Abstract

Engell, Miles Dean. The effect of endocrine disruptors on the monogamous pine vole (*Microtus pinetorum*). (Under the direction of Dr. John G. Vandenbergh)

Since the discovery in the 1960s that synthetic chemicals in the environment could alter normal hormonal functioning in wildlife, the study of endocrine disruptors has grown rapidly. Most xenobiotic endocrine disrupting chemicals (EDCs) arise from sources such as pesticides, industrial chemicals, and pharmaceuticals. Many of the chemicals either mimic estrogens or act as antiandrogens. Previous research has focused largely on physiological and anatomical endpoints, such as anogenital distance (AGD), organ weights, and alterations in sexual differentiation. The objective of this research was to examine the effects that EDCs have not only on these traditional toxicological measures of disruption but also on the behaviors associated with monogamy, which have the potential to alter an entire social system.

Female pine voles were orally administered an EDC or corn oil control throughout gestation and lactation of pups. EDCs used were antiandrogenic flutamide (positive control) or vinclozolin for effects on male pups; and estrogenic diethylstilbestrol (DES, positive control) or methoxychlor for effects on female pups. As adults, pre- and neonatally exposed pups were then examined for both behavioral and physiological alterations. AGD was lengthened and seminal vesicle weights were heavier in flutamide exposed males, suggesting that flutamide acted as androgen rather than antiandrogen in these experiments. Vinclozolin treated males exhibited a reduction in both AGD and

seminal vesicle weight, supporting vinclozolin's action as an antiandrogen. There were no differences in female AGD; methoxychlor exposed females had reduced uterine weights.

Following a cohabitation period, preference for the mate versus a stranger was assessed via a three-chambered preference test apparatus. After the birth of a litter, parental behavior was examined. In the male behavioral tests, flutamide males did not show a preference for their mate, but instead spent more time alone in the neutral chamber. Their parental behaviors were unaffected. Vinclozolin treated males' preference for the mate was no different from that shown by control males. Vinclozolin decreased paternal responsiveness, however.

DES exposed females spent more time with the stranger, most of it engaged in aggressive behavior, which was significantly greater in this treatment group than controls. The methoxychlor females resembled the flutamide males, in that they spent more time in the neutral chamber. There were no differences in maternal behavior among female groups.

Arginine vasopressin (AVP) receptor binding in males and oxytocin (OT) receptor binding in females was assessed by receptor autoradiography to examine any effect of EDCs on these behaviorally important neuropeptide systems. Among males, the cingulate cortex showed a reduction in AVP binding in the flutamide group only. The cingulate cortex was also affected in females, with methoxychlor exposure resulting in decreased OT binding in this region.

These findings demonstrate that exposure to endocrine disrupting compounds during pre- and neonatal development can alter adult brain neuropeptide receptor distribution and behavior related to pair bond formation.

THE EFFECT OF ENDOCRINE DISRUPTORS ON THE MONOGAMOUS PINE VOLE (*MICROTUS PINETORUM*)

by

MILES DEAN ENGELL

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Chair of Advisory Committee

"Happy are those who dream dreams and are willing to pay the price

to make them come true."

Unknown

I dedicate this dissertation to my parents, my husband,

and my dear daughter--

Each of whom endured the price with me, but made certain I never paid too much.

Biography

Although I was born in Indianapolis, Indiana, and immediately spent almost three years living in Frankfurt, Germany while my father served in the army, I consider myself a native North Carolinian. My brother and I, like both of our parents, were raised in Laurinburg, NC, in the southeastern part of the state. I attended Davidson College near Charlotte, NC, spending a semester abroad in Kenya studying wildlife conservation and management. In 1991 I graduated with a Bachelor of Science in Biology.

Following graduation, I returned to Laurinburg to teach biology at the same high school from which I graduated. I taught three levels of Biology and AP Biology from 1991- 1998. My daughter Munroe was born in December of 1994. In the fall of 1998 I entered the Department of Zoology at North Carolina State University as a doctoral student under Dr. John Vandenbergh. While pursuing my degree, I held a graduate research assistantship for one year, an NSF predoctoral fellowship for three years, and a supplemental fellowship from the Keck Center for Behavioral Biology at NCSU. I also taught the undergraduate Intro to Animal Behavior course at NCSU for two fall semesters. In addition to meeting wonderful colleagues and friends while at NCSU, I also met my best friend Adam, whom I married in June of 2000.

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Finally, and most importantly, a huge thank you to my family: My first and greatest teachers, my parents, who continue to spoil me with their generosity and caring, guide me with their wisdom and faith, and love and support me through everything; My Danish parents and family, whose interest, encouragement, and love are palpable even from thousands of miles away; My brother Brian, for help with centrifuges and geiger counters, for sharing my love of science and his incredible knowledge and understanding of it with me, and for being the friend that both he and his wife Lacey are; My precious daughter Munroe, who even after being dragged out of the house on a Saturday morning to go dose voles with me can still look up at me as we're holding hands walking across campus and say "I'm glad you're my Mommy"—thanks for

understanding, and making my *most* important job also my most fun one; and finally my wonderful-beyond-words husband Adam, who was brightening my days even before he moved across the ocean to become my own personal computer guru, writing the program for the preference tests from scratch and helping me with more computer "issues" than I can begin to count. His knowledge and insight astound me almost as much as his unending patience and support, but it's his depth of understanding and ability to keep me smiling and at peace for which I'm most grateful.

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Chapter One GENERAL INTRODUCTION

Monogamy

Monogamy is a complex and somewhat ambiguous concept. Definitions are often contradictory, and characteristics associated with monogamy vary from one species to another (reviewed Dewsbury 1987). Most definitions, including dictionary, state or imply exclusivity in mating between a given male and female (Alcock 1984; Carter *et al.* 1995; Webster 1960). However, such sexual exclusivity has yet to be proven for the majority of monogamous bird and mammal species (Bennett and Owens 2002; Carter *et al.* 1995; Griffith *et al.* 2002; SilleroZubiri *et al.* 1996). Thus monogamy is commonly defined as a social system rather than a mating system (Bennett and Owens 2002). Traits generally used to characterize either socially or sexually monogamous species include: (1) long-term selective association or pair bonds between one male and one female both during and outside reproductive periods; (2) an absence of adult, unrelated conspecifics from a pair's nest or territory, often accompanied by aggression toward unfamiliar conspecifics; (3) biparental care with high levels of paternal care, and alloparenting; (4) socially regulated reproductive processes, such as estrus induction; and (5) avoidance of incest via reproductive suppression of adult family members that remain in the group as "helpers" (Carter *et al.* 1995; Kleiman 1977).

Monogamy can be either *facultative* or *obligate.* Facultatively monogamous species show flexibility and will be monogamous where resources are scarce and polygynous where resources are common. Obligate monogamy, in contrast, generally occurs in species whose females always need help in rearing offspring. Thus, obligately monogamous males exhibit a high paternal investment and, in general, have stronger bonds with their mates (Kleiman, 1977). For example, the young of silverbacked jackals, which eat difficult-to-find small prey, require a great deal of parental care, and the adults maintain a very stable and strong pair bond. Closely related golden jackals, while also monogamous, do not require a great deal of parental care and accordingly will occasionally change mates (Moehlman, 1987).

Monogamy, while common in birds, is rare in mammals. Kleiman's (1977) exhaustive survey found that only about 3% of mammal species are monogamous. In contrast, approximately 90% of avian species practice pair bonding during a breeding season (Drickamer *et al.* 2002; Lack 1968). Breeding synchrony of female birds often makes it advantageous for a male to forego polygyny and help one mate instead (Ehrlich *et al.* 1988; Knowlton 1979; Emlen and Oring 1977). In addition, male birds have an opportunity to provide parental care both before and after hatching, through incubating eggs and feeding young (Orians 1969). In contrast, the more usual strategy of male mammals is to compete for a female, inseminate her, and then to seek another. Mammals have internal gestation, and after birth offspring are nourished by the mother's milk. Therefore mammals do not require an immediate and constant supply of food from the environment, and thus male assistance is less valuable to the female in the majority of mammalian species than it is in avian species (Orians 1969). Many monogamous males, however, show a level of investment that begins to approach that of females.

Those mammalian species whose members are usually or always monogamous have some striking similarities. The two sexes are often indistinguishable in size and general appearance (Alexander *et al.* 1979). In addition, both sexes of a monogamous species defend a territory or their nest and, without the male-male competition for mates or territories observed in harem or polygynous systems, little selection exists for large males endowed with uniquely male weaponry (Hrdy, 1999).

Evolution

Monogamy was probably not the primitive mating system for vertebrates, since anisogamy is an extremely primitive characteristic among sexually reproducing organisms, and anisogamy sets the stage for competition among males for access to

females and attempts by males to mate with as many females as possible (Trivers 1972). In fact, monogamy is thought to have evolved independently many times (Mock and Fujioka 1990; Mock 1985). Likely factors that have contributed to its common occurrence in birds are the parental care needs of the young, oviparity, dispersion of critical resources, and the absence of lactation.

In mammals, where monogamy is rare, various factors are commonly cited as resulting in a pair-bonded mating system. In environments where females are scarce and widely distributed, a male has greater reproductive success by remaining with and guarding one female when he finds her than continuing to search for others (Barlow 1988; Emlen and Oring 1977). In some mammalian species, a male is essential to offspring survival, due to limited food resources or nesting sites, extreme temperatures, or an extended time of young dependency (Gubernick and Teferi 2000; Gubernick *et al.* 1993). In monogamous species such as the dik-dik (*Madoqua kirkii*) there is no paternal care, and males are capable of defending territories that could support more than one female (Komers 1996). In these species, monogamy may have evolved because the cost of guarding more than one female from intruding males would be too great. The male dik-dik, for example, guards his female by covering up the scent of her territorial markers, thereby preventing other males from knowing when she is in estrus. (Brotherton *et al.* 1997). Finally, female aggression or suppression of the reproduction of other females in a group may prevent a male from mating with other females (Derix *et al.* 1993).

The ultimate or evolutionary causes of monogamy have been discussed at length (reviewed Borgia 1979; Mock and Fujioka 1990; Lott 1991). Mammalian obligate monogamy, as in pine voles, is thought by some to have evolved in species where help from the male is essential for capturing and delivering prey to a lactating female (Kleiman 1977). Thus the time and energy that a male devotes to feeding one female's offspring prevent him from devoting the same time and energy to another female (Kleiman and Malcolm 1981). Another hypothesis is that obligate monogamy evolved in

species such as the wild dog (*Lycaon pictus*) where female home ranges are small enough for males to defend, but large enough that males are unlikely to find another mate (Krebs and Davies 1993). As an obligately monogamous mammal, the pine vole could potentially fit either of these models.

Proximate Mechanisms

Within the past decade, attention has turned to the proximate or physiological mechanisms underlying monogamy (Williams *et al.* 1994; Winslow *et al.* 1993). That there even *is* a physiological basis to the characteristics of a mating or social system has not been widely recognized in the past (Carter *et al.* 1995). Specific reproductive and social behaviors in many species however, are modified by hormones and neurochemicals and, certainly, monogamy is a collection of such behaviors.

Much recent research (reviewed Wynne-Edwards 2001; Carter 1998; Insel 1997) has explored the endocrinological characters that can affect components of monogamy. While the California mouse (*Peromyscus californicus*) has been the focus of some research, the majority has been on microtine rodents, the voles. Voles, and their close relatives the lemmings, are the most common wild rodents in North America. Some 23 species occupy diverse habitats across the continent, eating grasses, grains, and much other plant material. They nest on or under the ground amid narrow runways and burrows. Most laboratory research concerning the proximate mechanisms for monogamy has used four primary species of vole: meadow vole (*Microtus pennsylvanicus*), montane vole (*Microtus montanus*), prairie vole (*Microtus ochrogaster*), and pine vole (*Microtus pinetorum*). These species can be maintained in laboratory facilities or outdoor enclosures and thus are well suited for controlled experiments. Particularly intriguing is the fact that, though closely related, they exhibit strikingly different social organizations and mating systems. Meadow and montane voles have

multiple mates and are uniparental, display very little affiliative behavior and only short-term bonds that do not last beyond mating. In contrast, prairie and pine voles are obligately monogamous with biparental care, are highly social and maintain enduring, selective pair bonds after mating. Thus, voles provide an excellent opportunity for comparative studies of the proximate mechanisms influencing monogamy.

Oxytocin and Arginine Vasopressin

The work of several laboratories (Williams *et al.* 1994; Winslow *et al.* 1993) has demonstrated that the neuropeptides oxytocin (OT) and arginine vasopressin (AVP) are involved in the behavioral changes of prairie voles as they go from virgin to having a pair-bond. These two nonapeptides are synthesized in the hypothalamus and released into the bloodstream through axon terminals in the posterior pituitary (Insel 1997). They are closely related structurally, differing in only two amino acids (Figure 1). Though homologues such as arginine vasotocin and isotocin exist in other vertebrates, with similarities in function, AVP and OT are found exclusively in mammals and are implicated in prototypically mammalian functions (Insel 1997). For example, OT has a large role in milk ejection during nursing and uterine contraction during labor. AVP (Anti-Diuretic Hormone) increases water resorption in the kidney. The traditional view of these peptides acting on peripheral organs as endocrine hormones has been revised, however, to include roles of neurotransmitters or neuromodulators as well, with the brain as target organ (Barberis and Tribollet 1996; Buijs and van Heerikhuize 1982; Sofroniew and Weindl 1981).

The neuroanatomical distribution of OT receptors (OTR) and V1a vasopressin receptors (V1aR) in the brain is virtually non-overlapping in monogamous and nonmonogamous voles, indicating that receptor distribution is related to the differences in social behavior between the species (Insel and Shapiro 1992; Insel *et al.* 1994). The receptors show similar binding characteristics (in terms of kinetics and specificity) in

voles with contrasting mating patterns, but are expressed within entirely different neural pathways (Insel *et al.*, 1992; 1994). Transgenic work supports the importance of the pattern of V1aR gene expression in the brain to the differing social behaviors of vole species. Mice that are transgenic for the (monogamous) prairie vole receptor gene have a neuroanatomical pattern of receptor binding similar to the prairie vole pattern, and also show increased affiliative behavior (Young *et al.* 1999).

FIGURE 1 – NEUROPEPTIDE STRUCTURE

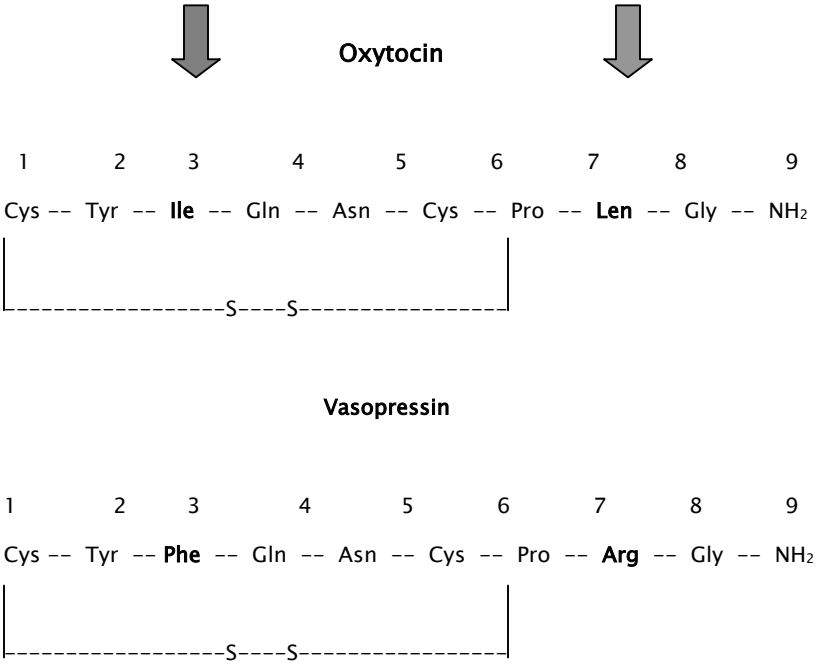


Figure 1: Nonapeptides oxytocin and vasopressin are closely related structurally. Arrows indicate amino acid differences.

In monogamous female voles (both prairie and pine), OTR density is high in the nucleus accumbens (NAcc) and prelimbic cortex (PLC) areas, whereas nonmonogamous female voles have virtually no oxytocin receptors in the NAcc, and very few in the PLC (Figure 2) (Insel and Shapiro, 1992). The NAcc and PLC are key components of the dopamine pathway, thought to be involved in the reinforcing or rewarding effects of natural stimuli and drugs of abuse (McBride *et al.*, 1999). Thus, it has been

hypothesized that oxytocin transmission in these areas may facilitate pair bonding in a female vole by reinforcing the association between the rewarding effects of NAcc activation and the male with whom she just mated (Young *et al.*, 2001). The pathway may also be important to maternal behaviors-- after parturition when the female montane vole becomes briefly parental, the pattern of OTR binding changes to resemble the pattern observed in the highly parental prairie vole (Insel *et al.*, 1992).

FIGURE 2- OXYTOCIN RECEPTOR BINDING

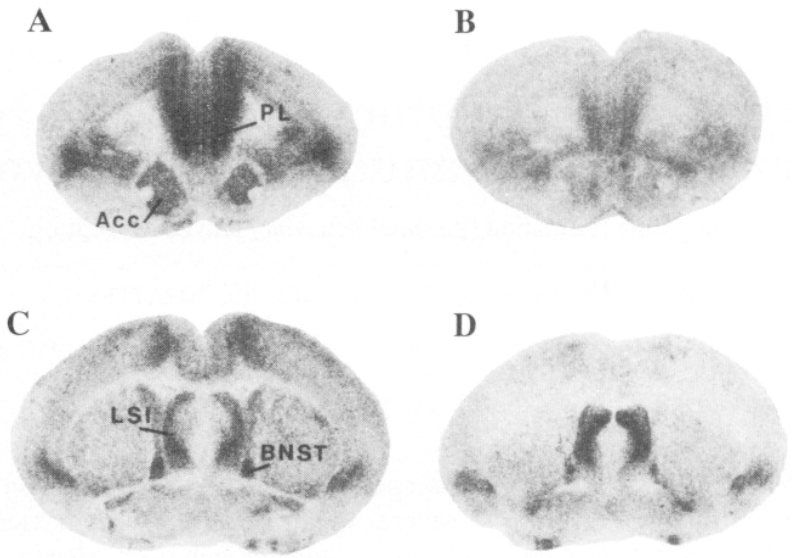


Figure 2: Photomicrographs displaying ^{125}I-oxytocin binding in paired brain sections from a prairie vole (A,C) and from a montane vole (B,D). PL=prelimbic cortex, Acc=nucleus accumbens, LS=lateral septum, BNST=bed nucleus of the stria terminalis From Insel and Shapiro, 1992

In male voles, the distribution of vasopressin receptors appears to contribute to the species differences in male social behavior. Monogamous voles have high densities of V1aR in a region of their forebrains located ventromedially to the NAcc, corresponding to the ventral pallidum (VP) (Young *et al.*, 2001). The VP, like the NAcc, receives dopaminergic input and is an important neurobiological substrate for the rewarding and reinforcing properties of natural stimulants and psychostimulants (McBride *et al.*, 1999). Thus, a release of AVP during mating may activate V1aR in the

VP, leading to a partner preference in a male vole. The lack of V1aR in the VP of nonmonogamous voles may explain their inabilities to form partner preferences after mating (Young *et al.,* 2001). In addition, the brain of a monogamous male has a higher density of V1aR in other brain regions, such as the diagonal band, while the brain of a nonmonogamous male has a greater density in the lateral septum (Figure 3) (Wang *et al.,* 1997).

FIGURE 3- ARGININE VASOPRESSIN RECEPTOR BINDING

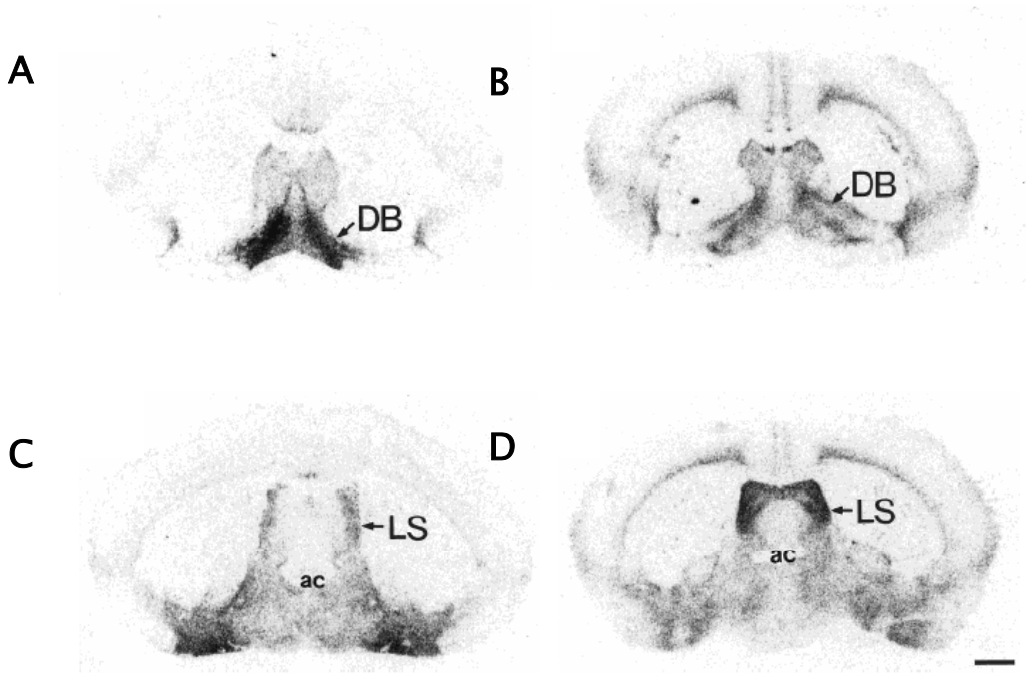


Figure 3: Photomicrographs displaying ^{125}I-linear-vasopressin (AVP) binding in paired rostral brain sections from a prairie vole (A,C) and from a montane vole (B,D). DB=diagonal band, LS=lateral septum, ac=nucleus accumbens. Wang et al., 1997

Central administration of OT and AVP further supports the role of the peptides in pair-bond formation. Specifically, in female prairie voles, an intracerebroventricular (icv) infusion of OT facilitates the formation of a partner preference and increases social

contact after cohabitation even in the absence of mating (Williams *et al.,* 1994; Cho *et al.*, 1999). In addition, an icv infusion of a selective antagonist (OTA) into female prairie voles blocks formation of a partner preference despite extended bouts of mating (Insel *et al.,* 1995). In male prairie voles, AVP plays an analogous role to OT in females: AVP administered centrally, even in the absence of mating, facilitates partner preference, increases affiliative behavior, and increases selective aggression toward intruders. As with females, the antagonist does not interfere with mating, but appears to block the consequences of mating (Winslow *et al.,* 1993; Cho *et al.*, 1999).

Vasopressin and oxytocin are also involved in parental behavior in voles. Infusion of vasopressin into the lateral septum of monogamous prairie voles increases paternal responsiveness (Wang *et al.* 1994a). In addition, both males and females of this species have increased vasopressin gene expression after the young are born, unlike males or females of either promiscuous species of vole (Wang *et al.* 2000). Oxytocin has long been known to play a critical role in the neuroendocrine system that regulates birth and lactation, as well as maternal behavior (Pedersen 1992). While the pattern of oxytocin receptor distribution in the brains of monogamous and promiscuous voles differs markedly in nulliparious females, postpartum promiscuous voles exhibit a significant increase in oxytocin receptors in specific brain regions that result in a much greater resemblance to the brains of monogamous voles. This increase in oxytocin binding is associated with a change to maternal behavior (Insel and Shapiro 1992).

Gonadal Steroid Involvement

The influence of gonadal steroid hormones on reproductive behaviors has been well documented (Beach, 1976; Brown, 1985; Meisel and Sachs, 1994; Pfaff 1997; Pfaff *et al.* 2000). In addition, gonadal steroids appear to play critical roles in the regulation of both OT and AVP. Investigations of rat maternal behavior have revealed that the steroid treatment regimen designed to facilitate maternal behavior artificially, 13 days of

17ß- estradiol (estrogen) and progesterone followed by 2 days of estrogen alone, is also the ideal schedule for inducing oxytocin gene expression in the hypothalamus (Crowley *et al.* 1995). Estrogen regulates the number of oxytocin receptors in the uterus and mammary epithelium, as well as those in the ventromedial nucleus (VMN) of the hypothalamus and the bed nucleus of the stria terminalis (BNST) (Crowley *et al* 1995). Binding of OT receptors increases 84% in the BNST at parturition, and 35% in the VMN at estrus (Insel, 1992). Additionally, an oxytocin antagonist attenuates the gonadal steroid induction of lordosis (Witt *et al.,* 1990). Such results demonstrate that changes in estrogen levels can alter receptor expression in specific brain areas.

The responsiveness of oxytocin to gonadal steroids in female prairie voles differs from that observed in female rats. In monogamous voles, female sexual receptivity is inhibited by central administration of OT at doses that typically facilitate sexual behavior in rats (Witt *et al.,* 1990). Likewise, the effects of estrogen in the brain are focused on the anterior olfactory nucleus (OAM) rather than the hypothalamus. Both exogenous and endogenous estrogen stimulation resulted in increased oxytocin receptor binding in the OAM. Because estrogen stimulation does not alter the affinity of OAM oxytocin receptors, it is thought that the observed increase in OT binding reflects an increase in the number of oxytocin receptors (Witt *et al.*, 1991).

Many of vasopressin's behavioral effects in rodents appear to be androgen-dependent, and testosterone has been shown to stimulate AVP mRNA expression in rats (Bluthe *et al.*, 1990; Miller *et al.*, 1989). In male prairie voles a mating-induced increase in circulating testosterone corresponds to increased AVP mRNA expression in the BNST (Wang *et al.,* 1994b). The BNST, along with the medial amygdaloid nucleus, forms the source of vasopressin-immunoreactive (AVP-ir) fibers in the lateral septum and lateral habenular nucleus. These fibers are directly influenced by testosterone (Wang and DeVries, 1993). Castration, in fact, causes the AVP-ir fibers to lose their immunoreactivity to AVP, and the BNST and medial amygdaloid nucleus to no longer be

labeled for AVP mRNA. Testosterone treatment of castrated voles prevents these changes (Wang and DeVries, 1993).

Endocrine Disruption

In the late 1960s, the effects attributed to the pesticide DDT led scientists in the USA to begin scrutinizing many ecological agents that had been designed to improve the quality of life. Declining bird populations and feminized male alligators in the 1990s (Guillette *et al.* 1994) helped to bring much attention to the potential hazards involved with adding chemicals to the environment. Initial studies of environmental pollutants were focused on the potential of toxicants to damage DNA directly and cause genetic abnormalities and/or cancer. Increases in testicular cancer (Jegou *et al.,* 2000) and breast cancer (Davis *et al.*, 1993) are still sited as examples for endocrine disruption effects in humans. It is now known, however, that many synthetic compounds in the environment mimic the actions of natural signaling molecules in animals, such as hormones and growth factors. These environmental hormones do not alter genes, but may change the way they are expressed.

Collectively, chemicals with the potential to interfere with the function of endocrine systems are called endocrine disrupting chemicals (EDCs). The EPA defines EDCs as exogenous agents that interfere with production, release, transport, metabolism, binding, action, or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes (*Research Plan* 1998). Such agents have been reported in semen, ovarian follicles, the uterine environment, and in breast milk at especially elevated concentrations. Each chemical appears to have its own mix of mechanisms of action and target sites (Colburn, 1995).

The best characterized EDCs are classified into two primary categories: environmental estrogens, also known as xenoestrogens, and antiandrogens.

Xenoestrogens have been defined as environmental substances that mimic or inhibit the action of endogenous estrogens (McLachlan, 1985). Interestingly, many of these synthetic, environmental compounds do not resemble the chemical structure of endogenous estrogen (Figure 4). Nevertheless, they are able to bind estrogen receptors within the cell nuclei, and subsequently bind to the regulatory regions of specific genes. They may then activate, repress, or alter the level at which the genes are expressed. Alternatively, xenoestrogens may act as antiestrogens, binding the estrogen receptor and blocking the natural hormone's access (McLachlan and Arnold, 1996).

Estrogenic EDCs do not produce equivalent estrogenicity in all target tissues, or in all species. They have been shown to have both organizational and activational effects in exposed individuals. The physiological and behavioral alterations observed in individuals exposed prenatally to diethylstilbestrol (DES) suggest that estrogenic compounds can alter the organization of the CNS in humans (Hines and Shipley, 1984). Rodent studies have further supported this possibility, with prenatal exposure to xenoestrogens resulting in masculinization or defeminization, depending on the species (Gray and Ostby, 1998).

The normal development of the reproductive system of a male mammal requires the action of androgen, primarily testosterone and dihydrotestosterone, mediated by the androgen receptor. Chemicals that inhibit androgen-mediated sex development via their antagonistic action with androgen receptors are known as antiandrogens. Such chemicals can bind to an androgen receptor without activating it, and simultaneously prevent binding of true androgens. Antiandrogens generally bind receptors with moderate affinity, inducing a conformational change that fails to protect the receptor against degradation or is incompatible with binding to the androgen response element DNA (Wong *et al.* 1995). Male laboratory rats (*Rattus norvegicus*) prenatally exposed to the antiandrogens flutamide or vinclozolin exhibit a variety of reproductive effects that

FIGURE 4- STRUCTURE OF ESTROGENIC COMPOUNDS

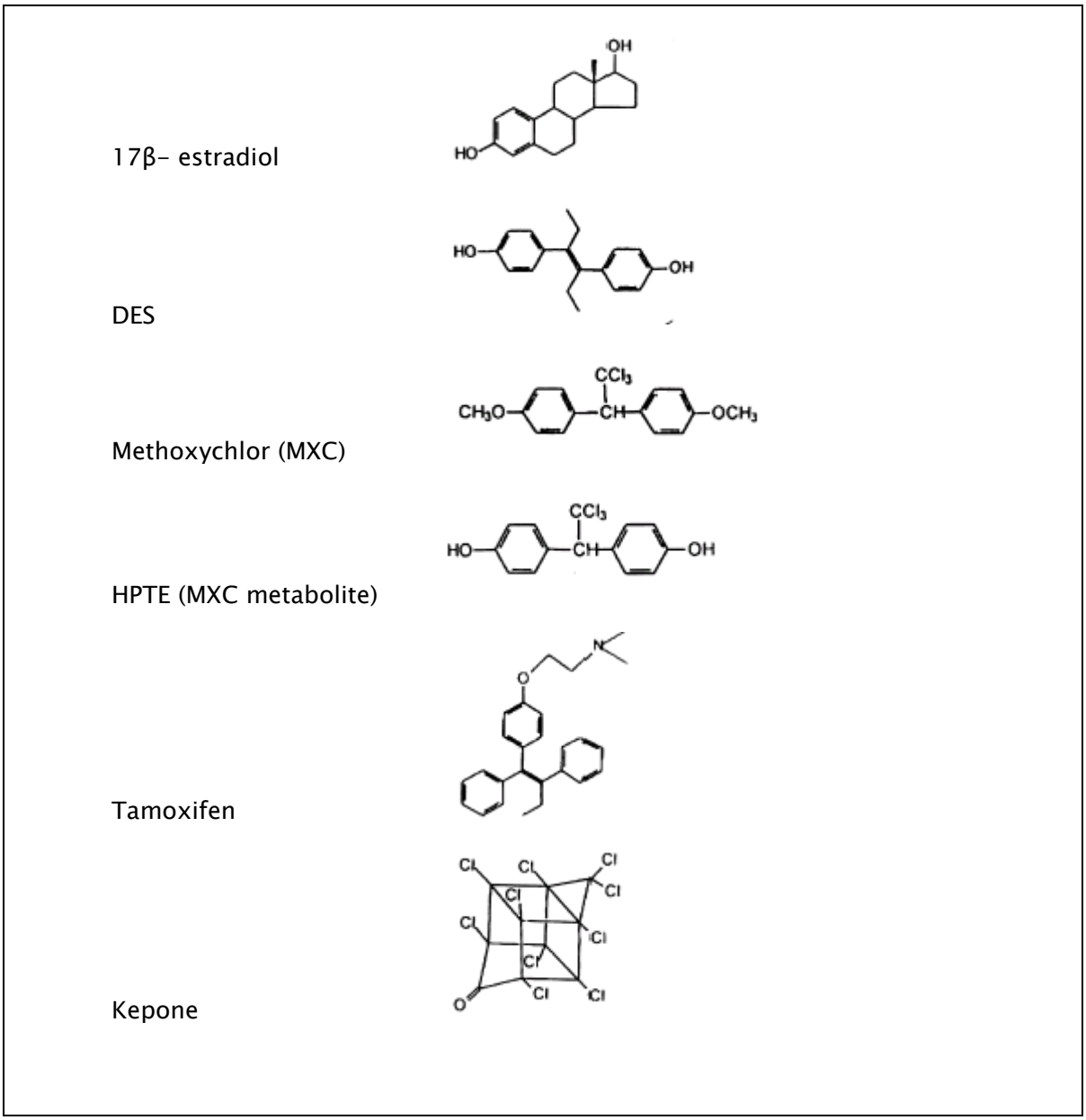


Figure 4: Many synthetic estrogens do not structurally resemble endogenous estrogen (17β- estradiol).

are characteristic of interference with androgen receptor action. Effects include reduction of anogenital distance to that characteristic of females, genital malformations including cleft phallus and hypospadias, retention of nipples, existence of vaginal pouches, prostate gland agenesis, delayed preputial separation, and reduced or absent

sperm production as judged by seminiferous tubule atrophy (Gray and Ostby, 1998; Gray *et al.,* 1994; Wolf *et al.*, 2000).

Though antiandrogens demasculinize and feminize the reproductive tract of male offspring via antagonistic interaction with androgen receptors, they do not appear to inhibit aromatization and estrogen-dependent CNS sex differentiation. For example, mounting behavior in rats is thought to result from the conversion of aromatizable androgens to estrogens in specific brain regions, rather than from a direct effect of androgens (Gray *et al.* 1994). When male rats are exposed to vinclozolin during sexual differentiation, they exhibit malformations and are unable to attain intromission or ejaculate normally, but they display normal mounting behavior (Gray and Ostby, 1998).

Antiandrogens also exhibit activational effects, largely in the form of delayed onset of puberty and reduced fertility (Kelce *et al.,* 1995). Seminal vesicle and prostate weights are reduced following antiandrogen exposure, as is fertility. Because the density of testosterone-dependent penile spines is reduced, it is likely that long-term, high dose antiandrogen treatment would alter the ability of the adult male rat to achieve intromission and ejaculate, since these spines provide critical sensory information for normal penile and ejaculatory function (Gray and Ostby, 1998). Finally, antiandrogens such as vinclozolin increase serum LH levels in male rats and alter androgen-dependent gene expression (Kelce *et al.* 1994; Gray and Ostby, 1998; Ostby *et al.* 1999).

This Research

Rationale/Hypothesis

The research presented here was prompted by the findings discussed above: OT in females and AVP in males are responsible for the behaviors associated with monogamy in pine voles; OT and OT receptors in female brains may be altered by estrogen levels; AVP in male brains may be altered by testosterone levels; and, finally,

estrogenic and antiandrogenic compounds in the environment are capable of disrupting normal levels and actions of steroidal hormones, especially when exposure occurs during the critical prenatal and neonatal periods. Thus, if pregnant and lactating voles are exposed to endocrine disrupting compounds, the development of their offspring may be altered in such a way as to disrupt the normal pair-bonding and parental behaviors characteristic of monogamy in adult pine voles.

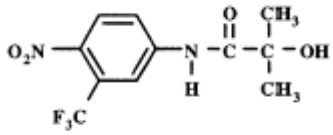
The endocrine disruptors used in this research were chosen for their known action and potency (Table 1). Flutamide is an antiandrogenic pharmaceutical that competes with androgens for the androgen receptor (AR), inhibits AR-DNA binding, and alters androgen-dependent gene expression *in vivo* and *in vitro* (Waller *et al.* 1996; Kelce *et al.* 1997; Ostby *et al.* 1999). It is meant to serve in this research as a positive control for antiandrogenic activity. Vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-oxazolidine-2,4-dione] is a systemic dicarboximide fungicide used in the United States and Europe for the control of fungal disease in grapes and other fruits, vegetables, hops, ornamental plants, and turf grass (Rankin *et al.* 1989). The two primary metabolites of vinclozolin, M1 and M2, compete for androgen receptor binding and inhibit androgen-induced transcription of androgen-related genes, by blocking the ability of the androgen receptor to bind androgen response element DNA (Kelce *et al.* 1997; Wong *et al.* 1995). The structures of M1 and M2 are remarkably similar to that of the potent flutamide metabolite hydroxyflutamide (Figure 5) and act in a very similar manner (Kelce *et al.* 1997).

TABLE 1 – SUMMARY CHART OF EDCS USED IN THIS STUDY

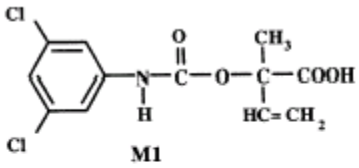
Chemical	Sex	Effect	Role
Corn Oil	Male/Female	---	Negative Control
Flutamide	Male	Antiandrogen	Positive Control
Vinclozolin	Male	Antiandrogen	EDC
DES	Female	Estrogenic	Positive Control
Methoxychlor	Female	Estrogenic/Antiandrogenic	EDC

Table 1: Summary chart of chemicals used in this research, showing modes of action, roles, and sex in which effects are investigated.

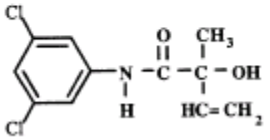
FIGURE 5– STRUCTURES OF ANTIANDROGENS



Hydroxyflutamide



M1



M2

Figure 5: Structures of the two primary metabolites of vinclozolin, M1 and M2, closely resemble the structure of the antiandrogen hydroxyflutamide.

Diethylstilbestrol (DES) is a potent estrogenic drug, whose action as a ligand for the estrogen receptor has been characterized across a wide range of doses (Greco *et al.* 1993; vom Saal *et al.* 1995, 1997). Here DES is used as a positive control for estrogenic activity. Methoxychlor [bis-*p*-methoxy o,p'DDT; 1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane] is an analog of o,p'DDT, but is far less persistent than o,p'DDT

and, thus, does not accumulate in animal tissues. It is widely used as an insecticide for pets, gardens, crops, and livestock. Methoxychlor (MXC) has estrogenic effects *in vivo* after demethylation in the liver to monohydroxymethoxychlor or bis-hydroxymethoxychlor (Kapoor *et al.* 1970). MXC has been reported to bind to androgen receptors as well, acting as an antiandrogen (Gray *et al.* 1999).

Experimental Design

I administered a treatment chemical or corn oil control to female pine voles orally throughout pregnancy and lactation. Upon weaning, I separated litters and housed voles in same-sex sibling groups.

My objective was to assess adult behavior of males that were prenatally and neonatally exposed to antiandrogenic compounds, and of females that were likewise exposed to estrogenic compounds. Thus the male offspring of flutamide and vinclozolin treated females, and the female offspring of DES and methoxychlor treated females became my research subjects.

At 60 days of age, I paired each prenatally and lactationally exposed individual, as well as each control, with an untreated member of the opposite sex. After sufficient time to establish a pair bond, I tested each for its preference of its mate versus a stranger. Following the birth of the first litter of pups, I evaluated maternal or paternal behavior.

At the completion of the behavioral tests, I sacrificed the individuals. I measured organ weights, removed and quick froze the liver in liquid nitrogen, and removed and quick froze the brain on dry ice. I later analyzed the livers for enzyme activity, and the brains for neuropeptide receptor number and distribution.

Thus I obtained both behavioral and physiological measures related to monogamy and reproduction for three groups of males (control, flutamide, and vinclozolin) and three groups of females (control, DES, and methoxychlor).

This thesis is divided into three sections. The first set of experiments (Chapter 2) describes the physiological and anatomical effects of prenatal and neonatal exposure to EDCs. Although most of these measurements were taken toward the end of my research after voles were sacrificed, I present these data first because they provide evidence that the chemical exposures were effective in altering steroid-dependent features. The second set of experiments (Chapter 3) examines the effects of prenatal and neonatal exposure to EDCs on adult affiliative and parental behaviors. The final set of experiments investigates the effects of developmental EDC exposure on neural distributions of OT and AVP receptors.

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Chapter Two ANATOMICAL AND PHYSIOLOGICAL EFFECTS OF PRENATAL AND NEONATAL EXPOSURE TO ENDOCRINE DISRUPTING COMPOUNDS IN THE PINE VOLE

General Methods

Animals and Husbandry

All animal care and treatment conformed to The Guide for the Care and Use of Laboratory Animals (NRC 1996).

Pine voles (*Microtus pinetorum*) used in these experiments were descendants of voles live-trapped in Henderson County, North Carolina. Food (Purina Mouse Diet 5015) and water were provided *ad libitum*. Animals were maintained on a 14L:10D light cycle with lights on at 0600hr. Breeding pairs were housed in 36 X 30 X 18-cm plastic cages with corn-cob bedding and shredded paper toweling for nest material. I weaned pups at 28 days of age. The target sample size for each of the treatment groups was 10. After weaning, I housed the pups in 18 X 29 X 12.5-cm plastic cages with same-sex siblings until they were approximately 60 days old (Solomon and Vandenbergh 1994). At this time, I then paired each with an unfamiliar, unrelated, conspecific of the opposite sex and allowed the pair to cohabitate for 48 hours prior to the start of behavioral tests (Williams *et al.* 1992). All behavioral tests were conducted between 1000 and 1700 hr.

At the conclusion of affiliative and parental tests (described in more detail in the following chapter), I anesthetized animals via sodium pentabarbitol injection (50mg/kg in saline), and decapitated them. I removed the brain, liver, and reproductive organs for weighing or further study. I collected trunk blood in heparinized tubes.

Maternal Treatment

I dissolved diethylstilbestrol (Sigma Chemical Co., St. Louis, MO), methoxychlor (Sigma Chemical Co., St. Louis, MO), flutamide (Sigma Chemical Co., St. Louis, MO), and vinclozolin (analytical grade, >99% purity, Crescent Chemical Co., Hauppauge, NY) in tocopherol-stripped corn oil (Cat. No. 901415, ICN, Aurora, OH). Vinclozolin mixtures

were stirred on a magnetic stir plate for three days to ensure complete dissolution. Females were administered 70 μl of corn oil daily, with or without added chemical. Because no reliable method for determining pregnancy in pine voles exists, administration began approximately three days after being paired with a male or three days after parturition (for postpartum estrus), and continued until the weaning of the pups. Thus all litters were exposed both in utero and through the mother's milk. I used a micropipet (Hamilton, Reno, NV) with attached gavage needle (Popper, 20/38mm, 2.25mm ball diameter) to deliver an accurate volume of corn oil into the mouth of each animal. Voles were restrained with a hand, and the gavage needle gently placed into the mouth. With the needle gently touching the back of the tongue, I ejected the oil from the syringe. Voles readily consume corn oil, and this modified gavage procedure was used to reduce the stress of intra-gastric gavage that can alter fetal development (vom Saal *et al.* 1990).

Control females were administered 70 μl of pure corn oil per day. Doses for the remaining groups of females were as follows per day: DES at 0.2 μg/kg body weight; MXC at 2000 μg/kg body weight; flutamide at 70 mg/kg body weight; or vinclozolin at 10 mg/kg body weight. Doses of DES, MXC, and vinclozolin were chosen based on observed low dose effects in the literature (vom Saal *et al.* 1997; Palanza *et al.* 1999; Gray *et al.* 1999). Flutamide was a higher dose, chosen to ensure the positive control would show effects (Imperatomcginley *et al.* 1992). I dosed an additional group of females with a lower dose of flutamide, 35 mg/kg, after reviewing results from the initial flutamide dose. I performed only select experiments with this lower dose of flutamide treatment group. Pregnant females were weighed twice during the administration period, with doses adjusted for average maternal body weight.

Sample sizes for groups varied from 7 to 12 (Figure 6).

FIGURE 6- SAMPLE SIZES OF TREATMENT GROUPS

	Control Male	Flutamide (70)	Flutamide (35)	Vinclozolin
n	12	11	7	10

	Control Female	Diethylstilbestrol	Methoxychlor
n	11	8	10

Figure 6: Sample sizes for male (top) and female (bottom) treatment groups.

Statistical Analysis

I used one-way analysis of variance to analyze results on Statistical Analysis Software (SAS version 8.02, Cary, NC). If overall analysis of variance was significant ($p \leq 0.05$), I used an LSMEANS post-hoc test to further investigate differences between the groups.

Measurements at Weaning

Introduction

A commonly used biomarker for prenatal exposure to androgens and antiandrogens in mice and rats is the anogenital distance (AGD). AGD refers to the distance separating the posterior aspect of the genital papilla and the anterior aspect of the anus. This region is organized by androgens prenatally, with males having an AGD approximately twice that of females (Clemens *et al.,* 1978; Vandenbergh and Huggett, 1995; Gray *et al.*, 1999). I used AGD to verify that the prenatal endocrine disruption treatments were physiologically effective.

Methods

At weaning, I measured AGD using calipers. Care was taken to ensure that the skin of the anogenital area was not stretched. I measured body weights with a digital balance accurate to 0.1gram. I then divided AGD by body weight to achieve the anogenital distance index (AGDI), a more accurate measure of AGD for comparison (Vandenbergh and Huggett 1995).

Results

Males:

Anogenital distance indices differed among treatments, and both flutamide and vinclozolin treatments differed from controls. AGDI were larger in flutamide exposed males, and smaller in vinclozolin exposed males than controls (Table 2).

TABLE 2 – MALE ANOGENITAL DISTANCE

	Control	Flutamide (70)	Vinclozolin
AGD (s.e.)	6.59 (0.40)	6.76 (0.58)	4.85 (0.42)
AGDI (s.e.)	**33.49** (1.30)	**39.19 *** (0.76)	**27.67*** (1.17)

Table 2: Anogenital distance (mm) and anogenital distance index in males exposed to oil, 70 mg/kg/day flutamide, or 10 mg/kg/day vinclozolin. * Significantly different from control ($p < 0.05$). Flutamide treatment increased while vinclozolin decreased AGDI in males compared to controls.

Females:

There were no differences in AGDI among female treatment groups (Table 3).

TABLE 3 – FEMALE ANOGENITAL DISTANCE

	Control	Diethylstilbestrol	Methoxychlor
AGD (s.e.)	4.47 (0.31)	5.46 (0.27)	4.73 (0.25)
AGDI (s.e.)	**24.34** (2.58)	**26.44** (1.75)	**23.09** (1.29)

Table 3: Anogenital distance (mm) and anogenital distance index in females exposed to oil, 0.2 µg/kg/day DES, or 2000 µg/kg/day MXC. There were no differences among treatment groups.

Discussion

The increase in AGDI of flutamide treated males was a surprising observation. Because AGD is a marker of exposure to androgens, with greater androgen exposure producing greater AGD, we expected to see a reduced AGDI in males exposed to a potent anti-androgen like flutamide. One explanation for this unexpected result is that the relatively high dose of flutamide (70 mg/kg/day), and the extended time of exposure, resulted in the flutamide actually acting as an androgen (Wong *et al* 1995). This has been implicated as a reason that many prostate cancers become resistant to antiandrogen (often flutamide) treatment after several years, possibly a consequence of androgen receptor (AR) mutation or increased interaction between the AR and its coactivator (Hara *et al.* 2003; Miyamoto *et al.* 1998).

Another possible explanation for the increased AGDI in flutamide males is that the external genitalia may have been demasculinized and reduced in size, giving a longer AGD. Wekesa (1995) noted that neonatal testosterone administration to female pine voles resulted in shorter AGDs than controls. This result is the opposite of what other studies have shown, that neonatal androgens masculinize, and thus increase, the AGD of females. Wekesa's reasoning for the contradiction was that the external genitalia of the androgen treated females were larger than controls, more closely resembling the male genitalia. Since AGD is measured from the base of the external genitalia, the enlargement masked the AGD measurement (Wekesa 1995). It may be possible, therefore, that the external genitalia of the flutamide males was reduced in size enough to increase the AGD. These results imply that perhaps pine vole AGD should be measured from the orifices rather than the base of the genitalia.

The vinclozolin exposed males showed the expected reduction in AGD following prenatal antiandrogen exposure. This effect of vinclozolin on AGD has been previously demonstrated in other species (Gray *et al.* 1999; Shimamura *et al.* 2002), and may result from interference with androgen receptors.

The lack of difference in AGDI among female groups was predicted, and consistent with other studies on the effects of prenatal exposure to xenoestrogens in females (Casanova *et al.* 1999; Kobayashi *et al.* 2002).

Reproductive Organ Weights

Introduction

In many mammals, alterations of the fetal androgen milieu can disrupt normal development of the reproductive system (Green *et al.* 1939; Rhees *et al.* 1997; Wolf *et al.* 2000). As a result of increased or decreased androgen exposure, organ sizes may be altered (Rhees *et al.* 1997; McCoy and Shirley 1992). Like AGD discussed above, measurements of reproductive organ weight can thus serve as an indicator of disruptions in levels of androgens during the developmental period (Gray *et al.* 1999, 2001).

Methods

Immediately after vole sacrifice, I removed the uterus or testes and seminal vesicles from the animal, trimmed the organs of fat and removed excess external fluid, and weighed them to the nearest 0.0001g. The prostate gland was not included in this research, since it is extremely difficult to locate and remove in a pine vole. To standardize organ weights to body weights, I followed a procedure outlined by Lepri and Vandenbergh (1986). Organ weights in mg were divided by body weight in g, and this quotient was then multiplied by 30, a typical body weight for a pine vole. This results in standardized values of mg organ/30 g body weight.

Results

Males:

Testes did not differ in weight among treatment groups (Figure 7).

The flutamide exposed males showed a trend toward heavier seminal vesicles than controls (p=0.07). Vinclozolin exposed males had lighter seminal vesicle weight than controls (p=0.05) (Figure 7).

FIGURE 7 – TESTES AND SEMINAL VESICLE WEIGHTS

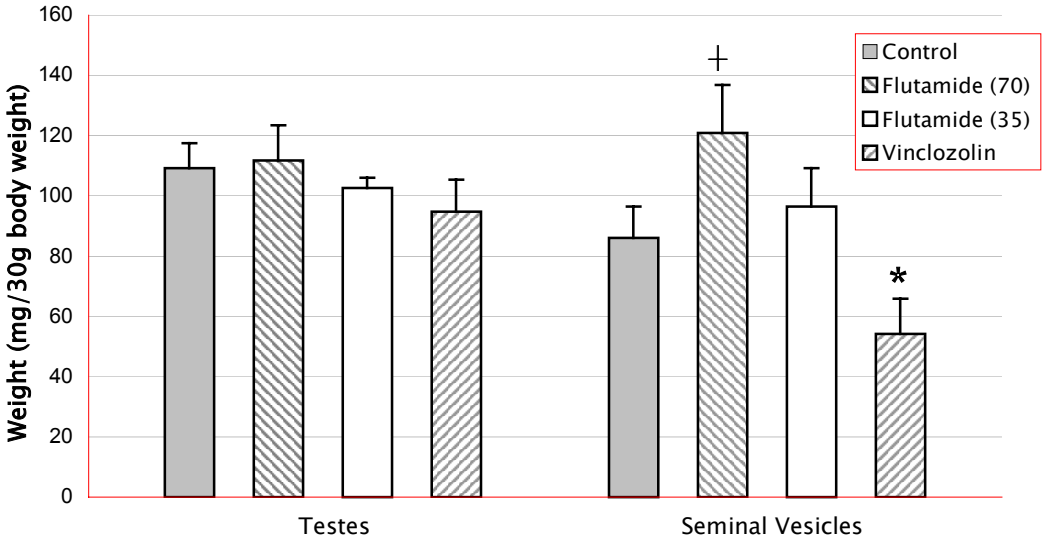

Figure 7: Testes and seminal vesicle weights of males exposed to oil, flutamide at 70mg/kg/day, flutamide at 35mg/kg/day, or vinclozolin at 10 mg/kg/day. Testes weight does not differ among treatment groups. Males exposed to 70mg flutamide had heavier, while vinclozolin exposed males had lighter, seminal vesicles than controls. * Significantly different from control at p=0.05. + 0.10> p > 0.05

Females:

Uterine weight did not differ between the control and DES exposed females. Methoxychlor exposed females had smaller uterine weights (Figure 8).

FIGURE 8 – UTERINE WEIGHTS

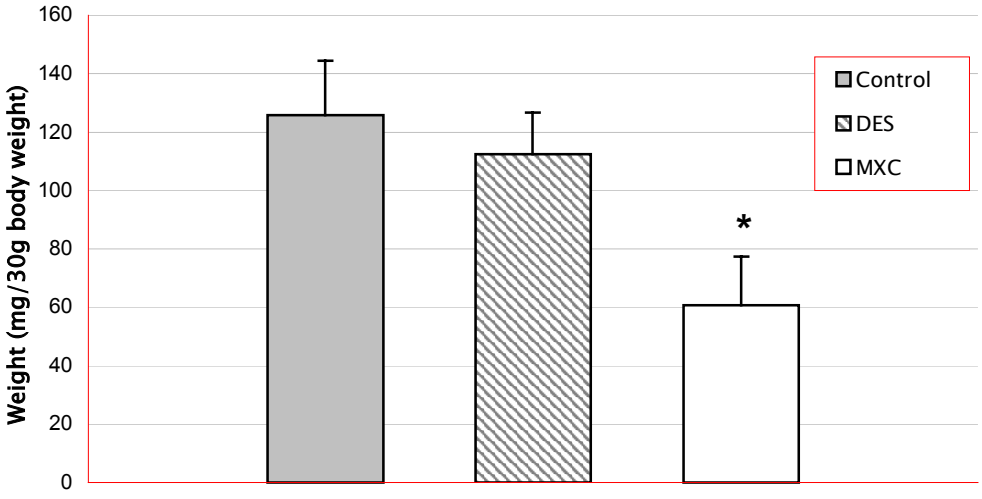

Figure 8: Uterine weight is unaffected in females exposed to DES, but lighter in females exposed to MXC than in control females. * Significantly different from control ($p<0.05$).

Discussion

Males:

Maintenance of the weight of the seminal vesicles, as well as other accessory sex organs such as the prostate, is regulated by the androgen receptor (AR). Thus those endocrine disruptors that act through the AR can affect the resulting size of these organs.

That the flutamide exposed males had greater seminal vesicle weights is consistent with stronger androgenic effects. There is a dose response of seminal vesicle weight to flutamide dose, with the higher dose producing heavier organs. This unexpected result follows the pattern of increased AGD in these same males, and gives further support to the idea that flutamide acts as an androgen agonist rather than antagonist when administered in the higher doses used here.

Vinclozolin treated males exhibited the predicted reduction in seminal vesicle weights following prenatal exposure to an antiandrogen. Seminal vesicle weight is

maintained by androgen receptor-regulated processes. Thus, interference with normal androgen receptor functioning via an antiandrogen should result in a decrease in seminal vesicle weight.

Females:

That DES produced no differences in uterine weight while MXC produced a reduction, was surprising. DES is a more potent estrogenic compound than is MXC (Folmar *et al.* 2002). However, difference in effects may relate to doses. Estrogenic chemicals in fetal life can result in enhancing or inhibiting effects, depending on the dose to which the animal is exposed (Alworth *et al.* 2002). Based on the uterine weight data, I would hypothesize that the dose of MXC was of greater strength than the dose of DES. The relative potency as estrogens of DES and MXC are debatable and vary greatly depending on type of test and species used (Folmar *et al.* 2002; Anderson *et al.* 1999; Ashby 1999; Shelby *et al.* 1996). Folmar *et al.* (2002) demonstrated that MXC was more potent *in vivo* than *in vitro*, and that the estimated potency of MXC was 400X less than that of estradiol. DES in one study was slightly more potent than estradiol (Folmar *et al.* 2002) and in another study DES was slightly less potent than estradiol (Anderson *et al.* 1999). Therefore, if DES is approximately of equal strength to estradiol, and MXC is 400X less potent than estradiol, the MXC dose was stronger than the DES dose used here, since a 10,000X greater dose of MXC was used.

It is possible that the uterine weight data can be explained by the inverted-U dose response curve that occurs at low doses of certain endocrine disrupting chemicals. Ashby (1999) observed the same unchanged uterine weight in mice (*Mus musculus*) exposed prenatally to the same dose of DES used here (0.2 μg/kg/day). Alworth *et al.* (2002) observed in mice that prenatal exposure to 0.1μg/kg/day resulted in heavier uteri, while exposure to 100 μg/kg/day resulted in lighter uterine weights. Thus the dose used in this research was intermediate to these opposite effects. That, plus the fact

that I exposed a different species, could account for the lack of observed effect on uterus weight in DES exposed female pine voles. The DES dose used could be on the "downward slope" of the inverted-U curve for this species.

The MXC dose may have been in an area of decreasing responsiveness as well, since in this case the uterus was not heavier, as expected at a very low dose, or even unchanged, but lighter. Alworth *et al.* (2002) found that MXC at 10 μg/kg/day resulted in a heavier uterus, while a dose of 10,000 μg/kg/day produced lighter uterine weights. The dose used here was 2000 μg/kg/day, and resulted in lighter uteri, like the higher dose in the Alworth *et al.* study. A lighter uterus is thought to be the result of down-regulation of estrogen receptors, a response to maximum receptor occupancy following estrogen administration. This down-regulation is permanent if it occurs during fetal life (NRC 1999; Medlock *et al.* 1991). Thus both DES and MXC results are consistent with earlier results of estrogenic compounds having stimulatory effects at low doses, and inhibitory effects at higher (but still considered low) doses.

Testosterone Biotransformation

Introduction

A sensitive indicator of exposure to androgen disruption in laboratory mice is the testosterone biotransformation profile. The activities of hepatic testosterone biotransformation enzymes, in particular 6α- and 15α-hydroxylase, are sexually dimorphic in mice (Wilson *et al.* 1999). The ratio of 6α to 15α-hydroxylase is significantly less in males than it is in females. This ratio increases (becomes more feminized) in males following either exposure to vinclozolin or a reduction in serum testosterone levels. Thus the 6α/15α-hydroxylase ratio is a reliable measure of androgen modulation in mice (Wilson *et al.* 1999).

No previous work on hepatic testosterone biotransformation profiles in pine voles exists. Thus, I examined all of the testosterone metabolites in both sexes to determine if there was a sex difference in hydroxylase activities in pine voles. Such a difference could then indicate prenatal androgen modulation, just as the 6α/15α-hydroxylase ratio does in mice.

Methods

I removed livers from sacrificed pine voles and immediately placed them in cold 1.15% KCl (fresh saline used for each liver). Livers were cut into several pieces (~1x1cm) while soaking, and then transferred to a small vial and frozen in liquid nitrogen. Livers were stored at -80°C.

I assayed testosterone hydroxylase activities in the livers as detailed by Wilson *et al.* (1999). Individual livers (male n=3, female n=4) were thawed and homogenized on ice in chilled buffer (0.1M HEPES, pH 7.4, 1mM EDTA, and 10% glycerol). Microsomes were prepared by differential centrifugation. Cytosolic supernatant was reserved, and microsomal pellets were resuspended in buffer (0.1M potassium phosphate, pH 7.4, 0.1 mM EDTA, and 20% glycerol). Protein concentrations were determined using commercially prepared reagents and bovine serum albumin as a standard.

Testosterone hydroxylase activities in livers were assayed using 400 μg microsomal protein and 40 nmol [^{14}C]testosterone as the substrate in 0.1M potassium phosphate buffer (pH 7.4). Reactions were conducted at 37°C and initiated with 1mM NADPH. Total assay volume was 400 μl. I terminated the reaction by adding 1ml ethyl acetate. Tubes were vortexed for 1 minute and then centrifuged for 10 minutes to separate the ethyl acetate and aqueous phases. Ethyl acetate fractions were transferred to a fresh tube. Extraction of the aqueous phase with ethyl acetate was repeated two more times to ensure complete recovery of all hydroxyl metabolites. Combined ethyl acetate fractions from each sample were evaporated under a stream of nitrogen until dry. The residue was resuspended in ethyl acetate and metabolites were separated by

thin-layer chromatography (TLC). Unmetabolized [^{14}C]testosterone and individual [^{14}C]metabolites were identified and then quantified by electro-autoradiography. Specific activity for the production of each metabolite was calculated.

Results

Only one metabolite, androstenedione, differed in rate of production between males and females (Table 4).

TABLE 4 – LIVER HYDROXYLASE METABOLITES

Metabolite	Male (s.e.)	Female (s.e.)
2	**0.667** (0.045)	**0.6925** (0.173)
3	**0.373** (0.267)	**0.3425** (0.06)
4	**0.75** (0.06)	**0.79** (0.164)
5	**0.787** (0.0820	**0.81** (0.104)
6	**1.347** (0.183)	**0.9925** (0.163)
7	**0.653** (0.02)	**0.6** (0.116)
8	**0.487** (0.033)	**0.425** (0.045)
9	**0.603** (0.047)	**1.09** (0.311)
Testosterone	**26.703** (0.462)	**26.93** (1.49)
Dihydrotestosterone	**0.39** (0.032)	**0.7725** (0.235)
Androstenedione	**2.33** (0.043)	**1.51** (0.219)*

Table 4: Average activities of testosterone, dihydrotestosterone, androstenedione, and eight testosterone hydroxylase metabolites in male (n=3) and female (n=4) pine voles. Metabolites 2-9 were not identified since no sex differences were observed. Androstenedione was the only metabolite that differed in activity between the sexes.

*Significantly different from male ($p<0.05$).

Discussion

The one observed metabolite that differed between the sexes, androstenedione, is commonly considered to be an inactive pool of testosterone precursor. In the presence of the enzyme 17β-hydroxysteroid dehydrogenase, it can be converted to testosterone and, likewise, testosterone can be converted back to androstenedione,

depending upon the relative abundance of the two steroids at the site of the enzyme. That males appear to have more androstenedione than females could mean that males preferentially store testosterone in this inactive form. This relatively small difference between the sexes (compared to sex differences in mouse hydroxylase levels) may also indicate that it is of no physiological relevance, however.

Based on these data, using the hydroxylase ratio as an indicator of prenatal androgen alteration is not reliable in the pine vole. It is possible, however, that further studies with greater sample size could lead to discovery of a reproducible sex difference in androstenedione, or even some other metabolite in pine voles.

The results of this chapter indicate that the pine voles were exposed to endocrine disrupting chemicals. Flutamide at the higher dose of 70mg/kg/day masculinized AGD and seminal vesicle weight compared to oil control. Vinclozolin demasculinized both of these measures. Methoxychlor exposure resulted in lighter uterine weights in females compared to controls. Only DES did not have a significant effect on any of these physiological endpoints, but DES did have a behavioral effect on female pine voles, as discussed in the following chapter.

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Chapter Three BEHAVIORAL EFFECTS OF PRENATAL AND NEONATAL EXPOSURE TO ENDOCRINE DISRUPTING COMPOUNDS IN THE PINE VOLE

Affiliative Behavior

Introduction

A strong and lasting association between a male and a female throughout breeding and nonbreeding seasons is a hallmark of monogamy (Carter *et al.* 1995). Monogamous pairs are said to be "bonded" to each other, meaning that they nest together, raise young together, and spend much time in direct contact. Though this bond does not necessarily translate to sexual exclusivity, as demonstrated by DNA testing (Carter et al. 1990), established bonded pairs often do show a sexual preference for each other as well (Getz *et al.* 1981). In addition, bonded pairs demonstrate a strong and reliable social preference for the mate, correlated with increased aggression toward any strange intruders (Carter and Getz 1993). Thus, pair bonding in the laboratory can be assessed through measurements of partner preference, selective social contact and aggression.

The effects of EDCs on behavior are relatively unexplored. Hotchkiss *et al.* (2002) reported alterations in rat play behavior following prenatal exposure to antiandrogens. Palanza *et al.* (1999, 2001) observed increased aggressive behavior in mice prenatally exposed to DES, and alterations in righting and cliff avoidance reflexes in mice prenatally exposed to MXC. This is the first study to investigate the effects of EDCs on pair bonding behavior. Considering the importance of steroid hormones in reproductive behaviors however (Beach 1976; Brown 1985; Meisel and Sachs 1994; Pfaff 1997; Pfaff *et al.* 2000), the potential most certainly exists for EDCs to alter pair bonding behavior.

Preference tests give animals a choice of a neutral chamber and two chambers each with a stimulus animal. Stimulus animals are confined to their chambers, and a test animal shows preferences via the amount of time spent in each chamber and via time spent fighting with or resting next to the stimulus animal. In general, about 20% of

their time is spent alone in the neutral chamber (Carter *et al.* 1995). Nonmonogamous voles remain alone approximately 90% of the time (Carter *et al.* 1995).

The formation of a measurable preference for the mate is achieved through cohabitation and mating. Prairie vole pairs that cohabit for 24 hours, with or without mating, develop a preference measurable in a test. If the pair mates, only 6 hours of cohabitation is needed to form a measurable preference (Williams *et al.* 1992). Longer cohabitation or mating periods do not affect the result of preference tests.

A preference test must last longer than needed for initial investigation of the test apparatus. Social preferences for mates are clearly evident after 1hr of testing and remain stable over a 24-hr testing period (Williams *et al.* 1992). A preference test of 3 hr duration is common procedure (Williams *et al.* 1994).

Although the primary measure of a preference test is time spent in each chamber, this may not be a reliable index of preference, since it includes both positive and negative social interactions. Most negative interactions, however, take place during the first 30 minutes of a preference test, when most exploration occurs (Williams *et al.* 1992). After this time, a monogamous vole will generally spend much time resting with its mate. Thus a 3 hr test allows sufficient time for a test animal to show social preference. Physical contact is the most sensitive measure of positive social interactions. Aggression, regarded as mate guarding in monogamous animals, is also recorded.

Methods

Pregnant and lactating voles were dosed as described in Chapter 2. Male offspring of flutamide and vinclozolin treated voles, female offspring of diethylstilbestrol and methoxychlor treated voles, and both males and females of oil control treated voles, were weaned at 28 days of age and reserved for testing.

When treated and control voles were approximately 60 days of age, I paired each with an untreated member of the opposite sex. Following a 48-hour cohabitation

period, social behaviors and preferences were assessed using a large, rectangular test apparatus (clear Rubbermaid box, 22 X 61 X 40.5 cm). The box was divided equally into three chambers (each 22 X 18 X 40.5 cm) by a double Plexiglas wall. The center chamber was connected to each end chamber via a 4 cm length of PVC pipe (7 cm diameter), located approximately 2.5 cm from the floor of the box. LED diodes (MED Associates, St. Albans, VT) within the walls of each PVC pipe were connected to a passive connection panel (MED Associates, St. Albans, VT), which allowed interface with a computer in an adjoining room. The two end chambers housed stimulus animals: the test vole's "mate" and a "stranger" (a conspecific similar in sex, age, social history and weight to the mate, but unfamiliar to the experimental animal). Each pair was tested once, and each stranger was used only once. I restricted the stimulus animals to their individual chambers by a plastic cable tie worn as a collar, with a small length left extending from it. The collar prevented the animals from passing through the PVC pipe. Placement of stimulus animals at one end or the other was randomized.

At the beginning of a test, the experimental vole was placed in the center (neutral) chamber and was free to move throughout the apparatus. Each vole was tested for 3 hours. A custom-made, data acquisition system recorded the total number of entries into and out of the two end chambers, as well as the when each passage occurred. Preference tests were recorded by a video camera (Hitachi model KP-D50) mounted on the ceiling and a time-lapse videocassette recorder (JVC model SR-9070U). Videotapes were scored during rapid playback with a 6:1 record/playback ratio. Frequencies or durations of the following behaviors were recorded by a neutral observer: time in each chamber, time in physical (side-by-side) contact, aggression, and sexual behavior. Aggression was defined as chasing, biting, or fighting. Sexual behavior was noted when the female assumed an immobile posture (lordosis) in response to mounting by the male.

Statistical Analysis

I used a MANOVA test for no differences in time spent in each chamber among treatment groups using Statistical Analysis Software (SAS version 8.02, Cary, NC). If overall analysis of variance was significant ($p \leq 0.05$), I used univariate and LSMEANS post-hoc tests to further investigate differences between the groups.

Results

Male data:

Males treated with the higher dose (70mg/kg/day) of flutamide spent less time in their mates' chambers, and significantly more time alone in the neutral chamber than did control males (Figure 9; Table 5). Treated males also spent less time in side-by-side contact with their mates and some time in side-by-side contact with the strange female. No control male ever was side-by-side with the strange female. Flutamide treated males engaged in fewer aggressive bouts with the strange female (Figure 10; Table 6). Vinclozolin treated males did not differ significantly from controls in any of these measures (Figure 9; Table 5; Table 6).

Control males mated their partners sometimes, as did flutamide males. Vinclozolin treated males did not mate at all (Table 7). None of these groups of males was observed mating with the strange female.

The additional group of males exposed to a lower dose (LD) of flutamide (35mg/kg/day) yielded results intermediate to those of control and higher dose flutamide males. LD flutamide males spent more time with the strange females, and less time in their mate's chamber than did control males (Figure 9; Table 5). Like control males, approximately half of the time that they were in their mate's chamber they were in side-by-side contact with her. Like higher dose flutamide males, some LD males spent time side-by-side with the stranger, and were less aggressive toward the stranger

as well (Table 6). Unlike any of the other male groups, LD males were observed mating with both their mate and the strange female (Table 7).

FIGURE 9 – MALE PREFERENCE TEST – PERCENTAGES

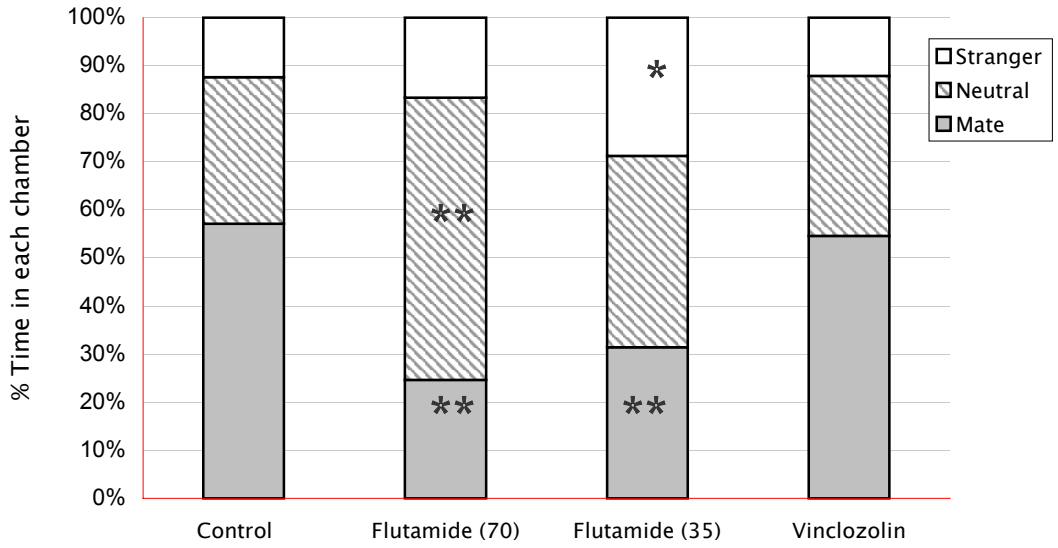

Figure 9: Percentage of 3 hour preference test that male treatment groups spent in each of the three chambers. Both groups of flutamide treated males spent less time in the mate's chamber than did control males. Males exposed to 70mg/kg/day flutamide spent more time alone in the neutral chamber than did controls, while those exposed to 35mg/kg/day flutamide spent more time in the stranger's chamber than controls. ** Significantly different from control ($p<0.001$). * Significantly different from control ($p<0.05$).

TABLE 5 – MALE PREFERENCE TEST– TIMES

Chamber	Control	Flutamide (70)	Flutamide (35)	Vinclozolin
Mate	**6165** (707)	**2665**** (425)	**3399**** (807)	**5892** (577)
Neutral	**3301** (640)	**6333**** (530)	**4293** (251)	**3600** (462)
Stranger	**1334** (304)	**1802** (422)	**3108*** (678)	**1308** (334)

Table 5: Average time (seconds) (± s.e.) that males exposed to oil (n=12), 70 mg/kg/day flutamide (n=11), 35 mg/kg/day flutamide (n=7), or 10 mg/kg/day vinclozolin (n=10) spent in each of the three preference test chambers.

**Significantly different from control ($p<0.001$). *Significantly different from control ($p<0.05$).

FIGURE 10 – MALE AFFILIATIVE AND AGGRESSIVE BEHAVIOR

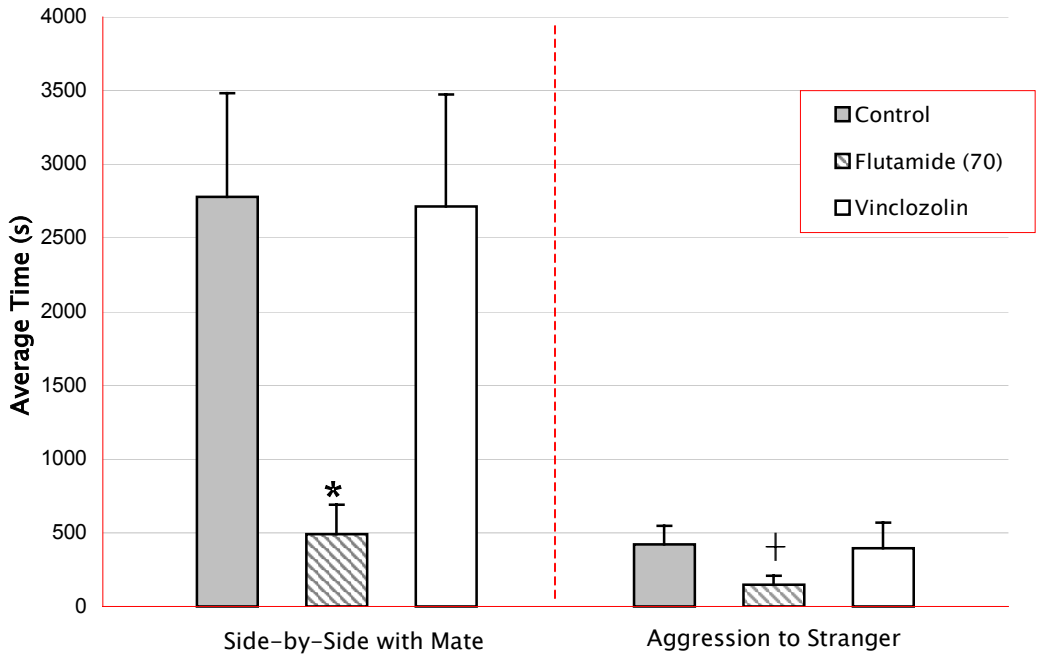

Figure 10: Average time (seconds) (± s.e.) that males exposed to oil, 70mg/kg/day flutamide, or 10mg/kg/day vinclozolin spent in side-by-side contact with the mate or actively aggressive toward the stranger. * Significantly different from control ($p<0.05$).

+ $0.10>p>0.05$.

TABLE 6 – MALE AFFILIATIVE AND AGGRESSIVE BEHAVIOR

	Control	Flutamide (70)	Flutamide (35)	Vinclozolin
Side-by-Side with Mate	2782	491*	2226	2716
Side-by-Side with Stranger	0	455	585	24
Aggression to Stranger	419	147+	109*	396

Table 6: Average time (seconds) that each male treatment group spent in side-by-side contact with a female, or acting aggressively toward the strange female.

*Significantly different from control ($p<0.05$). + $0.10>p>0.05$.

TABLE 7 – MALE MATING

	Control	Flutamide (70)	Flutamide (35)	Vinclozolin
With Mate	9	3	3	0
With Stranger	0	0	5	0

Table 7: Total number of observed mating encounters in each of the male treatment groups. Only males exposed to 35 mg/kg/day of flutamide were observed mating with the strange female.

Female data:

There were no significant overall differences in female preference test results. However, the overall ANOVA suggests a strong trend toward significance ($p=0.06$). Post hoc comparisons revealed trends toward significance in both mate ($p=0.06$) and neutral ($p=0.09$) chamber results. DES treated females showed a trend toward spending less time in their mates' chambers, and more time with the strange males than controls (Figure 11; Table 8). DES females spent the same amount of time in side-by-side contact with their mates as did controls, however. Aggression toward the stranger was significantly higher in the DES treated females than in controls (Figure 12; Table 9).

Methoxychlor (MXC) treated females showed a trend toward spending less time in their mates' chambers, and more time alone in the center neutral chamber (Figure 11; Table 8). MXC females spent the same amount of time in side-by-side contact with their mates as did controls. Aggression toward a strange male was significantly lower in MXC treated females than in controls (Figure 12; Table 9).

Females in all three treatment groups mated. One control female mated with the strange male once; otherwise all control females mated only with their mates. DES females mated only with their mates. MXC females mated with both their mates and the strangers (Table 10).

FIGURE 11 – FEMALE PREFERENCE TEST– PERCENTAGES

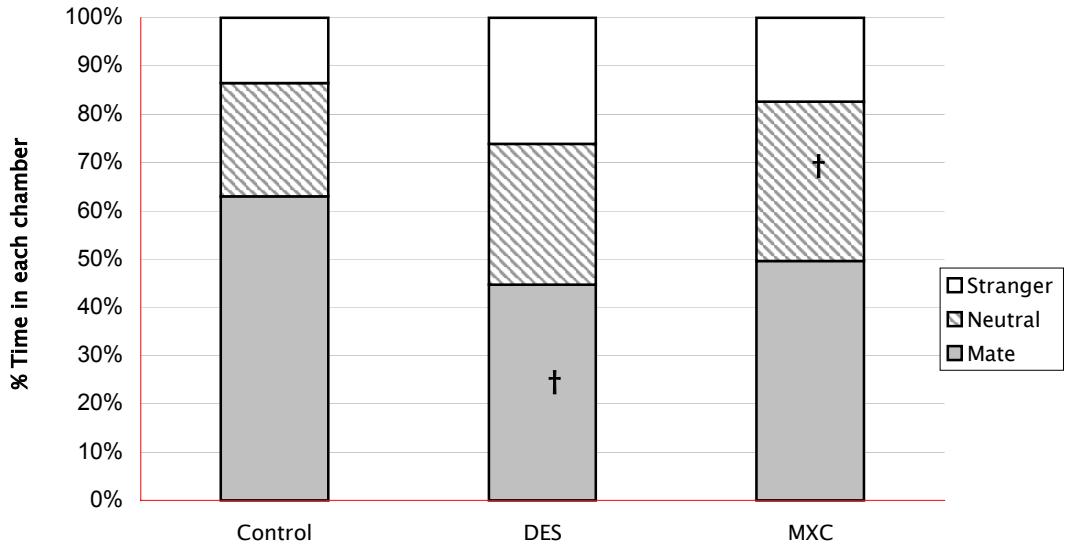

Figure 11: Percentage of 3 hour preference test that female treatment groups spent in each of the three chambers. Females exposed to DES spent less time in the mate's chamber than did controls. Females exposed to MXC spent more time alone in the neutral chamber than controls.

† 0.10>p>0.05.

TABLE 8 – FEMALE PREFERENCE TEST– TIMES

Chamber	Control	DES	MXC
Mate	**6807** (583)	**4831** (819)†	**5354** (343)
Neutral	**2537** (357)	**3153** (342)	**3566**(296)†
Stranger	**1455** (329)	**2816** (734)	**1879** (514)

Table 8: Average time (seconds) (± s.e.) that females exposed to oil (n=11), 0.2µg/kg/day DES (n=8), or 2000 µg/kg/day MXC (n=10) spent in each of the three preference test chambers. † 0.10>p>0.05.

FIGURE 12 – FEMALE AFFILIATIVE AND AGGRESSIVE BEHAVIOR

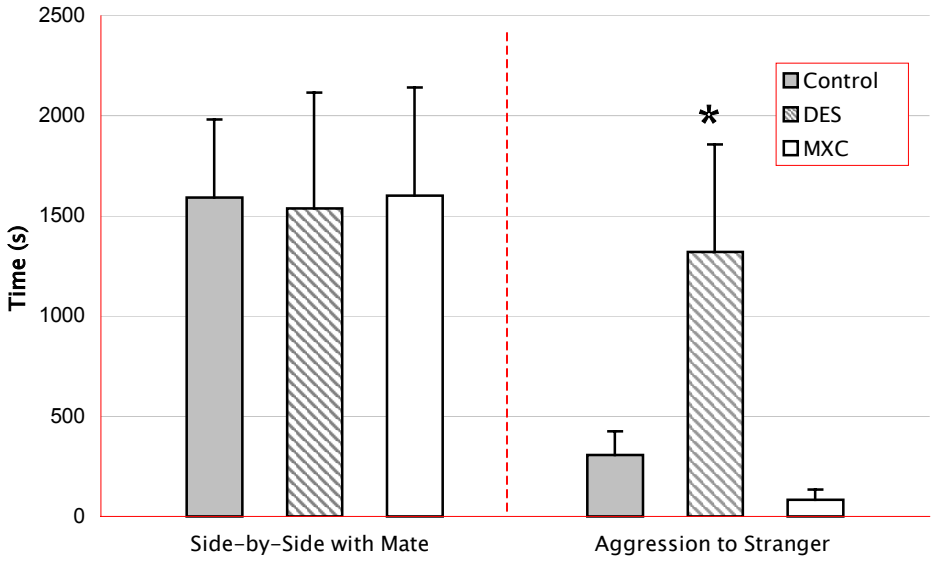

Figure 12: Average time (seconds) (± s.e.) that females exposed to oil, DES, or MXC spent in side-by-side contact with the mate or actively aggressive toward the stranger.

* Significantly different from control ($p<0.05$).

TABLE 9 – FEMALE AFFILIATIVE AND AGGRESSIVE BEHAVIOR

	Control	DES	MXC
Side-by-Side with Mate	1593	1538	1601
Side-by-Side with Stranger	27	0	422
Aggression to Stranger	305	1320*	84

Table 9: Average time (seconds) that each female treatment group spent in side-by-side contact with a male, or acting aggressively toward the stranger. *Significantly different from control ($p<0.05$).

TABLE 10 – FEMALE MATING

	Control	DES	MXC
With Mate	11	6	3
With Stranger	1	0	7

Table 10: Total number of observed mating encounters in each of the female treatment groups. MXC exposed females mated with the strange male more than their mate.

Discussion

Males:

In this study, both control males and females exhibited preference behaviors similar to those previously documented in other laboratories (Williams *et al.,* 1992; Carter *et al.* 1995, Insel *et al.* 1994). These corn oil treated individuals spent more time in their mates' chambers than either the strangers' or the neutral chambers ($p<0.0001$). About half of the time that they were in their mates' chambers, they were in close physical (side-by-side) contact with their mate. As stated previously, this side-by-side contact is a more reliable index of positive social interactions than the time measurement alone (Carter *et al.* 1990).

No aggression was shown toward the mate by any control male at any time. However, about a third of the time that a male was in a stranger's chamber, he exhibited aggressive behaviors (chasing, biting, rolling, vocalizations). Although I did not record it in detail, a great deal of the additional time spent in a stranger's chamber was spent in a "face-off", with the two voles staring intently at each other from opposite sides of the area. Often a female was near or completely in front of the entrance tunnel, preventing a male from exiting the chamber.

Approximately half of the control males engaged in sexual behaviors with their mates one or more times during the preference test. No sexual behaviors were exhibited with a strange female at any time by any control male.

Thus, each control male made a clear choice of their mate over the strange female—preferring to mate with her, huddle together with her, and spend time near her.

Many males even attempted to chew through and remove the plastic collar around their mates' necks! Likewise, aggression toward the stranger occurred and in some cases resulted in a superficial wound to one or both animals.

The vinclozolin treated males were very similar to the control males in almost all measures of the preference test. They most definitely preferred their mates' chamber over the stranger or the neutral chambers, spent a large amount of time in side-by-side contact with their mates, and exhibited a great deal of aggression toward strange females. The only difference between control and vinclozolin males was that no vinclozolin male was observed to mate during the test. It appears that prenatal and neonatal exposure to vinclozolin at the dose used in this study does not alter the pair-bonding behavior of male voles.

The higher dose (HD) flutamide treated males were very unlike the control and vinclozolin males. Though they did spend more time in their mates' chambers than in strangers' chambers, this difference was not highly significant ($p=0.08$). In addition, flutamide males spent much less time in side-by-side contact with their mates. Only about one-fourth of the time they were in their mates' chambers were they side-by-side. Thus, both time spent in their mates' chambers and percentage of time spent in physical contact with their mates were significantly reduced in these individuals.

The HD flutamide exposed males spent time alone in the neutral chamber, rather than with either of the two females. The preference to spend time alone in the neutral chamber very much resembles that of a nonmonogamous montane vole (Carter *et al.* 1995).

The preference test results of males exposed to a lower dose of flutamide (LD) were somewhat intermediate to those of control males and the HD flutamide males. Like the HD flutamide, these males spent the majority of their time alone in the neutral chamber, and thus appear to be much more nonsocial than a typical monogamous male pine vole. However, the LD males also spent more time with the strange females than did either controls or HD males. Thus LD males, while preferring to be alone more than

controls, also choose to be near a female, whether mates or strangers, more than the HD males.

Aggression toward a strange female was greatly reduced in both groups of flutamide males. There were half as many aggressive encounters between HD flutamide males and strangers as there were between the other male groups and strangers. The LD flutamide males showed even less aggression to strangers than HD males, while spending more time with strangers than HD males. Interestingly, three of the eleven HD flutamide males and three of the seven LD flutamide males spent some time in side-by-side contact with a strange female, something not seen in either control or vinclozolin males. Since aggression toward strangers increases upon formation of a pair bond, it would appear that flutamide males have not formed a strong pair bond with their mates.

The high dose of flutamide used here may possibly have resulted in flutamide having an androgenic rather than antiandrogenic effect. It is now well established that antiandrogens can act as agonists, dependent upon things such as ligand binding affinity, concentration, and presence of competing natural ligands (Miyamoto et al. 1998; Wong et al. 1995). For example, many men undergoing treatment for prostate cancer experience "flutamide withdrawal syndrome", a result of the androgenic properties of flutamide following prolonged exposure (Miyamoto et al. 1998; Wong et al. 1995). That higher levels of androgen in these flutamide exposed voles would result in behavior more typical of a promiscuous vole is not surprising, since polygynous voles have higher circulating testosterone concentrations than do monogamous voles (Klein and Nelson 1997).

Pair-bond formation was obviously disrupted in both groups of flutamide males. These males spend more time alone than with either female, atypical of a highly social monogamous vole. In addition, the reduction in aggression shown toward the strange female indicates that a strong pair-bond, which is always characterized by an *increase* in aggression toward members of either sex (Carter *et al.* 1995), has not been formed. Although both doses of flutamide resulted in males spending more time alone and less

time with their mates, the LD males appear to recognize their mates, and therefore spend more time with her than with a stranger. In contrast, it is questionable whether or not the HD males are even able to recognize their mates. If a change in AVP receptor distribution were the cause of this disruption in affiliative behavior, I would hypothesize that the higher dose more drastically alters the distribution, so that it perhaps more closely resembles the distribution of a promiscuous vole. Receptor distribution is further discussed in the next chapter.

Thus, it would appear that prenatal and neonatal exposure to flutamide significantly alters pair-bonding behaviors in pine voles. These males spent less time with their mates, more time alone, and were less aggressive toward a stranger.

Females:

As with the males, control females exhibited a previously documented preference for their mates, preferring to spend time in their mates' chambers rather than either of the other two chambers ($p<0.0001$), and spending about a quarter of that time in close side-by-side contact.

Female controls were aggressive toward the strange males. Fighting, biting, or chasing characterized roughly 20% of the time spent in the strangers' chambers. In addition, a substantial amount of time in the strangers' chambers was spent in "face-off" behavior. No control female was ever aggressive toward her mate, nor did any control female ever spend time in side-by-side contact with a stranger.

On one occasion, a female control was observed mating with the stranger. This observation is consistent with the idea that sexual exclusivity is not a characteristic of pine vole monogamy. On eleven separate occasions, however, a control female engaged in sexual behavior with her mate. The fact that the great majority of observed sexual encounters were with mates likewise is consistent with the claim that sexual preference very often follows social preference (Getz *et al.* 1981), and supports DNA fingerprinting field studies of monogamy in pine voles (Marfori *et al.* 1997).

DES females spent more time with the strange males, but they also exhibited significantly more aggressive behavior toward the stranger than control females. This heightened aggression resembles that shown by mice administered the same dose of DES (Palanza *et al.* 1999). Female mice prenatally exposed to DES were more reactive, more aggressive, and showed greater territoriality. The DES exposed voles here are behaviorally similar. Almost half of the time a female was in a stranger's chamber she was actively engaged in aggressive behavior toward him. This was the greatest proportional amount of time spent in aggressive behavior of any of the seven treatment groups. It appears then, that like DES exposed mice, these females showed a greater reactivity to aggression-inducing stimuli (a stranger in their territory) and a heightened tendency to attack. Since steroid hormones are involved in determining the number and sensitivity of brain receptors for steroids and neurotransmitters (Goetz *et al.* 1983; Pfaff *et al.* 1994), endocrine disruptors like DES might interfere with normal development of serotonin, dopamine, and GABA receptor systems, and thus alter responses to new or stressful situations.

DES may or may not have an additional direct effect on affiliative behavior. It would appear that the increased aggression is separate from any alteration in pair-bonding behavior, though it is of course possible that the increase in aggression may be due in part to an *increase* in affiliative behavior. If DES were to increase oxytocin receptors in areas of the brain associated with pair-bonding in female monogamous voles, then it is possible that an increase in aggressive behavior toward a stranger would be one expression of such an increase. Time spent side-by-side with the mate could support this idea, since although DES females showed a trend toward less total time in the mates' chambers than did controls, they did not decrease the amount of time spent in direct contact with their mate. Thus, DES females were side-by-side with their mate a greater proportion of time than were controls.

The MXC exposed females, on the other hand, showed a trend toward increased time alone and a corresponding decrease in pair-bonding behavior toward their mates. In addition, these females showed a trend toward being *non*-aggressive toward strangers. Also of interest is the fact that the MXC females on average had more mating encounters with a strange male than they did with their own mates, and like the flutamide males above, spent some time in side-by-side contact with a strange male. Though not as extreme as either of the flutamide exposed treatment groups, the MXC females are similar in their altered behaviors. They are much more antisocial, resembling a promiscuous vole more than a monogamous one, and show little to no aggression toward a stranger. The combination of these two characteristics points to a reduced preference for the mate, and a clear disruption in affiliative behavior.

The effects exhibited by MXC could be a result of its estrogenic or antiandrogenic properties. Because the DES and MXC females responded differently in a preference test, the two compounds may act in different ways (alternatively, the difference could be due to dose). Very little research exists on the effects of antiandrogens on females, and in particular female behavior. Certainly females do produce and require testosterone for normal functioning (Sands and Studd 1995; DeJonge *et al.* 1986; Vandepoll *et al.* 1986). Androgen levels of fetal rats are similar in males and females through much of gestation (Weisz and Ward, 1980; Baum *et al.*, 1991), and androgen receptor levels in females are comparable to those in corresponding males (George and Wilson, 1994). In addition, females are sensitive to exogenous androgens (Slob *et al.* 1983; Wolf *et al.* 2000). Thus, exposure to an antiandrogen during development could potentially alter behaviors influenced by testosterone, such as aggression, partner preference, receptivity, and proceptivity (DeJonge 1986; vom Saal *et al.* 1999).

Parental Behavior

Introduction

While the majority of female mammals are maternal at least to some extent, due to their possession of mammary glands essential to their offspring's survival, most male mammals are not paternal. Biparental care, where the male shares in parental responsibilities, is often associated with monogamous species (Insel 1997). A male with only one mate has an increased reproductive advantage if he ensures his offspring survive. Pine vole fathers spend much time in direct contact with pups, retrieving, grooming and crouching over their pups. Additionally, males invest in their pups indirectly through acquiring food, constructing and maintaining a nest, and defending nest and territory (Oliveras and Novak 1986). Male parents also continue to care for juvenile offspring and contribute to their behavioral development, even after the birth of a newer litter of pups (Wang and Novak 1992, 1994).

Female voles show all the patterns of parental behavior that are observed in other rodent species, such as nursing, huddling over, retrieving, grooming, and direct contact with the litter (McGuire and Novak 1984). In addition, female voles display other indirect parental behaviors, such as hoarding food, building nests, and constructing runways. None of these maternal behaviors differ among species that show differences in their social organization (McGuire and Novak 1984).

While investigating the effects of endocrine disruption on monogamy in the pine vole, the possibility of alterations in either paternal or maternal responsiveness and behavior was therefore important to assess.

Methods

Animals were returned to their home cages (with their mate) following the preference tests. Starting twenty-one days later, cages were checked daily for litters. Between postpartum days 3-5, I assessed parental behaviors of treated and control voles.

I quantified paternal behaviors as did Bamshad *et al.* (1994) and Wang *et al.* (1993, 1994). I removed male voles from their home cage to a similarly sized clean plastic cage with cob bedding and allowed them to acclimate for approximately 5 minutes. I cleaned a 3- to 5-day-old pup from an unrelated established breeding pair in the colony with a wet cotton ball to remove odors, and placed it into the cage at the opposite corner from the location of the male. The behavior of the male was recorded for 10 minutes. I defined paternal behavior as retrieving the pup, licking, brooding (huddling over), and remaining in direct body contact with the pup. Self-grooming, locomotion, and inactivity (remaining stationary, away from the pup) were recorded as nonsocial activities. Each pup was only used once, and returned to its home cage after being cleaned again with a wet cotton ball to remove odors of the experimental male. The sum of time spent with the pups and the sum of time spent in nonpup-directed (nonsocial) activities were measured.

I quantified maternal behavior as did Lonstein and DeVries (1998). Pups were removed from their parents and placed on a slide warmer at 32°C. Their mother was removed from the home cage and placed in a similarly sized clean plastic cage with cob bedding and a small amount of food. After a 2-hour separation, I weighed pups to the nearest 0.01g, and placed them in the test cage opposite to their mother. Behavior of mothers and pups was continuously observed for 45 minutes or videotaped for future observation or both. Pups were removed after testing, reweighed, and both parent and pups were returned to their home cage. Relative changes in litter weight were expressed in mg as

$$\frac{\text{(postobservation weight) – (preobservation weight)}}{\text{(preobservation weight)}} \times 1000$$

I recorded latency to retrieve pups, as well as the frequency and duration of active behavior directed towards pups (i.e. licking, grooming, crouching over pups, nursing, sniffing, and carrying them from one place to another). Nonpup-directed activities included self-grooming, exploration away from pups, digging, and eating, all of which were classified as nonsocial. I then measured the sum of time spent with the pups and the sum of time spent in nonsocial activities.

Because not all test voles produced litters, the sample size of parental tests varies from that of preference tests, and were as follows: control male n=10; flutamide (70 mg/kg) n=8; flutamide (35 mg/kg) n= 6; vinclozolin n=9; control female n=10; DES n=7; MXC n=8.

Statistical Analysis

I used one-way analysis of variance to analyze results on Statistical Analysis Software (SAS version 8.02, Cary, NC). If overall analysis of variance was significant ($p \leq 0.05$), I used an LSMEANS post-hoc test to further investigate differences between the groups.

Results

Males:

Control and flutamide-exposed males engaged in paternal activities approximately half of the time that they were with a pup (Figure 13). Latency to retrieve accounted for much of the nonsocial score. Males often retrieved the pup, and then left it for short times (~10-20 seconds) to go explore the cage. After initial retrieval of a

pup, however, control or flutamide males would always return to it frequently, and spend time in direct contact, often grooming it.

Vinclozolin exposed males had longer latency to retrieve times than did control or flutamide males (Figure 13). Three of the nine vinclozolin males *never* retrieved the pup. The vinclozolin males spent much more time away from the pup than did other groups. They explored the cage frequently and for long durations, and they spent much time self-grooming, often near a pup but not in direct contact with it.

FIGURE 13 – MALE PARENTAL BEHAVIOR

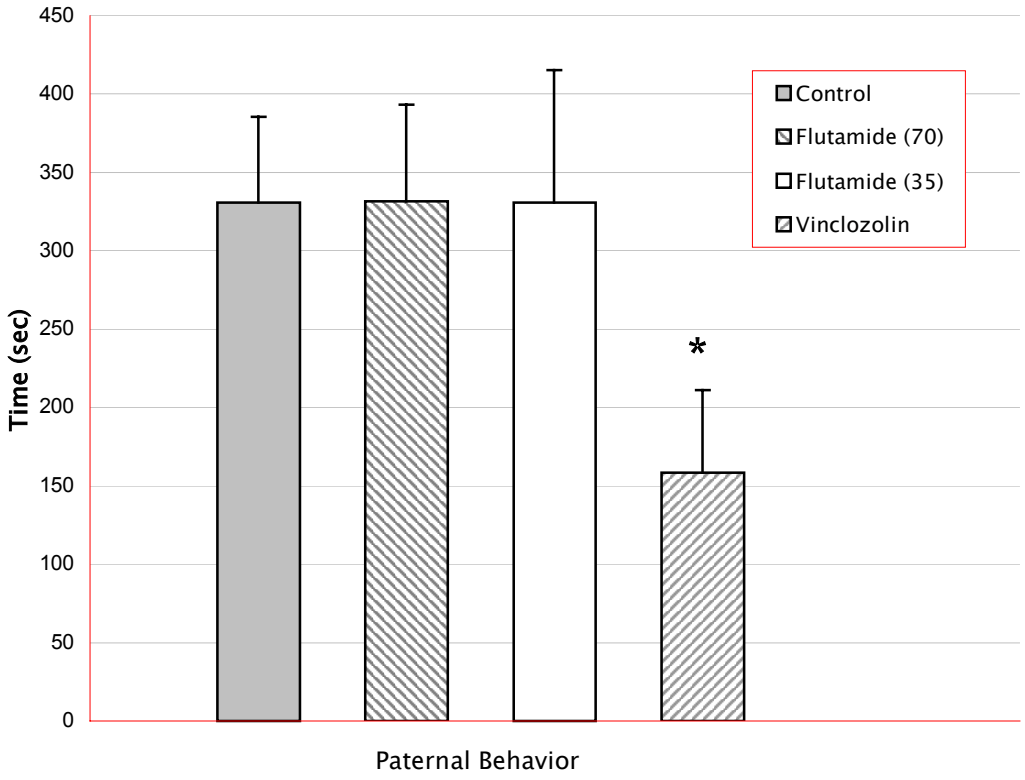

Figure 13: Average time (seconds) (± s.e.) that males exposed to oil, 70 mg/kg/day flutamide, 35 mg/kg/day flutamide, or 10 mg/kg/day vinclozolin were paternal toward pup. Vinclozolin exposed males differed from controls and flutamide exposed males, spending less time with pups and more time alone.
* Significantly different from control ($p<0.05$)

Females:

There were no observed differences in maternal behavior among the three groups of females (Figure 14). DES females showed a slight tendency toward more nonsocial behaviors such as exploration and self-grooming, but this difference was not significant.

There were no significant differences in pup weight gain (Table 11).

FIGURE 14 – FEMALE PARENTAL BEHAVIOR

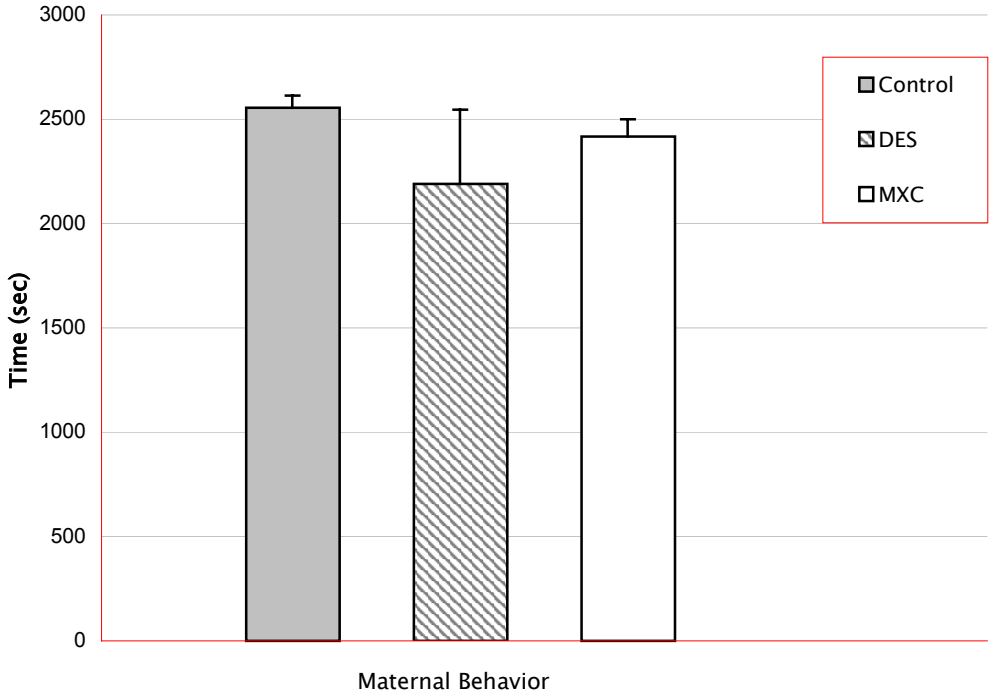

Figure 14: Average times (sec) (± s.e.) that females exposed to oil control, DES, or MXC were maternal toward pups. There were no differences among treatment groups.

TABLE 11 – STANDARD LITTER WEIGHT GAIN

	Control	DES	MXC
Weight Gain in mg (s.e.)	**2.81**(1.51)	**9.895**(6.05)	**9.681**(7.18)

Table 11: Average weight (mg) gained by litters of female treatment groups following 2 hour separation and 45 minute reunion with mother. There were no differences among treatment groups.

Discussion

Males:

One possible reason for a lack of flutamide effect on paternal behavior is that flutamide may have an inverted-U dose response curve (vom Saal *et al.* 1997; Gupta 2000; Alworth *et al.* 2002), as do many other endocrine disruptors. Thus the dose used here may result in no differences in paternal behavior, while a smaller (or much larger) dose potentially would alter the same set of behaviors. If this were the case, then vinclozolin's apparent effect in reducing paternal responsiveness could be explained in terms of its much smaller potency and dose, although the two chemicals may of course have different dose response curves.

My research is not the first to examine the effects of prenatal flutamide exposure on adult paternal behavior in a monogamous vole. Lonstein *et al.* (2000, 2002) reported results for a higher dose of flutamide (5mg/day/dam). The high doses of flutamide used in both this research and the above cited work may have resulted in flutamide having an androgenic rather than antiandrogenic effect, as described above. If flutamide were acting as an androgen, it would potentially be maintaining rather than reducing paternal behaviors in male pine voles. Previous studies with monogamous California mice and prairie voles have demonstrated that castration reduces paternal behavior while testosterone administration prevents this reduction (Trainor and Marler 2001; Wang and DeVries 1993). Thus adding androgenic activity to a system already under the influence of testosterone might have little or no obvious effect on resulting behavior. Further study has determined that the effect of testosterone in promoting paternal care in the

California mouse is a result of its aromatization to estrogen in the brain (Trainor and Marler 2002). However, there are very few estrogen receptors in the brains of adult prairie voles (Hnatczuk *et al.* 1994), so it seems unlikely that aromatization of testosterone is responsible for paternal behavior in the monogamous vole.

Part of the explanation for testosterone's importance in paternal behavior in these monogamous species is its conflicting effect on aggression. Because both California mice and prairie voles have postpartum estrus (as do pine voles), the male must guard his mate during this time (i.e. show heightened levels of aggression). Postpartum is also the time when males must be exhibiting parental behavior. Thus testosterone in these monogamous species is thought to foster *both* aggressive behavior and parental care (Trainor and Marler 2001). The results of this research are not consistent with the idea that flutamide acts as an androgen, since, although parental care was high in flutamide males, aggression toward strangers was markedly decreased. It is possible, however, that flutamide's particular mode of action is able to target one of these behaviors but not the other.

The explanation offered by Lonstein *et al.* for the observed lack of effect of flutamide on parental behavior in virgin male prairie voles was that prenatal exposure to gonadal hormones is not necessary for later paternal behavior in this species (Lonstein *et al.* 2000). Inhibition of gonadal hormone activity via flutamide had no effect on adult interactions with pups, and neonatal castration only reduces, but does not eliminate parental behavior in male prairie voles (Lonstein *et al.* 2000). This appears consistent with the vinclozolin exposed males here.

It is also possible that perinatal blocking of androgen activity by flutamide may have feminized paternal responsiveness, making it more dependent on hormonal stimulation. If so, then the presence of endogenous testosterone may have masked the effects of the treatment by stimulating parental behavior. Castrating adults could then potentially reduce paternal behavior (Lonstein *et al.* 2000).

Another explanation for the lack of flutamide effect on paternal behavior in monogamous voles is that this behavior may be influenced by a number of other testicular hormones, such as androstenedione, dehydroepiandrosterone, inhibin, anti-Mullerian hormone, and even estrogen (Lonstein *et al.* 2002). The combination of one or more of these products, acting directly or indirectly via other hormone systems (such as the hypothalamic- pituitary- adrenal axis), may contribute to the development of behavior in voles. Thus prenatal inhibition of androgenic activity alone would not result in a significant decrease in paternal behavior.

A final possibility regarding flutamide and paternal behavior involves the role of stress. Prenatal stress has been shown to feminize and increase paternal behavior in male rats (Kinsley 1990; Kinsley and Bridges 1988). This increase may be due to higher levels of adrenal hormones, and a resulting lower level of testosterone. In prairie voles, males receiving corticosterone (the most abundant adrenal corticoid for this species) pre- or perinatally were masculinized however, exhibiting levels of parental behavior as high or higher than controls (Roberts *et al.* 1996, 1997). Monogamous voles may thus rely on corticosterone and other adrenal steroid hormones rather than androgens to masculinize sexual behavior (Roberts *et al.* 1997).

The significant decrease in paternal behaviors observed in vinclozolin exposed males is due largely to the fact that three of the nine males never retrieved the pup. One control male never retrieved the pup. Previous researchers have noted that some virgin prairie voles will not quickly find and make contact with a pup, even though they are highly parental when the pup is offered to them directly (Lonstein *et al.* 2000). During a 15-minute parental test similar to the protocol used here, Lonstein moved pups to within an inch in front of the nonresponsive male after 3 minutes of no contact with the pup (Lonstein *et al.* 2000). At no time did I move the pups. If these three nonresponsive males and the one nonresponsive control male are removed from the data analysis, vinclozolin males no longer show a significant decrease in paternal behavior.

Accordingly, the above hypotheses concerning the lack of effect of flutamide on paternal behavior would apply to vinclozolin as well.

All of the experimental animals remained in the study and data analysis, however, thus the vinclozolin exposed males exhibited a significant reduction in paternal behavior. This could be attributable to the fact that the dose of vinclozolin was considerably less than the dose of flutamide. Vinclozolin potentially being at the low end of its dose response curve may result in effects different from those observed with flutamide, at the high end of its curve. Here again, if flutamide at such a high concentration is acting as agonist rather than antagonist, while vinclozolin at its much lower dose acts as antagonist, certainly this would explain why there are differences in the two treatments.

In rats, the masculinization of the brain is largely due not to the direct effects of testosterone, but instead to the conversion of testosterone to 17ß-estradiol by aromatase. Specific regions of the male brain contain aromatase, enabling the conversion to estrogen (Paup *et al.*, 1972; Reddy *et al.*, 1974). Thus the masculinization of many behaviors of the male rat is organized by estrogen, not testosterone, and as a result remains unaffected by antiandrogen administration. Certain behaviors in the male rat, however, are affected by androgens in the prenatal period, such as play behavior (Meaney *et al.,* 1983, 1986; Pellis and Pellis, 1997; Hotchkiss *et al.* 2002). It would appear that paternal behavior in the pine vole is organized by androgens rather than estrogen, since prenatal exposure to the antiandrogen vinclozolin affected its development. In addition, as stated above, the male monogamous vole has very few estrogen receptors (Hnatczuk *et al.* 1994).

Testosterone may influence paternal responsiveness by changing AVP release in the lateral septum. Injections of AVP into the lateral septum enhance parental care in male prairie voles, while antagonists impair it (Wang *et al.* 1994). The sources of the septal AVP-immunoreactive fibers are the bed nucleus of the stria terminalis (BST) and the medial amygdala (MA). These fibers are testosterone-dependent in prairie vole

males (Wang and DeVries 1993). After mating, there is an increase in both testosterone levels and AVP mRNA labeling in the BST (Gaines *et al.* 1985; Wang *et al.* 1993), suggesting that mating increases AVP release in the lateral septum, which in turn contributes to the increase in paternal responsiveness observed within three days of mating (Bamshad *et al.* 1993). The fact that castration in prairie voles inhibits both AVP synthesis and male paternal responsiveness supports this argument (Wang and DeVries 1993). Vinclozolin may thus be acting on this system, reducing the amount of active testosterone and consequently reducing paternal behavior.

Females:

That there were no differences observed among female treatment groups was not surprising. Estrogen is known to increase oxytocin receptor binding in certain regions of the female rat brain (Insel 1986; Johnson *et al.* 1989). Its effects on the vole brain however, are less striking, with only one brain region, the anterior olfactory nucleus (OAM), showing an increase in oxytocin binding following estrogen stimulation (Witt *et al.* 1991). The OAM receives input from the olfactory bulbs and projects to other brain areas that regulate behavior, such as the BNST and amygdala. Its involvement in vole maternal behavior is unclear. Although the connection between oxytocin receptors and estrogen is not completely clear, what is particularly interesting about oxytocin receptors in the female vole brain is that the promiscuous montane vole changes receptor distribution at parturition. Oxytocin receptors in a montane vole increase, primarily in the amygdala, to resemble the pattern observed in a prairie vole (Wang and Insel 1996). Thus if even nonmonogamous voles become maternal (in both brain and behavior) after giving birth, then we would not expect to see any differences in maternal behavior here.

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Chapter Four BRAIN NEUROPEPTIDE RECEPTOR DISTRIBUTION

Introduction

Extensive comparative studies of monogamous and promiscuous voles have revealed significant differences in the distribution of AVP (V_1a) and OT receptors (Insel and Shapiro 1992; Insel *et al.* 1994). Since AVP and OT are known to have critical roles in the control of social behaviors in voles, it seems likely that the receptor distribution differences are related to the differences in social behavior. Gonadal steroids interact with the AVP and OT receptors as well. The pre- and perinatal environment and hormonal milieu (i.e. estrogen and testosterone) can affect tissue-specific transcription factors, leading to the activation or inhibition of gene expression (Plotsky and Meaney 1993; Gorski 1985). Differences in gene expression account for the different receptor distributions in monogamous and promiscuous voles (Young *et al.* 1996a, 1996b). Thus, if changes in pine vole social behavior are observed following developmental exposure to endocrine disrupting compounds, it is possible that a change in neuropeptide receptor distribution is causally linked to the behavioral effects.

I examined six regions of the brains of male pine voles. Two of these regions, the lateral septum and ventroposterior thalamus, have increased V_1a receptor binding in polygamous montane and meadow voles. The other four areas examined, the cingulate cortex, diagonal band, ventral pallidum, and laterodorsal thalamus, show more V1a receptor binding in monogamous prairie and pine voles (Insel *et al.* 1994). I examined two regions of the brains of females, the cingulate cortex that has more OT binding in monogamous voles, and the lateral septum that has higher binding in polygamous voles. These regions were chosen based on prior comparative studies and the certainty with which particular anatomical distinctions could be identified.

Methods

I anesthetized and decapitated pine voles exposed to EDC treatment as described in Chapter 2. Brains were extracted and quick frozen on dry ice, and subsequently stored at -80 °C. I cut sections (16μm thick) at −15° C on a cryostat and thaw-mounted them on Superfrost plus slides (Fisher, Pittsburgh, PA), placing adjacent sections on separate slides. I then stored the sections at −80° C until use.

I conducted *in vitro* receptor autoradiography using previously published methods (Insel and Shapiro 1992; Wang *et al.* 1997). These employ a highly specific iodinated ligand for either oxytocin receptors, OTA: [^{125}I]d(CH_2)$_5$ [Tyr(Me)^2, Tyr-NH_2^{9}]ornithine vasotocin ([^{125}I]OTA); or V1a receptors: [^{125}I] linear V_1a receptor antagonist (HO- Phenylacetyl^1 -*D*- Tyr(Me)^2-Phe^3 -Gln^4 -Asn^5- Arg^6- Pro^7- Arg^8- NH_2). OTA was previously characterized in rats (Elands *et al.,* 1987). V_1a ligand has likewise been previously characterized in rats (Schmidt *et al.*, 1991; Johnson *et al.*, 1993). Both OTA and V1a have been employed extensively in vole studies (Insel and Shapiro 1992; Winslow *et al.* 1993; Wang *et al.* 1997; Young *et al.* 2001). Oxytocin receptors were labeled in both male and female brain samples, though I only analyzed oxytocin receptors in female sections. Vasopressin receptors were labeled in male sections only.

I thawed slides at room temperature (approximately 30 minutes), then fixed them for 2 minutes in 0.1% paraformaldehyde. After a double rinse (10 minutes each) in 50mM Tris-HCl (pH 7.4), brain sections were exposed to a 60-min incubation at room temperature, in a 50mM Tris-HCl solution containing 10mM $MgCl_2$, 0.1% bovine serum albumin, and a 50pM concentration of either [^{125}I] OTA (specific activity, 2200 Ci/mmol; New England Nuclear) or [^{125}I] V_1a receptor antagonist (specific activity, 2200 Ci/mmol; New England Nuclear). Slide chambers for this incubation with radioligand were constructed by Larry Dufour at Burlington Nuclear Engineering Labs, NCSU. Following incubation, I washed sections in 50mM Tris-HCl with 10mM $MgCl_2$ three times for 5 minutes each at room temperature, and then I washed them in the same solution with

stirring for 30 minutes at room temperature. Sections were dipped in water and then immediately dried under a stream of cool air.

After drying, I exposed slides to a phosphor imaging screen for 72 hours, after which time I analyzed the screens using a phosphor imager (Molecular Dynamics Storm System, Amersham Biosciences, Piscataway, NJ). Slides were then removed and exposed to Hyperfilm MP (Amersham, Buckinghamshire England) for 72 hours. For quantification, I included ^{125}I autoradiographic standards (Amersham, Arlington Heights, IL) in the cassette. Male OTA labeled sections were not put on film, due to a delay in receiving the microscale standards. Film was developed in a dark room using Kodak developer and fixer.

I also sectioned and performed autoradiography on a subset of male control and flutamide exposed brains at Emory University in the laboratory of Larry Young. The procedure was identical to the above, with the exception that the film was developed by an automated system, and the sections were not put on phosphor imaging screens. Due to differences in isotope lot, exposure time to film, and developing differences, the values of the two assays cannot be directly compared.

I later captured autoradiographic signal from film into a MacIntosh G3 computer, using Brain Software for Autoradiography (Version 3.0, Drexel University and the Computer Vision Center for Vertebrate Brain Mapping). Anatomical structures were matched by comparing adjacent AChE-stained sections with the ligand labeled male sections, and by counter-staining of female sections with cresyl violet. For each animal, three to five sections were averaged in each region to cope with gradients and variations due to plane of section.

Optical density values were converted to the natural log, and then to disintegrations per minute (DPM) by using a third order polynomial regression (Insel and Shapiro 1992).

Statistical Analysis

I used a MANOVA test for differences among treatments with Statistical Analysis Software (SAS version 8.02, Cary, NC). Since these tests were significant ($p \leq 0.05$) in both sexes, I then did univariate and LSMEANS tests for specific brain regions.

Results

Males:

Four regions of the brain investigated here show higher levels of AVP binding in monogamous voles (Insel *et al.* 1994). Of these regions, the cingulate cortex had significantly less binding in flutamide exposed males than in control males (NCSU processed $p=0.005$; Emory processed $p=0.03$). Vinclozolin treated males also showed a trend toward less binding in the cingulate cortex, though there was not a significant difference ($p=0.07$). The diagonal band and ventral pallidum showed trends toward reduced binding in both flutamide and vinclozolin males, but no significance. The laterodorsal thalamus binding was decreased significantly in flutamide males processed at NCSU, but not in the flutamide males processed at Emory University (Figure 15; Figure 16). Sample sizes were small in each case, due to the splitting of the treatment group between processing sites.

FIGURE 15 – MALE AVP RECEPTOR BINDING (NCSU PROCESSED)

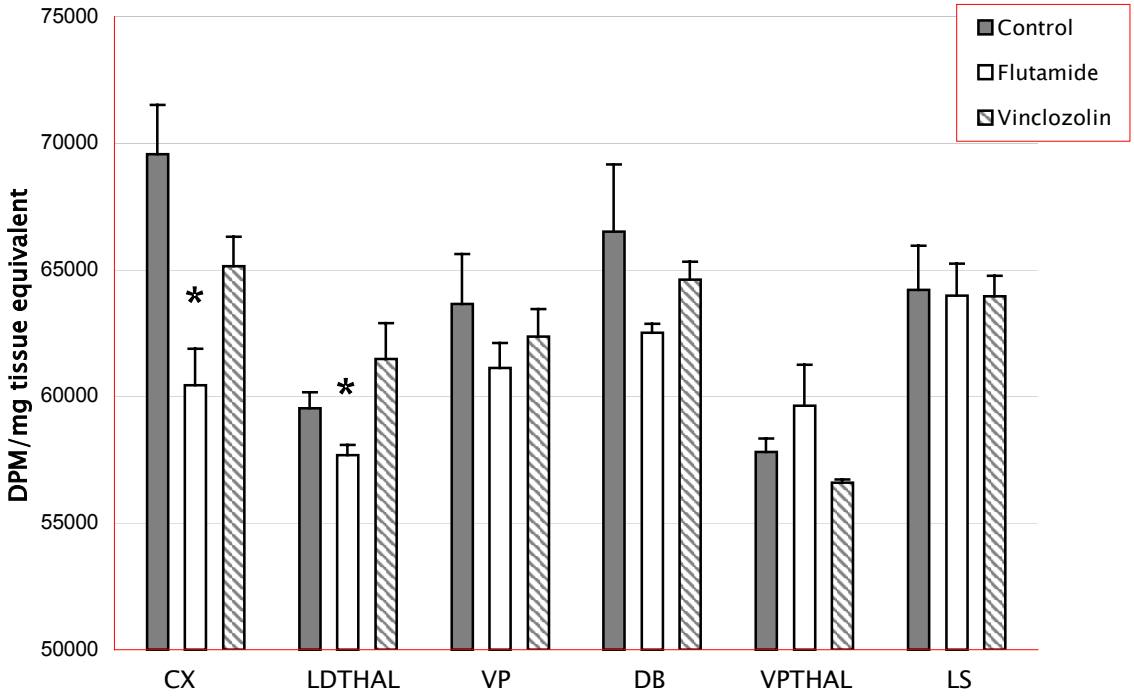

Figure 15: Average AVP receptor binding in six brain regions of males exposed to oil (n=4), 70 mg/kg/day flutamide (n=6), or 10 mg/kg/day vinclozolin (n=8). Flutamide treated males had reduced binding in the cingulate cortex and laterodorsal thalamus. DPM=disintegrations per minute; CX=cingulate cortex; LDTHAL=laterodorsal thalamus; VP=ventral pallidum; DB=diagonal band; VPTHAL=ventroposterior thalamus; LS=lateral septum.

* Significantly different from control at p=0.005.

FIGURE 16 – MALE AVP RECEPTOR BINDING (EMORY PROCESSED)

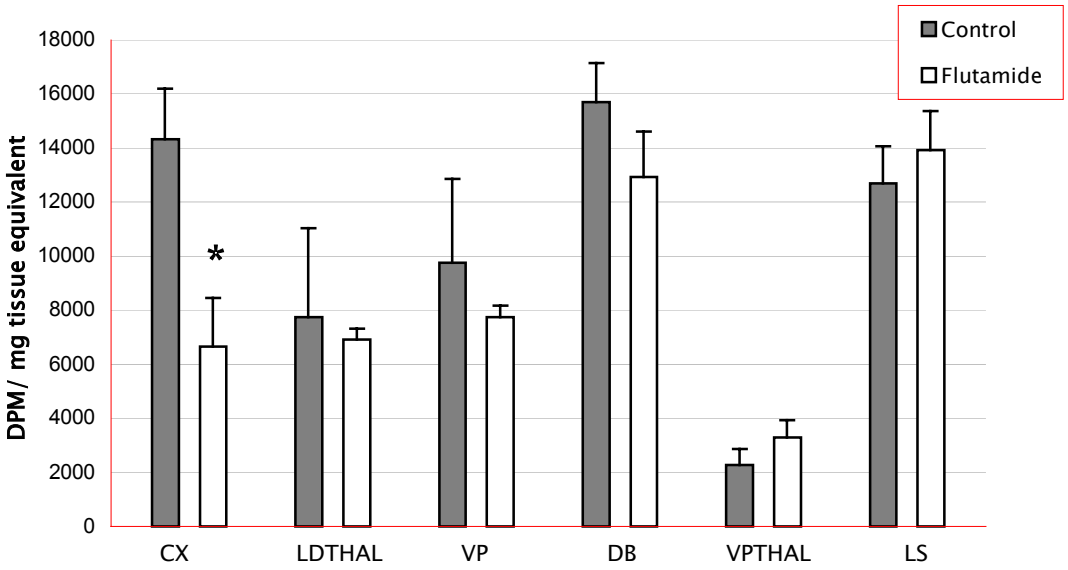

Figure 16: Average AVP receptor binding in six brain regions of males exposed to oil (n=5) or 70 mg/kg/day flutamide (n=5). Flutamide treated males had reduced binding in the cingulate cortex. DPM=disintegrations per minute; CX= cingulate cortex; LDTHAL=laterodorsal thalamus; VP=ventral pallidum; DB=diagonal band; VPTHAL=ventroposterior thalamus; LS=lateral septum.

* Significantly different from control at $p<0.05$.

Two regions of the brain show more binding in polygamous voles than monogamous: the lateral septum and the ventroposterior thalamus (Insel *et al.* 1994). The lateral septum showed no differences in binding among treatment groups in this study. The ventroposterior thalamus showed a trend toward increased receptor binding in flutamide exposed males, though sample sizes were small in both sets of data (Figure 15; Figure 16).

Females:

The cingulate cortex has higher levels of OTA binding in pine and prairie vole females than in meadow and montane vole females (Insel and Shapiro 1992). The DES exposed females did not differ from controls in cingulate cortex binding, but the MXC females had significantly less binding in this region (p=0.006) (Figure 17).

FIGURE 17 – FEMALE OT RECEPTOR BINDING

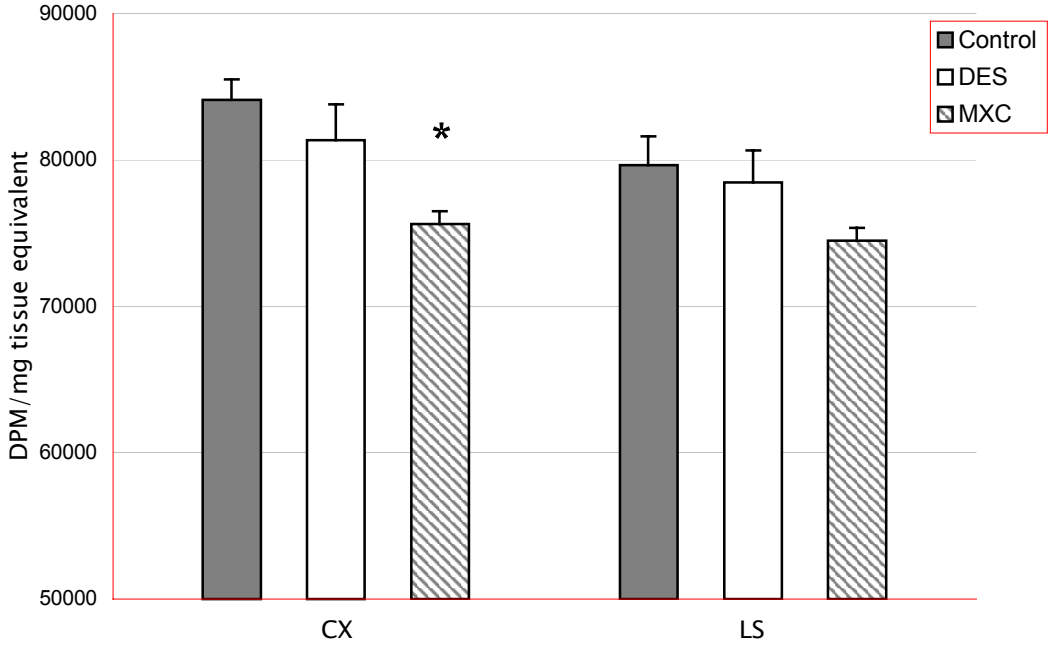

Figure 17: Average oxytocin receptor binding in females exposed to oil (n=8), 0.2µg/kg/day DES (n=7), or 2000µg/kg/day MXC (n=7). MXC exposed females had reduced binding in the cingulate cortex. DPM=disintegrations per minute; CX=cingulate cortex; LS=lateral septum. * Significantly different from control at p=0.006.

The lateral septum shows higher levels of binding in the meadow and montane voles (Insel and Shapiro 1992). Both DES and MXC showed slightly decreased binding relative to control females, but these differences were not near significance (p=0.13) (Figure 17).

Discussion

Males:

The fact that flutamide exposure significantly reduced V_1a receptor binding in the cingulate cortex, while vinclozolin exposure also resulted in less binding though not significantly so, is unexpected. The dose of flutamide used in this research appears to have had androgenic activity, as previously discussed. If true, this would mean that an elevated level of androgen pre- and perinatally significantly reduces V_1a receptor number and/or affinity in the pine vole cingulate cortex. Testosterone can act as either an enhancer or repressor of OT receptor binding in the brains of male mice, depending on the particular brain region considered (Insel *et al.* 1993). Thus it is possible that testosterone could likewise enhance or repress AVP receptor binding. One explanation for the observed flutamide result is that low levels of androgen are necessary for the monogamous pattern of V_1a receptor binding in the cingulate cortex. The androgenic effects of flutamide thus would have an inhibiting effect on gene expression, leading to a reduction in receptor binding. The antiandrogenic effects of vinclozolin, however, could have reduced the amount of androgen activity in the cingulate cortex slightly below the minimum threshold required for receptor induction, thus resulting in a less dramatic reduction in receptor binding.

Another possible explanation for the significant decrease in V_1a receptor binding in the cingulate cortex is that the androgenic properties of flutamide may have altered the synthesis and release of AVP itself, which could then potentially down-regulate its own receptors. Similar effects have been proposed for estrogen and oxytocin receptors, (Caldwell *et al.* 1989; Yamaguchi *et al.* 1979; Insel *et al.* 1992), and testosterone and oxytocin receptors (Insel *et al.* 1993). A flutamide effect on receptor expression would appear a more likely explanation if similar effects on receptor binding were found in all areas, but this was not the case. However, flutamide exposure resulted in a trend to *increased* binding, though not significant, in the ventroposterior thalamus.

Vinclozolin and flutamide effects on neuropeptide receptor binding could be directly due to androgenic or antiandrogenic effects. However, these compounds could also indirectly affect neuropeptide receptors through their influence on testosterone or other hormones or metabolizing enzymes.

In all four areas of the brain associated with high levels of V_1a receptor binding in monogamous voles, the trend was *de*creased binding in both flutamide and vinclozolin males. In the two areas associated with greater binding in the polygamous vole, binding was either completely unaffected (lateral septum) or slightly *in*creased (ventroposterior thalamus) in treated males. Although sample sizes are small, it appears that androgen levels outside of the normal developmental range for the monogamous pine vole may result in receptor binding more characteristic of a polygamous vole.

Females:

In the prairie vole, binding to OT receptors (via ^{125}I-OTA) is very high in the prelimbic cortex, nucleus accumbens, and bed nucleus of the stria terminalis (BST) (Insel *et al.* 1994; Insel and Shapiro 1992; Witt *et al.* 1991). In the pine vole, however, there is more binding in the cingulate cortex, and no apparent difference from polygamous species in binding in the nucleus accumbens or BST (Insel and Shapiro 1992). Thus I did not examine the prelimbic cortex, nucleus accumbens, or BST, but did examine OT receptor binding in the cingulate cortex.

That MXC resulted in a significant reduction in ^{125}I-OTA binding, while DES only slightly reduced it, supports the discussion in Chapter 2 that the administered doses of MXC and DES were not equivalent, and that MXC was in fact a stronger dose. This higher level of estrogenic compound resulted in a much more dramatic decrease in binding. As stated above, it is possible that this is due to estrogen altering the synthesis and release of oxytocin, which then in turn down-regulates its own receptor (Caldwell et al. 1989; Yamaguchi et al. 1979; Insel et al. 1992).

The other possible explanation as to why MXC reduces binding in the cingulate cortex while DES does not, is that the reduction is due not to MXC's estrogenicity, but its antiandrogenic properties. It is possible that the female brain requires a certain amount of testosterone present in order to produce the correct complement of transcription factors and activate the OT receptor gene. If this necessary amount of testosterone is relatively low, an antiandrogen could have a serious effect, regardless of how weak it is.

The other region examined in the female brain was the lateral septum. This area has a very high degree of binding in polygamous voles, but very little in monogamous voles, much like in males (Insel and Shapiro 1992). Though there were no significant differences in either treatment group, both DES and MXC resulted in decreased ^{125}I-OTA binding in this area as well. MXC in particular had much less binding than controls (MXC students *t*-test $p=0.03$; DES students *t*-test $p=0.69$). Again it would seem that MXC is the more potent estrogen in this case, due to dose, and thus it has a greater inhibitory effect on OT receptor binding.

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Chapter Five DISCUSSIONS AND CONCLUSIONS

During prenatal and early postnatal development, hormones involved in differentiation permanently turn on or off certain genes and determine rates of gene expression in a large number of cells, including those in the CNS (Holliday 1987; vom Saal *et al.* 1992; Lyn-Cook *et al.* 1995; Li *et al.* 1997). Exposure to endocrine disrupting chemicals (EDCs) during this critical and sensitive developmental time can thus result in permanent changes in gene expression, leading to subsequent changes in organ functioning and/or behavior.

I investigated the effects of developmental exposure to endocrine disruptors not only on the physiology of pine voles, but also on behaviors associated with monogamy. The effect of endocrine disruptors on behavior is a relatively new field, as the vast majority of EDC research has examined only physiological, pathological, or anatomical effects. Because behavior is the result of a number of integrated systems, even slight alterations in any of the systems will likely be reflected in the total behavioral phenotype. It follows, then, that behavior may be especially sensitive to disruptions of hormonal systems due to EDCs, and therefore should be a particularly relevant focus of endocrine disruption studies.

Any attempts at interpreting the results presented here must begin with an understanding of the properties of the particular endocrine disrupting compounds administered. Flutamide was intended to serve as a positive control for antiandrogenic activity. Based on AGD and seminal vesicle weight results, however, it appears to have had androgenic activity instead. It is not known if the effect of a compound (i.e., androgenic or antiandrogenic) can vary between different tissues in the same individual. If so, it could have profound implications for this and other research. Vinclozolin however produced the effects typical of an antiandrogen. As a result, these two compounds cannot be reliably compared to each other.

Methoxychlor (MXC) and diethylstilbestrol (DES) both appear to have had the expected estrogenic activity. The specific doses are most likely at different points on their dose-response curves, however, with MXC perhaps being slightly more potent.

Alternatively, MXC may have exhibited some antiandrogenic effects as well. Little evidence supports this, however, either in this research or previous investigations of vinclozolin effects on females (Gray *et al.* 1994).

One conclusion that can be drawn with relative certainty from the research presented here is that parental and affiliative behaviors are mediated by separate pathways, perhaps separate brain receptor fields, in pine voles. This idea has been suggested and supported by work with prairie voles (Insel and Shapiro 1992). Maternal care was unaffected in both DES and MXC exposed groups, although both groups exhibited some alterations in affiliative behavior and aggression. Likewise, flutamide exposed males exhibited extremely altered pair-bonding behavior, while remaining highly parental. Vinclozolin exposed males showed the opposite pattern: normal affiliative behavior, but disrupted paternal behavior. Thus in all treatment groups, either parental *or* affiliative behavior was affected, but not both (Table 15).

It is interesting that vinclozolin produced such a noticeable change in parental behavior with no associated change in the brain V_1a receptor pattern. This could be due to an alteration in OT receptors instead, though it is unlikely since current evidence does not support a role for OT in paternal behavior (Wynne-Edwards 2001). More likely is that vinclozolin had an effect on regions of the brain not analyzed here. For example, the medial preoptic area, bed nucleus of the stria terminalis, and medial amygdaloid nucleus have all been implicated in paternal behavior in prairie voles (Kirkpatrick *et al.* 1994a, 1994b; Shapiro *et al.* 1991). Additionally, testosterone influences AVP-ir fibers in these areas (Wang and DeVries 1993). More effective counterstaining could allow these regions to be located and analyzed, and perhaps determine the route whereby vinclozolin alters parental behavior in the pine vole male.

TABLE 12 – SUMMARY CHARTS

I Treatment	Dose/day	Weight	AGD	Uterus	Seminal Vesicles	Cingulate Cortex
Flutamide (70)	70mg/kg	---	⇑		(⇑)	⇓
Flutamide (35)	35mg/kg	---	---		(⇑)	
Vinclozolin	10mg/kg	⇑	⇓		⇓	(⇓)
Diethylstilbestrol	0.2µg/kg	---	---	(⇓)		---
Methoxychlor	2000µg/kg	---	---	⇓		⇓

II Treatment	Dose/day	Mate	Neutral	Stranger	Parental	Aggression
Flutamide (70)	70mg/kg	⇓	⇑	---	---	(⇓)
Flutamide (35)	35mg/kg	⇓	---	⇑	---	⇓
Vinclozolin	10mg/kg	---	---	---	⇓	---
Diethylstilbestrol	0.2µg/kg	(⇓)	---	(⇑)	---	⇑
Methoxychlor	2000µg/kg	(⇓)	(⇑)	---	---	(⇓)

Table 15: Summary of all major findings among male (flutamide and vinclozolin) and female (diethylstilbestrol and methoxychlor) treatment groups. Chart I: Anatomical effects. Chart II: Behavioral effects.
⇓ or ⇑ indicates significantly different from vehicle control ($p<0.05$).
(⇑) or (⇓) indicates trend toward significance.

The effect of DES on aggressive behavior in females is also worth further attention. The increase in aggression toward a strange male could be a result of a stronger pair bond with the mate, though there is no current evidence to support this. Alternatively, the increased aggression could be the result of heightened reactivity and territoriality. Studies of prenatal DES exposure in mice report similar increases in reactivity and aggressive behavior (Palanza *et al.* 1999a, 1999b, 2001). These changes suggest that DES exposure may lower the anxiety profile, since decreased reaction time

responding to unfamiliar objects or conspecifics has been associated with lower levels of anxiety (Rodgers and Cole 1993; Griebel *et al.* 1993). During development steroid hormones influence the number and sensitivity of brain receptors for neurotransmitters (Goetz *et al.* 1983), so it follows that endocrine disruptors might interfere with normal development of serotonin, dopamine, or GABA receptor systems, thereby altering responses to unfamiliar conspecifics. For example, DES decreases serotonin receptor function in rats (Cologer-Clifford *et al.* 1999). Specific tests to measure anxiety (elevated plus maze, open field, etc.) would help to identify the mechanism of this effect, as would neurotransmitter receptor studies. That both mice and pine voles have the same alterations in behavior following prenatal exposure to DES suggests that similar modes of action might be responsible, and thus that neurotransmitter pathways and function may be conserved across the different genera.

In both males and females, reduction in neuropeptide receptor binding in the cingulate cortex is associated with reduced aggression toward a stranger and more time spent alone (Table 12). I would be interested in knowing the role of the cingulate cortex in social behaviors. This region has been associated with separation distress in rats (Winslow and Insel 1993; Insel and Winslow 1991) and with reward-based decision making in humans (Bush *et al.* 2002). The cingulate cortex, like the diagonal band, ventral pallidum, and nucleus accumbens, may also be associated with reinforcement and conditioning. The nucleus accumbens, in particular, has been linked to reinforcing behaviors through both its OT and dopamine receptors (Wang *et al.* 1999; Gingrich *et al.* 2000; Young *et al.* 2001). It is possible that the cingulate cortex plays a part in the formation of a pair bond through the activation (via AVP or OT) of this reward pathway as well, and that a reduction in neuropeptide binding thus results in a reduction in affiliative behavior.

Alternatively, receptors in the cingulate cortex may be necessary for social memory. AVP and OT are involved in social recognition (Ferguson *et al.* 2002), and the behavior of the flutamide males and MXC females resembles that of OT knock-out mice

(Ferguson *et al.* 2000). In addition, V_1b knock-out mice exhibit decreased aggression and social recognition, with no other behavioral effects (Wersinger *et al.* 2002). If these individuals are unable to recognize their mate, they would show no increased mating with the mate (MXC females mated with the stranger more than the mate), no increased aggression toward a stranger following pair bond formation (as seen in both flutamide and MXC groups), and equal amounts of time spent with the stranger and mate (higher dose flutamide males spent equal amounts of time in side-by-side contact with mate and stranger). Thus it is possible that receptors in the cingulate cortex could have an effect on social memory.

In addition to examining the role of the cingulate cortex receptors in affiliative behavior, future research should also investigate *how* flutamide and methoxychlor were able to reduce receptor binding in this region, especially given that flutamide appears to be acting as an androgen, and methoxychlor is estrogenic. That endocrine disrupting compounds can alter receptor binding in the brain has profound implications, since brain receptors for steroid hormones, neuropeptides, and neurotransmitters play such critical roles in so many different behaviors.

In summary, the research presented here gives further support of the increasing need for more attention on functional changes, including behavioral responses, in toxicological studies. This research provides evidence that endocrine disrupting compounds are capable of altering not only traditional anatomical endpoints, but also receptor distributions in the brain, subsequently affecting reproductive behavior and possibly social systems as well.

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