EFFECTS OF ELEVATED TESTOSTERONE LEVELS ON REPRODUCTIVE NEUROENDOCRINE FUNCTION AND BEHAVIOR IN FEMALE RHESUS MACAQUES (*MACACA MULATTA)*

by

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A DISSERTATION

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CERTIFICATE OF APPROVAL

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This is to certify that the Ph.D. dissertation of

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ABSTRACT

Hyperandrogenemia (HA) is associated with several clinical disorders in reproductive-aged women. The most common disorder associated with HA is polycystic ovary syndrome (PCOS), which affects 4-8% of women and is characterized by elevated androgens and ovarian dysfunction. Other features associated with PCOS include neuroendocrine alterations such as increased frequency of pulsatile luteinizing hormone (LH) secretion, increased LH response to gonadotropin-releasing hormone (GnRH), and decreased sensitivity to progesterone negative feedback. Additionally, women with PCOS frequently are obese and have decreased insulin sensitivity compared with healthy women. The overall goal of this dissertation was to examine whether symptoms of PCOS would develop in female monkeys that were treated with low doses of testosterone (T), which were designed to mimic the circulating levels of T seen in women with PCOS, beginning just prior to puberty. To examine neuroendocrine function in these monkeys, pulsatile LH secretion, the LH response to GnRH, and sensitivity to progesterone negative feedback were assessed after the animals had gone through puberty and had been exposed to elevated T for 3 years. Additionally, ultrasounds were performed to assess ovarian function, and glucose tolerance testing and metabolic testing were performed to assess metabolic function. It was found that T treatment led to increased central drive to the reproductive axis, as evidenced by faster LH pulse frequency during the early follicular phase in the T-treated animals in addition to a larger LH response to exogenous GnRH. However, T treatment for 4 years did not result in any ovarian or metabolic changes that were indicative of a PCOS phenotype. After these initial assessments were made, the monkeys were fed a high-calorie, high-fat diet typical of

Western cultures (Western style diet; WSD) that resulted in increased adiposity in order to mimic the high prevalence of overweight and obesity in women with PCOS. Neuroendocrine, ovarian, and metabolic functions were assessed in the same manner as before the WSD. The frequency of pulsatile LH secretion measured during the early follicular phase increased in the control animals, such that T-treated and control animals both had approximately hourly LH pulses. It was also found that after 14 months on the WSD, all animals had peripheral compartmentalization of ovarian follicles, which is similar to the polycystic ovary phenotype in women with PCOS. Moreover, the T-treated animals had decreased insulin sensitivity compared with control animals after one year on the WSD, even when controlling for weight. These data indicate that T treatment, in combination with the WSD, leads to neuroendocrine, ovarian, and metabolic features that are typically seen in PCOS. Behavior was also examined in these animals to assess whether mild HA would lead to changes in aggression or anxiety (i.e., behavioral inhibition). No group differences were found in aggression, but the T-treated animals showed slightly less inhibited behavior, which may represent a greater degree of impulsivity, as they were more active than control animals when presented with a potentially threatening stimulus in a novel environment. This indicates that while low T might not increase aggression in female monkeys, it does lead to a decrease in behavioral inhibition, a common form of anxious behavior. Interestingly, there was also a strong correlation between decreased behavioral inhibition (being active in a novel environment) and weight gain on the WSD, suggesting that impulsive behavior may play an important role in determining who will become obese when a highly palatable diet is made available.

Chapter 1

INTRODUCTION

1.1 Hyperandrogenemia in Women of Reproductive Age

Hyperandrogenemia (HA) is a common endocrinopathy experienced by women of reproductive age. HA refers to the production and/or secretion of excess androgens, leading to levels that are elevated above those normally found in women. HA in women has been associated with an array of negative health-related consequences, such as type 2 diabetes (Ding et al. 2006), metabolic syndrome (Korhonen et al. 2003), dyslipidemia (Mudali et al. 2005), and male-pattern hair growth (Azziz et al. 2004a). Androgens are synthesized from cholesterol by a cascade of enzymes, and include testosterone (T), dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), androstenediol, and androstenedione (A4) (see Fig. 1.1). T can be aromatized into estradiol (E), and so may indirectly exert some of its effects by acting on E receptors (Naftolin et al. 1971; Bellino and Osawa 1974), in addition to acting on androgen receptors. T can also be converted into DHT, the only other androgen capable of acting directly on androgen receptors (Roy et al. 1999). In healthy women, most androgen synthesis occurs in the adrenals; however, ovarian theca and interstitial cells are responsible for the production of small amounts of T, and T is also produced by the systemic conversion of other androgens (Fortune and Armstrong 1977; Burger 2002). HA can result from adrenal or ovarian tumors, and is typically severe when neoplasm is the cause. Milder elevations in androgens occur in a variety of other conditions, including congenital adrenal hyperplasia, obesity, Cushing syndrome, and idiopathic HA (Unluhizarci et al. 2011). The most common cause of

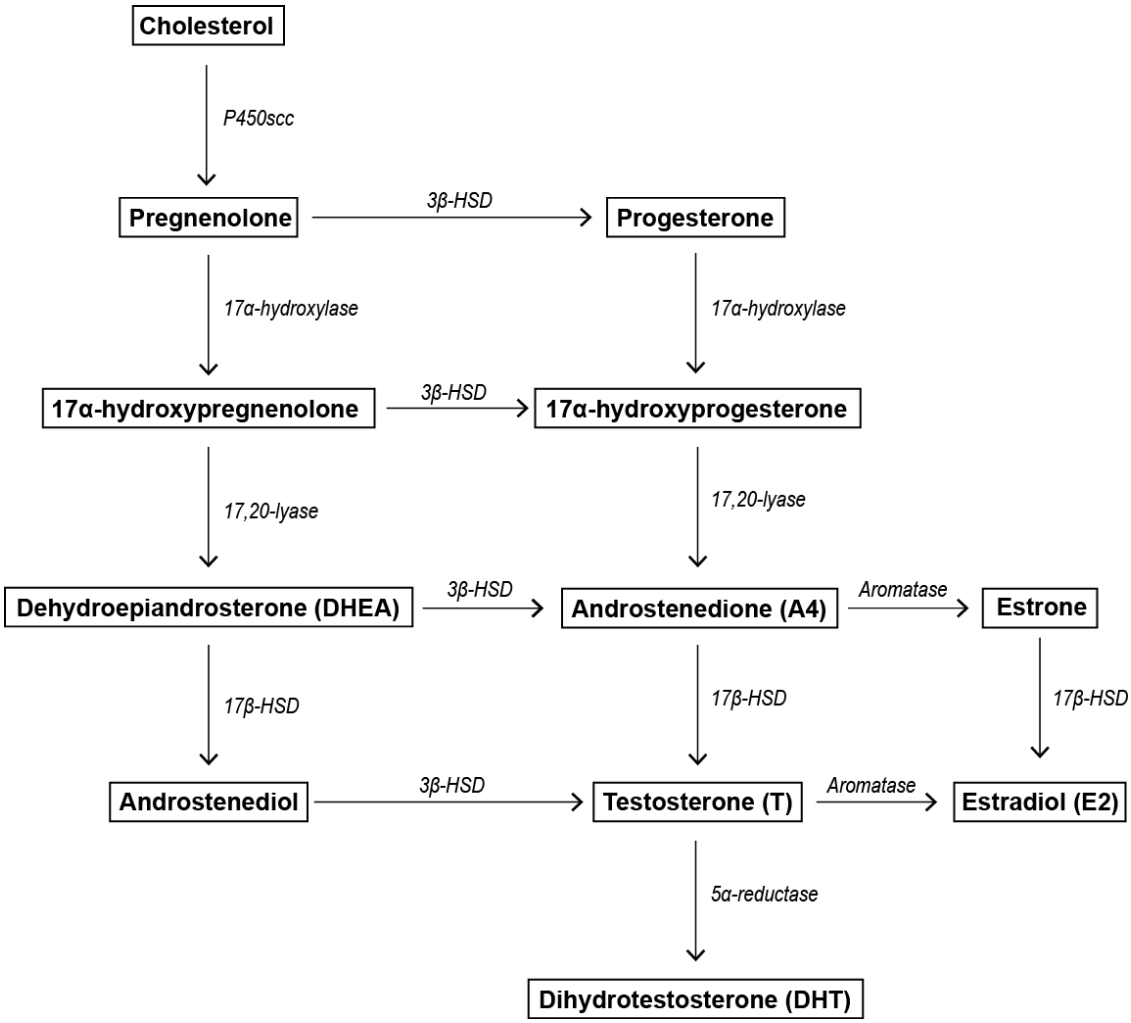

Figure 1.1 Steroidogenesis pathway. Steroids are in boxes, and the enzymes that catalyze each reaction are in italics. 3β-HSD=3β-hydroxysteroid dehydrogenase; 17β-HSD=17β-hydroxysteroid dehydrogenase

HA in women, however, is polycystic ovary syndrome (PCOS) (Azziz et al. 2004a; Carmina et al. 2006).

Congenital Adrenal Hyperplasia

A genetic disorder that often results in HA is congenital adrenal hyperplasia (CAH). CAH, which affects both women and men, is an autosomal recessive disorder caused by the mutation of one of several genes encoding enzymes involved in cortisol production. CAH is an uncommon disorder, with a prevalence of 1:15,000 for the severe type to 1:1,000 for the less severe type (Speiser et al. 1985; Pang and Clark 1993). In the most common forms of CAH, patients have a deficit of 21-hydroxylase, an enzyme necessary for the adrenal gland to convert 17-hydroxyprogesterone (17-OHP) into cortisol. As a result, the adrenals convert a greater proportion than usual of 17-OHP into androgens. The hypothalamic-pituitary axis, sensing a deficit of cortisol, secretes large amounts of corticotropin releasing factor (CRF) and adrenocorticotropin releasing hormone (ACTH), aggravating the condition with markedly elevated production of T and A4 by the adrenal glands (for review see Nimkarn et al. 2011). There are two types of CAH: classical, which results from a severe or complete enzyme deficiency, and nonclassical, which is more common and results from only a mild enzyme deficiency. In the classical form of CAH, exposure to androgens begins prenatally and often results in virilized or ambiguous genitalia in females at birth, while the nonclassical form is less severe and does not usually present until signs of HA occur in late childhood or early adulthood (New 2006). These signs include premature pubarche, acne, hirsutism,

deepening of the voice, and male-pattern alopecia. Another common finding in women with CAH is irregular or absent menstrual cycles. Additionally, neuroendocrine changes frequently occur in CAH patients. Hypothalamic function in people is typically assessed by measuring levels of luteinizing hormone (LH), which is released by the pituitary in response to the secretion of gonadotropin-releasing hormone (GnRH) by the hypothalamus (Clarke and Cummins 1982; Crowley et al. 1985). GnRH and LH are released in a pulsatile pattern (Butler et al. 1972; Gay and Sheth 1972; Yen et al. 1972; Carmel et al. 1976). In women with CAH, there is evidence of increased hypothalamic and pituitary function, as these women commonly have elevated LH levels, an increased LH:FSH ratio, and increased LH pulse amplitude (Levin et al. 1991; Trakakis et al. 2008). An increased LH response to exogenous GnRH is also seen in CAH, indicating increased sensitivity of the pituitary to GnRH (Levin et al. 1991). The mechanism by which these neuroendocrine alterations occur is not known, but a study in female mice found that DHT treatment increased GnRH neuron firing activity (Pielecka et al. 2006), so androgens may increase GnRH activity by acting directly on the hypothalamus in women with CAH. Additionally, androgens can be aromatized into estrogens in the brain, and therefore may function through activation of estrogen receptors (Naftolin et al. 1971). Local exposure to high levels of estrogen has been shown to increase pituitary sensitivity to GnRH, resulting in augmented LH release (Shaw et al. 1975; Wang and Yen 1975).

Obesity

Obesity, which is more prevalent than CAH, is also associated with HA in women. Approximately 65% of American women over the age of 20 are overweight (BMI ≥25) and 35% are obese (BMI≥30), percentages that have increased dramatically over the past 30 years (Kuczmarski et al. 1994; Flegal et al. 1998; Flegal et al. 2010). Obesity is becoming more common in adolescents as well, as the rate of obesity in girls aged 6-19 rose from approximately 5% in 1980 to almost 20% in 2007-2008 (Ogden and Carroll 2010). Women who are overweight or obese are more likely to have elevated T levels compared with lean women, and some studies have shown that the severity of HA is related to the degree of obesity (Strain et al. 2003; Taponen et al. 2003). Obesity during the pubertal transition in girls has also been associated with HA in many (van Hooff et al. 2000a; Reinehr et al. 2005; McCartney et al. 2006; McCartney et al. 2007), but not all (Bordini et al. 2009) studies.

The exact causes of HA in obesity are not well-understood. One study found that human adipose tissue can convert A4 to T, suggesting that increased adiposity could directly lead to elevated T levels (Quinkler et al. 2004). Others have found that obesity leads to decreases in levels of sex hormone-binding globulin (SHBG), the main binding protein of androgens, resulting in an increased amount of free, or unbound, androgen circulating in the system (Evans et al. 1983; Blouin et al. 2007). The consequences of HA in obese women are similar to those seen in women with CAH: hirsutism, acne, and alopecia. Insulin resistance and compensatory hyperinsulinemia are other common findings in obese, hyperandrogenic women (Kissebah et al. 1982; Dunaif et al. 1987). Some data suggest that childhood obesity may be related to early puberty, as girls who

had a high BMI for their age at 36 months went through puberty sooner than girls who were normal weight at 36 months (Lee et al. 2007). It is possible that this finding is due to androgenic stimulation of the GnRH pulse generator, leading to earlier central initiation of puberty. This theory is supported by the data from animal models showing that DHT increases GnRH neuron firing activity (Pielecka et al. 2006). Similar to adults, obese peripubertal girls also frequently suffer from hyperinsulinemia, and a positive correlation has been found between fasting insulin and free T levels in these girls (McCartney et al. 2006).

Polycystic Ovary Syndrome

Most women with HA (70-80%) suffer from polycystic ovary syndrome (PCOS) (Azziz et al. 2004a; Carmina et al. 2006). PCOS is a common reproductive disorder affecting 4-8% of reproductive-aged women worldwide (Knochenhauer et al. 1998; Asuncion et al. 2000; Azziz et al. 2004b). A heterogeneous disorder, PCOS can be difficult to identify and diagnose. Although what is now known as PCOS was first described in 1935 by Stein and Leventhal (1935), there have been many changes to its description over the years. There are currently three definitions of PCOS in use, all of which indicate that other causes of androgen excess should first be excluded. The first set of diagnostic criteria was decided upon by an expert panel convened by the National Institute of Child Health and Human Development, a branch of the National Institutes of Health (NIH), in 1990. The NIH criteria defined a woman as having PCOS if she had both chronic oligo- or anovulation AND clinical or biochemical signs of HA (Zawadzki

and Dunaif 1992). In 2003, a second expert panel met in Rotterdam, the Netherlands, at a conference held jointly by the European Society of Human Reproduction and Embryology and the American Society for Reproductive Medicine. The Rotterdam criteria defined PCOS as the presence of at least two of the following three symptoms: oligo- or anovulation, clinical or biochemical signs of HA, and polycystic ovaries (Rotterdam Consensus Group 2004). At a meeting of the Androgen Excess Society (AES) in 2006, a task force decided on the third set of PCOS diagnostic criteria. According to the AES, a woman has PCOS if she has clinical or biochemical signs of HA in addition to either polycystic ovaries OR chronic oligo- or anovulation (Azziz et al. 2006; 2009). While the exact causes of PCOS are unknown, potential etiologies are described in section 1.2 of this chapter.

In addition to the criteria that are used for diagnosis, there are a variety of other signs and symptoms commonly seen in PCOS patients. The features associated with PCOS can be categorized as ovarian, neuroendocrine, or metabolic in nature. One of the main signs of PCOS that is common to all its definitions is the presence of oligo- or anovulation. This lack of ovulation frequently occurs in women who have a polycystic ovary phenotype. Polycystic ovaries (PCO) were defined by the Rotterdam criteria as containing 12 or more follicles that are 2-9 mm in diameter and/or having an ovarian volume of 10 mL or more (Rotterdam Consensus Group 2004). Women with PCOS have a higher percentage of primary follicles and lower percentage of primordial follicles than healthy women (Webber et al. 2003). Although the mechanism is unknown, it appears that primordial follicles progress to the growing stages faster and have a reduced rate of atresia in women with PCOS compared with healthy women (Webber et al. 2003;

Webber et al. 2007). Follicular development is arrested and no dominant follicle is selected, thus preventing ovulation. Pelvic ultrasonography of women with PCOS frequently shows compartmentalization of these small follicles around the periphery of the ovary, referred to as the "string of pearls" phenomenon (Barber et al. 2010). Anovulation due to a PCO phenotype can cause sub- or infertility, and women with PCOS must often use assisted reproductive technology to become pregnant. Compared with anovulatory non-PCOS controls, women with PCOS undergoing *in vitro* fertilization (IVF) have to stop more IVF cycles prematurely due to complications (Kodama et al. 1995; Heijnen et al. 2006). Additionally, women with PCOS have more oocytes retrieved per cycle but a lower fertilization rate, ultimately leading to similar pregnancy and live birth rates between PCOS women and anovulatory controls (Heijnen et al. 2006). Several studies have also shown that women with PCOS have a higher risk of developing ovarian hyperstimulation syndrome during IVF compared with women without PCOS (MacDougall et al. 1992; Kodama et al. 1995). The risk of this complication has been reduced in recent years by using low-dose gonadotropin protocols in women with PCOS (White et al. 1996; Alsina 2003). However, once pregnant, PCOS patients have a higher rate of complications, including gestational diabetes, preeclampsia, and miscarriage (Wang et al. 2001; Dmitrovic et al. 2011; Kjerulff et al. 2011). In addition, women with PCOS show decreased uterine blood flow, which may be associated with some of these complications (Ajossa et al. 2001; Habara et al. 2002; Chekir et al. 2005). Nevertheless, long-term rates of successful childbirth in women who attempted to become pregnant are similar in PCOS patients and controls (87% and 92%, respectively) (Hudecova et al. 2009).

Neuroendocrine alterations are also commonly seen in PCOS, as patients typically have elevated LH levels and an increased frequency of LH pulses (Waldstreicher et al. 1988; Apter et al. 1994; Moret et al. 2009). Healthy women display variations in LH pulse frequency across the menstrual cycle, due primarily to the actions of progesterone (P) negative feedback on the hypothalamus. In the follicular phase, LH pulses occur about once every 60-90 min (Reame et al. 1984, 1986; Filicori et al. 1986). During the luteal phase, when P levels are high, LH pulse frequency slows to once every 3-4 hours or slower (Soules et al. 1984; Filicori et al. 1986; McCartney et al. 2002b). In contrast, women with PCOS, who are frequently anovulatory, usually have pulses that occur more consistently at about once per hour, without the typical variation seen in normal, ovulatory cycles (Waldstreicher et al. 1988). Additionally, results from several studies indicate that some women with PCOS are resistant to the normal feedback effects of ovarian steroids (Daniels and Berga 1997; Pastor et al. 1998). Pastor and colleagues (1998) gave E and P to women for 21 days beginning during the mid-follicular phase and found that women with PCOS showed less suppression of LH pulses than healthy women. LH and FSH production are dependent on the speed of the GnRH pulse generator, with fast pulses stimulating primarily LH synthesis and secretion, and slower pulses stimulating primarily FSH production (Gross et al. 1987; Spratt et al. 1987). Although women with PCOS are not always deficient in FSH, their levels tend to be on the low end of normal (Rebar et al. 1976; Taylor et al. 1997). Thus, women with PCOS often have an elevated LH:FSH ratio due to the fast frequency of the GnRH pulse generator (Rebar et al. 1976; Waldstreicher et al. 1988; Taylor et al. 1997). Another neuroendocrine feature commonly seen in PCOS is an exaggerated LH response to

exogenous GnRH administration (Eagleson et al. 2000; Patel et al. 2004; Bachelot et al. 2007). When given GnRH, women with PCOS release about twice the amount of LH compared with normal women, suggesting that PCOS patients have more sensitive pituitaries than healthy women. It is important to note, however, that these changes in neuroendocrine function are not always observed in PCOS patients. While many studies show that, on average, women with PCOS have higher LH levels than controls, examination of individual data reveals that some PCOS patients fall within the normal LH range (Taylor et al. 1997; Bachelot et al. 2007). Other studies have found increases in LH pulse amplitude in PCOS patients, with no change in pulse frequency (Kazer et al. 1987; Venturoli et al. 1988). Also, the finding of reduced suppression of LH pulses in response to P is only evident in a subset of the PCOS population (Pastor et al. 1998). These results illustrate the marked heterogeneity present in patients with PCOS. Despite this diversity, however, dysfunction of the neuroendocrine system is seen as a key feature in PCOS.

Neuroendocrine alterations consistent with adult PCOS are evident in earlier stages of development, as well. It has been documented that adolescents with HA, who are at high risk of developing PCOS, show an early transition from pubertal LH secretory patterns (with sleep-associated increases in LH) to adult patterns (with LH pulses occurring during both day and night) (Zumoff et al. 1983). Apter and colleagues (1994) found that HA adolescents made this transition almost 2.5 years earlier than their healthy counterparts. Similar to adults with PCOS, decreased sensitivity to P negative feedback has also been found in about half of HA adolescents studied (Chhabra et al. 2005; Blank et al. 2009).

In addition to reproductive and neuroendocrine changes, metabolic symptoms are also very common in PCOS patients. As many as 90% of women with PCOS are overweight or obese, and these women tend to have a central distribution of adiposity (Holte et al. 1994a; Legro 2000; Azziz et al. 2004a; Carmina et al. 2007). Abdominal obesity in particular is associated with an increased risk of type 2 diabetes, high blood pressure, and cardiovascular disease (Kissebah et al. 1982; Lapidus et al. 1984; Chuang et al. 2006). There is also a higher prevalence of the metabolic syndrome in PCOS patients compared with the general population (Apridonidze et al. 2005; Ehrmann et al. 2006), which is true even when comparing PCOS patients to BMI-matched controls (Gulcelik et al. 2008). Metabolic syndrome is a constellation of symptoms including abdominal obesity, hypertension, dyslipidemia, and insulin resistance (National Cholesterol Education Program 2002; Grundy et al. 2004). Approximately 50-70% of PCOS patients have at least some degree of systemic insulin resistance (Carmina et al. 1992; Meirow et al. 1995; Kauffman et al. 2002). Women with PCOS also have higher rates of insulin resistance in specific tissues, including adipocytes (Ciaraldi et al. 2009) and ovarian granulosa cells (Wu et al. 2003). Oftentimes, weight loss will help control some metabolic and reproductive symptoms of PCOS, although the resolution is not always complete (Holte et al. 1995; Huber-Buchholz et al. 1999; Bruner et al. 2006; Vigorito et al. 2007). For example, a study by Holte and colleagues (1995) examined the effects of weight loss on insulin sensitivity in obese, insulin-resistant women with PCOS. They found that losing 15% of their body weight partially normalized insulin sensitivity in PCOS patients, but these women continued to have an enhanced early insulin response to glucose. Other studies have found that some, but not all, PCOS patients resume

menstruation and ovulation after weight loss (Huber-Buchholz et al. 1999; Kuchenbecker et al. 2011).

There is also a subset of normal-weight PCOS patients, indicating that obesity is not a *necessary* component of the syndrome. However, lean women with PCOS still have higher rates of insulin resistance (Carmina et al. 2007) and are more likely to have abdominal adiposity (Kirchengast and Huber 2001) than lean controls. Additionally, one study found that the prevalence of the metabolic syndrome in lean women with PCOS was 16%, whereas it was only 4% in lean, BMI-matched controls (Attaoua et al. 2008). These data suggest that metabolic changes occur in PCOS somewhat independently of obesity, although normal-weight PCOS patients typically have less severe metabolic phenotypes than overweight PCOS patients (Dunaif et al. 1989; Coviello et al. 2006).

1.2 Potential Etiologies of PCOS

Hyperandrogenemia

The etiology of PCOS is unknown, although this has been an area of intense investigation over the past two decades. Elevated androgen levels are present in most women with PCOS, and the Androgen Excess Society has even suggested that this be a requirement for the diagnosis of PCOS (Azziz et al. 2009). As discussed below, there are several possible factors that may increase androgen levels in women, such as insulin resistance and genetics. Thus, elevated androgens may represent a final common pathway in the genesis of PCOS.

This theory is supported by evidence from girls and women with CAH. Elevated androgens are one of the key features of CAH. Women with CAH also frequently have other symptoms of a PCOS-like phenotype, including hirsutism, irregular menstruation, elevated LH levels, and an exaggerated LH response to GnRH (Levin et al. 1991; Pall et al. 2010; Trakakis et al. 2008), which could indicate that androgens are causing the reproductive and neuroendocrine changes seen in PCOS.

Results from studies of ovarian cultures have found that ovaries from PCOS women have abnormal androgen production compared with ovaries from healthy women. Gilling-Smith and colleagues (1994) found that theca cells from the ovaries of women with PCOS produced more A4 than theca cells from control women under both basal and LH-stimulated conditions. A study by Nelson and colleagues (1999) found similar results, as ovarian theca cells from PCOS patients produced more T compared with theca cells from control subjects. Interestingly, this study was performed after the cells had been passed through culture multiple times, indicating that the excess production of T did not result from the *in vivo* hormonal milieu, but rather likely arose from an intrinsic ovarian alteration. Another study found that ovarian stromal incubations from HA women were also more sensitive to insulin, producing significantly more A4, T, and DHT in response to insulin compared with stroma from healthy women (Barbieri et al. 1986). These results are interesting; however, they do not confirm whether HA plays a causal role in producing the PCOS phenotype, or whether these ovarian changes may occur in response to hyperinsulinemia or chronic exposure to elevated levels of androgens.

Chronic administration of androgens to women for non-therapeutic reasons is typically considered unethical, which makes it difficult to study the effects of androgens

on reproductive and neuroendocrine function in people. One interesting population that does allow us to study this is female-to-male (FTM) transsexuals. In a histological study of ovaries from androgen-treated FTM transsexuals, 68% were polycystic, compared to none of the ovaries studied from control women (Futterweit and Deligdisch 1986). Another study of FTM patients found enlarged ovaries in almost half and polycystic ovaries in 80% after 2-9 years of androgen treatment (Grynberg et al. 2010). In a study of agonadal FTM patients who had been on androgen therapy for at least 3 months, mean LH levels and LH pulse amplitude were higher compared with eugonadal women (Spinder et al. 1989a). A separate study found that eugonadal FTM patients who were tested before and 6 months after starting androgen treatment showed no change in LH pulse frequency or amplitude between the two time points (Spinder et al. 1989b). Taken together, the FTM literature indicates that androgens likely play a causal role in the morphological changes seen in the ovaries of PCOS patients; however, the results regarding LH secretion are less conclusive. The doses of androgens that FTM patients are exposed to are exponentially larger than the levels found in women with PCOS, so it is possible that the lower amounts of circulating androgens in PCOS patients may have a different effect on the hypothalamic-pituitary system. It is also possible that the age at time of exposure is important, as most FTM patients studied began androgen therapy in adulthood (Spinder et al. 1989a), while there is evidence that exposure to androgens in PCOS begins during puberty (Sir-Petermann et al. 2009).

Insulin Insensitivity

Consequences of Insulin Insensitivity on Ovarian Function

As the prevalence of overweight and obesity in PCOS is as high as 90% (Holte et al. 1994a; Legro 2000; Azziz et al. 2004a; Carmina et al. 2007), and as many as 70% of PCOS patients show some form of insulin insensitivity (Carmina et al. 1992; Meirow et al. 1995; Kauffman et al. 2002), numerous studies have focused on the role of insulin insensitivity in the etiology of PCOS. Insulin insensitivity is typically accompanied by an increase in the amount of circulating insulin present in one's system (Shanik et al. 2008). Insulin receptors are located in many tissues throughout the body, including in the ovary (Willis and Franks 1995). At high concentrations, insulin may also act on insulin-like growth factor I (IGF-I) receptors present in the ovary (Rechler et al. 1980; Steele-Perkins et al. 1988). The fact that women with PCOS frequently have hyperinsulinemia, in combination with the ability of insulin to act on either insulin or IGF-I receptors on the ovary has led to the proposal that insulin may play a causal role in the development PCOS (Barbieri and Ryan 1983; Poretsky and Kalin 1987).

Hyperinsulinemia can also inhibit production of IGF binding protein 1 (IGFBP-1) by the liver, which leads to elevated levels of bioavailable IGF-I (Suikkari et al. 1989). It is well-known that LH stimulates androgen secretion by ovarian theca cells (Fortune and Armstrong 1977; Hillier et al. 1991; Campbell et al. 1998), and IGF-I augments this process *in vitro* in cells from a variety of species, including rats (Cara and Rosenfield 1988), pigs (Caubo et al. 1989), and humans (Bergh et al. 1993). This too could be a

mechanism by which peripheral insulin insensitivity, and consequent hyperinsulinemia, may contribute to increased androgen production by the ovary.

The "insulin theory" of PCOS is supported by the fact that women with type 1 diabetes mellitus (DM1) have a higher incidence of PCOS than the general population, and the prevalence of PCOS or PCO morphology is higher in DM1 patients who use intensive insulin therapy as opposed to conservative insulin therapy (Codner et al. 2006). However, women with DM1 and PCOS generally have a different hormonal profile than women who have only PCOS. Women with both DM1 and PCOS generally have normal levels of SHBG and A4, and a normal LH:FSH ratio, while women with only PCOS tend to have decreased levels of SHBG, increased levels of A4, and a higher LH:FSH ratio compared with controls (Codner et al. 2007). This could indicate that the pathogenesis of HA is different in these two groups of patients, and that insulin insensitivity may only contribute to the etiology of PCOS in a subset of women.

Another piece of evidence supporting the insulin theory is that hyperinsulinemia seems to precede HA in adolescents. In a study of seventeen 4 to 18-year-old girls with obesity and acanthosis nigricans, all of them showed increased fasting insulin levels compared with controls, while only the post-menarcheal girls (n=12) had elevated T levels (Richards et al. 1985).

Consequences of Insulin Insensitivity at the Hypothalamus and Pituitary

Several studies have found evidence that insulin may affect the neuroendocrine system by stimulating LH secretion. Hypothalamic cell cultures from fetal mice showed

dose-dependently increased GnRH secretion when treated with insulin (Burcelin et al. 2003). Adashi and colleagues (1981) also showed that insulin enhanced LH release from cultured rat pituitary cells. These studies together indicate that insulin is capable of affecting gonadotropin release by acting at multiple levels of the hypothalamic-pituitary-gonadal (HPG) axis. Interestingly, however, the same study by Adashi et al. (1981) found that there was no insulin-mediated increase in LH release from pituitary cells that were cultured in serum-supplemented media, suggesting that a component present in the serum may lead to differing actions of insulin on the HPG axis *in vivo* as opposed to *in vitro*.

In vivo experiments have been inconclusive regarding the effect of insulin on LH secretion. Some *in vivo* work supports the finding that insulin enhances LH secretion, as Burcelin and colleagues (2003) found that LH levels were increased by 50-60% during a 3-hour insulin infusion in male mice. However, other studies have found no difference in GnRH-stimulated LH levels between female rats that were treated with insulin or vehicle twice daily for three weeks (Poretsky et al. 1988).

Studies in humans have also been inconclusive. Several studies have found that acute insulin does not affect LH secretion in women. In a study that assessed mean LH and LH pulse frequency and amplitude, none of these measures changed during a 12 h insulin infusion (Mehta et al. 2005). Similarly, a separate study found that the LH response to GnRH was also unchanged during an insulin infusion (Patel et al. 2004). Other studies have found that insulin decreases mean LH secretion over a 6- or 12-h period without affecting LH pulse frequency (Lawson et al. 2008). Interestingly, Moret and colleagues (2009) found that the LH response to insulin may depend on baseline LH function. Most of the control women in this study had a slower LH pulse frequency (7-8

pulses/12 h) at baseline than the women with PCOS. In these controls, pulse frequency increased during an insulin infusion (to 13 pulses/12 h), while there was no change in LH secretion in the control subject who already had a faster pulse frequency at baseline (13 pulses/12 h). The PCOS subjects, who had faster baseline pulse frequencies (10-13 pulses/12 h), also saw no change in LH secretion during the insulin infusion. Given the differences between in vitro and in vivo results, it is difficult to determine what the acute effects of insulin are on the reproductive neuroendocrine axis. Additionally, there has been little work done examining more chronic effects of exogenous hyperinsulinemia on the hypothalamic-pituitary control of the reproductive axis, as would occur in PCOS.

Insulin Sensitizers for the Treatment of PCOS

Because insulin has been hypothesized to play a role in the etiology of PCOS, one major area of research into treatments for PCOS has been the use of insulin sensitizers, such as metformin and the thiazolidinediones (pioglitazone, rosiglitazone, and troglitazone), to treat the reproductive abnormalities. In general, these drugs have been successful at improving some symptoms of PCOS; however, resolution of the reproductive problems is often incomplete.

As expected, treatment with insulin sensitizers typically leads to an improvement in insulin sensitivity (SI) in as little as 8 weeks (Velazquez et al. 1994; Dunaif et al. 1996; Moghetti et al. 2000; Mehta et al. 2005; Gambineri et al. 2006), although one study found no changes in SI in obese PCOS women with metformin treatment for 12 weeks (Ehrmann et al. 1997). Additionally, a meta-analysis found that metformin was only

successful at reducing fasting insulin in non-obese PCOS patients, while it had no significant effect on insulin concentrations in obese women with PCOS (Tang et al. 2010).

The most consistent improvement aside from that in insulin sensitivity is normalization of irregular menstrual cycles in PCOS patients treated with insulin sensitizers. Improvement in menstrual irregularity has been found when the drugs were administered for durations between 6 months and 1 year (Moghetti et al. 2000; Azziz et al. 2001; Gambineri et al. 2006). However, it is important to note that only about half of the women in these studies experienced normalization of their cycles, while the other half saw little or no improvement. A meta-analysis also found that while metformin improved ovulation and clinical pregnancy rates in women with PCOS, the frequency of live birth was not improved in these women (Tang et al. 2010). In one study (Azziz et al. 2001), it was found that the patients who ovulated during troglitazone treatment were less obese, had lower T, lower insulin, and higher SHBG before treatment began, suggesting that patients with a milder form of PCOS are more likely to benefit from insulin sensitizing agents.

Another area where improvement is often seen with insulin sensitizers is the lowering of circulating T levels. Studies have reported that free T may be reduced to levels seen in controls (Velazquez et al. 1994), or may be reduced compared with pre-treatment levels, but remain elevated compared with non-PCOS controls (Dunaif et al. 1996). Other studies have found no change in T values with insulin sensitizer treatment (Ehrmann et al. 1997; Ganie et al. 2004). The majority of studies, however, have seen decreases in T during insulin sensitizer treatment, but have no control (non-PCOS) group

with which to compare the values (Moghetti et al. 2000; Azziz et al. 2001; Gambineri et al. 2006). There are large variations in absolute values of T among the different commercially-available T assays, and the accurate detection of T can be a problem in women because levels of T are often close to the limit of assay detectability (Rosner and Vesper 2010; Vesper and Botelho 2010). It is therefore impossible to determine from the data presented for these studies whether T values returned to normal or were reduced but remained elevated above normal. In spite of decreases in T levels, most studies found only minor (Azziz et al. 2001; Ganie et al. 2004; Gambineri et al. 2006) or no (Moghetti et al. 2000) improvement in hirsutism in PCOS patients taking insulin sensitizers.

Gonadotropin levels are less likely to be altered by insulin sensitizers. Some studies have found that LH levels decreased during treatment with metformin (Velazquez et al. 1994) or troglitazone (Dunaif et al. 1996), although this was only seen with fairly high doses of the drugs. Most other studies have not seen any changes in LH levels during treatment with insulin sensitizing agents (Ehrmann et al. 1997; Moghetti et al. 2000; Mehta et al. 2005).

In spite of this evidence for improvement of some PCOS symptoms with insulin sensitizer treatment, it is important to note that there are PCOS patients who do not suffer from insulin resistance or hyperinsulinemia (Meirow et al. 1995; Kauffman et al. 2002), meaning that insulin is likely not playing a causal role in these cases. There are also women with hyperinsulinemia who do not have PCOS (Codner et al. 2006; Bizzarri et al. 2011). Thus, while insulin may play an important role in many cases, it seems likely that insulin insensitivity typically *aggravates* symptoms in women who are already predisposed to develop PCOS.

Genetic Factors

PCOS tends to affect members of the same family, indicating that there may be a genetic component to the syndrome. A large study of twins in the Netherlands determined that the heritability of PCOS is 72% (Vink et al. 2006). The prevalence of PCOS in non-twin sisters of women with PCOS is 40-50%, which is considerably higher than the 4-8% prevalence of PCOS in the general population (Carey et al. 1993; Knochenhauer et al. 1998; Kahsar-Miller et al. 2001; Azziz et al. 2004b). Sisters (who do not have PCOS) of women with PCOS have significantly higher levels of T and A4 compared with unaffected BMI-matched controls (Yildiz et al. 2003). Moreover, daughters of PCOS patients also have elevated levels of T beginning in late puberty (Sir-Petermann et al. 2009).

There is a higher risk of reproductive abnormalities in unaffected family members (i.e., those that don't have PCOS) of women with PCOS, as well. Polycystic ovaries have been found in up to 80% of sisters and mothers of PCOS patients (Norman et al. 1996). Daughters of PCOS patients also have increased ovarian volume beginning pre-pubertally (Sir-Petermann et al. 2009).

In addition to hormonal and reproductive abnormalities, female relatives of PCOS patients have higher rates of hyperinsulinemia and insulin resistance than the general population (Legro et al. 2002; Yildiz et al. 2003). Insulin insensitivity has been reported to appear during the pre-pubertal or pubertal period in daughters of women with PCOS (Sir-Petermann et al. 2007; Kent et al. 2008).

Hormonal and metabolic abnormalities extend to male relatives, as well. Hyperinsulinemia is more common in fathers and brothers of PCOS patients than in weight-matched control men (Yildiz et al. 2003). A study by Recabarren and colleagues (2008) examined sons of women with PCOS during infancy, childhood, or adulthood. They found that body weight was higher in sons of PCOS women than in sons of control women at all stages, and insulin resistance developed independent of body weight in PCOS sons during adulthood.

Premature baldness, or significant hair loss before the age of 30, has been suggested to represent a male phenotype of PCOS (Carey et al. 1993; Duskova et al. 2004). One study of five families found premature baldness in almost 90% of first degree male relatives of PCOS patients (Norman et al. 1996). In another study of relatives of PCOS patients, 22% of male relatives had premature baldness, compared with 5% of males from control families (Govind et al. 1999). Interestingly, the males with premature baldness had higher T levels than those who were not affected. This may indicate the presence of genetic differences in androgen production or metabolism in families of PCOS patients.

Genes Related to Androgen Function

A number of candidate genes have been proposed as playing a causal role in the development of PCOS. A major focus in the search for a PCOS gene has been on genes related to androgen production or action. *CYP11a* was studied as a candidate gene because it encodes the cholesterol side-chain cleavage enzyme, which is the rate-limiting

step in androgen biosynthesis (Chung et al. 1986; Morohashi et al. 1987). Results have been somewhat ambiguous regarding the role of *CYP11a* allelic variants in PCOS, with several studies showing an association (Gharani et al. 1997; Urbanek et al. 1999; Diamanti-Kandarakis et al. 2000), but others finding no association (San Millan et al. 2001; Gaasenbeek et al. 2004). As described earlier, SHBG regulates the amount of unbound circulating androgens in the body, and levels are frequently low in PCOS patients (Moran et al. 1999; Sieminska et al. 2004). For this reason, the SHBG gene was investigated as a candidate gene in PCOS patients. Although one study found no link (Urbanek et al. 1999), another study found that women with PCOS had a greater frequency of the longer (TAAAA)n allele in the promoter of the SHBG gene, while normal women typically had shorter alleles (Xita et al. 2003). The PCOS women with longer alleles had lower SHBG levels than PCOS women with shorter alleles, although all PCOS patients had lower SHBG levels than controls with either allele.

Genes Related to Insulin Function

Due to the prevalence of insulin resistance and hyperinsulinemia in PCOS, a second major focus in the search for a genetic cause of PCOS has been on genes related to insulin action, one of which is the insulin gene (*INS*) itself. The variable number tandem repeats (VNTR) in *INS* are embedded in the 5′ regulatory region of the gene. These alleles are grouped into three classes by size: class I alleles have 26-63 repeats, class II alleles have 64-140 repeats, and class III alleles have 141-209 repeats (Bell et al. 1982). The 5′ regulatory region containing the VNTR regulates transcription of *INS* and

the class III allele is associated with an increased risk of obesity, hyperinsulinemia and type 2 diabetes (Weaver et al. 1992; Ong et al. 1999). Waterworth and colleagues (1997) found a significant linkage between PCOS and the class III allele in a study of 17 affected families. However, two later studies failed to confirm this linkage (Urbanek et al. 1999; Powell et al. 2005). Urbanek and colleagues (1999) screened 37 candidate genes for PCOS and found that the strongest evidence for association was on chromosome 19p13.2 in an area containing D19S884, which was chosen as a marker for the insulin receptor. One study by another group later confirmed this relationship (Tucci et al. 2001), while results from a third study did not support it (Villuendas et al. 2003), illustrating the lack of consensus regarding a genetic component of the insulin resistance that is commonly seen in PCOS.

Conflicting results from association and linkage analyses may result from the small sample sizes of many studies, the limitation of diagnosis to reproductive-aged women, the variation in PCOS diagnostic criteria used, the lack of a well-defined male phenotype, and the possible existence of multiple etiologies. One must also consider that PCOS may arise from environmental factors (such as intrauterine conditions) or from an interaction between genes and environment. In fact, studies using prenatally androgenized rhesus monkeys (see section 1.3) found that genes involved in TGF-β signaling were differentially methylated in both infants and adults exposed to prenatal androgens (Xu et al. 2011), indicating that epigenetic changes may play a role in the development of PCOS.

1.3 Animal Models of Hyperandrogenemia

In order to isolate the effects of androgens on reproductive, neuroendocrine, and metabolic function, several animal models of HA have been utilized. Animal models have examined effects of HA prenatally, over adolescent development, and in adulthood.

Rodents

Rats have been used because they have short gestational periods, reach sexual maturity relatively quickly, and are inexpensive to maintain (gestation: 21-23 days; puberty at 35 days; Barrie 1938; Dudley 1981; Evans 1986). Wu and colleagues (2010) injected pregnant dams with T or DHT on days 16-19 of gestation, in order to mimic the endogenous surge of T that is observed in male rats during that time in prenatal development. Female offspring that were exposed to prenatal androgens were ovariectomized as adults and then a week later had blood samples collected to measure the frequency and amplitude of LH secretion. Basal LH levels, as well as LH pulse frequency and pulse amplitude, were all higher in T- or DHT-treated rats compared with controls. In addition, the androgen-treated rats had more preantral and antral follicles, fewer preovulatory follicles, and longer estrous cycles than control rats. These findings are all consistent with the PCOS phenotype seen in humans. However, the prenatally-treated rats also showed physical signs of masculinization (which is not seen in PCOS), such as increased anogenital distance and decreased numbers of nipples.

In another rat model of HA, animals received subcutaneous DHT implants beginning prepubertally (at 3 weeks of age) and continuing into adulthood (12-16 weeks of age) (Manneras et al. 2007). The DHT-treated animals displayed several reproductive

characteristics of PCOS, including irregular estrous cycles and increased numbers of cystic follicles compared with controls. These rats also had an increased percentage of body fat and decreased insulin sensitivity, which are other common features of PCOS. Neuroendocrine function was not evaluated in these animals. Another study looked at metabolic changes that occurred in rats given testosterone propionate (TP) injections starting post-pubertally, at 60 days of age (Perello et al. 2007). Rats were tested after two months of TP exposure and the androgen-treated animals had a significantly higher insulin response to glucose than control animals. Thus, in rodent models there is evidence that exposure to androgens, whether it occurs prenatally, peripubertally, or in adulthood, may play a role in the development of a PCOS-like phenotype.

Sheep

Another animal model of HA is the prenatally androgen-treated sheep. Pregnant ewes received injections of TP or DHT propionate (DHTP) twice a week from day 30-90 or day 60-90 of gestation, with full term gestation occurring at 147 days (Birch et al. 2003; Steckler et al. 2007; Manikkam et al. 2008). This paradigm is associated with a progressive loss of estrous cycles in adulthood. In one study, five out of seven ewes that were treated on gestational day (GD) 30-90 had normal reproductive cycles during their first breeding season, while none of them had any cycles during their second breeding season. Six of the seven ewes treated on GD60-90 had normal cycles their first breeding season, while only four had normal cycles their second breeding season (Birch et al. 2003). In one study that combined prenatal T-treatment with prepubertal overfeeding to

induce weight gain, luteal phase defects were found in adulthood (Steckler et al. 2009). All control animals (total n=13), including overfed controls (n=7), exhibited luteal increases in P in response to estrous synchronization with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) during their first breeding season (puberty at 28 weeks). Most (5/8) T-treated animals also showed a normal rise in P during the luteal phase, indicative of ovulation. However, only 1/7 overfed T-treated animals had a rise in P in response to estrous synchronization with $PGF_{2\alpha}$. These findings support observations that obesity aggravates the reproductive dysfunction found in women with PCOS (Huber-Buchholz et al. 1999).

Neuroendocrine changes consistent with PCOS are also seen in sheep treated prenatally with androgens. In male lambs, a pubertal rise in LH occurred by 9 weeks of age, whereas this rise did not occur until 33 weeks of age in control females, similar to results from previous studies (Echternkamp and Lunstra 1984; Huffman et al. 1987). In prenatally androgenized females, the LH rise occurred at an intermediate age, either at 22 weeks (GD60-90 treatment) or 12 weeks (GD30-90 treatment) (Birch et al. 2003). Prenatally T-treated sheep tested as adults also showed less suppression of LH pulses than controls in response to P administration (Robinson et al. 1999). Another study of female sheep treated with DHTP on GD30-90 examined pulsatile LH secretion both pre- and post-pubertally, and found evidence that these sheep had neuroendocrine function characteristic of PCOS patients (Manikkam et al. 2008). The androgenized sheep had elevated LH pulse amplitude, mean LH, LH:FSH ratio, and LH response to GnRH compared with controls at both time points, although LH pulse frequency did not differ between androgenized and control sheep. However, other studies have found an increased LH pulse frequency in prenatally androgenized sheep (Savabieasfahani et al. 2005;

Steckler et al. 2009). One of these studies found an increase in pulsatile LH frequency in prenatally T-treated sheep, as well as a milder increase in pulse frequency in overfed control sheep, although there was no interaction between T-treatment and overfeeding status (Steckler et al. 2009). This is further evidence supporting the finding that obesity can exacerbate the symptoms of PCOS. The study by Manikkam and colleagues (2008) also found increased mRNA expression of the GnRH receptor and decreased mRNA expression of estrogen receptor 1 (*ESR1*) in the pituitaries of prenatally androgenized female fetuses. Estrogen receptor α (coded by the *ESR1* gene) has been found to increase pituitary sensitivity to estrogen negative feedback (Dorling et al. 2003), so these findings are consistent with increased LH release in PCOS being due at least in part to the increased sensitivity of the pituitary to GnRH and decreased sensitivity to estrogen negative feedback.

Metabolic symptoms characteristic of PCOS have also been found in prenatally androgenized female sheep. A study of lambs treated with T on GD30-90 found higher fasting insulin levels in the treated animals compared with controls at 5 weeks of age (Recabarren et al. 2005). A later study by the same group extended these findings by showing that female animals treated with T on GD60-90 and tested as adults also had increased fasting insulin, in addition to a decreased insulin sensitivity index (Padmanabhan et al. 2010). Additionally, this study found an additive effect of overfeeding and T treatment on these measures. In one study, sheep were treated prenatally with T and then post-pubertally with rosiglitazone, an insulin sensitizer (Veiga-Lopez et al. 2010). Rosiglitazone decreased cumulative insulin secreted during a glucose tolerance test and increased insulin sensitivity scores after 12 weeks of treatment

in both controls and T-treated animals, although the insulin levels in T-treated animals were still higher than in controls. In the group that had been prenatally treated with T but did not receive rosiglitazone, animals had an increased percentage of long, irregular cycles in the second breeding season compared with the first breeding season. In the animals that were treated with T and then received rosiglitazone starting after their first breeding season, the number of long cycles during the second breeding season was decreased in comparison to the first. Importantly, however, the number of cycles occurring during the second breeding season in T + rosiglitazone animals was not as high as controls, indicating that although rosiglitazone improved the estrous cycles in these sheep, it did not completely normalize them.

Non-Human Primates

Due to their similarity to humans, many non-human primate species, including old world monkeys, are ideal animals in which to study reproductive and neuroendocrine function. As with women, rhesus macaques exhibit spontaneous ovulation and have menstrual cycles that are about 28 days in length (Knobil 1974). In order to study the effects of HA on female behavior and reproduction, several cohorts of monkeys were exposed to TP during gestation at the Wisconsin and California National Primate Centers (normal gestation=165 days; Neill et al. 1969; Bosu et al. 1973). Pregnant dams carrying female fetuses received injections of TP for 15-80 consecutive days, beginning either on GD40-44 (early-treated), or GD100-118 (late-treated) (Goy and Robinson 1982; Goy et al. 1988; Abbott et al. 2008), and levels of T were increased to those seen in male fetuses

(Resko et al. 1987). Animals from both groups have been studied throughout their lives and have been shown to exhibit many features that are characteristic of PCOS.

Although genital virilization is not seen in women with PCOS, monkeys that were prenatally androgenized during early gestation had virilized genitalia, with an empty scrotum and an immature phallus (Goy and Robinson 1982; Dumesic et al. 1997; Abbott et al. 2008). One question that arose from this prenatal androgenization paradigm in macaques was whether the animals would continue to have elevated levels of androgens in the absence of postnatal TP treatment. In blood samples taken from early-treated (GD40-80) fetuses, T and A4 were only elevated during the period when TP injections were given (Abbott et al. 2008). When infants were delivered by Cesarean section at term (160±2 days), they showed no baseline difference in T compared with controls, but had elevated A4 for the first 30 postnatal days (Abbott et al. 2008). Most studies of the early-treated females in adulthood found that they did not have elevated levels of T or A4 at baseline (Dumesic et al. 2002; Bruns et al. 2007), although one study found a trend toward higher T levels in the early-treated females (Zhou et al. 2007). Another study found that although baseline androgen values were not different between early-treated prenatally androgenized females and controls, both T and DHEA increased in response to an injection of human chorionic gonadotropin (hCG) in early-treated females, but not in controls (Eisner et al. 2002). While the prenatally androgenized monkeys tested as adults do not seem to mimic the constant elevation of androgens found in women with PCOS, a heightened responsiveness to hCG or a GnRH agonist is commonly seen in women with HA and is considered to be a marker of increased thecal cell steroidogenesis (Rosenfield et al. 1994; Gilling-Smith et al. 1997).

Body weights were not different when the early-treated prenatally androgenized females were born; however, the early-treated females were significantly heavier than controls by 8 weeks of age (Abbott et al. 2008; Abbott et al. 2010) and this difference continued through menarche (Goy and Robinson 1982). When prenatally androgenized animals were examined as adults and compared to weight- and BMI-matched controls, the early-treated group had a significantly higher amount of abdominal fat in general, and specifically, had a higher visceral fat mass (Eisner et al. 2003). This is consistent with data from women with PCOS showing that they have more abdominal adiposity than BMI-matched controls (Kirchengast and Huber 2001; Carmina et al. 2007).

Monkeys that were treated prenatally with TP also showed some aspects of menstrual dysfunction that are common in PCOS. The early-treated prenatally androgenized animals went through menarche about 6 months later than control animals (163 wks in early-treated monkeys vs. 133 wks in controls) (Goy and Robinson 1982). Both groups of animals had anovulatory cycles immediately following menarche, but by the twelfth cycle, there were no group differences in ovulation, as about 90% of the animals in each group had ovulated (Goy and Robinson 1982). Although there were also no group differences in the length of menstrual cycles during the first 12 cycles, the early-treated prenatally androgenized animals were more likely to have short luteal phases, an indication of abnormal P secretion from the corpora lutea (Goy and Robinson 1982). As adults, early-treated animals tended to have longer menstrual cycles compared with control monkeys (Bruns et al. 2007). Interestingly, one study found that a subset of early-treated prenatally androgenized females failed to have cyclic menses in spite of the fact that they showed hormonal evidence of ovulation (Dumesic et al. 1997).

Some of the prenatally androgenized females with irregular menstrual cycles were selected as subjects to test the effectiveness of the insulin sensitizer pioglitazone on normalizing menstrual cycle function (Zhou et al. 2007). Four out of five of the early-treated animals showed a normalization of menstrual cycles during pioglitazone treatment. In the monkeys that responded to treatment, the duration of the follicular phase was shortened, the luteal phase was lengthened, and luteal phase P values were increased, bringing all of these characteristics into the range of control animals. Early-treated animals have also been reported to have diminished oocyte quality, as evidenced by decreased numbers of zygotes that developed into blastocysts during a study that examined IVF success in prenatally androgenized monkeys (Dumesic et al. 2002). However, the fertilization rate was the same between prenatally androgenized monkeys and controls in this study. Women with PCOS who undergo IVF have lower fertilization rates compared with women without PCOS undergoing IVF, possibly indicating the presence of diminished oocyte quality in PCOS patients (Heijnen et al. 2006).

Neuroendocrine function has also been studied in the prenatally androgenized monkeys at various pre- and postnatal ages. In early-treated animals that were studied at the fetal and infant stages, the last day of TP injections was on GD80 (Abbott et al. 2008). Although LH levels were lower in prenatally androgenized monkeys than controls on GD80, they increased through GD120, at which point they were significantly higher than control animals. LH levels in controls did not change between GD80 and GD120. Although baseline LH levels were elevated in the early-treated monkeys on GD120, LH responsiveness to GnRH was not elevated compared with controls when measured on GD120 (Abbott et al. 2008). When tested as infants, LH levels remained significantly

higher in prenatally androgenized monkeys than controls, but there was again no difference in response to exogenous GnRH when animals were tested on postnatal day (PD) 30-37.

In a separate cohort of prenatally androgenized monkeys that were either early- or late-treated and then tested as adults, LH levels and the LH:FSH ratio were elevated across the entire menstrual cycle compared with controls (data from early- and late-treated monkeys were combined in this study) (Dumesic et al. 1997). However, another study from this same cohort of early- and late-treated prenatally androgenized females (it is unclear whether any of the same animals were used) found that basal LH levels and the LH:FSH ratio were not different from controls during the early follicular phase of a menstrual cycle (Zhou et al. 2007). Interestingly, the LH:FSH ratio was increased in these prenatally androgenized animals during treatment with pioglitazone. This is somewhat consistent with several studies that have found that LH levels remained elevated in PCOS women treated with pioglitazone or metformin (Ehrmann et al. 1997; Moghetti et al. 2000; Mehta et al. 2005). While there is evidence that the female prenatally androgenized monkeys are similar to PCOS women in that they have increased LH production and LH:FSH ratio, no studies were performed to assess hypothalamic function during the pubertal transition. Furthermore, studies were not carried out to determine LH pulse frequency and pulse amplitude in these animals.

The prenatally androgenized monkeys have also shown metabolic abnormalities that are characteristic of PCOS in some, but not all, studies. Early-treated prenatally androgenized animals did not show any differences in glucose or insulin at baseline or following an injection of glucagon directly into the fetal circulation on GD140 (Abbott et

al. 2010). Surprisingly, these same animals had increased insulin sensitivity (SI) scores compared with controls when tested as infants on PD45. Other studies have examined the disposition index (DI) in prenatally androgenized monkeys. The DI is the product of insulin secretion and insulin sensitivity values, and a lower score is associated with impaired glucose tolerance and the development of type 2 diabetes (Ahren and Pacini 1997; Larsson and Ahren 2000). Early-treated prenatally androgenized females that were tested as adults had lower DI scores compared with controls (Eisner et al. 2000). Based on the findings that women with PCOS have decreased SI and DI values, the early-treated females display some metabolic abnormalities that are characteristic of PCOS.

Fewer studies have been done examining the prenatally androgenized females that were treated in late gestation (starting on GD100-118). Similar to the early-treated females, most studies of late-treated prenatally androgenized females found that they did not have elevated basal levels of T or A4 (Dumesic et al. 1997; Dumesic et al. 2002; Bruns et al. 2007). Late-treated monkeys also had diminished oocyte quality in response to stimulation for IVF, although quality was not impaired to the same extent as in early-treated females (Dumesic et al. 2002). Contrary to what was seen in the early-treated females, however, the late-treated monkeys showed no evidence of virilization aside from clitoromegaly (Goy and Robinson 1982; Dumesic et al. 1997; Abbott et al. 2008). Also unlike the early-treated females, menarche was not delayed in the late-treated prenatally androgenized monkeys (Goy et al. 1988), and menstrual cycle length in the late-treated females was not different compared to controls as adults (Bruns et al. 2007). Additionally, late-treated females did not have impaired SI or DI compared with controls (Eisner et al. 2000). Overall, the early-treated monkeys show more symptoms

characteristic of PCOS compared with the late-treated prenatally androgenized monkeys, but with the caveat that they also have genital masculinization, which is not seen in women with PCOS.

A small number of studies have examined the effects of exogenous androgens on PCOS features when female monkeys are treated only during adulthood. In a series of studies, Billiar and colleagues (1985; 1987) gave adult (age unknown), normally cycling female monkeys A4 implants, increasing levels to 3-4 times those of controls. In the first 1-3 years after implantation, there were no differences in LH levels in the A4-treated animals vs. controls (Billiar et al. 1985). There were, however, more atretic follicles in the ovaries of A4-treated animals, and these animals were more likely to have anovulatory cycles during the summer months (a time when normal rhesus monkeys have an increased incidence of anovulation) (Billiar et al. 1985). After 2-4.5 years of treatment, there were no group differences in fasting glucose or insulin levels, and no differences in glucose or insulin response to a bolus injection of glucose (Billiar et al. 1987). Together, these results indicate that A4 treatment in adult monkeys does not induce metabolic or neuroendocrine changes similar to those seen in PCOS women; however, there were some mild ovarian differences indicative of a PCOS phenotype. In another study of prolonged androgen treatment, monkeys aged 4-12 years were given implants with one of two doses of T for 13-16 months (Faiman et al. 1988). Androgen levels were raised to 3 times that of controls in the lowest dose group and 5 times that of controls in the higher dose group. During the study, monkeys that were given the higher dose of T had slightly decreased numbers of menstrual cycles (10.6 cycles/yr in higher dose animals vs. 11.6 cycles/yr in controls), although all animals showed evidence of

ovulation (elevated serum P and/or fresh corpus luteum at laparoscopy) during most of the cycles that were studied. Using a different treatment paradigm, a study by Vendola and colleagues (1998) examined the effects of acute androgen treatment on monkeys aged 6-13 years. These animals were given high dose T (4 mg/kg/d for 3 d), mid-dose T (400 µg/kg/d for 10 d), low dose T (20 µg/kg/d for 5 d), or DHT (145 µg/kg/d for 5 d), followed by the removal of their ovaries for histological analysis. Compared with controls, serum T levels were raised by 83-fold, 35-fold, and 10-fold in the high-, mid-, and low-dose T groups, respectively. DHT levels were 42-fold higher in the DHT-treated animals than in controls, and DHT was not measured in the T-treated animals. Ovaries from monkeys in all of the androgen-treated groups were enlarged and had increased numbers of small follicles, including primary and small antral follicles. The animals were treated at various points of their menstrual cycles (6/8 controls and 10/16 androgen-treated monkeys were given implants during the follicular phase), and there did not appear to be an effect of menstrual cycle phase on the results. This study indicates that androgens stimulate the early stages of ovarian follicular growth, which is consistent with the increased numbers of small antral follicles seen in women with PCOS (Barber et al. 2010; Rotterdam Consensus Group 2004). Androgen treatment exclusively during adulthood in monkeys has been shown to produce some ovarian changes that are similar to those seen in PCOS, but this paradigm has been less successful at reproducing the neuroendocrine and metabolic features of PCOS.

Thus far, no studies have examined whether the neuroendocrine, ovarian, and metabolic effects of androgens beginning prepubertally would differ from the effects of androgens that are administered prenatally or in adulthood in monkeys. Puberty is a time

when the body is especially sensitive to the effects of hormones (Scott et al. 1974). Additionally, there is evidence that girls at high risk of developing PCOS do not have elevated androgens until the pubertal transition (Sir-Petermann et al. 2009), suggesting that exposure to androgens peri-pubertally (in the absence of prenatal exposure), may be sufficient to exert lasting effects on the neuroendocrine, reproductive, and metabolic systems. The overall aim of this dissertation was to determine the effects of peripubertal androgen administration in female monkeys. Monkeys used in these studies were given T implants starting prepubertally (1 year of age), and were followed until they were 7 years of age. A general timeline of the studies performed is shown in Fig. 1.2. In the studies performed in Chapter 2, I tested neuroendocrine, ovarian, and metabolic function after the animals had been exposed to chronic, slightly elevated T for 3-4 years. In the studies performed in Chapter 3, I examined how weight gain and increased adiposity influenced these parameters. The animals were fed a high-fat, high-calorie diet that is typical of Western cultures starting at 5.5 years of age. Neuroendocrine, ovarian, and metabolic function were measured after 6 months and again after 14 months on the high-fat diet.

1.4 Androgens and Behavior

Aggression

It is well-known that males of many species are more aggressive than females, and as a result, there has long been an interest in examining the roles of gonadal hormones, including androgens, on aggression. Gender differences in aggressive behavior in humans have been found as early as preschool, with boys of this age being more likely

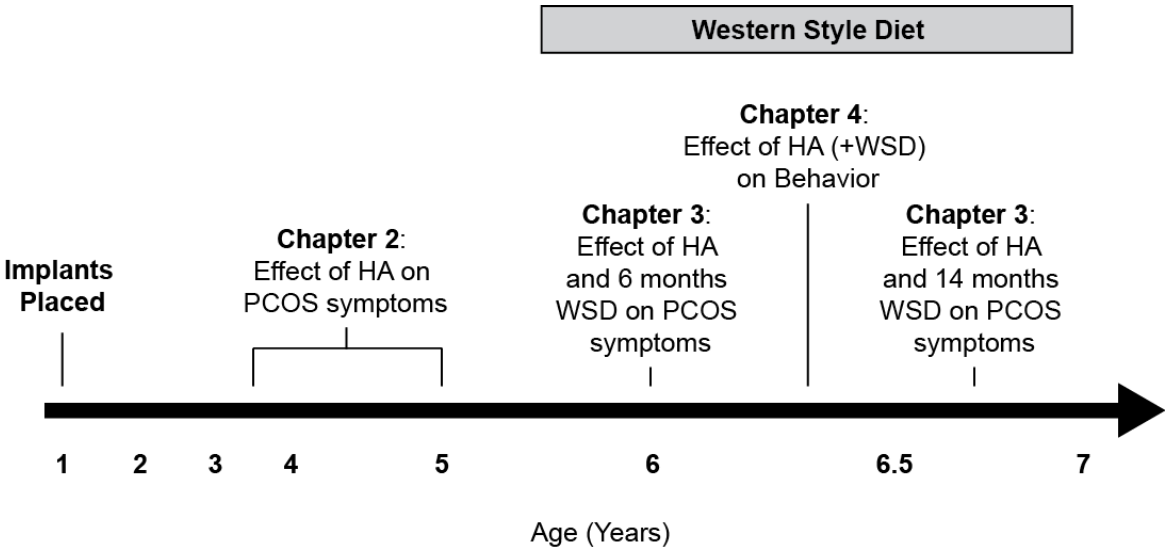

Figure 1.2 Schematic diagram of the experimental timeline, indicating the ages at which experiments were performed. Note that the timeline does not have a uniform scale.

to give and receive aggression than girls (Sanchez-Martin et al. 2000). There was also a correlation between T values and aggressive behaviors in boys, but not in girls, in this study. Because T levels were not different between preschool-aged boys and girls, it is possible that there may have been an effect of prenatal androgen exposure on the children's behavior, or differences may not be due to androgens but rather social correlates of rearing. The gender differences in aggressive behavior continued in older children, with 9-year-old boys scoring higher than girls of the same age in physical, verbal, and indirect aggression (Sanchez-Martin et al. 2011). After sex, the next best predictor of aggression in this study was T level. A study of children with disruptive disorder also found that T correlated positively with aggressive behavior as rated by teachers and staff (Scerbo and Kolko 1994).

In adulthood, there are numerous examples of gender differences in aggressive behavior. Male college students self-reported higher physical aggression than female students (Burton et al. 2007). Another study found that men scored higher than women on scales assessing hostility in addition to verbal and physical aggression (Buss and Perry 1992). Additionally, according to the 2007 Crime Victimization Survey, 75% of violent crimes were perpetrated by men, and only 20% by women, with gender unknown in the final 5% (United States Department of Justice 2010).

In men, the link between T and aggression has been fairly well-established, although not all studies confirm this relationship. In a longitudinal study, men with criminal records as adults had higher T at the age of 16, indicating that high T may be a cause, rather than a consequence, of this behavior (van Bokhoven et al. 2006). A study of male criminal offenders found that those who had committed violent crimes had higher T

than those who had committed non-violent or sexual crimes (Brooks and Reddon 1996). The relationship between T and aggression is likely more complicated, however, as a study by Coccaro and colleagues (2007) found no correlation between T and aggression in adult males with personality disorder. Additionally, another study found that men with a combination of high T and high cortisol levels had higher aggression compared with men who had low T and/or low cortisol, demonstrating that there are likely a variety of other factors playing a role in the production of aggressive behaviors (Kuepper et al. 2010).

In women, the relationship between T and aggression is still present, although it is less pronounced than that seen in men. One interesting study examined aggression in 13-year-old female twins with either a same-sex or opposite-sex co-twin, with the presumption that females with male co-twins were exposed to higher levels of androgens *in utero* (Cohen-Bendahan et al. 2005). Although T levels were not different at the time of the study, girls with male co-twins had higher scores than girls with female co-twins on a verbal, but not a physical, aggression scale. This difference may have been due to androgen exposure, as suggested by the authors of the study, but it is important to note that it could also arise from a social effect of the girls being raised with boys of the same age. Another study found that adolescent girls with conduct disorder and aggressive behavior had free T levels that were about twice as high as girls with conduct disorder who did not have aggressive behavior (Pajer et al. 2006), suggesting that T levels were associated with aggression and not general misconduct. In studies of female prisoners, a positive correlation between T and aggressive dominance has been found, as well as higher levels of T in violent as opposed to non-violent inmates (Dabbs et al. 1988; Dabbs

and Hargrove 1997). Because androgen production varies across the menstrual cycle in women, one study sought to compare aggressive behavior during different phases of the menstrual cycle (Dougherty et al. 1997). The authors found a positive correlation between T and aggression during the mid-follicular phase, but a strong negative trend at ovulation, when T levels were highest. There were no correlations immediately before or during menstruation, demonstrating that the relationship between T and aggression in women varies across the menstrual cycle.

Due to ethical considerations, most human studies of T and aggression are correlational, and many rely on participants' self-report of behavior. Animal studies, however, allow the manipulation of hormone levels, and thus, the evaluation of a potential causal role of androgens in aggression. Additionally, aggressive behavior can be observed and measured much more reliably in animals than in humans. A classic study by Barfield and colleagues (1972) looked at the effects of androgens on aggression in male mice. In one experiment, the animals were evaluated for aggressive behavior after castration, following supplementation with TP to reinstate normal physiological T levels, and then again two weeks after TP treatment was stopped. The rats had low rates of aggression when castrated and following the end of TP treatment, but high rates after two weeks of TP injections. The second experiment in this study measured aggression in males when they were intact, after castration, and again when they had T replaced. Consistent with the first study, these animals were more aggressive when intact and during T replacement, and less aggressive when tested after castration. This series of studies nicely demonstrates a role of T in causing aggression in male rodents.

This link is also evident in female rodents. Animals that were treated with DHT or TP at birth had a shorter latency to fight than did controls when both were exposed to chronic T treatment during adulthood (Schechter et al. 1981). The animals that were exposed to TP at birth also showed more fighting behavior in addition to the decreased latency to fight. Several studies have found that administration of T or DHT to adult ovariectomized female rats for 2-6 weeks induced excessive fighting behavior (Svare et al. 1974; Simon et al. 1985; Albert et al. 1989). Isolation is another factor that has been shown to induce aggression in mice, and T treatment has an additive effect on aggression when examined in combination with isolation (Howard et al. 1981).

While the exact mechanism by which T increases aggression is unknown, it is possible that T causes alterations to the serotonin (5-hydroxytryptamine, 5-HT) system. In a study where male mice were given anabolic androgenic steroids, the mice showed increased aggression as well as decreased 5-HT receptor mRNA in the amygdala and prefrontal cortex (Ambar and Chiavegatto 2009). As with rodents, 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) appear to have some influence on aggression in non-human primates. A study of a subset of male rhesus monkeys from a free-ranging breeding colony measured both T and 5-HIAA in CSF (Higley et al. 1996). Free T levels were positively correlated with overall aggression in these animals. Interestingly, low levels of 5-HIAA were also correlated with increased aggression, and it was found that animals with both low 5-HIAA and high T had the highest rates of aggression, indicating an additive effect of these two substances on promoting aggressive behavior.

Most of the non-human primate studies that have examined the relationship between T and aggression in females have administered T prenatally or when the animals were very young. Goy and Robinson (1982) exposed female rhesus monkey fetuses to levels of androgens typically found in male fetuses, and found that prenatally androgenized females showed more rough-and-tumble play as infants than females that were not exposed to exogenous androgens, although this behavior was still more frequent in males. Rough-and-tumble play was also increased in the first year of life in female marmosets that received T for 50 days after birth (Abbott and Hearn 1978). In another study of rhesus monkeys, females became hyperaggressive after being injected with TP three times per week from the age of 6.5 months to 14.5 months (Joslyn 1973).

Some correlative studies have also been done in non-human primate subjects. Beehner and colleagues (2005) followed a troop of baboons in Ethiopia and found that T levels were higher in females during pregnancy and the wet season, which correlates with the incidence of increased aggression in this population. T levels were also higher in females with a higher dominance rank, and overall aggression correlated with both dominance rank and T level. Correlational studies in both humans and animals have supported a link between higher T and increased aggressive behavior. Most studies that have administered high doses of exogenous androgens to females have found similar results, although the effect of chronic low doses of androgens on female behavior remains to be determined.

Mood (Anxiety and Depression)

The prevalence of mood disorders, particularly anxiety and depression, is high in women with PCOS. Studies have found that women with PCOS are 2-4 times more likely to have depressive symptoms compared with controls (Barnard et al. 2007; Jedel et al. 2010; Dokras et al. 2011), and are at least 4 times as likely to suffer from anxiety (Jedel et al. 2010; Moran et al. 2010; Dokras et al. 2012). The specific causes of these disorders in PCOS are unclear, but they may arise from a combination of obesity, infertility, and the inability to conform to society's ideals of femininity (due to hirsutism, acne, etc), in addition to a direct effect of elevated levels of androgens on mood.

Obesity has been shown to increase depression rates in healthy women (Istvan et al. 1992; Carpenter et al. 2000), but depression and anxiety scores are still higher in women with PCOS when compared with BMI-matched controls (Weiner et al. 2004; Ozenli et al. 2008). Similarly, when compared with a group of women who experienced non-PCOS related subfertility, women with PCOS had higher anxiety, depression, and anger suppression scores (Barry et al. 2011). Although T values did not directly correlate with any measures of mood in this study, T may interact with other factors to affect anxiety and depression in PCOS. In a study of 450 German women with PCOS, anxiety was especially increased in patients with acne, a clinical sign of HA (Benson et al. 2009).

T levels that were elevated 2-3 times above control levels have been associated with increased rates of depression in studies of otherwise healthy, pre-menopausal women (Vogel 1978; Baischer et al. 1995). However, low T has also been associated with depression in women, and treatment with low dose T (to more elevated levels that

were still within the normal range) relieved depressive symptoms in a majority of women with treatment-resistant depression (Miller et al. 2009). In addition, in a study of women with anorexia nervosa, there was a strong inverse relationship between T levels and severity of both anxious and depressive symptoms (Miller et al. 2007). Together, these studies may indicate that women with T levels at either the high or low extremes are at the highest risk of developing mood disorders. Although some studies have found relationships between T and anxiety in women, others have not. In a study of female college students, Maner and colleagues (2008) found no correlation between social anxiety and T levels either at baseline or after winning or losing a computerized competition. Further studies are needed to clearly understand the effects of varying levels of androgens on mood disorders in women.

Animal studies have been performed in order to better elucidate the effects of androgens on anxious and depressive symptoms in females. However, it must be kept in mind that contrary to the situation in humans, mood in animals must be inferred through behavior. This can make the interpretation of test results less straightforward. Given this, it is not surprising that the nature of the interaction between circulating androgen levels and mood remains unclear. In some studies, androgen exposure has led to increased anxiety-like behavior. For example, adolescent female mice that were treated with anabolic androgenic steroids (AAS) 5 days/week for 4 weeks showed a greater startle amplitude during the acoustic startle test than control mice (Costine et al. 2010), indicative of increased anxiety. The mice treated with AAS also had increased corticotropin-releasing factor (CRF) mRNA in the central amygdala, supporting the hypothesis that androgens might change anxious behavior by altering the function of the

hypothalamic-pituitary-adrenal (HPA) axis. Another study treated female rats with 83 μg/day of DHT either by continuous release implant for 90 days starting pubertally (21 days of age) or by daily injection for 1 week during adulthood (Feng et al. 2011). Animals were tested for 10 min in an elevated plus maze that was 73 cm above the floor. The maze has two closed arms with high walls (safe areas) and two open arms with only a short ledge to prevent falls (risky areas). The rats that were treated with DHT for 90 days through adolescence and early adulthood had increased anxiety-related behaviors as adults, defined as less time spent in the open arms of the elevated plus maze and decreased locomotor activity. The rats treated with DHT for only 1 week in adulthood also showed decreased locomotor activity, but not to the extent of the 90-day treatment group. This could indicate that either age and/or duration of exposure to androgens may play an important role in modulating anxious behavior.

Similar to the situation in people, some studies in animals have found that acute androgen exposure actually decreases anxious and depressive behavior in females. Rats that were treated with a single dose of T, DHT, or 5α-androstane-3α,17β-diol (3α-diol) had androgen levels that were increased 2-9 times above rats that were given vehicle (Frye and Lacey 2001), and all of the androgen-treated animals had decreased anxiety as they spent more time in the open arms of a plus maze 1 h after hormone administration. Anxiety was still decreased 24 h after the injection, when androgen levels were no longer elevated, as the animals spent more time with a novel as opposed to a familiar object when tested at this time point. In another study by the same group, aged male and female mice were treated with 1 mg/kg A4 or DHT 1 hour before undergoing a forced swim test, in which animals were placed into a glass cylinder filled with water and observed for

immobility, swimming, and struggling behavior. The androgen-treated animals spent less time immobile, indicating that androgen treatment may have anti-depressive effects in aged mice (Frye and Walf 2009). Of note however, all of these studies have looked at the relatively acute effects of androgens on behavior, which may well be different than more chronic effects of androgens.

The organizational effects of androgens on neural circuits that govern anxious behavior have also been studied. A study by Goel and Bale (2008) found varying effects of TP when mice were exposed at different ages and tested on different behavioral assays. To investigate the organizational effect of androgens, some mice were given a 100 µg TP injection on postnatal day (PD) 1. Other mice were given a TP implant after ovariectomy on PD 21 to assess the activational effects of T, and some animals were given a combination of both T treatments. The TP implants resulted in elevation of T values to the range of an intact male mouse. Behavioral tests, including the light-dark box, marble-burying, and tail suspension tests were performed between PD 67 and 78. Used to measure stress-provoked anxiety-like responses, the marble-burying test consisted of individual mice being placed in a cage with a layer of bedding and 12 marbles distributed across the surface of the bedding. The number of marbles buried in 30 min was considered a measure of anxiety. In this study, males and all TP-treated females buried more marbles than control females, indicating that T was associated with increased anxiety-like behavior. Interestingly, this was true even in the animals that only received TP at PD1 and did not have sustained levels of circulating androgens. Another measure of anxiety in rodents, the light-dark box, was also used in this study. Mice were placed into the dark side of a box that was divided into dark and light compartments, with

the ability to move between the compartments. Increased latency to move to the light compartment and decreased time spent in the light compartment are indicative of increased anxious behavior in rodents. Contrary to the results from the marble-burying test, there were no group differences when the animals were tested in the light-dark box. Depressive behaviors were examined using a tail suspension test (TST) in these mice, as well. For this test, animals were suspended by their tails 40 cm above the ground for 6 min. Mice that were given activational TP starting on PD21 (but that did not receive organizational TP) spent less time immobile during a tail suspension test, which is a sign of decreased depressive behavior in these animals. In addition, females given activational TP (regardless of whether they also received organizational TP) had corticosterone levels that were decreased below controls, to levels seen in males, again showing that TP may mediate behavior through its effects on the HPA axis. Differences in dose, timing of exposure, and duration of exposure to androgens in these studies are likely leading to the lack of consensus regarding androgens' effects on anxiety and depression, and more studies are needed before a complete understanding of this relationship will occur.

Impulsivity

Another behavior that may be affected by androgen levels is impulsivity, a general term describing decreased inhibitory control and action without foresight (reviewed in Evenden 1999; Arce and Santisteban 2006). Impulsivity in some instances can be viewed as being on the opposite end of the spectrum from anxiety, such that individuals displaying high levels of impulsivity have inappropriately low levels of

anxiety. One example of this is that the tendency to approach and explore a novel object has been described as an indicator of both decreased anxiety (Heisler et al. 1998), as well as increased impulsivity (Stansfield and Kirstein 2006).

Research regarding the relationship between T and impulsivity in humans is limited. One task commonly used to assess impulsivity in humans is the Iowa Gambling Task (IGT) (Bechara et al. 1994), where people must draw 100 cards from any of four different decks. Unbeknownst to the participant, two of the decks are considered disadvantageous, as they have high payouts, but even higher losses. The other two decks are advantageous and have low rewards but even lower losses. Consistently drawing from the advantageous decks will lead to overall gain of money, and normal controls eventually figure out and follow this strategy (Bechara et al. 1997). In a study that examined gender differences in performance on the IGT, men identified the advantageous decks faster and thus made more advantageous choices compared with women (Reavis and Overman 2001). This could lead to speculation that sex hormones play a role in decision-making and risk-taking behavior. Other studies have found that the gender difference is reversed, with men making more impulsive choices. For example, men have been found to discount money at higher rates than women (i.e. men preferred smaller immediate rewards to larger delayed rewards) during a delay discounting task (Kirby 1996), although not all studies have found this gender difference (Funder and Block 1989). These data together demonstrate that there may not be a linear relationship between T and impulsivity, and this relationship may differ based on how impulsivity is assessed.

Studies examining the effect of T on impulsivity in men have provided inconsistent results. In college-aged men, more advantageous choices in the IGT have been found to be negatively correlated with T levels, indicating that the men with higher T made more impulsive choices (Reavis and Overman 2001). Another study found a positive correlation between serum T levels and self-reported impulsivity in male prison inmates (Aluja and Garcia 2005). Other studies, however, have failed to find any relationship between T and impulsivity in men. Blanco and colleagues (2001) found that pathological gamblers had higher impulsivity scores as assessed by self-report, but not higher T levels, than control subjects. Furthermore, they found no correlation between T and impulsivity ratings in either the gamblers or the healthy controls.

Studies that have examined exclusively women are similarly inconclusive. One study showed that women made more impulsive choices on the IGT (i.e. drew more cards from the disadvantageous piles) when given a single dose of T that elevated their levels by 10-fold as opposed to when they were given placebo (van Honk et al. 2004). Another study found that T levels were positively correlated with commission errors (failure to withhold a response) during a delayed memory task, but not during an immediate memory task (Bjork et al. 2001). However, adolescent girls had no association between T levels and impulsivity as measured by a self-report questionnaire (Vermeersch et al. 2008). Additionally, post-menopausal women who were given T supplementation to elevate their T levels by 5-fold for 4 weeks did not differ from controls in impulsivity during a gambling task (Zethraeus et al. 2009). The small numbers of studies that have examined the effects of T on impulsivity in women, coupled with the conflicting results, solidify the need for further studies in this area.

Animal studies examining the effects of androgens on impulsivity are also scarce, with the majority focusing only on males. As with the research in humans, results from animal studies are inconsistent regarding the potential relationship between androgens and impulsivity. In one study, adult male rats were given subcutaneous T implants for 8 weeks or were given a single injection of T 24 hours before undergoing a Vogel conflict test (Bing et al. 1998). In this test, water-deprived rats were given an opportunity to drink from a bottle, but they experienced an electric shock upon every attempt to drink. Rats from both the acute and chronic T-treated groups accepted more shocks than controls during a 10-minute period, which the authors interpreted as a display of increased impulsivity (i.e. disregard for the consequences of actions) in these animals. In another study, male rats were gonadectomized as adults and given TP or E replacement in the form of implants for 90 days (Kritzer et al. 2007). These rats were trained on a differential reinforcement of low rates of responding paradigm during hormone exposure. In this experiment, rats were rewarded for withholding lever presses during a 12-sec waiting period, and lever presses made during this time were counted as errors and reset the waiting period. The gonadectomized group that did not receive any hormone replacement made more errors during the first 2 days of testing, although no group differences were found during the remaining 8 days of testing. A higher rate of errors is indicative of more impulsive behavior (inability to withhold responding), so the results from this study show that rats with higher T levels were actually less impulsive than the rats with lower T levels, although this relationship was only evident during the beginning of the study. In a study of the behavioral effects of androgens on heifers, 2-year-old cows were given TP injections for 100 days before testing (Boissy and Bouissou 1994). The T-

treated animals had shorter latencies than controls to enter a novel room, approach a novel apparatus, and eat in a novel environment, behaviors that are consistent with increased impulsivity. Interestingly, the T-treated heifers also had a lower cortisol response to exogenous ACTH administration than controls, so T may affect impulsive behavior through alterations to HPA axis function. The majority of non-human primate studies that have examined impulsivity have not assessed androgens, but in those that have, measures of impulsivity have not correlated with plasma T levels (Higley et al. 1996). Overall, studies examining the effects of androgens on impulsivity have had mixed results, but there seems to be a trend toward higher levels of androgens being associated with increased levels of impulsivity.

To better understand this relationship between androgens and behavior, studies performed in this dissertation examined the effects of chronic, slightly elevated T levels on behavior in young adult female rhesus monkeys (see Fig. 1.2). In Chapter 4, when the monkeys were 6.5 years of age, I examined how T affected aggression and behavioral inhibition. The Human Intruder Test was used to measure the response to a social stimulus, while the Novel Objects test was used to assess the response to novelty (Kalin and Shelton 1989; Mason et al. 2006; Sullivan et al. 2010).

Chapter 2

ELEVATED ANDROGENS DURING PUBERTY IN FEMALE RHESUS MONKEYS LEAD TO INCREASED NEURONAL DRIVE TO THE REPRODUCTIVE AXIS

2.1 INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common reproductive disorder affecting 4-8% of reproductive-aged women worldwide (Knochenhauer et al. 1998; Asuncion et al. 2000; Azziz et al. 2004b). Symptoms include evidence of hyperandrogenemia (HA), polycystic ovaries, and oligo- or amenorrhea (Knochenhauer et al. 1998; McCartney et al. 2006; Blank et al. 2008). Typically, pelvic ultrasonography reveals that women with PCOS have increased ovarian size, as well as greater numbers of antral follicles compared with healthy women (Chen et al. 2008; Shah et al. 2010). There is also an increased incidence of obesity and insulin insensitivity in women with PCOS (Legro et al. 1999; 2001; Ovalle and Azziz 2002), and obesity reportedly aggravates PCOS symptomology (Dunaif et al. 1989; Legro 2000). Characteristic neuroendocrine changes often seen in patients with PCOS include increased frequency of pulsatile LH secretion by the pituitary, increased pituitary responsiveness to GnRH, and decreased sensitivity of the hypothalamus to progesterone (P) negative feedback (Rebar et al. 1976; Pastor et al. 1998; Marshall and Eagleson 1999).

Interestingly, obese girls are often hyperandrogenemic even in early puberty, and this HA is associated with a rapid progression from pubertal to adult LH secretory patterns (Apter et al. 1994; McCartney et al. 2009). In addition, in girls with HA from

other causes, such as congenital adrenal hyperplasia (CAH), there is an increased incidence of elevated LH secretion and irregular menstrual cycles (Levin et al. 1991; Holmes-Walker et al. 1995). In animal studies, early exposure to elevated androgens in the prenatal period has been associated with increased neural drive to the reproductive axis. In both rats and sheep, HA in the prenatal period leads to an increased frequency of LH pulses and higher levels of circulating LH (Sharma et al. 2002; Foecking et al. 2005; Savabieasfahani et al. 2005). Similarly, previous studies in female monkeys have shown that excess androgen exposure during fetal development can lead to later HA, irregular or absent menstrual cycles, elevated LH levels, and polycystic ovaries as adults (Abbott et al. 1998; 2008; 2009). However, whether elevation of androgen levels in childhood through adolescence (in the absence of prenatal exposure to T) could lead to similar abnormalities in the neuroendocrine drive to the reproductive axis has not been examined.

In this study, I tested if an elevation in androgen levels in the peripubertal period (to levels similar to those seen in obese girls; McCartney et al. 2006) would result in alterations in pubertal LH secretory patterns that resemble those in hyperandrogenemic girls and women with PCOS. Female rhesus monkeys were exposed to low doses of testosterone (T) beginning prepubertally (1 year of age) and continuing into early adulthood (5 years of age). I hypothesized that if a slight elevation in peripubertal T leads to an increased central drive to the reproductive axis, then the T-treated animals would develop at least some characteristics seen in PCOS, including a faster LH pulse frequency, higher LH responsiveness to exogenous GnRH, and decreased sensitivity to P negative feedback. I further hypothesized that increased numbers of small antral follicles,

and decreased insulin sensitivity would occur along with neuroendocrine changes in T-treated animals compared with control (cholesterol-treated) animals.

2.2 MATERIALS AND METHODS

Animals

Given that this project began as a pilot study with limited funds, only 12 animals were studied in the following experiments. One-year-old female rhesus macaques (*Macaca mulatta*), weighing 1.7-2.4 kg, were obtained from the breeding corrals of the Oregon National Primate Research Center (ONPRC). They were housed in pairs in stainless steel cages (81 x 122 x 69 cm) in a temperature-controlled room (24±2°C), with lights on for 12 hr/day (0700h-1900h) during the first 2.5 years of the experiment. When the animals were 3.5 years of age, chronic indwelling venous catheters were implanted and the animals were then housed individually in single cages (81 x 61 x 69 cm). Monkeys were fed two meals of Purina LabDiet fiber-balanced monkey chow each day (no. 5000; Purina Mills, St. Louis, MO), supplemented with fresh fruits and vegetables. Monkeys were trained to approach the front of their cage so menses could be detected daily by swabbing the vaginal area with a cotton-tipped swab. The first day of menses was designated Day 1 of a menstrual cycle. All procedures in this study were reviewed and approved by the ONPRC Institutional Animal Care and Use Committee.

Testosterone Implants

Normal T levels in prepubertal female rhesus macaques were determined by assaying serum from four 12-month-old female monkeys in the ONPRC colony. The

average T value (0.4 ng/mL) was multiplied by three to achieve the lower limit (1.2 ng/mL), and by four to achieve the upper limit (1.6 ng/mL) of target values in the T-treated animals. This was based on the clinical evidence that obese girls with HA and women with PCOS have T levels about 3-4 times higher than controls (Eagleson et al. 2003; Silfen et al. 2003; McCartney et al. 2006). To determine the size of T implant needed, monkeys from the ONPRC colony, which were not used for this study, had implants of various lengths and T:cholesterol ratios placed subcutaneously under ketamine hydrochloride (Ketaset, 10 mg/kg i.m., Wyeth, Madison, NJ) sedation. Blood samples were taken daily to determine which implants resulted in a sustained 3-4-fold increase in serum T levels. Once an appropriate implant size was determined, all animals used in this study received either a T- or cholesterol-containing (n=6/group) implant at one year of age. Implants were made of Silastic tubing (Dow Corning, Midland, MI) and were initially 5 mm in length with an inner and outer diameter of 0.335 cm and 0.465 cm, respectively. Implants were filled with cholesterol (control animals), or a T/cholesterol mixture (T-treated animals), with a T:cholesterol ratio of 1:15 or 1:12 at the beginning of the experiment. As the animals grew, the length of the implant increased to 2 cm and the T:cholesterol ratio was increased gradually to 1:4 to maintain the desired serum T levels. Both cholesterol and T were purchased from Sigma Aldrich (St. Louis, MO).

Blood Collection and Steroid Hormone Assays

To collect blood samples for tracking serum T concentrations, animals were trained to jump from their cage into a portable transport box and were carried into a nearby room. The transport box door was opened and they were transferred to a specially

designed cage and trained to present their leg for blood collection from the femoral vein (Hunnell et al. 2007). Weekly blood samples (2 mL each) were collected from each animal, allowed to clot at room temperature for >1 h and refrigerated overnight. Samples were then centrifuged at 3000 rpm for 15 min at 4°C and serum was removed and stored at -20°C until assays were performed. Each week's samples were assayed for T and when an individual animal's serum T concentration fell below the threshold of 1.2 ng/mL, the implant was changed. Cholesterol implants were also changed regularly so that cholesterol-treated (i.e., control) animals received the same average number of implant surgeries as the T-treated animals. T was measured using a radioimmunoassay (RIA) kit (DSL-4100, Diagnostic Systems Laboratories, Inc, Webster, TX) by the Endocrine Services Core Laboratory at the Oregon National Primate Research Center. The sensitivity of the T assay was 0.05 ng/mL and the intra- and inter-assay coefficients of variation for the assays were 2.23% and 4.00%, respectively. Blood samples were drawn at times throughout the study to quantify serum estradiol (E) and progesterone (P) concentrations. Both E and P were assayed by the Endocrine Services Core using the Immulite 2000 platform. As with many validated clinical platforms, the Immulite 2000 runs three quality control (QC) serum pools daily and as such, no specific intra-assay QC data is available. The inter-assay coefficient of variation, reflecting variability in daily QC results over the period in which these assays were performed was 8.5% for E and 9.4% for P.

Nighttime LH Concentrations and LH Assay

In order to measure the sleep-associated rise in LH that would be indicative of puberty (Terasawa et al. 1984; Apter et al. 1989), nocturnal blood samples were collected from each animal once a month at approximately 2200h. These samples were collected as described for the weekly daytime samples, processed in the same manner, and then assayed for LH. LH was measured by RIA at the University of Pittsburgh assay core using recombinant cynomolgus monkey LH (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) as standards (Williams et al. 2001). The sensitivity of the LH assays was 0.1 ng/mL and the intra- and inter-assay coefficients of variation for the assays used in this study were 6.6% and 12.2%, respectively.

Catheterization

At 3.5 years of age, after all monkeys had experienced menarche, a chronic indwelling venous catheter was implanted by the ONPRC surgical staff using isoflurane anesthesia (Hospira, Lake Forest, IL) as described previously (Cameron and Nosbisch 1991). Briefly, the catheter was inserted into the subclavian or femoral vein and routed subcutaneously to exit in the mid-scapular region of the back. It was protected by a fitted nylon jacket worn by the monkey. The jacket was connected to a flexible metal tether and swivel which allowed the monkey to have full range of motion within its cage. Silastic tubing was routed through the wall into an adjacent room where blood samples were collected and drugs were infused without disturbing the monkeys or disrupting their normal activities. Previous studies established that female monkeys with chronic indwelling catheters display regular menstrual cycles (Herod et al. 2011). Catheters were

kept patent with a constant infusion of physiological saline (Baxter Healthcare, Deerfield, IL) containing heparin sodium (4 IU/mL) at a rate of approximately 100 mL/day. Animals were sedated weekly with ketamine in order to inspect the catheter system and replace a sterile dressing covering the exit site. No ketamine was administered in the 24 hours preceding any experiment. Following catheterization surgery, animals were allowed a minimum of 3 weeks to recover before any experiments were performed.

Experimental Protocols

PCOS-like symptoms were measured in seven experiments that took place over two breeding seasons (Oct-May). Neuroendocrine function was measured in four experiments. Pulsatile LH secretion was determined during the midfollicular phase of a menstrual cycle when the animals were both 4 and 5 years old. Based on clinical evidence that differences in LH secretion are larger during the early follicular phase when comparing PCOS patients with healthy women (McCartney et al. 2002a), pulsatile LH secretion was measured again during the early follicular phase when the animals were 5 years old. This was followed by the determination of the effect of P negative feedback on LH release. The LH response to exogenous GnRH was also measured when the animals were both 4 and 5 years of age. To examine the metabolic correlates of HA, glucose tolerance testing occurred when the animals were 4 years of age and basal metabolic rate was measured in a closed chamber when the monkeys were 4.5 years of age. Ultrasounds were performed when the animals were 5 years old to assess ovarian morphology. Figure 2.1 is a timeline of the experiments that were performed for this study.

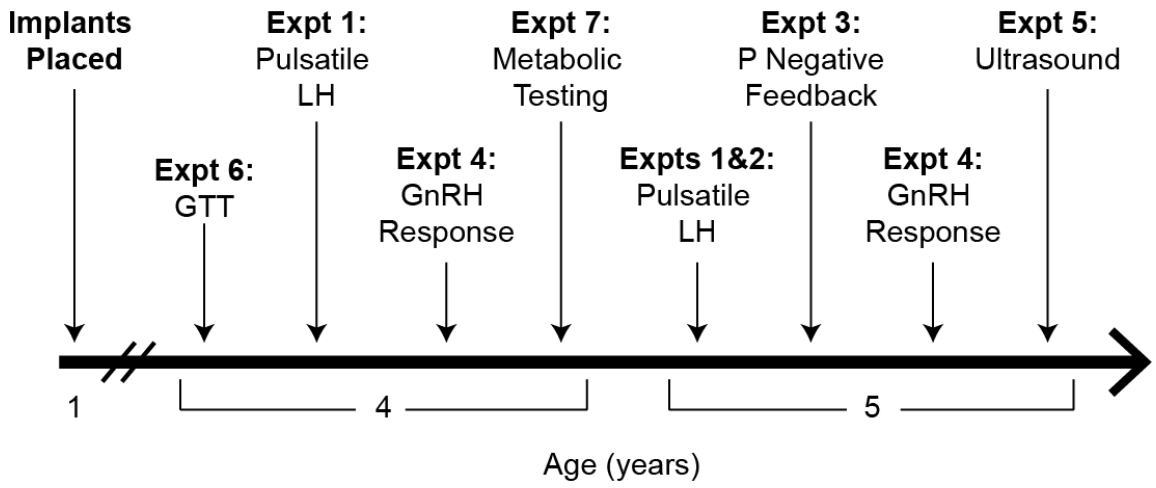

Figure 2.1 Schematic diagram of the experimental timeline for Chapter 2, indicating ages at which experiments were performed.

Experiment 1. Pulsatile LH Secretion during the Midfollicular Phase

Pulsatile LH secretion was measured during the midfollicular phase (D7-9) of the menstrual cycle when the monkeys were 4 and 5 years of age. These studies were done during the midfollicular phase so the data could be compared to pulsatile LH secretion measured in response to 7 days of E and P treatment (see Experiment 3). Blood samples (0.4 mL each) were collected into sterile heparinized syringes through the remote sampling system, every 10 minutes from 1300 h to 0100 h. This interval provided samples for 6 hours during the light phase and 6 hours during the dark phase of the day-night cycle in order to detect differences between daytime and nighttime LH secretion. Immediately after collection, the samples were placed into sterile plastic tubes and centrifuged at 3000 rpm for 15 min at 4°C. Plasma was removed and placed into plastic O-ring vials (containing 20 µL of a solution composed of equal volumes of 38% sodium citrate and 1,000 IU/mL sodium heparin to prevent clotting of plasma proteins) and stored at -20°C until assays were performed. Red blood cells were re-suspended in sterile saline and reinfused through the catheter system to the animal. Hematocrit was recorded at the beginning, middle, and end of the experiment to ensure that it remained in the normal physiological range.

Experiment 2. Pulsatile LH Secretion During the Early Follicular Phase

Pulsatile LH secretion was measured during the early follicular phase (D1-3) of the menstrual cycle in the animals when they were 5 years of age. The methods of this experiment were identical to Experiment 1, with the exception of the difference in menstrual cycle day. The early follicular phase was chosen because this is the time period

when differences in pulsatile LH secretion are most apparent between women with PCOS and healthy women (McCartney et al. 2002a).

Experiment 3. Effect of Progesterone Negative Feedback on LH Secretion

On the day following the D1-3 pulse bleed, animals had an E implant and a P implant (3 cm each) placed subcutaneously. Pilot studies were performed to determine the size of implant necessary to create levels of P that are similar to those seen during the mid-luteal phase of a normally cycling animal (pilot studies produced the following average levels: E=334 pg/mL; P=5.04 ng/mL). E was used to induce the upregulation of P receptors (Xiao and Goff 1999). After 7 days of hormone treatment, another LH pulse bleed was performed as described in Experiment 1. The protocol to measure P negative feedback was based on the clinical protocol developed by John Marshall and colleagues, which has been used in women with PCOS (Pastor et al. 1998; Blank et al. 2009).

Experiment 4. GnRH Stimulation

LH responsiveness to GnRH was measured between 0900 and 1000h on D8-10 of a menstrual cycle when the monkeys were 4 and 5 years of age. GnRH was obtained from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA), dissolved in 0.9% saline at 1µg/100µL, and stored in 200 µL aliquots at -20°C until use. Immediately before use, saline was added to individual aliquots to bring the concentration to 1µg/mL. GnRH (250 ng/kg, i.v.) was infused at time 0 and blood samples (0.4 mL) were collected at -15, -1, 15, 30, 60, and 90 minutes, as described previously (Cameron and Nosbisch 1991). This dose was chosen so that monkeys would

receive a physiological dose that caused a response, but a response that was sub-maximal to allow detection of individual differences in LH responsiveness. Samples were collected, centrifuged, and stored, and then red blood cells were reinfused as described for the pulsatile LH experiments.

Experiment 5. Ovarian Ultrasound

Ovarian ultrasounds were performed by Dr. Cecily Bishop on D1-3 of a menstrual cycle when the animals were 5 years of age, and blood samples were collected daily through the animals' catheter systems and assayed for E and P as described previously. Ultrasound was performed using a GE Medical Systems Voluson® 730 Expert Doppler ultrasound instrument (GE Healthcare, Waukesha, WI) with both 2D (4.5–16.5 MHz) and 4D (3.3–9.1 MHz) transabdominal probes. Methods were similar to previous studies in adult female macaques (Bishop et al. 2009). Animals were assigned random identifiers and Dr. Bishop was blinded to animal treatment as she assessed the follicle cohort present in each ovary and ovarian size. The 2D probe was used to orient image field to the uterus and identify the ovaries. The 4D probe was then used to generate a data file of each individual ovary which included a series of images collected in one scan through the entire ovary. Archived scans from each animal were analyzed at one time by Dr. Bishop, who remained blinded to treatment group. Ovaries were analyzed for ovarian area, circumference, and diameter, number of visible antral follicles on each ovary, the mean, maximum and minimum size of the antral follicles on each ovary, and the total number of antral follicles per female. Follicle counts and size of follicles were measured using

previously defined methods in adult female rhesus monkeys (Bishop et al. 2009). All parameters were then decoded for comparisons between treatment groups.

Experiment 6. Glucose Tolerance Testing

When the animals were 3.5 years, glucose tolerance testing (GTT) was performed during the early follicular phase of a menstrual cycle. For monkeys not showing regular menstrual cycles, the GTT was performed when a blood sample showed that E and P levels were low, indicating the absence of a dominant follicle or corpus luteum in the ovaries. Each animal was sedated initially with telazol (tiletamine hydrochloride and zolazepam hydrochloride, Fort Dodge Animal Health, Fort Dodge IA, USA) and subsequently with ketamine to maintain sedation, and the protocol was based on that designed by Richard Bergman (1979). Dextrose (300 mg/kg) was infused i.v. through the catheter system and blood samples were taken from 15 minutes before to 3 hours after the glucose infusion. Tolbutamide (5 mg/kg) was infused i.v. 20 minutes after the dextrose in order to stimulate the pancreas to secrete more insulin. All samples were immediately assayed for glucose using the YSI 2300 Stat Plus (YSI Inc, Yellow Springs, OH), and subsequently for insulin by RIA (Linco Human Insulin RIA, Millipore Corporation, Billerica, MA, USA). The sensitivity of the insulin assay was 1 µIU/ml and the intra-assay coefficient of variation was 2.7%.

Experiment 7. Metabolic Testing

Metabolic rate was measured over a 24-hour period when the animals were 4.5 years of age. The animals were transported into a sealed Lexan® and stainless steel

metabolic chamber (Columbus Instruments, Columbus, OH) at approximately 1000 h. Fresh air was pumped in and circulated with a 4-in fan. The amounts of oxygen consumed and carbon dioxide produced were measured using a computer-controlled open-circuit calorimeter, and total energy expenditure (kcal) was calculated using the Oxymax system (Columbus Instruments). The animals did not receive their normal meals during this time but were fed a 110-g banana at 1500 h.

Statistical Analyses

LH pulses were identified by Dr. Cliff Pohl using the Pulsar algorithm that was developed by Merriam and Wachter (1982), and used previously to detect LH pulses in monkeys (Cameron and Nosbisch 1991; Ramaswamy et al. 2007; Herod et al. 2011). Pulse frequency was defined as the number of Pulsar-detected pulses in 12 h and the following G-values were used: G(1): 50.00, G(2): 1.0, G(3): 0.40, G(4): 0.40, and G(5): 0.40. Pulse amplitude was calculated by subtracting the baseline LH level from the peak LH level of each pulse. For all analyses, LH values below the level of detectability for the assay were assigned the minimum detectable concentration of the assay (0.1 ng/mL). A Fisher's exact test was used to examine group differences in presence vs. absence of pulses when the monkeys were 4 years of age. An independent Student's *t*-test was used to determine group differences in number of pulses, proportion of daytime (vs. nighttime) pulses, and pulse amplitude when the animals were 5 years old. For the GnRH stimulation, LH area under the curve (AUC) was calculated, correcting for baseline LH levels. Due to abnormally distributed data, the non-parametric Mann-Whitney U test was used to assess group differences in LH response to GnRH.

The MINMOD Millenium computer program was used to determine glucose effectiveness, insulin sensitivity, acute insulin response, and disposition index values (Boston et al. 2003). This program was designed to calculate these values based on the glucose tolerance test protocol that was described by Bergman and colleagues (1979) and that was used in this study. Independent sample *t*-tests were used to determine group differences in GTT measures, and were corrected for multiple comparisons where necessary.

A Fisher's exact test was used when analyzing the presence or absence of follicles over 2.0 mm in diameter, and an independent *t*-test was used to analyze total follicle number, ovarian size, age at first nighttime rise in LH, and age at menarche.

Basal metabolic rate was calculated as the average number of kcal expended per kg per hour from 2300 h to 0300 h. This time period was chosen as it is when monkeys are typically asleep and their heart rate is slowest (Judy Cameron, personal correspondence). An independent *t*-test was used to assess group differences in basal metabolic rate. A mixed measures ANOVA was used to determine differences in weight across time. Statistical analyses were performed using IBM SPSS Statistics 20 (SPSS Inc, Chicago, IL). Values are presented as means ± SEM. Significance was set at $p<0.05$.

2.3 RESULTS

Plasma T concentrations in the T-treated animals were maintained in a narrow range at 3.7±0.2-fold higher than in the control animals from the time of first implant at 1 yr of age through 5.5 yrs of age (T-treated: 1.73±0.02 ng/mL; control: 0.50±0.05 ng/mL, $p=0.001$). Implants were replaced on a regular schedule, every 8.0±0.4 weeks throughout

the study. There were no differences in age at first nighttime rise in LH (T-treated: 33.0±2.8 months; control: 33.3±3.6 months) or age at menarche (T-treated: 32.1±1.4 months; control: 32.4±2.5 months). The groups also did not differ in the number of menstrual cycles nor in the number nor percentage of ovulatory menstrual cycles that occurred when they were three, four or five years of age (Table 2.1).

A mixed measures ANOVA (within-subjects factor: time; between-subjects factor: treatment) showed that all animals gained weight over the course of the study, which was expected as the animals matured. There was no effect of treatment and no treatment x time interaction when all weights were analyzed from ages 1-5.5 years ($p>0.1$). However, when only post-pubertal weights were assessed (3.5-5.5 yrs), there was a trend toward T-treated animals being heavier [$F(1,10)=4.48$, $p=0.06$; Fig. 2.2]. Food intake over the course of the study was the same in all monkeys.

Experiment 1. Pulsatile LH Secretion during the Mid-follicular Phase

In samples that were collected for 12 h on D7-9 of the menstrual cycle in 4-year-old monkeys, only 3/6 control animals showed detectable LH pulses, while 6/6 T-treated animals had detectable pulses. For this reason, presence vs. absence of pulses was analyzed and there was a trend toward a group difference ($p=0.09$), with T-treated animals being more likely to have LH pulses than controls. When the animals were five years old, 5/6 control animals and all T-treated animals had LH pulses on D7-9 of the menstrual cycle. There were no differences between the groups in number of LH pulses, proportion of pulses occurring during the daytime, or in average pulse amplitude ($p>0.1$).

Table 2.1 Numbers of menstrual cycles per year in control and T-treated monkeys from 3-5 years of age

		3rd Year		4th Year		5th Year	
	Animal	Number Menstrual Periods	Number Ovulatory Cycles	Number Menstrual Periods	Number Ovulatory Cycles	Number Menstrual Periods	Number Ovulatory Cycles
Control	A	2	0	3	0	4	0
	B	6	0	7	5	9	4
	C	0	0	8	0	6	4
	D	2	0	8	1	7	3
	E	4	0	4	0	7	3
	F	6	1	5	0	9	7
	mean	3.3±1.0	0.2±0.2	5.8±0.9	1±0.8	7.0±0.8	3.5±0.9
T-treated	G	7	0	8	1	9	3
	H	6	0	7	3	6	4
	I	5	0	5	0	4	0
	J	4	0	7	1	9	7
	K	3	0	7	3	10	5
	L	3	0	6	0	8	2
	mean	4.7±0.7	0	6.7±0.4	1.3±0.6	7.7±0.9	3.5±1.0

Menstrual periods are presented as recorded from September-May, as rhesus monkeys may be acyclic or anovulatory in the summer months. Therefore, number of menstrual periods ≥9 is considered normal for adults. Data are presented as mean ± SEM. There were no significant differences between groups for any measure.

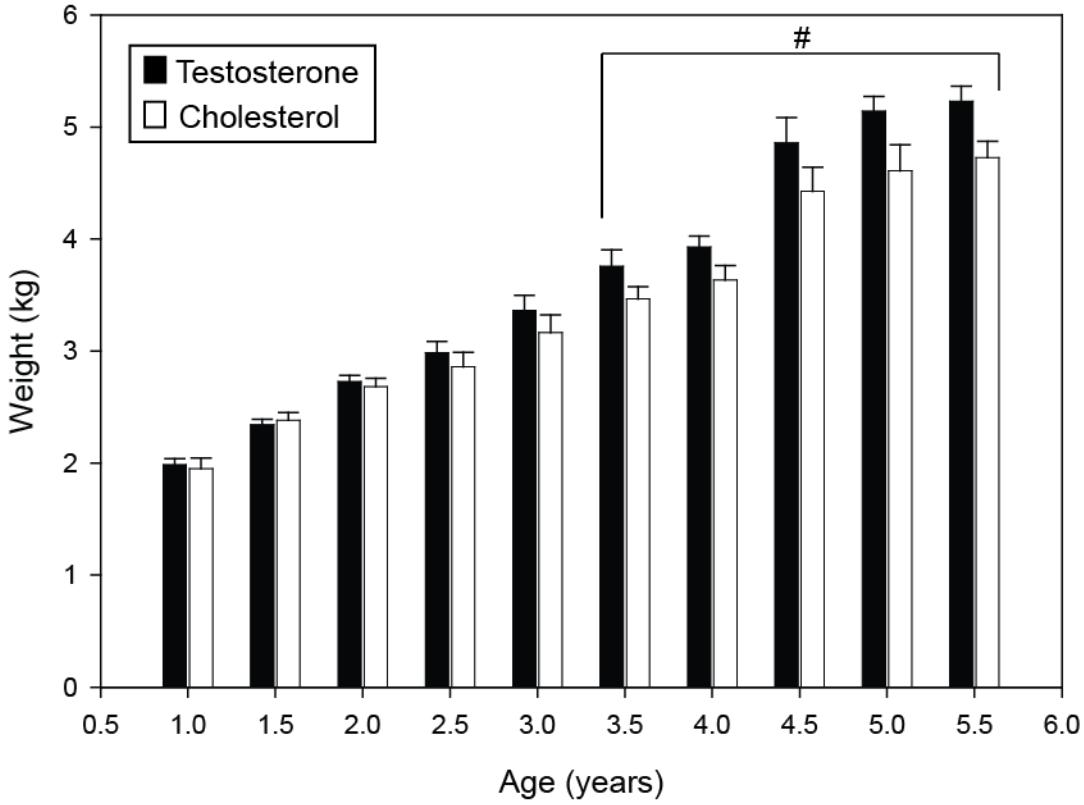

Figure 2.2 Body weight from 1-5.5 years of age. Values are mean ± SEM. # indicates a trend toward a group difference when post-pubertal weight gain was assessed (p=0.06).

Experiment 2. Pulsatile LH Secretion during the Early Follicular Phase

At 5 years of age, T-treated animals had a significantly greater number of LH pulses than control animals on D1-3 of the menstrual cycle [T-treated: 9.7±1.8 pulses; control: 3.7±1.8 pulses; $t(10)= -2.4$, $p=0.039$; Figs. 2.3, 2.4], though there was considerable individual variation in pulse frequency within each group. There was no correlation between LH pulse frequency and whether the monkey had ovulated in the prior menstrual cycle, and there were no group differences in pulse amplitude, proportion of daytime pulses, or E levels when pulsatile LH secretion was assessed (T-treated: 92±15 pg/mL; control: 81±14 pg/mL; Figs. 2.3 and 2.4).

Experiment 3. Effect of Progesterone Negative Feedback on LH Secretion

After one week of steroid hormone treatment, average plasma E levels were 216±37 pg/mL and average P levels were 4.08±1.05 ng/mL. These levels did not differ between the groups. When LH secretion was measured, none of the cholesterol-treated animals had any detectable LH pulses, and only 2/6 T-treated animals had LH pulses. There were no differences between the groups in presence vs. absence of pulses, as most animals from both groups completely suppressed LH secretion in response to the E and P treatment.

Experiment 4. GnRH Stimulation

One control animal was deemed an outlier and removed from this analysis because the LH AUC value was 2.5 standard deviations above the mean. T-treated animals had a significantly greater LH response to GnRH compared with control

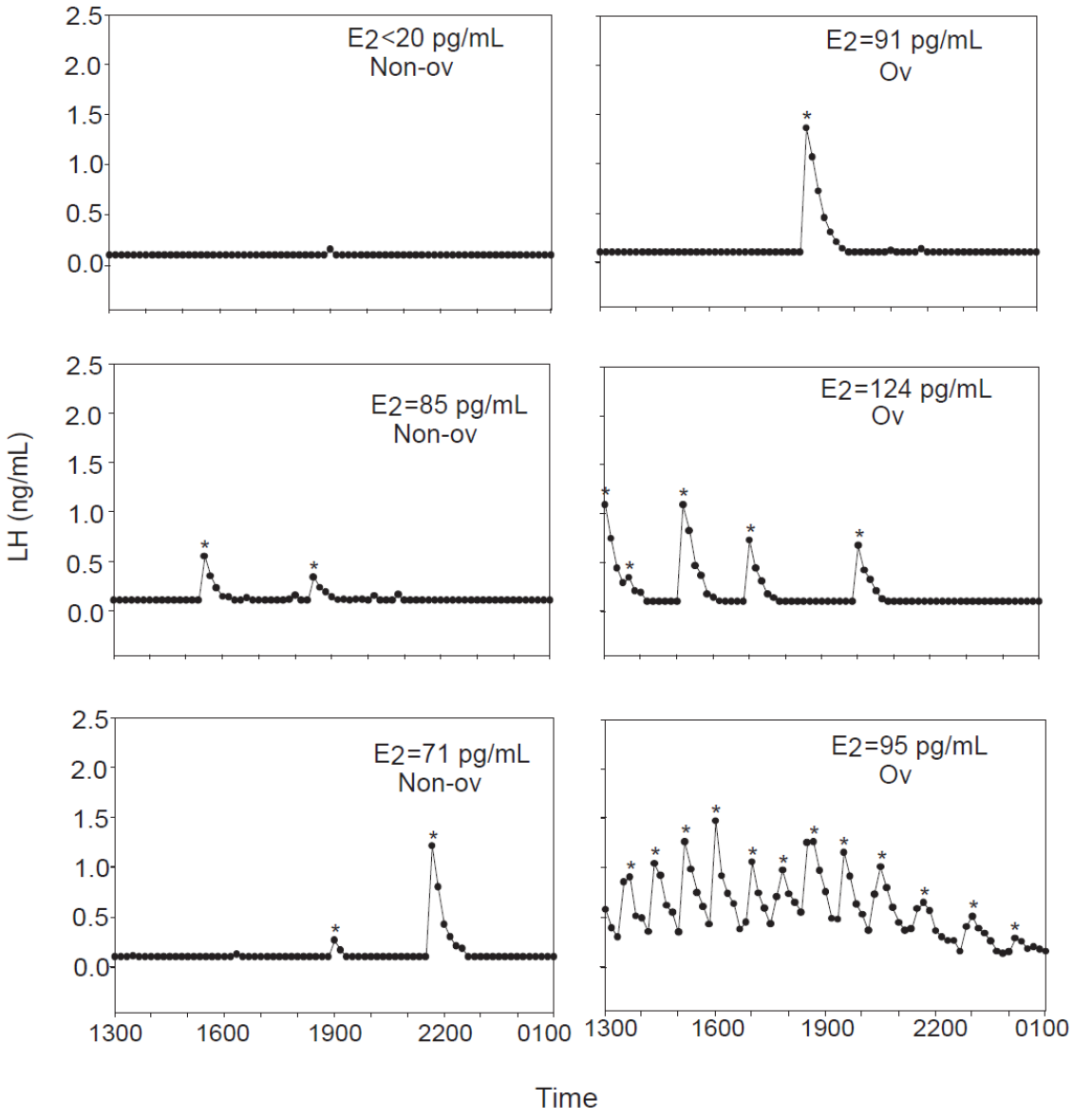

Figure 2.3 Pulsatile LH secretion in the six cholesterol-treated animals on D2-3 of the menstrual cycle at 5 years of age. Estrogen (E_2) at time of blood sampling is indicated for each animal. Each experiment is also labeled as occurring during either an ovulatory (Ov) or anovulatory (Non-ov) cycle. * Indicates LH pulse as detected by Pulsar analysis.

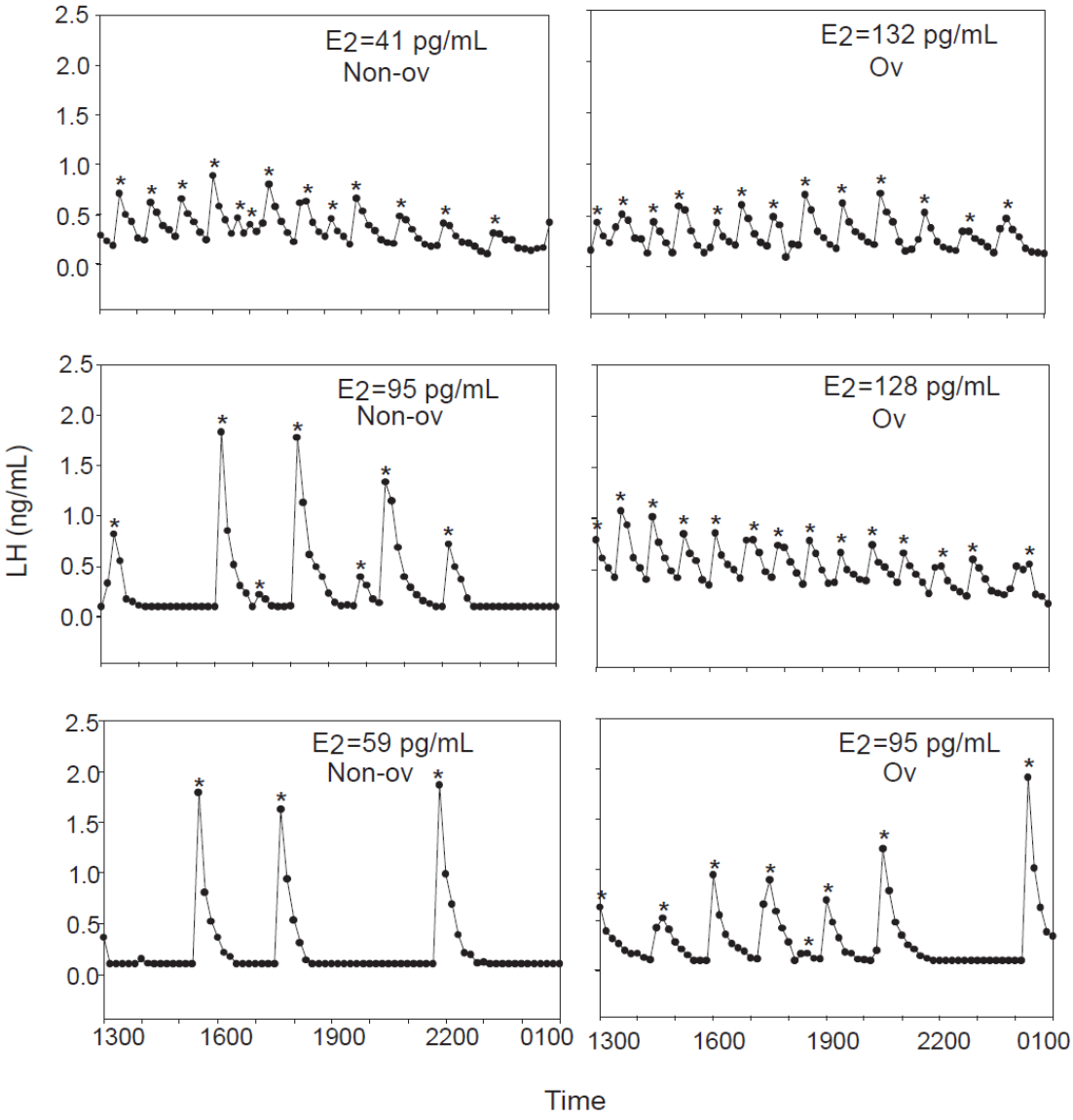

Figure 2.4 Pulsatile LH secretion in the six testosterone-treated animals on D2-3 of the menstrual cycle at 5 years of age. Estrogen (E_2) at time of blood sampling is indicated for each animal. Each experiment is also labeled as occurring during either an ovulatory (Ov) or anovulatory (Non-ov) cycle. * Indicates LH pulse as detected by Pulsar analysis.

monkeys on D8-10 of a menstrual cycle when the monkeys were 4 years of age (T-treated: 73.9±20.3 ng/mL/90 min; control: 17.9±10.8 ng/mL/90 min; p=0.042; Fig. 2.5). However, there was no group difference in LH response to GnRH when the monkeys were 5 years of age (T-treated: 47.7±22.4 ng/mL/90 min; control: 44.4±24.0 ng/mL/90 min; p=0.57).

Experiment 5. Ovarian Ultrasound

Ovarian ultrasounds were performed on D1-3 of a menstrual cycle when the animals were 5 years of age. There were no statistically significant group differences in ovarian area, circumference, or diameter (all p>0.1). There were also no differences in the total number of antral follicles on the ovaries, or in the mean, minimum or maximum size of the follicles (Table 2.2). There was, however, a trend toward control animals being more likely to have a follicle over 2.0 mm in diameter compared to T-treated animals (p=0.09), and follicles of this size were only present in control animals. As determined by daily hormone concentrations, there were no differences between the groups in length of follicular or luteal phase during the cycle in which ultrasounds were performed.

Experiment 6. Glucose Tolerance Testing

When the animals received glucose tolerance tests at 3.5 years of age, there were no differences in baseline or peak glucose, baseline or peak insulin, or insulin sensitivity, glucose effectiveness, acute insulin response, or disposition index as calculated by the MINMOD Millennium program (all p>0.1; data not shown).

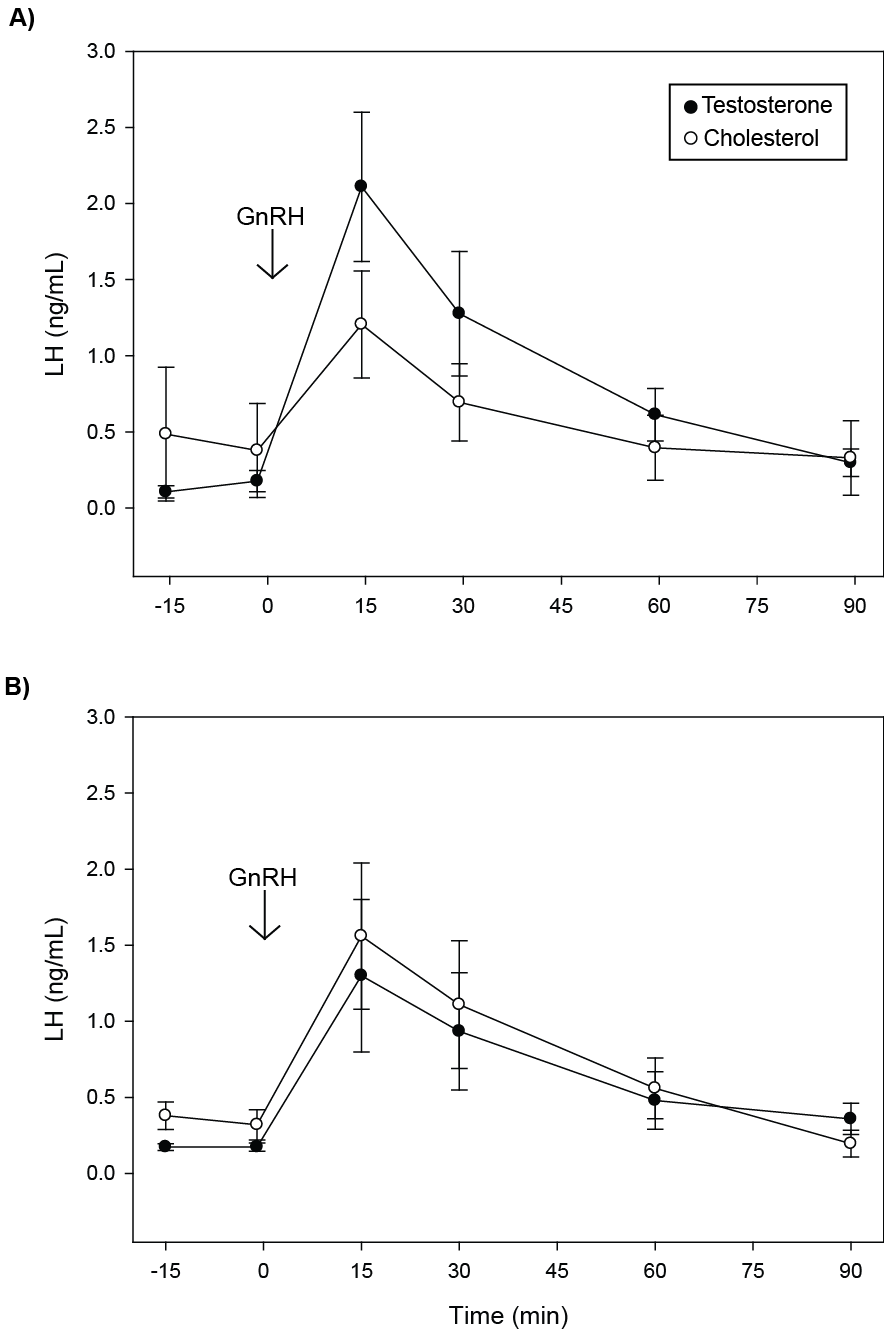

Figure 2.5 LH response to a bolus injection of GnRH at A) 4 years of age and B) 5 years of age. GnRH (250 ng/kg, i.v.) was infused at Time 0. T-treated animals showed a significantly greater response measured as area under the curve at 4 years of age (p=0.042) but not 5 years of age (p=0.57). Values are mean ± SEM.

Table 2.2 Ovarian Parameters as measured by ultrasound on D1-3 of the menstrual cycle at 5 years of age

	Avg. Ovary Diameter (mm)	Avg. Ovary Circumference (mm)	Avg. # Antral Follicles (both ovaries)	Max Follicle Size (mm)	# Animals with Follicles >2 mm
Control	3.7 ± 0.4	8.7±0.8	6.7 ± 0.9	2.0 ± 0.4	*3
T-Treated	2.8 ± 0.2	7.8±0.6	5.2 ± 0.5	1.4 ± 0.1	0

Data are presented as mean ± SEM. *Trend toward difference between groups (p=0.09)

Experiment 7. Metabolic Testing

There were no group differences in basal metabolic rate as measured between 2300 h and 0300 h (T-treated:1.5±0.16 kcal/kg/h; Control: 1.84±0.37 kcal/kg/h; p=0.4; data not shown). Table 2.3 shows a summary of the main findings from the experiments performed in Chapter 2.

2.4 DISCUSSION

Female monkeys treated with low doses of T during pubertal development (resulting in a 3.7-fold increase in T) showed earlier activation of the hypothalamic-pituitary-gonadal (HPG) axis, increased LH pulse frequency, as well as increased LH responsiveness to GnRH in early adulthood, all of which are key neuroendocrine features associated with hyperandrogenic states in women (Rebar et al. 1976; Levin et al. 1991; Apter et al. 1994; Pastor et al. 1998; Blank et al. 2009). This suggests that a modest increase in circulating androgen levels during the pre- to peripubertal interval in female monkeys can play a causal role in the greater activation of the central neural drive to the reproductive axis during early adulthood, and supports the hypothesis that HA may be pivotal in the development of reproductive dysfunction associated with obesity, PCOS and CAH.

The doses of androgens used in this study were based on clinical findings that obese girls and women with PCOS often have levels of T that are increased about 3-4 times above the levels seen in healthy girls and women (Eagleson et al. 2003; Silfen et al. 2003; Moran et al. 2004; McCartney et al. 2006). Although elevated, these increased levels of androgens are still relatively low compared to those typically present in men (3-

Table 2.3 Summary of main findings from Chapter 2

	Description	Age	p value	Direction of Significance
Expt 1	Pulsatile LH Secretion on D7-9	4 yrs	0.09	T>C
		5 yrs	NS	
Expt 2	Pulsatile LH Secretion on D1-3	5 yrs	0.039	T>C
Expt 3	Progesterone Negative Feedback	5 yrs	NS	
Expt 4	LH response to GnRH	4 yrs	0.042	T>C
		5 yrs	NS	
Expt 5	Ultrasound-presence of 2 mm follicle	5 yrs	0.09	C>T
Expt 6	Glucose Tolerance Testing	4 yrs	NS	
Expt 7	Basal Metabolic Rate	4.5 yrs	NS	

NS=not significant (p>0.1); T=T-treated animals; C=cholesterol-treated control animals

10 ng/mL; Evans et al. 1971; Piro et al. 1973). I was able to successfully mimic this modest increase in T in the female monkeys, with T levels still remaining lower than those observed in male macaques (5-20 ng/mL; Goodman et al. 1974). My findings of increased central neuroendocrine drive in T-treated animals support previous findings, which showed that monkeys exposed to high doses of androgens during early fetal development had elevated LH levels in adulthood (Dumesic et al. 2002). My study expands these findings by demonstrating that excess androgens do not need to be present during gestation in order for neuroendocrine changes to occur. Also, the doses of T used in the current study were smaller than had been used previously, indicating that a modest increase in T levels is sufficient to cause changes in neuroendocrine function.

There are several clinical conditions that produce both HA and increased pulsatile LH secretion, including PCOS and CAH. Women with PCOS reportedly have a consistently high rate of LH pulsatility (Rebar et al. 1976; Zumoff et al. 1983; Waldstreicher et al. 1988), while LH pulse frequency varies with the menstrual cycle in healthy women (Midgley and Jaffe 1971; Yen et al. 1972; Filicori et al. 1986). The difference in pulsatile LH secretion between women with PCOS and healthy women is most apparent during the early follicular phase of the menstrual cycle, when PCOS subjects have about one LH pulse per hour, compared with healthy women who may have an LH pulse frequency as slow as 1 pulse/90 min (Filicori et al. 1986; Waldstreicher et al. 1988; McCartney et al. 2002a). In one study comparing gonadotropin release in patients with either PCOS or CAH, women with PCOS showed both elevated androgens and increased frequency of pulsatile LH secretion compared with healthy controls. Women with CAH had an intermediate phenotype, showing levels of androgens and LH

pulse frequencies that were higher than controls but lower than PCOS patients (Levin et al. 1991). Other studies have also found elevated basal LH levels and an increased LH response to GnRH agonists in women with CAH (Barnes et al. 1994; Holmes-Walker et al. 1995), neuroendocrine changes that are similar to those seen in women with PCOS. Many CAH patients also experience premature pubarche, and in one study of women with premature pubarche, approximately 45% went on to develop polycystic ovaries, oligomenorrhea, and elevated LH levels post-pubertally (Ibanez et al. 1993). These girls also had elevated androgen levels at the time of their premature pubarche diagnosis, suggesting that increased levels of androgens during puberty may play a role in the development of later neuroendocrine and ovarian dysregulation.

In the current study, I found that the T-treated animals had significantly more pulses on D1-3 of the menstrual cycle as compared to control animals, despite the small sample size. There were no group differences in the percentage of menstrual cycles that were ovulatory (see Table 2.1), and looking at individual monkeys, there was not a relationship between LH pulse frequency and the incidence of ovulation in the previous menstrual cycle. The control monkeys had approximately one LH pulse per three hours in the early follicular phase. In contrast, the T-treated monkeys showed almost one pulse per hour in the early follicular phase, a pulse frequency about 3-fold greater than in the control animals, indicating greater central drive to the reproductive neuroendocrine axis.

The effect of P negative feedback on LH secretion was measured after one week of hormone exposure. I had hypothesized that the control animals would suppress pulsatile LH secretion to a greater degree than the T-treated animals, based on clinical studies that found decreased sensitivity to P negative feedback in women with PCOS

(Pastor et al. 1998; Blank et al. 2009). Instead, almost all of the animals in this study completely suppressed LH secretion. This was possibly due to the animals' relatively young age, as their menstrual cycles were potentially more prone to disruption by P treatment. E and P levels attained during treatment with the implants were within the range typically seen in ovulating macaques; however, E values approximated surge levels and were higher than typical for the luteal phase in rhesus monkeys (average luteal E: 50-80 pg/mL), so it is also possible that complete LH suppression resulted as a consequence of E negative feedback in combination with the P negative feedback.

I found that the T-treated animals had a significantly higher LH response to exogenous GnRH than control animals when tested at 4 years of age, as would be expected from clinical findings that women with PCOS secrete more LH in response to GnRH than controls (Yen et al. 1975; Patel et al. 2004; Bachelot et al. 2007). This difference was not apparent when the animals were tested at 5 years of age. However, previous studies have shown that LH responsiveness to GnRH is normalized in women with PCOS after spontaneous ovulation occurs (Blankstein et al. 1987). Four out of the six T-treated animals and two out of six controls had ovulatory cycles in the cycle before GnRH responsiveness was tested at five years of age (as indicated by elevated P on D20 of the previous cycle), so it is possible that recent ovulation led to a normalization of the LH response in those four T-treated animals.

Ovarian ultrasounds performed during the early follicular phase when these monkeys were 5 years of age showed no differences in numbers of small antral follicles or ovarian size between T-treated and control monkeys. This may indicate that the amount of ovarian T exposure in this study was not sufficient to induce changes in the

ovaries. Studies in female-to-male transsexuals have shown that extremely high doses of T can result in a PCOS phenotype replete with morphological changes in the ovaries (Pache et al. 1991). In addition, silastic implants that delivered high levels of T to nonhuman primates also showed an increase in ovarian follicle formation (Vendola et al. 1998). The dose of T in the current study was considerably lower than in these previous studies. By design in this study, I mimicked the circulating levels of T seen in women with PCOS; however, the ovary is a major source of androgens in PCOS and it is possible that higher levels of localized T or more prolonged exposure are needed to induce ovarian changes. Although no differences were found in follicle number, there was a trend toward control animals being more likely to display an antral follicle ≥2mm, a size that is indicative of selection of the dominant follicle during the early follicular phase of the cycle in rhesus monkeys (Bishop et al. 2009). Unlike the control group, none of the T-treated animals displayed antral follicles ≥2 mm. Importantly, pilot studies of adult breeding female rhesus monkeys found the average diameter of ovaries imaged during the early follicular phase to be 5.6±0.6 mm (Cecily Bishop, personal communication). This is much larger than ovaries of both control and T-treated females in the current study (see Table 2.2), suggesting that monkeys at five years of age are still developing reproductively. It is possible that ovarian changes resulting from increased T exposure may not occur until after the ovaries reach a normal adult size.

Approximately 80% of women with PCOS are overweight or obese (Azziz et al. 2004b), conditions that can aggravate PCOS symptoms such as insulin resistance (Legro 2000; Chang 2007). However, animals in this study were very lean when they underwent glucose tolerance testing at 3.5 years of age, so it may not be unexpected that group

differences in insulin sensitivity were not apparent. Metabolic testing was used to complement the GTT as a second method to examine the effects of T on metabolism. As with the GTT, the lack of group differences in basal metabolic rate may have been due to the lean state of the animals. Further studies are needed that examine the effect of HA on metabolic function in the presence of increased adiposity.

In summary, the increased pulsatile LH secretion and LH response to GnRH that I observed in T-treated monkeys indicates that HA during puberty could play a causal role in altering the neural drive to the reproductive axis, such as is seen in girls with HA and women with PCOS. In studies reported in Chapter 3, I examined whether additional neuroendocrine, ovarian, and metabolic changes may occur in response to chronic T exposure and/or weight gain associated with a high fat diet.

Chapter 3

EFFECTS OF HYPERANDROGENEMIA AND INCREASED ADIPOSITY ON NEUROENDOCRINE, OVARIAN, AND METABOLIC FUNCTION IN YOUNG ADULT FEMALE MONKEYS

3.1 INTRODUCTION

Polycystic ovary syndrome (PCOS) affects 4-8% of reproductive-aged women and is characterized by hyperandrogenemia (HA), irregular menstrual cycles, and polycystic ovaries, in addition to faster LH pulse frequency, increased LH response to GnRH, and decreased insulin sensitivity (Apter et al. 1994; Zawadzki and Dunaif 1992; Knochenhauer et al. 1998; Asuncion et al. 2000; Bachelot et al. 2007; Carmina et al. 2007). Studies have found that up to 90% of women with PCOS are overweight or obese, which is higher than the general population of the United States, where 65% of women over the age of 20 are overweight or obese (Legro 2000; Azziz et al. 2004a; Flegal et al. 2010). Women with PCOS also tend to have a central distribution of adiposity, which is associated with increased rates of high blood pressure and cardiovascular disease (Lapidus et al. 1984; Chuang et al. 2006).

Being overweight or obese aggravates many symptoms of PCOS. Basal production of T and free T have been reported to be elevated in obese, compared with non-obese, PCOS patients in many studies (Kiddy et al. 1990; Holte et al. 1994b; Acien et al. 1999; Moran et al. 2008). Hirsutism, a clinical marker of elevated androgens, is also worse in obese PCOS women (Kiddy et al. 1990). Obese women with PCOS have higher rates of oligo- and amenorrhea, infertility, and miscarriage than lean women with PCOS,

as well (Kiddy et al. 1990; Balen et al. 1995; Ozgun et al. 2011). Additionally, between 50 and 70% of women with PCOS suffer from some degree of insulin insensitivity, and insulin sensitivity scores tend to decrease (i.e. get worse) with increasing BMI in women with PCOS (Dunaif et al. 1989; Carmina et al. 1992; Meirow et al. 1995; Kauffman et al. 2002). Compensatory hyperinsulinemia follows a similar pattern, with obese PCOS women having the highest insulin secretion (Carmina et al. 2007). Many PCOS patients resume menstruation and ovulation with even mild weight loss, although this strategy is not always successful at normalizing reproductive function (Pasquali et al. 1989; Knochenhauer et al. 1998).

Obesity in peripubertal girls is associated with HA (McCartney et al. 2009), and this is thought to be a forerunner of adult PCOS (Franks 2002; Witchel 2006). Like adults with PCOS, obese girls with HA have increased levels of LH and decreased sensitivity to progesterone (P) negative feedback (Chhabra et al. 2005; Knudsen et al. 2010). Obese girls in late puberty have been reported to have increased frequency of pulsatile LH release compared with nonobese girls (McCartney et al. 2009). In addition, obese girls in the later stages of puberty were more likely to have irregular menstrual cycles compared with nonobese controls (McCartney et al. 2009). Like adults, hyperinsulinemia is more common in adolescents with PCOS, especially in those who are obese (Silfen et al. 2003).

Cumulatively, there is substantial evidence that PCOS is characterized by both HA and obesity. However, there are women with PCOS who are normal weight (Legro 2000; Azziz et al. 2004a), indicating that while obesity likely *aggravates* symptoms of PCOS, it is probably not the sole cause of PCOS. The experiments performed in Chapter

2 of this dissertation showed that elevated levels of T led to an increased central drive to the reproductive axis, similar to what is seen in women with PCOS (Waldstreicher et al. 1988; Apter et al. 1994; Bachelot et al. 2007). However, there were no robust group differences in ovarian or metabolic function after 4 years of continuous exposure to slightly elevated T. Thus, based on evidence that obesity aggravates PCOS symptoms in women, it was of interest to investigate whether weight gain would also lead to the development of ovarian or metabolic symptoms in the T-treated monkeys used for these studies. I hypothesized that there would be a continued effect of HA on neuroendocrine function, with T-treated animals having faster pulsatile LH secretion and a more pronounced LH response to GnRH. I also expected that T-treated monkeys would display less suppression of LH secretion in response to P negative feedback. I further hypothesized that T-treated, but not control animals, would develop a polycystic ovary phenotype, with T-treated animals having more small antral follicles, peripheral localization of follicles, and larger ovarian size. I expected all animals to gain weight and fat and have decreased insulin sensitivity scores compared to before the high fat diet, but I hypothesized that the T-treated monkeys would have lower insulin sensitivity compared with controls.

3.2 MATERIALS AND METHODS

Animals

Twelve female rhesus macaques (*Macaca mulatta*), 5-7 years of age, were utilized for this study. These were the same animals that were utilized in Chapter 2 of this dissertation when they were 3-5 years of age. All monkeys were housed individually in

single cages (81 x 61 x 69 cm) in a temperature-controlled room (24±2°C), with lights on for 12 hr/day (0700h-1900h). Monkeys were trained to approach the front of their cage so menses could be detected daily by swabbing the vaginal area with a cotton-tipped swab. The first day of menses was designated Day 1 of a menstrual cycle. All procedures in this study were reviewed and approved by the ONPRC Institutional Animal Care and Use Committee.

Western Style Diet

Until the monkeys were 5.5 years of age, they were fed two meals of Purina LabDiet fiber-balanced monkey chow each day (no. 5000; Purina Mills, St. Louis, MO), supplemented with fresh fruits and vegetables. When the animals were 5.5 years old, their diet was switched from the fiber-balanced monkey chow (15% calories from fat, 27% from protein, 59% from carbohydrates) to a diet that was specifically formulated to mimic the typical Western style diet (WSD, 33% calories from fat, 17% from protein, 51% from carbohydrates; 5A1F, Purina Mills, St. Louis, MO; Shadoan et al. 2003; Sullivan et al. 2010). The animals were fed *ad libitum* and each meal was supplemented with a high-sugar, high-calorie treat (e.g. a cookie, snack cake, graham cracker with peanut butter, etc). Previous studies have shown that monkeys eating this diet gain a significant amount of weight within the first 5-6 months of being on this diet (Sullivan et al. 2005; Sullivan et al. 2006).

Testosterone Implants

T implants were maintained as described in Chapter 2. Normal T levels in prepubertal female rhesus macaques were determined by assaying serum from four 12-month-old female monkeys in the ONPRC colony. The average T value (0.4 ng/mL) was multiplied by three to achieve the lower limit (1.2 ng/mL), and by 4 to achieve the upper limit (1.6 ng/mL) of target values in the T-treated animals. This was based on clinical evidence that obese girls with HA and women with PCOS have T levels about 3-4 times higher than controls (Eagleson et al. 2003; Silfen et al. 2003; McCartney et al. 2006). To determine the size of T implant needed, monkeys from the ONPRC colony that were not used for this study had implants of various lengths and T:cholesterol ratios placed subcutaneously under ketamine hydrochloride (Ketaset, 10 mg/kg i.m., Wyeth, Madison, NJ) sedation. Blood samples were taken daily to determine which implants resulted in a sustained 3-4-fold increase in T levels. Once an appropriate implant size was determined, all animals used in this study received either a T- or cholesterol-containing (n=6/group) implant at one year of age. Implants were made of Silastic tubing (Dow Corning, Midland, MI) and were initially 5 mm in length with an inner and outer diameter of 0.335 cm and 0.465 cm, respectively. Implants were filled with cholesterol (control animals), or a T/cholesterol mixture (T-treated animals), with a T:cholesterol ratio of 1:15 or 1:12 at the beginning of the experiment. As the animals grew, the length of the implant increased to 3 cm and the T:cholesterol ratio was increased gradually to 1:4 to maintain the desired serum T levels. Both cholesterol and T were purchased from Sigma Aldrich (St. Louis, MO).

Catheterization

At 3.5 years of age, after all monkeys had experienced menarche, a chronic indwelling venous catheter was implanted by the ONPRC surgical staff using isoflurane anesthesia (Hospira, Lake Forest, IL), as described previously (Cameron and Nosbisch 1991). Briefly, the catheter was inserted into the subclavian or femoral vein and routed subcutaneously to exit in the mid-scapular region of the back. It was protected by a fitted nylon jacket worn by the monkey. The catheter was run through a flexible metal tether attached to the jacket and connected to a swivel at the top of the cage, which allowed the monkey to have full range of motion within its cage. Silastic tubing was routed through the wall into an adjacent room where blood samples were collected and drugs were infused without disturbing the monkeys or disrupting their normal activities. Previous studies established that female monkeys with chronic indwelling catheters display normal, regular menstrual cycles (Herod et al. 2011). Catheters were kept patent with a constant infusion of physiological saline (Baxter Healthcare, Deerfield, IL) containing heparin sodium (4 IU/mL), at a rate of approximately 100 mL/day. Animals were sedated weekly with ketamine in order to inspect the catheter system and replace a sterile dressing covering the exit site. No ketamine was administered in the 24 hours preceding any experiment. Following catheterization surgery, animals were allowed a minimum of 3 weeks to recover before any experiments were performed.

Blood Collection and Steroid Hormone Assays

Blood samples were collected weekly from all animals to track serum T concentrations. Samples were collected into sterile heparinized syringes through the

remote sampling system, placed into sterile plastic tubes and centrifuged at 3000 rpm for 15 min at 4°C. Plasma was removed and placed into plastic O-ring vials (containing 20 µL of a solution composed of equal volumes of 38% sodium citrate and 1,000 IU/mL sodium heparin to prevent clotting of plasma proteins) and stored at -20°C until assays were performed. Red blood cells were sterilely re-suspended in saline and reinfused through the catheter system to the animal. Each week's samples were assayed for T and when an individual animal's serum T concentration fell below the threshold of 1.2 ng/mL, the implant was changed. Cholesterol implants were also changed regularly so that cholesterol-treated (i.e., control) animals received the same average number of implant surgeries as the T-treated animals.

T was measured using a radioimmunoassay (RIA) kit (DSL-4100, Diagnostic Systems Laboratories, Inc, Webster, TX) by the Endocrine Services Core Laboratory at the Oregon National Primate Research Center. The sensitivity of the T assay was 0.05 ng/mL and the intra- and inter-assay coefficients of variation for the assays were 2.23% and 4.00%, respectively. Blood samples were drawn at times throughout the study to quantify serum estradiol (E) and P concentrations. Both E and P were assayed by the Endocrine Services Core using the Immulite 2000 platform. As with many validated clinical platforms, the Immulite 2000 runs three QC serum pools daily and as such, no specific intra-assay QC data is available. The inter-assay coefficient of variation, reflecting variability in daily QC results over the period in which these assays were performed was 8.5% for E and 9.4% for P.

LH and FSH Assays

LH and FSH were measured by RIA at the University of Pittsburgh assay core using recombinant cynomolgus monkey LH and FSH (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) as standards (Williams et al. 2001). The sensitivity of the LH assay was 0.1 ng/mL and the intra- and inter-assay coefficients of variation for the assays used in this study were 6.6% and 12.2%, respectively. The sensitivity of the FSH assay was 0.05 ng/mL and the intra-assay coefficient of variation was 9.3 %.

Physical Activity

Physical activity levels were assessed continuously for the final 10 months of this study. Animals wore jackets that contained a pocket housing a three-way accelerometer (Actical®, Respironics, Bend, OR, USA). The monitors were programmed to record total activity counts per minute, and data was downloaded approximately once every three weeks.

Experimental Protocols

PCOS-like symptoms were measured in five experiments when the animals had been on the WSD between 3 and 16 months (see Fig. 3.1). In Experiment 1, neuroendocrine function of the reproductive axis was measured, as described in Chapter 2 before the animals were given the WSD. Measurements included determination of pulsatile LH secretion during the early follicular phase of a menstrual cycle, measurement of pulsatile LH secretion again during the luteal phase to determine the

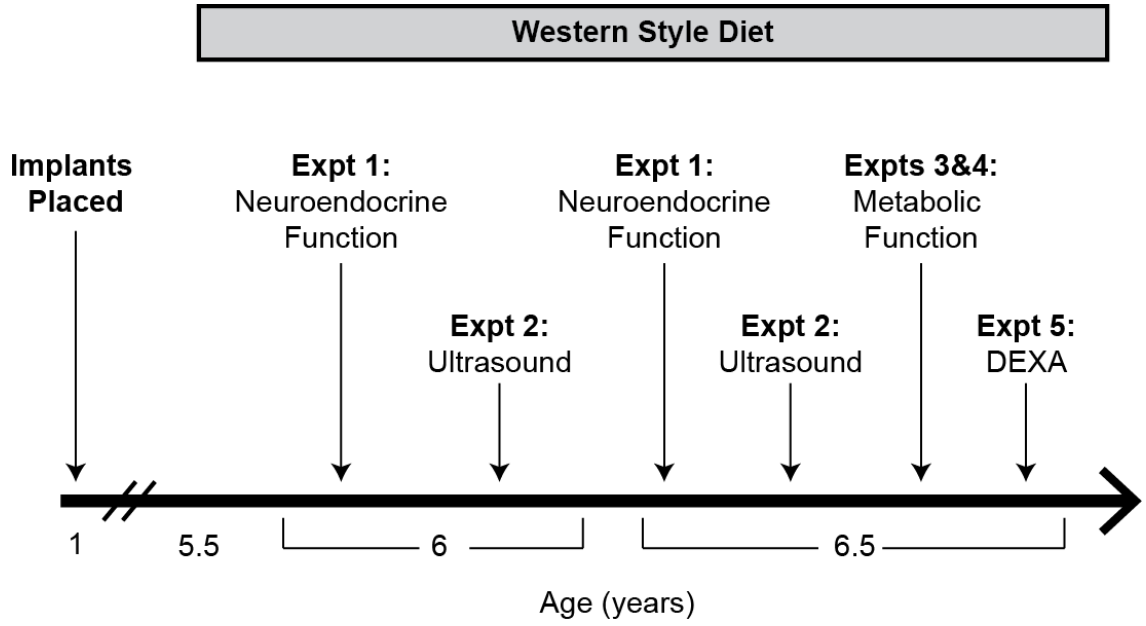

Figure 3.1 Schematic diagram of the experimental timeline for Chapter 3, indicating ages at which experiments were performed. Note that the timeline does not have a uniform scale.

effect of P negative feedback on LH release, and assessment of the LH response to exogenous GnRH. Experiment 1 was performed twice, when the animals were 6 and approximately 6.5 years of age. Experiment 2 utilized ovarian ultrasound to analyze follicular morphology and ovarian size, and was performed after neuroendocrine function was measured at both 6 and 6.5 years of age. To assess metabolic function, Experiments 3, 4, and 5 were performed. In Experiment 3, glucose tolerance testing was performed when the animals were 6.5 years of age in order to measure fasting glucose and insulin levels, and to measure glucose and insulin responsiveness to a bolus of glucose, which allowed the calculation of insulin sensitivity. Experiment 4 involved measurement of metabolic rate in a closed chamber when the monkeys were 6.5 years of age. Finally, in Experiment 5, monkeys were DEXA-scanned to evaluate body composition immediately before and again 16 months after starting the WSD.

Experiment 1. Evaluation of Neuroendocrine Function of the Reproductive Axis

Pulsatile LH secretion was measured during the early follicular phase (D1-3) of the menstrual cycle in the animals when they were 6 years, and again when they were approximately 6.5 years of age, after 6 and 14 months on the WSD, respectively. Blood samples were collected as described in Chapter 2 every 10 min from 1300 h to 0100 h. This interval provided samples for 6 hours during the light phase and 6 hours during the dark phase of the day-night cycle in order to detect differences between daytime and nighttime LH secretion. The early follicular phase was chosen because this is the time period when differences in pulsatile LH secretion are most apparent between women with

PCOS and healthy women (McCartney et al. 2002a), and when differences were seen between the groups in Experiment 2 of Chapter 2.

When the animals were 6 years old, and again when they were 6.5 years of age, pulsatile LH secretion was measured during the luteal phase of a menstrual cycle. Daily blood samples were taken beginning on D8 of the menstrual cycle and were assayed for P each day in order to track P levels on a day-by-day basis. On the first day that P levels were ≥2.0 ng/mL, pulsatile LH secretion was measured using the methods described above. A threshold of 2.0 ng/mL was chosen because it indicates a functional corpus luteum and is a standardized low level of P. Importantly, it is also a lower P level than was created using P implants in Experiment 3 of Chapter 2 (P values: 4.08±1.05 ng/mL), in which all pulsatile LH secretion was suppressed.

LH responsiveness to GnRH was measured between 0900 h and 1000h on D2-4 of a menstrual cycle when the monkeys were 6 and 6.5 years of age. GnRH was obtained from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA), dissolved in 0.9% saline at 1µg/100µL, and stored in 200 µL aliquots at -20°C until use. Immediately before use, saline was added to individual aliquots to bring the concentration to 1µg/mL. The protocol was the same as used in Experiment 4 of Chapter 2, with the exception that an additional sample was taken before GnRH administration in order to determine a more accurate baseline LH level. GnRH (250 ng/kg, i.v.) was infused at time 0 and blood samples (0.4 mL) were collected at -15, -8, -1, 15, 30, 60, and 90 minutes, as described previously (Cameron and Nosbisch 1991). This dose was chosen so that monkeys would receive a physiological dose that caused an LH response, but a response that was sub-maximal to allow detection of individual

differences in LH responsiveness. Samples were collected, centrifuged, and stored. At the end of the experiment, red blood cells were reinfused as described for the pulsatile LH experiments.

Experiment 2. Ovarian Ultrasound

Ovarian ultrasounds were performed by Dr. Cecily Bishop on D1-3 of a menstrual cycle, as described in Experiment 5 of Chapter 2. Ultrasounds were performed when the animals had been on the WSD for 3 months and again when they had been on the WSD for 14 months, when they were 5.75 and approximately 6.5 years of age, respectively. Ultrasound was performed using a GE Medical Systems Voluson® 730 Expert Doppler ultrasound instrument (GE Healthcare, Waukesha, WI) with both 2D (4.5–16.5 MHz) and 4D (3.3–9.1 MHz) transabdominal probes. Animals were assigned different random identifiers so that Dr. Bishop remained blind to treatment group. The follicle cohort present in each ovary and ovarian size were assessed. The 2D probe was used to orient image field to the uterus and identify the ovaries. The 4D probe was then used to generate a data file of each individual ovary which included a series of images collected in one scan through the entire ovary. Archived scans from each animal were analyzed at one time by Dr. Bishop, who remained blinded to treatment group. Ovaries were analyzed for circumference, number of visible antral follicles in each ovary, and the mean, maximum and minimum size of the antral follicles in each ovary. Follicle counts and size of follicles were measured using previously defined methods in adult female rhesus monkeys (Bishop et al. 2009). All parameters were then decoded for comparisons between treatment groups.

Experiment 3. Glucose Tolerance Testing

Glucose tolerance testing (GTT) was performed during the early follicular phase of a menstrual cycle when the animals were 6.5 years old and had been on the WSD for 12 months. For monkeys not showing regular menstrual cycles, the GTT was performed when a blood sample showed that E and P levels were low, indicating the absence of a dominant follicle or corpus luteum in the ovaries. Each animal was sedated initially with telazol (tiletamine hydrochloride and zolazepam hydrochloride, Fort Dodge Animal Health, Fort Dodge IA, USA) and subsequently with ketamine to maintain sedation. The protocol was based on that designed by Richard Bergman (1979). Dextrose (300 mg/kg) was infused i.v. through the catheter system and blood samples were taken from 15 minutes before to 3 hours after the glucose infusion. Tolbutamide (5 mg/kg) was infused i.v. 20 minutes after the dextrose in order to stimulate the pancreas to secrete more insulin. All samples were immediately assayed for glucose using the YSI 2300 Stat Plus (YSI Inc, Yellow Springs, OH), and subsequently for insulin by RIA (Linco Human Insulin RIA, Millipore Corporation, Billerica, MA, USA). The sensitivity of the insulin assay was 1 µIU/ml and the intra-assay coefficient of variation was 4.9%.

Experiment 4. Metabolic Testing

Metabolic rate was measured over a 24-hour period as described in Experiment 7 of Chapter 2 when the animals were 6.5 years of age. The animals were transported into a sealed Lexan® and stainless steel metabolic chamber (Columbus Instruments, Columbus, OH) at approximately 1000 h. Fresh air was pumped in and circulated with a 4-in fan. The amounts of oxygen consumed and carbon dioxide produced were measured using a

computer-controlled open-circuit calorimeter, and total energy expenditure (kcal) was calculated using the Oxymax system (Columbus Instruments). The animals did not receive their normal meals during this time but were fed a 110-g banana at 1500 h.

Experiment 5. DEXA Scanning

Percent body fat, percent central fat, fat mass in grams, and lean tissue mass were determined using dual-energy X-ray absorptiometry (DEXA) scanning. Monkeys were sedated with ketamine and positioned supine on the bed of a Hologic Dexa scanner (Discovery scanner, Hologic Inc, Bedford MA). Two to three scans were performed for each monkey in "infant whole body" mode and averages were calculated for each measure. To delineate central fat mass from peripheral fat mass, fat in the trunk (including both the subcutaneous and visceral compartments) and fat in the extremities were calculated using standard methodology (Clark et al. 2005). DEXA scanning was performed immediately prior to starting the WSD when the animals were 5.5 years old, and again when the animals had been on the WSD for 16 months.

Statistical Analysis

LH pulses were identified by Dr. Cliff Pohl using the Pulsar algorithm that was developed by Merriam and Wachter (1982), and used previously to detect LH pulses in monkeys (Cameron and Nosbisch 1991; Ramaswamy et al. 2007; Herod et al. 2011). Pulse frequency was defined as the number of Pulsar-detected pulses in 12 h and the following G-values were used: G(1): 50.00, G(2): 1.0, G(3): 0.40, G(4): 0.40, and G(5): 0.40. Pulse amplitude was calculated by subtracting the baseline LH level from the peak

LH level of each pulse. For all analyses, LH values below the level of detectability for the assay were assigned the minimum detectable concentration of the assay. Independent Student's *t*-tests were used to determine group differences in number of pulses, proportion of daytime (vs. nighttime) pulses, pulse amplitude, and LH response to GnRH. For the GnRH stimulation, LH area under the curve (AUC) was calculated, correcting for baseline LH levels.

The MINMOD Millenium computer program was used to determine glucose effectiveness (Sg), insulin sensitivity (SI), acute insulin response (AIRg), and disposition index values (DI) (Boston et al. 2003). This program was designed to calculate these values based on the GTT protocol that was described by Bergman and colleagues (1979) and that was used in this study. Sg indicates the capacity of glucose to mediate its own disposal, AIRg addresses the adequacy of insulin secretion, and SI quantifies the capacity of insulin to promote glucose disposal. DI is the product of AIRg and SI, and it therefore takes into account insulin concentration *and* action (Bergman 1989; Boston et al. 2003). Independent sample *t*-tests were used to determine group differences in GTT measures.

Basal metabolic rate was calculated as the average number of kcal expended per kg per hour from 2300 h to 0300 h. This time period was chosen as it is when monkeys are typically asleep and their heart rate is slowest (Judy Cameron, personal correspondence). Body mass index (BMI; kg/m^2) was calculated using the crown-rump length as height. An independent *t*-test was used to assess group differences in basal metabolic rate, percent fat, percent fat in the central region, and percent lean mass. Mixed measures ANOVAs were used to determine changes in measures across time. Post-hoc tests employed were paired t-tests with a Bonferroni correction for multiple comparisons.

Pearson r correlations were used to examine relationships between neuroendocrine, ultrasound, and metabolic parameters. Statistical analyses were performed using IBM SPSS Statistics 20 (SPSS Inc, Chicago, IL). Values are presented as mean ± SEM. Significance was set at $p<0.05$.

3.3 RESULTS

Plasma T concentrations in the T-treated animals were maintained in a narrow range, 4.2±0.2-fold higher than in the control animals from the time of first implant at 1 yr of age through 7 yrs of age (T-treated: 1.71±0.05ng/mL; control: 0.46±0.02ng/mL, $p=0.001$). Implants were replaced an average of every 9.2±0.4 weeks throughout the study.

The animals increased their daily caloric intake by an average of 140±13% when they switched from normal chow to the WSD (Before WSD: 467 kcal/day; After WSD: 1,121±62 kcal/day), and there were no differences between T-treated and control animals with regards to average kcal consumed per day ($p=0.95$). All animals gained at least 10% of their body weight (average percent gain for T-treated animals: 29.4 ± 7.5%, Controls: 27.3 ± 4.0%; Fig. 3.2). When weight gain over the entire post-pubertal period was analyzed (i.e. from 3.5-7 yrs of age), there was a significant effect of treatment [$F(1,10)=5.19$, $p=0.046$], with T-treated animals gaining more weight than controls, although percent weight gain on the WSD was not different between groups ($p>0.1$). Weight gain while on the WSD was significantly positively correlated with caloric intake when all of the animals were analyzed together ($r=0.781$, $p=0.003$; Fig. 3.3). When analyzed by treatment group, there was a significant correlation between caloric intake

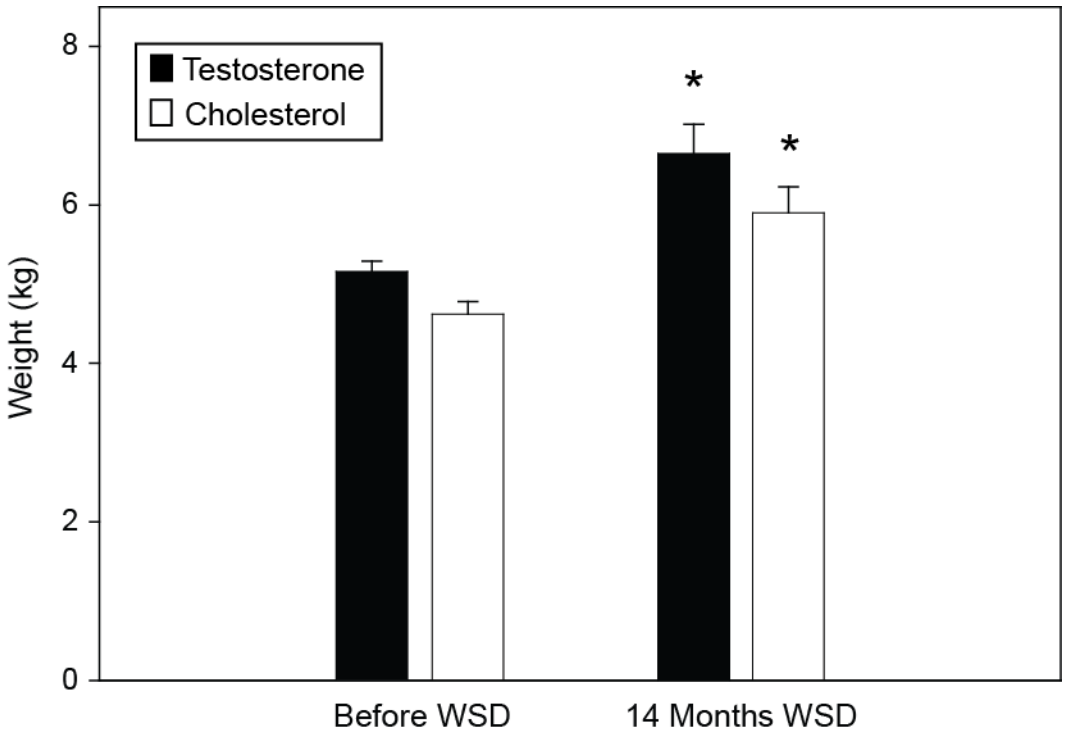

Figure 3.2 Animal weights from immediately before and 14 months after beginning the WSD. Closed bars are T-treated animals and open bars are cholesterol-treated controls. *indicates significant increase compared with values before WSD ($p<0.001$).

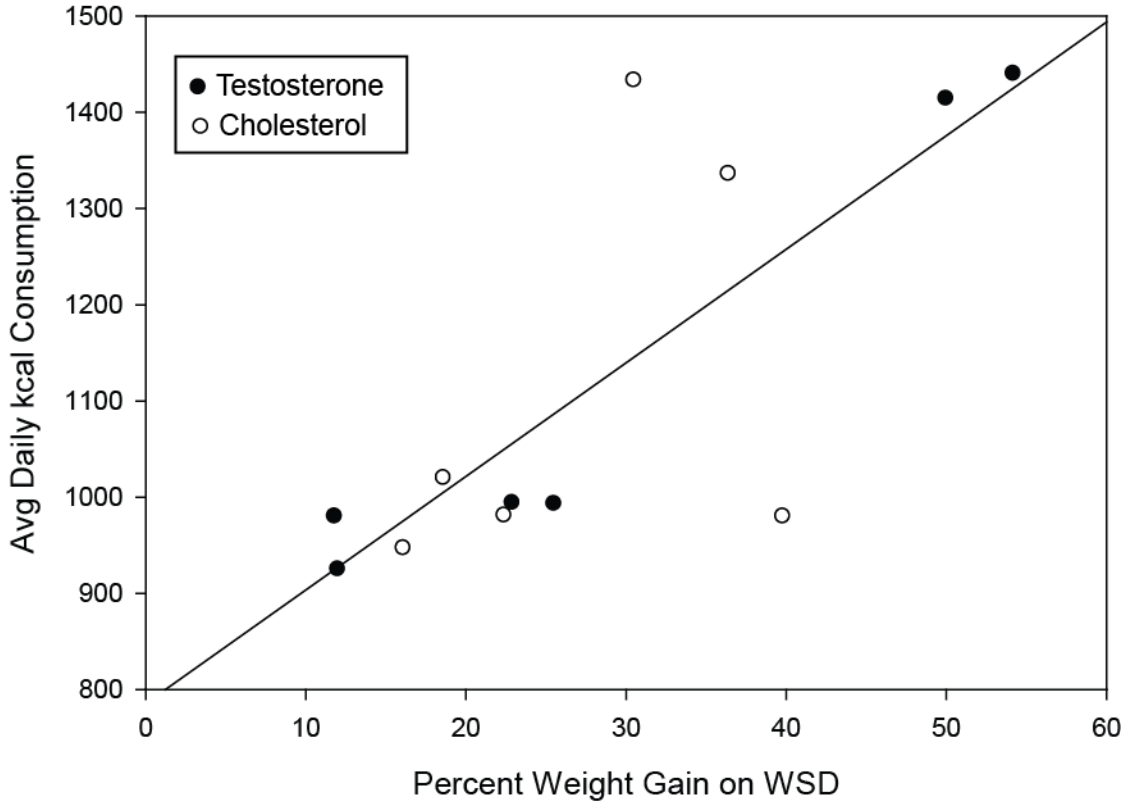

Figure 3.3 Correlation between percent weight gain during 14 months on the WSD and kcal consumed/day (r=0.781, p=0.003). Closed circles are T-treated animals and open circles are cholesterol-treated controls.

and weight gain in the T-treated animals (r=0.970, p=0.001) but not the control animals (r=0.457, p=0.362). BMI increased from 21.8±0.5 kg/m^2 before the WSD to 25.6±1.2 kg/m^2 after 16 months on the WSD (p=0.002), and was not different between the groups (p>0.1). There was a strong trend toward T-treated animals being less active than controls over the 10-month period that activity was measured [avg. activity counts/day: T-treated: 201,852±54,929; Control: 423,053±84,067; t(10)=2.203, p=0.052; Fig. 3.4]. However, there was no correlation between weight gain on the WSD and average activity (r= -0.167, p=0.60). There was also no correlation between post-pubertal weight gain and average activity (r= -0.001, p=0.997).

Experiment 1. Neuroendocrine Function of the Reproductive Axis

There were no group differences when analyzing number of pulses, average pulse amplitude, or proportion of pulses that occurred during the daytime on D1-3 of a menstrual cycle at either 6 or 6.5 years of age, after 6 or 14 months on the WSD, respectively. When assessing the change in pulse frequency from before to 14 months after starting the WSD, there was a significant diet x treatment interaction [F(1,9)=8.84, p=0.016). This resulted from an increase in pulse frequency in the control, but not the T-treated animals from before to 6 months after beginning the WSD [t(5)= -4.97, p=0.004; Fig. 3.5]. LH pulse number did not change in either group when comparing the data from 6 months to 14 months on WSD [Before WSD: T-treated: 9.67±1.78 pulses/12 h; Control: 3.67±1.80; 6 mo WSD: T-treated: 11.40±1.03; Control: 13.33±1.14; 14 mo WSD: T-treated: 13.00±1.05; Control: 12.33±1.02; Fig. 3.5]. There was a significant effect of diet on pulse amplitude, which decreased in both groups 6 months after the

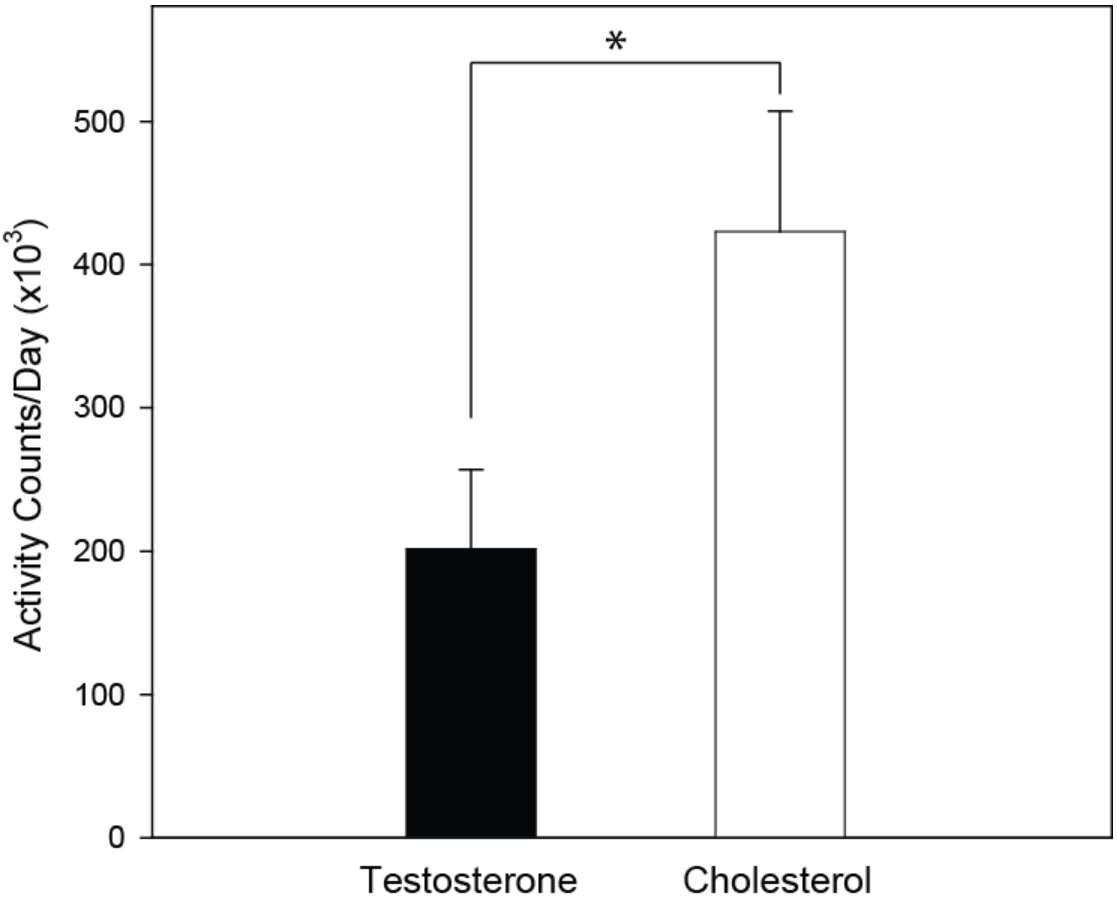

Figure 3.4 Average home cage activity over a 10 month period in T-treated and cholesterol-treated control animals. Data are presented as mean ± SEM. * indicates p=0.052

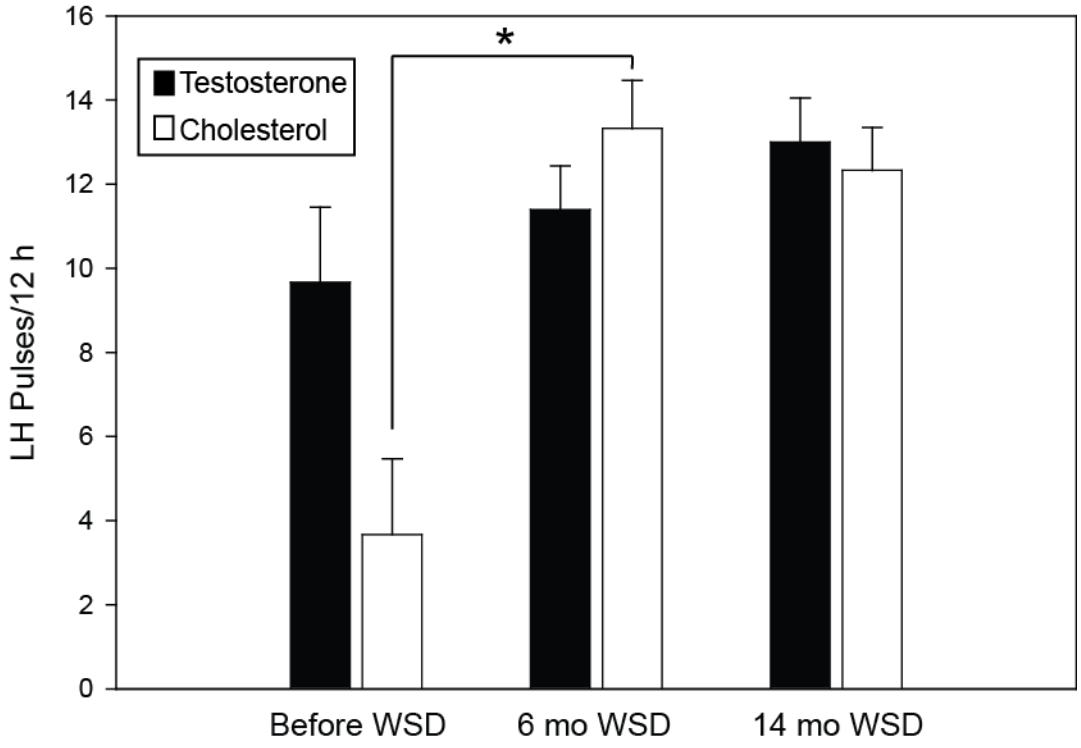

Figure 3.5 Number of LH pulses occurring in T-treated and control animals during a 12-h period on D1-3 of a menstrual cycle before (when monkeys were 5 yrs old), 6 months after (when monkeys were 6 yrs old), and 14 months after (when monkeys were 6.5 yrs old) starting the Western style diet (WSD). Closed bars are T-treated animals and open bars are cholesterol-treated controls. *Indicates p=0.004

animals switched to the WSD [Before WSD: T-treated: 0.73±0.21 ng/mL; Control: 0.70±0.15 ng/mL; 6 mo WSD: T-treated: 0.16±0.02 ng/mL; Control: 0.38±0.12 ng/mL; 14 mo WSD: T-Treated: 0.27±0.04 ng/mL; Control: 0.34±0.11 ng/mL; $F(1,8)=13.1$, $p=0.007$; Fig. 3.6].

There was no effect of time or treatment, nor was there a time x treatment interaction on LH AUC or peak LH response to exogenous GnRH (all $p>0.1$). The response to GnRH was measured at a different time of the menstrual cycle for these experiments compared with those performed in Chapter 2 (D2-4 in the current study vs. D8-10 in Experiment 4 of Chapter 2), so LH response to GnRH was not compared to the data that had been collected before the animals started the WSD.

Six months after starting the WSD, the effect of P negative feedback on pulsatile LH secretion was examined by comparing LH pulsatility measured during the early follicular phase (D1-3) to that measured during the luteal phase. Two control animals and one T-treated animal did not have this experiment performed due to either lack of ovulation or temporary catheter removal, so the analyses were performed on the remaining four cholesterol-treated controls and five T-treated monkeys. There were no group differences in pulse frequency, pulse amplitude, or P level measured during the luteal phase pulse bleeds (all $p>0.1$). Most animals showed a decrease in LH pulse frequency measured during the luteal phase compared with the values on D1-3, and there were no group differences in percent suppression [$t(7)= -0.875$, $p=0.4$; Figs. 3.7 and 3.8]. This experiment was performed again after 14 months on the WSD. Two T-treated animals did not undergo this experiment due to temporary catheter removal, so analyses were performed on the remaining four T-treated and six control animals. Similar to the

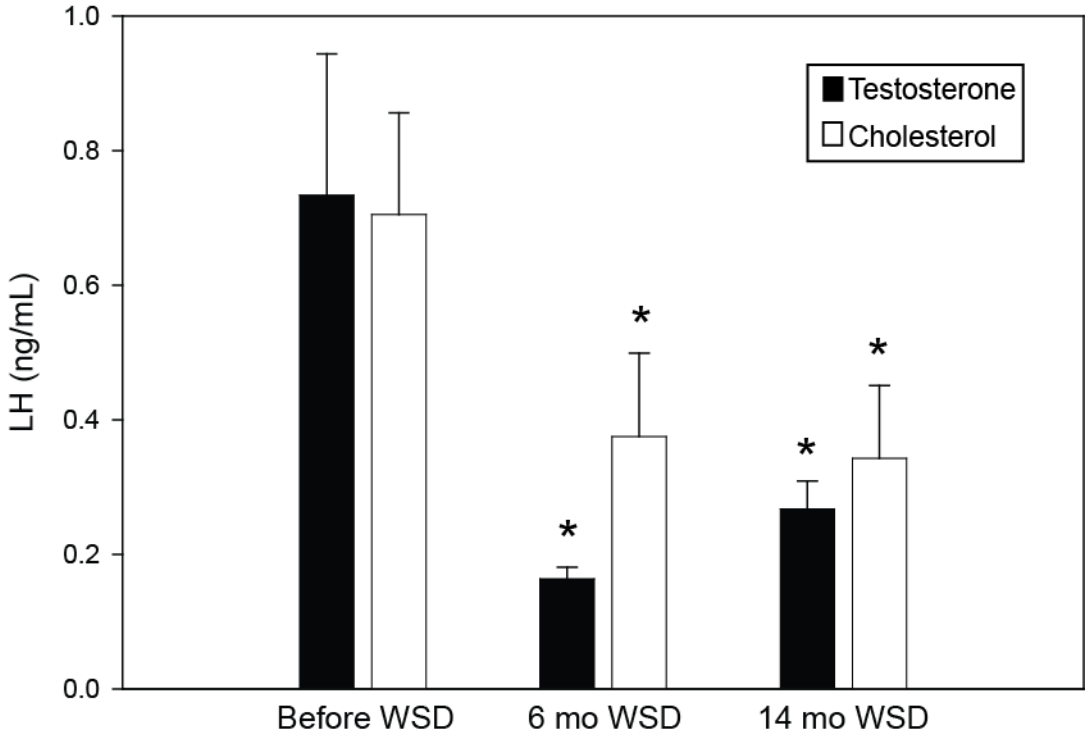

Figure 3.6 Average LH pulse amplitude during a 12 h period on D1-3 of a menstrual cycle before, 6 months, and 14 months after starting a WSD. Closed bars are T-treated animals and open bars are controls. * indicates significant difference from before WSD ($p<0.05$).

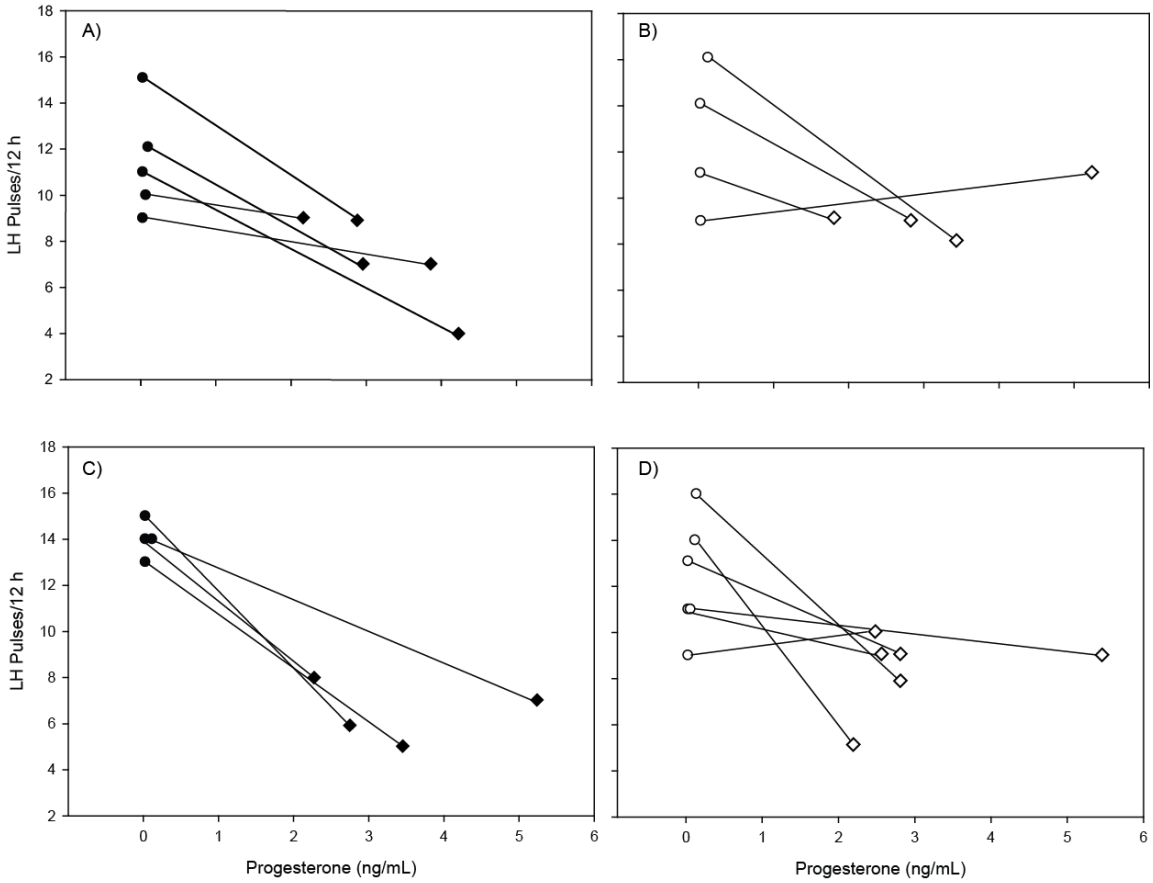

Figure 3.7 Suppression of LH pulsatility in the luteal phase compared to the early follicular phase of the menstrual cycle after 6 (A and B) and 14 (C and D) months on the WSD. Each panel shows LH pulse frequency on D1-3 (circles) and during the luteal phase (diamonds) as a function of average progesterone values on the day of blood sampling. Data from individual animals are connected by lines. Data is shown from A) T-treated animals, 6 mo WSD; B) control animals, 6 mo WSD; C) T-treated animals, 14 mo WSD; and D) control animals, 14 mo WSD.

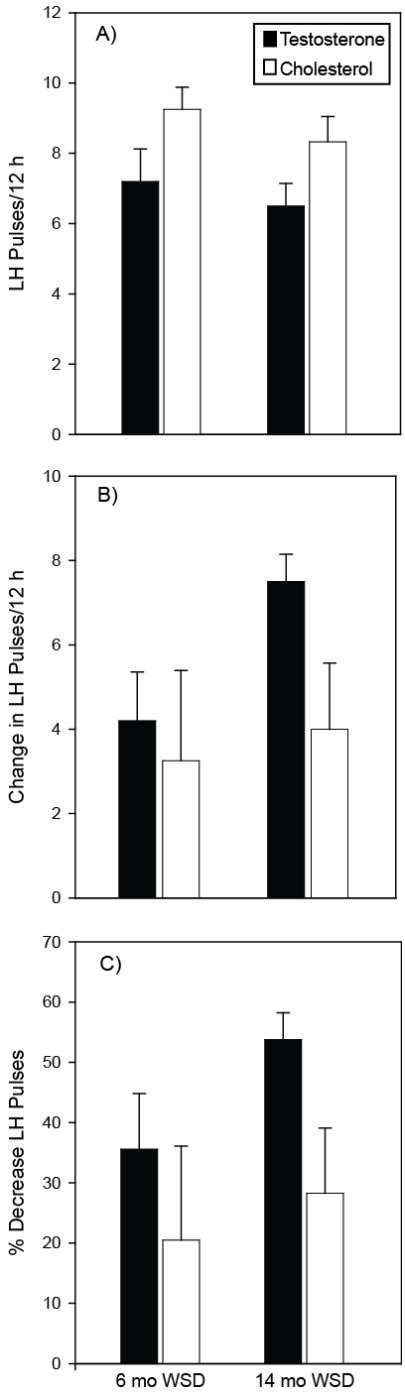

Figure 3.8 Suppression of LH pulses in response to progesterone as measured over 12 h during the early luteal phase of a menstrual cycle compared with the early follicular phase (D1-3). A) is the number of LH pulses that occurred in 12 hours during the luteal phase; B) shows the change in number of pulses when measured during the luteal phase vs. D1-3. C) shows the percent decrease in LH pulses in the luteal phase compared with D1-3. Closed bars are T-treated animals and open bars are cholesterol-treated controls.

results after 6 months on the WSD, most animals showed a decrease in pulse frequency during the luteal phase compared with the D1-3 data. Again however, there were no group differences in pulse frequency or amplitude, or percent suppression between the follicular and luteal measures (all $p>0.1$; Figs. 3.7 and 3.8).

Experiment 2. Ovarian Ultrasound

After 3 months, and again after 14 months on the WSD, the monkeys had transabdominal ultrasounds performed on D1-3 of a menstrual cycle. When analyzing data from before to 14 months after beginning the WSD, there was an increase in total follicle number [$F(2,20)=45.8$, $p<0.001$] and a decrease in maximum follicle size [$F(2,20)=7.92$, $p=0.003$; Fig. 3.9] in both groups, but there was no diet x treatment interaction and no main effect of treatment. There were no effects of time or T treatment on ovarian circumference ($p>0.1$; Fig. 3.9). After 3 months on the WSD, there was a trend toward T-treated animals being more likely to display peripheral compartmentalization of follicles, with 2/6 controls and 6/6 T-treated animals having peripheral compartmentalization ($p=0.06$). In the control animals that did display peripheral compartmentalization, only one ovary displayed this, while in the T-treated animals, 3/6 had compartmentalization in both ovaries. After 14 months on the WSD, 5/6 controls and 6/6 T-treated animals displayed peripheral compartmentalization of follicles, so there was no longer any indication of a group difference in this measure (Table 3.1).

T-treated and control animals did not differ in the number of menstrual cycles (T-treated: 14.2±0.9 cycles; Control: 13.8±0.6 cycles) or in the number or percentage of putative ovulatory cycles (as determined by elevated P during the luteal phase; T-treated:

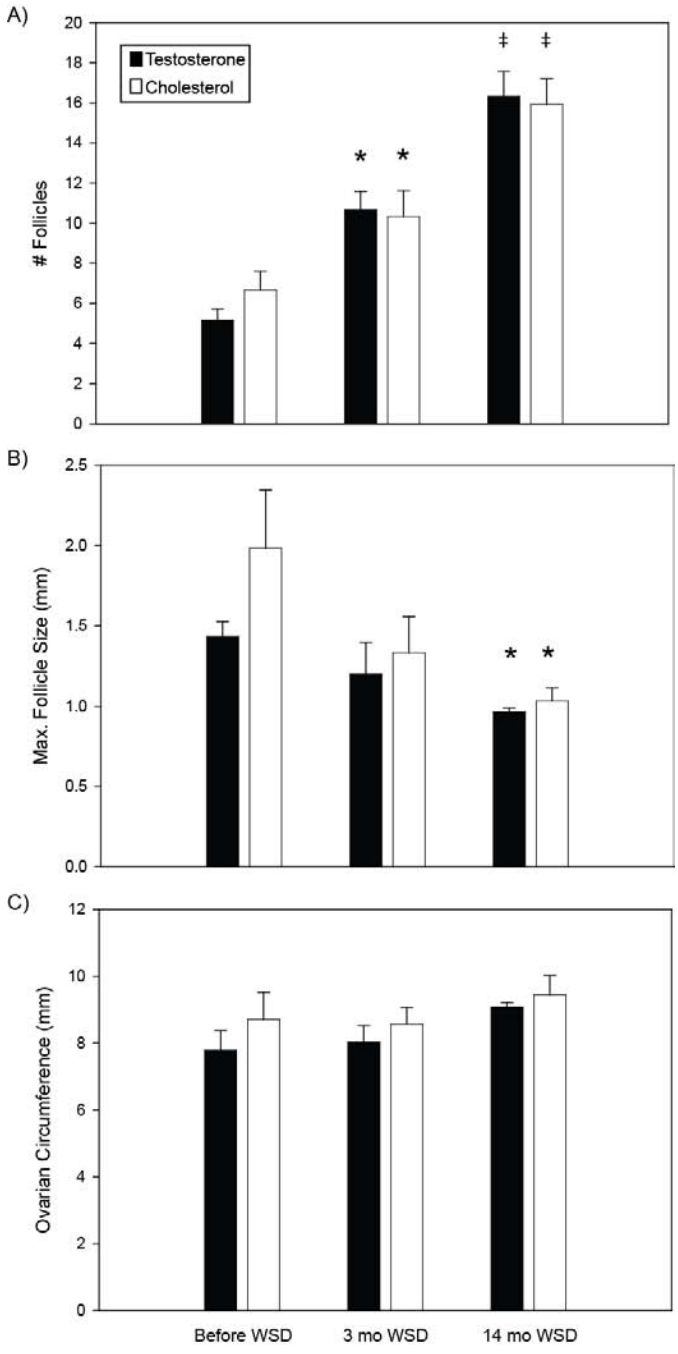

Figure 3.9 Changes in ovarian parameters from before to 14 months after starting WSD. Closed bars are T-treated animals and open bars are cholesterol-treated controls. *indicates significant difference ($p<0.05$) from before WSD, ‡ indicates significant difference ($p<0.05$) from before and 3 mo WSD.

Table 3.1 Number of animals displaying ovarian peripheral compartmentalization of follicles

		0 Affected Ovaries	1 Affected Ovary	2 Affected Ovaries
Before WSD	T-Treated	3	2	1
	Cholesterol	4	2	0
3 mo WSD	T-Treated	0	3	3
	Cholesterol	4	2	0
14 mo WSD	T-treated	0	4	2
	Cholesterol	1	2	3

84.7±4.9% ovulatory; Control: 87.7±3.1% ovulatory) that occurred when they were 5.5-7 years of age and on the WSD. In samples that were taken daily throughout one entire menstrual cycle after the animals had been on the WSD for 14 months, there was a significant group difference in peak P value, with T-treated animals having a lower peak compared with controls [T-treated: 6.8±1.1 ng/mL; Control: 12.1±1.6 ng/mL; $t(10)=2.0$, $p=0.02$; Fig. 3.10]. T-treated animals also had a lower area under the curve (AUC) when P values were assessed during the luteal phase [T-treated: 32.7±5.4 ng/luteal phase; Control: 52.4±6.0 ng/luteal phase; $t(10)=2.45$, $p=0.034$]. There were no group differences in E values measured on D1-3, peak E, FSH measured on D1-2, LH:FSH ratio on D1-2, follicular phase length, luteal phase length, or total cycle length (all $p>0.1$). These results did not change when controlled for weight.

There was no overall effect of 14 months of WSD on peak E or P or P AUC during the luteal phase when all of the animals were considered (T-treated and cholesterol treated), and this was still true when only analyzing animals that ovulated during both of the cycles that were examined (4 T-treated and 5 controls ovulated before WSD; all animals ovulated 14 mo after WSD). However, in ovulatory animals, there was a significant decrease in D1-3 E values after 14 months on the WSD [Before WSD: T-treated: 78.2±18.0 pg/mL; Control: 90.2±12.0 pg/mL; 14 mo WSD: T-treated: 53.4±10.7 pg/mL; Control: 49.8±6.1 pg/mL; $F(1,7)=5.57$, $p=0.05$; Fig. 3.10]. Cycle length also decreased significantly from before to 14 months after WSD [Before WSD: T-treated: 30.2±2.7 d; Controls: 30.6±1.9 d; 14 mo WSD: T-treated: 25.8±0.9 d; Controls: 26.8±0.6 d; $F(1,7)=8.66$, $p=0.022$; Fig. 3.10]. This was due mainly to a decrease in follicular phase length [Before WSD: T-treated: 17.0±4.1 d; Controls: 15.2±2.5 d; 14 mo WSD: T-

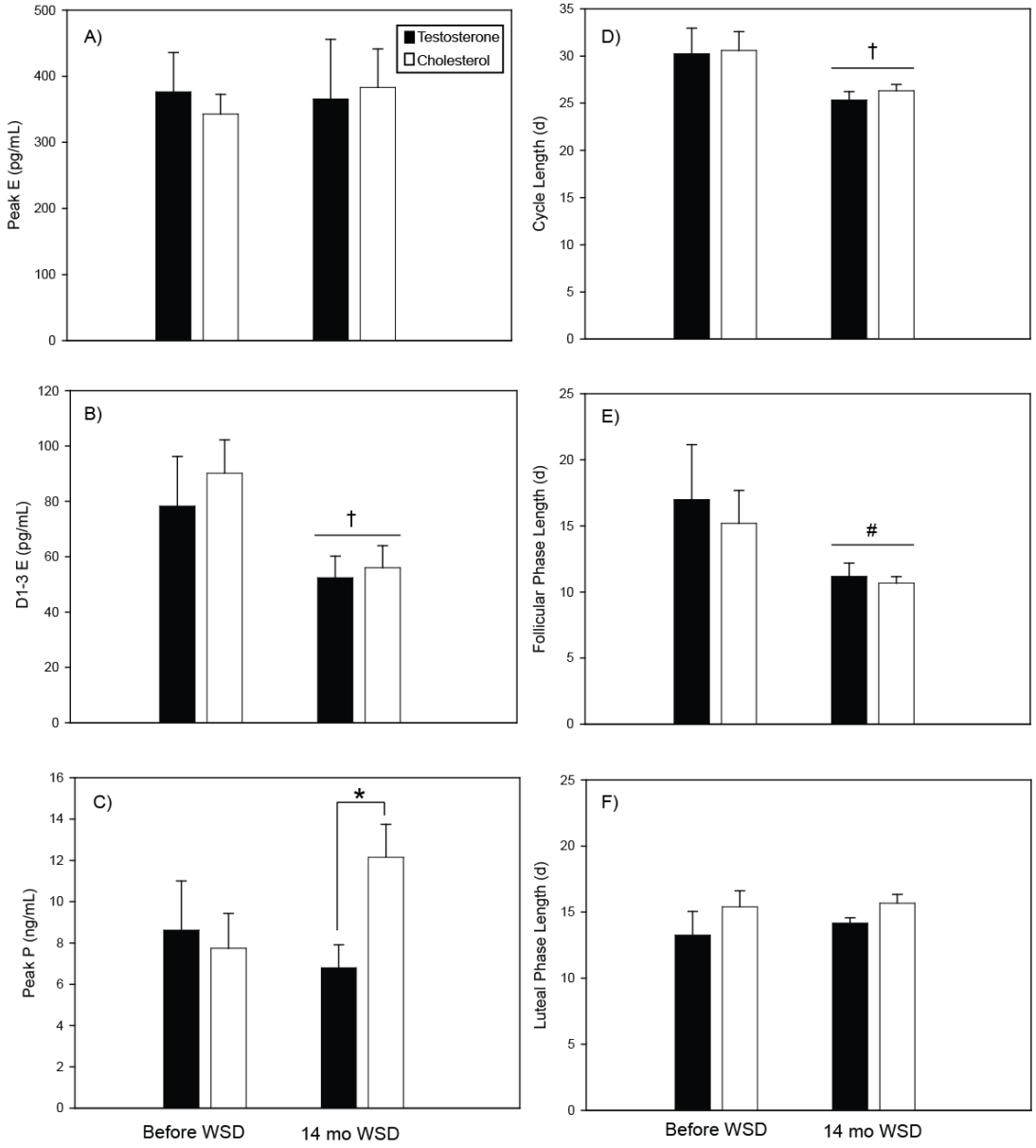

Figure 3.10 Characteristics of the menstrual cycle based on daily blood samples. Only animals that ovulated were included (n=4 T-treated and 5 controls before WSD; n=6/group 14 mo after WSD). A) Peak estrogen; B) Average estrogen from D1-3; C) Peak progesterone; D) Total menstrual cycle length; E) Follicular phase length; F) Luteal phase length. Closed bars are T-treated animals and open bars are controls. * indicates significant difference between groups, p=0.02; † indicates significant difference from before WSD, p<0.05; # indicates trend toward difference from before WSD, p=0.062.

treated: 11.5±1.2 d; Controls: 10.8±0.6 d; $F(1,7)=4.92$, $p=0.062$; Fig. 3.10], as there was no significant difference in luteal phase length. Luteal phase length was positively correlated with P AUC ($r=0.604$, $p=0.037$).

Experiment 3. Glucose Tolerance Testing

Glucose tolerance testing was performed when the animals were approximately 6.5 years old, after 14 months on the WSD. There were no differences in baseline or peak glucose, baseline or peak insulin, or in AIRg or Sg as calculated by MINMOD (all $p>0.1$). However, there was a significant difference in SI [T-treated: 5.3±1.8 $(mU/L)^{-1}min^{-1}$; Control:13.9±3.4 $(mU/L)^{-1}min^{-1}$; $t(10)=2.23$, $p=0.05$; Fig. 3.11]. There was also a significant difference in DI, the unitless product of SI and AIRg, with T-treated animals having a lower DI [T-treated: 4,223±791; Control: 7,567±1099; $t(10)=2.47$, $p=0.03$; Fig. 3.11]. These differences remained significant when controlling for weight. When compared with the GTT's that were performed when the animals were 3.5 years of age, prior to WSD consumption, there was a significant increase in peak glucose [$F(1,10)=8.0$, $p=0.018$], baseline insulin [$F(1,10)=27.1$, $p<0.001$], peak insulin [$F(1,10)=8.41$, $p=0.016$], and AIRg [$F(1,10)=10.2$, $p=0.01$]. However, there was no main effect of time on WSD on SI ($p=0.61$) or DI ($p=0.29$). Weight, percent fat, percent central fat, and changes in these variables while on the WSD were not correlated with the parameters measured using the GTT.

Luteal phase length when the animals had been on the WSD for 14 months was significantly positively correlated with SI ($r=0.909$, $p<0.001$; Fig. 3.12). There were no

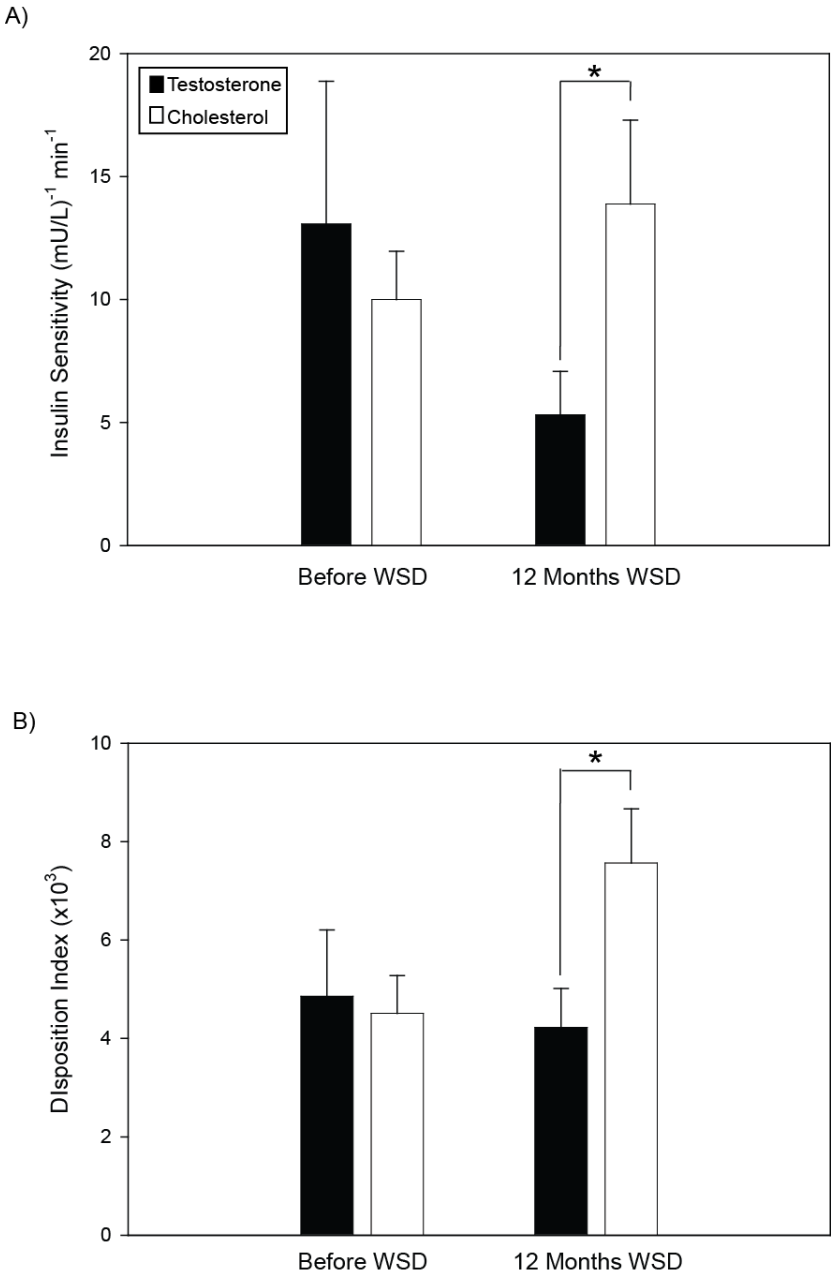

Figure 3.11 Insulin sensitivity and disposition index measured during a glucose tolerance test before and after 12 months of WSD consumption. Closed bars are T-treated animals and open bars are controls. * indicates significant group difference ($p<0.05$).

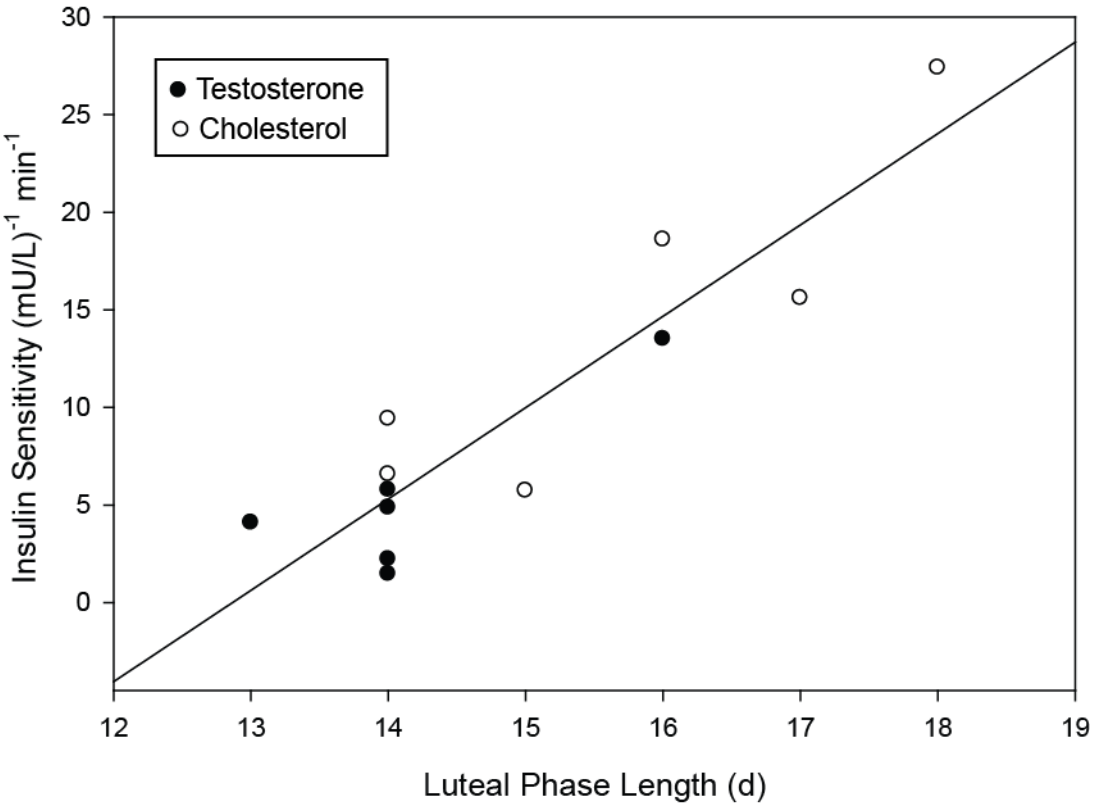

Figure 3.12 Correlation between insulin sensitivity as measured 12 months after starting WSD and luteal phase length measured during one menstrual cycle 14 months after starting WSD ($r=0.909$, $p<0.001$). Closed symbols are T-treated animals and open symbols are controls.

significant correlations between SI and follicular phase length, peak P, or P AUC (all $p>0.1$).

Experiment 4. Metabolic Testing

There were no group differences in basal metabolic rate as measured between 2300 h and 0300 h (T-treated: 1.21±0.08 kcal/kg/h; Control: 1.23±0.05 kcal/kg/h, $p=0.8$). There were also no group differences in metabolic rate measured over the rest of the 24-h period (all $p>0.1$, data not shown). There was a trend toward a main effect of time when data were compared from before and after one year of the WSD [$F(1,10)=4.192$, $p=0.068$]. Basal metabolic rate decreased in both groups over this time period (Before WSD: T-treated: 1.5±0.16 kcal/kg/h; Control: 1.84±0.37 kcal/kg/h).

Experiment 5. DEXA Scanning

There was a significant effect of diet on body composition, as percent fat and percent central fat increased significantly and percent lean mass decreased significantly during 16 months of WSD consumption (all $p<0.001$; Table 3.2). There was no effect of treatment group on percent fat, percent central fat, or percent lean mass when the monkeys were DEXA scanned before or 16 months after starting the WSD. Table 3.3 is a summary of the results from experiments performed in Chapter 3.

3.4 DISCUSSION

After being on the WSD for 16 months, the animals all showed increases in body weight and percentage body fat, as expected. While on the WSD and over the course of

Table 3.2 Body composition parameters before and 16 months after starting WSD

	Before WSD				16 Months WSD			
	Weight (kg)	% Fat	% Central Fat	% Lean Mass	Weight (kg)	% Fat	% Central Fat	% Lean Mass
Control	4.6	1.5	0.6	95.4	5.9	14.9	16.3	81.3
	(0.2)	(0.2)	(0.1)	(0.2)	(0.3)	(2.8)	(3.9)	(3.3)
T-	5.2	1.7	1.0	95.2	6.7	19.3	22.2	76.0
Treated	(0.1)	(0.3)	(0.3)	(0.3)	(0.4)	(3.8)	(4.9)	(3.6)

Data are presented as mean (SEM). There was a significant change in all parameters from before to after WSD, although there were no between-group differences.

Table 3.3 Summary of main findings from Chapter 3

Between Treatment Groups			
		p value	Direction of Significance
Experiment 1 Neuroendocrine	Pulsatile LH Secretion	NS	
	LH Response to GnRH	NS	
	P Negative Feedback	NS	
Experiment 2 Ultrasound	Follicle Number	NS	
	Max. Follicle Size	NS	
	Peripheral Compartmentalization	0.06	T>C
	Peak P	0.02	C>T
Experiment 3 GTT	Insulin Sensitivity	0.05	C>T
	Disposition Index	0.03	C>T
Experiment 4 Metabolic Testing	Basal Metabolic Rate	NS	
Experiment 5 DEXA Scanning	Weight	0.046	T>C
	% Fat	NS	
	% Central Fat	NS	
	% Lean Mass	NS	

Before vs. After WSD*			
Experiment 1 Neuroendocrine	LH Pulse Frequency (controls only)	0.004	increased
	LH Pulse Amplitude	0.007	decreased
Experiment 2 Ultrasound	Follicle Number	<0.001	increased
	Max. Follicle Size	0.003	decreased
Experiment 3 GTT	Baseline Insulin	<0.001	increased
	AIRg	0.01	increased
Experiment 4 Metabolic Testing	Basal Metabolic Rate	0.068	decreased
Experiment 5 DEXA Scanning	Weight	<0.001	increased
	% Fat	<0.001	increased
	% Central Fat	<0.001	increased
	% Lean Mass	<0.001	decreased

NS=not significant; T=T-treated animals; C=cholesterol-treated control animals; P=progesterone; *includes animals from both treatment groups unless otherwise specified

development, the central neuroendocrine drive to the reproductive axis in the control animals increased so that it was similar to that in the T-treated animals, indicating that T treatment led to earlier maturation of the GnRH pulse generator. After 14 months on the WSD, all T-treated and most of the control animals had peripheral compartmentalization of ovarian follicles, a feature that is common in women with PCOS (Barber et al. 2010; Faure et al. 1989). T-treated animals displayed compartmentalized follicles at an earlier time than controls, however, indicating that there was likely an additive effect of T and WSD on peripheral compartmentalization of ovarian follicles. T-treated monkeys also had significantly lower insulin sensitivity compared with controls, indicating that T in combination with increased adiposity had a negative effect on metabolic function. These findings indicate that the levels of androgens normally present in women with PCOS, coupled with consumption of a typical WSD, can lead to many of the neuroendocrine, ovarian, and metabolic symptoms associated with PCOS.

While consuming a WSD, there were no differences in early follicular phase LH pulse frequency or pulse amplitude between the groups. Compared with measurements taken before animals started on the WSD, LH pulse frequency increased in control animals but did not change in T-treated monkeys, such that monkeys from both groups had approximately hourly LH pulses after 6 months on the WSD, and maintained that pulse frequency after 14 months on the WSD. Women with PCOS have been found to consistently have about 1 LH pulse/hr, while women with normal, ovulatory cycles have changes in pulse frequency throughout the cycle (Filicori et al. 1986; Waldstreicher et al. 1988). Studies in healthy women, however, have also found individual variation in LH pulse frequency during the early follicular phase, with some studies reporting a frequency

of 1 pulse/90 min (Waldstreicher et al. 1988; Arroyo et al. 1997), and other studies reporting a frequency of 1 pulse/hr, not unlike what is seen in women with PCOS (Reame et al. 1984; Kazer et al. 1987). The presence of such variability in early follicular phase LH pulse frequency may have led to the lack of a group difference in pulse frequency in the current study.

The LH pulse frequency on D1-3 in control animals before the WSD (3.7±1.8 pulses/12 h) was significantly slower than any previous studies have found in healthy adult women or monkeys (Reame et al. 1984; Filicori et al. 1986; Waldstreicher et al. 1988), indicating that the GnRH pulse generator was not yet mature in these animals at 5 years of age. My finding that T-treated monkeys developed a pulse frequency of about 1 pulse/hr at a younger age than controls is indicative of earlier activation of the central neural drive to the reproductive axis, and thus faster maturation of the GnRH pulse generator in the T-treated animals. Similarly, in adolescents with HA, the transition from pubertal to adult patterns of LH secretion has been shown to occur about 2.5 years earlier than in control girls, even though age at menarche was not different (Apter et al. 1994).

It does not seem likely that the speeding of the GnRH pulse generator in controls was due to weight gain or consumption of the WSD, since LH pulse frequency in T-treated animals did not change while on the WSD. Rather, it appears that the pulse generator of control animals simply matured at a later age than in T-treated monkeys. However, it is also possible that the WSD did have an effect on maturation in the control animals, but no effect was seen in the T-treated animals because their LH pulse frequency was already close to 1 pulse/h when the WSD was begun. Studies examining the developmental increase in LH pulse frequency while maintaining animals on normal

monkey chow would be needed to determine if there was an effect of the WSD on the speeding of the GnRH pulse generator.

LH pulse amplitude significantly decreased in both treatment groups from before to 6 months after beginning the WSD, and remained lower after 14 months on the WSD. This finding is consistent with the literature from PCOS patients indicating that obesity is associated with a decrease in LH pulse amplitude (Arroyo et al. 1997; Taylor et al. 1997; Pagan et al. 2006), although not all studies have found such a change in LH pulse amplitude (Dunaif et al. 1988). Studies that have found a negative correlation between BMI and LH pulse amplitude in women with PCOS did not find correlations between BMI and LH pulse frequency (Taylor et al. 1997; Pagan et al. 2006). Therefore, the theory has been put forth that obesity acts to modulate LH release at the pituitary rather than at the hypothalamic level (Arroyo et al. 1997; Pagan et al. 2006). At least in the T-treated animals, pulse frequency was not significantly changed even though pulse amplitude decreased while on the WSD, which would support the theory that obesity modulates LH release at the level of the pituitary.

There were no differences in LH response to exogenous GnRH after either 6 or 14 months of the WSD. This was possibly due to the fact that all animals ovulated in the cycle before the response to GnRH was measured at both of these times. Blankstein and colleagues (1987) found that the LH response to GnRH was normalized in women with PCOS after ovulation was induced with human chorionic gonadotropin. It is also possible that T-treated animals had a greater LH response to GnRH only at 4 years of age (see Experiment 4 in Ch. 2) and not at any time after that because the GnRH pulse generator had fully matured in T-treated animals but had not yet reached maturity in controls at 4

years of age. By 4 years of age, the GnRH pulse generators in T-treated animals were more active, as evidenced by the higher likelihood of detecting LH pulses in T-treated animals (Experiment 1 in Chapter 2), and thus the pituitary was primed to release LH when exogenous GnRH was administered. Because the control animals were experiencing less frequent or absent LH pulses at 4 years of age, they presumably had smaller releasable stores of LH in the pituitary, so the control animals released less LH in response to GnRH than the T-treated monkeys. After the pulse generators reached full maturity in all animals, it is possible that there was no longer a detectable difference in LH response to GnRH because there were no group differences in the amount of LH stored in the pituitary.

The effect of P negative feedback on pulsatile LH secretion was also not different between the groups. Decreased sensitivity to P feedback is seen in about half of PCOS patients and HA adolescents (Pastor et al. 1998; Chhabra et al. 2005). Visual inspection of the current data (see Fig. 3.7) reveals that there are several animals that did not have a decrease in pulse number between D1-3 and the luteal phase of a menstrual cycle; however, these animals are distributed between the T-treated and control groups, so decreased sensitivity to P negative feedback was not more prevalent in T-treated compared with control animals. It is also unlikely that there was an effect of the WSD on the sensitivity of the hypothalamic GnRH neurons to P negative feedback, as the animals that had no change in LH pulse frequency were not the animals that gained the most weight or had the highest percentage fat after switching to the WSD (data not shown).

After 3 months on the WSD, there was a trend toward T-treated animals being more likely to have peripheral compartmentalization of small antral follicles in the ovary

when measured by ultrasound on D1-3 of a menstrual cycle. This is reminiscent of the 'string of pearls' phenomenon seen in women with PCOS, where follicles are localized to the periphery of the ovary and there is an enlarged central stroma (Faure et al. 1989; Buckett et al. 1999; Barber et al. 2010). After 14 months on the WSD, however, there was no longer a group difference in peripheral compartmentalization of follicles. The disappearance of the trend was due to more control animals displaying compartmentalization after 14 months on the WSD, such that only 1 animal (a control) did not display peripheral compartmentalization of follicles at this time. Importantly, a polycystic ovary phenotype is present in about 20% of healthy women who do not have other symptoms of PCOS (Polson et al. 1988; Michelmore et al. 1999). Similarly, in a group of 18 age-matched rhesus monkeys that were fed regular low-fat monkey chow and had ultrasounds performed as part of a separate study, 28% (5/18) showed peripheral compartmentalization of follicles on D1-3 of a menstrual cycle (Cecily Bishop and Mary Zelinski, unpublished data). This suggests that the increased prevalence of compartmentalization seen in the current study may be due at least in part to the WSD. There was also likely an additive effect of T, as 3 of the T-treated animals showed peripheral compartmentalization before beginning the WSD, and all of them displayed compartmentalization after only 3 months on the WSD, whereas the majority of the control animals did not display peripheral compartmentalization until 14 months of WSD consumption.

After 14 months on the WSD, T-treated animals had a lower peak P value and lower P AUC during the luteal phase compared with control animals as determined from samples that were taken daily throughout one menstrual cycle. Low levels of P during the

luteal phase in women are indicative of luteal phase defects (LPD) (Cook et al. 1983; Soules et al. 1989). LPD have been associated with abnormal gonadotropin secretion (Stouffer and Hodgen 1980; Cook et al. 1983; Homburg et al. 1988; Soules et al. 1989), poor follicular quality (DiZerega and Hodgen 1981), and stress during the follicular phase of the menstrual cycle (Xiao et al. 2002). Women with PCOS have been found to have lower levels of P than controls during the luteal phase after either spontaneous or induced ovulation, indicative of the presence of LPD in these women (Meenakumari et al. 2004). Similarly, Goy and Robinson (1982) also reported an increase in LPD in studies of female monkeys that were given high doses of androgens prenatally, even though ovulation rates were not different from control monkeys. These findings together could indicate that T may play a role in altering ovarian function in women with PCOS. Nevertheless, even though P levels in T-treated monkeys were significantly lower than control animals, they were still sufficient to maintain the secretory capacity of the uterine endometrium and allow implantation of a fertilized ovum if pregnancy were to occur. Indeed, P levels in control animals actually increased from before to after 14 months on the WSD, presenting the possibility that WSD consumption led to an increase in P secretion that was attenuated by T treatment. Future studies following the luteal phase P secretion in T-treated and control monkeys in the absence of the WSD would be needed to determine the role of the WSD on luteal phase P levels.

Metabolic changes were evident after the monkeys had been eating the WSD for 12-16 months. As expected, weight, percent fat, and percent central fat all increased with consumption of the WSD, and percent lean mass decreased. However, there was no difference in percent central fat between T-treated and control animals. Studies in

humans have found that higher T levels are associated with increased central adiposity in women (Kirchengast and Huber 2001; Lovejoy et al. 1996). Treatment with anti-androgens has been found to decrease visceral adiposity (Lovejoy et al. 1996; Gambineri et al. 2006), indicating that androgens are likely the cause of increased central fat, as opposed to the increased central adiposity leading to increased levels of androgens. I therefore expected the T-treated monkeys in this study to have higher levels of central adiposity compared with the control animals, but I found that this was not the case. It is possible, however, that if the animals had been kept on the WSD for a longer period of time and continued to gain weight, a group difference might have emerged.

When post-pubertal weight gain was assessed over time (3.5-7 yrs), the T-treated animals gained more weight than controls. Although T-treated monkeys did not eat more kcal than control animals, there was a strong trend ($p=0.052$) toward the T-treated animals being less active than controls during the last 10 months of this study. Studies in humans and animals have found that individuals that are less active tend to gain more weight (Klesges et al. 1992; Brown et al. 2005; Sullivan et al. 2006), which is consistent with the data presented here. In the rodent literature, it has been well-established that males are less active than females, leading to the hypothesis that gonadal hormones may play a role in determining physical activity levels (for review, see Lightfoot 2008). Few studies have examined the effects of T supplementation on activity in intact female rodents, but in those that have, no effect of T was found on physical activity level (Li and Huang 2006). Contrary to the rodent literature, the majority of research in humans, using both self-report questionnaires and accelerometers, has found that men are more active than women (Pate et al. 1994; Troiano et al. 2008), although some studies found no

gender differences in activity (Matthews et al. 2002). In monkeys, there do not appear to be gender differences in activity either pre-pubertally or post-pubertally (Ramsey et al. 2000; Cardenas et al. 2007). Regrettably, in the current study, activity levels were not assessed before T implants were placed, so it is unknown if the T-treated animals had lower levels of activity at baseline or whether the decreased activity levels occurred later as a result of chronic T treatment.

Metabolic changes were also evident between the groups after 12 months on the WSD. Insulin sensitivity scores were significantly lower in T-treated compared with control animals, even after controlling for weight. Similarly, women with PCOS have lower insulin sensitivity than weight-matched controls, and this relationship is present even in lean PCOS patients (Dunaif et al. 1989; Carmina et al. 2007). Unlike what is seen in PCOS women, I did not find any group differences in insulin sensitivity when the monkeys were tested at 3.5 years of age. This suggests that while T treatment alone did not lead to decreased insulin sensitivity in this study, T treatment in combination with adiposity did have a more detrimental effect on insulin sensitivity than adiposity alone.

Studies by Abbott and colleagues (1997) found insulin resistance and hyperinsulinemia in all monkeys they tested that had a high BMI. However, animals that were prenatally androgenized (PA) in addition to being obese and insulin resistant were more likely to be anovulatory than animals that were obese but hadn't been exposed to T during gestation (4/5 obese PA females were anovulatory vs. 0/4 obese controls). There were no differences in ovulation rates in PA and control females that were of average to low BMI. I did not see any differences in ovulation rates during the current study; however, a BMI under 41.2 kg/m^2 was considered "average to low" in Abbott's study, so

differences in ovulation were only seen in very obese animals. The average BMI after 16 months on the WSD in the current study was 25.6 kg/m^2, and the highest was 34.6 kg/m^2, so it is possible that some of the T-treated animals may have become anovulatory if they had continued to gain weight on the WSD.

Although I did not see any group differences in ovulation rates in the current study, there was a relationship between metabolic and ovarian parameters. The animals with lower insulin sensitivity also had shorter luteal phases, which can be an indicator of LPD (Soules et al. 1989). Decreased SI and anovulation are both common in women with PCOS, and when these women do ovulate, they often have LPD (Meenakumari et al. 2004). Thus, it is interesting that the animals with decreased SI were the same animals that had shorter luteal phases in this study, and it is possible that the T-treated monkeys may have developed higher rates of anovulation with continued exposure to elevated T. In the present study, SI was not significantly correlated with peak P or P AUC, other measures of luteal function. However, luteal phase length and P AUC were positively correlated, which suggests that a correlation between SI and P AUC might have emerged with a larger sample size or after a longer duration on the WSD.

There was a decrease in basal metabolic rate from before to 12 months after the animals began eating the WSD. This was likely due to aging, as metabolic rate tends to decline over puberty in people (Brown et al. 1996) and continues to decline with age (Henry 2000; Roberts and Dallal 2005; Ruggiero et al. 2008). The decrease in metabolic rate also could have been a result of a decrease in activity, as decreased activity is associated with decreased metabolic rate (Lawson et al. 1987; Poehlman and Danforth 1991). Activity levels were fairly stable over the 10 months that they were measured in

this study, but activity decreases over puberty in monkeys (Cardenas et al. 2007), so there is a possibility that activity decreased between the first time that metabolic rate was tested (when the animals were 4.5 years old) and when we started monitoring activity (when the animals were almost 6 years old). The lack of group differences in metabolic rate indicates that the change in metabolic rate over time is likely not attributable to T.

Obesity has been associated with increased levels of free T in women in some (Evans et al. 1983; Strain et al. 2003; Taponen et al. 2003), but not all (Bordini et al. 2009), studies. Taponen and colleagues (2003) found that levels of circulating sex hormone-binding globulin (SHBG), the main androgen-binding protein, decreased with increasing BMI in women, such that overweight women (25<BMI<30) had free T levels that were about 50% higher than normal weight women (BMI<25). Strain and colleagues (2003) found that obese women as a group had higher free T than lean women, but they did not find a correlation between BMI and free T. Not all studies have found a relationship between weight and free T, however. A study by Bordini and colleagues (2009) examined SHBG and free T in pubertal girls and found no differences between normal weight and overweight girls. Importantly, in the current study, there was no increase in total T level in the control animals after weight gain on the WSD. SHBG was not measured in this study due to the lack of availability of a reliable assay to measure SHBG in monkeys, so there is a small chance that free T levels did increase in control animals after weight gain. However, it is unknown whether SHBG is similarly affected by weight gain in monkeys as it is in humans.

Although increased adiposity as a result of the WSD did not appear to act in concert with T treatment to aggravate neuroendocrine dysfunction in the T-treated

animals, there were reductions in insulin sensitivity and P secretion compared with controls, indicating that even small increases in T can lead to deficits in reproductive and metabolic function. These deficits may have resulted from the extended length of T treatment, or due to the combination of T and WSD. The results from this study agree with previous findings that adiposity contributes to the severity of the PCOS phenotype. Due to the lack of a control group that was not fed the WSD, it is difficult to definitively state what role the WSD played in contributing to changes in insulin sensitivity and ovarian function in the T-treated group in the current study. It may have been that T-treatment alone for a prolonged time post-pubertally would have led both to luteal dysfunction and reductions in SI. Sheep treated prenatally with androgens have developed ovarian and metabolic abnormalities in the absence of excess adiposity (Veiga-Lopez et al. 2010). However, previous studies in monkeys have been less clear regarding the role of T vs. obesity. Monkeys that had been treated prenatally with T had decreased DI compared with weight-matched control animals in adulthood (Eisner et al. 2000); however, the animals used in that study had an average weight that was equivalent to the heaviest animal that was utilized in my study, suggesting that weight and adiposity may have also influenced the findings of Eisner et al. (2000). Furthermore, monkeys treated with chronic T in adulthood did not show changes in SI (Billiar et al. 1987). Monkeys exposed to androgens during gestation have been reported to experience delayed menarche and short luteal phases as adolescents (Goy and Robinson 1982), although again, weight was increased in the T-treated animals compared with the controls in that study. Monkeys treated with androstenedione during adulthood also had an increased incidence of anovulation in addition to increased numbers of ovarian follicles

compared with weight-matched controls (Billiar et al. 1985). Future studies that include both T-treated and control monkeys maintained on normal monkey chow would likely help clarify the role of adiposity compared with the role of normal pubertal development and aging on the neuroendocrine, reproductive, and metabolic parameters that were measured in the current study.

Chapter 4

MILD HYPERANDROGENEMIA IN FEMALE MONKEYS IS ASSOCIATED WITH SLIGHTLY LOWER LEVELS OF ANXIETY BUT NO INCREASE IN AGGRESSION

4.1 INTRODUCTION

Gender differences have been found in aggressive behavior in many species, including humans, with males typically showing more aggression than females (Barr et al. 1976; Rothstein and Griswold 1991; Bales and Carter 2003; Burton et al. 2007). This observation has led to the theory that increased levels of androgens are associated with aggression, a theory that has been supported by numerous studies in both animals and people (Brooks and Reddon 1996; Beehner et al. 2005; Pajer et al. 2006). Castrated male rats show decreased aggression and both male and female rodents given exogenous testosterone (T) as adults show an increase in aggressive behavior (Barfield et al. 1972; Albert et al. 1989). Female monkeys treated with T prenatally or as juveniles have also been shown to have increases in aggression (Goy and Resko 1972; Joslyn 1973). However, most studies that have examined this behavioral effect of T in females have used only high doses of androgens, with fewer studies (Trimble and Herbert 1968; Albert et al. 1989) focusing on the effects of the relatively mild elevations in androgens that are common in women with hyperandrogenemia (HA) (Eagleson et al. 2003; McCartney et al. 2006). The studies focusing on mild elevations in androgens have found mixed results, with some reporting an increase in aggression (Albert et al. 1989), and others failing to see any differences in aggression (Trimble and Herbert 1968).

There is a somewhat weaker link between T and another temperamental state, anxiety. A greater prevalence of generalized anxiety disorder and social phobia has been reported in women who have lower plasma levels of T compared with women who have higher plasma T levels (Giltay et al. 2012). One common measure used to assess anxiety in people and animals is behavioral inhibition, or a general aversion to novelty (Garcia Coll et al. 1984; Kagan et al. 1988; Rogers et al. 2008). There is evidence that young children who show increased levels of behavioral inhibition are more likely to suffer from anxiety disorders when they are older (Hirshfeld et al. 1992; Chronis-Tuscano et al. 2009). On the other end of the spectrum, a decrease in behavioral inhibition has been considered to be an indicator of higher levels of impulsivity (Evenden 1999; Congdon et al. 2008; Potter et al. 2012). Higher T has been linked to decreased behavioral inhibition in several studies using rodents (Feng et al. 2011) and people (Reavis and Overman 2001). However, as for aggression, very little is known about whether the elevation in androgens that occurs in women with mild HA will influence anxiety levels.

In this study, I asked if mild HA, resulting from a low but sustained increase in circulating T, could lead to changes in aggression or anxiety in female monkeys. Two well-studied and commonly utilized temperament tests, the Human Intruder and Novel Objects tests (Kalin and Shelton 1989; Mason et al. 2006; Sullivan et al. 2010), were used to examine temperamental characteristics of the monkeys. I hypothesized that T-treated animals would display more aggressive behavior and would also be less behaviorally inhibited than control animals. As part of the ongoing study described in Chapter 3, the animals used here were switched from a regimented amount of monkey chow to *ad libitum* feeding of a highly palatable and higher fat-containing diet one year before the

behavior testing was performed. Because obesity has been associated with increased impulsivity in many studies in people (Graziano et al. 2010; Pauli-Pott et al. 2010; Brogan et al. 2011), I also asked whether weight gain in these animals was correlated with any of the behavioral traits that were assessed in this study.

4.2 MATERIALS AND METHODS

Animals

Twelve female rhesus macaques (*Macaca mulatta*), 6.5 years of age, were utilized for this study, and were the same animals that were utilized in Chapters 2 and 3 of this dissertation. They were housed individually in single cages (81 x 61 x 69 cm) in a temperature-controlled room (24±2°C), with lights on for 12 h/day (0700h-1900h). Until the monkeys were 5.5 years of age, they were fed two meals of Purina LabDiet fiber-balanced monkey chow each day (no. 5000; Purina Mills, St. Louis, MO; 15% calories from fat, 27% from protein, 59% from carbohydrates), supplemented with fresh fruits and vegetables. At the time of these experiments, animals had been on a Western style diet (WSD; 33% calories from fat, 17% from protein, 51% from carbohydrates; 5A1F, Purina Mills, St. Louis, MO; Shadoan et al. 2003; Sullivan et al. 2010) for one year. The animals were fed the WSD *ad libitum* and each meal was supplemented with a high-sugar, high-calorie treat (i.e. cookie, snack cake, graham cracker with peanut butter, etc). All procedures in this study were reviewed and approved by the ONPRC Institutional Animal Care and Use Committee.

Testosterone Implants

Six monkeys were treated with low dose T (in the form of Silastic implants) continuously from one year of age through the completion of this study. The other six monkeys were controls that had Silastic implants filled with cholesterol over the same period of time. T implants were maintained as described in Chapters 2 and 3. Normal T levels in prepubertal female rhesus macaques were determined by assaying serum from four 12-month-old female monkeys in the ONPRC colony. The average T value (0.4 ng/mL) was multiplied by 3 to achieve the lower limit (1.2 ng/mL), and by 4 to achieve the upper limit (1.6 ng/mL) of target values in the T-treated animals. To determine the size of T implant needed, monkeys from the ONPRC colony, which were not used for this study, had implants of various lengths and T:cholesterol ratios placed subcutaneously under ketamine hydrochloride (Ketaset, 10 mg/kg i.m., Wyeth, Madison, NJ) sedation. Blood samples were taken daily to determine which implants resulted in a sustained 3-4-fold increase in T levels. Once an appropriate implant size was determined, all animals used in this study received either a T- or cholesterol-containing (n=6/group) implant at one year of age. Implants were made of Silastic tubing (Dow Corning, Midland, MI) and were initially 5 mm in length with an inner and outer diameter of 0.335 cm and 0.465 cm, respectively. Implants were filled with cholesterol (control animals), or a T/cholesterol mixture (T-treated animals), with a T:cholesterol ratio of 1:15 or 1:12 at the beginning of the experiment. As the animals grew, the length of the implant increased to 3 cm and the T:cholesterol ratio was increased gradually to 1:4 to maintain the desired serum T levels. Both cholesterol and T were purchased from Sigma Aldrich (St. Louis, MO).

Blood Collection and Steroid Hormone Assays

To collect blood samples for tracking serum T concentrations, animals were trained to jump from their cage into a portable transport box and were carried into a nearby room. The transport box door was opened and they were transferred to a specially designed cage and trained to present their leg for blood collection from the femoral vein (Hunnell et al. 2007). Weekly blood samples (2 mL each) were collected from each animal, allowed to clot at room temperature for >1 h and refrigerated overnight. Samples were then centrifuged at 3000 rpm for 15 min at 4°C and serum was removed and stored at -20°C until assays were performed. Each week's samples were assayed for T and when an individual animal's serum T concentration fell below the threshold of 1.2 ng/mL, the implant was changed. Cholesterol implants were also changed regularly so that cholesterol-treated (i.e., control) animals received the same average number of implant surgeries as the T-treated animals. T was measured using a radioimmunoassay (RIA) kit (DSL-4100, Diagnostic Systems Laboratories, Inc, Webster, TX) by the Endocrine Services Core Laboratory at the Oregon National Primate Research Center. The sensitivity of the T assay was 0.05 ng/mL and the intra- and inter-assay coefficients of variation for the assays were 2.23% and 4.00%, respectively. Blood samples were drawn to quantify serum estradiol (E) and progesterone (P) concentrations. Both E and P were assayed by the Endocrine Services Core using the Immulite 2000 platform. As with many validated clinical platforms, the Immulite 2000 runs three QC serum pools daily and as such, no specific intra-assay QC data is available. The inter-assay coefficient of variation, reflecting variability in daily QC results over the period in which these assays were performed was 8.5% for E and 9.4% for P.

Cortisol Measurement

Cortisol was measured in blood samples that were taken as part of the experiments described in Chapter 3 to assess pulsatile LH secretion. Samples were taken approximately 2 months after behavior testing occurred. Briefly, blood sampling was performed through the remote sampling system and occurred on D1-3 of a menstrual cycle when the animals were in a non-stressed state. Samples were taken over a 12-hour period, at 1300h, 1600h, 1900h, 2200h, and 0100h. Cortisol was measured by electrochemiluminescence immunoassay using a Cobas e411 (Roche Diagnostics, Indianapolis, IN). The intra-assay coefficient of variation was 2.7%.

Physical Activity

Physical activity levels were assessed continuously from 6 months before until 4 months after the behavior testing was performed. Animals wore jackets with a pocket housing a three-way accelerometer (Actical®, Respironics, Bend, OR, USA). The monitors were programmed to record total activity counts per minute. Data was downloaded approximately once every three weeks.

Experimental Protocols

Experiment 1. Human Intruder Test

The Human Intruder Test (HIT) was initially designed by Kalin and Shelton (1989) to measure behavioral responses to both threatening and non-threatening social stimuli. Between 1000 h and 1200 h, monkeys were transferred into a single cage (81 x 61 x 69 cm) mounted at eye level in a novel room and were left alone for a 10-min

acclimation period. Animals were videotaped from behind a blind for the duration of the experiment. After the acclimation period, the test was broken into five 2-min periods, the first of which was a control period with no human present (Control 1). Following this, a human intruder (whom the animals had never seen) entered the room and stood 1 m from the cage, presenting their facial profile to the animal (non-threatening stimulus) and taking care not to make eye contact with the monkey (Profile). The intruder exited the room and the monkey was left alone for another control period (Control 2). The human intruder then entered the room again and stood in the same location, this time maintaining direct eye contact with the animal, which is a threatening social stimulus (Stare). The test concluded with the animal being left alone for a final control period (Control 3).

Experiment 2. Novel Objects Test

Immediately following the HIT, the Novel Objects test was performed. The same human intruder presented the animal with a series of five objects, which were left with the monkey for five min each. For the presentation of each object, the intruder quickly entered the room, removed the old object from a tray mounted on the front of the cage (except the fruit if the monkey had taken it into the cage or eaten it), introduced the new object, and quickly left the room, avoiding eye contact with the animal at all times. The first object was a piece of novel fruit (kiwi). The second object was a piece of apple, which is a familiar and highly palatable food. The third novel object was a toy with large eyes that were directed toward the monkey [Mr. Potato Head (Hasbro, Pawtucket, RI, USA), with only the eyes and feet attached]. This was a potentially threatening stimulus, as direct eye contact or staring is seen as a threat by monkeys (Hinde and Rowell 1962;

van Hooff 1962). The fourth object was a coiled rubber snake that was presented with a piece of apple on top, arranged so the monkey had to reach over the snake (potentially threatening novel object; Vitale et al. 1991) to obtain the apple (desired object). The final novel object was a colorful plastic toy (a non-threatening novel object) that was hung on the outside of the front of the cage. The order of presentation of the objects was not counterbalanced due to the small sample size. Figure 4.1 is an illustration of the sequence of events in the HIT and Novel Objects test.

Behavioral and Statistical Analyses

Videos were scored using Observer software (v.5.0, Noldus, the Netherlands), and previously described ethograms (Williamson et al. 2003; Coleman et al. 2003; Sullivan et al. 2010). Behaviors scored included movement, vocalizations, responses to the human intruder or novel objects, and latency to inspect, touch, or eat (fruit tests only) the objects.

Independent sample *t*-tests were used to examine group differences. If a variable had a non-normal distribution, logarithmic, square root, or arcsine transformations were tested to determine if they would normalize the distribution. If transformations were unsuccessful at normalizing the data, then a non-parametric Mann-Whitney U was performed. To compare the same measure across different epochs of the testing period, repeated measures ANOVAs were performed using paired t-tests for post hoc follow-up. Exploratory analyses were performed to look at correlations between behavioral measures assessed here and weight gain when the animals were fed *ad libitum* as part of the ongoing study described in Chapter 3. Pearson r correlations were used to examine relationships between normally distributed variables, and Spearman's rho was used to

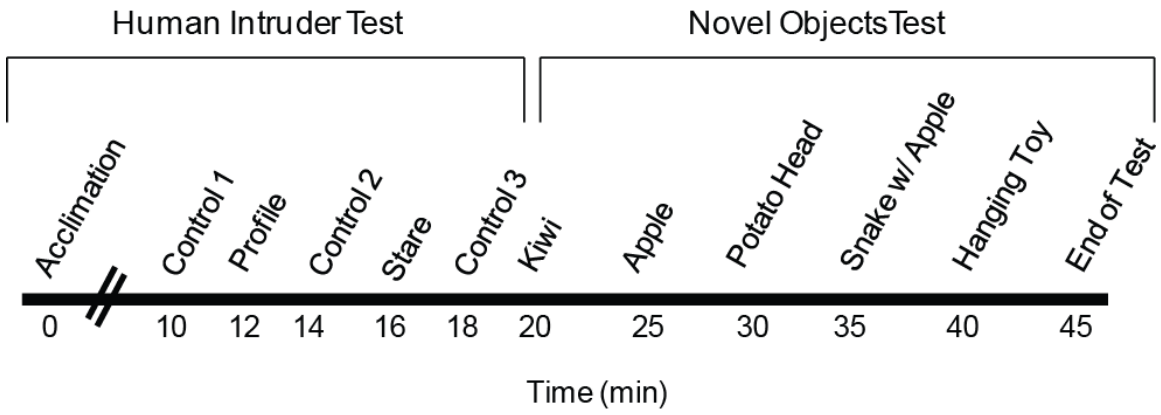

Figure 4.1 Sequence of events during the Human Intruder and Novel Objects tests.

examine relationships between non-normally distributed variables. A mixed measures ANOVA was used to assess weight gain over time. Statistical analyses were performed using IBM SPSS Statistics 20 (SPSS Inc, Chicago, IL). Values are presented as mean ± SEM. Significance was set at $p \leq 0.05$.

4.3 RESULTS

Aggression

There were no group differences in aggressive behavior (threatening facial expressions, lunging at intruder, shaking cage) during the Stare portion of the HIT (percent time displaying aggressive behavior: T-treated: 24.9±8.5%; Control: 19.2±7.0%; $p>0.1$). No animals from either treatment group showed aggressive behavior toward the Mr. Potato Head toy when it was presented during the Novel Objects test.

Group Differences in Behavioral Inhibition

Behavioral inhibition during the 10-min acclimation period, measured as percentage of time spent immobile, was not different between groups (T-treated: 74.5±6.6%; Controls: 76.5±3.3%, $p=0.79$). Animals in both groups spent an increased percentage of time immobile during the Profile portion of the HIT compared with the acclimation period, and this was not different between groups (T-treated: 94.1±3.1%; Controls: 97.4±1.4%, $p=0.001$ vs. acclimation). There was no effect of treatment, but there was a main effect of period when examining percent time spent immobile across the three control periods [Control 1: 86.5±2.7%; Control 2: 81.4±3.8%; Control 3: 67.8±6.4%; $F(2,10)=6.47$, $p=0.007$]. This was driven by a decrease between Control 2

and Control 3 in percent time spent immobile [t(11)=2.46, p=0.032]. Compared with control monkeys, T-treated monkeys spent a slightly (but significantly) smaller portion of their time immobile during the presentation of Mr. Potato Head [T-treated: 71.1±4.9%; Control: 85.8±4.1%; t(10)=2.47, p=0.033; Fig. 4.2]. There was also a trend toward T-treated animals having a shorter latency to touch the apple when it was presented alone during the Novel Objects test [T-treated: 3.8±1.6 s; Control: 96.7±56.9 s; t(10)=2.00, p=0.073, data not shown]. There were no group differences in any other measures (latency to inspect, touch, or eat the fruit or other novel objects; all p>0.1).

Cortisol

The nadir in cortisol values occurred in the 1900h or 2200h sample in all monkeys, and these values were not different between the groups (T-treated nadir: 26.2±4.4 ng/mL; Control: 32.6±9.1 ng/mL; p>0.1). There was also no difference in area under the curve over the 12-hour period (T-treated: 621.9±50.7 ng/mL/12h; Control: 761.1±90.3 ng/mL/12h; p>0.1). Cortisol levels were not significantly correlated with any measures of aggression or behavioral inhibition (all p>0.1; data not shown).

Relationship between Behavioral Inhibition and Weight Gain

There was a strong correlation between activity during the acclimation period of the HIT (i.e., decreased behavioral inhibition) and percent weight gain that occurred

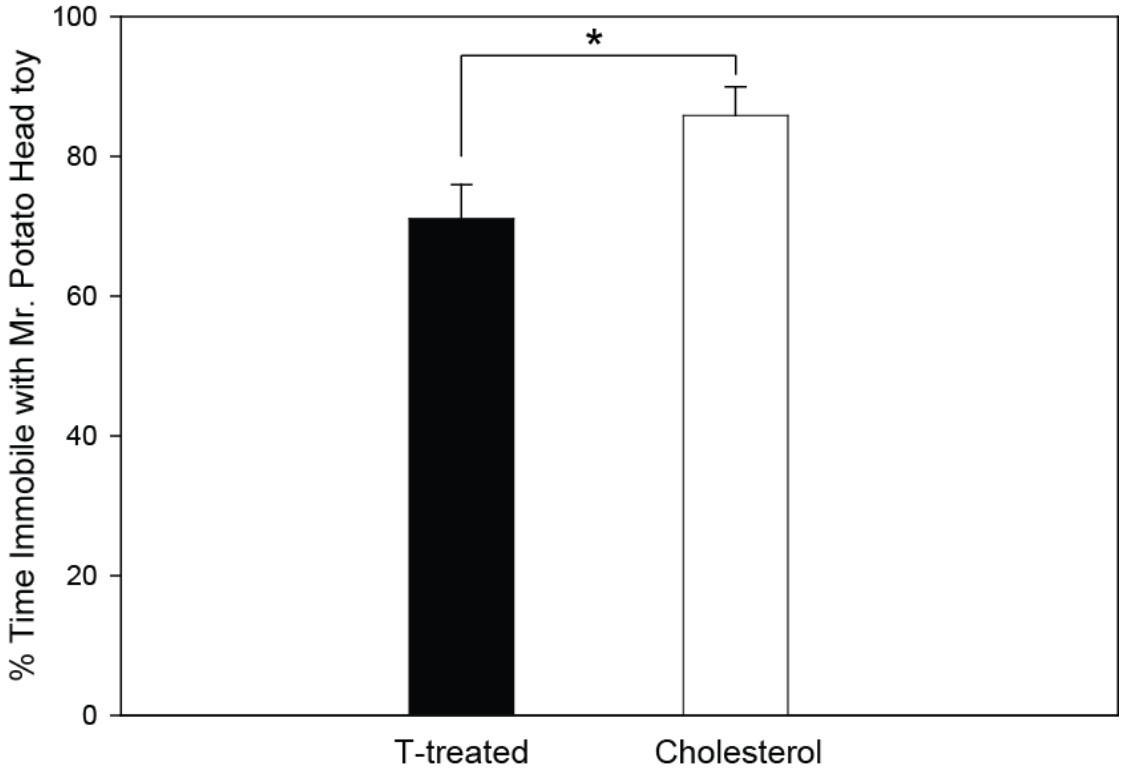

Figure 4.2 Percent time spent immobile during the 5-min presentation of Mr. Potato Head. * indicates p=0.033

during *ad libitum* feeding (r= 0.810, p=0.001; Fig. 4.3A), such that animals that were more active during the acclimation period gained more weight on the WSD. There was also a correlation between caloric intake on the WSD and activity during the acclimation period of the HIT when the animals from both groups were analyzed together (r= 0.704; p=0.011, Fig. 4.3B). When the treatment groups were analyzed separately, the correlation was significant in the T-treated animals (r=0.880, p=0.021), but not the control animals (r=0.617, p=0.192). There was no correlation between activity during the acclimation period and home cage activity for the 2-week period surrounding the HIT (r=0.115, p=0.722). There was also no correlation between average home cage activity and weight gain on the WSD. Spearman's rho revealed that there was a significant negative correlation between latency to inspect the snake with the apple and activity during the acclimation period (r=-0.648, p=0.023), such that monkeys that were less behaviorally inhibited during the acclimation inspected the snake with the apple faster. Table 4.1 is a summary of the findings from the experiments performed in Chapter 4.

4.4 DISCUSSION

The results from this study did not support my hypothesis that the T-treated animals would show more aggression than control animals, indicating that the small elevation in T levels may not have been sufficient to lead to increased aggressive behavior. However, my hypothesis that the T-treated monkeys would have lower levels of behavioral inhibition compared with control animals, as measured by their responses to novelty, was supported. The T-treated animals were more active when exposed to a potentially threatening stimulus (Mr. Potato Head) and were faster to touch a piece of

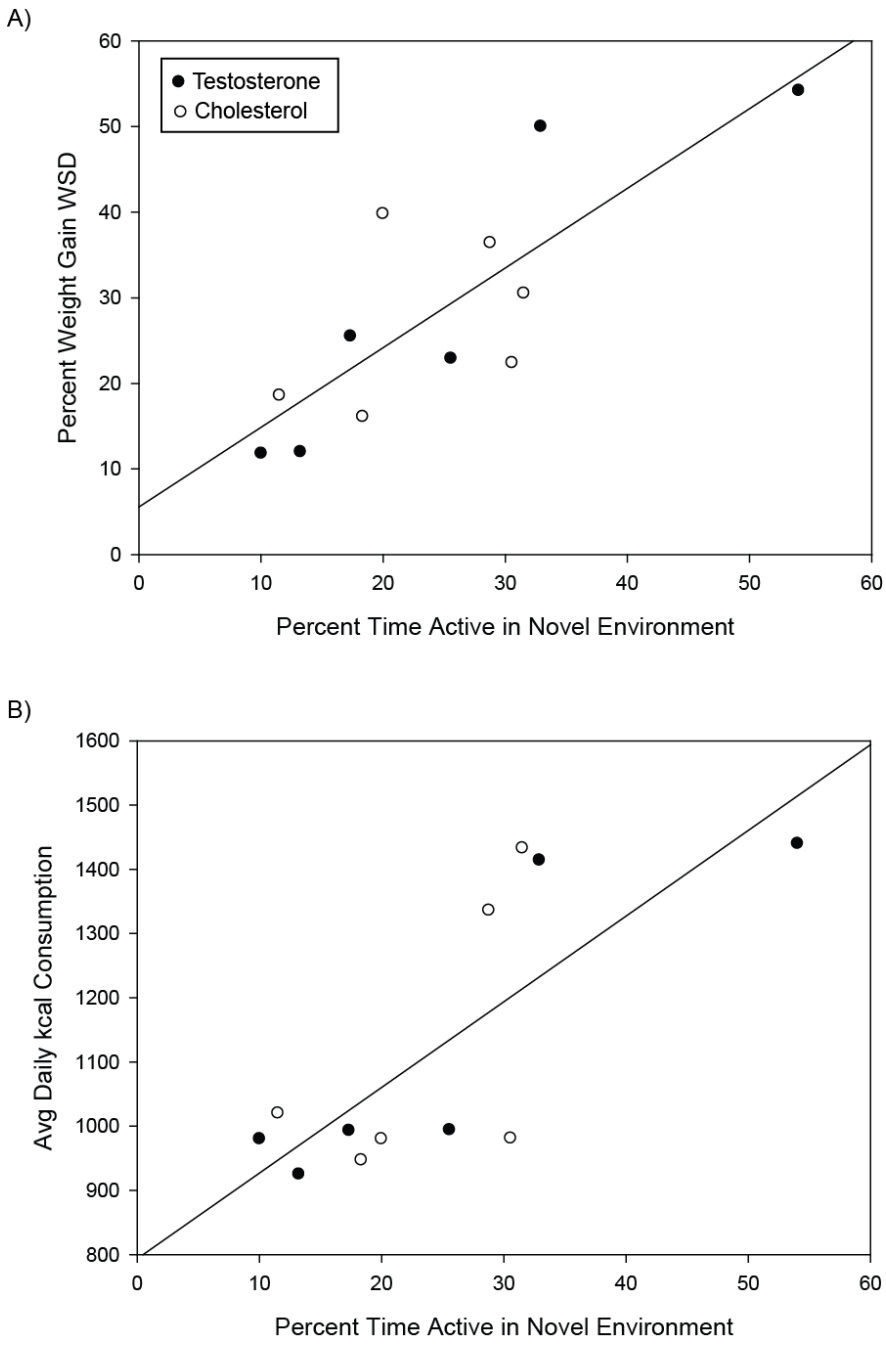

Figure 4.3 A) Correlation between percent weight gain on WSD and activity during the first 10 minutes in a novel environment (r= 0.813, p=0.001). B) Correlation between average daily kcal consumption and percent time active in a novel environment (r=0.704, p=0.011). T-treated animals are shown in closed circles and controls in open circles.

Table 4.1 Summary of main findings from Chapter 4

Measure	**p value**	**Direction of Difference**
Aggression	NS	
Immobility during Mr. Potato Head Epoch	0.033	C>T
Latency to Touch Apple	0.073	C>T

Correlations with Activity in Novel Environment	**r value**	**p value**
Weight Gain on WSD	0.810	0.001
Latency to Inspect Snake with Apple	-0.648	0.023

NS=not significant; T=T-treated animals; C=cholesterol-treated control animals

food (apple) in a novel environment when there was no threatening stimulus present. Both of these findings could be interpreted as the T-treated animals displaying increased levels of impulsivity compared with control animals. Interestingly, although it was not a main hypothesis of this study, I also found a very strong correlation between decreased behavioral inhibition in a novel environment and increased food intake and weight gain when the animals were fed the WSD *ad libitum*. This correlation was present in both groups, indicating that the phenomenon was not related to T treatment, but rather appears to be an underlying behavioral trait. Animals that were less behaviorally inhibited in the novel environment were also significantly faster to inspect a potentially threatening stimulus (the snake with apple), suggesting that these monkeys were more impulsive, and that more impulsive animals are more likely to eat more and gain more weight when a novel, palatable food is made available.

Animals from both treatment groups displayed aggressive behavior (predominantly threatening facial expressions) during the Stare portion of the HIT, but there were no differences between the groups in the number of animals that displayed threatening behavior or in the amount of time that the animals spent displaying threatening behavior. Additionally, none of the animals from either treatment group displayed any aggression toward the Mr. Potato Head toy. It was initially hypothesized that aggressive behavior might occur toward the toy due to its prominent eyes, because eye contact is seen as a direct threat by the monkeys (Hinde and Rowell 1962; van Hooff 1962), and monkeys and baboons have both been reported to show aggression toward similar toys (J. Rogers and E. Sullivan, personal communications). It is possible, however, that because the toy could not move to maintain direct eye contact with the

animals, it was perceived as being more similar to the Profile portion of the HIT (when animals remained stationary and were not reacting to the intruder) than the Stare portion of the HIT (when animals displayed aggressive behaviors toward the intruder). The association between high levels of T and increased aggression is fairly consistent in the literature (Barfield et al. 1972; Schechter et al. 1981; Brooks and Reddon 1996; Pajer et al. 2006). In female monkeys that were treated prenatally with T and exposed to levels comparable to those seen in male fetuses, the females made more threatening facial expressions compared with control females at 12 months of age (Goy and Resko 1972). High doses of T (25 mg/day for 3 weeks) in ovariectomized adult female monkeys also increased aggression towards males, while lower levels of T (1 or 5 mg/day for 3 weeks) did not increase aggressive behaviors towards males (Trimble and Herbert 1968). Similarly, in the current study, I found no evidence that a very mild increase in T level was sufficient to increase aggressive behavior in intact young adult female monkeys.

The HIT has been widely employed and is successful at eliciting aggressive behaviors from monkeys (Kalin and Shelton 1989; Coleman et al. 2003; Sullivan et al. 2010). Most of the monkeys in this study did show aggressive behavior toward the human intruder, with the greatest response to the intruder being the display of aggression for 47% of the Stare period. Nevertheless, it is possible that the elicitation of aggressive behavior was not as great in this test as if the monkeys had been exposed to other unfamiliar monkeys. Behavior testing was also not performed before T treatment commenced; therefore, it is unknown whether baseline differences in aggression between animals may have masked an effect of T treatment on aggression.

The T-treated monkeys did display less behavioral inhibition during the Novel Objects test. When the animals were presented with Mr. Potato Head, the T-treated monkeys spent significantly less time immobile than controls. Freezing or immobility in response to a threat in the wild may prevent animals from being attacked by predators (Edut and Eilam 2003; Searcy and Caine 2003). With its large eyes, Mr. Potato Head is a potentially threatening stimulus to the monkeys (Hinde and Rowell 1962; van Hooff 1962), so immobility is not an unexpected reaction. The T-treated monkeys did spend a significant portion of their time immobile in the presence of Mr. Potato Head (71%), but this was significantly less time than the controls spent immobile (85%). There was also a trend toward the T-treated animals touching the apple (familiar food) sooner than controls when it was presented alone (i.e. without the snake). This effect is similar to what was seen in heifers that were chronically treated with T before having their feeding behavior assessed in a novel environment (Boissy and Bouissou 1994). The T-treated heifers were faster to enter a novel room, and were quicker to feed in the novel area than controls, similar to the monkeys in this study being slightly faster to reach for food in a novel environment. The findings that T-treated monkeys were less behaviorally inhibited in the presence of a potentially threatening novel object and were more willing to eat a familiar food in a novel environment could indicate that the T-treated animals have lower levels of anxiety or increased levels of impulsivity.

There is a well-established relationship between the hypothalamic-pituitary-adrenal (HPA) axis and anxiety (Stenzel-Poore et al. 1994; Timpl et al. 1998; Risbrough and Stein 2006). Studies have found that androgens can modulate this relationship, and while the exact mechanism is unknown, T generally suppresses HPA activity (Handa et

al. 1994; Costine et al. 2010; Goel and Bale 2010). A study by Handa and colleagues (1994) found that castrated male rats had increased corticosterone (CORT) responses to stress compared with intact males, and T or DHT replacement decreased the CORT response. Female mice were shown to have greater CORT responses than males both basally and in response to stress (Goel and Bale 2010), and treatment with TP decreased the CORT levels in these females. Another study found that anabolic androgenic steroids increased corticotropin-releasing factor (CRF) mRNA in the central amygdala of mice, but serum CORT levels in this study were not altered (Costine et al. 2010). In the current study, baseline (non-stressed) cortisol values were not different between the T-treated and control animals, indicating that treatment with low levels of T did not suppress basal HPA functioning. However, the HIT and Novel Objects tests exert some amount of stress on the monkeys, but the cortisol response to stress was not measured in this study. It is therefore possible that mild HA in these animals may have led to changes in the HPA response to stress, and this was associated with the decreased behavioral inhibition seen in the T-treated monkeys.

The finding of a correlation between decreased behavioral inhibition during the acclimation period and increased weight gain was not predicted *a priori*, but is in agreement with previous studies. In people, a lack of inhibition, or increased impulsivity, has been associated with obesity across all ages studied. Children who had less inhibited and more impulsive behavior at 2 years of age had higher BMI's at 5.5 years compared with children who were more behaviorally inhibited at 2 years of age (Graziano et al. 2010). A study of 8-year-old children (Pauli-Pott et al. 2010) also found a strong correlation between BMI and impulsivity (defined as fast and inaccurate responses on a

go/no-go task), such that more obese children showed greater impulsivity. A separate study found that compared with age- and education-matched controls, obese adults made more impulsive decisions during the Iowa Gambling Task, as evidenced by the obese subjects drawing more cards from the disadvantageous decks during the test (Brogan et al. 2011). In the current study, the animals that were less inhibited, or were more active and exploratory in a novel environment, also showed less inhibited feeding behavior. These findings confirm those from the human literature that impulsivity and body weight are positively related and suggest that monkeys may be a useful model for helping us understand the factors that contribute to impulsive behavior and overeating in humans.

Interestingly, activity in the novel environment was not correlated with home cage activity during the 2-week period surrounding the behavior testing. Thus, it would not be accurate to interpret these results as an indication that the more active animals gained more weight. This disparity between activity in the home cage and activity in a novel environment has been previously reported in rodents (Zombeck et al. 2011; Langford-Smith et al. 2011). In a study by Langford-Smith and colleagues (2011), mice with a lysosomal storage disorder were tested for activity during 1 h in an open field test and these results were compared to activity over a 24-h period in the home cage. The results showed that mice with the lysosomal disorder spent significantly more time moving faster than 90 mm/sec compared with controls during the open field test, but there was no difference between the groups on this measure during the 24-h period in the home cage. Another study by Zombeck and colleagues (Zombeck et al. 2011) examined the correlation between open field and home cage activity in two outbred strains of rats that were being selectively bred for increased home cage activity. They found that the two

measures of activity were not correlated in individuals from one strain and only weakly correlated in the other strain of rats. The authors concluded that the neural circuits governing home cage activity were different from those involved in open field activity, which may explain why I found no correlation between home cage activity and activity in a novel environment in the current study.

Anxious behaviors have been linked to perturbations in the serotonin (5-hydroxytryptamine; 5-HT) system. This relationship is partially modulated by genetics, as many studies in monkeys and people have found that individuals homozygous for the short allele of the 5-HT transporter (SERT) gene display increased levels of anxiety (Lesch et al. 1996; Bethea et al. 2004; Lee et al. 2005; Lin 2007). While the relationship between the 5-HT system and anxiety is complex, increased anxious behavior has often been linked to decreased activity of the 5-HT system (Beaulieu et al. 2008; Sullivan et al. 2010), although this is not always the case (Jennings et al. 2006; Fernandez and Gaspar 2012). Selective serotonin reuptake inhibitors (SSRI's), which increase the amount of 5-HT present in synapses, are the most commonly prescribed drugs to treat anxiety disorders (Koen and Stein 2011). The frequent success of these drugs has further implicated the 5-HT system in the expression of anxious behavior.

Impulsivity has also been linked to 5-HT function. In rats, depletion of 5-HT via i.c.v. administration of 5-dihydroxytryptamine led to increased premature responding on a 5-choice serial reaction time test, which is indicative of increased impulsivity after 5-HT depletion (Harrison et al. 1997). Higley and colleagues (1996) also found that monkeys with lower CSF levels of the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) made more long and dangerous leaps between trees, a measure of impulsivity.

These seemingly paradoxical findings that decreased 5-HT can lead to both increased anxiety, as well as increased impulsivity, may arise from the complexity of the 5-HT system and the implication of the SERT as well as various 5-HT receptor subtypes in the control of different aspects of anxious and impulsive behaviors (Griebel 1995; Fernandez and Gaspar 2012). Markers of 5-HT function were not analyzed in the current study, but the inclusion of these measures in future studies may help to elucidate the effects of a potential interaction between T and 5-HT on anxious and impulsive behavior.

One possible limitation of this study was that the phase of the menstrual cycle was not controlled for during the performance of the behavioral temperament tests. Some studies have indicated that there may be changes in aggressive and impulsive behavior across the menstrual cycle in monkeys and women (Howard et al. 1988; Rapkin et al. 1995), although other studies have not supported this claim (Dougherty et al. 1997). There also appears to be significant individual variation in how hormones affect mood. For example, premenstrual syndrome can actually present at any time during the menstrual cycle, and may last for varying amounts of time, making it difficult to determine the exact influence of E and P on mood in women (Reid 1985; Kiesner 2011). In order to eliminate any possible effects of hormones on the behaviors tested here, the experiments for the current study were performed in July, which is in the middle of the summer season when most rhesus monkeys are anovulatory (Walker et al. 1983; 1984). However, blood samples taken at the time of behavior testing indicated that four animals had elevated P (>1.0 ng/mL), indicative of recent ovulation. No animals had elevated E levels at the time of testing. When the data were re-analyzed to control for P values, the

results were not changed, suggesting that variation in ovarian hormones was not driving differences in behaviors that were measured in this study.

In conclusion, the results from this study show that chronic treatment with low doses of T decreased behavioral inhibition in adult female monkeys, but did not affect aggressive behavior. Studies that measure HPA axis and 5-HT function in the presence of T may help to elucidate the mechanisms by which T alters behavior. Additionally, animals that were less behaviorally inhibited gained more weight when fed *ad libitum*, suggesting that strategies aimed at controlling impulsive behaviors may contribute to more successful weight regulation in the human population.

Chapter 5

GENERAL DISCUSSION

5.1 Integration of Findings in Monkeys with Hyperandrogenemia with Our Clinical Understanding of Polycystic Ovary Syndrome

The most common cause of hyperandrogenemia (HA) in women is polycystic ovary syndrome (PCOS), which is a complex and heterogeneous disorder. Symptoms associated with PCOS fall into three broad categories: neuroendocrine, ovarian, and metabolic. The neuroendocrine changes associated with PCOS include alterations in the function of the reproductive neuroendocrine axis, specifically an increased frequency of pulsatile LH secretion, increased LH responsiveness to GnRH, and a decreased sensitivity to progesterone (P) negative feedback (Pastor et al. 1998; Patel et al. 2004; Bachelot et al. 2007; Moret et al. 2009). Ovarian changes associated with PCOS include increased numbers of small antral follicles that tend to be localized to the periphery of the ovary, increased ovarian size, oligo- or amenorrhea, and an increased incidence of anovulation (Zawadzki and Dunaif 1992; Rotterdam Consensus Group 2004; Barber et al. 2010). Metabolic features associated with PCOS include increased central adiposity, decreased insulin sensitivity, dyslipidemia and metabolic syndrome (Dunaif et al. 1989; Carmina et al. 1992; Apridonidze et al. 2005; Carmina et al. 2007). Although various combinations of these diverse symptoms have been identified in women with PCOS, it is unclear how the signs and symptoms are related to each other. HA and obesity are two key components of PCOS, but they likely have differential effects on neuroendocrine, ovarian, and metabolic function. By employing an animal model of HA, and adding a

Western-style diet (WSD), it has been possible to begin to tease apart these interactions. The work presented in this dissertation, using monkeys to study the consequences of mild HA (with levels designed to model those seen in women with PCOS) in combination with the WSD, has increased our understanding of how neuroendocrine, ovarian, and metabolic symptoms associated with PCOS may relate to each other. Figure 5.1 summarizes the results from the studies presented in this dissertation, indicating which findings occurred in the presence of hyperandrogenemia alone compared with hyperandrogenemia plus the WSD or the WSD alone. Further studies (see section 5.2) are needed to elucidate whether the metabolic and ovarian alterations seen in the T-treated animals on the WSD resulted from continued exposure to T or from the combination of T+WSD. Based on findings emanating from the research in my dissertation in combination with results of previous clinical studies and those from other animal models, a diagram of the proposed relationships between the different facets of PCOS is shown in Figure 5.2. These relationships are explained in more detail below.

My studies showed that the first effect of HA was increased neuroendocrine drive to the reproductive axis. This effect of HA was described in detail in Chapter 2. T-treated animals began to show detectable LH pulses at an earlier age than the control animals, when pulsatile LH secretion was measured within the first year post-menarche, at 4 years of age. The T-treated animals continued to show faster LH pulses than control animals at 5 years of age. At 4 years of age, T-treated animals also had a greater LH response to a GnRH challenge than control animals, further demonstrating an increased activation of the reproductive neuroendocrine axis at a younger age compared with control animals.

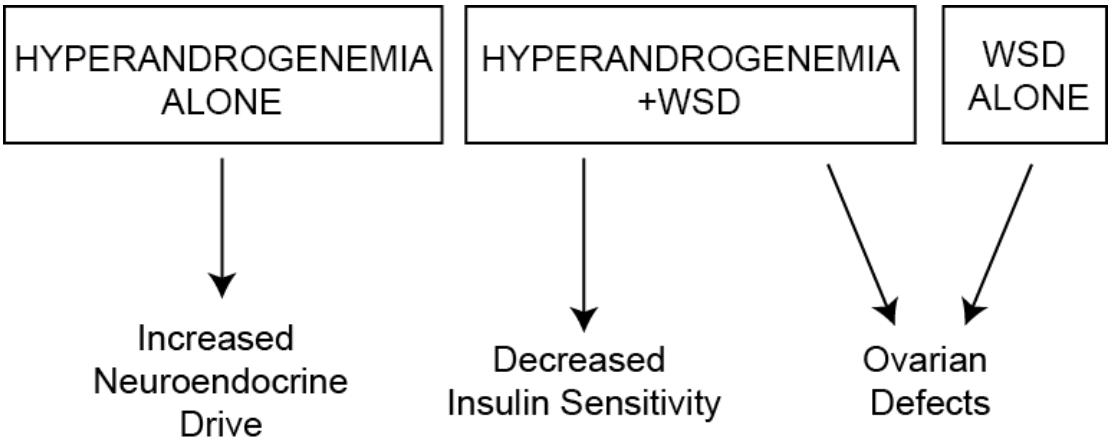

Figure 5.1 Summary of findings from the studies performed for this dissertation.

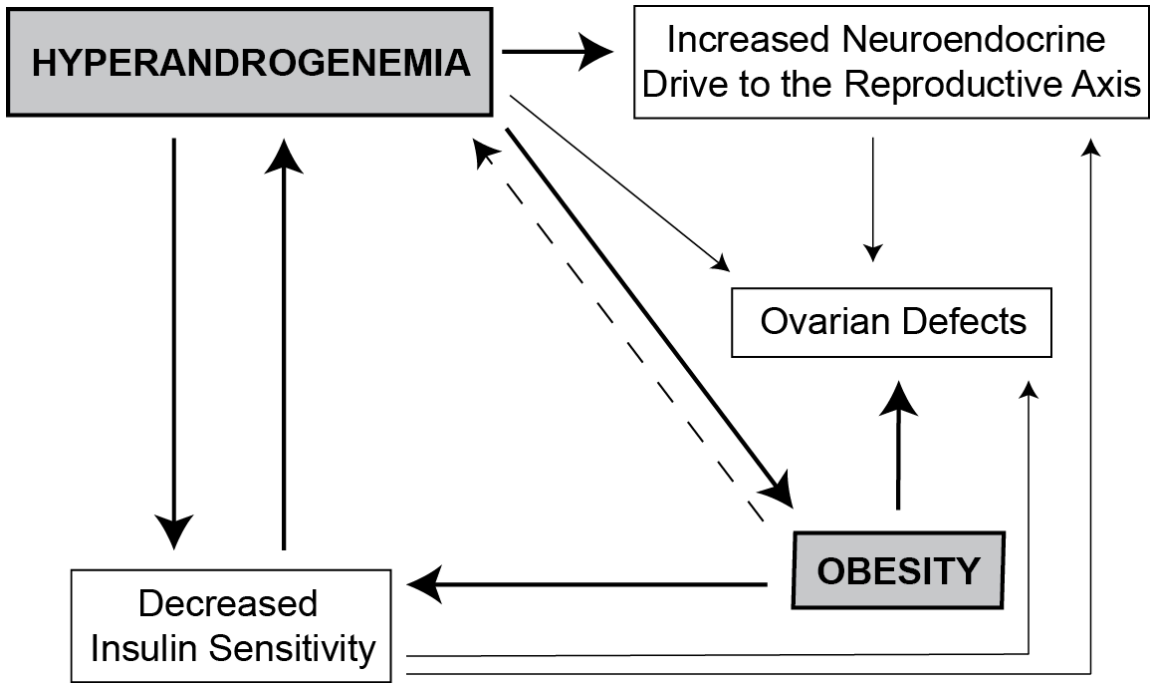

Figure 5.2 Proposed pathways showing the interrelatedness of PCOS symptoms. Thick solid lines indicate relationships that are well-established in the literature and/or I found evidence to support. Thin solid lines indicate relationships that are tentatively supported by my data or the literature. The dashed line indicates a relationship that has been reported in the literature but was not supported by the current studies.

However, as the animals got older (6-7 years of age), there was no evidence for a group difference in the degree of activation of the reproductive neuroendocrine axis. As detailed in Chapter 3, pulsatile LH secretion measured at 6 or 6.5 years of age did not differ between control and T-treated monkeys. Nor were any differences in LH responsiveness to GnRH apparent at 6 or 6.5 years of age. It is important to note, however, that the animals were all put on a WSD at 5.5 years of age, so neuroendocrine function at 6 and 6.5 years of age was assessed in the presence of increased adiposity due to the WSD. In both treatment groups, LH pulse amplitude decreased while on the WSD as compared with before the WSD. In women with PCOS, obesity has been reported to decrease LH pulse amplitude without affecting pulse frequency (Arroyo et al. 1997; Taylor et al. 1997; Pagan et al. 2006), so it is unlikely that the lack of a group difference in pulse frequency during adulthood in the current study was due to the WSD. Future studies using animals that were not maintained on the WSD would confirm this.

Similar to my findings, other studies have found that girls with HA experience a faster progression of pulsatile LH patterns from those typical of puberty to those typical of adulthood (Apter et al. 1994). Additionally, women with PCOS who have been studied into their mid-30's (approximately equivalent to a 12-year-old monkey) have been shown to have slightly greater LH secretion, due to a slight increase in pulse frequency and an increase in pulse amplitude (Waldstreicher et al. 1988; Arroyo et al. 1997; Patel et al. 2004). For example, Arroyo et al. (1997) found that control women (n=32) had 16.5 pulses/24 hours, while the PCOS patients (n=33) had 22.8 pulses/24 hours ($p<0.0001$). My studies found that the T-treated monkeys also had approximately 1 pulse/hour, similar to the PCOS women in these studies. However, the cholesterol-treated control

monkeys also had an LH pulse frequency of about 1 pulse/hour, rather than 1 pulse/90 min. A survey of previously published data examining LH pulse frequency in the early follicular phase shows that some studies report a pulse frequency of 1 pulse/hour in both normal women (Reame et al. 1984; Kazer et al. 1987; Venturoli et al. 1988) and normal monkeys (Norman et al. 1984; Herod et al. 2011). Thus, there appears to be some variability in the early follicular phase LH pulse frequency in normal females, and this appears to account for my failure to see that T-treated monkeys have faster pulsatile LH secretion than controls, as there was no decrease in LH pulse frequency in the T-treated monkeys as they reached adulthood. A larger study with a greater sample size (of both T-treated and control monkeys) would likely be needed to determine if there is a significant difference in neuroendocrine function between T-treated and control monkeys in adulthood.

Evidence from clinical studies and my data suggest that there is a bi-directional relationship between HA and obesity. Women who are obese are more likely to suffer from HA compared with their lean counterparts (Strain et al. 2003; Taponen et al. 2003). It has been shown that obesity may lead to increased levels of biologically active androgens directly via the capability of adipose tissue to synthesize T from androstenedione (Quinkler et al. 2004). There is also evidence that obesity decreases the levels of circulating SHBG, which leads to an increased amount of free T (Evans et al. 1983; Blouin et al. 2007). While I did not see an increase in total T levels in the control animals used for the current study after 16 months on the WSD, I was unable to measure SHBG due to the lack of a validated assay to measure this in monkeys.

Therefore, I cannot state with certainty whether obesity affected free androgen levels in the monkeys used in the studies of this dissertation.

In Chapters 2 and 3, however, I did report that T-treated animals gained more weight post-pubertally than control animals, indicating that HA appeared to promote weight gain. Interestingly, I found that T-treated animals gained more weight than controls even though there were no group differences in caloric intake either before or during WSD consumption. I did, however, find in Chapter 3 that T-treated animals were less active than control animals during the last 10 months of this study. This could indicate that T treatment led to a decrease in physical activity levels that subsequently led to weight gain, although this conclusion cannot be verified from the current data because physical activity was not measured before T treatment began. Previous findings support this theory, however, since decreased physical activity has been linked to weight gain in both monkeys (Sullivan et al. 2006) and people (Klesges et al. 1992; Brown et al. 2005). Another mechanism by which T may have led to weight gain was by decreasing behavioral inhibition. In Chapter 4, I reported that T-treated monkeys showed less inhibited behavior, supported by the finding that they were more active when exposed to a potentially threatening stimulus (Mr. Potato Head) in a novel environment, which could also be interpreted as being less behaviorally inhibited. I also found that animals that were more active (i.e. less behaviorally inhibited) in a novel environment gained more weight while on the WSD. Studies in humans have also found that decreased inhibition is related to increased weight gain (Pauli-Pott et al. 2010; Brogan et al. 2011). Taken together, these results could indicate that T-treatment may lead to increased weight gain by increasing impulsive behavior. Although in my studies, I did not find that T-treated

monkeys gained more weight while on the WSD compared with controls, a group difference may have become apparent with a larger sample size or a longer period of time on the WSD.

Clinical studies have found a link between HA and metabolic abnormalities and my results confirmed this relationship. Insulin is important for the growth and development of ovarian follicles (Campbell et al. 1995; Young and McNeilly 2010; Chaves et al. 2011). However, excess insulin may lead to the overproduction of T by binding to insulin and IGF-I receptors on ovarian theca cells (Bergh et al. 1993; Willis and Franks 1995; Campbell et al. 1998). For example, increased ovarian T production has been seen in women who use insulin therapy to treat type 1 diabetes (Codner et al. 2006; Codner et al. 2007). There is a complex relationship between HA and insulin sensitivity (SI), as evidence suggests that excess levels of androgens may also be able to alter SI. In women with CAH, a disease that has androgen excess as its primary symptom, SI is significantly decreased compared with control women (Speiser et al. 1992; Charmandari et al. 2002). Additionally, I reported in Chapter 3 that the T-treated animals had decreased SI compared with control animals after 12 months on the WSD, while no difference was apparent when the monkeys were 3.5 years of age and maintained on a diet of typical monkey chow. It is possible that a group difference in SI did not emerge until the animals were older because a more prolonged exposure to T was necessary to induce metabolic defects. It is also possible that the decreased SI did not become apparent until the animals were tested at 6.5 years of age because decreased SI was dependent on the interaction between T and weight gain while on the WSD. It is well-established that obesity leads to decreased SI in both monkeys and humans (Bodkin et al.

1993; Abbott et al. 1997; Martyn et al. 2008). The T-treated animals had decreased SI compared with control animals, even when statistically controlling for weight, so it is possible that it was the *interaction* between HA and increased adiposity that resulted in decreased SI in the T-treated animals. Unlike my finding that T-treated monkeys did not have decreased SI compared with controls when the monkeys were tested before WSD consumption, lean women with PCOS have been reported to have reduced SI compared with lean controls (Chang et al. 1983; Dunaif et al. 1989). This suggests that there is another contributing factor (perhaps something such as a genetic tendency toward insulin insensitivity) in addition to HA that causes metabolic abnormalities in the lean subset of PCOS patients.

I also found an effect of HA and WSD on ovarian morphology and function. In Chapter 2, before the animals started the WSD, I reported that T-treated animals were less likely to have a follicle over 2.0 mm during the early follicular phase compared with control animals, although this was not a significant difference. In Chapter 3, I found further evidence for an effect of HA on ovarian dysfunction in that the T-treated monkeys were somewhat (although again not statistically) more likely to have peripheral compartmentalization of follicles than controls after 3 months on the WSD. In addition, T-treated monkeys also had lower peak P values during the luteal phase of a menstrual cycle after 14 months on the WSD, indicative of decreased luteal function (Soules et al. 1989; Bukulmez and Arici 2004). It is unclear from my studies whether the ovarian changes were due to direct effects of HA on the ovary, or whether these changes were somehow mediated by the faster maturation of the GnRH pulse generator or the decreased insulin sensitivity that was present in T-treated animals when they were on a

WSD. Some studies have reported ovarian dysfunction that was associated with HA in the absence of decreased SI in women, suggesting that androgens may have the ability to affect ovarian function directly (van Hooff et al. 2000b). However, other studies have also described a subset of women with HA who have ovulatory menstrual cycles and normal insulin levels and normal SI (Dunaif et al. 1987), indicating that HA does not always lead to decreased metabolic or ovarian function. These results suggest that a variety of factors likely give rise to ovarian defects in women with PCOS.

I also reported in Chapter 3 that after 14 months on the WSD, the control animals had the same prevalence of peripherally compartmentalized follicles as the T-treated animals, suggesting that obesity alone may also affect ovarian function in the absence of HA. Although the mechanism is unknown, obese women experience subfertility at a higher rate than lean women (Brewer and Balen 2010). Obese women are more likely than lean women to suffer from menstrual disturbances (Lake et al. 1997), and obese women who do have normal menstrual cycles have still been reported to experience impaired fertility compared with lean women (Gesink Law et al. 2007). My findings in combination with those from previous studies suggest that obesity and T have an additive effect on producing ovarian abnormalities.

5.2 Future Directions for Studying the Effects of Androgens on Behavior and Reproductive, Neuroendocrine, and Metabolic Function

In the experiments of this dissertation, I examined the effects of T and increased adiposity on symptoms that are characteristic of women with PCOS, with all animals

being tested before and after weight gain associated with the consumption of a WSD. Some group differences did not become apparent until after the animals had gained weight on this diet. For example, the T-treated animals had decreased SI compared with controls after 12 months on the WSD, as reported in Chapter 3. Additionally, the T-treated animals had lower peak P and P area under the curve during the luteal phase of a menstrual cycle compared with control animals after 14 months on the WSD. It is unknown whether these differences arose due to continued exposure to T or due to the interaction between T and increased adiposity. Due to financial constraints, only 12 animals were studied for this dissertation project, which did not allow for the inclusion of all desired control groups. For this reason, it would be of interest to expand my findings by performing a similar study in a larger cohort of monkeys with additional treatment groups. Ideally, there would be 4 groups of animals that would begin treatment prepubertally: 1) T-treated animals that are fed the WSD for the entire study, 2) cholesterol-treated animals that are fed the WSD for the entire study, 3) T-treated animals that are fed normal low-fat monkey chow for the entire study, and 4) cholesterol-treated animals that are fed normal low-fat monkey chow for the entire study. By organizing the monkeys into these 4 treatment groups, one could more easily determine whether the findings are related to T-treatment, increased adiposity as a result of the WSD, or an interaction between the two factors.

Another interesting experiment would be to test fertility in the 4 groups of animals described above. Although all of the monkeys in the studies for this dissertation were displaying ovulatory cycles, this finding may not translate directly to normal fecundity. Studies have found that women with PCOS have decreased uterine blood flow

and increased rates of pregnancy complications (Ajossa et al. 2001; Wang et al. 2001; Chekir et al. 2005; Kjerulff et al. 2011). Decreased blood flow to the uterine artery has been found in women with recurrent pregnancy loss (Habara et al. 2002), and has also been associated with preeclampsia and fetal growth restriction (Takata et al. 2002; Gomez et al. 2006; Klein et al. 2011). Interestingly, a correlation has been found between P levels and uterine blood flow (Habara et al. 2002), such that women with higher P also had higher blood flow. In Chapter 3, I found that the T-treated animals had lower P levels compared with controls, which might indicate that the T-treated animals also would have decreased uterine blood flow. Thus, although the T-treated monkeys had similar rates of ovulation compared to controls, they might have a higher rate of pregnancy complications and pregnancy loss compared with control animals. This also may hold true for animals that consume the WSD but are not given exogenous T, as people who are obese have been reported to have decreased fertility, even if they experienced regular menstrual cycles (Gesink Law et al. 2007; Brewer and Balen 2010). Based on these data, I would hypothesize that T-treated animals that are also fed the WSD would experience a lower fertility rate compared with control animals eating a low fat diet, or those that are only treated with T or only given the WSD.

My research also has implications for clinical studies related to behavior in women with PCOS. The high prevalence of mood disorders in women with PCOS has only recently become the focus of attention, but impulsivity levels have not been comprehensively investigated in women with PCOS. In Chapter 4, I found that T-treated animals displayed less inhibited behavior that could also be characterized as being more impulsive. Several studies in humans have found an association between increased

impulsivity and increased weight gain (Pauli-Pott et al. 2010; Brogan et al. 2011). This association is present throughout life, and is apparent as early as 2 years of age (Graziano et al. 2010). Similarly, I also reported a correlation between decreased behavioral inhibition and increased weight gain in monkeys in Chapter 4 of this dissertation. Because women with PCOS typically have elevated androgens and are frequently overweight or obese (Legro 2000; Azziz et al. 2004a), it would be interesting to examine levels of impulsivity in PCOS patients compared with healthy controls. I would expect that PCOS patients would show more impulsive behavior, and that impulsivity would be positively correlated with BMI. If this hypothesis was supported, then monkey studies could be expanded to determine whether antiandrogen drugs (e.g. flutamide) would decrease impulsivity levels in PCOS patients, which could potentially help obese women control their caloric intake. Because PCOS symptoms are more severe in overweight or obese patients (Dunaif et al. 1989; Kiddy et al. 1990), any treatment that has the potential to encourage weight loss would be incredibly useful in a clinical setting.

5.3 Summary and Conclusions

The studies in this dissertation have expanded the body of literature describing the effects of HA on reproductive, neuroendocrine, and metabolic function in women. It was the overall goal of my dissertation to test the hypothesis that increased levels of androgens over puberty would lead to the development of symptoms associated with PCOS. Previous studies from *prenatally* androgenized rats, sheep, and monkeys have found that neuroendocrine, ovarian, and metabolic function are altered in a manner

similar to that seen in women with PCOS (Goy and Robinson 1982; Robinson et al. 1999; Birch et al. 2003; Abbott et al. 2008; Wu et al. 2010). This has been attributed to the organizational effects of T on the developmental programming of these physiological systems (Phoenix et al. 1959; Thornton et al. 2009). However, it has been proposed that puberty is another period of time when the body is particularly susceptible to the effects of gonadal hormones (Scott et al. 1974). Additionally, studies in girls at high risk of developing PCOS (daughters of women with PCOS) have found that elevated androgens do not appear until during the pubertal transition in these girls, indicating that the presence of HA *during puberty* may be critical in the development of PCOS symptoms. Whether symptoms characteristic of PCOS could be induced by exposure to elevated androgens beginning just prior to puberty had never been examined in monkeys before the studies were performed for this dissertation.

One of the main findings from these studies was that slightly elevated levels of T that first occurred in the prepubertal period increased the speed with which the GnRH pulse generator matured in pubertal monkeys. Although there were no differences observed between T-treated and control animals when neuroendocrine function was assessed during adulthood, this finding indicates that prepubertal T treatment, in the absence of prenatal T exposure, is capable of altering central neural drive to the reproductive axis. This may indicate that the GnRH pulse generator is especially sensitive to the influence of elevated androgens during puberty, regardless of prenatal exposure to androgens.

Another main focus of this work was to examine the effects of adiposity on the development of symptoms that are characteristic of PCOS. I found evidence to support

findings from previous studies that increased adiposity worsens the ovarian and metabolic phenotypes of women with PCOS (Dunaif et al. 1989; Kuchenbecker et al. 2011), as T-treated animals had decreased insulin sensitivity and decreased P secretion compared with control animals after 14 months on the WSD. However, I also found that increased adiposity, in the absence of T treatment, led to the peripheral compartmentalization of ovarian follicles, confirming findings from clinical studies that obesity alone can have detrimental effects on ovarian function (Lake et al. 1997; Gesink Law et al. 2007). These results indicate that while obesity did not seem to *cause* a complete PCOS phenotype in these monkeys, the interaction between T and weight gain did *aggravate* metabolic and ovarian dysfunction. Although weight loss is often difficult for people to maintain (Kramer et al. 1989; Stern et al. 1995), my studies provide support for the clinical recommendation that a primary target for overweight or obese women with PCOS should be to lose weight (Hoeger 2001; Moran and Norman 2004).

The third goal of my dissertation was to determine the effects of HA on behavior. Although there were no group differences in aggression, T-treated monkeys did show slightly less inhibited behavior than control monkeys, indicating that androgen treatment decreased anxiety or increased impulsivity. Additionally, there was a strong correlation between activity in a novel environment and weight gain on the WSD, such that less inhibited animals consumed more food and gained more weight when a palatable diet was available. This finding is in agreement with results from the human literature showing that higher levels of impulsivity are associated with increased body weight (Pauli-Pott et al. 2010; Brogan et al. 2011). Therefore, a monkey model may be a useful

tool to help elucidate the factors that contribute to this relationship between impulsivity and overeating in humans.

In conclusion, I found that slightly elevated levels of T over puberty led to increased central drive to the reproductive axis in female monkeys. Furthermore, I found evidence that weight gain and increased adiposity in combination with T exposure resulted in disturbances of ovarian and metabolic function. This work, in combination with other studies, will lead to a better understanding of the specific contributions of HA and obesity to the development of symptoms that are characteristic of PCOS, which could result in improved treatment strategies for women affected by PCOS.

Chapter 6

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