from research with rhesus monkeys to suggest that the enzyme responsible for the conversion of DHED to active estradiol is expressed only in the brain in primates, as well as in rodents. Our lab, in collaboration with Dr. Laszlo Prokai at the University of North Texas at Fort Worth and Dr. Istvan Merchenthaler at the University of Maryland, Baltimore, investigated the effects of chronic DHED treatment on brain and peripheral tissues in OVX rhesus monkeys. DHED was detected in the urine of all DHED-treated subjects; however, no DHED was detected in the urine of vehicle-treated controls. DHED was not detectable in the serum of any of the DHED-treated subjects, suggesting that DHED rapidly exits circulation and enters tissues. DHED treatment increased estradiol concentrations in both the cortex and mediobasal hypothalamus as compared to vehicle (Figure 1); conversely, serum estradiol concentrations did not significantly differ between DHED-treated subjects and vehicle-treated controls (Figure 2). In mammals, expression of the kisspeptin-1 gene in the POA decreases following ovariectomy, and increases in response to treatment with exogenous estradiol (Adachi et al., 2007; Smith et al., 2005). DHED treatment significantly increased kisspeptin-1 gene expression in the POA of DHED-treated subjects as compared to vehicle-treated controls (Figure 3). Taken together, these results support the view that, in both rodents and primates, DHED (a) crosses the blood-brain barrier, (b) is

converted to estradiol in the brain, and that (c) estradiol generated from DHED remains confined to the central nervous system. Thus, DHED may represent a brain-specific estradiol pro-hormone, capable of delivering effective amounts of estradiol to the brain without having peripheral effects on estrogen-sensitive tissues, including breast and uterine tissues. The development of a brain-specific estrogen therapy would be a major advance in the treatment of hypoestrogenic conditions, and could potentially improve quality of life for millions of women worldwide.

1.4. Mechanisms by which androgens may influence women's sexual desire

Because of concerns about the potential carcinogenic effects of high-dose estrogen therapies, physicians frequently prescribe testosterone therapy for the treatment of low libido in postmenopausal women (Krapf & Simon, 2009). Testosterone, however, is not approved by the Food and Drug Administration (FDA) for the treatment of low sexual desire in women, and physiological levels of testosterone on their own do not meaningfully increase women's sexual desire. Evidence indicates that low-dose estrogen therapies are generally more effective at increasing sexual desire in hypogonadal woman when administered in combination with supraphysiological testosterone; however, it remains unclear how and why testosterone has this effect. One possibility is that testosterone enhances the effectiveness of a low-dose estrogen therapy at increasing women's sexual desire via its own conversion to estradiol. While the gonads are the primary source of sex steroids in both men and women, estrogens can be synthesized in a number of extragonadol tissues via the aromatization of androgens (Simpson, 2002). Testosterone is directly converted to estradiol in adipose, breast, bone, and brain tissue (amongst others) via the enzyme aromatase (Simpson, 2002). As such, the aromatization

of testosterone to estradiol in various areas of the brain could account for testosterone's ability to enhance the effectiveness of a low-dose estrogen therapy at increasing women's sexual desire. In this view, the addition of testosterone to a low-dose estrogen therapy would increase the amount of estradiol in the brain, resulting in increased sexual desire.

Only one study has directly investigated whether aromatization is required for androgens to exert influence over women's sexual desire. Davis et al. (2006a) administered testosterone (as a topical gel) alone or in combination with an orally-administered aromatase inhibitor (letrozole) to 76 postmenopausal women currently taking, but dissatisfied with, an estrogen therapy. The authors found no difference in self-reported levels of sexual desire between the testosterone-only and testosterone+letrozole treatment groups, and concluded that testosterone was capable of increasing women's sexual desire without first being aromatized to estradiol. However, no data were presented showing that the dosage of letrozole administered in this study fully suppressed the aromatization of testosterone. As evidence to the contrary, levels of circulating estradiol did not differ between the testosterone-only and testosterone+letrozole treatment groups, even though estradiol levels should have been lower in the group receiving the aromatase inhibitor – as was found in a similar study of men treated with testosterone or testosterone plus an aromatase inhibitor (Bagatell et al., 1994). Furthermore, as the authors did not include a placebo control group that did not receive testosterone, it is not possible to confirm that their testosterone treatment, with or without letrozole, increased participants' sexual desire as compared to placebo. Today, it remains unclear whether aromatization is critical for testosterone's ability to influence women's sexual desire.

A second, although little explored, possibility is that testosterone enhances the effectiveness of a low-dose estrogen therapy at increasing women's sexual desire by preferentially binding sex hormone binding globulin (SHBG). SHBG is a binding-protein found in all vertebrates, apart from birds, which circulates in the blood and reversibly binds both estradiol and testosterone, although it binds testosterone with twice the affinity that it binds estradiol (Burke & Anderson, 1972; Hammond & Bocchinfuso, 1995; Mean et al., 1977). Steroids in blood circulate in three forms: (1) bound to SHBG, (2) bound to albumin (a high capacity, low affinity serum binding protein), or (3) unbound (Pardridge, 1988). The majority of estradiol and testosterone in the blood circulates bound to SHBG or albumin at any given time, and only the relatively small unbound (free) fraction (1-3%) of either steroid is considered biologically active (Burke & Anderson, 1972; Mean et al., 1977). The equilibrium between bound and free concentrations of estradiol and testosterone is modulated by the two steroids' differential binding affinities for SHBG. Because SHBG preferentially binds testosterone, testosterone is capable of displacing and liberating SHBG-bound estradiol (Burke & Anderson, 1972; Mean et al., 1977). As a result of this dynamic system (Burke & Anderson, 1972), the addition of testosterone to an estrogen therapy would theoretically increase circulating levels of unbound and biologically active estradiol, resulting in increased sexual desire (Wallen, 2001). There is currently little evidence to support either the aromatization or SHBG explanation for the enhanced effectiveness of a low-dose estrogen therapy in combination with supraphysiological testosterone, and the two possibilities are not mutually exclusive; testosterone may enhance the effectiveness of a low-dose estrogen therapy at increasing

women's sexual desire via its aromatization to estradiol, or via its preferential binding to SHBG, or both.

Unlike testosterone, the androgen 5-α-dihydrotestosterone (DHT) cannot be aromatized to an estrogen, and DHT can therefore be used to elucidate whether aromatization is required for androgens to exert influence over women's sexual desire. Furthermore, SHBG has a stronger affinity for DHT than it does for either testosterone or estradiol (Hammond & Bocchinfuso, 1995; Mean et al., 1977), and thus DHT can be used to determine whether androgen therapy increases circulating levels of free versus SHBG-bound estradiol. Understanding the mechanism by which testosterone enhances estradiol's influence over women's sexual desire may lead to the development of safer and more effective treatments for low libido in hypogonadal women, and help elucidate the role that androgen's play in the maintenance and modulation of women's sexual desire.

1.5. Hormones and female primate sexual behavior

The present dissertation explored the effects of DHED and DHT on female sexual motivation and behavior in rhesus monkeys. Female rhesus monkeys share many aspects of their reproductive biology in common with women, including a roughly 28 day menstrual cycle, with nearly identical patterns of hormonal fluctuation (Wallen et al., 1984; Wilson et al., 1982). In naturally cycling female rhesus monkeys, as with women, circulating estradiol levels increase steadily throughout the follicular phase of the menstrual cycle, peak at midcycle roughly 24h prior to ovulation, and subsequently drop off and remain at midfollicular levels throughout the luteal phase of the menstrual cycle (Wallen et al., 1984; Wilson et al., 1982).

Ball and Hartman (1935) were the first to study female rhesus monkey sexual behavior across the menstrual cycle. They used rates of female-initiated sexual behavior as a proxy for female sexual motivation, and reported that rates of female sexual initiation (i.e. the frequency with which females approached males, solicited attention from males, and presented their hind-quarters to males) fluctuated across the menstrual cycle, and peaked at midcycle just prior to ovulation. In the early 1940's, C. R. Carpenter studied female rhesus monkey sexual behavior across the menstrual cycle in free-ranging rhesus monkeys on the island of Cayo Santiago, Puerto Rico. Like Ball and Hartman (1935), Carpenter (1942) reported that female rhesus monkeys displayed a pronounced midcycle peak in sexual motivation, as indicated by the eagerness with which females approached and solicited sexual behavior from males. The results of Ball and Hartman (1935) and Carpenter (1942) suggested that ovarian steroids modulated female primate sexual motivation in such a way as to maximize female mating efforts at ovulation.

Despite early evidence to indicate that female rhesus monkey sexual motivation fluctuated across the menstrual cycle, years of subsequent laboratory studies involving timed observations of male-female rhesus monkey pairs in controlled testing environments ("pair-tests") produced conflicting results (for review see Wallen, 1990). Early researchers employing the pair-test paradigm reported that female rhesus monkeys mated across the menstrual cycle, with only a slight and non-significant increase in copulation frequency at ovulation (e.g. Johnson & Phoenix, 1978). Female primates, however, are physically capable of engaging in mating behavior at any time, irrespective of their hormonal condition or level of sexual motivation, making copulation frequency an unreliable proxy for female primate sexual motivation. In addition, pair-tests were

conducted in confined testing enclosures that offered females limited opportunity to avoid or refuse the sexual advances of males; thus, copulation frequency under these testing conditions was almost certainly influenced by male sexual motivation (Wallen, 1990; 2001).

Researchers who modified the pair-test experimental paradigm to give rhesus monkey females greater control over the frequency of sexual interactions with males reported a more striking relationship between female primate sexual behavior and the menstrual cycle (Keverne, 1976; Michael & Bonsall, 1977; Pomerantz & Goy, 1983). Pomerantz and Goy (1983), for example, placed the pair-test male on a short tether, allowing the female to control both her proximity to the male and the frequency of sexual interactions. They reported that, under these testing conditions, females approached and solicited sexual behavior from males significantly more frequently during the follicular versus the luteal phase of the menstrual cycle. Keverne (1976) and Michael and Bonsall (1977) both employed an operant paradigm which required females to press a bar 250 times to gain access to a male, and both reported that females exhibited the fastest access times just prior to ovulation. Keverne (1976) further reported that females stopped bar-pressing entirely following ovariectomy, but that bar-pressing could be reinstated by treatment with exogenous estradiol. Wallen and Goy (1977) employed the pair-test paradigm, but used considerably larger cages than had previous studies, and used female sexual initiation (as opposed to copulation frequency) as a proxy for female sexual motivation. In agreement with Keverne (1976), Wallen and Goy (1977) found that treatment with estradiol benzoate increased the frequency with which OVX female rhesus monkeys approached and solicited attention from males. In summary, when researchers

manipulated the laboratory pair-test paradigm to allow for independent expression of male and female sexual motivation, they revealed a clear relationship between ovarian steroids and female primate sexual motivation (Wallen, 1990; 2001).

Researchers studying female rhesus monkey sexual behavior in more ecologically relevant social contexts provided further evidence to indicate that female primate sexual motivation was modulated by ovarian steroids. Wallen et al. (1984) measured the frequency of 10 different female-initiated male-directed sexual behaviors across the menstrual cycle in naturally cycling female rhesus monkeys living in large species-typical social groups. They found that 9 out of 10 female-initiated sexual behaviors were positively correlated with circulating estradiol prior to ovulation, 10 out of 10 behaviors were negatively correlated with circulating progesterone after ovulation, and that none of the 10 behaviors were significantly correlated with circulating testosterone at any point during the ovarian cycle. Zehr et al. (1998) administered estradiol to OVX female rhesus monkeys living in large species-typical social groups. The authors reported the female-initiated sexual behavior decreased following ovariectomy, but that estradiol treatment significantly increased the frequency with which OVX females approached, contacted, groomed, and presented to males. Zehr et al. (1998) further reported that the frequency of female-initiated sexual behavior was positively correlated with circulating estradiol and negatively correlated with circulating progesterone for both the estradiol-treated OVX females and the intact/nonpregnant controls.

Taken together, studies of female rhesus monkey sexual behavior in both laboratory and more ecologically relevant settings have demonstrated that female rhesus monkey sexual motivation (as measured by the frequency of female-initiated sexual

behavior) fluctuates across the ovarian cycle and peaks at midcycle just prior to ovulation (Ball & Hartman, 1935; Michael & Bonsall, 1977; Pomerantz & Goy, 1983), decreases following ovariectomy (Keverne, 1976; Zehr et al., 1998), and increases in response to treatment with exogenous estradiol (Keverne, 1976; Wallen & Goy 1977; Zehr et al., 1998). As such, increased female sexual initiation serves as both a behavioral marker for the actions of estradiol and as an indicator of increased sexual motivation in female rhesus monkeys. Female sexual initiation in rhesus monkeys therefore represents an appropriate behavioral endpoint with which to test the effects of estrogens and androgens on female primate sexual motivation.

2. SPECIFIC AIMS

The present dissertation, comprising two separate studies, investigated the roles of estrogens and androgens in the hormonal modulation of female primate sexual motivation in rhesus monkeys (*Macaca mulatta*). Study One investigated the effects of estradiol, DHED (a brain-specific estradiol pro-hormone), and DHT (a non-aromatizable androgen) on serum estradiol levels and rates of female sexual initiation in group-living, estradiol-primed OVX rhesus monkeys. Study Two investigated dynamic changes in serum concentrations of free versus SHBG-bound estradiol following an acute injection of DHT in chronic estradiol-treated OVX rhesus monkeys. Taken together, these two studies will further elucidate the roles that estrogens and androgens play in the hormonal modulation of female primate sexual motivation, and hopefully contribute to the development of safer and more effective hormone therapies for women for the treatment of symptoms associated with hypogonadal conditions, including natural and surgical menopause.

Specific aim 1: Investigate the effects of estrogens and androgens on female primate sexual motivation (Study One).

Specific aim 1a: Determine whether acute treatment with estradiol increases female sexual initiation in estradiol-primed OVX rhesus monkeys.

Rationale: We hypothesized that acute estradiol treatment, producing periovulatory levels of serum estradiol, would increase rates of female sexual initiation in estradiol-primed OVX rhesus monkeys, which would provide further evidence that estradiol (at periovulatory levels) is capable of increasing female primate sexual motivation without the co-administration of an androgen. Given

the substantial literature supporting a relationship between estradiol and female primate sexual motivation (for review see Wallen & Tannenbaum, 1997), in the present study, acute estradiol treatment served as a positive control.

Specific aim 1b: Determine whether acute treatment with DHED (a brain-specific estradiol pro-hormone) increases rates of female sexual initiation in estradiol-primed OVX rhesus monkeys.

Rationale: Preliminary data indicate that DHED has no receptor-mediated biological activity in its native form (Prokai-Tatrai et al., 2008), but that DHED can be converted to active estradiol by an enzyme expressed only in the brain (Prokai et al., 2015; Prokai-Tatrai et al., 2008). Female sexual initiation in rhesus monkeys is sensitive to levels of estradiol (for review see Wallen & Tannenbaum, 1997). We hypothesized that acute DHED treatment would increase rates of female sexual initiation in estradiol-primed OVX rhesus monkeys, which would provide further evidence that DHED acts as estradiol in the brain.

Specific aim 1c: Determine whether acute treatment with DHT (a non-aromatizable) increases female sexual initiation in estradiol-primed OVX rhesus monkeys.

Rationale: A growing body of evidence suggests that low-dose estrogen therapies are generally more effective at increasing sexual desire in postmenopausal women when co-administered with supraphysiological levels of testosterone (reviewed in Cappelletti & Wallen, 2016); however, the mechanism by which testosterone influences women's sexual desire in the presence of estradiol remains unclear. We

hypothesized that acute DHT treatment would increase rates of female sexual initiation in estradiol-primed OVX rhesus monkeys, which would provide evidence to indicate that aromatization to estradiol is not required for androgens to exert influence over female primate sexual motivation.

Specific aim 2: Measure dynamic hormonal changes in response to acute estrogen and androgen treatments.

Specific aim 2a: Determine whether acute DHED treatment increases serum estradiol concentrations in estradiol-primed OVX rhesus monkeys (Study One).

Rationale: Preliminary data indicate that (1) DHED is converted to estradiol only in the brain, and (2) that the estradiol generated from DHED remains confined to the central nervous system (Prokai-Tatrai et al., 2008, Prokai et al., 2015). We hypothesized that acute DHED treatment would not increase serum estradiol concentrations in estradiol-primed OVX rhesus monkeys, which would provide further evidence to indicate that the enzyme that converts DHED to estradiol is expressed only in the brain, and that the estradiol generated from DHED *in situ* remains confined to the central nervous system.

Specific aim 2b: Determine whether an acute injection of DHT results in a transient increase in serum concentrations of free versus SHBG-bound estradiol in chronic low-dose estradiol-treated OVX rhesus monkeys (Study Two).

Rationale: The mechanism by which testosterone influences female sexual motivation in the presence of estradiol remains unclear. One possibility is that

testosterone influences female sexual motivation by preferentially binding SHBG, liberating SHBG-bound estradiol, and increasing circulating levels of biologically active, free estradiol (Burke & Anderson, 1972; Wallen, 2001). We hypothesized that acute treatment with DHT would temporarily increase serum levels of free- versus SHBG-bound estradiol in OVX rhesus monkeys receiving chronic estradiol therapy, which would provide support for the view that androgens influence female primate sexual motivation (at least in part) by preferentially binding SHBG and liberating SHBG-bound estradiol.

3. STUDY ONE: THE EFFECTS OF ESTRADIOL (E2), ESTRADIOL PARA-QUINOL (DHED), AND DIHYDROTESTOSTERONE (DHT) ON FEMALE SEXUAL INITIATION IN RHESUS MONKEYS

3.1. METHODS

3.1.1. Subjects and housing

Subjects were OVX adult rhesus monkey females (n = 6) living in two age-graded social groups (3 in one group, 3 in the other) at the Yerkes National Primate Research Center (YNPRC) Field Station in Lawrenceville, GA. Each social group was comprised of 15-20 individuals, including one adult male and multiple intact, cycling females. Housing consisted of a large outdoor enclosure (~230 m^2) with an attached heated and cooled indoor quarters (~16.5 m^2). All subjects had *ad libitum* access to low-fat high-fiber monkey chow (Ralston Purina Company, St. Louis, MO) and water throughout the study, and received fresh fruit and vegetables daily. All subjects had given birth at least once prior to study assignment, and ranged in age from 6 to 17 years at study onset. In an attempt to minimize the potential confounding impact of rank on female sexual initiation, the highest and lowest ranking females in each group were excluded as subjects. A seventh subject had originally been assigned to the study, but was released from the study prior to completion of data collection due to declining health and poor body condition. Data collected on this subject are not included in any of the hormonal or behavioral analyses reported here. All research was conducted in accordance with the National Institutes of Health (NIH) standards and guidelines, and was approved by the Emory University Institutional Animal Care and Use Committee (IACUC).

In order to induce a hypoestrogenic condition, similar to that associated with natural and surgical menopause in women, all subjects underwent bilateral ovariectomy. All subjects were determined to be non-pregnant prior to ovariectomy via consultation with Colony Management and Veterinary Staff, including a pre-assignment physical with abdominal ultrasound when necessary. Laparoscopic ovariectomy was performed by YNPRC veterinarians. The ventral abdomen and either an arm or leg were clipped for catheter implantation, and the animal was placed on isoflurane anesthesia administered via an enodotracheal tube and intravenous (IV) crystalloid therapy at a rate of 10 mL/kg/hr. A respiratory ventilator was used based on veterinarian discretion. The animal was positioned in dorsal recumbency. The abdomen was repeatedly scrubbed with a surgical scrub solution, and sterilely draped. A 1cm skin incision was made above the umbilicus using a scalpel blade with a stab incision into the abdominal wall. A Verres needle was used to insufflate the abdomen with carbon dioxide gas, generally to 15mm Hg pressure. Once sufficient insufflation had occurred, the Verres needle was replaced with a trocar and sheath for insertion of a fiber-optic telescope for visualization. The animal was then moved into Trendelenburg positioning. Two stab incisions were made into the abdominal wall with a scalpel blade, guided by visualization from the telescope, allowing for the placement of bilateral paralumbar accessory ports. The ovarian vascular pedicle was grasped using electrocautery capable scissors introduced through the contralateral accessory port. The utero-ovarian ligament and vascular pedicle were transected by a combination of cutting and coagulating current, with preservation of the fimbria and oviduct. Monopolar cautery was used to complete the ovarian resection. Resected tissues were removed via the accessory port. Visualization from the telescope

was used to assure hemostasis. The procedure was then repeated for the contralateral ovary. The abdomen was lavaged with warmed, sterile saline to remove hemorrhagic fluid. Laparoscopic instruments were removed and incisions are closed with 3-0 vicryl sutures in the muscular fascia and 4-0 polydioxanone sutures (PDS) in the skin, with either simple continuous, cruciate, or horizontal mattress sutures.

3.1.2. Experimental design

Rhesus monkeys both in the wild and housed in captivity in seminaturalistic environments exhibit a seasonal breeding cycle, which coincides with annual fluctuations in gonadotropin secretion and gonadal steroid levels. Rhesus females exhibit ovulatory cycles and fertility during the August through April breeding season, but cease ovulating during the May through July nonbreeding season, when gonadotropin secretion is reduced and serum gonadal steroid levels are low (Walker et al., 1983). The present study was conducted between October and April, during the rhesus monkey breeding season, when the adult males in the social groups containing the study subjects would have been in breeding condition, and when the intact group females would have been experiencing ovulatory cycles.

Our lab has demonstrated previously that an acute injection of estradiol producing periovulatory levels of circulating estradiol (400-800 pg/mL) increases female sexual initiation in OVX rhesus monkeys when administered on a background of midfollicular estradiol (100-200 pg/mL; Graves & Wallen, Unpublished). In the present study, subjects received each of four acute treatments in a counterbalanced order on a background of midfollicular estradiol. The four acute treatments included: (1) estradiol (10 µg/kg; Product E-8875, Sigma Aldrich Corp), (2) DHED (100 µg/kg), (3) DHT (10 µg/kg;

Product D-073, Sigma Aldrich Corp.), and (4) vehicle. This repeated-measures design allowed for each female to act as their own control, and maximized statistical power. All acute treatments were dissolved in pharmaceutical grade corn oil, and corn oil served as the vehicle control. All acute treatments were administered as intramuscular injections (IM) between 8:30AM and 10:30AM on treatment days.

To produce background levels of midfollicular estradiol, subjects were treated with estradiol benzoate (EB; 3 μg/kg; Product E-8515, Sigma Aldrich Corp.) once-daily on the four days leading up to each acute treatment day. In a preliminary investigation, we confirmed that this dosage of EB produced midfollicular levels of serum estradiol (100-200 pg/mL; Appendix 1). EB is an esterified form of estradiol. Esterification refers to the process by which a natural steroid is combined with a fatty acid (ester), making the steroid more lipophilic. Esterified steroids are more readily taken up by adipose tissue than are natural steroids, which slows the rate of steroid degradation and excretion. EB is therefore metabolized more slowly than is native estradiol, and acute treatment with EB produces a more gradual and prolonged increase in serum estradiol than does acute treatment with native estradiol. EB was administered IM, between 8:30AM and 9:30AM on treatment days.

In summary, subjects received once-daily EB on days 1-4 of each treatment week, and then received one of four acute treatments on day 5. There were six hormone-free washout days after each acute treatment day, allowing serum steroid levels to return to baseline between treatment weeks, and a total of 11 days between acute treatments. There were more than five months between ovariectomy and the first treatment week, allowing

subjects time to fully recover from surgery, and for their gonadal steroid levels to reach baseline.

The dosage of the acute estradiol treatment (10 ug/kg) was selected because it has been shown to produce periovulatory levels of circulating estradiol (400-800 pg/mL; Graves & Wallen, Unpublished). The dosage of the acute DHT treatment (10 μg/kg) was selected to match that of the acute estradiol treatment. We did not know the percentage of DHED administered that could be expected to be converted to estradiol within the window of behavioral observation following acute treatment administration; therefore, the dosage of the acute DHED treatment (100 μg/kg) was selected to be ten-times higher than that of the acute estradiol treatment.

DHED is not currently commercially available. DHED used in this study was synthesized and supplied by Dr. Laszlo Prokai and his associates at the University of North Texas Health Science Center at Fort Worth, TX, and the Emory IACUC policy for non-pharmaceutical grade drugs was followed. Various procedures have been developed for the synthesis of DHED (Gold & Schwenk, 1958; Lupon et al., 1983; Prokai-Tatrai et al, 2007). The method adopted in the present study was a “one-pot” transformation (Prokai et al., 2003; Solaja, 1996), which uses meta-chloroperbenzoic acid (m-CPBA) as an oxidant, dibenzoyl peroxide ($PheCO^2O^2$) as a radical initiator, and visible-light irradiation that, with a refluxing aprotic solvent, has be shown to produce excellent yields. DHED is a stable chemical compound; Dr. Prokai’s laboratory prepared their first batch of DHED in 2001, and regular analyses by high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) have not revealed the formation of impurities during storage to this point. DHED synthesized

in this fashion has now been used in several studies in rodents (Prokai et al., 2015), and no complications have been described thus far.

Prior to implementation of the treatment paradigm described above, a different treatment paradigm had been implemented and subsequently abandoned due to suspected treatment carryover effects that were seemingly suppressing behavior. A full description of the abandoned treatment paradigm is included as Appendix 2. More than eight weeks elapsed between the last treatment of the abandoned treatment paradigm and the first treatment of the revised (final) treatment paradigm, giving subjects' hormone levels ample time to return to baseline.

3.1.3. Behavioral testing

A 60-minute behavioral test was conducted 24h prior to acute treatment administration and 24h after acute treatment administration, and a 120-minute behavioral test was conducted immediately following acute treatment administration. In an attempt to minimize the potential confounding impact of time of day on behavior, all behavioral tests were conducted between 9:30AM and 12:30PM. Rhesus monkeys are crepuscular, and are therefore most active during the time periods immediately after dawn and immediately before dusk. Behavioral tests were conducted in the morning, as opposed to the afternoon, in an attempt to maximize behavior during the period of observation. The social group was locked in the outdoor portion of the enclosure during all behavioral tests. The adult male was sequestered in an indoor area away from the rest of the group for 45 min prior to each behavioral test, to maximize sexual behavior during the period of observation.

All behavioral tests were conducted by the same observer, who watched the animals and called out behavior codes to a typist, who entered the codes into a portable computer. Both the observer and typist were blind to treatment. The adult male of the group was followed, and all interactions between the male and group females (both subjects and nonsubjects) were recorded, including sexual behavior, aggression, grooming, and other social behaviors. A complete list of all behaviors recorded and their definitions is included as Appendix 3. Behavioral data were recorded in an actor-behavior-recipient format using the “WinObs” program (Center for Behavioral Neuroscience, Atlanta, GA), which collects event-sequential data with an elapsed time in thousandths of a minute from the test start, allowing for collection of information regarding both frequency and duration of behavior. Behavioral data files were transferred to a custom data extraction program (Datsummary, Center for Behavioral Neuroscience, Atlanta, GA), and frequencies, durations, and interaction matrices were generated in a format compatible with SPSS data files.

Prior to study onset, intra-observer reliability tests were performed using previously recorded video footage of rhesus monkey social groups. The observer watched the same three videos three times each (with at least two weeks between viewings), following the adult male of the group and recording all interactions between the male and group females. For all three videos, intra-observer reliability exceeded 85%.

To allow the observer to administer the acute treatments and still remain blind to treatment, each acute treatment was randomly assigned a letter code by a lab member not involved with behavioral testing. All acute treatment solutions were stored in identical glass injection vials, and the same lab member covered the label on each vial with a label

bearing the randomly assigned letter code for each acute treatment. The observer knew which subjects were scheduled to receive which letter codes on each acute treatment day, but did not know which letter codes referred to which acute treatments.

The present study investigated the effects of estrogens and androgens on female sexual motivation, and the primary behavioral endpoints of interest were female proceptive behaviors, female-initiated sexual behaviors that demonstrate active interest in the group male (Wallen et al., 1984). Analysis focused on six female proceptive behaviors, all of which have previously been shown to be positively associated with serum estradiol levels in naturally cycling female rhesus monkeys (Wallen et al. 1984; Lovejoy & Wallen, 2001): approach to within one meter of the male (approach), approach to within arms distance of the male (prox), staccato head and hand gestures directed towards the male (solicit), presentation of hindquarters to the male (present), persistent trailing of the male (follow), and grooming of the male (groom). Full definitions of all six proceptive behaviors can be found in Appendix 3. Analysis of the "present" behavior was limited to what has been referred to as "noncontact" or "spontaneous" present, where presentation of the hindquarters is not initiated as a result of the behavior of the recipient (Wallen & Winston, 1984), as opposed to a present in response to the approach of the male (present to approach) or present in response to a hiptouch (present to hiptouch). Male-initiated sexual behaviors, including hiptouches, mounts, and ejaculations, were recorded, but were not analyzed, given that these behaviors reflect both male and female sexual motivation (Wallen, 1990; 2001), and we were specifically interested in effects of treatment on female sexual motivation.

3.1.4. Blood sampling

A 3 mL blood sample was collected from each subject immediately before acute treatment administration, and after the 120-minute behavioral test following acute treatment administration. A 3 mL blood sample was also collected immediately before the 60-minute behavioral observation 24h after acute treatment administration. All subjects were trained to separate from their social group on command and to enter an indoor area. Once sequestered inside, subjects were individually transferred to a modified cage with a small window, which allowed for the presentation of a leg for an awake, unanaesthetized blood draw. All blood samples were collected from the saphenous vein on the back of the calf. The calf was clipped and wiped with a disposable antiseptic wipe prior to venipuncture. Blood samples were collected into 4 mL glass serum separating vacutainer tubes (Product CVN-8881302072, Fisher Scientific), and centrifuged for 15 min at 3000 RPM at 4°C. Plasma was then transferred to 3 mL plastic cryovials and stored at -20°C while awaiting analysis. Blood sample collection remained within the limit of 10 mL/kg/month for each subject.

All hormone assays were conducted by the YNPRC Biomarkers Core Laboratory (Emory University, Atlanta, GA). Estradiol was measured using an immunoassay produced by DRG International Inc. (Springfield Township, NJ) for the Hybrid XL platform (reference number HYE-5349). Samples and standards were analyzed in duplicate, with a maximum tolerated coefficient of variation (CV) of 12%. The lower level of detection limit was 18.2 pg/mL. No inter-assay CV was determined, as all samples were run at the same time to minimize between-assay variability.

3.1.5. Group stability

Rhesus monkey social groups, both in captivity and in the wild, self-organize into linear dominance hierarchies based on matrilineal kinship (referred to as matrilines), which maintain group stability (Lindburg, 1971; McCowan et al., 2008). Rhesus monkey matrilines are comprised of related adult females and their juvenile offspring, and all members of the highest ranking matriline outrank all members of the second highest ranking matriline, and so on and so forth. Rhesus monkeys inherit the social rank of their mothers at birth and, because female rhesus monkeys typically remain in their natal groups, female social rank remains relatively stable across the lifespan (McCowan et al., 2008). Aggression is frequently used by both male and female rhesus monkeys to establish and reinforce social position within the dominance hierarchy (Bernstein & Ehardt, 1985; Lindburg, 1971), and group instability (as characterized by ambiguous dominance relationships between group members) has been shown to be associated with increased levels of both aggression and wounding (Capitanio & Cole, 2016; McCowan et al., 2008).

The two social groups containing the study subjects (S1 and S8) were progressively formed over the course of the summer immediately prior to study onset, and were largely comprised of unrelated and unfamiliar adult females. Group formation began in April of 2014, but additional individuals were progressively introduced into the groups over the next six months, and the adult males were not introduced into each of the groups until October of 2014, the month that the present study began. As such, these two social groups were likely relatively unstable during the present study, given that these unrelated females would have had to establish a *de novo* linear dominance hierarchy

following group formation, without the support of female kin. To assess group stability, levels of wounding during the breeding season immediately following group formation (October 2014 - April 2015; breeding season 1), during which the present study was conducted, were compared to levels of wounding during the subsequent breeding season (October 2015 - April 2016; breeding season 2), more than a year after group formation. Wounding was defined as wounds serious enough to warrant hospitalization for veterinary attention (McCowen et al., 2008). The total number of days that each female spent (at least a portion of the day) out of their social group and in the veterinary hospital as a result of wounding between October 1st and April 30th was calculated for each breeding season. Analysis was limited to females who were members of a given social group during both breeding seasons.

3.1.6. Statistical analyses

All analyses used SPSS software (version 23, IBM Corp., Armonk, NY). All tests were two-tailed, and were considered significant with a p value less than 0.05. Differences in serum estradiol concentrations between treatment conditions immediately before acute treatment administration (at baseline) and after the two-hour behavioral observation immediately following acute treatment administration (2h post-injection), were assessed using repeated-measures analysis of variance (ANOVA). Post hoc analyses examining differences in mean serum estradiol concentrations at 2h post-injection between the estradiol treatment condition and the DHT, DHED, and vehicle treatment conditions used the paired samples t-test. The paired samples t-test was also used to examine differences in mean serum estradiol concentrations at baseline and at 2h post-injection for each treatment condition. For all paired sample t-tests, the magnitude of

differences between means was assessed by calculating Cohen's *d* as an indicator of effect size.

Behavioral data were analyzed using means for all six subjects and, because behavioral test duration was variable, all behavioral frequencies and durations were converted to rates per hour. A one-way ANOVA was first performed to examine effect of treatment order on behavior. If no order effects were found, data were collapsed across treatment order and analyzed for treatment effects. Treatment effects were examined in several ways. First, repeated-measures ANOVA was used to assess differences in behavior between treatment conditions during the two-hour behavioral test immediately following acute treatment administration. Next, repeated-measures ANOVA was used to assess differences in behavior between the three behavioral tests of each treatment week (24h prior to acute treatment administration, immediately following acute treatment administration, and 24h after acute treatment administration), and between treatment conditions, where behavioral test and treatment condition both represented within subjects variables. Finally, to examine the possibility of acute changes in behavior in response to acute changes in hormone levels, repeated-measures ANOVA was used to assess differences in behavior between the first and second hour of the two-hour behavioral test immediately following acute treatment administration, and between treatment conditions, where test hour and treatment condition both represented within subjects variables.

Differences in wounding between breeding season 1 (immediately following group formation) and breeding season 2, and differences in wounding between the two social groups (S1 and S8), were assessed using repeated-measures ANOVA, where

breeding season represented a within subjects variable and social group represented a between subjects variable.

There is currently no consensus on the most appropriate estimator of effect size for analyses of variance involving repeated-measures (Bakeman 2005; Cohen, 1973; Keppel, 1991; Olejnik & Algine, 2003). Partial eta squared has been proposed as an appropriate effect size statistic for repeated-measures ANOVA (Cohen, 1973; Keppel 1991), and is commonly reported in the social sciences for studies involving repeated-measures designs (e.g. Allin et al., 2008; Hands, 2008, Ssewamala et al., 2008; Starr et al., 2004). Partial eta squared is based on the variance within a single factor, and is calculated by dividing the sum of squares (SS) for each factor in a given model by the SS for that factor plus the SS for the error associated with that factor. As such, partial eta squared for a particular factor takes into account the variance associated with that factor, but (unlike eta squared) does not take into account the variance associated with other factors in the model (Olejnik & Algine, 2003). In the present study, partial eta squared (η_p^2) was used as an indicator of effect size for all repeated-measures analyses. Sphericity is an *a priori* assumption of repeated-measures ANOVA, and refers to the condition where the variances of the differences between all possible pairs of groups (i.e., levels of the independent variable) are equal. If the assumption of sphericity is violated, then variance calculations will be distorted, leading to an inflated F-ratio and an increased risk of Type 1 error. Mauchley's Test of Sphericity was used to check for violations of sphericity for all repeated-measures analyses. If the Mauchley's Test of Sphericity and the overall ANOVA were both significant, then the Greenhouse-Geisser correction was used to adjust the degrees of freedom to account for the violation of sphericity.

3.2. RESULTS

3.2.1. Hormonal data

The estradiol benzoate (EB) treatment produced slightly higher background estradiol levels than anticipated; however, background estradiol levels remained within physiological range for the follicular phase of the ovarian cycle (M = 314.84, SD = 236.58 pg/mL). Hormonal data were analyzed using means for all six subjects, as shown in Figure 4. Serum estradiol concentrations measured immediately prior to acute treatment administration (at baseline) did not differ between the four treatment conditions ($F(3,15) = 0.44$, $p = 0.73$, $\eta_p^2 = 0.08$); however, serum estradiol concentrations measured after the two-hour behavioral test immediately following acute treatment administration (2h post-injection) significantly differed between treatment conditions ($F(3,15) = 139.12$, $p < 0.01$, $\eta_p^2 = 0.97$). Post hoc analyses revealed that serum estradiol concentrations at 2h post-injection were significantly higher in the estradiol condition as compared to the DHT ($t(5) = 14.98$, $p < 0.01$, $d = 6.404$), DHED ($t(5) = 11.69$, $p < 0.01$, $d = 5.90$), and vehicle conditions ($t(5) = 29.28$, $p < 0.01$, $d = 13.514$). As expected, the acute estradiol treatment significantly increased serum estradiol concentrations as compared to baseline ($t(5) = 15.18$, $p < 0.01$, $d = 9.03$), but serum estradiol concentrations were not affected by treatment with vehicle ($t(5) = 0.63$, $p = 0.55$, $d = 0.40$). Importantly, the acute DHED treatment did not increase serum estradiol concentrations as compared to baseline ($t(5) = -0.83$, $p = 0.45$, $d = 0.26$). Mean serum estradiol levels numerically decreased from baseline to 2h post-injection in the DHT treatment condition. This decrease in serum estradiol following acute DHT treatment administration did not reach statistical significance ($t(5) = -2.18$, $p = 0.08$, $d = 1.14$); however, the large effect size estimate

suggests that this lack of significance may reflect low statistical power in the present study.

3.2.2. Behavioral data

Overall, very low levels of female proceptive behavior were observed. Five of the six subjects were observed to approach within one meter of the male and to approach within proximity of the male during at least one behavioral test, but only four of the six females were observed to groom the male and only three of the six were observed to follow the male. Most surprisingly, only one subject (Ef6) was ever observed to present to the male and only one subject (Vu12) was ever observed to solicit the male (Table 1). Because of the very low levels of female proceptive behavior, a composite behavioral variable “female sexual initiation” was created, combining frequencies for five female proceptive behaviors: approach, prox, solicit, present, and follow. A second composite behavioral variable “time spent near male” was created, combining duration of female-initiated time spent within one meter of the male and duration of female-initiated time spent within proximity of the male.

Both groom duration and groom frequency were measured; however, groom duration was decided to be the more reliable measure of female sexual interest. This decision was based on observed variation in grooming style between study subjects. Some females were noted to be very consistent groomers, in that once they initiated a grooming session with the male, they groomed consistently until the end of the grooming session. In contrast, other females were inconsistent groomers, in that after they initiated a grooming session with the male, they stopped and restarted grooming repeatedly throughout the grooming session. An “end groom” was coded each time a female stopped

grooming, even if only for a short duration of time, and a "groom" was coded each time a female restarted grooming, in accordance with our behavioral ethogram (Appendix 3). In consequence, an inconsistent groomer would theoretically end up with a much higher groom frequency for a given behavioral test than would a consistent groomer, even if the actual duration of time spent grooming did not differ between the two. Groom duration was therefore initially analyzed instead of groom frequency.

3.2.3. Analysis of behavioral means

Behavioral data were first analyzed using means for all six subjects. Rates of female sexual initiation ($F(3,68) = 1.67$, $p = 0.18$, $\eta_p^2 = 0.07$), duration of time spent near the male ($F(3,68) = 0.85$, $p = 0.47$, $\eta_p^2 = 0.04$), and duration of time spent grooming the male ($F(3,68) = 0.92$, $p = 0.44$, $\eta_p^2 = 0.04$) did not significantly vary across successive treatment weeks, indicating a lack of order effects. During the two-hour behavioral test immediately following acute treatment administration, there were no significant differences between treatment conditions in rates of female sexual initiation ($F(3,15) = 0.66$, $p = 0.59$, $\eta_p^2 = 0.12$), duration of time spent near the male ($F(3,15) = 1.67$, $p = 0.23$, $\eta_p^2 = 0.25$), or duration of time spent grooming the male ($F(3,15) = 0.66$, $p = 0.59$, $\eta_p^2 = 0.12$).

Rates of female sexual initiation did not differ across observation days (24h prior to acute treatment administration, immediately following acute treatment administration, and 24h after acute treatment administration) for any given treatment ($F(2,10) = 0.98$, $p = 0.41$, $\eta_p^2 = 0.16$), or between treatments ($F(3,15) = 0.42$, $p = 0.74$, $\eta_p^2 = 0.08$), and there was no interaction between observation day and treatment ($F(6,30) = 0.38$, $p = 0.89$, $\eta_p^2 = 0.07$). Duration of time spent near the male did not differ across observation days

($F(2,10) = 0.81$, $p = 0.47$, $\eta_p^2 = 0.14$), or between treatments ($F(3,15) = 0.28$, $p = 0.84$, $\eta_p^2 = 0.05$), and there was no interaction between observation day and treatment ($F(6,30) = 2.05$, $p = 0.09$, $\eta_p^2 = 0.29$). Duration of time spent grooming the male did not differ across observation days ($F(2,10) = 1.87$, $p = 0.21$, $\eta_p^2 = 0.27$), or between treatments ($F(3,15) = 0.25$, $p = 0.86$, $\eta_p^2 = 0.05$), and there was no interaction between observation day and treatment ($F(6,30) = 1.13$, $p = 0.37$, $\eta_p^2 = 0.19$).

Rates of female sexual initiation did not differ between hour one and hour two of the two-hour behavioral test immediately following acute treatment administration ($F(1,5) = 2.76$, $p = 0.16$, $\eta_p^2 = 0.36$), or between treatments ($F(3,15) = 0.66$, $p = 0.59$, $\eta_p^2 = 0.16$), and there was no interaction between observation hour and treatment ($F(3,15) = 0.91$, $p = 0.46$, $\eta_p^2 = 0.15$). Duration of time spent near the male did not differ between hour one and hour two ($F(1,5) = 2.84$, $p = 0.15$, $\eta_p^2 = 0.36$), or between treatments ($F(3,15) = 1.63$, $p = 0.23$, $\eta_p^2 = 0.25$), and there was no interaction between observation hour and treatment ($F(3,15) = 1.28$, $p = 0.32$, $\eta_p^2 = 0.20$). Duration of time spent grooming the male did not differ between hour one and hour two ($F(1,5) = 3.75$, $p = 0.11$, $\eta_p^2 = 0.43$), or between treatments ($F(3,15) = 0.66$, $p = 0.59$, $\eta_p^2 = 0.12$), and there was no interaction between observation hour and treatment ($F(3,15) = 0.94$, $p = 0.45$, $\eta_p^2 = 0.16$).

3.2.4. Reanalysis of behavioral means

One subject (It13) never displayed any of the female proceptive behaviors of interest (Table 1). In contrast, all other subjects displayed at least three of the six female proceptive behaviors of interest at some point during the study. Because of this female's complete absence of proceptive behavior, which may or may not have been related to hormonal condition, all behavioral data were reanalyzed without this female.

Behavioral data were analyzed using means for the five subjects who displayed at least some female proceptive behavior during the study. During the two-hour behavioral test immediately following acute treatment administration, there were no significant differences between treatment conditions in rates of female sexual initiation ($F(3,12) = 0.65$, $p = 0.60$, $\eta_p^2 = 0.14$; Figure 5), duration of time spent near the male ($F(3,12) = 1.67$, $p = 0.23$, $\eta_p^2 = 0.30$; Figure 6), or duration of time spent grooming the male ($F(3,12) = 0.65$, $p = 0.60$, $\eta_p^2 = 0.14$; Figure 7).

Rates of female sexual initiation did not differ across observation days (24h prior to acute treatment administration, immediately following acute treatment administration, and 24h after acute treatment administration) for any given treatment ($F(2,8) = 0.98$, $p = 0.42$, $\eta_p^2 = 0.20$), or between treatments ($F(3,12) = 0.41$, $p = 0.75$, $\eta_p^2 = 0.09$), and there was no interaction between observation day and treatment ($F(6,24) = 0.37$, $p = 0.89$, $\eta_p^2 = 0.08$; Figure 8). Duration of time spent near the male did not differ across observation days ($F(2,8) = 0.80$, $p = 0.48$, $\eta_p^2 = 0.17$), or between treatments ($F(3,12) = 0.27$, $p = 0.84$, $\eta_p^2 = 0.06$), and there was no interaction between observation day and treatment ($F(6,24) = 2.15$, $p = 0.09$, $\eta_p^2 = 0.35$). Duration of time spent grooming the male did not differ across observation days ($F(2,8) = 1.93$, $p = 0.21$, $\eta_p^2 = 0.33$), or between treatments ($F(3,12) = 0.24$, $p = 0.87$, $\eta_p^2 = 0.06$), and there was no interaction between observation day and treatment ($F(6,24) = 1.14$, $p = 0.37$, $\eta_p^2 = 0.22$).

Rates of female sexual initiation did not differ between hour one and hour two of the two-hour behavioral test immediately following acute treatment administration ($F(1,4) = 2.98$, $p = 0.16$, $\eta_p^2 = 0.43$), or between treatments ($F(3,12) = 0.65$, $p = 0.60$, $\eta_p^2 = 0.14$), and there was no interaction between observation hour and treatment ($F(3,12) =$

0.91, $p = 0.47$, $\eta_p^2 = 0.19$; Figure 9). Duration of time spent near the male did not differ between hour one and hour two ($F(1,4) = 3.08$, $p = 0.15$, $\eta_p^2 = 0.44$), or between treatments ($F(3,12) = 1.67$, $p = 0.23$, $\eta_p^2 = 0.30$), and there was no interaction between observation hour and treatment ($F(3,12) = 1.29$, $p = 0.32$, $\eta_p^2 = 0.24$). Duration of time spent grooming the male did not differ between hour one and hour two ($F(1,4) = 4.23$, $p = 0.11$, $\eta_p^2 = 0.51$), or between treatments ($F(3,12) = 0.65$, $p = 0.60$, $\eta_p^2 = 0.14$), and there was no interaction between observation hour and treatment ($F(3,12) = 0.93$, $p = 0.46$, $\eta_p^2 = 0.19$).

Although unlikely, it was nonetheless possible that a given acute treatment increased the expression of certain female proceptive behaviors included in the female sexual initiation composite variable and decreased the expression of others, in which case the inclusion of all of five proceptive behaviors in the composite variable would mask the effects of treatment on behavior. Each behavior included in the female sexual initiation composite variable (approach, prox, solicit, present, follow) was therefore analyzed individually, using means for the five subjects who displayed female proceptive behavior during the study. For completeness, groom frequency was analyzed as well. During the two-hour behavioral test immediately following acute treatment administration, rates of approach, prox, solicit, present, follow, and groom did not significantly differ between treatment conditions (Table 2).

3.2.5. Individual subjects

To address the possibility of individual differences in response to treatment, rates of female sexual initiation during the two-hour behavioral test immediately following treatment administration were examined separately for each of the five subjects who

displayed some female proceptive behavior during the study. These data are shown in Figures 10. Four of the five subjects showed no clear relationship between rates of female sexual initiation and treatment condition. One subject (Vu12), however, showed what appeared to be a clear response to treatment (Figure 10). For this subject, rates of female sexual initiation were approximately three times higher during the estradiol and DHED treatment conditions as compared to the vehicle condition, and rates of female sexual initiation were more than five times higher during the DHT condition as compared to the vehicle condition. This female also showed the highest overall rates of female sexual initiation as compared to all other subjects.

3.2.6. Male behavior

Although male sexual behavior was not a primary endpoint of interest in the present study, it was nonetheless noted that very low levels of male sexual behavior were observed in both social group throughout the course of the study. The male in S1 (Hm) was observed to mount only six times throughout the study, and the male in S8 (Hv) was never observed to mount. Furthermore, no ejaculations were observed during the study. It should be emphasized that these very low levels of male sexual behavior reflect behavior directed at all group females, not just study subjects.

3.2.7. Wounding data

Group size did not considerably differ between breeding seasons one (October 2014 – April 2015) and two (October 2015 - April 2016) for either of the two social groups containing our subjects (S1 and S8). One social group (S1) contained the exact same number of individuals during each breeding season (n = 17), and the other social

group (S8) contained only one more individual during breeding season two (n = 17) as compared to breeding season one (n = 16). Furthermore, the resident adult males were the same for each social group during both breeding seasons. There were 25 females in total who were members of a given social group during both breeding seasons (13 in S1, 12 in S8). These 25 females (which included our six subjects) spent a total of 417 days hospitalized for wounding during breeding season one (M = 16.68, SD = 20.92 days), as compared to 178 days during breeding season two (M = 7.12, SD = 10.66 days). Wounding data are shown graphically in Figure 11. Overall, group females spent significantly more days hospitalized for wounding during breeding season one as compared to breeding season two ($F(1,23) = 4.96$, $p = 0.04$, $\eta_p^2 = 0.18$). Conversely, days spent hospitalized for wounding did not significantly differ between the two social groups ($F(1,23) = 0.31$, $p = 0.58$, $\eta_p^2 = 0.01$), and there was no interaction between social group and breeding season ($F(1,23) = 0.04$, $p = 0.84$, $\eta_p^2 < 0.01$).

3.3. DISCUSSION

Apart from the statistically significant increase in serum estradiol levels in response to the acute estradiol treatment, none of the hormonal or behavioral comparisons in the present study reached statistical significance; however, because this study was under powered, we did not want to ignore the possibility that there may have been effects of treatment that failed to reach significance because of sample size (Sullivan & Feinn, 2012). In the discussion that follows, we therefore consider all comparisons for which the effect size was moderate ($\eta_p^2 > 0.30$), while recognizing that none of these comparisons reached statistical significance.

As we had hypothesized, and in agreement with the results of previous studies with rodents (Prokai et al., 2015), in the present study, acute DHED treatment did not increase serum estradiol concentrations in OVX rhesus monkey. These results suggest that DHED is not converted to estradiol in the periphery and that, if DHED is converted to estradiol in the brain, then estradiol generated from DHED *in situ* remains confined to the central nervous system.

Although not statistically significant, we found a large (and unexpected) effect of acute DHT treatment on serum estradiol levels, whereby serum estradiol levels actually decreased following acute DHT treatment administration. This decrease in serum estradiol levels in response to acute DHT treatment may reflect a previously undiscovered and/or unreported effect of androgen therapy on circulating estradiol. For example, it is possible that androgen receptor occupation somehow upregulates estradiol metabolism, but that this effect of androgen receptor occupation has not been seen in previous studies that administered testosterone because testosterone is aromatized to estradiol, which would offset the decrease in serum estradiol due to increased estradiol metabolism. Although purely speculative at this point, the possibility that androgen therapy may alter estradiol metabolism warrants future investigation.

Based on preliminary data collected previously in our lab, but never published, we had hypothesized acute changes in serum hormone levels during the two-hour behavioral test immediately following acute treatment administration. We therefore compared behavior during the first and second hour of the two-hour behavioral test, to investigate the possibility of acute changes in female proceptive behavior. We had anticipated that female proceptive behavior would be higher during the second hour of the test, in

response to acute changes in serum hormone levels. Although not statistically significant, there was a moderate effect of test hour on rates of female sexual initiation, time spent near the male, and time spent grooming the male across treatment conditions; however, these effects were in the opposite direction than had been predicted. For all three behavioral measures, females showed more behavior during the first hour of the behavioral test as compared to the second hour. These higher levels of behavior during the first hour of the test may have reflected an initial increase in behavior in response to our accessing the group to administer the acute treatments, or generally higher levels of social activity earlier in the day, but do not seem to have been associated with acute changes in hormone levels.

In the present study, neither the acute DHED nor the acute DHT treatment had any measurable impact on rates of female sexual initiation. Unfortunately, the effects (or rather lack of effects) of DHED and DHT treatment on female sexual initiation were rendered uninterpretable, owing to the fact that the acute estradiol treatment, which had been intended to serve as a positive control, also had no measurable impact on female sexual initiation. There are several possible explanations as to why estradiol failed to influence female sexual initiation in the present study. The first, and perhaps most conspicuous, possibility is that estradiol is not associated with the expression of female sexual initiation in rhesus monkeys. This possibility seems unlikely, however, given that previous studies have demonstrated that female rhesus monkeys stop initiating sexual interactions with males following ovariectomy (Keverne, 1976; Zehr et al., 1998), and that female sexual initiation can be reinstated by treatment with exogenous estradiol (Keverne, 1976; Pazol et al., 2004; Pope et al., 1987; Wallen & Goy, 1977; Zehr et al.,

1998). Indeed, our lab has previously demonstrated increased rates of female sexual initiation in OVX rhesus monkeys in response to acute estradiol treatment under the exact treatment paradigm employed in the present study (Graves & Wallen, Unpublished). Furthermore, in the present study, preliminary data indicate that estradiol therapy increased the expression of female sexual initiation during the initial (abandoned) treatment paradigm. As described in the Methods section above, prior to implementation of the treatment paradigm used in the present study, an initial treatment paradigm had been implemented and subsequently abandoned due to suspected treatment carry-over effects (Appendix 2). During the abandoned treatment paradigm, 91% of recorded solicits occurred when study subjects were receiving chronic estradiol therapy – only 21 of 227 solicits occurred when study subjects were not receiving exogenous estradiol. Of the total solicits recorded over the course of both the abandoned and final treatment paradigms, 92% of solicits occurred during the abandoned treatment paradigm, and 83% of those solicits occurred during the first two weeks of the abandoned treatment paradigm. The EB injections used in the final treatment paradigm produced serum estradiol levels (M = 314.84, SD = 236.58 pg/mL) similar to those produced by the silastic estradiol capsules used in the abandoned treatment paradigm (M=269.42, SD = 114.77 pg/mL), and the acute estradiol treatment used in the final treatment paradigm significantly increased serum estradiol concentrations during the two-hour behavioral test immediately following acute treatment administration. Taken together, these data suggest that, in the present study, subjects were initially responsive to estradiol therapy, but that estradiol responsivity decreased across the abandoned treatment paradigm, and never returned during the final treatment paradigm.

The fact that estradiol therapy failed to influence female sexual initiation during the final treatment paradigm, yet seemingly influenced female sexual behavior during the abandoned treatment paradigm, warrants critical evaluation. One possibility is that, during the final treatment paradigm, the effects of estradiol on female sexual initiation were negatively impacted by prior exposure to the anti-estrogen tamoxifen (TAM), which had been administered during the abandoned treatment paradigm. In the abandoned treatment paradigm, estradiol, DHED, and DHT were administered both alone and in combination with TAM. During the course of the abandoned treatment paradigm, we became concerned that the TAM treatment was attenuating the effects of subsequent treatments on female sexual initiation. We therefore made the decision to drop the TAM treatment from the study entirely, and to proceed with the revised (final) treatment paradigm. There were more than eight weeks between the final treatment of the abandoned treatment paradigm and the first treatment of the final treatment paradigm. The half-life of TAM in serum is approximately 10-12 hours in both rats and mice (Robinson et al., 1991); however, at least one study has reported effects of TAM on estrogen receptor availability in uterine tissue in rats even up to five weeks after TAM treatment cessation (Gottardis & Jordan, 1987). It is therefore conceivable, although improbable, that the TAM administered in the abandoned treatment paradigm may have attenuated the effects of estradiol on female sexual initiation during the final treatment paradigm. However, our lab has previously demonstrated effects of acute estradiol treatment on female sexual initiation in OVX rhesus monkeys only two weeks after TAM treatment, at the same dosage of TAM used in the abandoned treatment paradigm (Graves & Wallen, Unpublished). Furthermore, all study subjects were noted to mense during the

six-day washout interval between treatment weeks in the final treatment paradigm, indicating that the EB injections resulted in estrogenic stimulation and growth of the uterine endometrium, which would have theoretically been blocked by lingering TAM in circulation or in uterine tissue. We cannot rule out the possibility of TAM carryover in the present study; however, TAM carryover seems unlikely to be able to fully account for the complete lack of observed treatment effects on female sexual initiation in the final treatment paradigm.

Another possibility is that the expression of female sexual behavior was inhibited by the sexual composition of the social groups containing the study subjects, and more specifically by the number of adult males in each social group. We had proposed to conduct the present study with rhesus females living in multi-male/multi-female social groups; however, this was not possible, and there was only one adult male in each of the social groups containing the study subjects during the study. Previous research has demonstrated that the sexual composition of a rhesus monkey social group impacts the extent to which social rank influences the expression of female sexual behavior (for review see Wallen, 1990). In stable, multi-male/multi-female rhesus groups, low-ranking females copulate as frequently, and with as many individual males, as do high-ranking females (Wilson, 1981). Furthermore, females are equally likely to receive aggression from other group females during the mating and non-mating portions of the menstrual cycle (Walker et al., 1983), indicating that aggression received is unrelated to the expression of sexual behavior. Conversely, in one-male/multi-female rhesus groups, females are more likely to receive aggression from other group females during the periovulatory phase of the menstrual cycle, when they are displaying the highest levels of

female sexual initiation (Wallen & Winston, 1984), indicating that aggression received is likely related to the expression of sexual behavior. Thus, within the one-male/multi-female social context, a rhesus female seemingly puts herself at considerable social risk by interacting with the group male, which constrains the expression of female sexual behavior (Michael & Zumpe, 1984; Wallen, 1990; 2001). This exaggerated female-female competition likely accounts for why lower-ranking females in one-male/multi-female rhesus groups only mate during the periovulatory phase of the menstrual cycle, when their levels of estradiol are highest and when their corresponding levels of sexual motivation are powerful enough to overcome their fear of other group females (Wallen & Winston, 1984; Wallen et al., 1984; Wallen, 1990; 2001). In the present study, it is possible that the study subjects experienced repeat agonistic interactions with other group females after initiating sexual interactions with the group male, which ultimately inhibited the expression of female sexual initiation over time.

Perhaps the most likely explanation as to why acute estradiol treatment had no measureable impact on female sexual initiation in the present study is that the two social groups containing the study subjects were relatively unstable throughout the entire study period, and that the expression of female sexual behavior was profoundly impacted by social group instability. We had proposed to conduct the present study with rhesus females embedded within established, natal social groups – social groups that the females had been born into, and which contained their adult female kin. Unfortunately, this was not possible, and the study subjects were conversely embedded in newly formed social groups, largely comprised of unrelated and unfamiliar adult females. As described in the Methods section above, these two social groups were formed during the summer

immediately prior to study onset, and the resident adult males were not introduced into each social group until October of 2014, the same month that the present study began. Studies of both captive and free-ranging primate groups have demonstrated increased rates of wounding following new group formation (Goo & Sassenrath, 1980; Gust et al., 1993) and during transient periods of group instability, as defined by ambiguous dominance relationships between group members (Capitanio & Cole, 2015; McCowen et al., 2008; Sapolsky, 1983). Increased rates of wounding can therefore be used as an indicator of group instability in nonhuman primate social groups. Levels of wounding were extremely high in both of the social groups containing the study subjects throughout the breeding season immediately following group formation, during which the present study took place; between October 1, 2014 and April 30, 2015, animals in these two social groups spent a combined total of 467 days hospitalized for wounding, corresponding to an average of more than 15 days spent hospitalized for wounding per group member. In comparison, animals living in a long-term, stable multi-male/multi-female social group (D2) at the YNPRC Field Station spent an average of less than 5 days hospitalized for wounding per group member during that exact same time period. Furthermore, there was an overthrow in one of the two social groups (S1) containing the study subjects during the study, whereby a lower-ranking female actually deposed the alpha female of the group, drastically altering the group dominance hierarchy. Finally, levels of wounding were significantly lower during the subsequent breeding season for both social groups, even though group size and composition were essentially identical between the two breeding seasons. Taken together, these data strongly suggest that the two social groups containing the study subjects were relatively unstable throughout the

present study. In this seemingly volatile social environment, study subjects may have avoided initiating sexual interactions with the group male in an effort to avoid conflict, and to minimize their risk of attack from other group females and/or from the group male himself.

It is additionally possible that the effects of estradiol on female sexual initiation may have been attenuated by exposure to the chronic psychosocial stress associated with group instability. Both new group formation and group instability have been shown to be potent sources of psychosocial stress in nonhuman primates. Studies have demonstrated increased cortisol levels and suppressed immune function following new group formation, and during transient periods of group instability, in both male and female rhesus monkeys (Capitanio & Cole, 2015; Goo & Sassenrath, 1980; Gust et al., 1991). Increased adrenal responsiveness to adrenocorticotropic hormone (ACTH) is associated with heightened social stress in rhesus monkeys (Sassenrath, 1970), and one study reported persistent increased adrenal responsiveness to ACTH in female rhesus monkeys even up to 13 weeks after relocation into a new single-male social group (Goo & Sassenrath, 1980). Cortisol levels have been shown to correlate with social rank in established, stable primate groups, wherein higher-ranking individuals have lower baseline cortisol levels than do lower-ranking individuals (Gust et al., 1993; Sapolsky, 1983); however this relationship between cortisol and rank disappears during transient periods of group instability (Sapolsky, 1983), and is not present in newly formed social groups (Gust et al., 1993), likely reflecting the psychological stressfulness of group instability (Sapolsky, 1992). There is also evidence to suggest that rank instability, even within an overall stable dominance hierarchy, can be psychologically stressful for

nonhuman primates. In one study of free-ranging olive baboons, for example, a higher percentage of dominance reversal interactions between a given individual and the three individuals ranked below that individual in the otherwise stable dominance hierarchy predicted elevated cortisol levels (Sapolsky, 1992). Interestingly, ambiguous dominance relationships between an individual and the three individuals ranked above that individual did not predict elevated cortisol levels, suggesting that the stressfulness of rank instability depends on whether an individual is rising or falling in the dominance hierarchy (Sapolsky, 1992). In summary, there is considerable evidence to suggest that both new group formation and social group instability are psychologically stressful for nonhuman primates. Because the two social groups containing the study subjects were formed immediately prior to study onset, and because both groups were seemingly relatively unstable during the study period, it seems reasonable to conclude that the study subjects were exposed to elevated levels of psychosocial stress throughout the study.

There is evidence from studies of various mammalian species, including rats, sheep, and rhesus monkeys, to suggest that the activational effects of estradiol on female sexual behavior can be inhibited by exposure to psychosocial stress. For example, female rats (and other rodents) display a sexual behavior called the lordosis reflex, characterized by a swaying of the back, during the periovulatory portion of the ovarian cycle. Expression of the lordosis reflex is under strict hormonal control; female rats stop showing lordosis following ovariectomy, but lordosis can be reinstated by treatment with exogenous estradiol followed by progesterone (Powers, 1970). Studies have shown that five minutes of physical restraint, a source of mild stress, significantly decreases the expression of lordosis in estradiol benzoate-progesterone (EB-P) primed OVX rats (Truitt

et al., 2003; White & Uphouse, 2004). Five minutes of physical restraint has also been shown to decrease the amount of time that EB-P primed OVX rats spend in the male's compartment of a paced mating chamber, a chamber with two compartments separated by a barrier through which only the female can pass (Uphouse et al., 2005). Increased expression of corticotropin-releasing factor (CRF) in the central nucleus of the amygdala, a recognized factor in the pathophysiology of many stress-related disorders, decreases the expression of female proceptive behaviors (hops and darts) in EB-P primed OVX rats (Keen-Rhinehart et al., 2009). Psychosocial stress has also been shown to inhibit the effects of estradiol on the expression of female sexual behavior in sheep (Papargiris et al., 2011; Pierce et al., 2008). Similar to the lordosis reflex in rats, ewes display a behavior called immobilization when in estrus, wherein females become immobile in response to a male's attempts to mount, which allows for mating to occur. Ewes stop showing immobilization following ovariectomy, but immobilization can be reinstated by treatment with exogenous progesterone followed by estradiol (Tilbrook et al., 1990). Studies have shown that exposure to acute or chronic stress-like levels of cortisol inhibits the effects of estradiol on immobilization in estrus-induced OVX ewes (Papargiris et al., 2011; Pierce et al., 2008). Furthermore, estrus-induced OVX ewes exposed to a layered psychosocial stress paradigm approach rams significantly less often, and spend less total time near rams, than do unstressed ewes (Pierce et a., 2008). And finally, the psychosocial stress associated with social subordination (i.e. low social rank) has been shown to inhibit the effects of estradiol on female sexual initiation in OVX rhesus monkeys living in one-male/multi-female social groups (Reding et al., 2012). Given the fact that the social groups containing the study subjects were seemingly unstable throughout the study, and

given the fact that both group formation and group instability have been shown to be psychologically stressful for nonhuman primates (Capitanio & Cole, 2015; Goo & Sassenrath, 1980; Gust et al., 1991; 1993; Sapolsky, 1983; 1992), in the present study, it is possible that the effects of estradiol treatment on female sexual initiation were attenuated by prolonged exposure to the chronic psychosocial stress associated with social group instability.

In the present study, subjects were living in a seemingly unstable and psychologically stressful social environment, and the impact of social instability was ostensibly reflected in the low levels of female sexual behavior observed throughout the study. However, group instability would not have been expected to uniformly impact all patterns of female proceptive behavior. For example, fear of invoked aggression from other group females would have been expected to disproportionately impact the expression of more conspicuous female proceptive behaviors. In agreement with this view, there was no measurable effect of treatment on behaviors that required close proximity to the male, or that were conspicuous indicators of sexual interest (i.e. solicit and present), which would have conceivably put subjects at higher risk of invoked aggression from other group females, or from the group male. However, while not statistically significant, there was a moderate effect of treatment, in a direction consistent with our hypotheses, on time spent near the male, which only required subjects to be within one meter of the male, and on the frequency with which subjects followed the male, which did not require any particular nearness to the male. Both of these behaviors reflected interest in the male, but both could be done at a distance, and were therefore presumably less risky than other, more conspicuous, patterns of female sexual initiation.

Similarly, the psychosocial stress associated with group instability would not have been expected to uniformly impact all study subjects; conversely, the degree of stress experienced by individual subjects would have been expected to vary based on factors such as rank, aggression received, and wounding sustained, amongst others. Compellingly, the only subject who showed what appeared to be a clear behavioral response to treatment, and who showed the highest levels of female sexual behavior throughout the study, was the only subject who was never hospitalized for wounding during the study (Vu12). These data highlight the fact that steroid hormones modulate, but do not regulate, female sexual motivation in nonhuman primates, and further emphasize the degree to which the relationship between steroid hormones and female primate sexual behavior can be influenced by social context.

4. STUDY TWO: DYNAMIC CHANGES IN FREE ESTRADIOL FOLLOWING AN ACUTE INJECTION OF DIHYDROTESTOSTERONE (DHT) IN ESTRADIOL-TREATED FEMALE RHESUS MONKEYS

4.1. METHODS

4.1.1. Subjects and housing

Subjects were OVX adult female rhesus monkeys (n = 10) housed in two large, age-graded social groups (5 in one group, 5 in the other) at the Yerkes National Primate Research Center (YNPRC) Field Station in Lawrenceville, GA. Both social groups were comprised of 60-80 individuals, including multiple adult males and intact, cycling adult females. Housing consisted of a large outdoor enclosure (~900 m^2) with an attached heated and cooled indoor quarters (~20 m^2). Subjects were fed a standard commercial low-fat high-fiber monkey chow diet (Ralston Purina Company, St. Louis, MO), and received fresh fruit and vegetables daily. One subject was ovariectomized two months prior to study onset, but all other subjects had been ovariectomized over a year prior to study onset. All subjects had given birth at least once prior to ovariectomy, and ranged in age from 5 to 15 years at study onset. All research was conducted in accordance with the National Institutes of Health (NIH) standards and guidelines, and was approved by the Emory University Institutional Animal Care and Use Committee (IACUC).

All subjects were implanted with two 4.5cm silastic capsules (ID=0.132in, OD=0.183in) containing crystalline estradiol (Product E- 88775, Sigma Aldrich Corp.), previously shown to produce midfollicular levels of circulating estradiol (100-200 pg/mL). Silastic capsules were implanted by YNPRC veterinarians under Ketamine anesthesia (10mg/kg IM). After the animal was anesthetized, the area between the

scapulae was clipped, repeatedly cleansed with an antiseptic solution and alcohol, and sterilely draped. A small 1-2cm skin incision was made between the scapulae using a scalpel blade, and a small subcutaneous pocket was created via blunt dissection with hemostats. The silastic capsules were inserted subcutaneously into the pocket. After implantation, the incision site was closed with subcutaneous and skin sutures using a resorbing suture material. Banamine (1 mg/kg) or Ketoprofen (2 mg/kg IM) was given immediately after implantation to provide analgesia and to relieve any discomfort.

4.1.2. Experimental design

The present study was conducted between May and July, during the rhesus monkey summer nonbreeding season. All ten females served as both treated subjects and vehicle controls. On each of the two acute treatment days, subjects received an acute injection (IM) of either 5-alpha-dihydrotestosterone (DHT; 10 µg/kg; Product D-073, Sigma Aldrich Corp.) or vehicle. Subjects were randomly assigned to receive DHT on acute treatment day one or two, and there were two weeks between the two acute treatment days. This repeated-measures design allowed each subject to act as their own control, and maximized statistical power. DHT was dissolved in pharmaceutical grade corn oil, and corn oil served as the vehicle control. All subjects received their DHT or vehicle injections between 8:00AM and 9:00AM on acute treatment days. A 6 mL blood sample was collected from each subject immediately prior to acute treatment administration, and 6 mL blood samples were collected at 30, 60, 120, and 360 min after acute treatment administration. There were more than two months between silastic capsule implantation and the first acute treatment day.

All subjects were trained to separate from their social group on command and to enter an indoor area. Once sequestered inside, subjects were individually transferred to a modified cage with a small window, which allowed for the presentation of a leg for an awake, unanaesthetized blood draw. All blood samples were collected from the saphenous vein on the back of the calf. The calf was clipped and wiped with a disposable antiseptic wipe prior to venipuncture. Blood sample collection remained within the limit of 10 mL/kg/month for each subject. Blood samples were collected into 6 mL glass serum separating vacutainer tubes (Product CVN-8881302072, Fisher Scientific), and centrifuged for 15 min at 3000 RPM in a refrigerated centrifuge at 4°C. Plasma was then transferred to 3 mL plastic cryovials and stored at -10°F while awaiting analysis.

4.1.3. First hormone assays run

All hormone assays were conducted by the Wisconsin National Primate Research Center (WNPRC) Assay Services (University of Wisconsin-Madison, Madison, WI). For the first assay run, free estradiol was analyzed for five of the ten subjects (all housed in the same social group) under the DHT condition only.

Free estradiol was separated from bound estradiol via centrifugal ultrafiltration. Serum was transferred to a clean glass test tube and 1 mL of saline was added. The tube was vortexed and the entire volume transferred to a Vivaspin 2 centrifugal concentrator (30,000 MWCO; Sartorius Stedim Biotech, Concord, CA). The samples were then put into a centrifuge at room temperature, and spun at 4000 x g for 10 minutes.

Filtrate from centrifugal concentration and serum (200 uL) were extracted for estradiol according to the protocol previously described (Kenealy et al., 2013; 2016). Unknowns, standards, and quality control samples were diluted in 500 µL of ultrapurified

bottled water (Fisher Scientific), and internal standard (50 pg d5-estradiol) was added to each sample. One milliliter of methyl tert-butyl ether (MTBE; Fisher Scientific) was then added. Tubes were then vortexed vigorously, and centrifuged at 1500 RPM for 3 min. The top organic phase containing estradiol was transferred to a new tube with a glass pipette, evaporated to dryness via air stream and heated water bath (60°C), and then resuspended in 100 μL of ethanol and 500 μL of water. A second liquid–liquid extraction was performed with dichloromethane (Fisher Scientific). The lower dichloromethane organic phase containing estradiol was transferred into a clean test tube and evaporated to dryness. Samples were then resuspended in 25 μL of NaHCO3 buffer and estradiol was derivitized with 25 μL of dansyl chloride (200 mg/mL in acetone; Fisher Scientific), heated at 40°C for 4 min, and transferred into minivials for LC-MS/MS analysis.

Free estradiol were measured via liquid chromatography/tandem mass spectroscopy (LC-MS/MS). Samples were analyzed on a QTRAP 5500 quadruple linear ion trap mass spectrometer (AB Sciex, Framingham, MA) equipped with an atmospheric pressure chemical ionization source. The system included two Shimadzu LC20ADXR pumps and a Shimadzu SIL20ACXR autosampler. A sample of 30 μL was injected onto a Phenomenex Kinetex 2.6u C18 100A, 100 × 2.1 mm column (Phenomenex, Torrance, CA) for separation using mobile phase: water with 1% formic acid (Solution A) and acetonitrile with 1% formic acid (Solution B), at a flow rate of 200 μL/min. Three-percent Solution B was held for 3 min followed by 50% Solution B for the next 0.10 min, then maintained for 2.9 min, followed by an increase to 67% Solution B for 15 min and an increase to 100% Solution B over the next 3 min. This was held for 7 min before the system was returned to initial conditions of 3% Solution B over 0.1 min and held for the

final 9.9 min of each run. Mass spectrometer results were generated in positive-ion mode with the following optimized voltages: corona discharge current, 3 V; entrance potential, 10 V. The source temperature was 500°C. The gas settings were as follows: curtain gas, 30 psi; nebulizing gas, 20 psi; collisionally activated dissociation gas, medium. Quantitative results were recorded as multiple reaction monitoring (MRM) area counts after determination for the response factor for each compound and internal standard. The concentrations for the calibration curve for estradiol were 312.5, 156.25, 78.125, 39.06, 19.53, 9.77, 4.88, 2.44, 1.22, 0.61, 0.30, and 0.15 pg. The linearity was $r > 0.9990$, and the curve fit was linear with 1/x weighting. No estradiol was detected in blank or double blank samples. Interassay coefficient of variation was determined by a pool of human serum and was <13%.

4.1.4. Second hormone assay run

The first run of the centrifugal ultrafiltration-LC-MS/MS free estradiol assay produced highly variable free estradiol results, and we became concerned that the observed variability was indicative of issues with the assay. We subsequently worked with the WNPRC Assay Services Team to improve the reliability of the free estradiol assay. During the initial assay run, centrifugal ultrafiltration had been conducted at room temperature; however, SHBG's binding affinity has been shown to be temperature sensitive, and SHBG shows increased binding affinity at lower temperatures (Obminski, 1998; Ray et al., 2012; Shanbhag & Sodergard, 1985; Van Uytfanghe et al. 2004). Therefore, when temperature is uncontrolled, there is likely to be increased variance in serum free estradiol concentrations. For the second assay run, centrifugal ultrafiltration was conducted using a temperature controlled centrifuge heated to a constant temperature

of 37°C (approximating body temperature) for all serum samples. Furthermore, during the initial assay run, centrifugal ultrafiltration was conducted using inconsistent aliquot volumes across serum samples, in an attempt to maximize aliquot volume for each sample and to increase the chances of detecting free estradiol. We became concerned, however, that the flow rate through the filter may have been affected by aliquot volume, with larger volumes resulting in proportionally less free estradiol getting through the filter, as the filter accumulated greater amounts of serum proteins. For the second assay run, a consistent aliquot volume (1 mL) was used for all serum samples. Apart from these two methodological changes, centrifugal ultrafiltration and LC-MS/MS procedures for the second assay run were identical to those outlined above for the first assay run.

For the second assay run, total estradiol was measured via LC-MS/MS in addition to free estradiol, to allow for the calculation of percent free estradiol. Free and total estradiol were analyzed for the other five subjects (all housed in the same social group) under the DHT condition, and free and total estradiol were analyzed for three of those five subjects (selected at random) under the control condition.

4.1.5. Statistical analyses

All analyses used SPSS software (version 23, IBM Corp., Armonk, NY). All tests were two-tailed, and were considered significant with a p value less than 0.05. Differences in serum concentrations of free and total estradiol across time points were assessed with repeated-measures ANOVA. The magnitude of differences between means was assessed by calculating partial eta squared (η_p^2) as an indicator of effect size. Post hoc analyses were conducted to compare individual means, and the Sidak-Bonferroni correction was employed to adjust the alpha level to account for multiple comparisons.

4.2. RESULTS

4.2.1. First hormone assay run data

Free estradiol data were analyzed using means for all five subjects. Serum concentrations of free estradiol did not significantly vary across the five time points ($F(4,16) = 0.48$, $p = 0.75$, $\eta_p^2 = 0.11$). Free estradiol data for assay run one are shown in Figures 12 and 13.

4.2.2. Second hormone assay run data

Unfortunately, due to complications during the assay process, during the second assay run, four serum samples were lost. Consequently, we were left with complete free estradiol data for only three of the five subjects under the DHT condition, and only one of the three subjects under the control condition.

Hormonal data were analyzed using means for all three subjects for whom we had complete data under the DHT treatment condition. For three of the fifteen serum samples included in these analyses, free estradiol levels were below the limit of detection for the centrifugal ultrafiltration-LC-MS/MS free estradiol assay, in which case the lower limit of the assay (0.2 pg/mL) was substituted for the free estradiol value, although this value likely represented an overestimate. As anticipated, the silastic estradiol capsules produced midfollicular levels of total estradiol (M = 198.53, SD = 151.11 pg/mL). Serum concentrations of total estradiol did not significantly differ across the five time points under the DHT treatment condition ($F(4,8) = 1.17$, $p = 0.39$, $\eta_p^2 = 0.37$). Total estradiol data for assay run two are shown in Figure 14. Serum concentrations of free estradiol also did not significantly differ across the five time points under the DHT treatment condition ($F(4,8) = 0.96$, $p = 0.48$, $\eta_p^2 = 0.33$). Free estradiol data for assay run two are shown in

Figures 15 and 16. Finally, percent free estradiol (free estradiol divided by total estradiol x 100) did not significantly differ across the five time points under the DHT treatment condition ($F(4,8) = 0.89$, $p = 0.51$, $\eta_p^2 = 0.31$). Percent free estradiol data for assay run two are shown in figures 17 and 18. Although not statistically significant, there was a moderate effect of time point on free estradiol, and the numerical changes in free estradiol following DHT treatment administration were consistent with our hypothesis. As predicted, mean serum free estradiol levels increased following DHT treatment administration, peaked at 60 min post-injection, and had returned to baseline by 360 min post-injection. At 60 min post-injection, mean serum free estradiol levels had more than doubled as compared to baseline (Figure 15). We also conducted a crude comparison of serum estradiol levels across the five time points for the one subject (Ea4) for whom we had complete data under both the DHT and vehicle treatment conditions. For this female, serum free estradiol levels numerically increased following DHT treatment administration, and subsequently returned to baseline by 360 min post-injection, but there was no clear pattern of variation in free estradiol across the five time points under the vehicle treatment condition (Figure 19).

4.3. DISCUSSION

The data produced by the second run of the centrifugal ultrafiltration-LC-MS/MS free estradiol assay were generally encouraging. The numerical changes in free estradiol following acute DHT treatment were not statistically significant; however, this was not surprising given our very small sample size, and correspondingly low statistical power. Nonetheless, there was a moderate effect of time point on serum free estradiol levels, and the numerical changes in free estradiol following DHT treatment administration were

consistent with our hypothesis. Free estradiol levels numerically increased in response to an acute injection of DHT, and subsequently returned to baseline within three hours of DHT treatment administration. This transient increase in serum free estradiol following an acute injection of DHT is consistent with the idea that DHT preferentially binds SHBG, and liberates SHBG-bound estradiol.

The data from the second assay run raise several important questions. For example, although not statistically significant, there was a moderate effect of time point on serum total estradiol levels. This study was conducted with OVX rhesus females receiving chronic estradiol therapy, and serum total estradiol levels should have remained relatively constant across the three hour time span encompassing the five measurement time points. The moderate effect size for differences in serum total estradiol between time points therefore suggests that the WNPRC LC-MS/MS estradiol assay may not have been entirely reliable. Furthermore, while the changes in free estradiol following acute treatment with DHT were consistent with our hypothesis, the absolute amount of free estradiol detected was much smaller than anticipated. Previous studies have proposed that 1-3% of total estradiol circulates unbound at any given time; however, in the present study, mean percent free estradiol varied between 0.3 and 0.9%. It is unclear whether these lower than anticipated values represent actual free estradiol levels, or conversely reflect an issue with the sensitivity of the free estradiol assay. However, the concentrations of serum free estradiol detected during the second assay run were comparable to serum free estradiol concentrations that have been reported for monkeys (Koritnik & Maschke, 1986), as measured by centrifugal ultrafiltration-radioimmunoassay, and for women across the menstrual cycle, as measured by

equilibrium dialysis-LC-MS/MS (Ray et al., 2012), the technique generally agreed to provide the most accurate measurement of free steroid concentrations (Rosner, 2015). There is also evidence to suggest that indirect methods for the measurement of free hormone, such as titrated dialysis, which have generally been used in the past, substantially overestimate free estradiol levels (Ray et al., 2012). It is therefore possible that the proportion of total serum estradiol that circulates unbound at any given time is actually much lower than was previously believed.

These data also raise the question of whether changes in free estradiol can be expected to meaningfully modulate female primate sexual motivation, when free estradiol is seemingly present in such minimal quantities to begin with. In the present study, serum free estradiol concentrations increased by roughly 1 pg/mL following an acute injection of DHT, and the biological relevance of such a small amount of free estradiol remains unclear. However, it has been suggested that levels of free estradiol measured *in vitro* may not be representative of actual levels of free estradiol in the brain. Pardridge (1988) proposed that rapid interactions between the surface of the SHBG protein and the surface of the microcirculation cause conformational changes of the SHBG steroid binding site, which increases the rate of steroid dissociation from the binding protein. Thus, within the capillary bed of the hypothalamus, for example, where blood has almost continuous contact with the surrounding neural tissue, rates of estradiol disassociation from SHBG, and levels of free estradiol, may be substantially higher than in the periphery. Therefore, serum free estradiol levels measured *in vitro,* in the absence of protein-microcirculation interactions, may represent a gross underestimation of actual free estradiol levels within the cellular compartment in the brain.

5. LIMITATIONS

There are several limitations to the present dissertation. For Study One, we cannot know the extent to which the TAM administered in the abandoned treatment paradigm influences female sexual behavior during the final treatment paradigm. Furthermore, the study subjects were arguably housed under unnatural and psychologically stressful social conditions. The study subjects were embedded within single-male/multi-female social groups, which are less naturalistic than multi-male/multi-female social groups. Furthermore, the social groups containing the study subjects were largely comprised of unrelated adult females, whereas free-ranging female rhesus monkeys are typically embedded within their natal matrilines, and surrounded by female kin. The social groups containing the study subjects were formed immediately prior to study onset, and both groups were seemingly unstable, with abnormally high levels of wounding, throughout the study. We cannot know the extent to which group composition and social instability influenced the expression of female sexual behavior in Study One, and it is therefore unclear whether the results of Study One are generalizable to rhesus monkeys housed under more naturalistic/stable social conditions.

For Study Two, the measurement of free estradiol in serum posed a considerable methodological challenge. The centrifugal ultrafiltration-LC-/MS/MS free estradiol assay produced highly variable results, and the reliability of the assay is still in question. Finally, both Studies One and Two were under powered, making interpretation of null findings difficult. Given the limitations of small sample size, the lack of statistical significance for comparisons with a moderate effect size may have been the result of low statistical power.

6. CONCLUSIONS

The findings of the present dissertation have implications for both primate behavioral research and women's health. The hormonal results of Study One contribute to the growing body of evidence suggesting that the compound DHED, a naturally occurring metabolite of estradiol, is not converted to estradiol in the periphery, and that estradiol generated from DHED *in situ* remains confined to the central nervous system. Because female rhesus monkeys share many aspects of their reproductive biology with women, these results have implications for women's health. In short, these results support the view that DHED may represent a brain-specific estradiol pro-drug, capable of delivering effective amounts of estradiol to the brain without having peripheral effects on estrogen-sensitive tissues, such as breast and uterine tissue. DHED therefore holds promise as a possible brain-specific estrogen therapy for women for the treatment of symptoms associated with hypoestrogenic conditions, including natural and surgical menopause.

The hormonal results of Study Two, although preliminary, nonetheless suggest that an acute injection of DHT may result in a temporary increase in circulating levels of free estradiol in female rhesus monkeys. These data offer tentative support for the notion that androgens influence female primate sexual motivation, at least in part, by preferentially binding SHBG, and liberating SHBG-bound estradiol. These data also suggest that the proportion of total serum estradiol that circulates unbound at any given time may be lower than previously believed. Resolving these issues will require a larger and more involved study that can compare the centrifugal ultrafiltration and equilibrium dialysis methods for the separation of free from SHBG-bound estradiol, and that can

address the biological relevance of small changes in serum concentrations of free estradiol.

The behavioral results of Study One contribute to the already robust literature indicating that the relationship between gonadal steroids and female primate sexual behavior is highly sensitive to social context. Estradiol seemingly increased female sexual motivation during the first two weeks of the initial treatment paradigm, immediately following the introduction of the adult males into the two social groups; however, estradiol responsivity subsequently declined, and there was no measurable impact of estradiol therapy on female sexual initiation during the final treatment paradigm. It is not possible to know exactly why the females in this study stopped responding to estradiol therapy; however, it seems likely that the effects of estradiol on female sexual initiation were ultimately inhibited by a combination of social and contextual factors, including prolonged exposure to the chronic psychosocial stress associated with group instability. This theory finds support in the human literature, where chronic psychosocial stress has been shown to be associated with decreased sexual desire and impaired sexual functioning in women (Bodenmann et al., 2006; Dennerstein et al., 1999; Hartmann et al., 2000; ter Kuile et al., 2007). The behavioral results of Study One have significant implications for primate behavioral research, and emphasize the critical importance of social context in the study of primate sexual behavior.

Unfortunately, the lack of effects of DHED and DHT treatment on female sexual initiation in Study One were rendered uninterpretable, because the estradiol treatment, which had been intended to serve as a positive control, also failed to influence female sexual behavior. It therefore remains unknown whether DHED therapy is capable of

influencing estrogen-responsive behavioral systems in female primates, and whether aromatization to estradiol is required for androgens to exert influence over female primate sexual motivation. Future studies should investigate the effects of DHED and DHT therapy on female sexual motivation under more naturalistic social conditions – i.e. with female rhesus monkeys embedded within their natal matrilines, in long-term, stable, multi-male/multi-female social groups.

For the vast majority of mammalian species, gonadal steroids regulate the ability to mate, such that females are only physically capable of engaging in mating behavior while fertile (Wallen, 2001). In primates (with the exception of some prosimians), however, the ability to mate has been emancipated from hormonal control. Ovarian steroids function to modulate female primate sexual motivation in such a way as to maximize female mating efforts during the most fertile portion of the ovarian cycle, but social context can significantly impact the expression of female primate sexual behavior (Wallen 1990; 2001). For example, in rhesus monkeys, the expression of female sexual behavior has been shown to vary with contextual factors such as enclosure size, group composition, and social rank (for review see Wallen 1990; 2001). As with other female primates, women are physically capable of engaging in sexual intercourse under any hormonal condition, and irrespective of their levels of sexual desire. In consequence, women can (and do) engage in sexual behavior for reasons other than sexual desire (Meston & Buss, 2007), and conversely women's sexual desire is not necessarily manifested in sexual behavior (Spector et al., 1996). Furthermore, humans are capable of modifying their sexual habits to accommodate a wide range of cultural conventions (Wallen, 2001). For example, sexual intercourse is often intentionally avoided during

menstruation in response to cultural taboos (Stanislaw & Rice, 1988), and at midcycle in an effort to avoid pregnancy (Tsui et al., 1991). In Western nations, intercourse frequency has been shown to vary consistently with external factors such as the days of the week, and reported levels of both sexual desire (Roney & Simmons, 2013) and sexual behavior (Palmer et al., 1982; Wilcox et al, 2004) increase on the weekends. As such, human sexual behavior varies in response to a combination of hormonal, cultural, and contextual factors.

The evolutionary pressures that resulted in the emancipation of the ability to mate from hormonal control in anthropoid primates remain unknown (Wallen, 2001; Wallen & Zehr, 2004); however, female primates arguably benefit from the ability to engage in mating behavior outside of the 12 or-so hours per month surrounding peak fertility. For example, the ability to mate under any hormonal condition allows for sexual behavior to be used in a number of nonreproductive social contexts, such as alliance formation, conflict resolution, and reconciliation (Kappeler & van Schaik, 2002; Vasey, 1995). In primate social groups, nonreproductive sexual behavior may function to relieve tension and /or to reinforce affiliative relationships between nonrelated individuals (de Waal, 1995; Parish 1994). The ability to engage in mating behavior outside of the period of peak fertility also allows female primates to confuse paternity certainty by mating with multiple males throughout the ovarian cycle, which may decrease the risk of infanticide for their offspring (Heistermann et al., 2001; Kappeler & van Schaik, 2002). In summary, it remains unclear exactly how and why the physical capacity to mate became emancipated from hormonal control in primates (Wallen, 2001; Wallen & Zehr, 2004),

but the resultant behavioral flexibility makes the study of female primate sexual motivation a complex, and sometimes frustrating, endeavor.

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Appendix 1: Validation of estradiol benzoate dosage

Prior to implementation of the treatment paradigm used in Study One (see section 3.1.2), the dosage of the estradiol benzoate (EB) treatment (3 ug/kg) was validated in two randomly selected subjects (Mq11 and Vu12). The goal of the EB treatment was to produce midfollicular levels of serum estradiol. Both subjects received an acute injection of EB (IM) at 8:30AM, and 3 mL blood samples were subsequently collected from each subject at 2, 4, 6, and 24 hours post treatment. Blood sampling methods and hormone assays were identical to those described for experiment one (see sections 3.1.4 and 3.1.5). Serum estradiol concentrations had already increased by a factor of 4 for both subjects at 2 hours post-treatment, and serum estradiol concentrations remained elevated even at 24 hours post treatment (Table 3). Mean serum estradiol concentrations were within midfollicular range (100-200 pg/mL) at 2, 4, and 6 hours post treatment, and were just below midfollicular range at 24 hours post treatment (M = 96.10, SD = 3.25 pg/mL). Based on these preliminary data, it was decided to go forward with the 3ug/kg EB dosage in Study One.

Appendix 2: Abandoned treatment paradigm

Prior to implementation of the final treatment paradigm used in experiment one, an initial treatment paradigm had been implemented and subsequently abandoned due to suspected treatment carryover effects. Seven subjects were included in the abandoned treatment paradigm, but one subject was released from the study due to declining health prior to implementation of the revised treatment paradigm. Apart from inclusion of this seventh subject, subjects and housing conditions were identical during implementation of the abandoned and final treatment paradigms (see section 3.1.1).

The abandoned treatment paradigm was intended to address two specific aims: (1) to determine whether acute treatment with estradiol, DHED (a brain specific estradiol pro-hormone), and/or with DHT (a nonaromatizable androgen) increased female sexual initiation in chronic low-dose estradiol-treated OVX rhesus monkeys, and (2) to determine whether the co-administration of the estrogen receptor blocker tamoxifen (TAM) attenuated the effects of estradiol, DHED, and/or DHT on female sexual initiation in chronic low-dose estradiol-treated OVX rhesus monkeys.

The abandoned treatment paradigm differed from the revised treatment paradigm in two critical ways; in the abandoned treatment paradigm, the four acute treatments (estradiol, DHED, DHT, and vehicle) were administered (1) both with and without concurrent TAM treatment, and (2) both on a background of chronic estradiol and without background hormone. Therefore, in total, the abandoned treatment paradigm included eight acute treatments: (1) estradiol (10 μg/kg; Product E-8875, Sigma Aldrich Corp), (2) DHED (100 μg/kg), (3) DHT (10 μg/kg; Product D-073, Sigma Aldrich Corp.), (4) vehicle, (5) estradiol (10 μg/kg) + TAM (480 μg/kg; Product T-5648, Sigma

Aldrich Corp), (6) DHED (100 μg/kg) + TAM (480 μg/kg), (7) DHT (10 μg/kg) + TAM (480 μg/kg), and (8) vehicle + TAM. The study was divided into two treatment periods, and subjects were randomly assigned to receive the eight acute treatments on a background of estradiol in treatment period I or in treatment period II.

At the beginning of treatment period I, subjects scheduled to receive their acute treatments on a background of estradiol were implanted with two 4.5cm silastic capsules (ID=0.132in, OD=0.183in) containing crystalline estradiol (Product E- 88775, Sigma Aldrich Corp.), previously shown to produce midfollicular levels of circulating estradiol (100-150 pg/mL). Silastic capsules were implanted by YNPRC veterinarians under Ketamine anesthesia (10mg/kg IM). After the animal was anesthetized, the area between the scapulae was clipped, repeatedly cleansed with an antiseptic solution and alcohol, and sterilely draped. A small 1-2cm skin incision was made between the scapulae using a scalpel blade and a small subcutaneous pocket was created via blunt dissection with hemostats. The silastic capsules were inserted subcutaneously into the pocket. After implantation, the incision site was closed with subcutaneous and skin sutures using a resorbing suture material. Banamine (1 mg/kg IM) or Ketoprofen (2 mg/kg IM) was given immediately after implantation to provide analgesia and to relieve any discomfort.

There were at least 14 days between silastic capsule implantation and the first acute treatment of treatment period I, and seven days between acute treatments for the rest of the treatment period thereafter. All acute treatments were administered IM, between 8:30AM and 9:30AM on treatment days. The dosage of the acute TAM treatment (480 μg/kg; Product T-5648, Sigma Aldrich Corp.) was selected to reflect the dosage of TAM used to treat estrogen receptor positive breast cancer in pre- and

postmenopausal women (Dardes & Jordan, 2000). This dosage of TAM has also previously been shown to antagonize estrogenic facilitation of HPA responsivity (Wilson et al., 2003) and to attenuate the effects of estradiol on sexual initiation (Graves, 2006) in OVX rhesus monkeys. All acute treatments were dissolved in pharmaceutical grade corn oil, and corn oil served as the vehicle control.

A 120-minute behavioral observation was conducted directly following acute treatment administration, and a 60-minute behavioral observation was conducted 24h after acute treatment administration. A 3 mL blood sample was collected from each subject immediately before acute treatment administration, and after the 120-minute observation following acute treatment administration. A 3 mL blood sample was also collected before the 60-minute observation 24h after acute treatment administration. Behavioral data collection and blood sampling methods were identical to those described for experiment one (see sections 3.1.3 and 3.1.4).

At the end of treatment period I, a one-way ANOVA was performed to confirm the absence of carryover effects across the first eight treatment weeks. Surprisingly, overall rates of female sexual initiation significantly differed across successive treatment weeks, despite the counterbalanced treatment design, indicating potential treatment carryover effects ($F(7,104) = 2.12$, $p = 0.05$; Figure 20). We became concerned that the TAM treatment was impacting the effects of subsequent treatments on female sexual initiation. We had originally decided that seven days was sufficient between acute treatments, given that the half-life of TAM in serum is approximately 10-12 hours in both rats and mice (Robinson et al., 1991). However, at least one study has reported effects of TAM on estrogen receptor availability in rats even up to five weeks after TAM treatment

cessation (Gottardis & Jordan, 1987). Given time and budgetary constraints, it was not possible to start the study over from the beginning and to allow for a longer duration of time between acute treatments. We therefore made the decision to abandon the initial treatment paradigm, drop the TAM component from the study entirely, and to proceed with the revised (final) treatment paradigm outlined in the Methods section for Study One. There were more than eight weeks between the last treatment of the abandoned treatment paradigm and the first treatment of the final treatment paradigm, giving subjects' hormone levels ample time to return to baseline.

Appendix 3: Behavioral ethogram

BEHAVIORAL ETHOGRAM		
Distance Codes		
a1	approach to within 1m	Animal initiates an approach when it comes within 1 meter of another animal and remains stationary there for several seconds. Terminated by *lb* or *px.*
px	approach to within proximity (prox)	Animal initiates *px* when it comes within arm's reach of another animal's body and remains there. Animal may be in *px* with multiple animals at a time. Not scored for animals in motion, or passing by each other. If in *px* with multiple animals, each *px* needs to be terminated by *lb*.
co	contact	Two animals with significant portions of their bodies in contact. Does not include touching, grooming, or momentarily moving across another animal.
pc	proximity to contact	Scored when contact is initiated between two animals already in proximity.
lp	leave to proximity	Two animals end contact but remain in proximity.
lb	leave beyond	Animals separate to a distance beyond 1 meter.
l1	leave within 1m	Scored when proximity is ended by animals separating to beyond proximity but within 1 meter of each other. Terminated by *px* or *lb*.
Other Duration Codes		
fl	follow	Persistent trailing of another animal. Both animals must be in motion. Not defined by distance between the two animals, but by the inferred intention of the follower. The follower is clearly attending to the movements of the animal being followed. The distance between the animals must be greater than arms' reach. Terminated by *f-* or *fp*.

f-	end follow	Ends a follow. Makes no assumption about the animal's ending location; if follow ends with animals in proximity, *px* should be scored simultaneously.
gm	groom	One animal combing through the hair of another with hands or mouth. The two animals must be in proximity. Not scored as *co* unless substantial parts of the body are touching.
gg	genital groom	Animal grooming another animal's genitals. Not rhythmic. If using mouth to groom genital region, score as *og*.
g-	end grooming	Ends all grooms. All grooms are terminated if action stops for any amount of time.
Agonistic/Dominance/Submissive/Other Behaviors		
tc	threat w/ contact aggression	Aggressive contact between two individuals (i.e. hit, bite, grab, etc.). A threat w/contact does not require proximity. If proximity precedes the threat with contact, it should be scored. If an animal hits another while passing, proximity is not scored.
tn	threat non-contact aggression	Lunge or open mouth threat or barking (no contact between animals). Threat non-contact makes no assumption regarding the distance between animals. Proximity/leave beyond should also be scored, if appropriate.
ch	chase	Both animals are running, not in a context of play. Animals do not need to be in proximity at initiation, but if they are, *lb* is scored at the start of *ch*.
gr	grimace	Animal pulls back lips to reveal teeth with a closed jaw. If a recipient cannot be identified, or is a non-monkey, score as actor = recipient.
wd	withdraw	Animal is clearly avoiding another animal. Does not imply any distance.

gs	groom solicit	Animal assumes posture to solicit grooming from another animal. Animal can solicit grooming from multiple animals simultaneously.
ls	lipsmack	Animal opens and closes lips rapidly. If a recipient cannot be identified, or is a non-monkey, score as actor = recipient.
Sexual Behaviors		
pr	present	Animal directs hindquarters towards a recipient, often with tail deviated. Gaze should be directed towards the recipient.
ht	hiptouch	Animal places two hands on hips of a recipient; as if to position them into a present.
hp	ht following a pr	Animal hiptouches a recipient after the recipient presents. *pr* must be coded before *hp.*
sl	solicit	Animal makes a staccato head or hand gesture, the inferred intention of which is to attract the attention of a recipient. May involve a hand slap, head bob, crouch, or threat-away.
ph	pr following a ht	Animal presents in response to being hiptouched. *ht* must be coded before *ph.*
pa	pr to approach	Animal presents in response to recipient's approach. Occurs prior to *px.*
si	sex invite	Lifting of muzzle, gaze directed at recipient over shoulder or through legs, head bobbing in recipient's face and/or prancing.
sn	sniff genitals	Animal sniff's the genital region of a recipient.
nn	no footclasp/no thrusting	Animal mounts a recipient with both feet on the ground. Animal's pelvis is oriented towards the hindquarters of the recipient. Typically, the animal grabs the recipient's waist.
nt	no footclasp/thrusting	As above, but with pelvic thrusting.

mn	footclasp mount/no thrusting	Animal mounts a recipient with one or both feet clasped on the recipient's ankles. Animal's pelvis is oriented towards the hindquarters of the recipient. Typically, the animal grabs the recipient's waist.
mt	footclasp mount/thrusting	As above, but with pelvic thrusting.
am	abortive mount	Incorrectly oriented mount with thrusting.
an	abortive mount no thrusting	Incorrectly oriented mount without thrusting.
in	intromission	Scored once during a given mount sequence, when thrusting becomes deeper and rhythmic. Usually accompanied by penile insertion into the vagina.
ej	ejaculatory reflex	Release of ejaculate or the behavioral response which characterizes such.
og	oral-genital contact	Any contact of mouth to genitals. May be solitary or partnered, homo- or heterosexual.
o-	end oral-genital contact	Ends all oral-genital contact.
m1	ht, ph, mt	Expanded by lab program.
m2	pr, hp, mt	Expanded by lab program.
m3	ht, ph, mn	Expanded by lab program.
m4	pr, hp, mn	Expanded by lab program.

FIGURE 1. Estradiol (E2) concentrations in the brain following 12 days of chronic treatment with DHED (20 ug/kg/day) or vehicle (both s.c. via Alzet osmotic minipump). DHED treatment increased E2 concentrations in the cortex and mediobasal hypothalamus (MBH) as compared to vehicle.

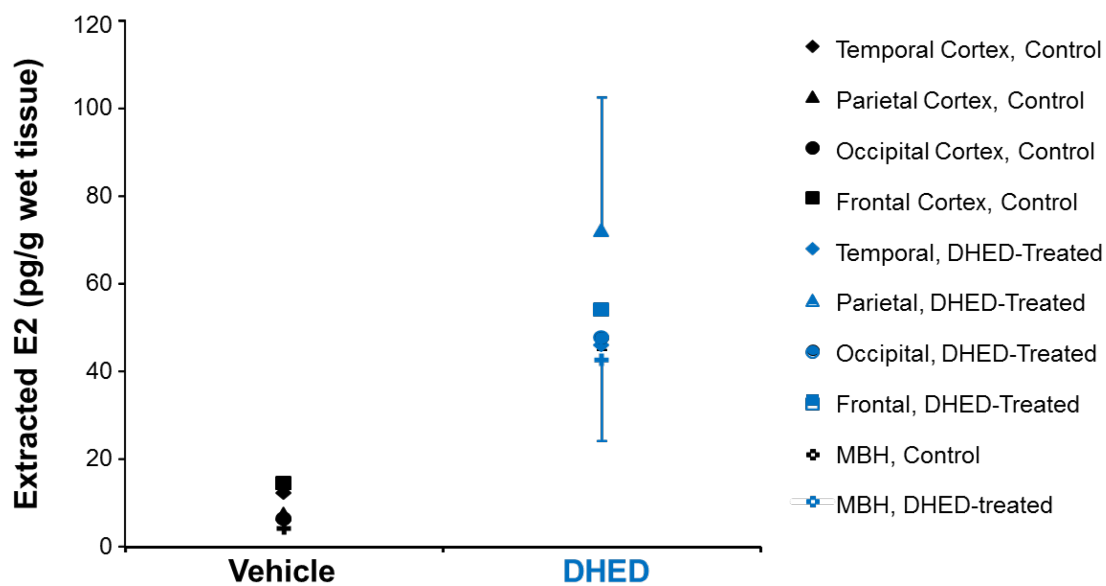

FIGURE 2. Mean (± SD) serum estradiol (E2) concentrations across 12 days of chronic treatment with DHED (20 ug/kg/day) or vehicle (both s.c. via Alzet osmotic minipump). Serum E2 concentrations did not significantly differ between DHED-treated subjects and vehicle controls.

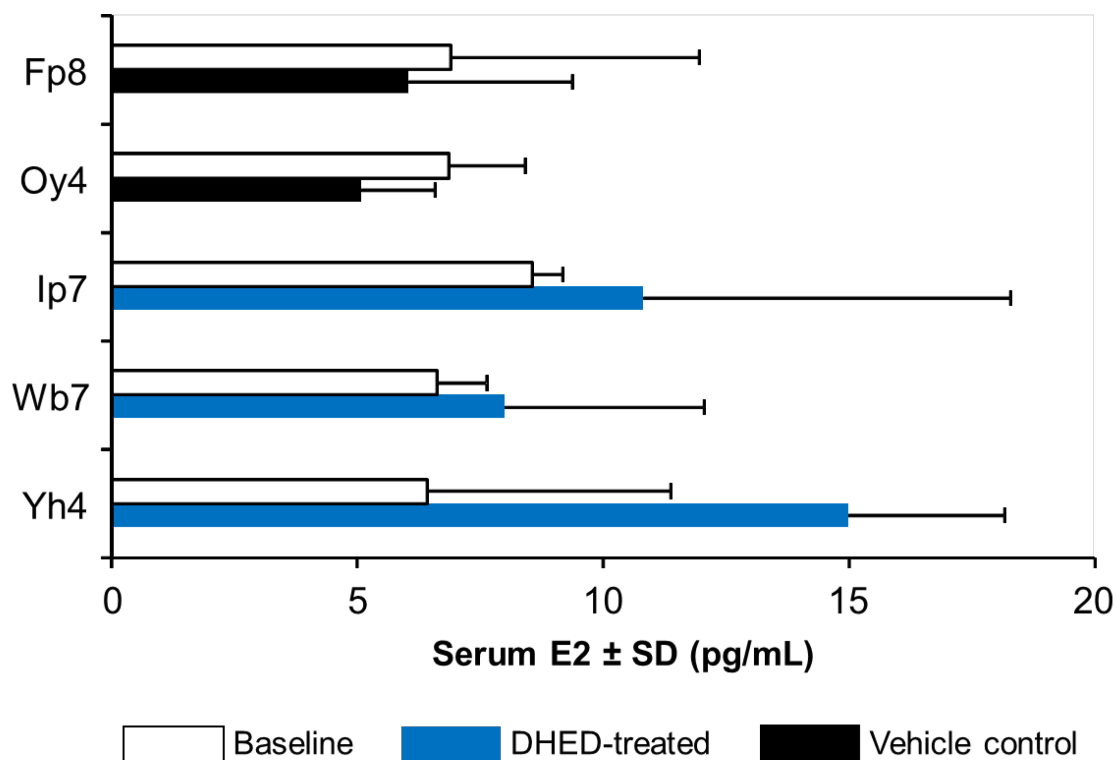

FIGURE 3. Kisspeptin-1 gene expression in the preoptic area (POA) of the hypothalamus following 12 days of chronic treatment with DHED (20 ug/kg/day) or vehicle (both s.c. via Alzet osmotic minipump). DHED treatment increased kisspeptin-1 gene expression in the POA as compared to vehicle.

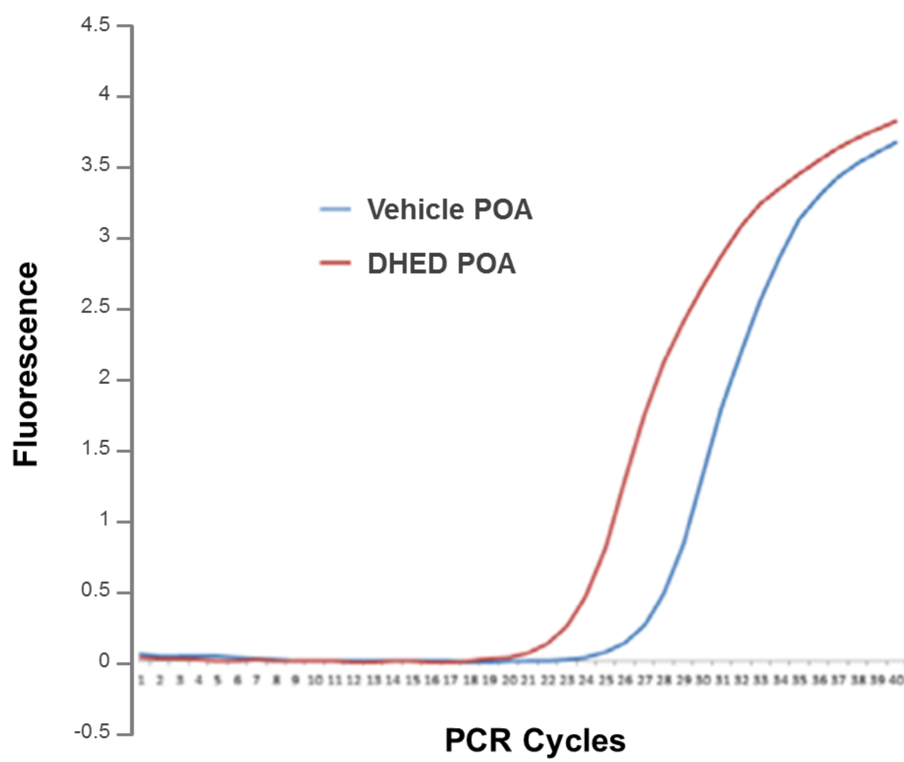

FIGURE 4. Mean (± SEM) serum estradiol concentrations measured immediately prior to acute treatment administration (pre-injection) and after the two-hour behavioral test immediately following acute treatment administration (2h post-injection). Only the acute estradiol treatment significantly increased serum estradiol concentrations as compared to baseline. Baseline estradiol concentrations did not differ between acute treatment conditions.

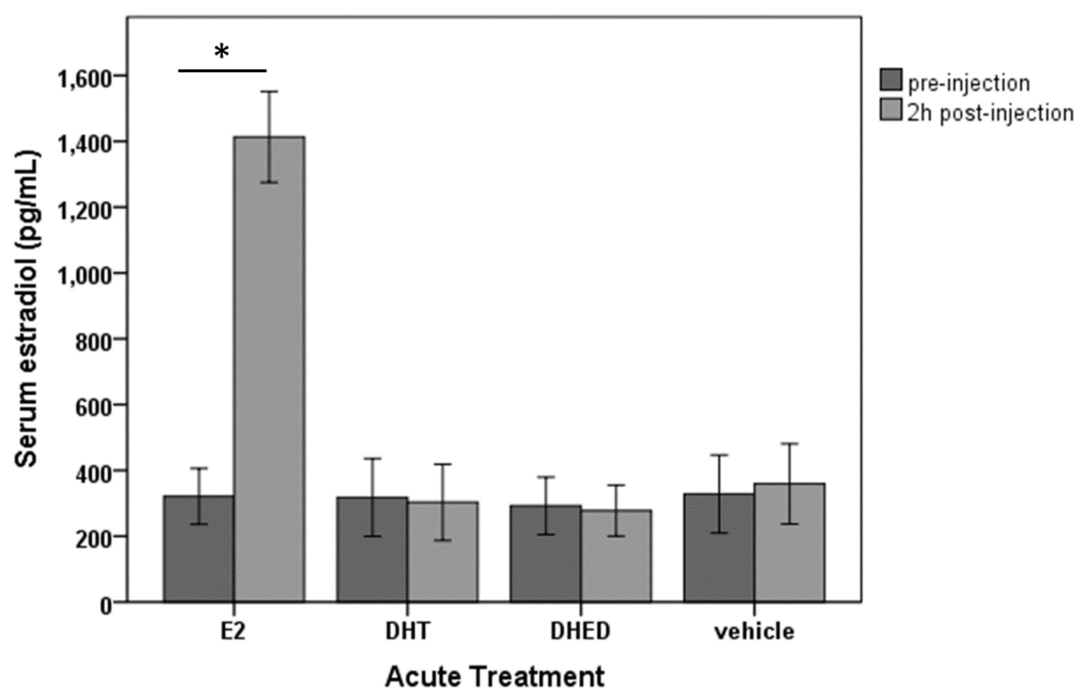

FIGURE 5. Mean (± SEM) rates of female sexual initiation during the two-hour behavioral test immediately following acute treatment administration for the five subjects who showed female proceptive behavior during the study. Rates of female sexual initiation did not significantly differ between treatment conditions.

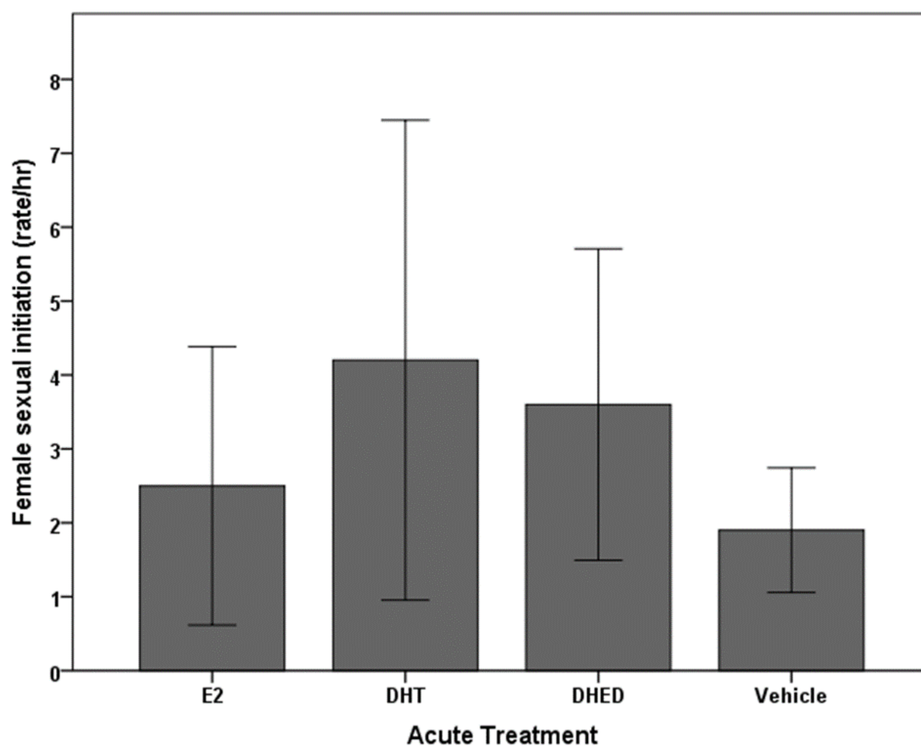

FIGURE 6. Mean (± SEM) duration of time spent near the male during the two-hour behavioral test immediately following acute treatment administration for the five subjects who showed female proceptive behavior during the study. Duration of time spent near the male did not significantly differ between treatment conditions.

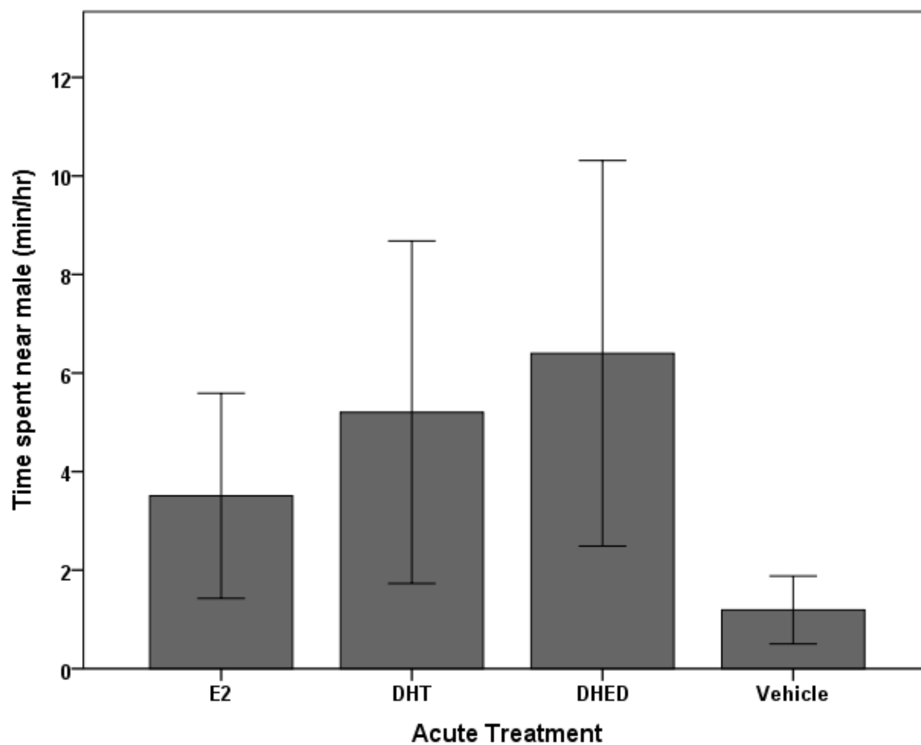

FIGURE 7. Mean (± SEM) duration of time spent grooming the male during the two-hour behavioral test immediately following acute treatment administration for the five subjects who showed female proceptive behavior during the study. Duration of time spent grooming the male did not significantly differ between treatment conditions.

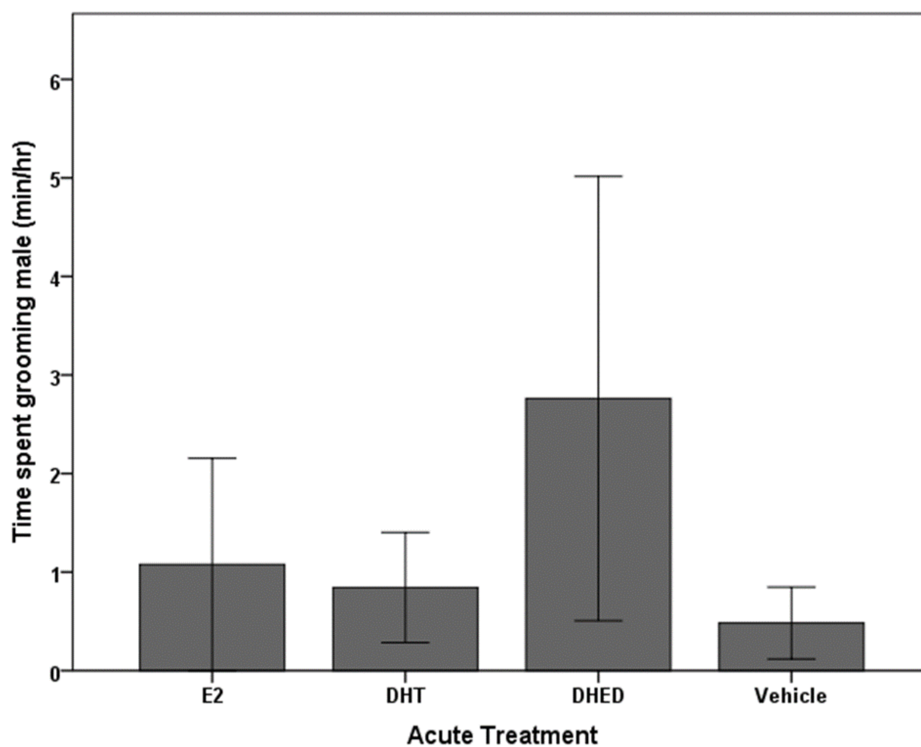

FIGURE 8. Mean (± SEM) rates of female sexual initiation across the three observation days of each treatment week for the five subjects who showed female proceptive behavior during the study. Rates of female sexual initiation did not significantly vary across observation days for any given treatment, or between treatments, and there was no significant interaction between observation day and treatment.

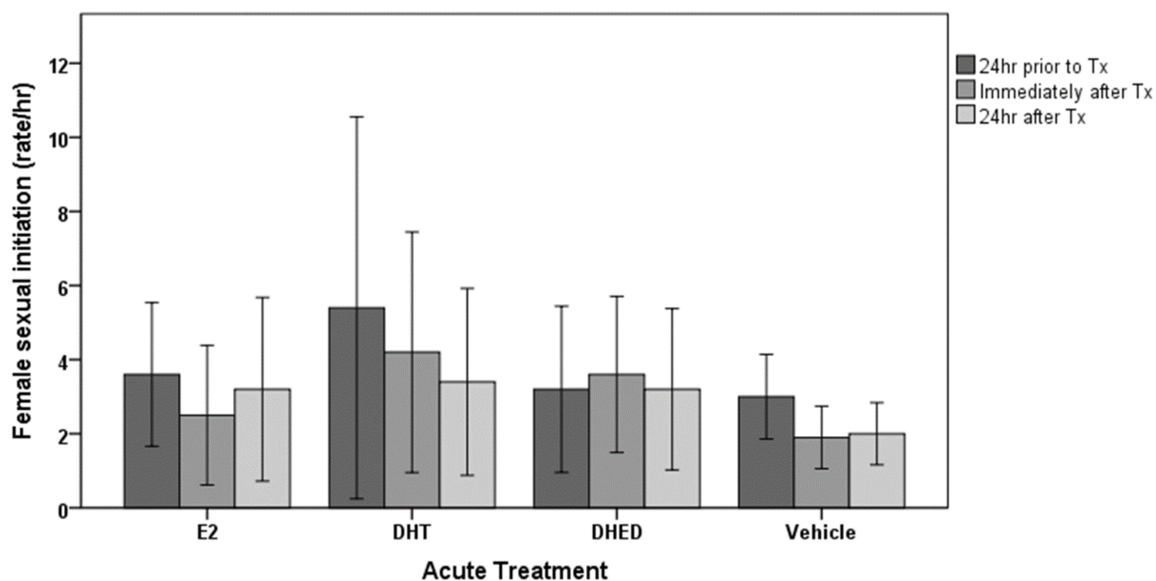

FIGURE 9. Mean (± SEM) rates of female sexual initiation across hours one and two of the two-hour behavioral test immediately following acute treatment administration for the five subjects who showed female proceptive behavior during the study. Rates of female sexual initiation did not significantly differ between hours one and two for any given treatment, or between treatments, and there was no significant interaction between observation hour and treatment.

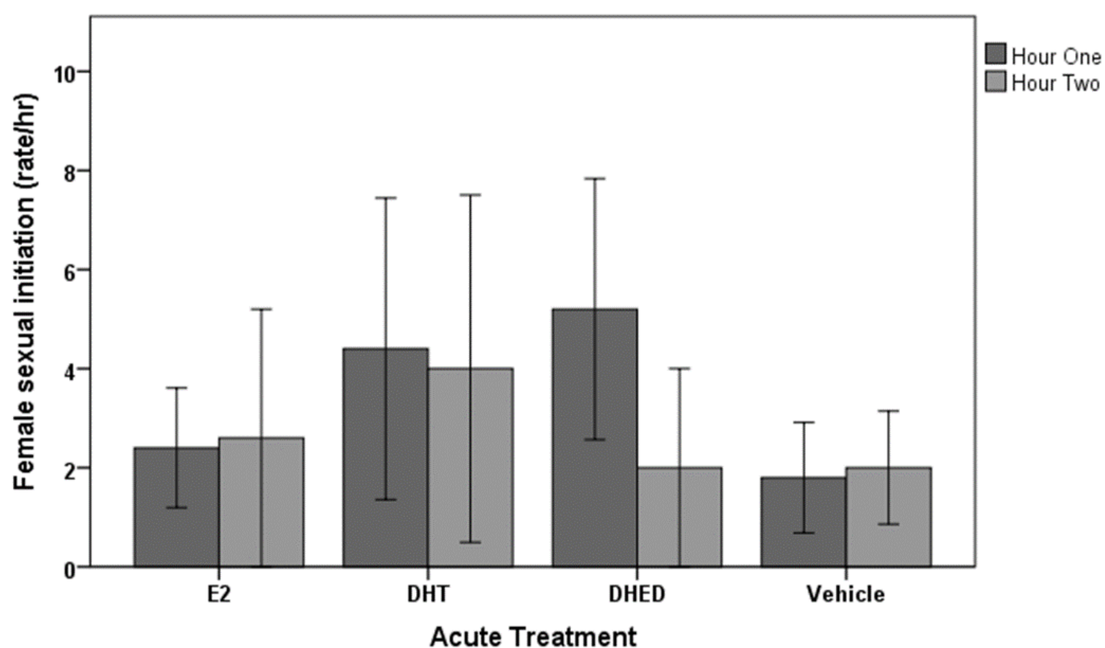

FIGURE 10. Rates of female sexual initiation by subject during the two-hour behavioral test immediately following acute treatment administration for the five subjects who displayed proceptive behavior.

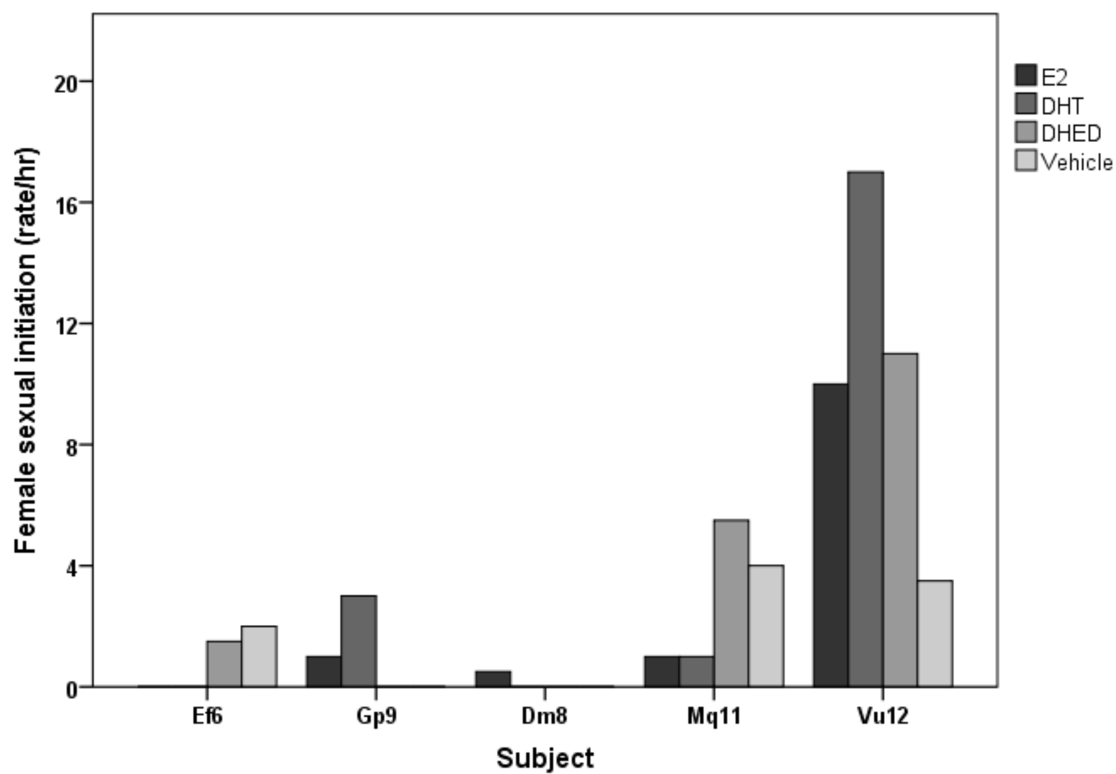

FIGURE 11. Mean (± SEM) number of days that group females spent hospitalized for wounding during the breeding season (Oct-Apr) immediately following group formation (breeding season 1) and during the subsequent breeding season (breeding season 2). Females spent significantly more days hospitalized for wounding during breeding season 1 as compared to breeding season 2, and days spent hospitalized for wounding did not significantly differ between the two social groups.

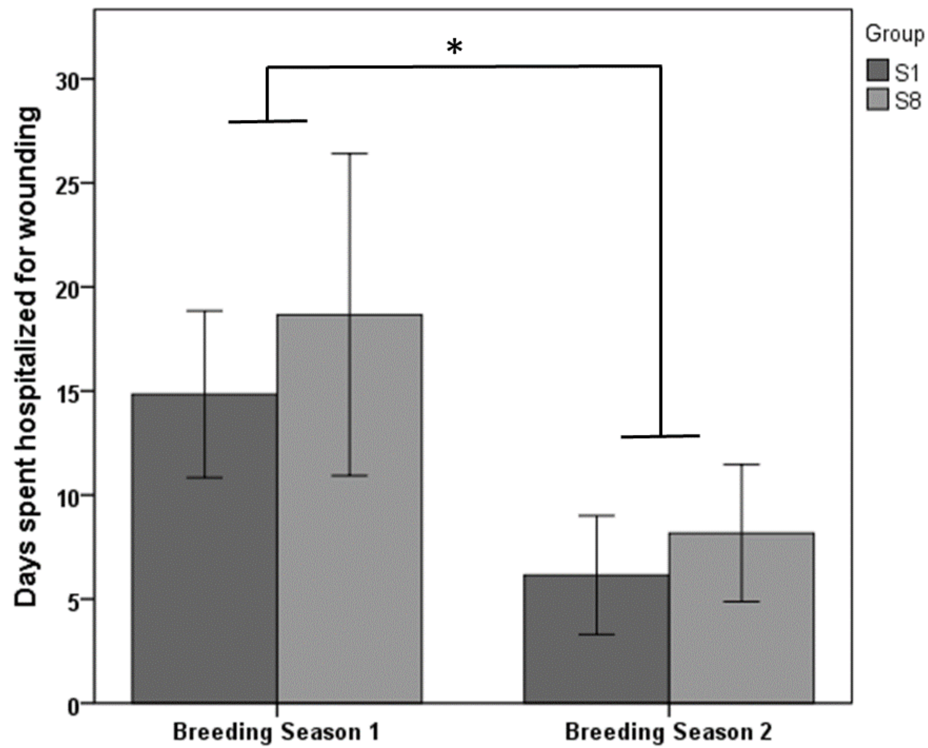

FIGURE 12. Mean (± SEM) serum free estradiol concentrations across the five measurement time points for assay run one.

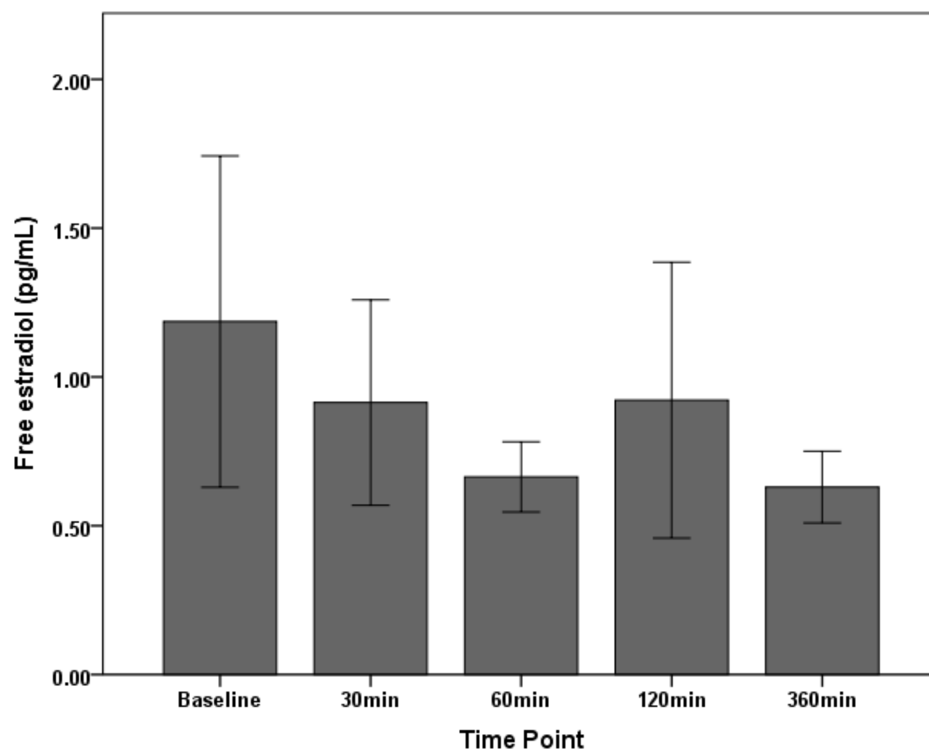

FIGURE 13. Serum free estradiol concentrations by subject across the five measurement time points for assay run one.

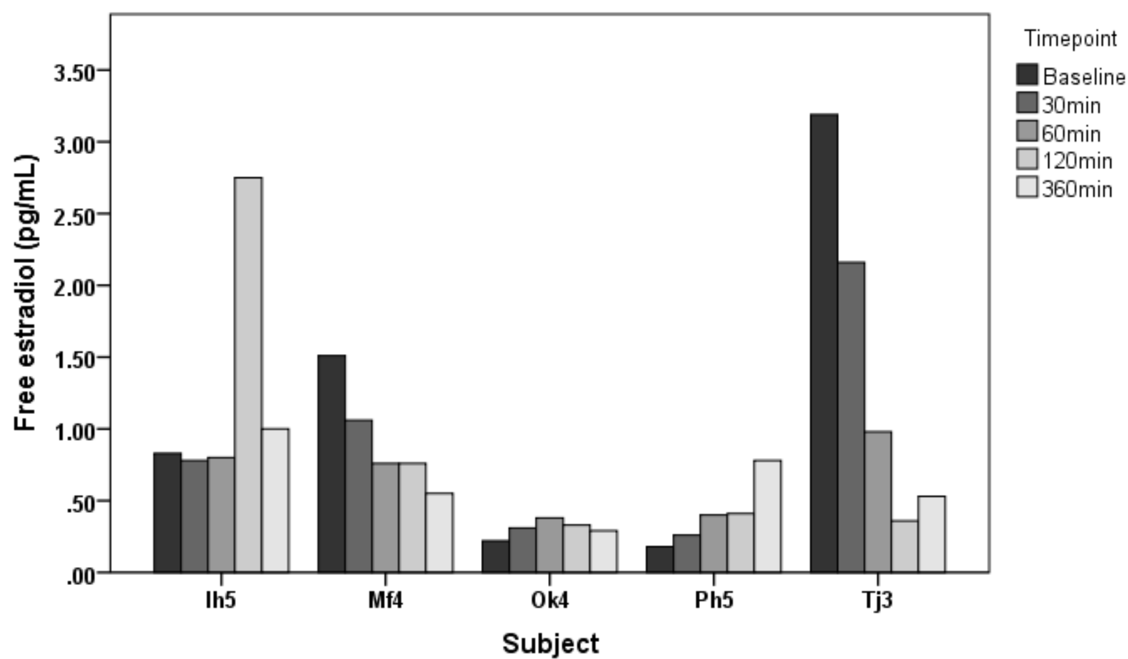

FIGURE 14. Mean (± SEM) serum total estradiol concentrations across the five measurement time points for assay run two.

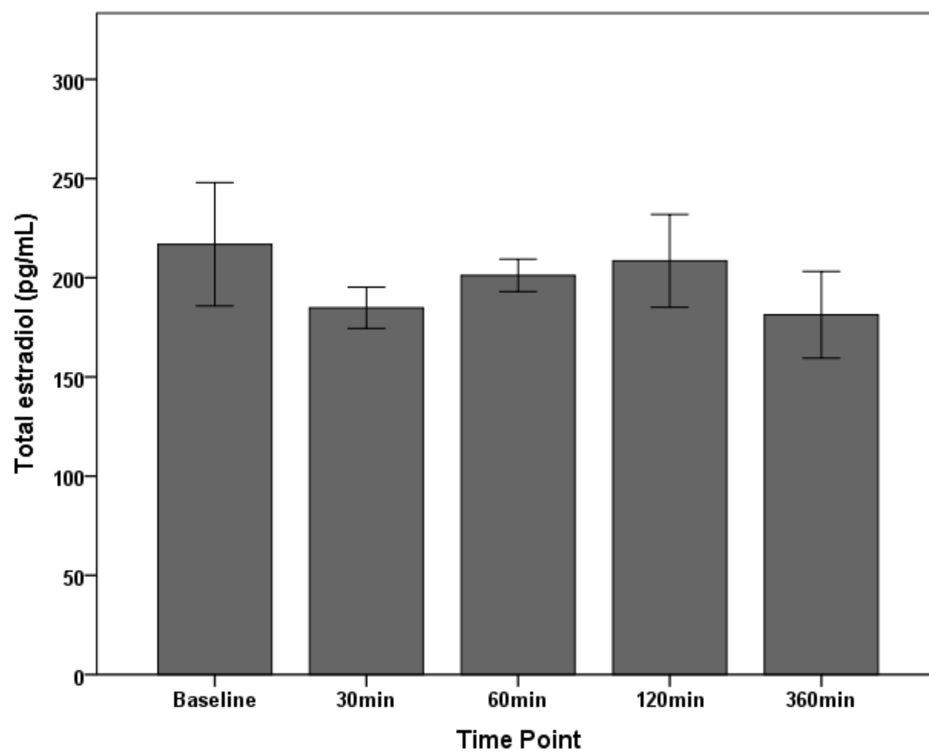

FIGURE 15. Mean (± SEM) serum free estradiol concentrations across the five measurement time points for assay run two.

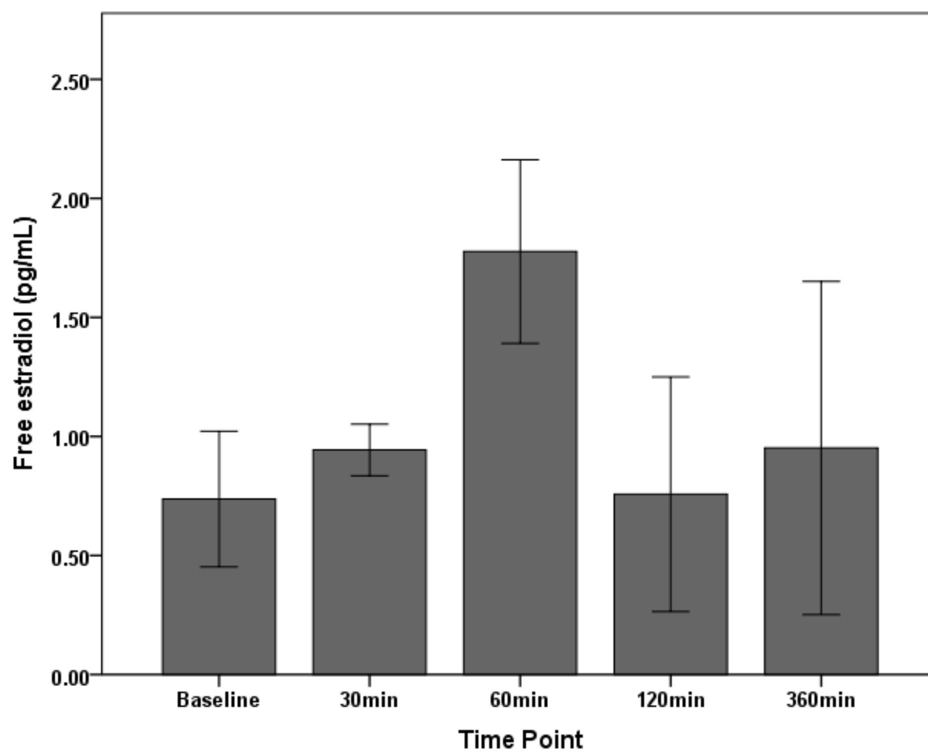

FIGURE 16. Serum free estradiol concentrations by subject across the five measurement time points for assay run two.

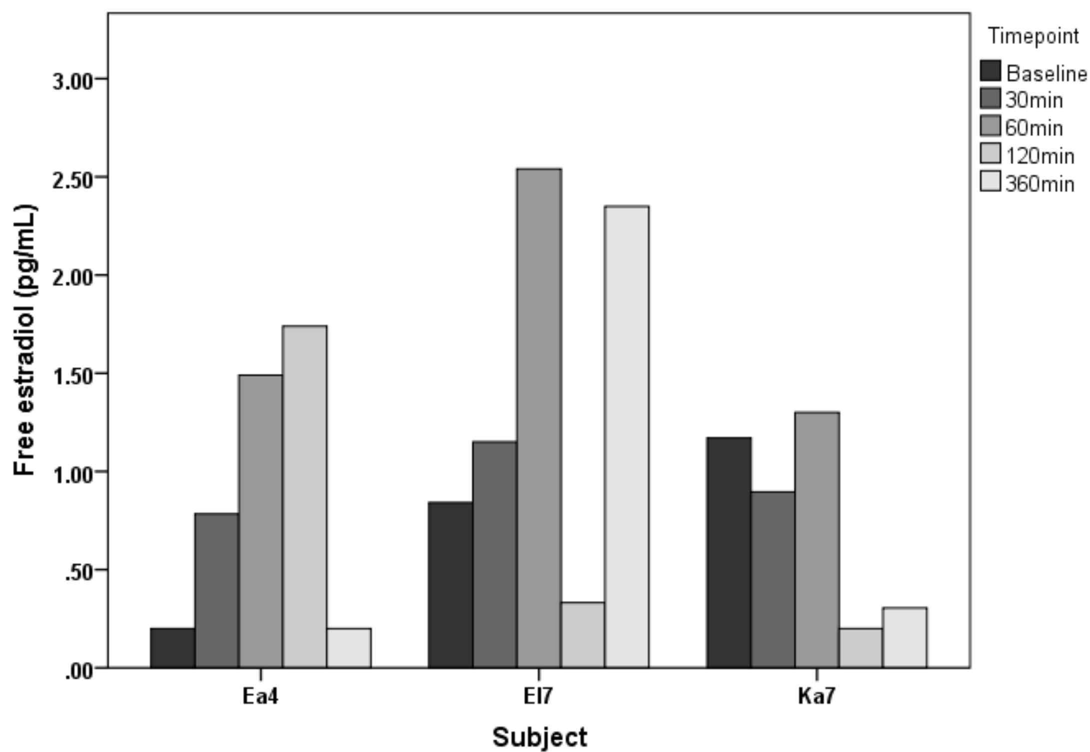

Figure 17. Mean (± SEM) percent free estradiol in serum across the five measurement time points for assay run two.

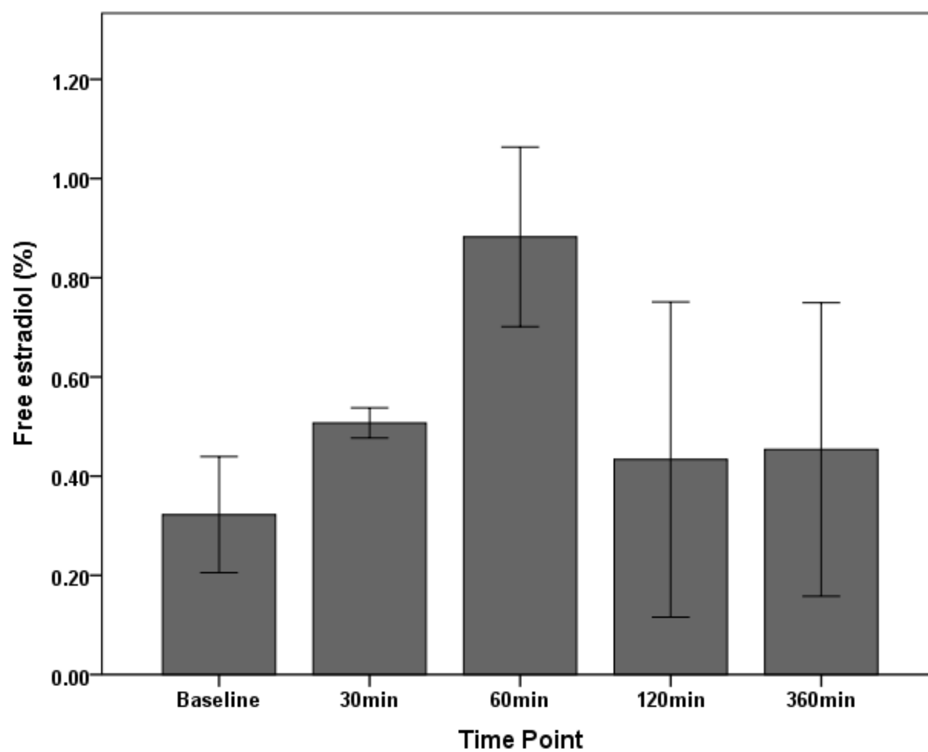

FIGURE 18. Percent free estradiol in serum by subject across the five measurement time points for assay run two.

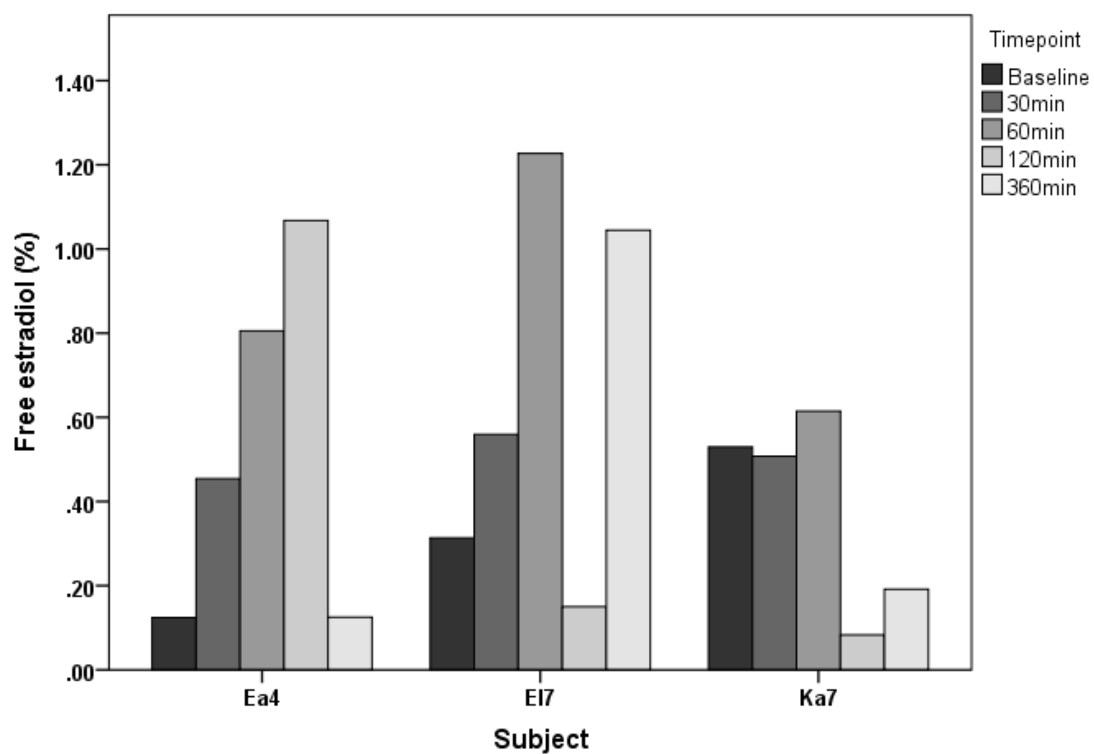

Figure 19. Serum free estradiol concentrations for subject Ea4 across the five measurement time points for assay run two, under the DHT and vehicle treatment conditions.

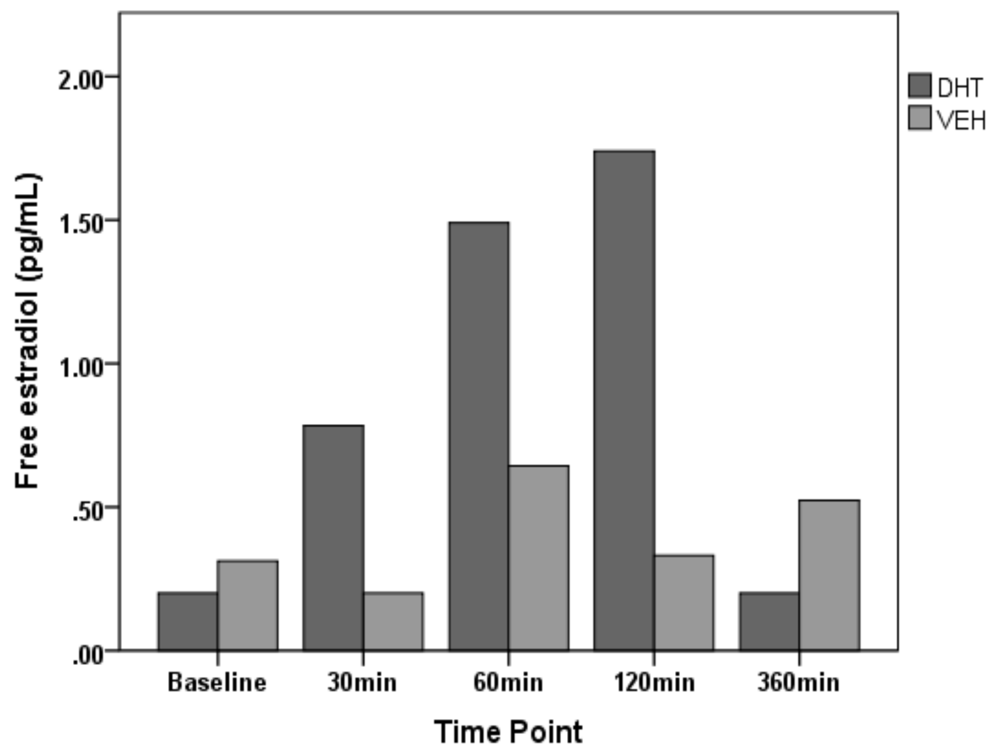

FIGURE 20. Mean (± SEM) rates of female sexual initiation for the seven subjects originally assigned to the study across the eight weeks of the abandoned treatment paradigm. Rates of female sexual initiation significantly varied across the eight study weeks, indicating possible treatment carryover effects. Post hoc analyses revealed that rates of female sexual initiation were significantly higher during week one as compared to weeks three through eight, and rates of female sexual initiation did not vary across weeks three through eight.

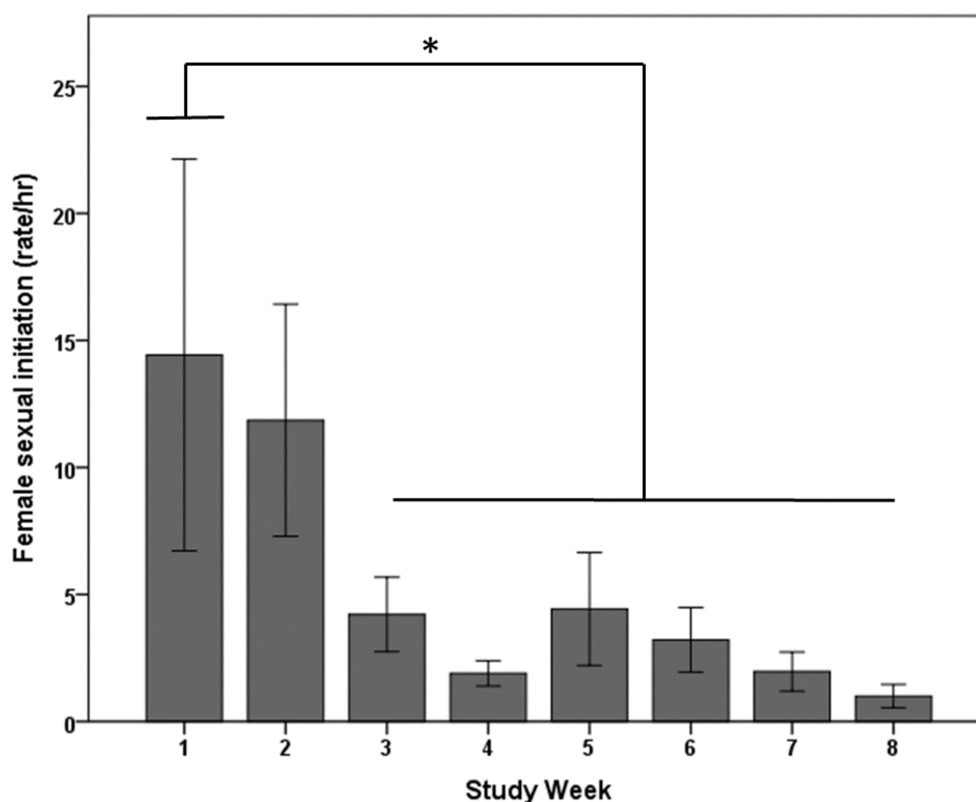

TABLE 1. Female proceptive behaviors displayed by each subject during the study.

	Solicit	Present	Follow	Groom	Approach	Prox	Total # of behaviors
Ef6		x		x	x	x	4
Gp9			x		x	x	3
It13							0
Dm8				x	x	x	3
Mq11			x	x	x	x	4
Vu12	x		x	x	x	x	5
Total # of females	1	1	3	4	5	5	

TABLE 2. Repeated-measures ANOVA results for rates of female proceptive behaviors during the two-hour behavioral test immediately following acute treatment administration across treatment conditions.

Behavior	F(3,12)	*p*	Partial eta squared
Approach	0.17	0.92	0.04
Prox	1.54	0.26	0.28
Follow	2.29	0.13	0.36
Present	1.00	0.43	0.20
Solicit	1.00	0.43	0.20
Groom	0.93	0.46	0.19

TABLE 3. Serum estradiol concentrations (pg/mL) for subjects Mq11 and Vu12 following an acute injection of estradiol benzoate (3 ug/kg; IM).

Time Point	Mq11	Vu12	Mean	SD
Baseline	47.6	20.5	34.05	19.16
+2 hours	201.0	88.3	144.65	79.69
+4 hours	163.0	89.7	126.35	51.83
+6 hours	157.0	123.0	140.00	24.04
+24 hours	98.4	93.8	96.10	3.25