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Adrenal Expression of cyp17 and hsd3b1 mRNA in Peromyscus maniculatus bairdii

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ADRENAL EXPRESSION OF *CYP17* AND *HSD3B1* mRNA IN *PEROMYSCUS MANICULATUS BAIRDII*

A Thesis

Presented to

The Faculty of the Department of Biology

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Brian Thomas Lovitt

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APPROVAL SHEET

This thesis is submitted in partial fulfillment of

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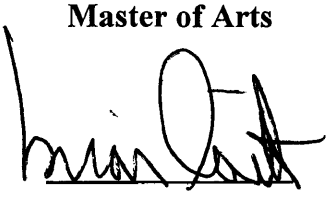

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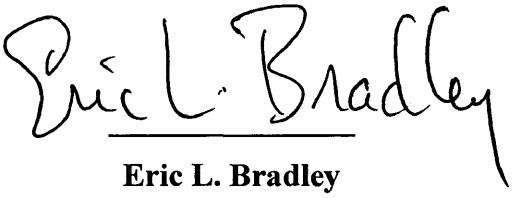
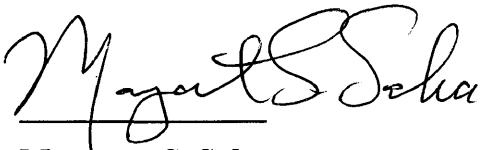


Eric L. Bradley



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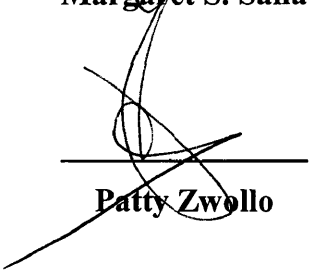

Patty Zwollo

DEDICATION

This thesis is dedicated to my parents, Tom and Pam Lovitt, who have been nothing but supportive through all my endeavors. I have been provided with a wealth of opportunity and encouragment, and hope that I have made the most of the sacrifices my parents have made for my betterment. I will always appreciate all that they have done, and all that they continue to do, to help me reach all my goals.

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ABSTRACT

The primary physiological pathway active during puberty is the hypothalmic-pituitary-gonadal axis, but the factors or mechanisms involved in the activation of this pathway are not well understood. Several mechanistic hypotheses have been set forth, with this investigation focusing on the possible role for the adrenal glands in the theory of adrenarche.

Peromyscus maniculatus has evolved a mechanism of population control that appears to involve a disruption in pubertal transition. Previous work by our laboratory has found distinct morphometric changes in the adrenal associated with puberty, and a differing pattern in reproductively inhibited *Peromyscus*. To investigate these changes, this study measured the expression of two genes involved in the steroidogenic pathway of the adrenal before, during, and after normal pubertal transition, and in the special case of the disrupted puberty found in reproductively inhibited animals.

Ribonuclease protection assays demonstrated increased adrenal expression of *hsd3b1* and *cyp17* at the time of puberty. In reproductively inhibited animals, *hsd3b1* expression was not different than in normally maturing animals, but *cyp17* expression was statistically significantly reduced compared to similarly aged control animals.

The increased levels of *hsd3b1* and *cyp17* suggest increased adrenal production of glucocorticoid and/or weak androgen at puberty. Normal levels of *hsd3b1* suggest normal levels of corticosterone and aldosterone in inhibited animals, while reduced levels of *cyp17* suggest reduced levels of cortisol and/or weak androgen. The adrenal gland appears to be involved in pubertal transition and the disrupted pubertal transition seen in reproductively inhibited animals, but the exact role and extent of involvement is still not clear.

ADRENAL EXPRESSION OF *CYP17* AND *HSD3B1* mRNA IN *PEROMYSCUS MANICULATUS BAIRDII*

Introduction and Literature Review

Overview

The significant evolutionary investment in reproduction is undeniable on any level of biological study. Behaviors have evolved that propagate reproductive success, from courting rituals to aggression for mate selection in the animal kingdom; from various forms of taxis to parasitism in the kingdom monera; from chemical herbicides and insecticides to symbiosis with bacteria or insects in the plant kingdom. These behaviors have all evolved to ensure that the individual will survive to obtain the resources necessary to reproduce. Physiologies governing reproduction are also often complex and extensive, with nearly all bodily systems participating in some aspect of reproduction. Entire organs or features are devoted solely to preparing for, participating in, and/or ensuring or maintaining reproductive success. These features, organs, or systems often require a prodigious allocation of resources, an allocation that often places reproductive success before personal long-term welfare. The genetic and molecular/ cellular machinery devoted or associated with reproduction is equally impressive. The complex cellular process of meiosis has evolved expressly to allow contribution of an individual's genes to a new generation.

In humans, there is great medical interest in understanding processes directly or indirectly involved in reproduction. Ensuring a timely, disease-free pregnancy and birth are the goals of nearly all prospective parents. Additionally, as in nearly all species, the human body becomes increasingly catabolic following the reproductively fertile period. All beings pass their own genetic information on to new generations. Once the success of

this transfer is ensured, there is little selective pressure to lengthen lifespan. There is an obvious human desire to slow this genetically predisposed catabolism to extend lifespan, and mechanisms underlying this senescence will be discussed in greater detail in later sections.

While selective pressure on lifespan may be weak after reproduction, the attainment of reproductive capability has been strongly selected for, resulting in elaborate maturational strategies. A first priority in these species appears to be the attainment of that capability through maturation of the machinery primary to, or accessory to reproduction. This may involve the development of organs specific to reproduction, or simply attainment of the size or shape necessary to attract a mate or fend off threats to reproductive success. This progression from reproductive adolescence to reproductive maturity takes many forms, from the metamorphosis seen in insects to the pubertal transition seen in mammals. While this reproductive maturation is nearly universal, and critical to the propagation of life, the mechanisms governing its initiation and advance are often poorly understood.

This thesis aims to advance our knowledge of the mechanisms underlying reproductive maturation through the study of components of pubertal transition in a natural model, the prarie deermouse, *Peromyscus maniculatus bairdii*. Specifically, two genes encoding enzymes essential to adrenal steroidogenesis are studied in relation to pubertal transition to investigate a possible role in reproductive maturation and/or the disruption of that maturation. Before presenting these investigations, it is necessary to review pubertal transition in mammals generally, as well as the systems and processes believed to be involved in puberty and reproduction in the deermouse.

Puberty

Puberty is not a single event, but rather a developmental period distinguished by the attainment of sexual maturity and fertility from an adolescent state. Mammalian puberty is characterized by the growth of reproductive organs, development of secondary sexual characteristics, and the acceleration of growth (Sizonenko, 1987). In humans, pubertal development is a process that lasts several years, with the majority of development occurring between pubarche, or the first appearance of pubic and axillary hair, to menarche, or the first menstruation (in females) (Wilson et al., 1998).

The physiological pathway that is primarily responsible for pubertal transition is the hypothalamic-pituitary-gonadal (HPG) axis (Figure 1). In mammals, this axis is very active in fetal life and immediate postnatal life, in particular when sexual differentiation of the fetus takes place (Brook, 1999). After approximately the second year of age in humans, the HPG axis enters a quiescent period, only becoming active again at approximately ten years of age, or just prior to puberty. At this age, a pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus begins, with largest spikes occurring at night (Sizonenko, 1987). These nocturnal peaks increase in amplitude and frequency, causing the anterior pituitary to release increasing amounts of follicle stimulating hormone (FSH), growth hormone (GH), leutenizing hormone (LH), but not adrenocorticotropic hormone (ACTH). FSH and LH act to promote growth of the testes and ovaries, which correspondingly produce and release increasing amounts of androgens and estrogens. These gonadal steroids propogate the development of the sexual organs, and primarily through augmentation of GH release, promote skeletal growth. This skeletal growth is mediated by growth hormone's stimulation of insulin-like growth

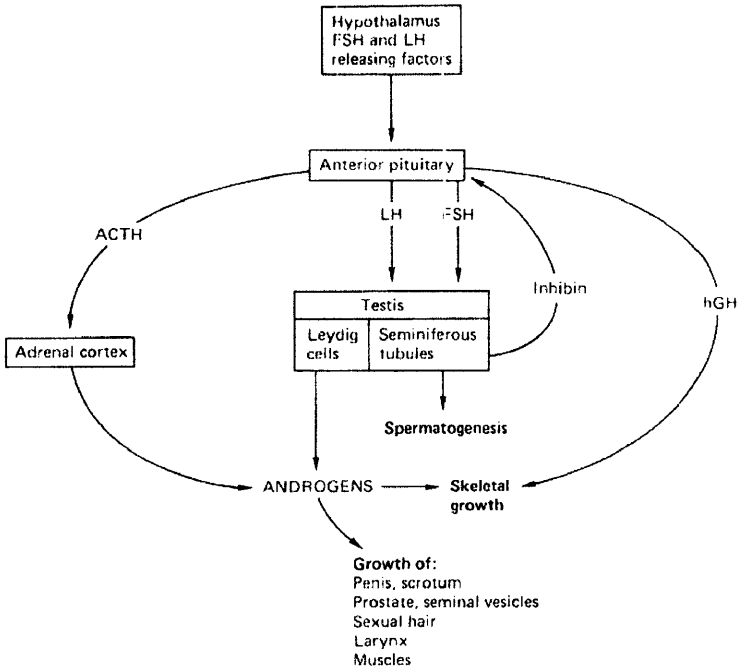


Figure 1 - Hypothalmic-Pituitary-Gonadal (HPG) Axis. The physiological pathway that is primarily responsible for pubertal transition is the hypothalamic-pituitary-gonadal axis. LH=leutenizing hormone, FSH=follicle stimulating hormone, ACTH=adrenocorticotropic hormone, hGH=human growth hormone. From Sizonenko, 1987.

factor 1 (IGF-1 or somatomedin-C) production (Wilson et al., 1998).

The mechanism and factors responsible for the initiation of puberty are not entirely understood, but likely involve a host of genetic and environmental factors including nutrition and stresses. Reactivation of the HPG axis after adolescent senescence is clearly the control point for pubertal initiation, with the CNS being the likely target of initiation factors. Hypothalamic GnRH releasing neurons begin their pulsatile release of pituitary stimulating hormone after a near decade (in humans) of silence. While all mammals share a similar HPG mechanism for puberty, the initiators of that reactivation are widely varied, representing diverse adaptive strategies in evolution (Wilson et al., 1998). These initiators are currently a popular topic of investigation.

Receiving particular attention is the critical mass or critical fat theory of pubertal initiation. The observation that obese humans reach menarche earlier, while malnourished or extremely athletic individuals have delayed menarche, led to a critical mass theory. Quite simply, the body will not undergo puberty if it does not have the necessary energy stores. This was tested in rats nearly 40 years ago (Kennedy and Mitra, 1963), and has been documented in a host of mammals since (Foster and Olster, 1985; Frisch, 1994). Frisch (1977) showed that rats raised on a high fat diet reached first estrus earlier than controls, but both groups had similar body fat composition once reaching first estrus (Frisch et al., 1977). Frisch (1994) also showed that female humans reach menarche at different weights and heights, but tend to have similar body fat percentages upon reaching menarche. The search for a molecule in adipose tissue capable of altering the hypothalamus timing of puberty led to the discovery of leptin (Zhang et al., 1994). Zhang (1994) found that it was a homozygous mutation in the leptin gene (*ob*) that was responsible for the obesity of obese mice. Cunningham (1999) found that these mice are also reproductively incapable, but can be restored to reproductive fertility through peripheral injections of recombinant leptin (Cunningham et al., 1999). However, initiation of premature puberty in rats and mice through administration of exogenous leptin has not been reproducable (Ahima et al., 1997; Cheung et al., 1997; Cunningham et al., 1999). Taken together, it appears that leptin is essential, but not sufficient, for pubertal initiation. A wide body of investigations into leptin's influence on the hypothalamus has been undertaken. While leptin receptors have been found in the hypothalamus, there is no evidence that leptin directly influences GnRH secreting neurons (Cunningham et al., 1999). Most likely, leptin relays the body's metabolic state

to GnRH neurons indirectly, by influencing other hypothalamic peptides (Cunningham et al., 1999). Leptin also plays a role in the adrenal expression of the steroidogenic enzyme cyp17. This influence will be discussed in a later section.

Obtaining proper levels of body fat is an important prelude to pubertal initiation, but it is not the only determinant of age of initiation. Siberian hamsters, like other seasonal breeders, also rely on detection of photoperiod to determine time of pubertal development. Adam et al. (2000) studied the regulation of puberty and several neuropeptides and their receptors in short and long day hamsters. Hamsters reared in a short-day photoperiod normally show restricted growth and do not go through puberty, while long-day animals grow rapidly and undergo pubertal transition. Adam found differences in leptin signalling and in activities of hypothalmic neuropeptide and receptor systems in long and short day animals (Adam et al., 2000). Notably, higher levels of leptin mRNA in adipose tissue, and leptin receptor mRNA in the hypothalamus of long-day animals was detected by Northern blot and *in situ* hybridization. Unfortunately, these higher levels were not detected before the two groups diverged in body mass, making it difficult to determine directionality of leptin/ leptin receptor and body mass.

Leptin signalling of a critical fat level does appear to be an important initiator of puberty. But like most physiological systems, this pathway is likely not the sole contributor to pubertal initiation. The adrenal glands are also known to play a role in puberty, though the extent of that role is still not fully understood. The theory of adrenarche, or the puberty of the adrenals, has received much attention in primates (Cutler et al., 1978; Miller, 1999). Briefly, a major developmental change in the adrenals prior to puberty leads to the substantial increase in adrenal production of several weak

androgens. Adrenal androgens can be converted to testosterone and/or estrogens in peripheral tissues, and are believed to contribute to pubic hair growth, gonadal development, and skeletal growth (Sizonenko, 1987). The extent of this contribution and the role of adrenal androgens in the maturation of the HPG axis remain speculative. But before discussing adrenarche more fully, it is necessary to discuss the anatomy and function of the mammalian adrenal glands.

Adrenal Gland

The adrenal is an essential endocrine gland responsible for the production of a variety of hormones. In primates, the adrenals are located adjacent to the rostral surface of the kidney. The anatomy of the mammalian adrenal can be divided into two functionally and embryologically distinct tissues; a medulla consisting of neural crest derived chromaffin, and a steroidogenic cortex arising from coelomic mesoderm of the genital ridge (Figure 2) (Wilson et al., 1998). The medulla is responsible for the production of the catecholamines epinephrine and norepinephrine, while the cortex is responsible for the production of the mineralcorticoid aldosterone, the glucocorticoids cortisol and corticosterone (rodents), and the weak androgen dehydroepiandrosterone (DHEA) (Wilson et al., 1998). All adrenal cortex products are produced from the substrate cholesterol, with the subsequent specific hormone production being dependant upon the zone specific genetic expression of substrate altering enzymes (Figure 3) (Wilson et al., 1998).

The initial and rate limiting step in all adrenal steroid production is the cytosolic transport of free cholesterol to the inner mitochondrial membrane. While adrenal cells

are capable of producing cholesterol *de novo* from acetate or intracellular cholesteryl ester, nearly 80% of utilized cholesterol is provided by circulating low density lipoprotein cholesterol (Wilson et al., 1998). This substrate is internalized via cell surface, receptor-mediated endocytosis. The invaginated vesicle fuses with lysosomes, within which cholesterol is liberated by the hydrolysis of cholesteryl esters (Wilson et al., 1998).

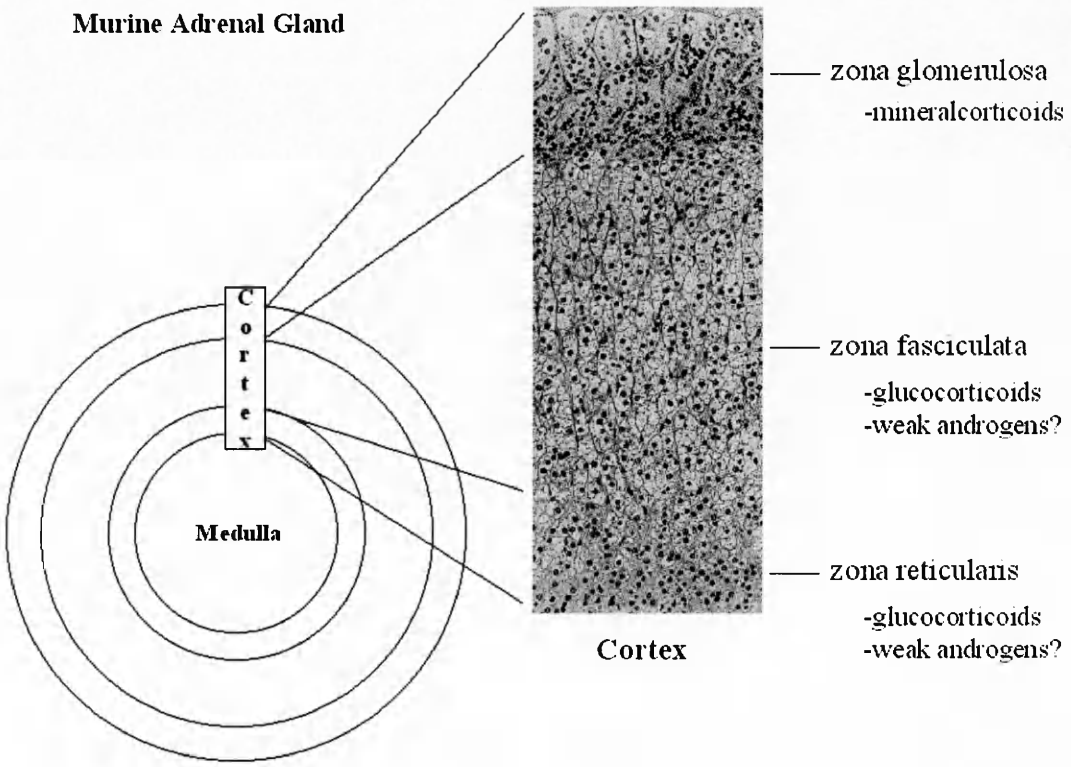


Figure 2 - Murine Adrenal Gland. The mammalian adrenal is divided into a functionally distinct medulla and cortex. The medulla is responsible for the production of the catecholamines, while the further subdivided cortex produces a variety of steroids. The production of weak androgens in murine species is not resolved.

Adrenal Steroidogenic Pathway

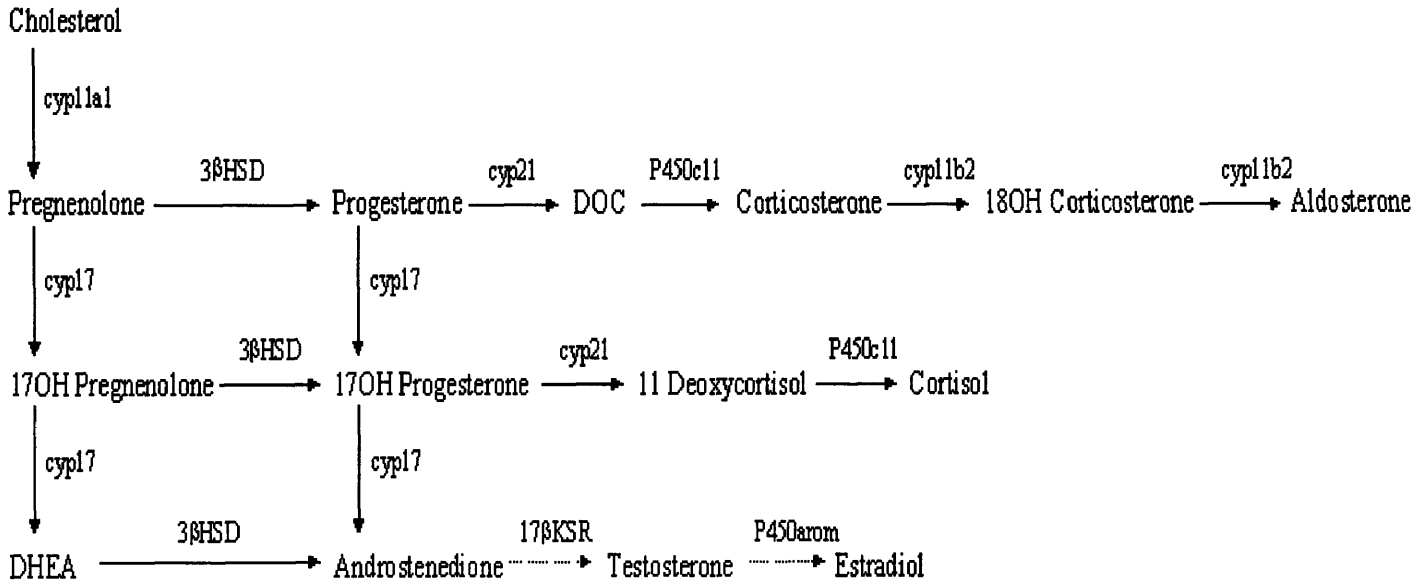


Figure 3 - Adrenal Steroidogenic Pathway. The steroids produced from cholesterol are dependent upon the zone specific distribution of enzymes. The lack of cyp17, and presence of 3βHSD and cyp11b2 causes the production of the mineralcorticoid aldosterone in the *zona glomerulosa*. In the mouse, the presence of 3βHSD, but not cyp17 in the *zona fasciculata* facilitates the production of the glucocorticoid corticosterone. The presence of cyp17, but little 3βHSD in the *zona reticularis* facilitates the production of the weak androgen DHEA. Schematic adapted from Wilson, 1998.

Cholesterol delivery to the inner mitochondrial membrane is enhanced by the presence of several factors, most notably by the steroidogenic acute regulatory proten (StAR) through an as yet undetermined pathway (Miller, 1998). ACTH also increases delivery by increasing the number of LDL cell-surface receptors and increase of cholesterol esterase (Wilson et al., 1998). Conversion of cholesterol to pregnenolone procedes via the cleavage of the side chain at C_{20}. This reaction is catalyzed by the NADPH dependant enzyme CYP11A1, the single product of the *CYP11A1* gene. Further conversion of pregnenolone is dependant upon the zone-specific distribution of enzymes. In the outer-most *zona glomerulosa*, the lack of the enzyme CYP17 (*CYP17* gene product), and the presence of 3βHSD Type II (*HSD3B2* gene product), causes the conversion to proceed to progesterone. The NAPDH dependant, microsomal enzyme 3βHSD catalyzes the dehydration of the 3-hydroxyl group of pregnenolone and the isomerization of the double bond at C_5 to produce progesterone. The glomerulosa is the only layer of cells to possess the enzyme CYP11B2, the product of the gene *CYP11B2*. This tissue specific distribution of enzymes allows the glomerulosa to be the sole producer of the mineralcorticoid aldosterone through the DOC to corticosterone pathway.

In the *zonae fasciculata and reticularis*, the presence of the enzyme CYP17 allows for the conversion of pregnenolone to 17OH pregnenolone or progesterone to 17OH progesterone through hydroxylation of C_{17}. The same enzyme is also responsible for the cleavage of the carbon chain at C_{17}, producing DHEA from 17OH pregnenolone or androstenedione from 17OH progesterone. The lyase conversion to DHEA or androstenedione is determined by the presence or absence of the necessary electron donor

P450 Oxidative Reductase (OR) (Miller, 1998). Alterations in the relative areas and hormone output of these two zones prior to puberty has raised debate regarding the adrenals role in puberty. Albright (1947) first set forth the concept of adrenarche, which implicates the adrenal in the pubertal transition.

Adrenarche

In humans, 'adrenarche' refers to the prepubertal rise in the secretion of the adrenal androgens dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate (DHEAS), and androstenedione (Albright, 1947; Miller, 1999). The onset of human adrenarche corresponds approximately to the prepubertal rise in body mass index (BMI) and the gradual increase in plasma insulin and insulin-like growth factor I (IGF-I) (Rolland-Cachera, 1993; Smith et al., 1989). Insulin and IGF-I augment adrenal androgen production through altered expression of adrenal steroidogenic enzymes *in vitro*, and an increase in BMI has been shown to reflect alterations in the biological activities of these peptides (Attia et al., 1998; Forbes et al., 1989; Frystyk et al., 1995). In a longitudinal study of 42 children, Remer (1999) found a significant correlation between changes in BMI and onset of adrenarche, providing evidence for possible *in vivo* influences of insulin and IGF-I on adrenal steroidogenic enzyme expression and adrenal androgen production.

Levels of plasma DHEA(S) in humans demonstrate a biphasic pattern of prepubertal rise and postpubertal decline that is paralleled by IGF-I. DHEA(S) levels remain low in children 2-8 years of age, then rise dramatically at adrenarche to peak by

age 20-25, followed by a steady decline until levels return to prepubertal levels by age 70 (Figure 4; Miller, 1999).

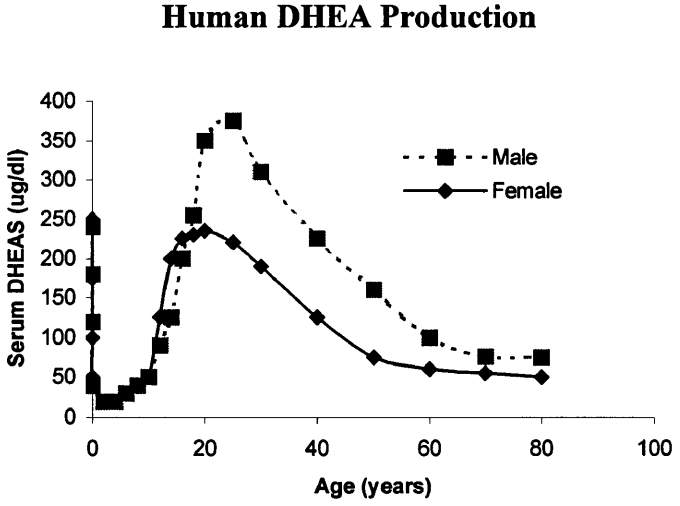


Figure 4 - Human DHEA Production. Human DHEA production in males and females drops significantly at birth, remaining at low levels until adrenarche. The significant rise at adrenarche continues until peaking at age 20-25, then declines dramatically for the remainder of life. This decline has been termed adrenopause. Figure redrawn from data by Miller, 1999.

Clinical studies have shown that neither the gonad nor gonadotropins are necessary for the onset of adrenarche (Sklar et al., 1980), and so the physiological role of adrenarche in humans remains unclear. It has not yet been determined whether adrenarche is a necessary prelude to puberty or a coincidental event. Recent studies of DHEA replacement have shown that DHEA appears to enhance immunological functions, stimulates bone formation, and increases the bioavailability of IGF-I (Kasperk et al., 1997; Khorram et al., 1997). DHEA(S) levels increase with prepubertal BMI increases, possibly providing a homeostatic feedback to the increasingly anabolic body through immunologic protection, bone formation, and increased IGF-I availability

(Kasperk et al., 1997; Khorram et al., 1997). Possible correlations with increasing adrenal DHEA production and increasing levels of IGF-1 and BMI make the adrenal an attractive candidate in the mechanism of puberty, but the extent of these interactions is still confusing. The influence of BMI on the adrenal and/or IGF-1 suggests a role for leptin, and that role may be in the stimulation of the hypothalamus GnRH pulse generator. Adding to the confusion regarding a role for the adrenal in puberty is the observation that normal puberty can develop without an adrenarche event (Sklar, 1980; Miller, 1999). These include primary adrenal insufficiency and idiopathic precocious puberty (without precocious adrenarche) (Sklar et al., 1980). Clearly, greater study of adrenarche must occur to clarify a role for the adrenal in puberty.

Unfortunately, studying adrenarche and then the subsequent drop in adrenal androgens, or adrenopause, in humans is difficult due to the length of normal humans lifespan. The biphasic pattern of DHEA(S) production in humans has not been found in rats, guinea pigs, hamsters, sheep, pigs, goats, horses, cows, or chickens (Cutler et al., 1978). Rabbits and dogs have been found to show modest twofold changes, but only the chimpanzee has been found to have the dramatic shifts in DHEA found in humans (Cutler et al., 1978). *Peromyscus* species may have a DHEA pattern not seen in other rodent species. Recent work by Hurst (1998) has shown adrenal expression of *cyp17*, the gene necessary for the production of DHEA and the weak androgens, in *P. leucopus* (Hurst, 1998).

In addition to being a possible animal model for adrenarche, *Peromyscus* have a mechanism of population growth control that involves the disruption of normal pubertal development. Whether or not this disruption involves a possible adrenarche is not

known, but elucidation of the overall mechanism in *Peromyscus* may provide a clearer understanding of reproductive disorders in humans.

Reproductive Inhibition in Peromyscus maniculatus

The prairie deermouse, *Peromyscus maniculatus bairdii*, exhibits little population density fluctuation in both natural and laboratory populations (Terman, 1973b). Population growth reaches a density-related, but not density-dependant growth asymptote in laboratory populations provided with excess food, water, and shelter through reduced survivability of young, or more commonly, through cessation of reproduction (Terman, 1973b). More than 90% of young born into these populations and surviving for at least 90 days will exhibit reproductive failure, with reproductive organ mass (ovaries, uteri, testes, seminal vesicles) and body mass of both male and female deermice remaining significantly lower than in control populations (Terman, 1973b). The reduced body and reproductive organ mass of population deermice represents an incomplete sexual maturation. A pubertal transition normally occurs between 40 to 60 days after birth in control laboratory animals. However, more than 90% of the young born into and maintained within laboratory populations will remain juvenile in appearance and gonad development (Bradley and Terman, 1981a; Terman, 1973b), and in addition will show a reduction in active and resting metabolism rate for an indefinate period (Staubs and Bradley, 1998). This inhibition is reversible, with nearly 50% of inhibited animals reproducing within 30 days, and 75% within 80 days of removal from a population context and pairing (Terman, 1973a). Accompanying the recovery of reproductive capability is the onset of adult patterns of metabolism and reproductive organ and total

body mass consistent with a post-pubertal transition (Staubs and Bradley, 1998; Terman, 1973a). This reversable inhibition indicates that the mechanism does not involve a permanent early alteration to the hypothalamic-pituitary axis.

In a previous study by Teague and Bradley (1978), the existence of puberty accelerating pheromone was discovered in the urine of male *P. maniculatus*. This factor was found to be testis dependent, as the urine from castrated males did not elicite weights different from controls. A later study by Terman (1984) found that the urine of adult females did not significantly alter the gonadal development of immature females, but found physical contact significantly retarded growth of ovaries and uteri. Contact with reproductively proven females or their urine was found to accelerate the reproductive maturation of immature males (Babb and Terman, 1982). Creigh and Terman (1988) found that the presence of reproductively proven females or their urine also increased the testis and seminal vesicle mass of inhibited males, promoting the reproductive recovery of those males in a fashion similar to the immature males studied by Babb and Terman (1982).

A further study found a significant reduction in the serum testosterone of inhibited animals compared to controls (Bradley and Terman, 1981b). This reduction was much less (63% from controls) than expected from the severely reduced testis masses seen in inhibited animals, leading the authors to speculate a possible role for the adrenals of inhibited animals in producing androgen precursors. A comparison of adrenal histology found a reduced mean adrenal mass in inhibited animals, but a trend towards larger relative adrenal masses in these inhibited animals (Bradley and Terman, 1981a). Corticosterone levels were also found to be significantly elevated in inhibited animals

compared to controls of both sexes. To investigate whether it is chronically elevated ACTH levels or corticosterone levels that are contributing to reproductive inhibition, Coppes and Bradley (1984) examined the effects of exongenous ACTH and glucocorticoid on the adrenal ultrastructure of inhibited and control male *P. maniculatus*. They found that exogenous ACTH caused a hypertrophy of the adrenal, and a corresponding reduction in testis and seminal vesicle weight similar to that seen in inhibited animals. However, the ultrastructure of the inhibited male's adrenal *zona fasciculata* differed drastically from that of control animals and animals treated with exogenous ACTH. Most notable was a gross alteration of mitochondrial structure in inhibited animals that was not seen in either other group. Serum ACTH levels were not found to be significantly different in inhibited animals compared to controls. These findings suggested that ACTH alone does not promote the reproductive inhibition, however it may play some role.

The depression in general growth and development of population animals prompted Pitman and Bradley (1984) to investigate the role of the thyroid in inhibition. Total serum thyroxine (T3) and thriiodothyronine (T4) were significantly lower in both male and female inhibited animals when compared to controls. A functional hypothyroidism was found in both male and female inhibited animals through gravimetric measurements. Kirkland and Bradley (1986) found a reduced mean serum prolactin level in inhibited males and females. They suggested that these reduced levels may be a consequence of the previously observed reduction in thyroid hormones and elevation of corticosterone, both of which have been shown to inhibit synthesis and

secretion of prolactin. Whether the reduced levels of prolactin were the cause or consequence of reproductive inhibition was not determined.

In some other rodent species, density-related reproductive inhibition is accompanied by a significant stress-induced adrenal gland hypertrophy (Calhoun, 1962; Christian, 1956; Christian, 1955; Christian et al., 1965). However, reproductively inhibited *P. maniculatus* have been shown to exhibit gonadal regression and elevated serum corticosterone concentrations, but without gravimetric evidence of the adrenal hypertrophy seen in other rodent species (Bradley and Terman, 1981c; Sung et al., 1977). The apparent *Peromyscus* exception to the murine adrenal-associated population-stress paradigm prompted Cherry (1998) to look more closely at the adrenal histology of reproductively capable and inhibited animals before, during, and after the predicted pubertal transition. Cherry reported histogical changes in the adrenal that were associated with the pubertal transition in control animals, as well as a distinctly contrasting *zona reticularis* histology in reproductively inhibited deermice compared with controls. In particular, reproductively capable control animals had significantly larger mean relative *zona reticularis* areas at prepubertal ages, followed by a decreased *zona reticularis* post puberty. In contrast, reproductively inhibited animals maintained a proportionally larger *zona reticularis* area at ages more than twice that of the time of normal puberty. Hence, there appears to be an association of changed adrenal activity with a delayed pubertal transition.

Cherry's (1998) morphological observations suggest that the adrenals of maturing *P. maniculatus* undergo functional changes, and that reproductively inhibited animals have a different functional capacity. To investigate these possible functional changes, the

study of two genes involved in different steps of adrenal steroidogenesis were studied. The expression of these genes, *cyp17* and *hsd3b1*, can be used to infer changes in steroidogenesis of the adrenals through normal puberty, and in the special case of the disrupted puberty that is responsible for reproductive inhibition.

CYP 17

Biochemistry

Human cytochrome 17α-hydroxylase/17,20-lyase (CYP17; also P450c17) catalyzes the 17α-hydroxylation of pregnenolone to 17α-hydroxypregnenolone and progesterone to 17α-hydroxyprogesterone. The same enzyme also catalyzes 17,20-lyase activity to convert 17α-hydroxypregnenolone to dehyroepiandrosterone (DHEA), but only in rodents is the 17α-hydroxyprogesterone to androstenedione conversion found to be of significance (Figure 5) (Lin et al., 1993).

Cloning

Cyp17 is encoded by a single gene, *CYP17* (*cyp17* in rodents), and a single species of mRNA (Miller, 1998). Human *CYP17* has been mapped to chromosome 10q 24.3, and is found to exist as a single copy (Matteson et al., 1986). The cloning and expression patterns of *CYP17* (*cyp17*) have been studied in a variety of animals. *Cyp17* has been found in the gonads of all animals studied, in the central nervous system of embryonic mice and rats (Compagnone, 1995), as well as in the brain of mice and rats (Stromstedt, 1995). Cloutier (1997) cloned hamster *cyp17*, and found the cDNA to be 76% homologous in sequence to human *CYP17*.

Cyp17 Steroid Conversions

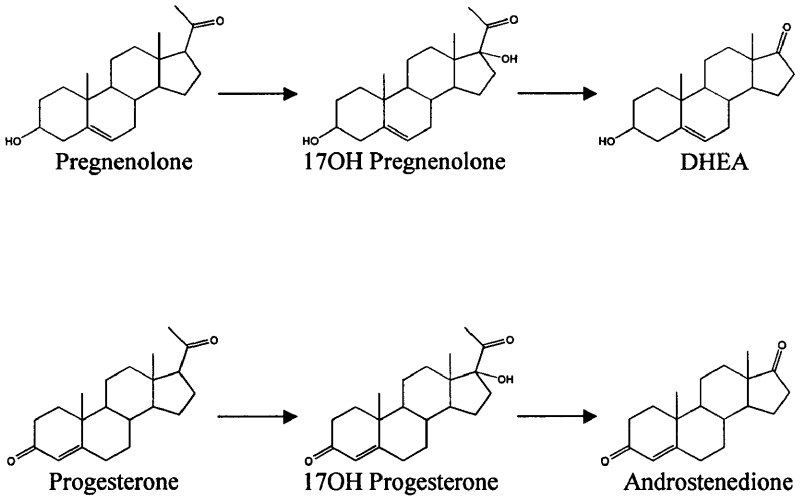


Figure 5 - Cyp17 Steroid Conversions. The first conversion involves the hydroxylation of carbon 17. The second (independent) conversion involves the lyase conversion of carbons 17 and 20. The upper series of conversions are referred to as the Δ^5 pathway, while the lower are referred to as the Δ^4 pathway, in reference to the double bond position in ring 1. Schematic produced with CS Chemdraw Ltd. 3.1 (CambridgeSoft Corporation).

Transcriptional Regulation

Like other steroidogenic enzyme genes, the promoter of *cyp17* contains a consensus binding sequence for steroidogenic factor-1 (SF-1). Binding of SF-1 activates transcription in *cyp17* and other steroidogenic genes. Hanley (2001) found three distinct promoter elements for SF-1 binding and transcriptional regulation in human, rat, mouse, pig, and cow. The nuclear orphan receptor DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia congenital, critical region on the X chromosome gene 1) has been shown to colocalize with SF-1, and repress transcription of steroidogenic enzyme genes through recruitment of corepressors (including nuclear receptor corepressor N-CoR) (Hanley et al., 2001). Interestingly, DAX1 has no transcriptional suppressive effect when only one SF-1 binding site is ocupied. When two are occupied, weak suppression occurs, with

strong repression occurring only when all three sites are occupied (Hanley et al., 2001). Hanley suggests that fine tuning of transcription could occur through not only binding of SF-1, but also other factors that could effectively compete for binding sites.

Through a cAMP mediated pathway, ACTH is known to upregulate transcription of *cyp17* mRNA within 4 hours of exposure, but the exact mechanism is unknown (Wilson et al., 1998). One proposed mechanism is through the interactions of SF-1 with the cAMP-responsive proximal sequence (CRS2) and multiple coactivators (cAMP binding protein and steroid receptor co-activator) or corepressors (DAX1 and chicken ovalbumin upstream promoter-transcription factors) (Lehmann et al., 2000). ACTH is not believed to affect the 17,20-lyase activity of the cyp17 enzyme, as ACTH levels do not increase with the corresponding increase in 17,20-lyase activity at adrenarche (Biason-Lauber et al., 2000).

Functional Studies

Cyp17 is a microsomal protein found in cells of the gonads and the adrenal *zonae fasciculata* and *reticularis*, with only gonad and *reticularis* cells having both 17α-hydroxylase and 17,20-lyase activities in humans. This distribution permits the *fasciculata* to produce C-21 steroids and cortisol, but not the C-19 steroids that may be only synthesized by cells of the *reticularis* and gonad. The dual enzymatic functions of cyp17 are under independent regulatory control. Cortisol is synthesized throughout the lifetime of humans, necessitating a constant expression of *cyp17*. The adrenarche and adrenopause phenomena suggest a temporal control of the 17, 20 lyase activity of cyp17 necessary to synthesize DHEA. A second hypothetical peptide hormone (cortical

androgen stimulatory hormone) was originally suggested to account for this independantly regulated 17,20-lyase activity (Parker and Odell, 1980). Independent control of 17,20-lyase activity is now known to be accomplished through regulation of cyp17 and P450 oxidoreductase (OR) interaction, an interaction necessary to mediate the electron transfer from NADPH that is essential to 17,20-lyase enzymology (Lin et al., 1993). Studies increasing the molar ratio of OR to cyp17 in transfected mammalian cells resulted in increased ratios of lyase to hydroxylase activity (Lin et al., 1993). This is also the condition found in the testis, where a high molar ratio of OR to cyp17 leads to the exclusive production of C-19 steroids. Confirming this are studies inducing mutations in the OR binding site, which resulted in decreased lyase activity without a paralleled decrease in hydroxylase activity (Kitamura et al., 1991). Regulation of cyp17 and P450 OR interaction is mediated through phosphorylation of cyp17 serine and threonine residues by cAMP-regulated kinases (Miller, 1998). These reactions alter the electrostatic charge on the surface of the cyp17 protein in the region of the OR binding site, allowing greater OR interaction efficiency, and a subsequent increase in lyase activity independent of hydroxylase activity (Miller, 1998).

Cytochrome b_5 has recently been implicated as an additional potential component in the regulation of the 17,20-lyase activity of cyp17 (Auchus et al., 1998; Mapes et al., 1999). Mapes et al. (1999) performed a immunohistochemical analysis of cyp17, 3βHSD, and b_5 distribution in the adrenals of rhesus macaques. Cyp17 was found throughout the *fasciculata* and *reticularis*, 3βHSD was restricted to the *fasciculata*, and b_5 was restricted to the *reticularis*. This distribution of b_5 is in accordance with the necessary lack of 17,20-lyase activity requisite to a cortisol-producing primate

fasciculata, and abundance requisite to a weak androgen-producing primate *reticularis*. Auchus et al. (1998) engineered yeast with inducible promoters to express human cyp17, OR, or b_5. Co-expression of cyp17 with b_5 resulted in a 10 fold increase in V_{max} for pregnenolone and progesterone conversion, but this increase existed only in the 17,20-lyase portion of the reaction. Through analysis of xray crystal structures of OR and cyp17, Auchus (1998) proposed a model by which b_5 acts as an allosteric effector protein, optimizing the geometry of the cyp17-OR complex for the more sensitive 17,20-lyase reaction. Hence, it appears that b_5 is not an essential component of 17,20-lyase activity, but rather acts to enhance the activity of OR.

The peptide hormone leptin has also recently received attention for its possible role in the independent control of 17,20-lyase activity. Patients with leptin deficiency or leptin resistance exhibit normal ACTH and cortisol levels, but do not produce sex steroids, or subsequently undergo sexual development (Clement et al., 1998; Montague et al., 1997). This suggests that the 17α-hydroxylase activity of cyp17 is intact, but the 17,20-lyase activity is somehow impaired. Biason-Lauber et al. (2000) studied the effects of leptin on cyp17 activity in human adrenocortical cells. They found a direct effect of leptin on 17,20-lyase activity that was dependent upon intact leptin receptor and an intact receptor signal transduction pathway. The increased 17,20-lyase activity to leptin treatment was immediate, and therefore appears to involve posttranslational modification of cyp17 (Clement et al., 1998; Montague et al., 1997) or an associated protein such as OR, rather than transcriptional or translational alterations. Addition of the protein synthesis inhibitor cycloheximide did not alter DHEA production, supporting the authors hypothesis of posttranslational involvement. Leptin acts through a cell

surface receptor, with transcriptional actions mediated through the JAK-STAT protein phosphorylation pathway (Biason-Lauber et al., 2000). Phosphorylation is known to be crucial to 17,20-lyase activity, and it may be that leptin's effect on cyp17 involves portion(s) of this pathway (Clement et al., 1998; Montague et al., 1997).

Cyp17 has been implicated in the induction and maintenance of adrenarche and adrenopause in humans. Miller (1999) has proposed that the upregulation of 17,20-lyase activity necessary to produce DHEA could be promoted by an increased biosynthesis of OR, but this has thus far been unproven. In addition, IGF-1, whose levels parallel those of DHEA production, is also known to elicit numerous adrenal intracellular expression changes through phosphorylation pathways and therefore may play a regulatory role in cyp17 modifications in a fashion similar to serine/threonine kinases (Miller, 1999). Regulation of b_5 may also play a role, although no positive correlation between *CYB5* (the gene encoding b_5) expression and 17,20-lyase activity has yet been made (Biason-Lauber et al., 2000).

Most rodent species produce corticosterone as their primary glucocorticoid, therefore not requiring expression of *cyp17* in the adrenals. *Cyp17* has been found in the adrenals of hamsters (Cloutier, 1997), guinea pigs (Tremblay, 1994), in *Peromyscus leucopus* (Hurst, 1998) and now in this study, *Peromyscus maniculatus*. The presence of *cyp17* in the adrenals of hamsters is not surprising, as cortisol is their primary glucocorticoid. Using immunolocalization of anti-rat cyp17 antibody, Briere et al. (1997) found the *zona fasciculata* to be the sole producer of the cyp17 enzyme in the hamster. A dose dependent increase in cyp17 expression with ACTH stimulation was found through immunolocalization as well as western blotting assays, with the *zona fasciculata* again

being the significant zone of expression in hamsters (Briere et al., 1997). Additionally, immunocyctochemistry with colloidal gold showed a predominance of cyp17 present in intermitochondrial spaces corresponding to the endoplasmic reticulum (ER). ACTH stimulation showed an increased ER staining with no increase in *zona fasciculata* area, suggesting increased cellular activity of *cyp17* without cellular proliferation (Briere et al., 1997). Cloutier et al. (1997) characterized hamster adrenal *cyp17* through cloning and transient expression studies, and cyp17 through immunoblotting studies. They found that hamster *cyp17* cDNA was 76% homologous to that of human, and when transfected in an expression vector into COS 1 cells it was capable of converting pregnenolone to 17α-hydroxypregnenolone to DHEA as well as progesterone to 17α-hydroxyprogesterone to androstenedione. Using cell suspensions and microsomal preparations, Cloutier found both DHEA and pregnenolone were produced, but found their intermediates (17α-hydroxypregnenolone and 17α-hydroxyprogesterone) produced at much higher molar ratios. These results indicate that the hamster adrenal is both a cortisol and DHEA producer, with the maintenance of 17α-hydroxylase activity significantly higher than 17,20-lyase activity, suggesting that more cortisol is produced compared to DHEA.

Cortisol is also the primary glucocorticoid produced by the guinea pig. In a study of zonal distribution of cyp17 in the guinea pig adrenal, Shinzawa et al. (1988) found immunolocalization of cyp17 in both the *zonae fasciculata* and *reticularis*, with the majority located in the outer *fasciculata*. Addition of ACTH to microsomal fractions resulted in increased 17α-hydroxylase activity in both the *zonae fasciculata* and *reticularis*, but did not affect 17,20-lyase activity in either zone. These data suggest that the synthesis of cortisol, but not weak androgen, is upregulated in the intact guinea pig

adrenal upon ACTH stimulation. Tremblay et al. (1994) performed similar distribution and substrate studies on the *cyp17* gene in guinea pig adrenals. To determine enzyme activities and substrate specificity, full length *cyp17* cDNA was inserted into an expression vector and transfected into COS-1 cells. Tremblay found both 17α-hydroxylase and 17,20-lyase activity when incubated *in vitro* with pregnenolone or progesterone, though progesterone conversion was extremely low. To confirm the Δ^4 pathway (pregnenolone substrate) preference *in vivo*, the authors transfected the full length cyp17 cDNA into primary adrenocortical cell culture while blocking the 3βHSD activity through administration of trilostane. The result was only a 12% conversion of the Δ^5 pregnenolone to 17α-hydroxypregnenolone, confirming the Δ^4 substrate specificity that is thus far novel to the guinea pig. Interestingly, very low levels of the Δ^4 pathway intermediate 17α-hydroxyprogesterone were detected, leading the authors to suggest the 17,20-lyase reaction to proceed more rapidly than the 17α-hydroxylation reaction. Supporting this is the significantly higher cyp17 reaction V_{max} of 24.4 pmole/min·mg for 17α-hydroxyprogesterone, versus 1.4 pmole/min·mg for progesterone. The authors hypothesize that it is the 17α-hydroxylation that is rate-limiting, and that the cyp17 enzyme does not dissociate from the substrate between 17α-hydroxylation and 17,20-lyase reactions.

Preliminary gene expression studies by Hurst (1998) and Powers (1999) suggest that *cyp17* is expressed in the adrenals of *Peromyscus* species. The presense of *cyp17* in the adrenals of *Peromyscus* suggests that either low levels of cortisol, or possibly C-19 steroids such as DHEA, are being produced by the *Peromyscus* adrenal. The significance

of this gene expression or hormone synthesis by the *Peromyscus* adrenal is currently under evaluation.

HSD3B1

Biochemistry

3β-hydroxysteroid dehydrogenase/isomerase (3βHSD) catalyzes the conversion of Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids, an obligate step in the biosynthesis of all biologically active adrenal steroid hormones. In the adrenal cortex, 3βHSD is necessary for the production of the mineralcorticoid aldosterone, the glucocorticoids cortisol and corticosterone, and the weak androgen androstenedione (Wilson, 1998). In the gonads 3βHSD is required for conversion of the weak androgen DHEA to the more active testosterone or estrogen. The dehyrogenase and isomerase activities are linked on a single dimeric protein, with no isolatable intermediates found during the Δ^5-3β-hydroxysteroid to Δ^4-3-ketosteroid conversion (Mason et al., 1998). 3βHSD is an NAD^+ or NADPH dependent, membrane-bound protein closely associated with the endoplasmic reticulum and mitochondrial membrane of traditional steroidogenic tissues (adrenal, gonads, and placenta) (Mason et al., 1997). The enzyme is additionally found in nonsteroidogenic tissues such as the kidney and liver, where it appears to catalyze the formation or degradation of the 5α-androstanes and 5α-pregnanes (Zhao et al., 1991).

Cloning

At this time two functional and five pseudogenes have been characterized for *HSD3B1* in the human genome (McBride, 1999; McCartin, 2000), while five and six

functional genes and one pseudogene have been identified in the rat and mouse genomes respectively (Payne et al., 1997). Human *HSD3B* exists as a cluster of genes located on chromosome 1p13.1 (McCartin, 2000), while the seven mouse *hsd3b* genes have been found in a cluster on chromosome 3 between *Tshb* and *Gba* (Payne et al., 1997). The *HSD3B* gene family is thought to have arisen through gene duplication of a single ancestral *HSD3B* gene (McBride et al., 1999). Six murine cDNAs (from six separate genes) exhibiting a high degree of amino acid identity (between 72 and 93% amino acid identity) have been isolated to date (Figure 6) (Abbaszade et al., 1997; Payne et al., 1997).

***Mus musculus* 3βHSD Amino Acid Homologies (%)**

	Type II	**Type III**	**Type IV**	**Type V**	**Type VI**
Type I	84	83	77	74	84
Type II		93	75	72	91
Type III			73	72	91
Type IV				93	74
Type V					72

Figure 6 - *Mus musculus* 3βHSD Amino Acid Homologies. The six cDNAs arise from six separate *hsd3b* genes, and fall into two groups based on amino acid homology and functionality. cDNAs I, II, III, and VI act as NAD^+ dependent dehydrogenase/ isomerases and are 83-93% similar in amino acid sequence. cDNAs IV and V function as NADPH dependent 3-ketosteroid reductases and share 93% amino acid sequence identity. Figure from data by Abbaszade, 1997.

All of the murine cDNAs contain a 1119 bp open reading frame encoding a protein of 373 amino acids (Payne et al., 1997). The six murine cDNAs fall into two groups based on substrate specificity and activity (Figure 7). cDNAs I, II, III, and VI function as NAD^+ dependent dehydrogenase/ isomerases and are 83-93% similar in amino acid

sequence (Payne et al., 1997). cDNAs IV and V function as NADPH dependent 3-ketosteroid reductases and share 93% amino acid sequence identity (Payne et al., 1997). cDNAs were named based on their chronological discovery creating confusion, with murine type I being homologous to human type II, and mouse type VI being homologous to rat type IV and human type I. Murine type I (human type II) 3βHSD is predominantly found in the adrenals and gonads, where it is responsible for the biosynthesis of active steroids. Type I is additionally found in the kidney, fat tissue, and placenta of rodents (Payne et al., 1997). Type II and III have been isolated from murine kidney and liver tissue, with type III being the dominant cDNA expressed in the adult liver (Payne et al., 1997). Type III is the major NAD^+ dependent cDNA expressed in the adult rodent liver, and may be important in the production of steroid hormones in the case of adrenal or gonadal genetic deficiencies (Payne et al., 1997). In mice, type IV is found exclusively in the adult kidney cortex, with a predominance of expression in the proximal convoluted tubles (Payne et al., 1997). Type V is expressed only in the male rodent liver, where, like type IV in the kidney, it is most likely involved in the deactivation of active steroid hormones (Zhao et al., 1991). Mouse type VI (rat type IV, human type I) is principally expressed in the placenta and sebaceous glands of skin (Abbaszade et al., 1997). In the placenta, type VI functions to locally synthesize progesterone from pregnenolone, a conversion that is essential to blastocyst implantation and maintenance of pregnancy (Abbaszade et al., 1997).

3βHSD Steroid Conversions

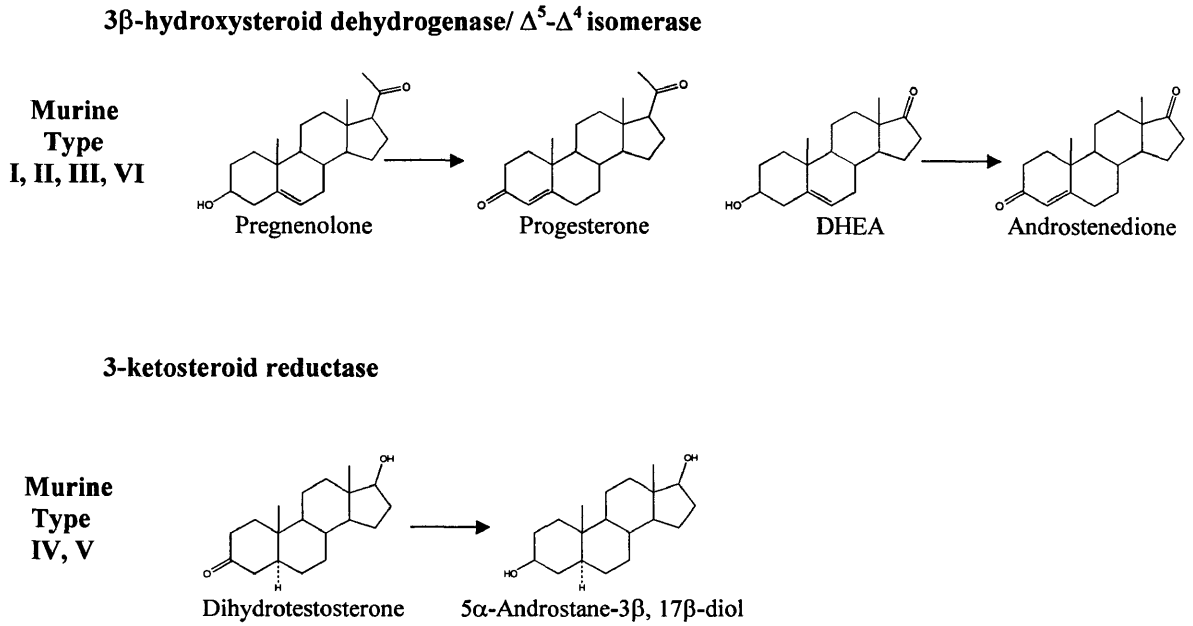


Figure 7 - 3βHSD Steroid Conversions. cDNAs I, II, III, and VI act as NAD^+ dependent dehydrogenase/ isomerases, while cDNAs IV and V function as NADPH dependent 3-ketosteroid reductases. Schematic produced with CS Chemdraw Ltd 3.1 (CambridgeSoft Corporation).

Expression

Like other adrenal steroidogenic enzyme genes, 3βHSD contains a putative consensus binding element for the transcription factor, steroidogenic factor 1 (SF-1) (Mason et al., 1997). This 9 base pair element (TCAAGGTAA) lies 64 to 56 base pairs upstream of the human type II promoter but not that of human type I. The functionality of this element has been demonstrated through transient transfection with promoter-CAT constructs containing intact or mutated element sequence (Mason et al., 1997). Leers-Sucheta et al. (1995) found that expression was only induced by phorbolester in the

presence of intact SF-1 binding element constructs. The authors also found expression to be enhanced by cotransfection with a SF-1 encoding expression vector, lending support to the element's putative transcriptional role (Leers-Sucheta et al., 1995). The lack of this element in human type I is in accordance with its expression in only placental tissues, where the trancription factor SF-1 is not found. Interestingly, the rat adrenal cDNA (type I) sequence demonstrates significant divergence in the area of the SF-1 bindings site from human (type II) (Leers-Sucheta et al., 1995). The crucial core sequence (AAGG) is found at other places in the first intron, and it is not known if these areas, or the divergent putative binding site are able to mediate SF-1 transcription effects (Leers-Sucheta et al., 1995). As in *cyp17*, DAX1 is capable of repressing *hsd3b1* transcription by binding to SF-1 and recruiting corepressors. However, the single SF-1 binding domain found in human and mouse adrenal *hsd3b* is not significantly repressed (Lalli et al., 1998). Hanley's (2001) observation that an increased number of SF-1 elements binding to the promoter of *cyp17* increased DAX1 inhibition suggests that the single *hsd3b* consensus sequence allows it and *cyp17* to be differentially transcribed by SF-1 and DAX1 interaction.

Doi et al. (2001) investigated the effects of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) on *HSD3B* (human type II, murine type I) and *CYP17* expression in human adrenocortical carcinoma cells. They found that both EGF and bFGF induced mRNA production of 3βHSD while suppressing CYP17 production. Similar to the time course seen in ACTH-induced expression of 3βHSD and CYP17, EGF and bFGF induction did not occur until approximately 6 hours after treatment (Doi et al., 2001). This induction was determined to require *de novo* protein synthesis, as

treatment with the protein synthesis inhibitor, cycloheximide, abolished these effects (Doi et al., 2001). The pathways by which these factors effect *HSD3B* and *CYP17* expression are still not understood, though CaMK II and MAPK signalling pathways are indicated to be important based on results from treatments manipulating pathway intermediates (Doi et al., 2001).

Functional Studies

In humans, fifteen missense mutations have been reported in the gene encoding 3βHSD type II that result in altered adrenal and gonadal ouput (McCartin et al., 2000). Several null mutations lead to congenital adrenal hyperplasia (CAH), characterized by reduced levels of glucocorticoids, sex steroids, and loss of aldosterone, with subsequent severe salt wasting (McCartin et al., 2000). Less severe mutations have been identified that allow sufficient production of aldosterone, but not that of the sex steroids. The loss of testosterone production in these males leads to pseudohermaphroditism, while the loss of estrogens in these females can lead to virilization or premature puberty (McCartin et al., 2000). Interestingly, McCartin et al. (2000) reported the first case of premature adrenarche in a male with 3βHSD type II deficiency. This observation supports Miller's (1999) hypothesis that a 3βHSD decline results in the elevated adrenal DHEA paramount to adrenarche. Residues Cys182 and His261 are believed to be the active sites of substrate binding and isomerase activity on human type II 3βHSD, as evidenced from mutation studies (Strickler et al., 1993) and affinity labelling with radiolabelled substrate (Thomas et al., 1993). Of the fifteen missense mutations clinically reported thus far, six are adjacent to one of these residues (McCartin et al., 2000).

In adult humans, 3βHSD type II is located predominantly in the *zonae glomerulosa* and *fasciculata*. A lack of 3βHSD activity in the adult *reticularis* predicates the production of the weak androgen DHEA and not cortisol. This is also the condition found in rhesus macaques and other nonhuman primates (Mapes et al., 1999). Two studies have provided evidence that adrenarche is related to age-related changes in the expression of 3βHSD type II (murine type I) in the human adrenal. Gell et al. (1998) utilized an antibody for 3βHSD to study changes in enzyme expression in the adrenals of 38 autopsy patients from 4 months to 56 years in age. They found a significant decline in 3βHSD production in the *reticularis* of children age 8-13, and a continued gradual decline in *reticularis* 3βHSD beyond 13 years of age, with no corresponding change in either the *fasciculata* or *glomerulosa* (Gell et al., 1998). Dardis et al. (1999) examined total adrenal RNA extractions of 11 individuals for 3βHSD type II expression using RT-PCR. They found a similar decrease in 3βHSD expression in the older of the two groupings, suggesting the same overall decline in 3βHSD production following adrenarche and puberty that Gell (1998) observed (Dardis et al., 1999). In a study of premature pubarche (the appearance of pubic hair), Marui et al. (2000) identified two mutations in *HSD3B2* that led to premature pubarche and elevated ACTH-stimulated 17OH-pregnenolone production. This elevated 17OH-pregnenolone is likely a result of decreased 3βHSD competition on CYP17, and given the subsequent premature pubarche, provides additional support for the importance of altered 3βHSD expression with adrenarche.

In contrast to humans, adrenal expression of 3βHSD is found in all three cortical zones of rats. As in humans, rat adrenal 3βHSD is upregulated by ACTH and downregulated by glucocorticoid administration (Pignatelli et al., 1999). Pignatelli et al. (1999) utilized an antibody for 3βHSD type I (human type II) to study the postnatal ontogeny of 3βHSD in the adrenal glands of rats. The inner and outer *fasciculata* were found to exhibit 3βHSD activity from birth, with both increasing in staining intensity to pubertal ages. The *glomerulosa* and *reticularis* were slower to develop, with the *reticularis* showing no 3βHSD activity until 10-18 days after birth, and both showing increasing activity until time of predicted puberty. However, this study did not focus on the pubertal transition, and lacked any conclusive data relating 3βHSD activity to pubertal transition.

3βHSD was found to be distributed throughout the hamster adrenal cortex through the use of immunohistochemistry with a rabbit anti-human placental 3βHSD antibody (Rogerson et al., 1998). The kinetics of pregnenolone and DHEA conversion by 3βHSD in hamster adrenal cell homogenates was also measured. Interestingly, 3βHSD displayed a higher affinity for DHEA than pregnenolone as substrate. Rogerson (1998) suggests that DHEA levels in hamster adrenals are significantly lower than pregnenolone, as evidenced by the substantially lower levels of cyp17 compared to other steroidogenic enzymes, necessitating a high affinity for efficient conversion of DHEA. This is not the case in rat and mouse, where cyp17 is not present, and affinity of 3βHSD is much higher for pregnenolone (Simard et al., 1996).

Concluding Remarks and Hypothesis

The physiological differences Terman (1973), Bradley and Terman (1981a,b,c), and Staubs and Bradley (1998) found between reproductively capable controls and reproductively inhibited *Peromyscus maniculatus* collectively suggest that reproductive inhibition acts in part through a delayed pubertal transition. The adrenal differences Bradley and Terman (1981c) and Cherry (1998) described between reproductively capable controls and reproductively inhibited *P. maniculatus* suggests an association of changed adrenal activity with a delayed pubertal transition. However, the nature and consequence of these adrenal morphometric changes is not understood. Presumably, alterations in the zonation of the *Peromyscus* adrenal results in altered output as other investigators have shown in other species (Colby et al., 1993; Dardis et al., 1999; Gell et al., 1998; Mapes et al., 1999). The small size of the *P. maniculatus* adrenal makes the study of hormone production difficult by current practices. However, through the study of the genes requisite to their production, alterations in adrenal output can be inferred. Previous to this study, the adrenal expression patterns of *hsd3b1* and *cyp17* were unknown in *Peromyscus maniculatus*.

While these genes and their proteins have been studied in a number of other species, current documentation of gene and protein expression correlated with puberty is limited (Figure 8). In humans, adrenarche and the onset of puberty have been correlated with decreased expression of *hsd3b* and 3βHSD in the *zona reticularis*. Concurrently, the expression of *cyp17* does not change, but the relative 17,20-lyase activity of cyp17 increases dramatically in the *zona reticularis* (Miller, 1999). A pubertal time course of

hsd3b or its protein in the guinea pig is currently unpublished. Enzyme activity assays have found an increase in cyp17 activity in the *zona fasciculata*, while a decrease was found in the *zona reticularis* (Colby et al., 1993). This study was limited to observations made at 35, 100, and 200 days of age, and therefore only included prepubertal, and post pubertal observations, and only the 17α-hydroxylase activity of cyp17 was measured (to investigate cortisol production), therefore neglecting possible DHEA production through additional 17,20-lyase activity. There are no reports in the literature of studies of *cyp17* in the adrenals of rat and mouse. 3βHSD has been studied in the adrenals of both species, but only in rat has pubertal transition been addressed. Pignatelli (1999) found increased levels of 3βHSD as rats approached puberty, but, this study ended at 40 days of age, and therefore did not detect possible pubertal changes.

Currently Documented Patterns of Adrenal cyp17 and hsd3b in Pubertal Transition

	cyp17	*hsd3b*	Comments
Human	No differences in protein or mRNA	↓ protein in ZR ↓ mRNA in total adrenal	change in cyp involves ↑ 17,20-lyase activity, not expressional changes
Guinea Pig	↑ in ZF ↓ in ZR (enzyme activity)	Currently unknown	cyp enzyme assays on 35d & 100d animals only (prepubertal & adult only)
Rat	*cyp expression not reported	↑ protein in ZF & ZR	study ended at 40d of age (likely prepubertal)
Mouse	*cyp expression not reported	Currently unknown	
Hamster	Currently unknown	Currently unknown	

Figure 8 - Currently Documented Patterns of Adrenal *cyp17* and *hsd3b* in Pubertal Transition. While *cyp17* and *hsd3b* have been studied in all species above, current literature documenting pubertal changes in adrenal *cyp17* and *hsd3b* are limited. Relative levels of protein and mRNA at puberty are listed above when reported (↑ indicates increased levels at puberty, ↓ indicates decrease levels at puberty). ZR= zona reticularis, ZF= zona fasciculata, **cyp17* is not expressed in rat or mouse adrenals, studies are cited in text.

Clearly, there is a lack of studies investigating the changes in *cyp17* and *hsd3b1*, and their enzyme products in the adrenals of murine species. The *hsd3b1* gene product (3βHSD) is essential to the production of all active adrenal steroids, and is therefore believed to be an indicator of overall adrenal activity. The *cyp17* gene product (cyp17) is essential to the production of adrenal cortisol and/or weak androgen. These two genes represent an important branch point in steroidogenesis, and their expression may indicate functional changes in the adrenal during both maturation and reproductive inhibition.

The expression pattern of these genes will be investigated in the adrenals of normally maturing male *Peromyscus maniculatus* before, during, and after pubertal transition using the ribonuclease protection assay. Because puberty is likely to be a time of increased metabolic demand requiring glucocorticoid, and possibly weak androgen production for sexual maturation, we hypothesize that both adrenal *hsd3b1* and *cyp17* will show increased expression during normal pubertal transition. We will also study the expression pattern of the same two genes in a naturally occurring mechanism of pubertal dirsuption (reproductive inhibition) and compare their expression to the normal maturational time course expression. Because reproductive inhibition does not disrupt the ability of the adrenal to maintain mineral balance or to produce corticosterone (Bradley and Terman, 1981c), our second hypothesis is that expression of *hsd3b1* in reproductively inhibited animals will not be significantly different than similarly aged controls. However, due to the impaired sexual maturation characteristic of reproductive inhibition, we hypothesize that reduced expression of weak androgen producing *cyp17* will be found in reproductively inhibited animals compared to controls.

Materials and Methods

Animal Maintenance

Animals used in this study were prairie deermice (*Peromyscus maniculatus bairdii*) obtained from our outbred production colony (Bradley and Terman, 1981a; Bradley and Terman, 1981b; Bradley and Terman, 1981c). All animals were maintained within an annual temperature range of 23±4°C and exposed to fluorescent lighting that averaged 120 lux at the floor and 160 lux at the height of individual cages. Lighting for all studies was provided for 14 hours followed by a daily dark period between 2000h and 0600h. All production colony and selected control animals were maintained in wiretop plastic cages measuring 12.8 x 27.8 x 14.5 cm on wood shavings in same-sex sib groups following weaning (21d). Food (Prolab Rat, Mouse, Hamster 3000, Agway, Inc.) and water were provided *ad libitum.*

Reproductively capable control animals constituted non-sibling males selected from different litters at the specific ages of 30, 50, 70 (±2), 90 (±3), and 120 (±5) days. To create a representative sample and to permit continued production of animals within a fairly constant population context only two animals were sampled from any single litter.

Reproductively inhibited animals were obtained from growing "laboratory populations" by methods that have been previously described in detail (Bradley and Terman, 1981a; Bradley and Terman, 1981b; Bradley and Terman, 1981c). Briefly, two populations were established within 1.5m diameter enclosures with four pairs of reproductively proven animals. Young produced within the population were checked

every two weeks for reproductive condition. Reproductively inhibited animals were selected by lot for study from among appropriately aged animals only if they had never displayed a scrotal testes. To create a representative sample and to permit continued production of animals within a fairly constant population context only two animals were sampled from any single litter.

Organ Sampling

Adrenal glands were removed from control male *P. maniculatus* at ages 30, 50, 70 (±2), 90 (±3), and 120 (±5) days (n=5 per age group) and reproductively inhibited male *P. maniculatus* at 130 (±10) days (n=5). Animals were sacrificed by cervical dislocation with decapitation and immediately weighed prior to adrenal removal. A longitudinal abdominal incision was made and the adrenals were removed, cleaned of fat and connective tissue, and then weighed to the nearest 0.1mg before rapid freezing in liquid nitrogen. Testes and seminal vesicles were dissected and fixed in 10% buffered formaldehyde and weighed to the nearest 0.1mg after complete dehydration. There were never more than two siblings sampled for each age group. Animals were kept undisturbed in their home cage until the moment of sacrifice to limit possible adrenal stress response.

RNA Isolation

Total RNA from male *P. maniculatus* adrenal and testis pairs was extracted with GITC (Sambrook et al., 2001). Briefly, RNA was extracted with phenol/chloroform and precipitated in isopropanol. DNA was removed with 0.5µl RNase-free DNase (10U/µl,

Promega, Madison, WI), followed by a second round of phenol/chloroform extraction and ethanol precipitation. The concentration and purity of total RNA was determined through denaturing gel electrophoresis and optical density measurement at 260 and 280 nm with a GeneQuant Pro (Amersham Pharmacia Corp.).

DNA Isolation

Genomic DNA from male *P. maniculatus* brain and testis was extracted by a protocol from Sambrook et al., 2001. Briefly, tissues were homogenized in a digestion buffer containing 0.1mg/ml proteinase K (Promega, Madison, WI). DNA was extracted with phenol/chloroform twice and ethanol precipitated. RNA was removed with 2µl RNase A (40µg/ml, Ambion, Austin, TX), followed by phenol/chloroform extraction, chloroform/isoamyl alcohol extraction, and ethanol precipitation. The concentration and purity of genomic DNA was determined through gel electrophoresis.

Cloning

A 129 base pair fragment from exon 3 of murine Type I *hsd3b* (*hsd3b1*) was isolated through PCR amplification of *P. maniculatus* genomic DNA with the forward primer 5' TAGTGTGTTCACCTTCTCCTA 3' and reverse primer 5' TGTCTCCTTGTGCTGCTCCAC 3' and cloned into Bluescript KS^+ vector (Stratagene, La Jolla, CA). A 252 base pair fragment, comprising 81bp of 5' upstream sequence and 171bp of exon 1 of *cyp17*, was isolated through PCR amplification of *P. maniculatus* genomic DNA with the forward primer 5' AAGAGATAACACAACTTCAAG 3' and reverse primer 5' CCGTGTCTGGGGAGAAACGGC 3' and cloned into Bluescript SK^+

vector (Stratagene, La Jolla, CA). Sequence information was confirmed through manual sequencing by the Sanger dideoxynucleotide method (Sanger et al., 1977) using the Sequenase 2.0 kit (USB Corp., Cleveland, OH), as well as through automated sequencing using a Licor Model 4200 (Licor, Lincoln, NE) and Sequitherm Excel II Long-Read DNA Sequencing Kit LC (Epicentre Technologies, Madison, WI) according to instructions provided by the manufacturers. Sequences were analyzed with Omiga 2.0 (Oxford Molecular) and were found to be highly homologous to published rodent and human sequences for adrenal *hsd3b* and *cyp17* (complete sequences and alignments can be found in Appendix A).

Ribonuclease Protection Assay

The *hsd3b1* and *cyp17* containing recombinant plasmids were linearized with BamHI and labeled with ^{32}P-αUTP (Perkin Elmer/ NEN, Boston, MA) using T3 RNA polymerase for *hsd3b1* and T7 RNA polymerase for *cyp17* to yield antisense probes (BamHI, T3, T7, Promega, Madison, WI). The internal control, pTRI RNA 18S (an antisense 18S ribosomal probe, Ambion, Austin, TX), was transcribed using the T7 Shortscript Kit (Ambion, Austin, TX) according to manufacturers instructions. Sense probes for *hsd3b1* and *cyp17* were transcribed by the same method, using HindIII linearizations for both, T7 polymerase for *hsd3b1,* and T3 polymerase for *cyp17*. Probes were purified on a 6% polyacrylamide denaturing gel, excised, and eluted for 4 hours. Five to 20μg testis total RNA and complete adrenal pair total RNA (typically 10-15μg) were hybridized with $5x10^4$ cpm of *cyp17* (365bp probe; 171bp protected fragment) and *hsd3b* (242bp probe; 129bp protected fragment) cRNA probes, and 1260ng pTRI RNA

18S cRNA (120 bp probe; 80bp protected fragment) probe for 10-12 hours at 50°C. Digestion with RNase A and T1 (40μg/ml and 2μg/ml respectively, Promega, Madison, WI) at 37°C for 30 minutes was followed by enzyme deactivation, phenol/chloroform extraction, and precipitation. RNA was separated on a 6% polyacrylamide denaturing gel, with autoradiography (Fuji RX) exposed at -80°C with an intensifying screen (complete RPA procedure can be found in Appendix B).

Ribonuclease Protection Assay Analysis

Films for the four assays were scanned as tif files and then quantified through scanning densitometry using NIH ImageJ v1.2h (see Appendix B for complete protocol). Briefly, regions of interest were selected to include the differing shapes of protected fragments, and therefore differed in length and height accordingly. However, as the analysis software determines average pixel density, regions of interest were identical in the total number of pixels. Sense transcripts were run as negative controls, as were lanes containing all probes with no RNA. The protected fragments for each *cyp17* and *hsd3b1* band were normalized for RNA loss by comparison with the corresponding 18S ribosomal band density, and expressed as a ratio with the ribosomal signal. The first of the four assays utilized 3μCi of ^{32}P-labelled UTP in the transcription of the ribosomal probe. To optimize protected fragment visualization, ^{32}P-labelled UTP was increased to 10μCi for the remaining 3 assays. To allow for comparison of the four runs, ribosomal values for the first assay were therefore normalized to the other assays by multiplying by 3.33. The manufacturer of the ribosomal control reports this probe to often produce two closely migrating protected fragments, the larger at 80bp, due to the rehybridization of a

portion of the antisense probe following denaturation. This phenomena appears to be correlated to the large mass of 18S ribosomal RNA in reactions containing more than 0.5μg total RNA, and the manufacturer suggests including both bands when quantifying the intensity of signal.

The masses of protected fragments were evaluated in duplicate in a separate assay hybridizing known masses of the sense transcripts of *hsd3b1* and *cyp17* with excess antisense probe (Figure 9).

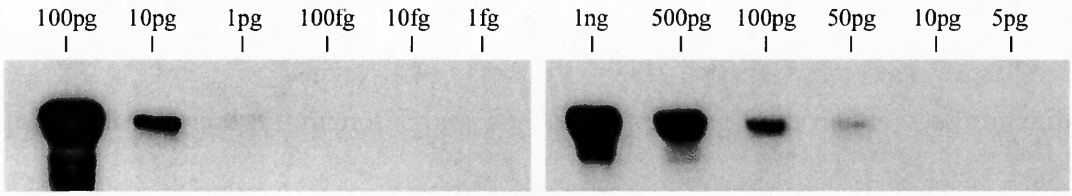


Figure 9 - Quantitative RPA. Protected fragment masses were determined through a separate assay utilizing optical density measured sense transcript templates of A) *cyp17* and B) *hsd3b1* performed in duplicate. Sense transcripts of known masses were hybridized with excess antisense probe. *Cyp17* exposure of 2 weeks, *hsd3b1* exposure of 6 hours.

Statistical Analysis

All data were analyzed by separate one-way ANOVA. Tukey's multiple comparison tests were performed on positive F values obtained from ANOVA to locate significant differences. Student's t-tests were restricted to comparing reproductively inhibited and similarly aged reproductively capable groups. Data with $p<0.05$ were considered statistically significant. Raw data and statistical analysis can be found in Appendix C.

All work involving animal subjects has been approved by the Institutional Animal Care and Use Committee, College of William and Mary. All work involving infectious agents and/or recombinant DNA has been approved by the Institutional Biosafety Committee, College of William and Mary. All work involving radioactive materials has been approved by the Radiation Safety Officer, Department of Biology, College of William and Mary.

Results

Body, Reproductive Organ, and Adrenal Weights

The mean body, reproductive organ, and adrenal weights tended to increase with age in control male animals at each of the four ages (30, 50, 70, and 90 days old, n=5 in each age group) studied (Table 1). Mean paired testis mass of 50 day control males was significantly larger ($p<0.05$) than control males at 30 days of age (18.70 ± 1.99mg; 8.04 ± 1.51mg, respectively). Likewise, 90 day mean testis mass was significantly larger ($p<0.01$) than control males at 70 days of age (36.74 ± 1.40mg; 23.12 ± 3.37mg, respectively). Adrenal mass relative to body mass was not significantly different for any age group.

Reproductively inhibited males had significantly smaller mean body mass (13.94 ± 0.96g; 18.89 ± 0.75g, respectively), adrenal mass (2.06 ± 0.12mg; 3.48 ± 0.45mg, respectively), and testis mass (14.98 ± 1.94mg; 30.02 ± 2.70mg, respectively) than reproductively capable control males of similar age ($p<0.01$, $p<0.05$, $p<0.01$ respectively). Seminal vesicle mass tended ($p<0.1$) to be lower in inhibited males than similar aged controls (7.46 ± 1.74mg; 12.76 ± 3.10mg, respectively). Adrenal mass relative to body mass (mg adrenal mass per 100g body mass) tended to be lower ($p<0.1$) in inhibited males than similar aged controls (14.91 ± 0.88mg/100g; 18.28 ± 1.83mg/100g, respectively).

Table 1 - Body, adrenal, and gonad weights of control and inhibited male *P. maniculatus* of different ages. Values are presented as the mean (±SEM) for the total body, adrenal pair, testis pair, and seminal vesicle pair. n=5 in each age category. Groups that are marked with different letters were significantly different at $p<0.05$ (maturation study and inhibition study are compared separately in this scheme).

Age (days)	Body Mass (g)	Adrenal Mass (mg)	Testis Mass (mg)	Sem Ves Mass (mg)
Maturation Study				
30	14.75 a ±1.19	2.94 a ±0.38	8.04 a ±1.51	4.33 a ±2.84
50	16.33 a ±0.98	3.38 a ±0.42	18.70 b ±1.99	5.84 a ±1.31
70	18.03 a ±0.57	3.70 a ±0.20	23.12 b ±3.37	11.50 a ±1.33
90	19.71 a ±0.87	4.26 a ±0.53	36.74 c ±1.40	15.28 a ±1.05
Inhibition Study				
Control	18.89 a ±0.75	3.48 a ±0.45	30.02 a ±2.70	12.76 a ±3.10
Inhibited	13.94 b ±0.96	2.06 b ±0.12	14.98 b ±1.94	7.46 a ±1.74

Maturation Study

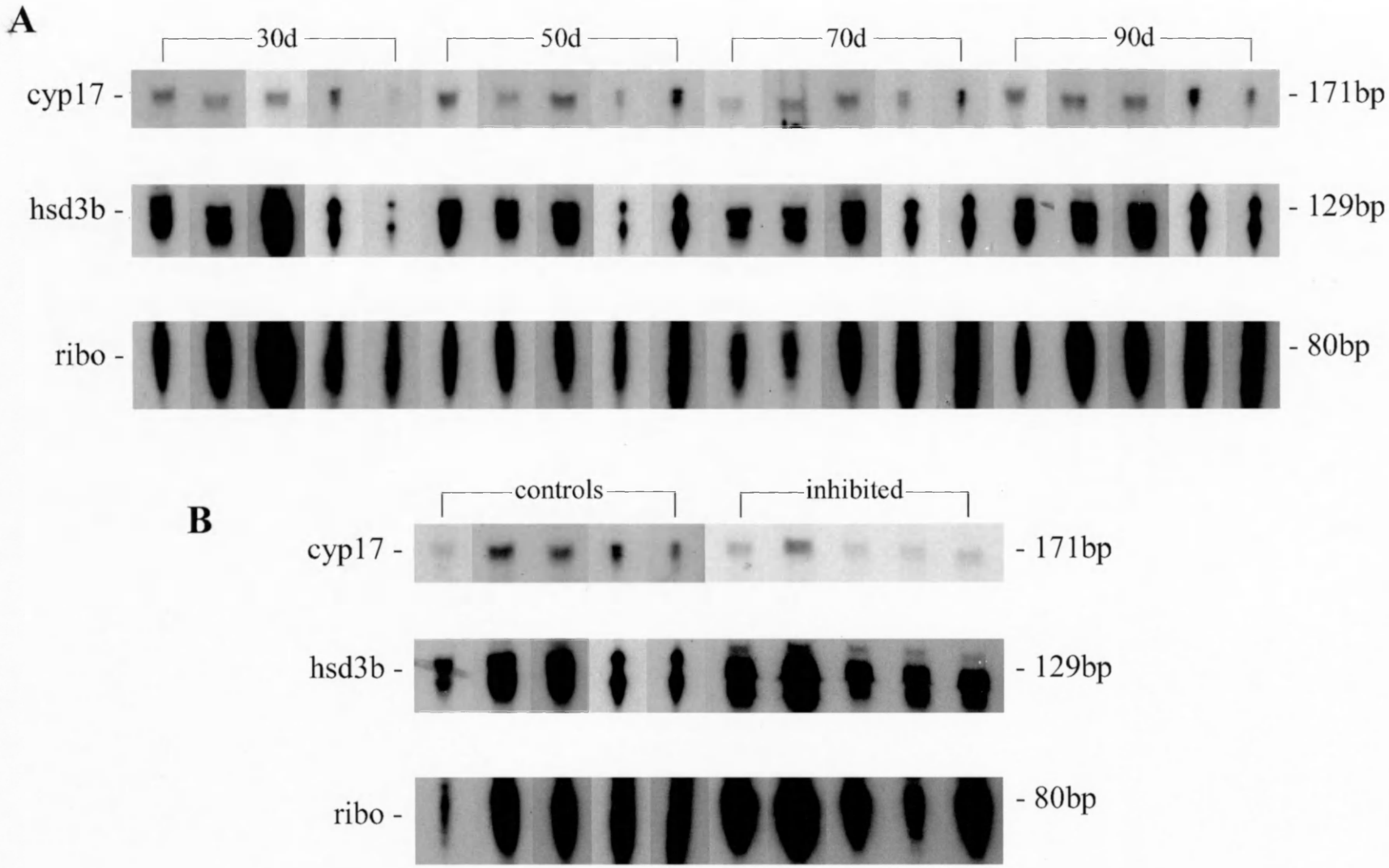
Ribonuclease protection assays were performed to determine the expression patterns of *hsd3b1* and *cyp17* in the adrenals of sexually maturing *P. maniculatus*, at time points before (30 day), during (50 day), and after (70 and 90 day) the time of predicted pubertal transition (n=5 per age group) (Figure 10a).

hsd3b1 Expression

Like the ribosomal probe, the probe for *hsd3b1* also consistently protected two closely migrating fragments, the 129bp fragment predicted from sequence analysis, and a second fragment 2bp smaller. To assure that a phenomena similar to the ribosomal pattern was not overlooked, two *hsd3b1* expression analyses were performed; one considering only the largest protected fragment, and a second including both protected fragments. No significant differences were found between largest fragment analysis and analysis including the second fragment. Data reported here include both fragments after 6 hours of exposure to xray film with an intensifying screen at -80°C.

To determine total adrenal *hsd3b1* expression, *hsd3b1* signal was normalized with the ribosomal internal control (expressed as the ratio *hsd3b1* / 18S ribosomal). The mean values increase from the prepubertal (30d) to pubertal (50d) age group (1.236 to 1.784), followed by mean values in adulthood that return to near prepubertal levels (1.372 at 70d; 1.314 at 90d). However, the range of values for the *hsd3b1* expression at each of the ages sampled are such that comparisons between the four age groups do not show any significant differences (Figure 11a).

Figure 10 - RPA Analysis. Figures are composites of lanes taken from four RPAs at indicated ages. **a)** Maturation Study; **b)** Inhibition Study. *Cyp17* exposure of 2 weeks, *hsd3b1* and 18S ribosomal RNA control probe exposure of 6 hours. Data obtained from image where measured using scanning densitometry, with RNA losses being corrected for by the 18S ribosomal internal control (not corrected in this composite).


A
30d
50d
70d
90d
cyp17 -
- 171bp
hsd3b -
- 129bp
ribo -
- 80bp
B
controls
inhibited
cyp17 -
- 171bp
hsd3b -
- 129bp
ribo -
- 80bp

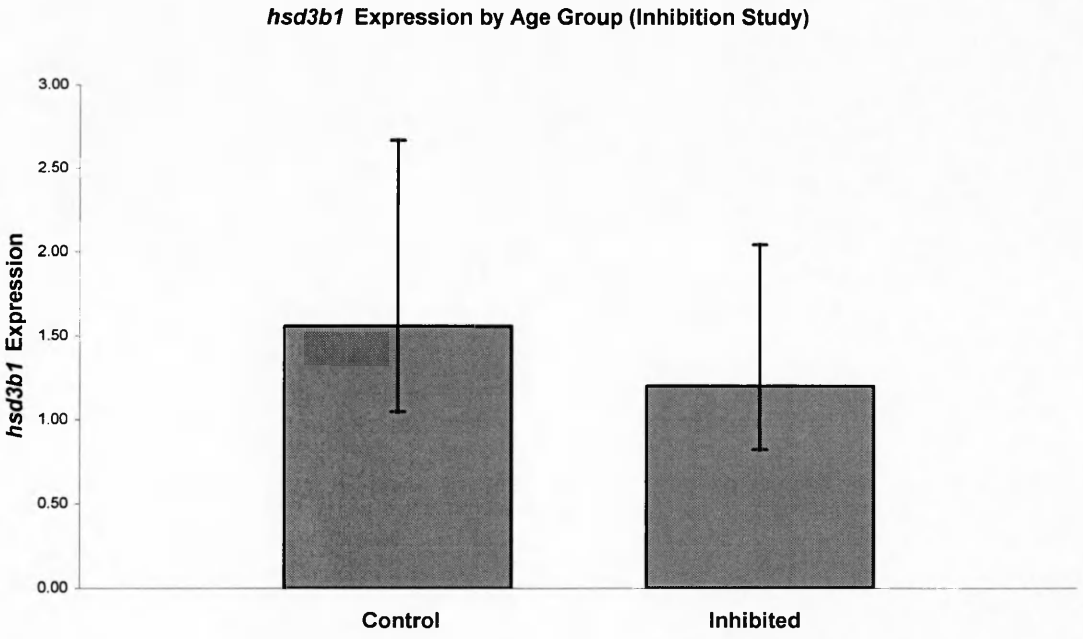


Figure 11 - Expression of adrenal *hsd3b1* in male *P. maniculatus* during normal maturation and in reproductive inhibition. **a)** Mean expression of adrenal *hsd3b1* increased during time of expected pubertal transition, returning approximately to prepubertal levels in adulthood. **b)** Mean adrenal *hsd3b1* expression was not significantly different in reproductively inhibited males and similarly aged control males. Bar height represents mean with range bars included. n=5 per group in both studies.

pattern of *hsd3b1* might be related to the degree of reproductive development.. In fact, no statistically significant correlations were demonstrated between the body weight, adrenal weight, or any gonad comparison with expression of *hsd3b1* (see Appendix C for correlation analyses).

cyp17 Expression

The probe for *cyp17* protected a single species of mRNA at 171bp, as expected from sequence analysis. Measurements for expression were taken after 2 weeks of exposure to xray film with an intensifying screen at -80°C.

To determine total adrenal *cyp17* expression, *cyp17* signal was also normalized using the ribosomal internal control (expressed as the ratio *cyp17* / 18S ribosomal). The mean values increase from the prepubertal (30d) to pubertal (50d) age group (from 0.244 to 0.384), followed by mean values in adulthood that remain just above prepubertal levels (0.354 at 70d; 0.288 at 90d). However, the mean values and the range of values for the expression of *cyp17* at each of the ages sampled were such that comparisons between the age groups do not show any significant differences (Figure 12a). To determine if the expression pattern of *cyp17* might be related to the amount of reproductive development, correlation analyses of the level of gene expression versus the testes and seminal vesicle weights were made and no correlations were demonstrated between the body weight, adrenal weight, or any gonad comparison with expression of *cyp17*.

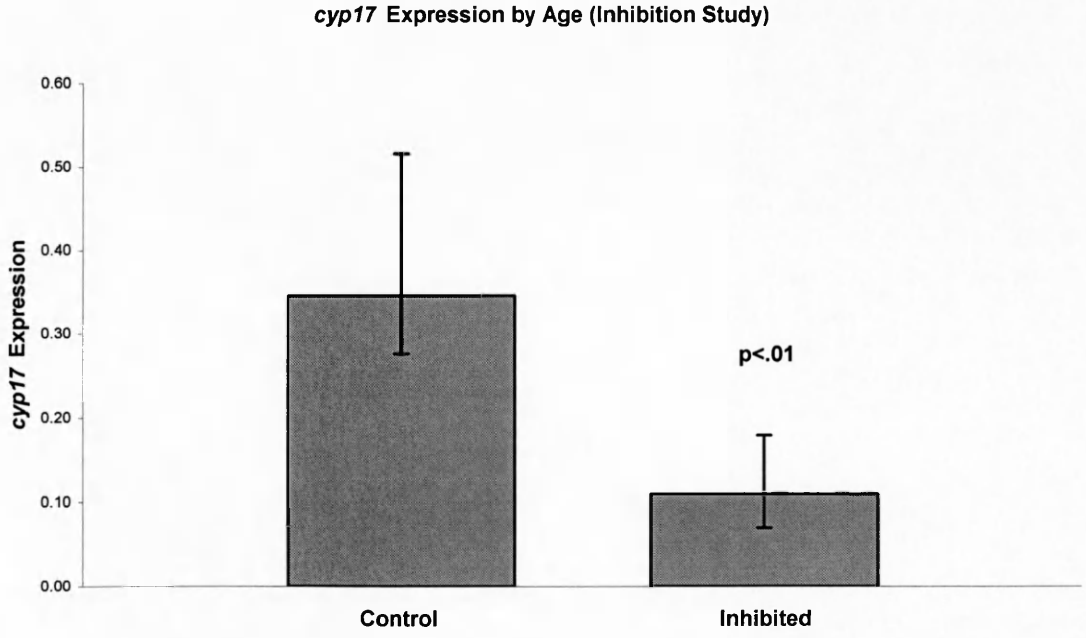


Figure 12 - Expression of adrenal *cyp17* in male *P. maniculatus* during normal maturation and in reproductive inhibition. **a)** Mean expression of adrenal *cyp17* increased during time of expected pubertal transition, remaining elevated above prepubertal levels into adulthood. **b)** Mean adrenal *cyp17* expression was significantly lower ($p<0.01$) in reproductively inhibited males than in similarly aged control males. Bar height represents mean with range bars included. n=5 per group in both studies.

cyp17 / hsd3b1 Expression

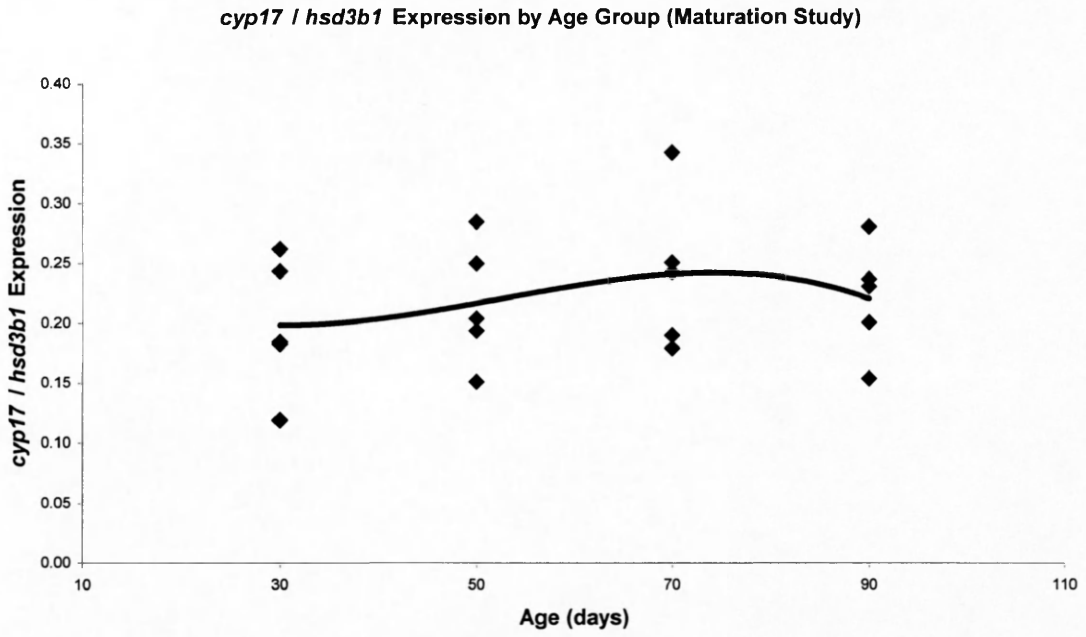
The ratio of the mean relative *cyp17* to *hsd3b1* expression at each age group tends to increase from the prepubertal to pubertal age group (from 0.198 to 0.216), with a continued increase at the postpubertal age of 70 days (0.242), and a subtle decline at 90 days of age (0.220). However, the range of values for the ratio of *cyp17 / hsd3b1* expression at each of the ages sampled are such that comparisons between the age groups do not show any significant differences between age groups (Figure 13a). No significant correlations were demonstrated between the body weight, adrenal weight, or any gonad comparison with expression of *cyp17 / hsd3b1*.

Reproductive Inhibition Study

Ribonuclease protection assays (RPAs) were also performed to determine the expression patterns of *hsd3b1* and *cyp17* in the adrenals of 130 (±10) day old reproductively inhibited males, and 120 (±5) day old reproductively proven controls (n=5 per group) (Figure 10b).

Hsd3b1 Expression

Although the mean *hsd3b1* expression in reproductively inhibited males is lower than in similarly aged reproductively proven control males (1.203 and 1.557, respectively) these mean values were not statistically significantly different (Figure 11b). The range of expression is from 1.05 to 2.67 in control animals, and from 0.82 to 2.04 in reproductively inhibited animals.



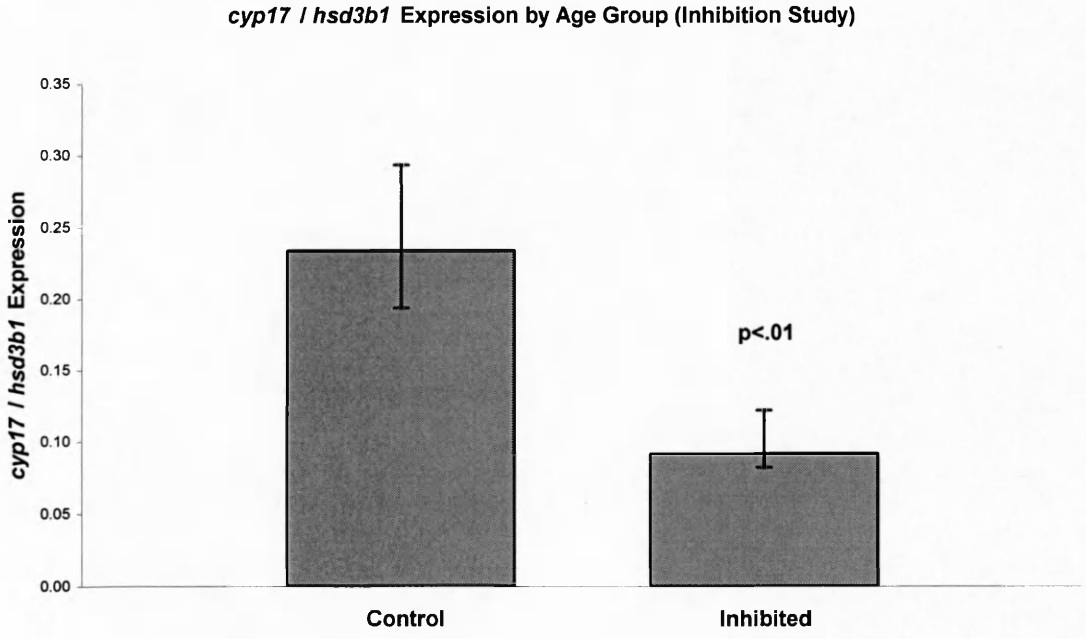


Figure 13 - Expression of adrenal *cyp17 / hsd3b1* in male *P. maniculatus* during normal maturation and in reproductive inhibition. **a)** Mean expression of adrenal *cyp17 / hsd3b1* increased throughout the maturational timecourse. **b)** Mean adrenal *cyp17 / hsd3b1* expression was significantly lower ($p<0.01$) in reproductively inhibited males than in similarly aged control males. Bar height represents mean with range bars included. n=5 per group in both studies.

The mean expression and range of expression of control animals was most similar to the values of 70 day adult animals from the maturation study (mean - 1.372, range from 0.74 to 2.44). The mean expression and range of expression of reproductively inhibited animals was similar to 90 day adult animals from the maturation study (mean - 1.314, range from 0.89 to 1.99), as well as to the 30 day prepubertal age group from the maturation study (mean - 1.237, range from 0.67 to 1.81). A statistically significant positive correlation between *hsd3b1* expression and seminal vesicle weight was found ($p<0.05$), but not for body, adrenal, or testis weights.

Cyp17 Expression

The mean *cyp17* expression expression value in reproductively inhibited males was approximately one third that of the mean reproductively capable control male value (0.111 and 0.345, respectively), a difference that was found to be statistically significant ($p<0.01$) (Figure 12b). The *cyp17* mean expression value for the reproductively inhibited animals was also significantly reduced when compared with each of the age groups in the maturation study (Figure 12a). The range of expression in inhibited animals was also approximately half that of similar aged controls (inhibited from 0.07 to 0.18; control from 0.28 to 0.52). The mean expression and range of expression of control animals was consistent with values of 90 day adult animals from the maturation study (mean - 0.288, range from 0.18 to 0.46). The mean expression and range of expression of inhibited animals was different from all age group from the maturation study. Statistically significant positive correlations were found between *cyp17* expression and body, adrenal, testis, and seminal vesicle weights ($p<0.01$, $p<0.05$, $p<0.01$, $p<0.05$, respectively).

cyp17 / hsd3b1 Expression

The mean ratio of *cyp17 / hsd3b1* expression in reproductively inhibited males was less than one half that of the corresponding mean ratio in reproductively capable control male value (0.091 and 0.233, respectively), a difference that was found to be statistically significant ($p<0.005$) (Figure 13b). This significant reduction in the mean *cyp17 / hsd3b1* expression ratio was also seen when comparing reproductively inhibited animals with the corresponding ratios for 30, 50, 70, and 90 day males ($p<0.01$ for each, from Figure 13a). The range of expression in inhibited animals was also approximately half that of similar aged controls (inhibited from 0.08 to 0.12; control from 0.19 to 0.29). The mean expression and range of expression of control animals was consistent with values of 90 day adult animals from the maturation study (mean - 0.221, range from 0.15 to 0.28). The lower mean expression and range of expression of inhibited animals was significantly different from all other age groups in the maturation study. Statistically significant correlations were found between *cyp17 / hsd3b1* expression ratio and the body, adrenal, and testis weights ($p<0.01$, $p<0.05$, $p<0.01$, respectively).

Mass Approximation

A separate RPA was conducted to estimate the mass of the expressed mRNA for *cyp17* and *hsd3b1*. This assay relates the optical density measured for known masses of a sense sequence hybridized with excess antisense probe to experimental hybridization reactions. The mass of the protected fragment of the sense sequence was the same as the mass of the antisense probe after digestion; 171bp for *cyp17* and 129bp for *hsd3b1*. The

full length cDNA for *cyp17* is reported to be 1724bp in hamster (Cloutier et al., 1996) and the full length cDNA for *hsd3b1* is reported to be 1119bp for mouse (Payne et al., 1997). The percentage differences between the sizes of the probes and the sizes of the full length mRNA were 10.1% for *cyp17* and 11.5% for *hsb3b1*. Therefore, the estimated masses were corrected by these factors, as well as for RNA loss through the ribosomal RNA internal control as described above. The *cyp17* mRNA was estimated to be as low as 7.9pg in inhibited animals, and as high as 61.4pg in control animals. The estimated mass of the *hsd3b1* mRNA was estimated to be as low as 523pg in prepubertal (30d) animals, and as high as 2080pg in pubertal (50d) animals.

Discussion

Maturation Study

The presence of 3βHSD is obligate to the production of all biologically active adrenal steroids including the essential mineralcorticoid aldosterone, and the glucocorticoid corticosterone (Wilson, 1998). As expected, *hsd3b1* was expressed in the adrenals of all animals in this study (Figure 11). The mean value for the expression of *hsd3b1* at each of the four ages evaluated showed a non-significant increase at 50 days and then returned to prepubertal levels by 70 and 90 days (Figure 11a). The rise in *hsd3b1* expression during the expected time of puberty (50 days) demonstrates that *hsd3b1* expression is not necessarily a function of increasing adrenal size because the adrenals continue to increased in mass at 70 and 90 days without increasing *hsd3b1* expression. Furthermore, the ratio of adrenal mass relative to body mass does not change significantly at any of the ages studied (Table 1). Taken together, this increase in *hsd3b1* expression at 50 days suggest that overall adrenal steroid activity is slightly elevated during the pubertal transition.

The expression of *cyp17* is not required for the production of the critical rodent hormones corticosterone and aldosterone. However, if cortisol or weak androgens are made the enzyme must be present. Unlike 3βHSD, cyp17 has not been previously reported in the adrenal of any *Peromyscus* species. This present study can not differentiate between the possible products of cyp17 action. Because the cyp17 enzyme catalyzes both 17α-hydroxylase and 17,20-lyase activities, its presence may lead to the

production of either cortisol and/or weak androgens, depending upon the presence and activity of P450 oxidative reductase (OR) (Cloutier et al., 1997; Miller et al., 1998).

We have radioimmunoassay data from our laboratory indicating that low serum concentrations of both cortisol and the weak androgen DHEA exist in *P. maniculatus* (Cherry, 2001), although it is not possible to determine whether these steroids are of adrenal or gonadal origin. Our present report of low levels of *cyp17* expression in the adrenals of all age groups (Figure 12), suggests that the adrenal is at least contributing to some of the observed serum cortisol and/or DHEA.

The pattern of *cyp17* expression generally was similar to *hsd3b1* throughout pubertal development, with a modest and transient increase in mean expression at the time of puberty (50 days), followed by a return to prepubertal levels at 70 and 90 days (Figure 12a). These data suggest that there may be some increase in weak androgen and/or cortisol production during puberty. However, the lack of a statistically significant increase in either *hsd3b1* and/or *cyp17* expression in this maturation study suggests that there is likely no adrenarche-like event linked with pubertal transition equivalent to the condition reported in humans (Remer and Manz, 1999; Miller, 1999).

The enzyme 3βHSD has been shown to compete with cyp17 for the substrate pregnenolone (Gell et al., 1998). This observation prompted our examination of the ratio of *cyp17* to *hsd3b1* at each of the four age groupings to elucidate whether the pathway leading to the weak androgens and cortisol may be increased over the production of adrenal glucocorticoids and mineralcorticoids. Although no statistically significant changes were found between these ratios for any age grouping, some increase in the mean ratio was seen during pubertal development, which peaked at 70 days before a

subtle decline at 90 days of age. This small change suggests an increase in the adrenal production of cyp17-dependant hormones during the pubertal transition, however the lack of statistically significant differences between the four age groups seems to argue against a major effect of the adrenal in the pubertal transition.

The wide range in the levels of *hsd3b1* and *cyp17* expression within each age grouping prompted a consideration of whether the range of expression might reflect a lack of strict correlation of expression with chronological age. Indeed, the ranges in testis and seminal vesicle mass in each age group supports the notion that chronological age is not a strict indicator of developmental age. Positive relationships between the increased expression of either *hsd3b1* or *cyp17*, and both the testis and the seminal vesicle mass, were found when all of the maturational age group data were combined in a single regression analysis. However, none of these analyses were shown to be statistically significantly correlated. This suggests that during normal maturation at the ages evaluated there is not a strict dependence on the size of the testes or the seminal vesicles with adrenal steroid activity. Clearly then other factors are likely to also influence this relationship. It is likely that the range of expression values observed at each age reflects, among other things, the varying frequency of the release of aldosterone and corticosterone in response to changes in activities such as feeding, drinking, and response to stress. It is therefore reasonable to expect that expression of *hsd3b1* and *cyp17* will change throughout the day. Although we collected all adrenals during a set period of the inactive/ sleep cycle, it is possible that this 2-3 hour collection period could include a range of physiological conditions requiring different levels of adrenal activity that would not be related to sexual maturatin, per se.

An additional complication may result from the likelihood that animals start a pubertal transition at different chronological ages. Hence, sampling adrenal tissue at only 30 and 50 days old will condense the continuum of gonad maturation stages into only two groups making the possibility of detecting transient adrenal-gonad changes more difficult.

Inhibition Study

As in previous studies of *P. maniculatus* (Terman, 1973b; Bradley and Terman, 1981a,b,c), reproductively inhibited animals in this study exhibited body, gonad, and adrenal masses that were all significantly smaller than similarly aged reproductively capable controls (Table 1). When compared with these earlier studies of organ mass and histology, as well as hormone profiles, these data suggest that the animals selected from laboratory populations in this study are indeed reproductively inhibited, even at an age approximately three times the normal age of the pubertal transition.

Although the masses of adrenals from reproductively inhibited animals were statistically significantly less than controls (Table 1), the reduction in *hsd3b1* expression was not statistically significantly different from the control value. This suggests that the adrenals of reproductively inhibited animals are not different from similarly aged controls in terms of their ability to produce corticosterone and aldosterone (Figure 11b). However, the mean value for the expression of *cyp17* was statistically significantly lower in reproductively inhibited animals compared with controls of similar age (Figure 12b), as well as every other mean expression value for each of the age groups in the maturation study (Figure 12a). These findings indicate a disruption in *cyp17* expression in

reproductively inhibited animals that is not seen even in the 30 day prepubertal group which have small adrenal masses that are not different from inhibited animals. In addition, the statistically significant positive correlations found between *cyp17* expression and reproductive organ masses within the group of inhibited males suggests some role for adrenal *cyp17* in reproductive inhibition.

Based on the statistically significant reduction of *cyp17* expression in reproductively inhibited animals compared with all other groups, it was not unexpected to find a similar statistically significant reduction in the *cyp17 / hsd3b1* expression ratio (Figure 13b). This reduction indicates that there is likely to be an altered pattern of hormone production in reproductively inhibited animals compared to similarly aged normally maturing animals. Although statistically significantly lower than for all age groups from the maturation study, the *cyp17 / hsd3b1* expression value of reproductively inhibited animals most closely approximated the low values seen in 30 day immature animals. These data indicate that reproductive inhibition involves a drastic reduction in the production of either cortisol and/or weak androgens, but not in the mineralcorticoid or glucocorticoid production levels.

It is clear from this study that reproductive inhibition involves a drastic reduction in adrenal *cyp17* expression in *Peromyscus.* What is not clear is whether the adrenal changes contribute directly to the pubertal transition as they may in human adrenarche when adrenal androgen production increases dramatically (Miller, 1999). This study was not designed to differentiate between the production of cortisol versus weak androgens that might be produced by the dual enzymatic activities of cyp17. It may be that increased *cyp17* expression at puberty is related to developmental factors that are more

basic than sexual maturation *per se*. If general metabolic changes must occur prior to or concomitant with puberty, then there may be an increased need for the minor glucocorticoid cortisol. Alternatively, an increase in adrenal weak androgens could be important to processes that are not directly related to gonadal maturation. Such is the case in humans, where adrenal androgens are known to contribute to secondary sex characteristics such as pubic hair growth (Wilson et al., 1998). Additional studies will be necessary to determine which of the *cyp17*-dependent hormone products is actually being produced before these questions can be answered.

The finding in this study that the *hsd3b1* expression in reproductively inhibited animals is not significantly different from controls indicates that there may be no large differences in the levels of production of corticosterone (or aldosterone). This observation should be considered relative to several earlier reports finding serum corticosterone to be significantly elevated in reproductively inhibited animals, despite the fact that these animals show no gravimetric evidence of adrenal hypertrophy (Sung et al., 1977; Bradley and Terman, 1981c; Ransone and Bradley, 1992). To resolve this apparent paradox of elevated hormone levels without adrenal hypertrophy, it was earlier suggested that the elevations in serum corticosterone concentration may not be due to stress-elevated pituitary activation of the adrenal, but rather due to either an increase in protective serum protein binding and/or a reduction in the metabolic clearance rate of the steroid (Bradley and Terman, 1981c). The present findings add cosiderable support to this explanation.

The adrenal zonal distribution of *cyp17* in *P. maniculatus* is currently unknown. The mRNA and enzyme have been found in the *zona fasciculata* in hamster (Briere et al.,

1997), in the *zonae fasciculata* and *reticularis* in the guinea pig (Shinzawa et al., 1988; Tremblay et al., 1994), and in the *zonae fasciculata* and *reticularis* in primates (Mapes et al., 1999). The significant reduction in *cyp17* expression by reproductively inhibited animals coincides with the significant increase in small, presumably dysfunctional, pycnotic cells in the *zona reticularis* of reproductively inhibited *P. maniculatus* reported by Cherry (1998), suggesting that the *zona reticularis* is, at least in part, responsible for expression of *cyp17* in normal *P. maniculatus*.

In summary, the finding of no significant differences in the adrenal expression of *hsd3b1* between reproductively inhibited animals and controls indicates that these adrenals from inhibited animals are not compromised with respect to the production of corticosterone and aldosterone, but produce far less of the *cyp17*-dependent additional hormones characteristic of normally maturing animals over 30 days of age. Whether these weak androgens and/or cortisol are responsible for, or the consequence of, the pubertal transition is not presently known, but these data clearly support an association of changes in adrenal *cyp17* activity with pubertal transition.

A future study sampling animals at a larger number of time points around the expected time of puberty might elucidate more transient increases in *hsd3b1* and/or *cyp17* expression to ensure that a critical period of significantly altered mRNA production is not overlooked. In addition, to determine temporal-spatial distribution of the enzymes encoded by these genes, antibodies against cyp17 and 3βHSD should be used in immunohistochemical investigations. Also, the simultaneous study of the serum concentrations of cortisol and corticosterone through RIA should be employed to determine if cortisol is more than a minor glucocorticoid in *Peromyscus* species. To

expand on previous RIA results indicating the presence of DHEA (Cherry, 2001), additional assays should be performed to determine if additional C19 steroids are being made by the low levels of *cyp17* expression discovered in this study. Such future investigations would help to further clarify the potential influence(s) of the changing expression levels of *hsd3b1* and *cyp17* in normally maturing and reproductively inhibited *P. maniculatus* presented by this study.

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Appendix A - Sequences & Alignments

Clone Sequences

Hsd3b1 (Murine Type I, Portion of Exon 3)

```
TAGTGTGTTC  ACCTTCTCCT  ACAAGAAAGC  TCAGCGAGGT  40
CTGGGCTATG  AGCACCTATC  AGCTGGGAGG  AAGCCAGACA  80
GAAAACTTCA  CAGTGGATTG  GGTCACTAGT  GGAGCAGCAC  120
AAGGAGACA  129
```

Cyp17 (5' Upstream Sequence and Portion of Exon 1)

```
aagagataac  acaacttcaa  ggtgacaacc  agggaggcct  40
tttaaaagtc  ccccttctcc  gttagagtcg  ctgagcctct  80
cCACGCTGGC  CATCTGCTGA  CACCTGGCTA  CCATGTGGGA  120
ACTCGTGTGT  CTCTTGCTGC  TCATCCTAGC  CTTTCTCTTT  160
TGGCCCAAGC  CAAAGACATT  TGATGCCAAG  TTTCCCAGGA  200
GCCTCCCATT  CCTGCCCCTT  GTGGGCAGCC  TGCCGTTTCT  240
CCCCAGACAC  GG  252
```

*lower case indicates 5' upstream sequence

*underline indicates 5'UTR

*bold faced bases indicate start site of translation (CDS)

PTRI RNA 18S (Ambion; Austin, TX)

TCCCAAGATC CAACTACGAG CTTTTTAACT GCAGCAACTT 40
TAATATACGC TATTGGAGCT GGAATTACCG CGGCTGCTGT 80

GAPD

TCAGGTTGTC TCCAGCGACT TCAACAGTGA CCCCCACTCT 40
TCCACCTTCG ATGCTGGGGC TGGAATTGCC CTCAATGACA 80

***17βKSR* Clone**

GCTGACCCCT TATTCTGTTT CAACTCCAGT GGCAAAGTAC 40
CTAAAAACCA ACATGGTGAC CAAGACTGCC GATGAGTTTG 80
TTAAAGAGTC CTTGAAATAT ATCACGATCG GAGCTGAAAC 120
CTGTGGCTGC CTTGCTCATG AAATC 145

Hsd3b1 Alignment (Rodents Type I, Human Type II)

```
P. maniculatus TGTCTCCTTGTGCTGCTCCACTAGTGACCCAATCCACTGTGAAGT 45
M. auratus     ---TC----------------C--------G-------C------
M. musculus    -------C------------------T---G-------C---G--
R. norvegicus  -------C------------------T---G-------CC--G--
H. sapiens     G------------C-G-----A--G--A----C-----CCACG--

P. maniculatus TTTCTGTCTGGCTTCCTCCCAGCTGACAGGTGCTCATAGCCCAGA 90
M. auratus     -----CC---------------------A-G--------------
M. musculus    ------CT--------------------A----------------
R. norvegicus  ------CT--------------------A-G--------------
H. sapiens     ------CT-----------------TA--TG---T---CG-----

P. maniculatus CCTCGCTGAGCTTTCTTGTAGGAGAAGGTGAACACACTA 129
M. auratus     T-------------------------------------
M. musculus    T-------------------------A----GT------
R. norvegicus  T---T---------------------A------G-----
H. sapiens     -----------C--------A------------------
```

GAPD Alignment

```
P. maniculatus TCAGGTTGTCTCCAGCGACTTCAACAGTGACCCCCACTCTTCCAC 45
M. auratus     -------------T----------G------T-------------
M. musculus    -------------T-------------CA--T-------------
R. norvegicus  -------------T-T-----------CA--T----T--------
H. sapiens     C-----G------TCT-----------C---A-------C-----

P. maniculatus CTTCGATGCTGGGGCTGGAATTGCCCTCAATGACA 80
M. auratus     ---T--------------C-----T----------
M. musculus    ---------C--------C-----T----------
R. norvegicus  ---T--------------C-----T----------
H. sapiens     ---T--C-----------C-----------C---C
```

Cyp17 Alignment (Portion of Exon 1)

```
P. maniculatus CACGCTGGCCATCTGCTGACACCTGGCTACCATGTGGGAACTCGT   45
M. auratus     GCA--G-------------///////T-G-------------T--
M. musculus    ACA-------------CT----------G-------------T--
R. norvegicus  ACA-------------CT----------G-------------T--
H. sapiens     ACT----T-T----//////////G-C-----------G-----

P. maniculatus GTGTCTCTTGCTGCTCATCCTAGCCTTTCTCTTTTGGCCCAAGCC   90
M. auratus     -GC----C---------C---G----A-T--------T-----T-
M. musculus    ----------------------G----A-T--------------T-
R. norvegicus  -G------------------------A-T--------GT----T-
H. sapiens     -GC------------T-C------T-A-T-G------------AG

P. maniculatus AAAGACATTTGATGCCAAGTTTCCCAGGAGCCTCCCATTCCTGCC  135
M. auratus     ------G-G--G--------CG----A-----------------
M. musculus    -------CC-A----------C-----------T----------
R. norvegicus  ------GCCC-G-------C-C-----------T----C------
H. sapiens     --G-TGCCC--G--------AC----A--------TG-C------

P. maniculatus CCTTGTGGGCAGCCTGCCGTTTCTCCCCAGACACGG   171
M. auratus     G--------------------A-------------
M. musculus    ---G-----T--T--A--------------------
R. norvegicus  ---G--------T----------------------
H. sapiens     ---G--------------A--C-------------
```

**Bold faced bases indicate start site of CDS, underline indicates 5'UTR*

**Full clone also includes 81bp of 5' upstream sequence, not included in this alignment (252bp total length with 5'upstream sequence included)*

*Hash marks indicate significant divergence of sequence (ie. missing or additional bases)

Appendix B - Protocols

RPA Protocol

Updated 2-13-01 BL

A. Probe synthesis

	4.0µl 5x transcription buffer
	1.0µl sddH_2O
1- set up transcription rxn	2.0µl 100mM DTT
@ rm temp:	4.0µl r(CAG)TP Mix → → 10µl each of 10mM rATP,
	2.4µl 100µM UTP carrier rCTP, rGTP +10µl sddH_2O
	2.0µl linearized DNA template
	0.6µl RNase Block (RNasin)
*behind shield from here on→	3.0µl ^{32}P-αUTP (800ci/mmol)
	1.0µl appropriate RNA polymerase (T3/T7/SP6)

2- flick mix, pulse spin down, and incubate @ 37°C (behind shield) for 90 minutes (see #4)

3- make & pour 6% polyacrylamide gel (see recipe page) using inch long spacers to make wells (use more spacers than necessary - wells sometimes break when pulling spacers out)

4- after first 40 min of transcription rxn, add an additional 0.5µl RNA polymerase to increase yield (doping)

5- add 1.0µl DNase, flick mix, incubate for 15 more minutes @ 37°C

6- prepare gel for running - make 2L 0.5x TBE, assemble apparatus, clean wells

7- add 10µl RPA-formamide dye to rxns, boil 3 min to denature, place on ice, load all

8- run gel @ 2000v (normal setting) for about 40-50 min (fast dye ~2/3 from top)

9- turn on 65°C water bath for upcoming elution

B. Probe Purification

1- carry plate to darkroom, separate with licor wedge, and cover gel with saran wrap (hold upper plate between you and gel for radio protection - may want to work behind shield if many probes)

2- turn off lights and place film atop saran wrap and below upper plate, add some weight to top plate and expose for 2 minutes (be sure to place film in an orientation that you will not forget!)

3- develop film - 2 min developer, 30 sec water, 3 min fixer, 30 sec water

4- lights on - place film on bench and place plates on top using alignment from previous exposure (#2)

5- slide top plate off first band, pull saran back, and excise band with razor blades wiped with DEPC H_2O soaked kim wipe and place in epp containing 400µl elution buffer (be sure band is immersed -- also - remember ALARA)

6- elute in 65°C water bath for 3-4 hours

7- after elution, transfer eluates but not gels to new epps and remove 1µl to count with scinti (spin gel to bottom briefly - allows easier eluate removal, and allows liquid to cool - more accurate 1µl this way)

8- add 1ml 100% EtOH to each epp and precip @ -80°C for ≥ 30 min -- turn H_2O bath to 50°C

9- spin @ +4°C for 20 minutes (max), remove super to radio waste -- turn heat block to 85°C

10- resuspend pellets in an amount of hyb that will approximately get you 50k cpm/ µl (ex. if you had 100k cpm/ µl before, use 800µl hyb - doubling previous 400µl and therefore halving per µl count)

11- remove another 1µl and count in scinti

C. Hybridization Rxns

1- thaw RNA on ice

2- label epps for each hyb and aliquot xµl RNA to each (check with ELB/ MSS for RNA mass & volume you should use)

3- dry down RNA in speedvac (but don't overdry - want them just dry ~5-20 minutes usually)

4- each hyb rxn will be 20µl total volume - want to add ~50k cpm of each probe, and enough hyb for 20µl total volume (use scinti counts to determine volume necessary to achieve 50k)

5- add hyb first, then add probes (hyb first will allow pellet to begin going into solution and make adding small volumes easier)

6- flick mix, pulse spin down, and denature @ 85°C for 10 minutes, transfer immediately to 50°C water bath (take entire heat block to water bath and transfer directly)

7- hyb for at least 10 hours

---------- end day I ----------

D. Digestion Rxns

1- make digestion mix (just enough - see #2 - expensive RNases!)

		50µl 1M Tris
		50µl 0.5M EDTA
10 mM Tris	ex. 5mls →	300µl 5M NaCl
5 mM EDTA		4392µl sddH2O
300 mM NaCl		8µl RNase T1
sddH_2O		200µl RNase A
2µg/ml RNase T1		
40µg/ml RNase A	*RNases used only in hood - be excessively careful	

2- set heat block to 50°C -- transfer epps to this heat block and add 350µl digestion mix to each epp (in hood, and still in heat block -- this assures RNase digestion at prescribed stringency)

3- flick mix and transfer to 37°C block (also in hood) for 30 minutes -- thaw Proteinase K & tRNA on ice

4- make and pour 6% polyacrylamide gel using large sharkstoothe comb (red) inverted to form well

5- stop digestion by adding 20µl 10% SDS followed by 10µl Proteinase K (5mg/ml), flick mix and incubate an additional 15 min @ 37°C

6- add 400µl P/C, vortex 3 sec, place on ice, then spin for 10 min @ +4°C (12k rpm)

7- during spin, add 2.7µl (9.8mg/ml) tRNA to new epps, transfer aq to these new tubes

8- flick mix well, then add 1ml Ab EtOH to each epp & precip @ -80°C for ≥ 30 min

9- spin 20 min @ max in +4°C, remove & discard supers by pouring into radio waste & speedvac just dry

E. Running Samples

1- resuspend samples in 2µl TE @ rm temp -- and prepare gel for running (assemble, clean wells, insert cleaned comb, make 0.5x TBE, begin prerunning gel)

2- add 2µl RPA-formamide dye to samples -- begin boiling water

3- prepare 'probe only' samples -- 2µl probe + 4µl RPA-form dye -- also, get ladders out

4- place samples in nalgene plastic racks in desired loading order and boil 3 min, place on ice

5- after about 1 min ice time, pulse spin samples down and load

6- run gel so that slow dye front is approximately 8 inches from well bottom (1:45 - 2:30 total)

7- separate plates with licor wedge (carefully) and apply 3MM paper (take a good minute to assure gel is firmly attached to paper) peel up, cover with saran, and gel dry @ 78°C for 2- 2 ½ hours

8- remove saran, and in dark room, add to cassette -- gel , film, intensifying screen (reflective side towards film) and place in -80°C for 6 hrs - 2 wks depending on expression (ask ELB/ MSS for advice)

---------- end ----------

see next page for RPA solution recipes

RPA Recipes

Hybridization Buffer

(4mls) 3350µl deionized formamide
320µl 5M NaCL
320µl 0.5M PIPES
8µl 0.5M EDTA

store @ +4°C (make fresh each RPA)

Elution Buffer

(10mls) 8313µl sddH2O
1000µl 10% SDS
667µl 7.5M NH4Ac
20µl 0.5M EDTA

store @ +4°C

RPA-Formamide Loading Dye

(1ml) 800µl deionized formamide
20µl 0.5M EDTA
90µl xylene cyanol (10mg/ml)
90µl bromophenol blue (10mg/ml)

store @ +4°C

6% Polyacrylamide Gel

46.0g urea
40ml sddH_2O
15ml 40% polyacrylamide
5ml 10x TBE

1- stir on lowest heat (turn heat off after a few minutes of stirring) with foil covering top of beaker
2- prepare 10% APS (1ml sddH_2O + 10mg APS) (often aliquotted in -20°C)
3- add 700µl 10% APS & 18µl TEMED, stir for additional 20 sec and pour gel

RPA Image Analysis Procedure *Used by BLovitt - Outlined Feb 23, 2001*

* The following protocol was developed using **ImageJ (v.2.1)** with **SpecifiROI** plugin - both downloadable @ http://rsb.info.nih.gov/ij/ SpecifiROI saved into 'ImageJ plug-ins' folder on hard drive

* ImageJ is public image analysis software, and SpecifiROI is a plugin that allows you to specify a ROIs pixel dimensions.

1- Film scanned into ELBs computer as black & white 'image only'

2- After preview was generated, resolution was changed to 600dpi, and 'perform rescan if necessary' box was checked.

3- A region of interest was drawn to exclude nonessential parts of the film, and the 'OK' button was checked - this brings up the 'save as' dialog box - save pictures as 'noncompressed tiffs'

4- These files are very large (>30mb possibly), and ImageJ may have trouble opening them/ working efficiently with them, so I used Photoshop 5.0 (any image analysis program will work) to crop the film into three pictures - corresponding to the areas of my probes (ie. first crop contains all ribosomal bands, with an inch above and below bands in crop; second & third containing second and third probes with appropriate room above and below)

   a- to keep track of cropped images, I used the typing tool in Photoshop to label the pictures directly (in a corner out of the way) and saved them with appropriate corresponding filename

5- Using ImageJ, open the crop that corresponds to the control bands (ribo for mine)

6- Use the magnifying glass to enlarge to preference (may have to enlarge window by dragging on corner after magnifying)

7- Click on rectangle tool on ImageJ main window.

8- Select 'plug-ins' - 'utilities' - 'panel' the SpecifiROI window will appear

9- On opened image, draw a rectangle slightly larger than largest band of interest, then click on 'specify ROI' button of newly opened 'panel' - the pixel dimensions of your rectangle will be shown. This allows you to manipulate the rectangle size (by entering new values) or record the pixel dimension you intend to use for every other similar band (ie. same gene)

10- Once you have chosen a dimension, you can fine tune the position of the rectangle by dragging it, or using the keyboards arrows.

11- When positioned correctly, chose 'analyze' - 'histogram' and a histogram of the pixels luminescence within the ROI will appear - record the 'mean'.

12- Move the ROI onto an immediately adjacent area of the film that represents the background (ie. no exposure showing) and repeat 'histogram' function - recording again the 'mean'

13- Subtracting the control bands mean from the background mean will give you the '*darkness*' or '*expression*' of the band corrected for background.

14- I then repeated this same procedure for every hybridization on the films crop.

Appendix C - Data & Statistical Analyses

Statistical Analyses

One way analysis of variance (ANOVA), Tukey's multiple pairwise comparison, and paired t-test performed using StatsDirect Statistical Software v.1.9.0 (copyright 3/1/2001). Available @ http://www.statsdirect.com

Maturation Study - Body Mass by Age

One way analysis of variance

Variables: 30, 50, 70, 90 day control male *P. maniculatus*

Source of Variation	Sum Squares	DF	Mean Square
Between Groups	68.7415	3	22.913833
Within Groups	68.8669	16	4.30418
Corrected Total	137.6084	19	

F (variance ratio) = 5.323623 P = 0.0098

Tukey multiple comparisons

Critical value (Studentized range) = 4.046093, |q*| = 2.861082

Pooled standard deviation = 2.074652

Comparison	**Mean Difference L (95% CI)**	**|L/SE(L)|**	
30 vs 90	-4.96 (-8.71 to -1.20)	5.34	P=0.008
50 vs 90	-3.38 (-7.13 to 0.37)	3.64	P=0.085
30 vs 70	-3.28 (-7.03 to 0.47)	3.54	P=0.098
50 vs 70	-1.7 (-5.45 to 2.05)	1.83	P=0.579
70 vs 90	-1.68 (-5.43 to 2.07)	1.81	P=0.588
30 vs 50	-1.58 (-5.33 to 2.17)	1.70	P=0.633

Maturation Study - Adrenal (Pair) Mass by Age

One way analysis of variance

Variables: 30, 50, 70, 90 day control male *P. maniculatus*

Source of Variation	Sum Squares	DF	Mean Square
Between Groups	4.63	3	1.543333
Within Groups	12.792	16	0.7995
Corrected Total	17.422	19	

F (variance ratio) = 1.930373 P = 0.1654

Tukey multiple comparisons

Critical value (Studentized range) = 4.046093, |q*| = 2.861082

Pooled standard deviation = 0.894148

Comparison	**Mean Difference L (95% CI)**	**|L/SE(L)|**	
30 vs 90	-1.32 (-2.93 to 0.29)	3.30	P=0.131
50 vs 90	-0.88 (-2.50 to 0.74)	2.20	P=0.430
30 vs 70	-0.76 (-2.38 to 0.86)	1.90	P=0.55
70 vs 90	-0.56 (-2.18 to 1.06)	1.40	P=0.757
30 vs 50	-0.44 (-2.06 to 1.18)	1.10	P=0.863
50 vs 70	-0.32 (-1.94 to 1.30)	0.80	P=0.941

Maturation Study - Testis (Pair) Mass by Age

One way analysis of variance

Variables: 30, 50, 70, 90 day control male *P. maniculatus*

Source of Variation	Sum Squares	DF	Mean Square
Between Groups	2119.018	3	706.339333
Within Groups	391.532	16	24.47075
Corrected Total	2510.55	19	

F (variance ratio) = 28.864638 P < 0.0001

Tukey multiple comparisons

Critical value (Studentized range) = 4.046093, |q*| = 2.861082

Pooled standard deviation = 4.946792

Comparison	**Mean Difference L (95% CI)**	**|L/SE(L)|**	
30 vs 90	-28.7 (-37.65 to -19.75)	12.97	P<0.0001
50 vs 90	-18.04 (-26.99 to -9.09)	8.15	P=0.0002
30 vs 70	-15.08 (-24.03 to -6.13)	6.82	P=0.001
70 vs 90	-13.62 (-22.57 to -4.67)	6.16	P=0.0025
30 vs 50	-10.66 (-19.61 to -1.71)	4.82	P=0.017
50 vs 70	-4.42 (-13.37 to 4.53)	2.00	P=0.509

Maturation Study - Seminal Vesicle (Pair) Mass by Age

One way analysis of variance

Variables: 30, 50, 70, 90 day control male *P. maniculatus*

Source of Variation	Sum Squares	DF	Mean Square
Between Groups	329.538039	3	109.846013
Within Groups	125.746667	16	9.672821
Corrected Total	455.284706	19	

F (variance ratio) = 11.356151 P = 0.0006

Tukey multiple comparisons

Critical value (Studentized range) = 4.150866, |q*| = 2.935169

Pooled standard deviation = 3.110116

Comparison	**Mean Difference**	**L (95% CI)**	**|L/SE(L)|**	
30 vs 90	-10.95	(-17.61 to -4.28)	6.81	P=0.0017
50 vs 90	-9.44	(-15.21 to -3.67)	6.79	P=0.0017
30 vs 70	-7.17	(-14.14 to -0.19)	4.27	P=0.0432
50 vs 70	-5.66	(-11.78 to 0.46)	3.84	P=0.074
70 vs 90	-3.78	(-9.90 to 2.34)	2.56	P=0.3115
30 vs 50	-1.51	(-8.17 to 5.16)	0.94	P=0.9089

Inhibition Study - Body Mass

Paired t test

For differences between control and inhibited:

Mean of differences = 4.948

Standard deviation = 2.498794

Standard error = 1.117495

95% CI = 1.845338 to 8.050662

df = 4

t = 4.427762

One sided P = 0.0057

Two sided P = 0.0114

Inhibition Study - Adrenal (Pair) Mass

Paired t test

For differences between control and inhibited:

Mean of differences = 1.42

Standard deviation = 1.241773

Standard error = 0.555338

95% CI = -0.121865 to 2.961865

df = 4

t = 2.557003

One sided P = 0.0314

Two sided P = 0.0628

Inhibition Study - Testis (Pair) Mass

Paired t test

For differences between control and inhibited:

Mean of differences = 15.04

Standard deviation = 7.376517

Standard error = 3.298879

95% CI = 5.880845 to 24.199155

df = 4

t = 4.559125

One sided P = 0.0052

Two sided P = 0.0103

Inhibition Study - Seminal Vesicle (Pair) Mass

Paired t test

For differences between control and inhibited:

Mean of differences = 5.3

Standard deviation = 8.555992

Standard error = 3.826356

95% CI = -5.323667 to 15.923667

df = 4

t = 1.38513

One sided P = 0.1191

Two sided P = 0.2383

Maturation Study - *Hsd3b1* Expression by Age

One way analysis of variance

Variables: 30, 50, 70, 90 day control male *P. maniculatus*

Source of Variation	Sum Squares	DF	Mean Square
Between Groups	0.8888	3	0.296267
Within Groups	5.51348	16	0.344593
Corrected Total	6.40228	19	

F (variance ratio) = 0.859759 P = 0.48197

Tukey multiple comparisons

Critical value (Studentized range) = 4.046093, |q*| = 2.861082

Pooled standard deviation = 0.58702

Comparison	**Mean Difference L (95% CI)**	**|L/SE(L)|**	
30 vs 50	-0.544 (-1.61 to 0.52)	2.07	P=0.4798
50 vs 90	0.468 (-0.59 to 1.53)	1.78	P=0.5995
50 vs 70	0.412 (-0.65 to 1.47)	1.57	P=0.6889
30 vs 70	-0.132 (-1.19 to 0.93)	0.50	P=0.984
30 vs 90	-0.076 (-1.13 to 0.99)	0.29	P=0.9968
70 vs 90	0.056 (-1.01 to 1.12)	0.21	P=0.9987

Maturation Study - *Cyp17* Expression by Age

One way analysis of variance

Variables: 30, 50, 70, 90 day control male *P. maniculatus*

Source of Variation	Sum Squares	DF	Mean Square
Between Groups	0.057535	3	0.019178
Within Groups	0.50632	16	0.031645
Corrected Total	0.563855	19	

F (variance ratio) = 0.606046 P = 0.620603

Tukey multiple comparisons

Critical value (Studentized range) = 4.046093, |q*| = 2.861082

Pooled standard deviation = 0.17789

Comparison	**Mean Difference L (95% CI)**	**|L/SE(L)|**	
30 vs 50	-0.136 (-0.46 to 0.19)	1.71	P=0.6303
50 vs 70	-0.11 (-0.43 to 0.21)	1.38	P=0.7638
50 vs 90	0.092 (-0.23 to 0.41)	1.15	P=0.8452
70 vs 90	0.066 (-0.26 to 0.39)	0.83	P=0.9347
30 vs 90	-0.044 (-0.37 to 0.28)	0.55	P=0.979
50 vs 70	0.026 (-0.30 to 0.35)	0.33	P=0.9955

Maturation Study - *Cyp17 / Hsd3b1* Expression by Age

One way analysis of variance

Variables: 30, 50, 70, 90 day control male *P. maniculatus*

Source of Variation	Sum Squares	DF	Mean Square
Between Groups	0.004935	3	0.001645
Within Groups	0.04844	16	0.003028
Corrected Total	0.053375	19	

F (variance ratio) = 0.543353 P = 0.659581

Tukey multiple comparisons

Critical value (Studentized range) = 4.046093, |q*| = 2.861082

Pooled standard deviation = 0.055023

Comparison	**Mean Difference**	**L (95% CI)**	**|L/SE(L)|**	
30 vs 70	-0.044	(-0.14 to 0.06)	1.79	P=0.5972
50 vs 70	-0.026	(-0.13 to 0.07)	1.06	P=0.8765
30 vs 90	-0.024	(-0.12 to 0.08)	0.97	P=0.8995
70 vs 90	0.020	(-0.08 to 0.12)	0.81	P=0.9362
30 vs 50	-0.018	(-0.12 to 0.08)	0.73	P=0.9537
50 vs 90	-0.006	(-0.11 to 0.09)	0.24	P=0.9981

Inhibition Study - *Hsd3b1* Expression

Paired t test

For differences between control and inhibited:

Mean of differences = 0.354

Standard deviation = 0.941504

Standard error = 0.421053

95% CI = -0.815032 to 1.523032

df = 4

t = 0.840748

One sided P = 0.2239

Two sided P = 0.4478

Inhibition Study - *Cyp17* Expression

Paired t test

For differences between control and inhibited:

Mean of differences = 0.236

Standard deviation = 0.126214

Standard error = 0.056445

95% CI = 0.079284 to 0.392716

df = 4

t = 4.181086

One sided P = 0.007

Two sided P = 0.0139

Inhibition Study - *Hsd3b1 / Cyp17* Expression

Paired t test

For differences between control and inhibited:

Mean of differences = 0.142

Standard deviation = 0.044944

Standard error = 0.0201

95% CI = 0.086194 to 0.197806

df = 4

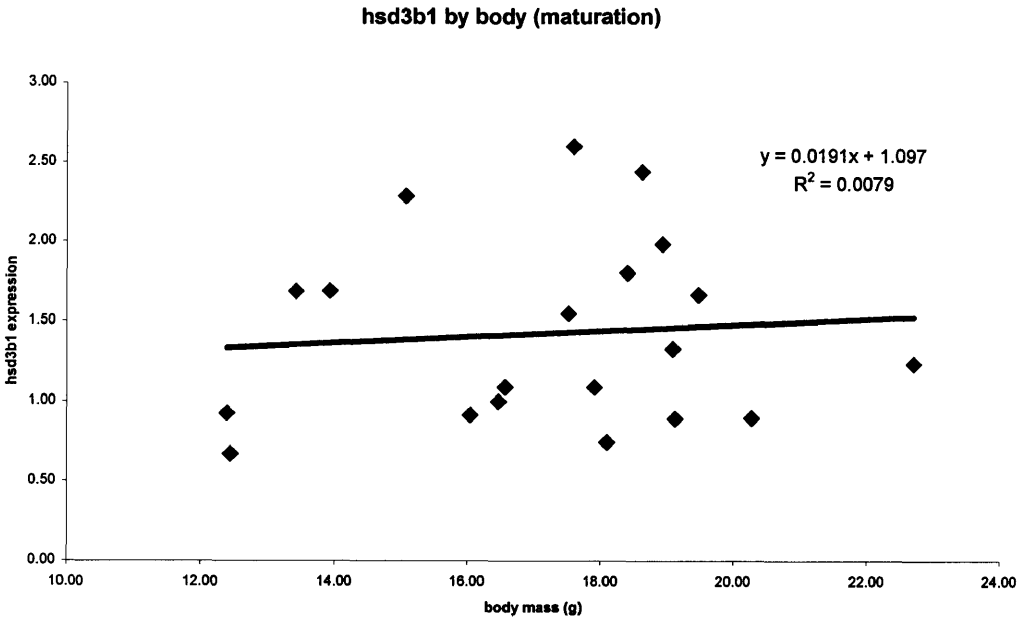
t = 7.064764

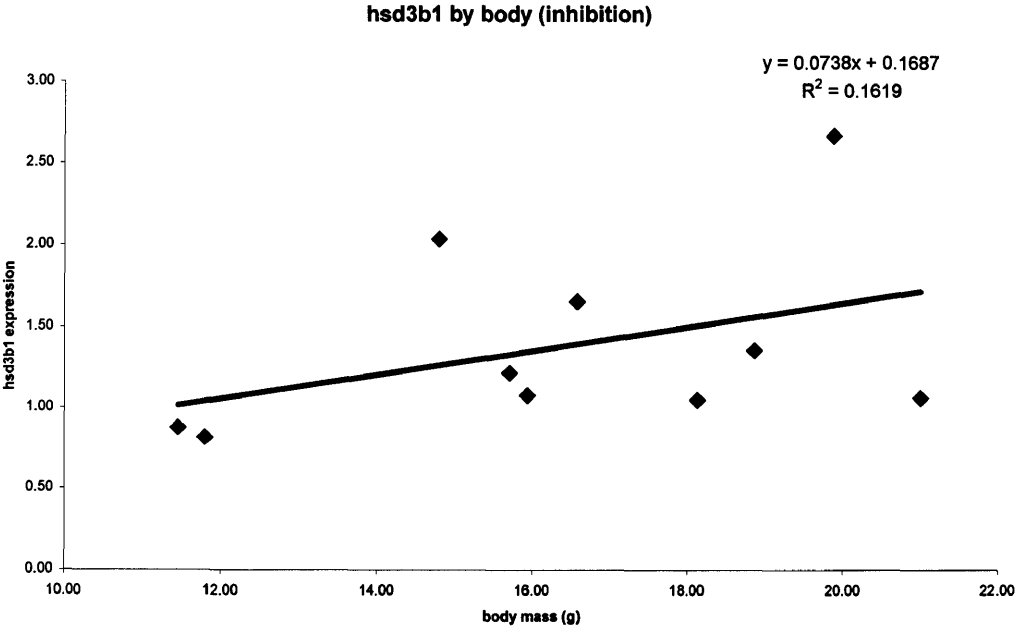
One sided P = 0.0011

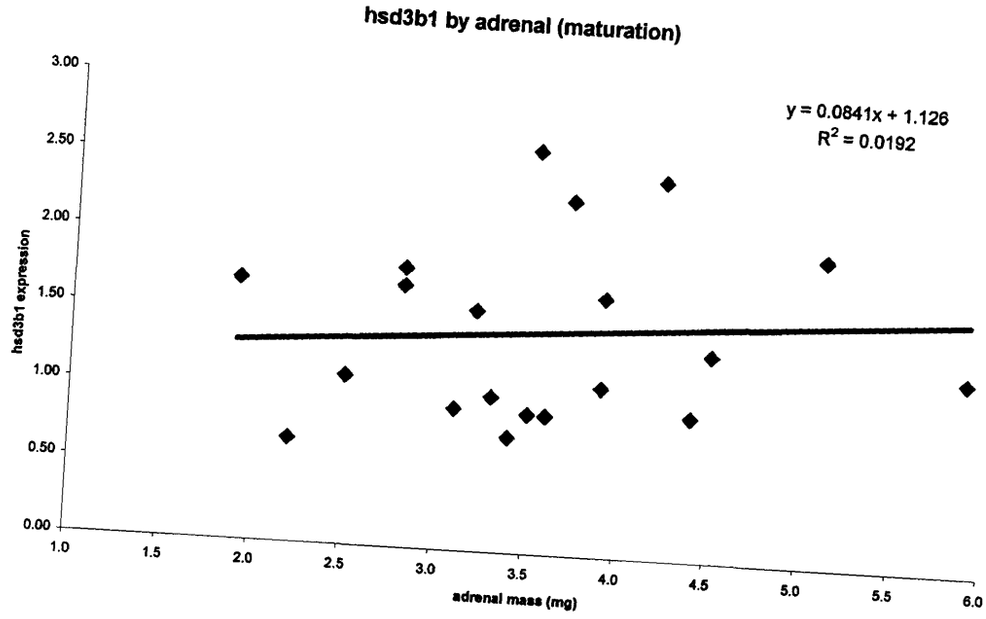
Two sided P = 0.0021

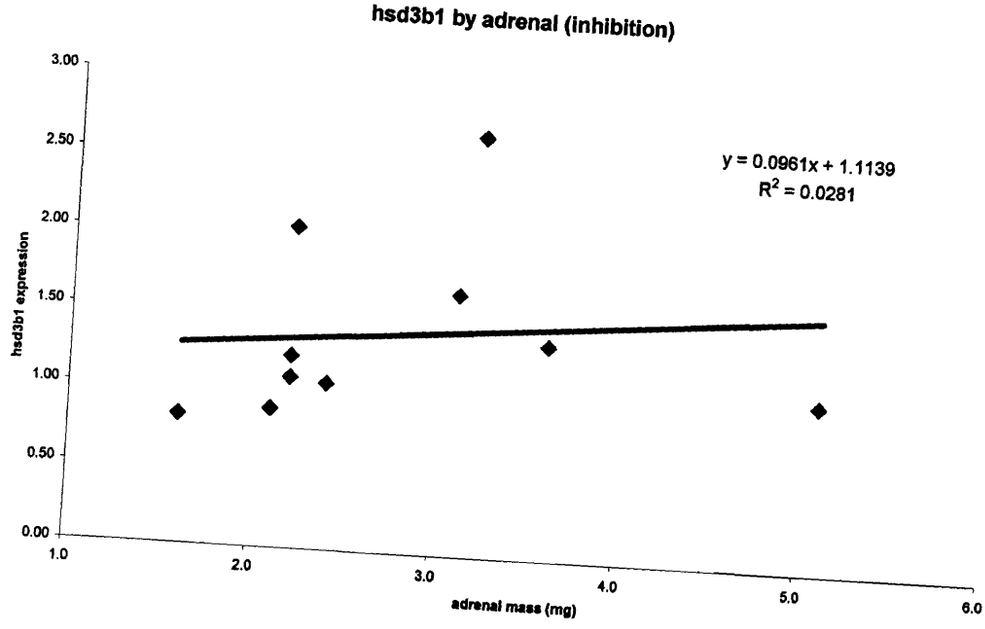
Correlation Analyses

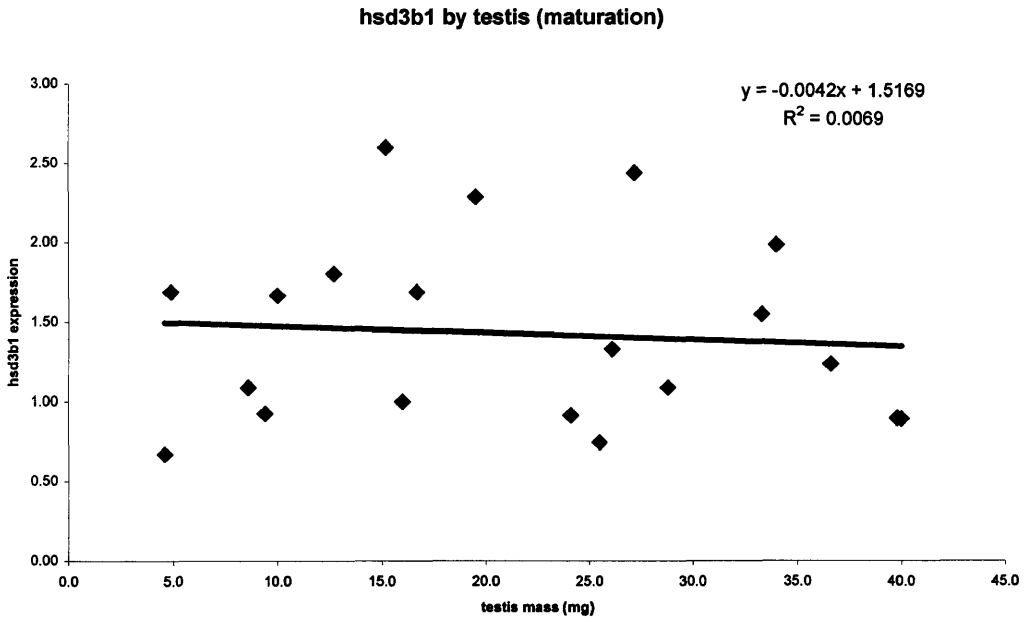
Correlation analyses performed with Microsoft Excel. Slope and R^2 value included on each graph. P-value included when statistical significance was found.

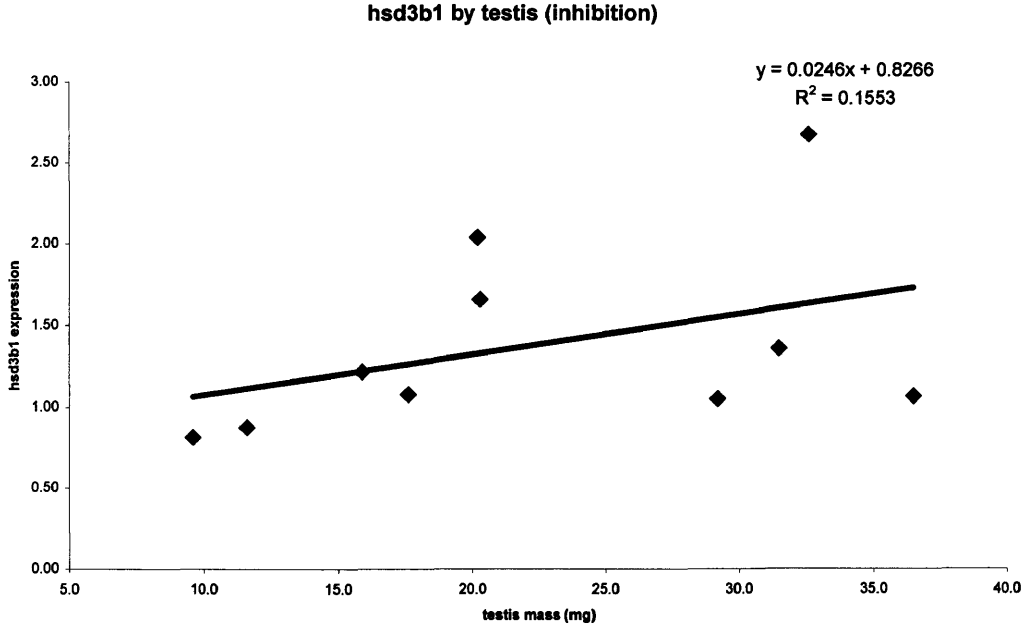

hsd3b1 by body (maturation)
y = 0.0191x + 1.097
R2 = 0.0079
hsd3b1 expression
body mass (g)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00

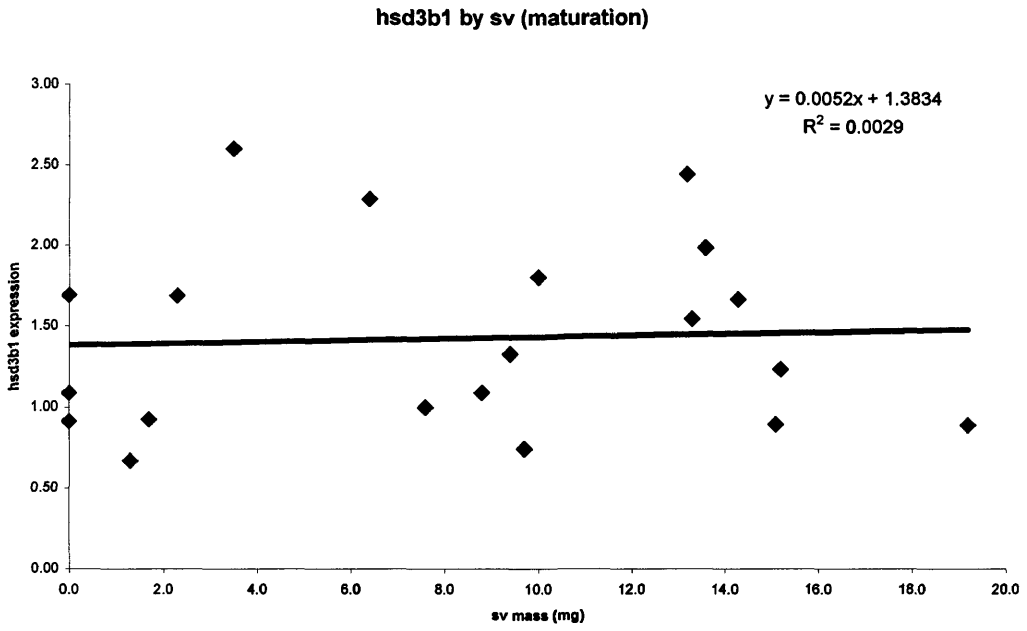

hsd3b1 by body (inhibition)
y = 0.0738x + 0.1687
R2 = 0.1619
hsd3b1 expression
body mass (g)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00

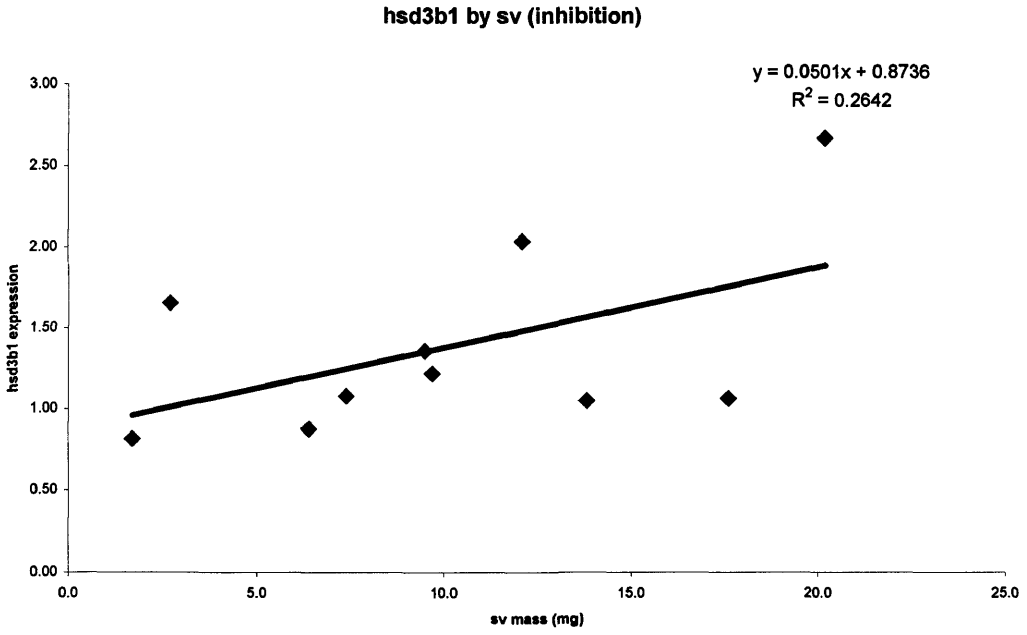

hsd3b1 by adrenal (maturation)
y = 0.0841x + 1.126
R2 = 0.0192
hsd3b1 expression
3.00
2.50
2.00
1.50
1.00
0.50
0.00
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
adrenal mass (mg)

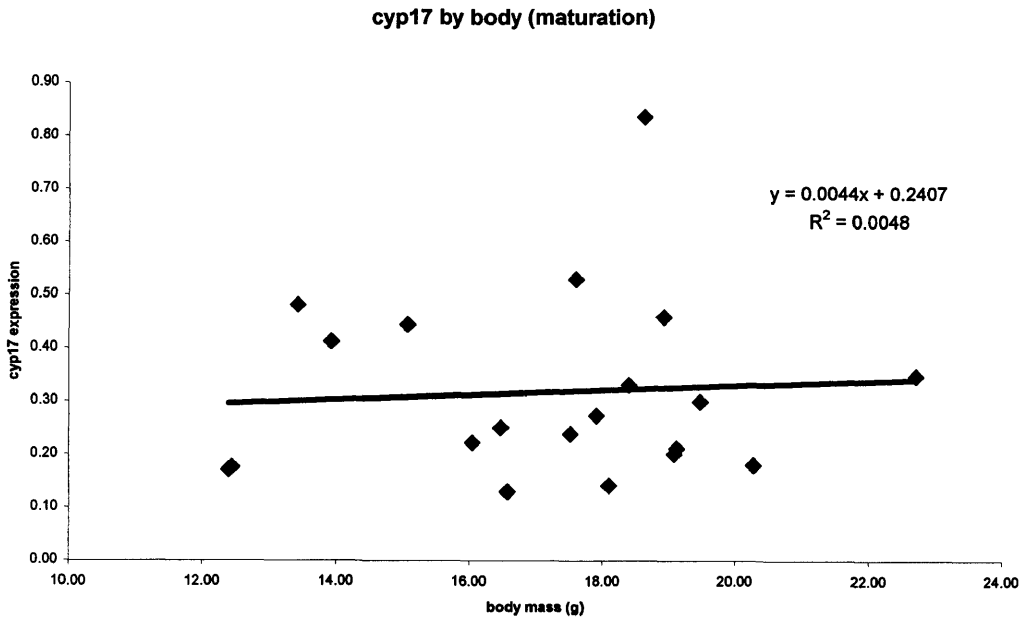

hsd3b1 by adrenal (inhibition)
y = 0.0961x + 1.1139
R2 = 0.0281
hsd3b1 expression
3.00
2.50
2.00
1.50
1.00
0.50
0.00
1.0
2.0
3.0
4.0
5.0
6.0
adrenal mass (mg)

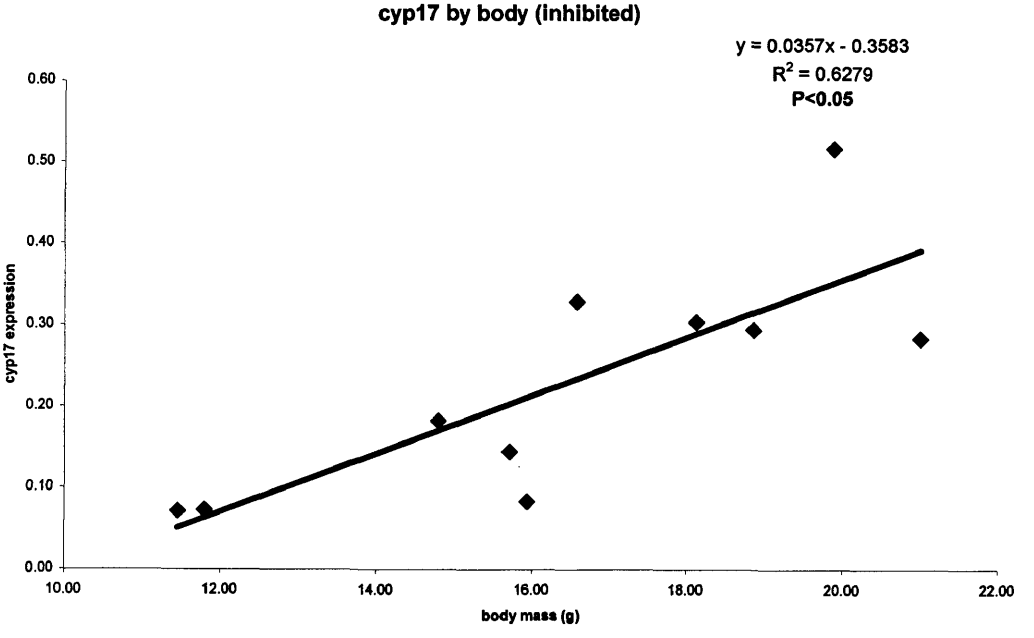

hsd3b1 by testis (maturation)
y = -0.0042x + 1.5169
R2 = 0.0069
hsd3b1 expression
testis mass (mg)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0

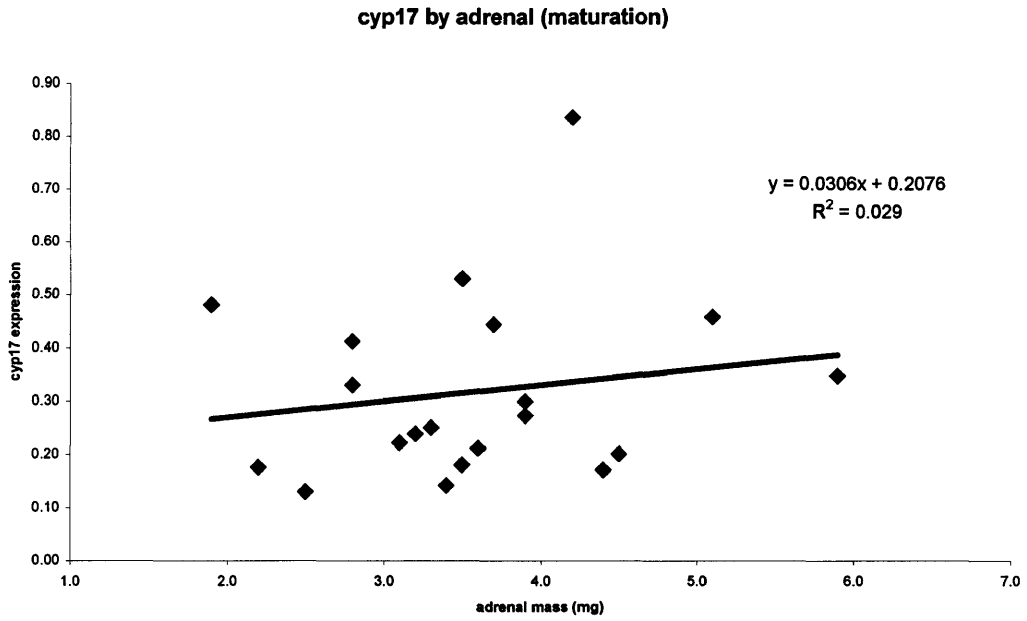

hsd3b1 by testis (inhibition)
y = 0.0246x + 0.8266
R2 = 0.1553
hsd3b1 expression
testis mass (mg)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0

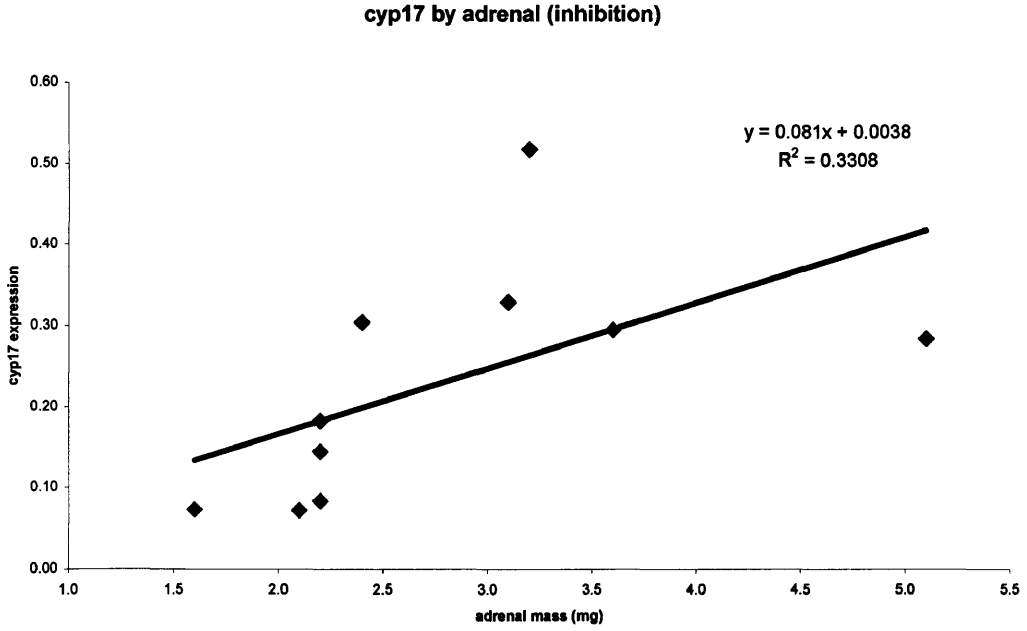

hsd3b1 by sv (maturation)
y = 0.0052x + 1.3834
R2 = 0.0029
hsd3b1 expression
sv mass (mg)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0

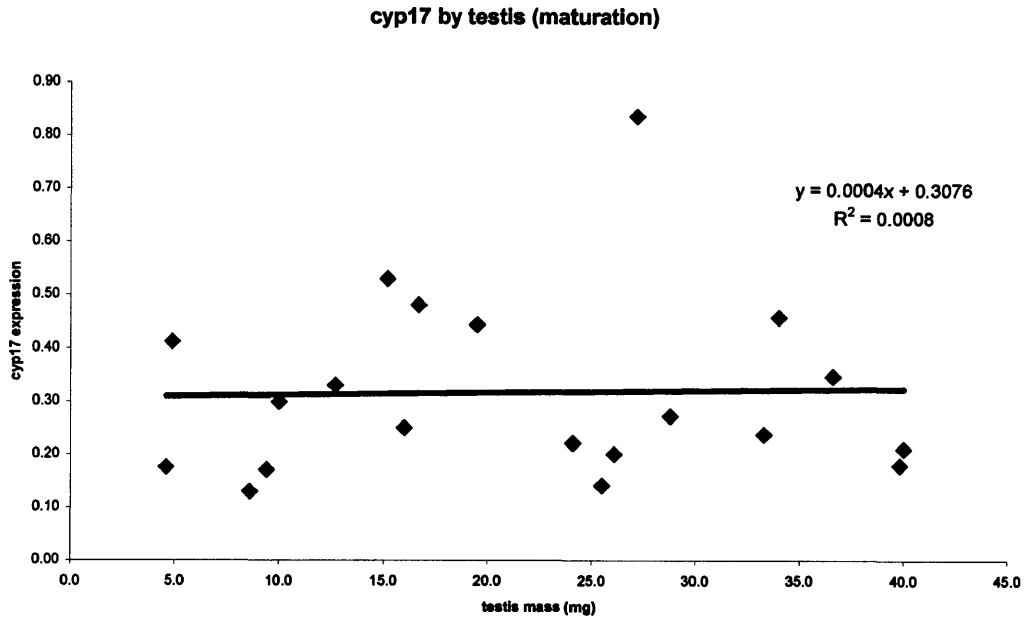

hsd3b1 by sv (inhibition)
y = 0.0501x + 0.8736
R2 = 0.2642
hsd3b1 expression
sv mass (mg)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.0
5.0
10.0
15.0
20.0
25.0

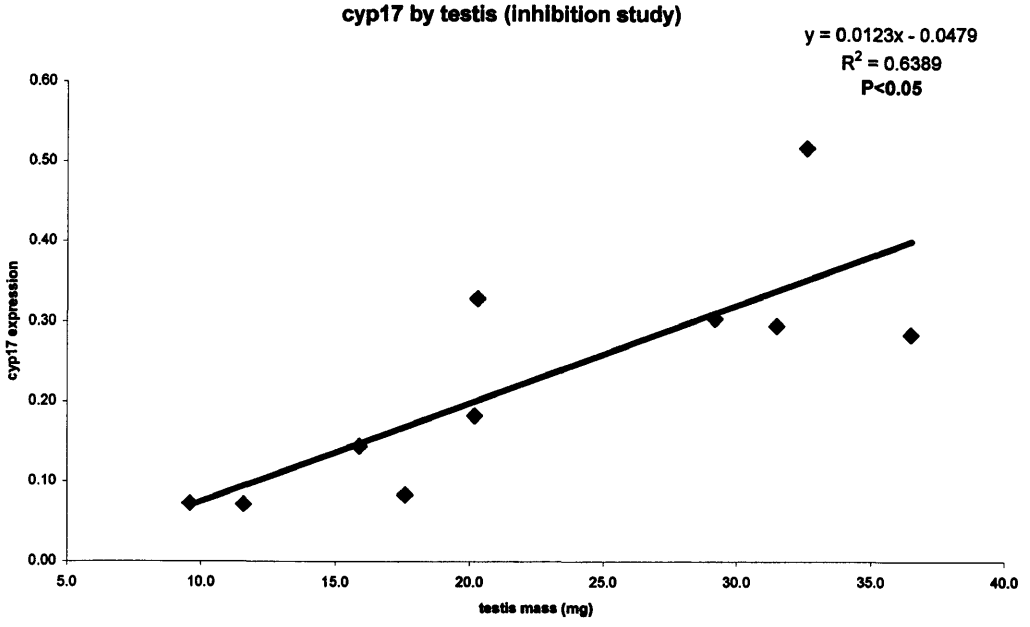

cyp17 by body (maturation)
y = 0.0044x + 0.2407
R2 = 0.0048
cyp17 expression
body mass (g)
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00

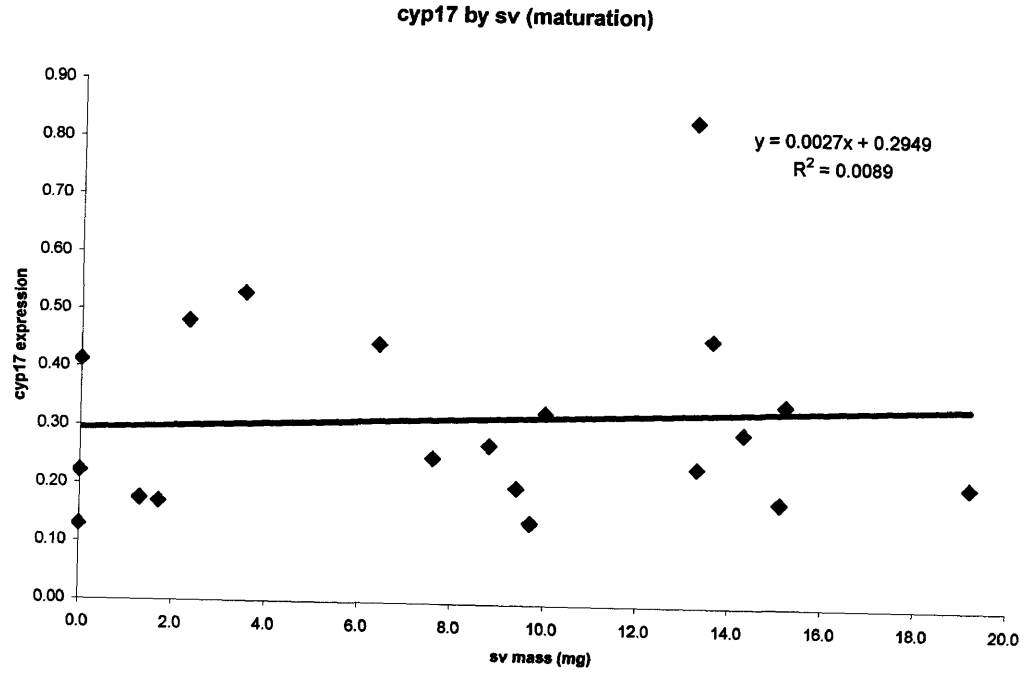

cyp17 by body (inhibited)
y = 0.0357x - 0.3583
R2 = 0.6279
P<0.05
cyp17 expression
body mass (g)
0.00
0.10
0.20
0.30
0.40
0.50
0.60
10.00
12.00
14.00
16.00
18.00
20.00
22.00

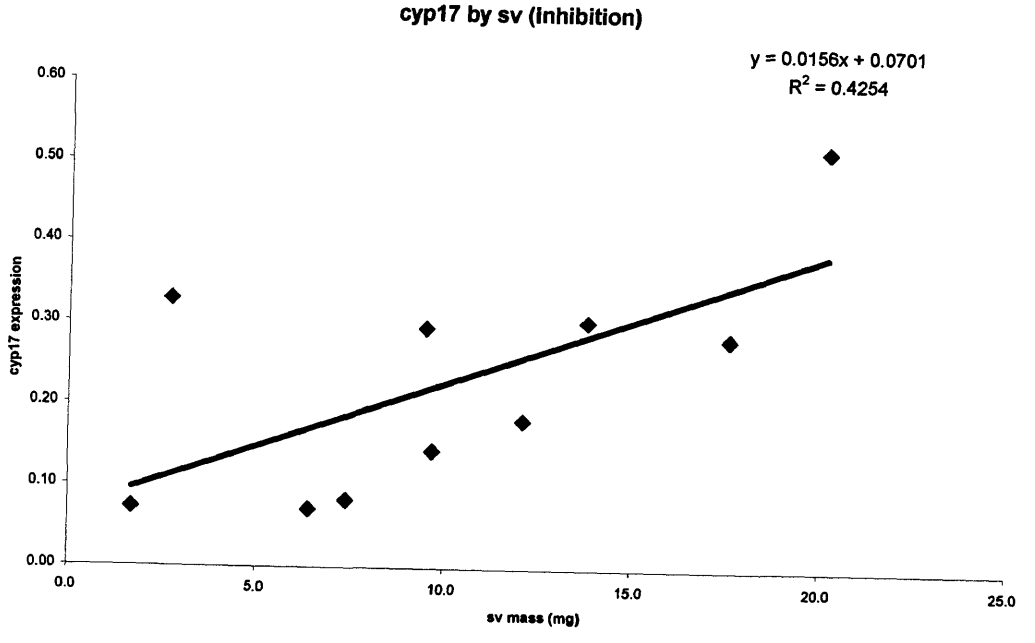

cyp17 by adrenal (maturation)
y = 0.0306x + 0.2076
R2 = 0.029
cyp17 expression
adrenal mass (mg)
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
1.0
2.0
3.0
4.0
5.0
6.0
7.0

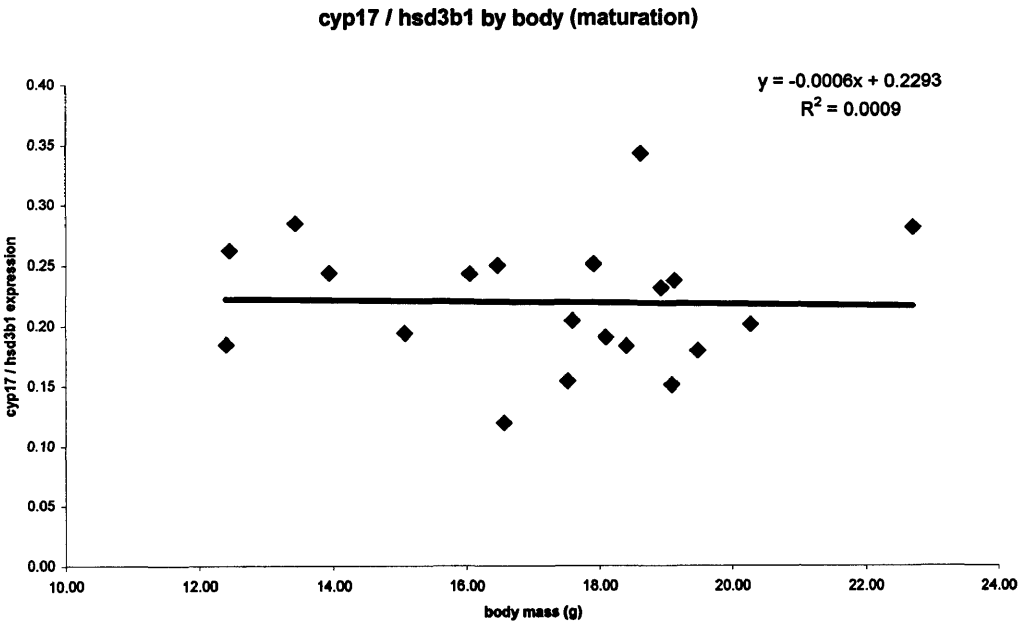

cyp17 by adrenal (inhibition)
y = 0.081x + 0.0038
R2 = 0.3308
cyp17 expression
adrenal mass (mg)
0.00
0.10
0.20
0.30
0.40
0.50
0.60
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5

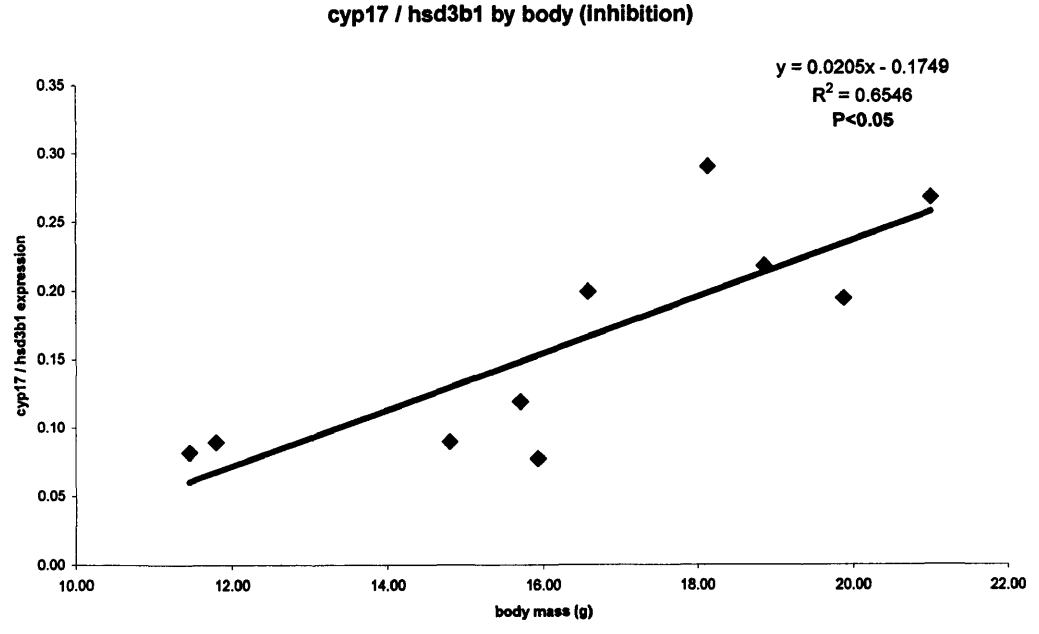

cyp17 by testis (maturation)
y = 0.0004x + 0.3076
R2 = 0.0008
cyp17 expression
testis mass (mg)
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0

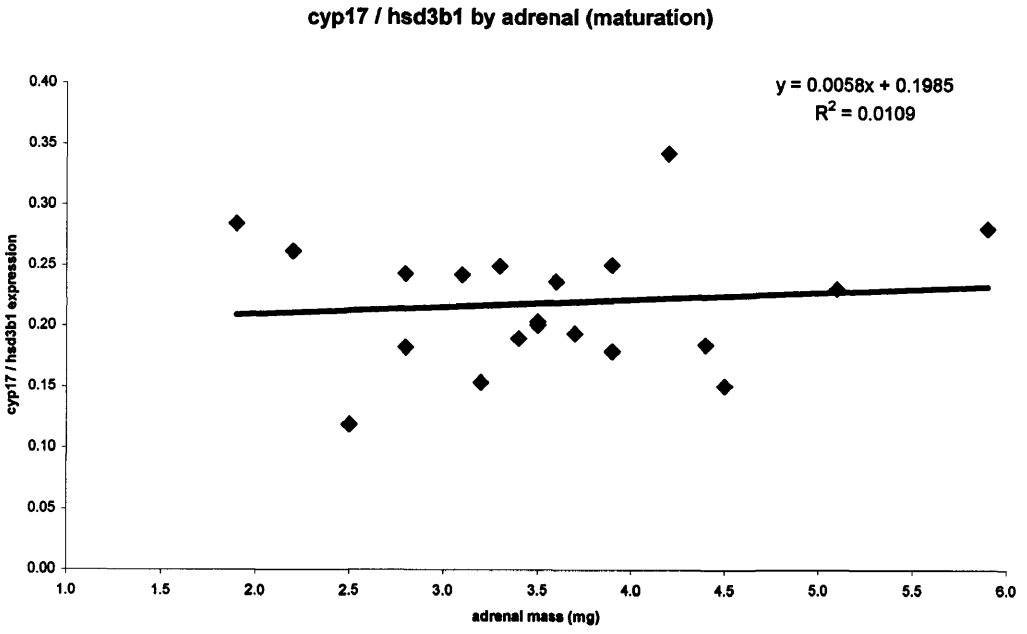

cyp17 by testis (inhibition study)
y = 0.0123x - 0.0479
R2 = 0.6389
P<0.05
cyp17 expression
testis mass (mg)
0.00
0.10
0.20
0.30
0.40
0.50
0.60
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0

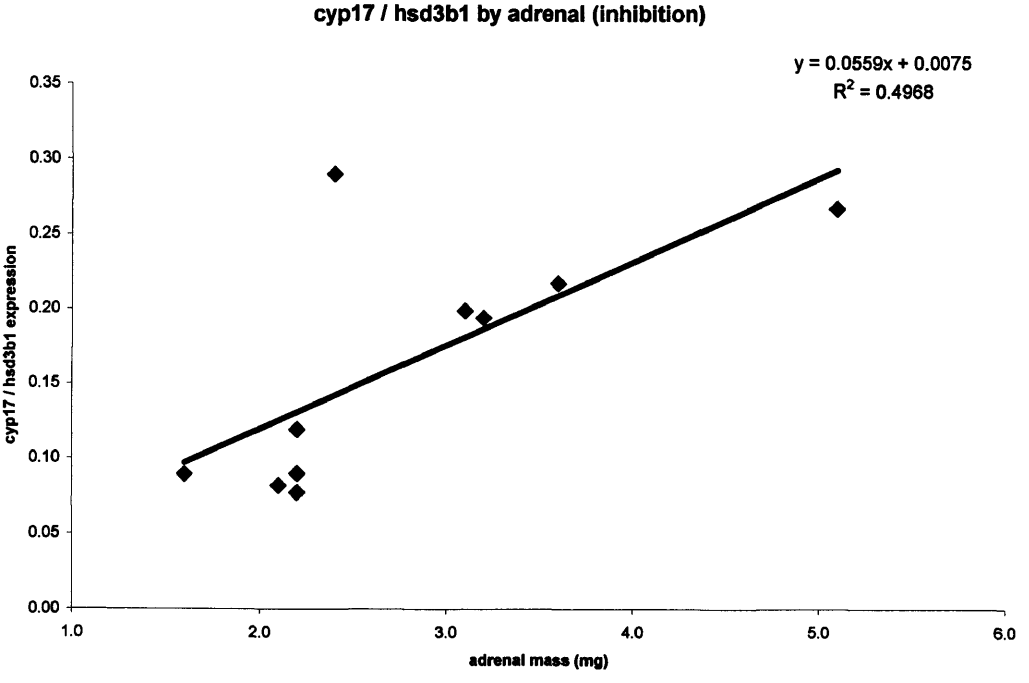

cyp17 by sv (maturation)
y = 0.0027x + 0.2949
R2 = 0.0089
cyp17 expression
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
sv mass (mg)

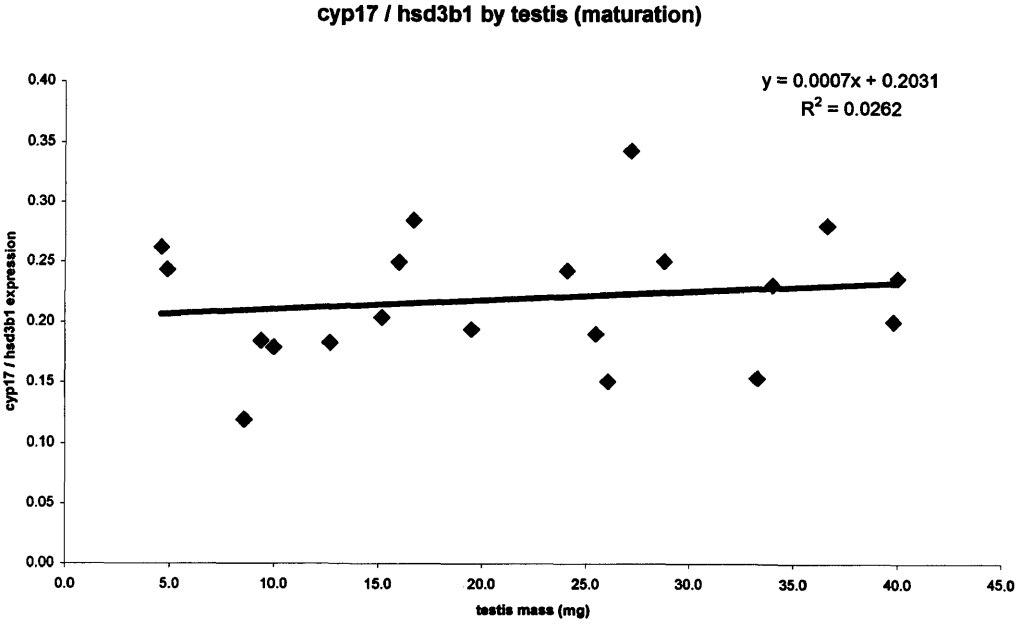

cyp17 by sv (inhibition)
y = 0.0156x + 0.0701
R2 = 0.4254
cyp17 expression
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.0
5.0
10.0
15.0
20.0
25.0
sv mass (mg)

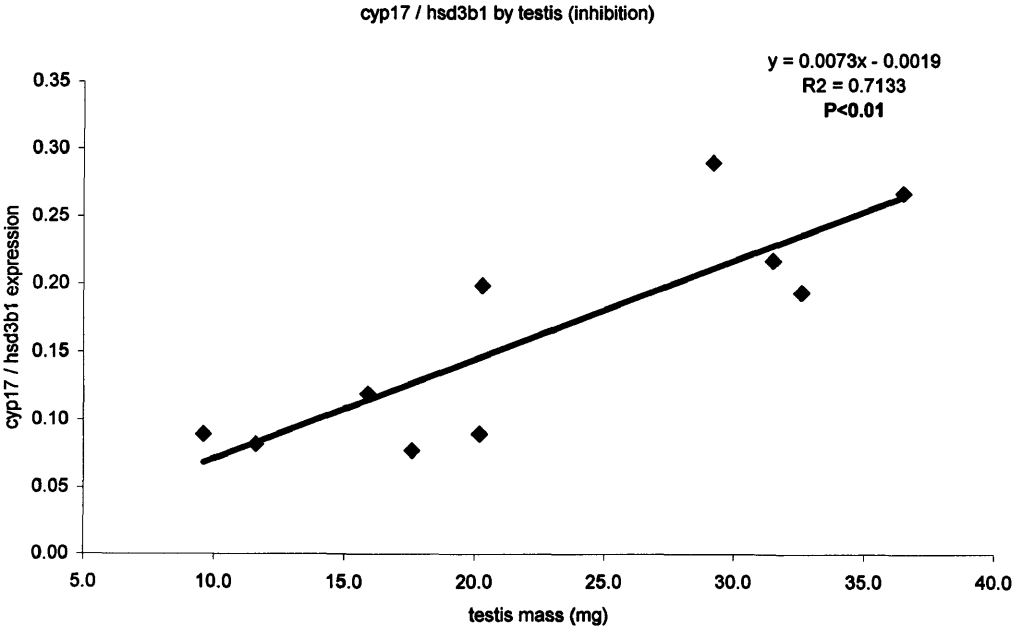

cyp17 / hsd3b1 by body (maturation)
y = -0.0006x + 0.2293
R2 = 0.0009
cyp17 / hsd3b1 expression
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
body mass (g)

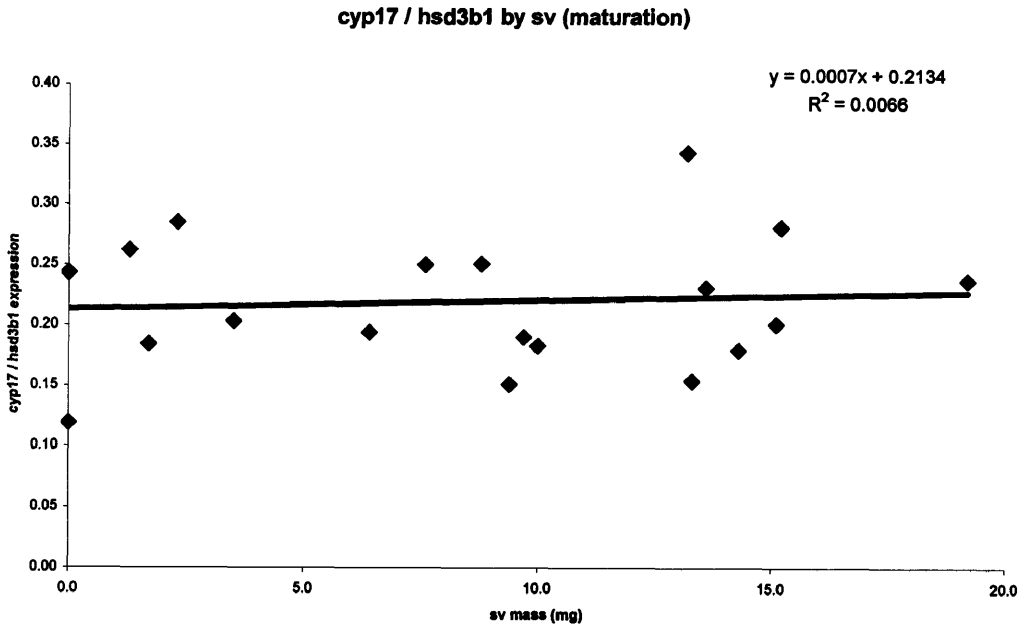

cyp17 / hsd3b1 by body (inhibition)
y = 0.0205x - 0.1749
R2 = 0.6546
P<0.05
cyp17 / hsd3b1 expression
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
body mass (g)

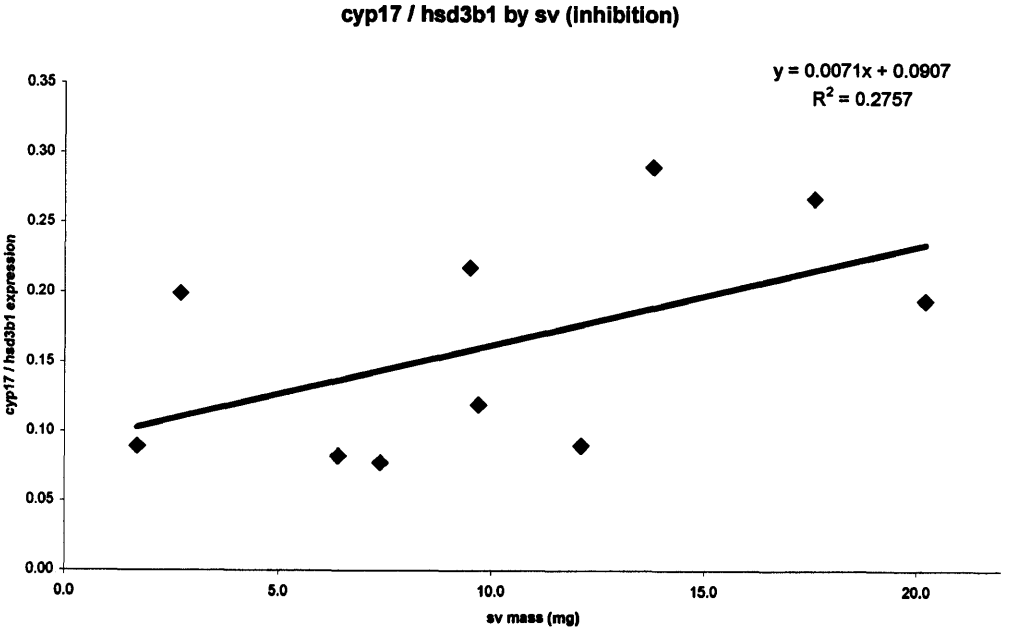

cyp17 / hsd3b1 by adrenal (maturation)
y = 0.0058x + 0.1985
R2 = 0.0109
cyp17 / hsd3b1 expression
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
adrenal mass (mg)


cyp17 / hsd3b1 by adrenal (inhibition)
y = 0.0559x + 0.0075
R2 = 0.4968
cyp17 / hsd3b1 expression
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
1.0
2.0
3.0
4.0
5.0
6.0
adrenal mass (mg)


cyp17 / hsd3b1 by testis (maturation)
y = 0.0007x + 0.2031
R2 = 0.0262
cyp17 / hsd3b1 expression
testis mass (mg)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0


cyp17 / hsd3b1 by testis (inhibition)
y = 0.0073x - 0.0019
R2 = 0.7133
P<0.01
cyp17 / hsd3b1 expression
testis mass (mg)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0


cyp17 / hsd3b1 by sv (maturation)
y = 0.0007x + 0.2134
R2 = 0.0066
cyp17 / hsd3b1 expression
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.0
5.0
10.0
15.0
20.0
sv mass (mg)


cyp17 / hsd3b1 by sv (inhibition)
y = 0.0071x + 0.0907
R2 = 0.2757
cyp17 / hsd3b1 expression
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.0
5.0
10.0
15.0
20.0
sv mass (mg)

VITA

Brian Thomas Lovitt

Born in Coldwater, Michigan, August 31, 1976. Graduated from Huron High School in Huron, Ohio, June 1994. Graduated from The University of Wisconsin, Madison, Wisconsin, 1998 with a B.S. in Zoology. In August 1998, entered The College of William and Mary as a graduate student in the Department of Biology. Defended thesis entitled: *Adrenal Expression of Cyp17 and Hsd3b1 mRNA in Peromyscus maniculatus baidii*, May 2001. All requirements for the degree of Master of Arts have been completed.