ABSTRACT

**Ryan, Bryce Clair.** Developmental exposure to environmental estrogens alters adult behavior in female rodents. (Under the direction of Dr. John G. Vandenbergh)

Humans and wildlife are exposed to numerous anthropogenic drugs and pollutants. Many of these compounds are hormonally active and recent evidence strongly suggests that the presence of these endocrine disruptors can permanently alter normal development and physiology in a variety of vertebrate species. The experiments in this project investigated the effects of two common estrogenic pollutants. Bisphenol A, is a monomer of polycarbonate plastic used to make resins for the food and dental industries. Ethinyl estradiol is used pharmaceutically as the active estrogen in the oral contraceptive pill. The majority of past research on these chemicals has focused on reproductive physiology. The focus of my research in on the behavioral consequences of developmental exposure to these compounds. Estrogens will feminize the reproductive system but will masculinize the rodent nervous system, so I focused on identifying whether females would show masculinization of sexually dimorphic traits. The effects of these compounds were studied on two commonly used laboratory species, the mouse (*Mus musculus domesticus*) and the rat (*Rattus norvegicus*). The test animals were exposed to environmentally relevant levels of these compounds (ranging from 2 – 200 µg/kg/day for bisphenol A and 0.05 to 50 µg/kg/day for ethinyl estradiol) throughout prenatal and early postnatal development. After this exposure, the animals were allowed to reach adulthood and then observed in a variety tests measuring sexually dimorphic behaviors. These include short-term spatial memory, anxiety, saccharin preference, motor activity and lordosis. Developmental exposure to ethinyl estradiol
was found to masculinize every behavior measured in both species in a dose-dependent fashion. Bisphenol A disrupted selected behaviors, namely anxiety and motor activity, and was active in both rodent species, but did not always follow a clear dose response. These results indicate that sexually dimorphic behavior can be exquisitely sensitive to endocrine disruption. In addition, these experiments suggest that both humans and wildlife are presently being exposed to levels of these endocrine disrupting compounds that are sufficient to disrupt the development of the nervous system and that may have permanent consequences on sexually dimorphic behaviors.
DEVELOPMENTAL EXPOSURE TO ENVIRONMENTAL ESTROGENS
ALTERS ADULT BEHAVIOR IN FEMALE RODENTS

by

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Chair of Advisory Committee
I, Bryce Clair Ryan, was born and raised in San Carlos, California by my parents Mark and Veda Ryan. After attending The Menlo School, in Atherton, California, I went on to Claremont McKenna College where I majored in Psychobiology. While at Claremont, I spent two years studying human visual and verbal memory under the supervision of Dr. Harvey Wichman. This project culminated in my senior thesis entitled “Testing the Dual Coding Theory of Memory.” After graduation, I accepted a position as a middle and high-school science teacher at Gaston Day School in Gastonia, North Carolina. During my time at Gaston Day, I developed curricula for and taught classes in subjects ranging from A.P. Biology to Chemistry. In addition to my teaching load, I was also responsible for organizing the annual science fair and served as the assistant varsity baseball coach. After three years at Gaston Day School, I accepted a position as a graduate student and research assistant in Zoology under the supervision of Dr. John Vandenbergh at N.C. State. As a graduate student, I have served as both Vice President and President of the Zoology Graduate Student Association, and was fortunate enough to be awarded the best Ph.D. level oral presentation at the annual Zoology/Botany Graduate Student Symposium in 2004. In 2003, I was also awarded a NCSU/EPA Cooperative Predoctoral Training Fellowship and under this fellowship spent the last two years working at the EPA in the laboratories of Dr. L. Earl Gray and Dr. Kevin Crofton.
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LIST OF SYMBOLS AND ABBREVIATIONS

AGD: anogenital distance
BPA: bisphenol A
CAH: congenital adrenal hyperplasia
CO: corn oil
EE: ethinyl estradiol
EPA: Environmental Protection Agency
ER: estrogen receptor
GD: gestational day
IUP: intrauterine position
PND: postnatal day
SO: tocopherol-stripped corn oil
µg or ug: microgram
1. GENERAL INTRODUCTION

Sexual differentiation in mammals is regulated primarily by steroid hormones throughout development. In eutherian mammals, exposure to these hormones can be influenced by the intrauterine positioning of fetuses. Despite the protected developmental environment of a uterus, fluctuations in intrauterine hormonal concentrations do occur, and when severe, they can disrupt the development of the fetus. This project investigates the disruption of sexual differentiation that can arise from exposure to external hormonal sources during pregnancy and lactation.

In addition to external hormonal inconsistency, another source of hormonal variation exists. The source of this variability is not external, but rather internal. Many mammals give birth to large litters. The animals from a litter develop together within the uterus, and this proximity to each other causes each individual to be exposed to slightly different hormonal environments. The hormonal differences between individuals are not great; however, developing mammals have a number of sensitive periods to the actions of hormones and are exquisitely sensitive to any fluctuations. As a result, hormonal variability that may otherwise be considered quantitatively trivial can have significant permanent effects on sexually dimorphic traits. The evidence for this comes from a body of work in litter-bearing mammals referred to as “intrauterine position effects.” Reviewing this literature indicates how easily the process of sexual differentiation can be disrupted. This focused review also identifies sexually dimorphic endpoints sensitive to developmental disruption. A comprehensive review of this literature is beyond the scope of this chapter. For a more complete review on intrauterine position effects, see Ryan and Vandenbergh (2002).
1.1 Intrauterine Position Effects

In litter-bearing mammals, pups from the same litter must share space in their mother’s uterus. This space sharing results in pups from large litters developing in slightly different environments from each other. For example, a mouse or swine fetus that occupies a position at either end of a uterine horn receives more nutrient-rich blood and is subsequently heavier at birth than other fetuses (Bulman-Fleming and Wahlsten, 1991; vom Saal and Dhar, 1992; Wise and Christenson, 1992). A rat fetus located at the cervical end of the uterus receives maternal blood flow prior to fetuses in other uterine positions (Gorodeski et al., 1995; Lipton et al., 1998). Any fetus not located at an end of a uterus will be located between two males (2M), two females (0M), or one male and one female (1M) (Figure 1.1). This intrauterine position (IUP) has significant and wide-ranging effects on the development of the fetus (Ryan and Vandenbergh, 2002).
Figure 1.1: Schematic illustrating fetus positions in a rodent womb. The 0M-2M classification refers to the number of males flanking the fetus. This classification is the most common categorization system used in the literature.

In rodents, such as the house mouse (Mus musculus) or the Norway rat (Rattus norvegicus), male fetuses produce testosterone earlier and in higher amounts than females. Females, on the other hand, produce higher amounts of estradiol later in development. These hormones can diffuse through the membranes and amniotic fluid between fetuses. As a result, both male and female 2M mouse fetuses (flanked by males) have higher blood concentrations of testosterone and lower blood concentrations of estradiol than 0M fetuses (flanked by females).
(vom Saal et al., 1990). This mechanism of hormone transfer among mouse fetuses has become fairly well understood and accepted.

In addition to diffusion through the amniotic fluid, hormones may also travel among fetuses through the bloodstream of the mother. In pregnant rats, uterine blood flows predominantly in the caudal to distal direction (i.e. from the cervix to the ovaries) (Gorodeski et al., 1990). Fetuses located distally to male pups will thus be subjected to higher levels of testosterone and fetuses located caudally to male pups will not be subjected to high testosterone levels. Pregnant mice, in contrast to rats, show bi-directional blood flow through the uterus (vom Saal and Dhar, 1992). This makes a one-way movement of hormones among mouse fetuses unlikely. It is not known if the differences in circulation play some predictable role in developmental differences or IUP differences between species. However, this physiological difference between rat and mouse circulation has resulted in most investigators classifying rat fetuses by the number of males located upstream (caudal male hypothesis), whereas mice and gerbil researchers have maintained the classical 0M-2M designation (contiguous male hypothesis).

Regardless of the exact mechanism, it is clear that same-sex fetuses from the same litter will be subjected to different concentrations of steroid hormones. Many mammals seem to rely on this variable uterine environment to develop normally. Both gerbils and mice that do not have any littermates mature abnormally and reproduce poorly (Clark et al., 1997; Gandelman and Graham, 1986). IUP, therefore, serves as a source of non-genomic variability in these animals.

Sexual differentiation in mammals is largely mediated by androgens early in development. Variable concentrations of androgens will therefore alter this process of
differentiation. 2M pups exposed to high levels of androgens will show increased
masculinization and 0M pups exposed to low levels of androgens will show increased
feminization. Steroid hormone transfer among fetuses not only alters development, but it
increases variability among individuals from the same litter. From an experimental perspective,
variability is unwanted and may mask potentially significant results. For example, IUP alters
sensitivity to certain endocrine disruptors (Howdeshell et al., 1999; Howdeshell and vom Saal,
2000) and therefore serves as an additional source of variation. Laboratory rodents are
commonly used experimentally because of their genetic and phenotypic homogeneity. It is
important to understand the potential implications of this IUP effect in order to control and
minimize this endogenous, non-genetic, variability. Controlling both genetic variability and IUP
variability will result in a more tightly controlled phenotype. This reduction in variability will
result in a more powerful model for studies in developmental biology and toxicology.

The recent public and scientific concern over the effect of hormone mimics in the
environment has simulated a vast amount of work in the relatively new field of endocrine
disruption. Similar concerns regarding low dose and mixture effects of certain toxicants have
surfaced in recent years as well (Bigsby et al., 1999; Hotchkiss et al., 2004). IUP, by altering
prenatal endogenous hormone exposure, has the potential to interact with experimental
manipulations using endocrine disruptors.

IUP can influence aggression, dispersal and mating in wild populations (Drickamer,
1996; Zielinski et al., 2001) and could therefore affect studies dealing with the population
biology of mammals. IUP can also influence immunological responses as well as enzyme levels
in the body (Wise and Christenson, 1992) and could therefore become important for general
animal health and human health. The work on IUP serves as an excellent overview of the
potential impact of a variable hormonal environment during development. For the purpose of this review, different IUP effects will be organized into physiological, morphological and behavioral categories. Effects shown on non-laboratory animals, including humans, will be covered as well.

1.1.1 Physiology

A 2M female mouse fetus has a higher concentration of testosterone in both her blood and her amniotic fluid than does a 0M female (vom Saal and Bronson, 1980; vom Saal et al., 1990). A female rat fetus located distally to males also has a high concentration of plasma testosterone (Houtsmuller et al., 1995), although this has not been shown in every case (Hernandez-Tristan et al., 1999). These differences appear to originate from the male pups surrounding a fetus and not the mother’s circulation. In mice, the difference in hormone levels disappears by adulthood (vom Saal and Bronson, 1980). The influence of IUP on estradiol concentrations is less clear. In one study, 0M female mice had higher fetal concentrations of estradiol than 2M mice (vom Saal et al., 1983). Another study found no significant differences (vom Saal and Bronson, 1980). Differing concentrations of prenatal testosterone will alter the organizational function of this hormone and have effects that persist through adulthood. In addition, differing concentrations of adult steroid levels will obviously influence any activational steroid function as well.

Female rats located distally to male rats in utero possess an increased sensitivity to testosterone as adults, showing an increase in mounting behavior, a known response to androgen treatments (Houtsmuller et al., 1995; Slob and Vreeburg, 1985). 2M female rats also show increased mounting behavior (Clemens et al., 1978) and these females became sterile faster and anovulatory sooner than 0M females when injected with testosterone, again showing an
increased sensitivity to testosterone (Tobet et al., 1985). Sensitivity to estrogen does not appear to be correlated with IUP, as measured by uterine weight and luteinizing hormone levels in mice (vom Saal and Bronson, 1978). However, more 0M males are mounted after treatment with estradiol than are 2M males, indicating that 0M males are more feminized than are 2M males (vom Saal, 1983). A pup’s prior IUP can clearly influence adult sensitivity to androgens. This sensitivity difference could theoretically alter any and all functions of the steroid hormones in adult animals.

IUP can influence the onset of puberty and reproductive capacity. 0M female mice show an earlier age at vaginal opening and earlier age at first estrus than do 2M females (Clark et al., 1991; Clark and Galef, 1988; Clark et al., 1993b; McDermott et al., 1978; Vandenbergh and Huggett, 1995; vom Saal, 1989b). Anogenital distance is a morphological measurement sensitive to prenatal testosterone levels (see 1.1.2 Morphology). A female rat with a short anogenital distance (more likely to be 0M) experiences vaginal opening and first estrus at an early age (Zehr et al., 2001). 0M female mice also produce more potent estrous delaying pheromones than do 2M mice, but do not appear to differ in sensitivity to males’ estrous promoting cues (Vandenbergh and Huggett, 1995; vom Saal, 1989b; vom Saal and Bronson, 1978; vom Saal et al., 1981).

A female mouse with a short anogenital distance is more likely to become pregnant (Drickamer, 1996) and 2M females produce fewer viable litters overall and cease giving birth at younger ages than do 0M females (vom Saal and Moyer, 1985). 2M females tend to produce larger litters, however, especially later in life (Vandenbergh and Huggett, 1995; vom Saal et al., 1991), whereas 0M mice produce more young in their first litter (Kinsley et al., 1986a).
IUP alters secondary sex ratios (i.e. the ratio of male pups to female pups at birth). 2M female gerbils give birth to almost 60% male pups. Conversely, 0M females, and singleton females, give birth to about 40% male pups. 1M mothers produce the traditional 50% sex ratio (Clark et al., 1993b; Clark and Galef, 1995; Clark et al., 1997). Similar reproductive outputs are also seen in mice (Hirlemann et al., 1990; Vandenbergh and Huggett, 1994; Vandenbergh and Huggett, 1995). These effects do not seem to be caused by selective cannibalism because the litter sizes of 0Ms and 2Ms are similar, and the ratios remain if the pups are delivered via a cesarean section. The mechanism behind this phenomenon is unknown; however, the timing of insemination may cause these shifts in the sex ratio. Female rats inseminated close to the time of ovulation produce more females than males, while females inseminated a few hours before or after ovulation produce more males than females (Hendricks and McClintock, 1990). Interestingly, a similar “U” shaped insemination curve has been reported in humans as well (for a review, see James, 1987). This phenomenon has never been reported in mice or gerbils, but 0M female mice (and rats) have shorter estrous cycles and are more sensitive to the presence of pheromones that may alter the length of the estrous cycle (vom Saal, 1989b; vom Saal and Bronson, 1978; vom Saal and Bronson, 1980b; vom Saal et al., 1981; vom Saal et al., 1990; Zehr et al., 2001).

This phenomenon of altered secondary sex ratio may serve as a form of non-genetic inheritance from mother to daughter. 0M females give birth to a high number of females and therefore, these females will more likely be surrounded by females in utero. In other words, a 0M female is likely to produce high numbers of 0M female. Similarly, a 2M female will be more likely to produce 2M offspring than will other mothers.
0M female mice are more sensitive to the environmental estrogen bisphenol A than are 2M females. When exposed to 2.4 µg/kg of this chemical prenatally, 0M females show an abnormally short interval between vaginal opening and first estrus. In addition, both male and female 0M and 1M mice are heavier at weaning than 2M mice (Howdeshell et al., 1999; Howdeshell and vom Saal, 2000). These findings demonstrate that pups developing in the 0M position are more sensitive to bisphenol A than other pups. These results are quite relevant to this project.

1.1.2 Morphology

Anatomically, a 0M female mouse has a shorter distance between the anus and the genital papilla than does a 2M female. This anogenital distance (AGD) difference is not surprising, as male rodents generally have longer AGDs than females. 2M female mice subjected to high levels of testosterone have a more masculine AGD. The AGD is simple to measure and, in mice, has become widely used as a marker for a female pup’s prior IUP or androgen exposure (Gandelman et al., 1977; Jubilan and Nyby, 1992; McDermott et al., 1978; Palanza et al., 1995; Vandenbergh and Huggett, 1995; vom Saal and Bronson, 1978; vom Saal and Dhar, 1992; vom Saal et al., 1990; Zielinski et al., 1991).

In addition to mice, female rats located downstream from males have longer AGDs than other females (Clemens, 1974; Houtsmuller et al., 1995; Richmond and Sachs, 1984) as do 2M females (Clemens, 1974; Tobet et al., 1982). This increase in AGD is most likely due to increased testosterone levels in utero because in rats, treatment with the anti-androgen flutamide abolishes the effect (Clemens, 1974; Clemens et al., 1978). In contrast to the findings in mice and rats, 2M male gerbils possess longer AGDs than 0M male gerbils, with females showing no
difference (Clark et al., 1990). It is unclear why male gerbils appear to be more sensitive to fetal steroids than female gerbils, while in other rodents females show a higher sensitivity.

In mice and rats, some of the variability present in the AGD can be explained by the weight of the animal being measured. Heavier animals tend to have longer AGDs than lighter animals. Therefore, a more accurate measurement can be obtained by dividing the AGD by weight, yielding an anogenital distance index (AGDI). The AGDI can, in some cases, serve as an accurate marker for a mouse pup’s prior IUP (Graham and Gandelman, 1986; Vandenbergh and Huggett, 1994; Vandenbergh and Huggett, 1995) and for a rat pup’s prior IUP (Meisel and Ward, 1981). A number of studies, however, have found that variations in weight do not account for a significant portion of the variability in AGD measurements (Palanza et al., 2001; vom Saal and Dhar, 1992). A sensible policy would be to use an analysis of covariance to evaluate the importance of weight to the variability seen in AGD measurements before calculating an AGDI. The AGD-IUP correlation allows for the identification of an adult animal’s prior fetal position and in some cases eliminates the need for cesarean sections. Using the AGD as a biomarker for intrauterine hormone exposure is simple and non-invasive and can be used on wild caught animals. This technique makes field research on IUP effects a possibility (see Massey and Vandenbergh, 1980 and Massey and Vandenbergh, 1981 for examples of controlled experiments in field rodent populations and Drickamer, 1996; Palanza et al., 1995; Zielinski et al., 1991 for examples of IUP research on field rodent populations).

2M male mice have larger seminal vesicles and smaller prostate glands than do their 0M brothers. These 2M mice also show lower androgen binding levels in their prostates but no difference in estrogen binding levels (Nonneman et al., 1992). Similarly, 2M male rats (van der Hoeven et al., 1992) and gerbils (Clark et al., 1990; Clark et al., 1993a) possess heavier testes
than do 0M males. In contrast, male testis weights in swine do not vary with IUP (Rohde Parfet et al., 1990).

1.1.3 Behavior

Adult 2M female mice injected with testosterone show greater frequencies of chasing and biting, and initiate fights more often than do 0M females receiving the same treatment. 2M females also begin displaying aggression after a shorter duration of testosterone treatment than do 0M females (Gandelman et al., 1986). This again shows that 2M females are more sensitive to testosterone as adults than are 0M females. IUP not only alters sensitivity to testosterone, but it also influences endogenous levels of aggression. 2M female mice initiate more fights than do 0M mice, 2M mothers fight for a longer duration than do 0M mothers and pregnant 2M mice show more intense aggression than do 0M mice (Kinsley et al., 1986b; Quadagno et al., 1987; vom Saal and Bronson, 1978). One study contradicts this, however (Hirlemann et al., 1990). These differences in aggression may be related to lower anxiety levels, as masculinized 2M female mice show less shock avoidance than 0M females (Hauser and Gandelman, 1983).

Similarly to females, castrated 2M male mice require a shorter duration of testosterone treatment to induce aggressive behavior than do 0M males. This suggests that 2M male mice are more sensitive to testosterone than are 0M males despite the fact that 2M male fetuses have never been shown to be exposed to higher levels of testosterone in utero (vom Saal et al., 1983). Conversely, castrated 2M males show greater parental behaviors than castrated 0M males (vom Saal, 1983; vom Saal, 1989). These results seem conflicting, but may simply indicate that prenatal androgen exposure stimulates parental behavior and permits later androgen treatment to induce aggression (see vom Saal, 1983 for a discussion of this). In contrast, IUP does not appear to correlate with maternal behavior in female mice (Kinsley et al., 1986a). Even though no
studies have demonstrated a correlation between IUP and AGD in male mice, males with large AGDs are more aggressive than males with small AGDs (Drickamer, 1996).

0M male mice prefer greater amounts of a saccharin solution than do 2M males (Bushong and Mann, 1994) and 0M females are more likely to compete for and steal food from another animal (Quadagno et al., 1987). This seems to conflict with other work that shows 2M mice to be heavier and more aggressive than their 0M siblings (Kinsley et al., 1986b; vom Saal et al., 1983). Differing metabolism in the 0M and 2M mice, however, could explain these discrepancies. 0M animals may have an increased motivation for food that drives them to be aggressive in certain situations.

When treated with testosterone, 2M female mice (Quadagno et al., 1987; Rines and vom Saal, 1984) and rats (Clemens et al., 1978; Meisel and Ward, 1981) are more likely to exhibit mounting behavior than are 0M females, as are female rats located distally to male rats (Clemens, 1974; Clemens and Coniglio, 1971; Houtsmuller and Slob, 1990). A few studies, however, have found no correlation between mounting behavior and IUP in rats (Slob and van der Schoot, 1982; van de Poll et al., 1982). These studies, however, used crude classifications of IUP and a lengthy period of hormone treatment, factors which may have obscured any potential differences.

0M female mice show a higher lordosis quotient and are more likely to receive a male’s first ejaculation than are 2M mice (Rines and vom Saal, 1984). Similarly, female rats located downstream from at least two males show a decreased lordosis quotient (Houtsmuller and Slob, 1990). In contrast, however, Zehr, et al. (2001) found that female rats with a short AGD had less intense lordosis responses. This discrepancy in results may arise from the method of inducing lordosis (male-induced vs. manually palpated) or by the method of classifying the rats.
(downstream from males vs. short AGD). Yet another study found no effect of IUP on lordosis (van de Poll, 1982). This study used an imprecise method of calculating IUP (number of males per litter), however.

Compared to a 2M female, a 0M female mouse is more likely to be chosen by a male, mounted by a male and is more likely to be attacked when rejecting a male’s advance (vom Saal and Bronson, 1978; vom Saal and Bronson, 1980a). Male mice are also more attracted to the odors of females with short AGDs (Drickamer et al., 2001). 0M female mice mate at a younger age than do 2M females (vom Saal, 1989b), but do not differ in their ultrasonic mating vocalizations (Jubilan and Nyby, 1992). These results suggest that 0M females are more attractive to males than their 2M sisters. This attraction may be mediated olfactorally.

Both 2M male and female pups receive more attention (i.e. anogenital licking) from their parents relative to their 0M brothers and sisters (Clark et al., 1989). High levels of anogenital licking may explain the increased number of motor neurons observed in the spinal nucleus of the bulbocavernosus of 2M female gerbils (Forger et al., 1996), as increased stimulation increases these motor neurons in rats. Interestingly, anogenital stimulation also promotes hippocampal synaptogenesis and mRNA expression important for brain functioning in rats (Liu et al., 2000). This difference in attractiveness between 0M and 2M individuals could alter mate selection and potentially influence the number of offspring an individual produces, as well as higher level brain functioning.

1.1.4 Other Species

The vast majority of work on IUP effects has been conducted on laboratory mammals. This poses a potential problem. Laboratory animals are generally inbred and therefore have low
genetic diversity. The reported IUP effects could be construed purely as a laboratory phenomenon. The genetic diversity of a wild population could theoretically mask the IUP effects that are seen in laboratory colonies. Fortunately, a number of studies have been conducted on non-laboratory species, including humans, which may clarify this issue. The results of these studies show that IUP effects can influence heterogeneous populations. I will review the limited work completed to date in this field. More studies must be completed, however, before the true importance of IUP effects in humans or wild populations can be determined.

Female wild house mice from a 2M position have a longer AGD than mice from a 0M position (Palanza et al., 1995; Zielinski et al., 1991). Females with long AGDs also elicited more urine marking in male mice (Palanza et al., 1995) and females with short AGDs were more likely to be pregnant and more likely to be in estrus (Drickamer, 1996). In addition, males and females with long AGDs were more aggressive than mice with short AGDs (Drickamer, 1996; Palanza et al., 1995). Males with long AGDs are more likely to disperse than males with short AGDs (Drickamer, 1996) and 2M males and females maintain larger home ranges than 0M mice (Zielinski et al., 2001). These results suggest that both male and female mice with long AGDs are masculinized in a similar fashion to laboratory mice. The parallel between these results and the results seen in laboratory colonies indicate that IUP effects are not masked by the genetic diversity of a wild population.

Large litters of four pups (the average litter size is 2) in the California mouse (Peromyscus californicus) have a high proportion of males. This could be due to selective resorption or cannibalism in large litters to favor males, which are slightly smaller at birth and may be less costly to raise (Cantoni et al., 1999). This is similar to the phenomenon seen in
many animals, including swine, where one sex of offspring is favored in times of stress (Chen and Dzuik, 1993). In addition, the California mouse shows a similar trend in anogenital morphology as other rodents. The AGDs of females from male biased litters were longer than the AGDs of females from female biased litters (Cantoni et al., 1999).

A study with laboratory-bred hamsters (presumably *Mesocricetus auratus*) showed elevated estrogen levels and depressed androgen levels in males located downstream from two or more females. Unlike many of the other species studied, however, androgen levels in female hamsters did not correlate with the number of males located upstream (Vomachka and Lisk, 1986). It is unclear why androgen levels do not correlate with the number of males in utero. It is possible that male hamster fetuses produce abnormally low levels of testosterone throughout development or produce high testosterone at a different time than was measured (gestational day 14).

Female grey-sided voles (*Clethrionomys rufocanus*) from male biased litters show a greater tendency to disperse than females from female biased litters (Ims, 1989; Ims, 1990). This result shows a possible masculinization effect, as dispersal is more common in male voles. Meadow voles (*Microtus pennsylvanicus*) from male biased litters performed better on a water-maze task than voles from female-biased litters (Galea et al., 1994). This suggests that IUP alters nervous system development in voles, as seen in other rodent species.

Very few IUP studies investigating non-rodent species exist. The studies that have been completed produced interesting results, and this gap in the IUP literature should be addressed. Sheep (presumably *Ovis aries*) normally give birth to one offspring, however litters of two or three are not uncommon. Avdi and Driancourt (1997) investigated a number of reproductive parameters with these twin and triplet offspring. They found that a lamb born with one or two
male siblings had abnormally high embryonic mortality when pregnant as adult in comparison to lambs born with females siblings.

Female swine from female biased litters (thus more likely to be 0M) are inseminated more often and have fewer pregnancy failures than females from male biased litters (more likely to be 2M) (Drickamer et al., 1997) and have more teats than females from male-biased litters (Drickamer et al., 1999). 0M female swine are also more receptive to male advances (Rohde Parfet et al., 1990). Consequently, females exposed to few males in utero should have increased reproductive capabilities as adults.

Female ferrets (presumably Mustela furo) located downstream from at least two males contain higher levels of androgens than other females (Krohmer and Baum, 1989). With differing steroid levels, it is possible that female ferrets show some of the IUP effects seen in other mammals. To date, however, no studies have demonstrated these potential effects.

1.1.5 Humans

A small number of studies conducted on human twins have produced interesting results. Humans show dental asymmetry, with males generally possessing larger teeth in the right jaw. In opposite sex dizygotic twins, however, this sexual dimorphism disappears. In addition, females who had an opposite sex twin show high levels of sensation seeking (sky-diving, for example) (Resnick et al., 1993). Other minor differences have been found as well (for a review, see Miller, 1994). These studies should be interpreted cautiously because they are based more on human judgment than scientific quantification, or use non-randomly chosen participants.
Loehlin and Martin (1998) completed an extensive survey of characteristics from a large pool of Australian twins. They found some minor differences between opposite sex twins and same sex twins, such as a higher rate of premature babies in opposite sex twins. Resnick et al. (1993) also found that female, opposite sex dizygotic twins showed higher levels of sensation seeking than did female, same sex dizygotic twins.

Researchers have also investigated spontaneous otoacoustic emissions (SOAEs) and click-evoked otoacoustic emissions (CEOAEs) in opposite sex twins. SOAEs are continuous, tonal sounds produced naturally in the cochlea whereas CEOAEs are sounds produced in the cochlea in response to a click stimulus. Females generally exhibit a higher frequency of SOAEs and CEOAEs than males. McFadden (1993) found that females with a male twin demonstrated significantly less SOAEs than other females. Females with a male twin also demonstrated less CEOAEs, although the difference did not reach significance (McFadden, 1996).

Taken as a whole, the research conducted to date on humans suggests that twins may be subjected to some minor hormonal influences in utero. These hormonal influences do not appear to cause the same level of modifications in humans as they do in the other mammals with larger litters.

In comparison with rodents, few IUP effects have been reported in humans. As a result, it could be construed that humans are simply not sensitive to intrauterine hormonal insult. A body of research on congenital adrenal hyperplasia however, indicates that humans are indeed sensitive to the prenatal hormonal environment.
1.2 Congenital Adrenal Hyperplasia

The adrenal gland is responsible for production of glucocorticoids, mineralcorticoids and androgens. Disruption of adrenal functioning, therefore, can alter profiles in each of these hormone classes. One such adrenal disorder is congenital adrenal hyperplasia (CAH). In CAH, the activity of one enzyme, 21-hydroxylase, is reduced or completely eliminated via alteration or deletion of the CYP21 gene (New, 2004).

CAH is typically split into three broad categories. Salt wasting CAH (SW-CAH) occurs when the activity of 21-hydroxylase is completely eliminated. Simple virilizing CAH (SV-CAH) is associated with enzyme activity less than 10% of normal and non-classical CAH (NC-CAH) occurs when enzyme activity is below 50% of normal (New, 2004; Therrell et al., 1998).

All forms of CAH show abnormally low levels of glucocorticoid release by the adrenal gland (in addition, SW-CAH results in low levels of mineralcorticoids). This low glucocorticoid level results in elevated concentrations of corticotrophin releasing factor and resulting high concentrations of adrenocorticotropic hormone (ACTH). ACTH stimulates the adrenal gland and results in elevated levels of adrenal androgen production. The androgens produced by the adrenal are typically androstenedione and dehydroisoandrosterone. Both of these androgens can be metabolized into testosterone and dihydrotestosterone (Migeon and Wisniewski, 2003).

Abnormal androgen production by the adrenal begins during development of the fetus and continues until treated. Treatment typically consists of corticosteroid replacement (and mineralcorticoid replacement in SW-CAH). This reestablishes the normal negative feedback loop present in the hypothalamus-pituitary-adrenal axis and restores normal adrenal function. This hormone replacement, however, cannot begin until after birth. Therefore, individuals with CAH are exposed to high levels of androgens throughout development in utero.
Physical symptoms of CAH vary depending on the severity of the disorder, and are typically seen only in females. NC-CAH symptoms range from nothing to mild virilization of the genitalia that develops postnatally, and sometimes as late as puberty. SV-CAH symptoms include moderate virilization of the genitalia, including an enlarged clitoris and reduced vaginal opening and are present at birth. SW-CAH symptoms are similar but more severe and may result in incorrect sex assignment at birth (with an XX individual being designated male). Although controversial, surgical reconstruction of the virilized genitalia is common in both SV-CAH and SW-CAH in an effort to create a “normal” female vagina (Edmonds, 2003).

The classical forms of CAH (SW-CAH and SV-CAH) occur in approximately 1 out of every 15,000 live births (Therrell et al., 1998), and individuals with classical CAH have been studied to determine the psychological effects of exposure to elevated levels of prenatal androgens. This work is loosely based on a body of research (including IUP effects) showing abnormal behavior in female rodents exposed to elevated prenatal androgens. CAH allows researchers the rare opportunity to investigate how sexually-dimorphic behaviors are organized in humans.

To date, researchers have made a number of intriguing findings by studying the behavior of girls with CAH. Girls with CAH appear to possess slightly skewed gender identity scores, with CAH females possessing a more masculine score than control females (Berenbaum and Bailey, 2003; Meyer-Bahlburg et al., 2003). Other research has directly linked prenatal testosterone to gender identity, (Hines et al., 2002), as well as indirectly to a variety of other behaviors (for a review, see Hines, 1998).

Gender identity shows a very strong sexual dimorphism in the general population. However, less markedly dimorphic measures can be influenced by CAH as well. Girls with
CAH show improved performance on certain spatial tasks when compared to control girls (Hampson et al., 1998). In addition, girls with CAH show less interest in infants than do control girls (Berenbaum, 1999a). Both of these measures show only mild sexual dimorphism in the general population, but are nonetheless still influenced by CAH.

Girls with CAH also tend to play more with toys typically favored by boys (Berenbaum et al., 2000; Berenbaum and Hines, 1992; Nordenstrom et al., 2002). This finding would seem likely to be caused primarily by social factors; however similar findings have also been reported in nonhuman primates (Alexander and Hines, 2002), indicating that sexually dimorphic play behavior may have some biological root causes.

Female adolescents with CAH prefer male-typical activities (sports, hunting, manual labor) more often than do control girls (Berenbaum, 1999b; Berenbaum et al., 2000). CAH girls also choose to play with boys a higher percentage of the time than do control girls, although the level of rough and tumble play is the equivalent in CAH girls versus control girls (Hines and Kaufman, 1994). In addition, female CAH adolescents and adults (but not children) score higher in a variety of aggression indices (Berenbaum and Resnick, 1997).

It has been hypothesized that elevated prenatal testosterone levels can predispose an individual to developing learning disorders. Some research indicates that CAH children may have an elevated risk for learning disabilities and associated brain abnormalities that correlate with some learning disabilities (Plante et al., 1996); however, this study showed this elevated risk was present in the entire family, including unaffected siblings.

Not all studies on CAH females have had such positive findings. Many early studies showed mixed results, however it has been argued that many of these studies had methodological
flaws (for a review of these as well as a more complete review of older CAH research see: Berenbaum, 1999a; Berenbaum et al., 1995).

It might be expected that boys with CAH would also show some behavioral abnormalities. Males are normally exposed to high levels of androgens prenatally, but only during specific periods of development. In contrast, boys with CAH are exposed to high levels of androgens throughout development. Despite this lengthened exposure to androgens, males with CAH show behavior that is generally not different from controls (Berenbaum, 1999b). This likely indicates the presence of sensitive periods to androgens. Male fetuses are normally exposed to high levels of androgens starting approximately halfway through development in utero. Fetuses with CAH are exposed to androgens much earlier in development, but if the fetus is not sensitive to their effects at these times, then this abnormal exposure would not produce any behavioral or physiological abnormalities. This appears to be the case in boys with CAH.

A few exceptions need to be noted, however. CAH boys show less rough-and-tumble play than control boys (Hines and Kaufman, 1994). This finding was unexpected and its effect was in the opposite direction than would be anticipated (i.e. CAH boys, exposed to high androgens, were demasculinized). A possible explanation emerges from a discussion on sensitive periods. Male fetuses with CAH are exposed to high levels of androgens early in development. This exposure may not directly alter behavior, but may down-regulate androgen (or estrogen) receptors in certain brain areas, making them less responsive to the masculinizing effect of androgens during the later sensitive periods. This would result in a demasculinized fetus. The receptor down-regulation could be quite modest, and might impact only the most sensitive behaviors such as rough-and-tumble play, which has been shown to be very sensitive to anti-androgen treatment in the rat (Hotchkiss et al., 2003). In addition, CAH boys have smaller
(more feminine) amygdalas than do control boys (Merke et al., 2003). This seems to lend support to the idea that CAH may, in fact, slightly demasculinize males. It is possible that boys with CAH may have other behavioral abnormalities that have gone unnoticed because they are not in the expected direction.

Females also have sensitive periods in their development, presumably. However, female fetuses are normally exposed to very low concentrations of androgens throughout development. So, in normal development, female fetuses are exposed to low concentrations of androgens during their sensitive periods, unlike males. Females with CAH, therefore, have no protection from the developmental effects of abnormally high androgen levels. This likely explains why females seem more susceptible to CAH than males, even though both are exposed to abnormal patterns of androgens.

CAH studies have received a fair amount of criticism. In general, most of the criticism does not focus on whether behavioral differences exist, but rather on the cause of these differences. Most researchers believe that the differences in behavior arise due to abnormal levels of prenatal androgens, masculinizing the girls with CAH (Berenbaum, 1999a). Critics of this “hormonal hypothesis” assert that the differences in behavior arise from different social pressures placed on girls with CAH. For example, the parents of girls with CAH are certainly aware of their daughters’ condition and therefore may (even unconsciously) treat them differently. In addition, girls with CAH themselves will know they are different, due to their virilized genitalia and/or having undergone corrective surgeries. This knowledge of being different may cause girls with CAH to act differently and result in others treating them differently (Fausto-Sterling, 2000). These confounding variables are difficult to control. For example, the degree of genital virilization seems to be strongly correlated with the level of
prenatal androgen exposure (although this has not been specifically tested) (Therrell et al., 1998). Therefore both the “social hypothesis” and the hormonal hypothesis would predict the same individuals to be the most masculine. For these and other reasons, proponents of the social hypothesis contest that it is inaccurate to ascribe the differences in behavior simply to abnormal hormonal levels in utero (see Fausto-Sterling, 2000 for a more complete critique of CAH research). Recent CAH research, however, has taken into consideration these criticisms. For example, Berenbaum et al. (2000) measured and found no effect of social factors such as parental influence. Given the strong scientific support of prenatal androgens altering behavior in non-human species, as well as the general consistency of results in the CAH field, it seems highly unlikely that the effects of CAH on behavior are mediated predominantly through social factors, as Fausto-Sterling (2000) contends.

It is clear that many species, including humans, are sensitive to prenatal hormonal insult. If exogenous sources of hormones existed, there may be cause for concern; as developmental exposure to these compounds could lead to permanent alterations in physiology, morphology and behavior in humans as well as wildlife. This theory of “endocrine disruption” was championed by Theo Colborn who first introduced the idea to the scientific community (Colborn et al., 1993) and then to the general public in her 1996 book, Our Stolen Future.

An endocrine disruptor is any chemical not normally found in the body, but can nonetheless interact with the endocrine system and interfere with normal endocrine function. Such compounds can be naturally occurring or man-made and could theoretically interact with the endocrine system by binding directly to hormone receptors, altering receptor number, or altering production, transport and metabolism of hormones. Endocrine disruptors have been identified that interact with the thyroid, androgen and estrogen pathways, among others. This
project, however, focuses on estrogenic endocrine disruptors, otherwise known as environmental estrogens.

1.3 Environmental Estrogens

Both natural and man-made environmental estrogens have been identified. The majority of natural environmental estrogens fall into the category of phytoestrogens. Phytoestrogens, as the name suggests, are environmental estrogens found in plants. Man-made estrogens come from a variety of sources; however many of these compounds are used in the pharmaceutical and the plastics industries. This project focuses on one chemical from each of these man-made sources, bisphenol A (a component of plastic) and ethinyl estradiol (a pharmaceutical).

1.3.1 Bisphenol A

Bisphenol A (BPA) is a synthetic compound used to make polycarbonate plastic and epoxy resins. It was first synthesized in 1905 by the German scientist Thomas Zincke. In the 1930’s, it was discovered that BPA was estrogenic when fed to ovariectomized rats (Dodds and Lawson, 1936). It was not until the development of polycarbonate plastics in the 1950’s however, that BPA began being used industrially. BPA itself is a relatively simple molecule consisting of two phenol rings connected by a central propane (See Figure 1.2).
This simple molecule can polymerize across its hydroxy functional groups and this polymer forms the basis for polycarbonate plastic. Introducing this polymer to water will reverse some of the ester bond formations, resulting in free monomers of BPA. This process is enhanced by heat and an alkaline environment (Sajiki and Yonekubo, 2004). Therefore, the usage of polycarbonate plastic and epoxy resins will introduce BPA into the environment.

Significant levels (up to 1900 ng/L) of bisphenol A have been measured in surface water sources (Rippen, 1999), with higher levels from certain point sources, such as paper manufacturing mills (up to 70 µg/l) (Furhacker et al., 2000). Most, but not all, of this BPA is removed during wastewater treatment (Drewes et al., 2005). Consequently, BPA has been detected in European drinking water at levels up to 2 ng/l (Kuch and Ballschmiter, 2001).

In addition to the water supply, humans living in industrialized (i.e. plasticized) countries are subjected to BPA from a variety of sources. Dental sealants contain BPA, and this BPA can leach into the saliva. This leaching is highest immediately after receiving the sealants, and salivary levels have been conservatively measured as high as 40 ng/ml saliva (Olea et al., 1996; Sasaki et al., 2005).

Many metal cans, especially those used in the food industry, contain an inner resin lining. This lining contains BPA which will migrate to the contained food during processing and storing.
(Goodson et al., 2004; Howe et al., 1998; Kang and Kondo, 2002). Plastic stretch film, used in many food applications, also leaches BPA up to 30µg/dm² (Lopez-Cervantes and Paseiro-Losada, 2003).

New polycarbonate baby bottles leach BPA and continue to leach it at higher levels (up to 8 µg/l) as they are used and washed (Brede et al., 2003). Powdered infant formula also contains BPA at levels ranging from 40-100 ng/g (Kuo and Ding, 2004).

The prevalence of BPA in the water supply, paired with the levels of BPA found in plastics and resins, results in the exposure of nearly all individuals in developed countries to this compound. As a result, BPA appears in 95% of U.S. urine samples nationwide with a mean level of 1.33 µg/L (Calafat et al., 2005) and is common in Japanese urine samples as well (Matsumoto et al., 2003; Ouchi and Watanabe, 2002). In addition, a study of pregnant women in Germany consistently found BPA in the placental tissue (12.7 ng/g), maternal plasma (3.1 ng/ml) and fetal plasma (2.3 ng/ml) (Schonfelder et al., 2002). Furthermore, pregnant women in Japan possess amniotic fluid levels of BPA around 8 ng/ml (Ikezuki et al., 2002).

In mammals, orally administered BPA is metabolized primarily to its monoglucuronide by the liver, although unchanged BPA, sulfated BPA and hydroxylated BPA are also excreted. This was first reported in rats (Knaak and Sullivan, 1966) and has been more recently confirmed in rats (Jaeg et al., 2004; Pottenger et al., 2000), monkeys (Kurebayashi et al., 2002) and humans (Volkel et al., 2002). The major metabolite, BPA-monoglucuronide, has negligible estrogen activity (Matthews et al., 2001).

BPA has a low affinity for plasma binding proteins in comparison to estradiol (Nagel et al., 1997; Nagel et al., 1999). This results in more of the compound remaining free, and active in the body. This also means that in vitro assays of estrogenicity (such as a breast cancer cell line,
yeast assay or estrogen receptor binding assay) may underestimate its \textit{in vivo} estrogenicity, as these assays do not account for the ratio of bound to free chemical in the blood. Plasma binding proteins protect a chemical from metabolism, so BPA’s low affinity for these proteins also contributes to its short half-life.

In humans, the half-life of BPA is approximately five and one half hours, with all the BPA from a single dose being excreted after twenty-four hours (Volkel \textit{et al.}, 2002). Interestingly, it has been shown that pregnant rats excrete BPA at a lower rate than non-pregnant rats, most likely because the activity of microsomal UDP-glucuronosyltransferase is cut in half during this time (Inoue \textit{et al.}, 2005; Matsumoto \textit{et al.}, 2002). This places fetal animals at a higher risk for exposure.

Not only do pregnant rats excrete BPA at a lower rate, but they also pass this BPA onto their developing offspring. BPA has been detected in fetuses within one hour of injecting the mother with the compound (Shin \textit{et al.}, 2002) and BPA has been shown to activate estrogen receptors within fetuses eight hours after the mother is exposed (Lemmen \textit{et al.}, 2004). BPA levels in the uterus are 20% higher than levels in the plasma. In addition, the blood/brain barrier does not prevent the movement of BPA (Kim \textit{et al.}, 2004) in adults and is unlikely to in fetuses. BPA is also passed through the mammary gland and excreted in milk (Hong \textit{et al.}, 2004; Yoo \textit{et al.}, 2001), placing nursing animals at risk.

The general consensus of the published literature is that the exposure to BPA from any one source is quite low. Pairing this exposure with the fact that BPA is rapidly cleared from one’s system might lead to the conclusion that BPA is of little concern to the general population. However, individuals are likely exposed to multiple sources of BPA on a regular basis. This explains the high prevalence of BPA in urine samples found by Calafat \textit{et al.} (2005). There are
certain to be individuals who are exposed to higher levels of BPA (those exposed to high levels of plastics, for example). In addition, among those individuals who are exposed to higher than average levels, there are certain to be some who are more sensitive to endocrine disruption (such as developing fetuses or infants). Following this logic, it is almost a certainty that individuals are exposed to levels of BPA that will disrupt normal endocrine function. The next step is to determine the effects of this exposure.

Bisphenol A has been shown to have weak, but significant, estrogenic effects which can influence rodent physiology when administered at biologically relevant doses impacting varied endpoints such as the onset of puberty (Howdeshell et al., 1999), meiosis (Hunt et al., 2003), the morphology of the prostate in males (Timms et al., 2005; Welshons et al., 1999) and genitalia in females (Markey et al., 2005). BPA does not appear to be androgenic or anti-androgenic in mammals (Kim et al., 2002; Yamasaki et al., 2003).

It is assumed that BPA works primarily via the two mammalian intracellular estrogen receptors, stimulating dimerization of these receptors (Schwartz-Mittelman et al., 2005) which in turn triggers down-stream effects on gene expression of the cell. It is unclear however, if BPA binds preferably to one ER over the other (see Kuiper et al., 1997 and Kurosawa et al., 2002, for example). Interesting recent research indicates that ERα can be located on the cell membrane and that BPA can bind to this receptor, triggering rapid calcium influxes (Wozniak et al., 2005).

A main goal of this project is to see if environmentally relevant doses of bisphenol A can specifically alter mammalian behavior, an endpoint that had been neglected in earlier studies. Since the inception of this project, new research has been published which shows that bisphenol A can indeed alter behavior.
Adolescent female rats exposed developmentally to BPA show altered play behavior (Dessi-Fulgheri et al., 2002; Porrini et al., 2005), altered novelty seeking (Adriani et al., 2003), an altered response to pain (Aloisi et al., 2002), altered maternal behavior (Palanza et al., 2002) and altered activity levels (Kubo et al., 2003). Fewer studies have shown developmental effects on male rodents; however male mice exposed developmentally to BPA do show increased aggression (Kawai et al., 2003). In addition, nursing rat dams exposed to BPA show decreased maternal behavior (Seta et al., 2005), which could negatively impact development of the young and have permanent effects.

It is important to continue research to investigate the behavioral consequences of developmental exposure to BPA. Behavior, being the end coordinated result of a variety of inputs and body systems, may be uniquely sensitive to endocrine disruption. A slight alteration in any of the involved systems could drastically impact the end behavior.

1.3.2 Ethinyl Estradiol

Another common estrogenic endocrine disruptor is ethinyl estradiol (EE). This compound is identical to beta estradiol with the addition of an ethinyl group at the seventeenth carbon (See Figure 1.3).
EE became widely available pharmaceutically in the 1960’s and has remained in use since then. This compound is most commonly used as the active estrogenic component of the contraceptive pill, but it is also prescribed to reduce the effects of menopause. EE is readily absorbed by the gastrointestinal tract and is resistant to metabolism in the gut due to the ethinyl functional group. As a result, metabolism is slow compared to other estrogens, with a half life in humans between thirteen and twenty-seven hours. Ethinyl estradiol is metabolized primarily via hydroxylation and the formation of methyl esters (Hardman, 1996).

The widespread use of EE in pharmaceuticals leads to the introduction of this chemical into the environment. EE has been detected in European effluents and surface water in concentrations ranging from 0.5 to 7 ng/L but as high as 50 ng/L in some cases (Desbrow et al., 1998; Larsson et al., 1999; Routledge et al., 1998; Ternes et al., 1999). With the widespread use of EE and its presence in surface water, wildlife and accidental human exposure certainly occurs.

EE is a strong estrogen, comparable in strength to 17β-estradiol (Folmar et al., 2002). Rodents exposed developmentally to EE show disrupted morphology and physiology such as
alterations in the testis (Fisher et al., 1999), deficits in spermatogenesis (Atanassova et al., 1999), and altered prostate growth (Thayer et al., 2001) in males as well as altered morphology (Sawaki et al., 2003) and gene expression (Naciff et al., 2002) in the female reproductive system. EE does not appear to have any androgenic or anti-androgenic qualities (Yamasaki et al., 2003).

Despite its prevalence in the environment and its clear estrogenicity, almost no studies in mammals have investigated the behavioral repercussions of developmental exposure to the chemical. Rats exposed developmentally to EE do show increased activity and anxiety-like behavior (Arabo et al., 2005; Dugard et al., 2001), however. This obvious gap in the literature must be addressed.

Past research on estrogenic endocrine disruptors has focused on reproductive endpoints. However, estrogens can influence many body systems, not just the reproductive organs. This project focuses on the non-reproductive, behavioral changes due to environmental estrogen exposure.

1.4 Project Goals

The main goal of this project is to determine the effects on behavior of developmental exposure to environmentally relevant levels of estrogenic endocrine disruptors. To date, the majority of studies on endocrine disruptor action have been based on anatomical and physiological reproductive endpoints. Studying behavior has a number of advantages. Steroids and steroid modulators have vastly different effects on the reproductive system in comparison to the nervous system. Estrogens, for example, will masculinize the rodent nervous system and feminize the reproductive system. Studying behavior therefore, will potentially uncover new
consequences of endocrine disruptor exposure, new modes of action for known endocrine
disruptors, and even identify altogether new endocrine disrupting compounds.

Mammalian behavior is the end result of a large number of stimuli from a variety of body
systems. These inputs are integrated by the nervous system and result in an action also utilizing
multiple body systems. A slight disruption in any one of these components (incoming stimuli,
integration, various outputs) can alter the end result, making behavior potentially quite sensitive
to disruption.

In this project, I take a first step in pairing endocrine disruption and behavior by
surveying a number of different behavioral assays in two commonly used mammalian model
organisms: rats (*Rattus norvegicus*) and mice (*Mus musculus domesticus*). The experiments in
this dissertation focus entirely on estrogenic endocrine disruptors. These choices were not meant
to minimize the importance of other model organisms or other classes of endocrine disruptors.

Using this approach, I was able to determine the appropriateness of using behavioral
assays. I also gained some insight into the sensitivity of these assays as well as the sensitivity of
the two model organisms used. Lastly, and most importantly, any increase in the overall
understanding of endocrine disruptors allows for more accurate extrapolations into their effects
on wildlife and human health.
2. GENERAL METHODOLOGY

I investigated two different environmental estrogens at multiple doses. It is important to include some environmentally relevant doses, so that conclusions can be drawn as to the relative risk these chemicals pose to humans and wildlife, as well as gain important dose response information on each chemical. The experiments in this project followed the dose ranges used in prior studies, most notably Sawaki et al. (2003) for ethinyl estradiol (EE) and vom Saal et al. (1998) for bisphenol A (BPA).

Prior research with endocrine disruptors has generally utilized a prenatal or perinatal administration schedule in order to maximize the organizational effects of the chemicals. In contrast, prior behavioral research with estrogens has generally utilized an adult administration schedule to focus on the activational effects of these compounds. This discrepancy poses a potential problem. For this experiment, I used perinatal administration and focus on organizational effects. When appropriate however, I tested the compounds on adult animals. This allowed me to compare adult and prenatal sensitivities to these estrogens.

In rodents, the presence of estrogen during development masculinizes the nervous system. Since male gonads produce high levels of androgens (and low levels of estrogen) throughout development, estrogen is converted from testosterone in the appropriate tissues of the nervous system by the enzyme aromatase. Female gonads secrete low levels of estrogens and androgens during development, leaving the nervous system devoid of any exposure to masculinizing agents. For more complete reviews of this aromatization process see Gorski (1985) and Hutchinson (1997).
The prenatal presence of estrogens masculinizes rodent behavior. For this reason, female rodents are the more appropriate sex to use in this study, as they are not normally exposed to masculinizing agents prenatally. Male rodents would most likely be less sensitive to masculinizing agents and are therefore less appropriate model in this case.

Not all behaviors will be sensitive to endocrine disruption, however. For this reason, I have chosen to investigate sexually dimorphic behaviors which are organized prenatally or early postnatally through the actions of steroids. These behaviors should be the most sensitive to endocrine disruption.

Since these sexually dimorphic behaviors are organized during development, exposure to endocrine disruptors should also occur during development. Therefore, exposure occurred *in utero* and continued into early postnatal life. This exposure encompassed the period of sexual differentiation and early nervous system development. It is difficult to directly expose developing fetuses to external agents. Instead, the compounds were delivered orally to the mother, who then passed these compounds onto her litter. This most closely approximates a natural route of exposure for a developing organism.

This project did not focus exclusively on behavior. Reproductive endpoints were also measured. This allowed for comparisons of sensitivity between behavioral and reproductive endpoints. This also allows the study to be compared to and placed in context with past studies using similar experimental designs.

When studying rodents, the housing conditions are important to consider. The rodents in this study were housed in standard polycarbonate cages. Polycarbonate cages have been shown to leach environmental estrogens, including bisphenol A, under certain conditions (Howdeshell *et al.*, 2003), as have plastic rodent water bottles (Yoshida *et al.*, 2004), and this leaching can
disrupt cell functioning (Hunt et al., 2003). Since no alternative caging or bottle material was readily available at the time of the study, care was taken to ensure that all materials used were in good condition and showed no signs of degradation such as cracking or hazing.

Rodent chow is another important variable to consider. Standard rodent pellets (i.e. Purina Lab Diet 5001) contain a significant level of phytoestrogens (Thigpen et al., 2004) as well as bisphenol A (Yoshida et al., 2004). These compounds could potentially confound this study, by elevating the amount of estrogens to which control animals are exposed. Dietary estrogen exposure however, has little or no effect on the sensitivity of the uterotrophic assay to EE or BPA (Owens et al., 2003; Wade et al., 2003; Yamasaki et al., 2002), or with the toxicity of EE (Takagi et al., 2004).

Alternative rodent pellets utilizing casein as the main source of protein are available. However, these diets also have negative effects on rodents, increasing the likelihood of fat deposition and obesity (FS vom Saal, personal communication). These diets are also significantly more expensive than standard diets. Considering the expense, the imperfect nature of the alternative, as well as the lack of evidence supporting a diet-endocrine disruptor interaction, standard rodent chow was used in this project. The possible confounding factors associated with this diet would result only in increasing the likelihood of false negative results.

This project investigated estrogen-sensitive behaviors in adult female rodents. Estrogen levels cycle in an adult rodent, and this cyclicity would negatively impact the integrity of the studies. To eliminate this source of variability, the animals were surgically ovariectomized. This procedure is relatively simple. Under anesthesia, two small incisions are made in the flank of the animal through the skin and underlying musculature into the body cavity. Each ovary is identified, ligated with suture, and removed. The remaining portions of the uterine horns are
repositioned back in the body cavity and each incision is closed with a wound clip. An inhaled anesthetic was used to induce the proper surgical plane, and a moderate dose of buprenorphine was administered immediately before surgery to serve as an analgesic. The wound clips were removed one week after surgery, and the animals were given one additional week of recovery before being used in any behavioral assays.

All statistical analyses were completed on PC SAS, version 8.2 (Cary, NC). In every experiment, I had multiple treatment groups, so to calculate main effects I used the proc glm statement paired with “by” “class” and “model” statements as appropriate. When the p value for the main effect reached significance (p < 0.05), a post-hoc lsmeans statement was used to determine specific differences within the treatments.

There was a large number of different treatment groups used throughout these experiments. For simplicity, the treatment groups are abbreviated in this dissertation. For example, a 200 µg/kg/day bisphenol A treatment group would be abbreviated BPA 200. Similarly, a treatment group of 0.5 µg/kg/day ethinyl estradiol would be abbreviated EE 0.5.
3. METHODOLOGY - MOUSE STUDIES

Many different strains of laboratory mouse are widely available. One must be careful in choosing a strain to study, as different strains of mice have been shown to have differing levels of responsiveness to estrogen treatments (Spearow et al., 1999). CD-1 mice are less responsive to estrogen than many other strains. In one sense, therefore it would be beneficial to use this strain for the experiment. Any behavioral changes in CD-1 mice caused by estrogenic action would most likely be seen in other strains as well. By using CD-1 mice, though, it is more likely that no behavioral change will be detected. This makes negative results difficult to explain. Therefore, as this project is considered a first step in linking behavior and endocrine disruption, it would be best not to use CD-1 mice.

C-57/Bl-6 mice are more responsive to estrogen treatments than CD-1 mice (Spearow et al., 1999). In addition, C-57/Bl-6 mice are often used in radial maze experiments, one of the chosen assays for this project. For these reasons, the C-57/Bl-6 strain was used in this project.

The bedding used in the cages is an important variable to consider as well. Dried corn cob bedding (i.e. Bed-o’Cobs) has been shown to act as a mitogenic agent and disrupt cycling (Markaverich et al., 2002). Cellulose bedding (i.e. Alpha-Dri) has a compromised ability to absorb urine, resulting in a strong ammonia odor within the cage if the bedding is not changed every few days. These bedding materials were deemed inappropriate for this study, and instead, hardwood chip (i.e. Beta Chip) bedding was used, as no potentially confounding factors have been identified with this material.

Nulliparous female mice, originally obtained from Charles River Laboratories, were housed with a stud male and checked daily for vaginal plugs. When a plug was detected, the
male was removed from the cage. The female was considered to be pregnant and to be on gestational day one (GD-1). On GD-3, each female was randomly assigned to a treatment group and the exposure period was initiated. Each pregnant female was orally gavaged once daily with the appropriate compound dissolved in tocopherol-stripped corn oil. Oral administration occurred from GD-3 through weaning on postnatal day 21 (PND-21).

Multiple doses of bisphenol A were administered. A low, biologically relevant, dose of 2 µg/kg per day, as well as a higher dose of 200 µg/kg per day were chosen for this study. A third group of mice received ethinyl estradiol at a dose of 5 µg/kg per day. One final group received only tocopherol-stripped corn oil. These doses were based on past research (vom Saal et al., 1998) and will allow this study to be compared to other bisphenol A studies. Ideally, more doses would have been used to allow for a more accurate representation of dose responsivity; however the low-throughput nature of the behavioral assays used in the mouse studies made more dose groups unfeasible.

All mice were weaned on PND-21 and females were housed individually from this day forward. One week after weaning, the mice were surgically ovariectomized as described earlier. After a two week recovery period, each mouse, now an adult, was run through each behavioral assay. All mouse studies were conducted at the Biological Resources Facility at North Carolina State University, and every aspect of these studies was reviewed by the institutional animal care and use committee at North Carolina State University.
4. INVESTIGATION OF THE VEHICLE CONTROL – MICE

4.1 Introduction

Corn oil has previously been used as the vehicle for many experiments in our laboratory. Corn oil is derived from plant material, and it is possible that phytosterogens or other active endocrine substances may be present in the oil at sufficient levels to alter estrogen sensitive biomarkers in the mouse. Therefore, before corn oil is used as a vehicle in this project, its potential estrogenic activity needs to be investigated.

The immature mouse uterotrophic assay is a quick and simple test for estrogenic activity. After weaning (starting on day 22), young female animals were orally dosed with the compound of interest for three days. On the fourth day, the animals are killed and the uteri are weighed. The uterus in a prepubescent animal will be small; however, in the presence of an estrogen the uterus will enlarge and swell. Simply weighing the uterus will give an indication of estrogen exposure.

4.2 Methods

Eighteen C57/Bl-6 mice were dosed orally for three days with one of four corn oil solutions:

- Tocopherol-stripped corn oil
- Mazola brand corn oil
- ½ stripped, ½ Mazola corn oil
- 5µg/kg ethinyl estradiol (EE) dissolved in tocopherol-stripped corn oil
Each mouse received 20µl of the appropriate solution via oral gavage for three days. This volume of liquid was chosen because it approximated the amount of liquid that would be given to the pregnant dams in the other mouse experiments. On the fourth day, the animals were weighed, killed and the uterus was removed. When removing the uterus, it is important not to lose any fluid, so the uterus was first cut at the cervix and raised with forceps. With the cervical end of the uterus held and elevated with one hand, the uterus was cleared of excess fat and then cut away from the ovaries at the fallopian tubes. This method of excising the uterus minimizes any fluid loss. The uterus, including any retained fluid, was then weighed. This uterine weight was then divided by the body weight to yield a uterine index.

4.3 Results

There was a main effect of treatment on both the uterine weight (p < 0.0001) and the uterine index (p < 0.0001). Post hoc analyses showed that exposure to Mazola brand corn oil increased the uterine weight when compared to the stripped-oil control. This difference approached significance (p = 0.066) when the uterine weight was measured alone (Figure 4.1) and this uterine weight difference reached significance (p = 0.036) when the uterine weight was divided by the body weight (Figure 4.2). In both cases, the EE exposed, positive control animals had a significantly (p < 0.0001) increased uterine weight when compared to the stripped-oil control.
Figure 4.1: Uterine weight in 25 day old mice after three days of exposure to Mazola brand corn oil

Mean ± the standard error

+ p = 0.066 vs. stripped oil control

*** p < 0.0001 vs. stripped oil control

Mazola brand corn oil appears to have a uterotrophic effect which approached significance despite the small sample size.
Figure 4.2: Uterine Weight Index (Uterine Weight/Body Weight x 100) in 25 day old mice after three days of exposure to Mazola brand corn oil

Mean ± the standard error

* p = 0.036 vs. stripped oil control

*** p < 0.001 vs. stripped oil control

The trend seen in Figure 1 reaches significance when the body weight is factored into the analysis.
4.4 Discussion

Mice appear to show some sensitivity to estrogenic corn oil compounds. The mice exposed to Mazola brand corn oil showed a dose-dependent enlargement of the uterus. The fact that this response was seen at relatively low volumes (20 µl) is surprising. This volume is approximately the same volume that was planned for use in future behavioral mouse studies. For this reason, it was decided to use tocopherol-stripped corn oil for all future mouse studies in this project.

It is assumed that the uterotrophic activity of corn oil is caused by a naturally occurring phytoestrogen in the corn. Tocopherol-stripped corn oil is steamed to remove the vitamin content (tocopherol is vitamin E) in the oil (ICN Biomedicals Technical Services, personal communication); therefore the estrogenic agent could be tocopherol or some other molecule that is removed during the steaming process. This would not be the first implication of an endocrine disrupting chemical in corn. Markaverich et al., (2002) identified an agent in corn cob bedding, and later in corn tortillas, which was mitogenic in breast and prostatic cancer cell cultures.

Alternately, the estrogenic agent could have leached from the plastic bottle holding the Mazola oil, as the tocopherol-stripped oil was stored in a glass bottle. It would be interesting to further investigate the estrogenicity of corn oil. With the number of potential variables however, it was beyond the scope of this project.
5. GENERAL REPRODUCTIVE ENDPOINTS - MICE

5.1 General Introduction

A first step in determining any compound’s toxicity is to collect general, easily gathered information. Carefully chosen endpoints can offer insight into the general effects of a chemical without extensive effort. Such commonly used endpoints in rodent toxicology are litter size and pup weight. Toxic compounds will tend to decrease both of these measures. In addition to these measures of toxicity, any easily measured endpoints which may be sensitive specifically to endocrine insult would also be worth collecting. Two such endpoints are the onset of puberty, which can be accelerated by estrogen exposure, and the anogenital distance, which can be lengthened by exposure to androgens.

5.2 AGD and Litter Size

5.2.1 Introduction

Anogenital distance (AGD) is a measurement of the length of the tissue between the anus and the genital papilla. This distance is sexually dimorphic in mammals, with male animals possessing a longer AGD than female animals. This distance is influenced by androgens, so any toxicant that interferes with the androgen pathway would be expected to alter AGD. The AGD is partially correlated with body size, and dividing the AGD by body weight (yielding an AGD index or AGDI) controls for some of this variability (Vandenbergh and Huggett, 1995). Estrogens do not directly alter androgen pathways in the genitalia, however AGD is simple to
measure, and it is possible that high levels of strong estrogens may interfere with the
development of the genitalia, resulting in skewed AGD scores, as estradiol can bind to the
androgen receptor at very high doses (Bigsby et al., 1999)

Strongly toxic compounds can cause pup mortality in pregnant dams. Estrogens are not
generally considered toxic, but exposure to high levels of a strong estrogen during development
can cause pup death. Sawaki et al. (2003) found that developmental exposure to 50 µg/kg/day
EE increased pup mortality in rats. It is possible that mice are more sensitive to the toxic effects
of estrogens than rats. Recording litter size will give a good indication of this sensitivity.

5.2.2 Methods

Mice were housed and exposed to environmental estrogens as previously described. At
weaning (postnatal day 21), the litter size was counted, and pup weight and AGD was measured
and recorded. The AGD was measured by restraining the mouse in one hand, exposing the
abdomen. The thumb and forefinger were used to restrain the animal by the scruff of the neck
while the tail was held securely between the pinky finger and the base of the thumb. The AGD
was defined as the distance from the anterior end of the anus to the posterior end of the genital
papilla, and the measurement was taken with dial calipers accurate to 0.1 millimeters. The
AGDI was then calculated by dividing AGD by body weight. The same experimenter, blind to
the treatment groups, made these measurements in every mouse throughout the project.

5.2.3 Results

The developmental exposure had no effect on any of the measured variables.
AGD in females: p = 0.2812, in males: p = 0.6183 (data not shown).
AGDI in females: $p = 0.1611$, in males: $p = 0.5953$ (Figures 5.1 and 5.2).

Body weight at weaning in females: $p = 0.7709$, in males: $p = 0.7448$ (Figures 5.3 and 5.4).

Litter size: $p = 0.2271$ (Figure 5.5).
Figure 5.1: AGDI (AGD/body weight) by treatment in female mice.

Mean ± the standard error.

No group differed significantly from the stripped oil control.
Figure 5.2: AGDI (AGD/body weight) by treatment in male mice

Mean ± the standard error.

No group differed significantly from the stripped oil control.
Figure 5.3: Weight at weaning by treatment in female mice

Mean ± the standard error.

No group differed significantly from the stripped oil control.
Figure 5.4: Weight at weaning by treatment in male mice

Mean ± the standard error.

No group differed significantly from the stripped oil control.
Figure 5.5: Litter size at weaning by treatment in mice

Mean ± the standard error.

No group differed significantly from the stripped oil control.
5.2.4 Discussion

Neither BPA nor EE had any effect on the AGD, AGDI, body weight or litter size in the mouse. This strongly indicates that such measures are insensitive to the effects of estrogens and are not appropriate measures of their potential action in mice. Oral administration of EE and BPA to pregnant dams has been shown to be maternally toxic and toxic towards developing mouse fetuses (Morrissey et al., 1987; Yasuda et al., 1981), but at doses much higher than used in this study. It is possible that other general health or reproductive endpoints may have been more sensitive to the low dose exposure used here. Prostate morphology, for example, appears to be quite sensitive to low dose estrogen exposure (Timms et al., 2005). It was beyond the scope of this project, however, to investigate a large number of reproductive endpoints.

5.3 Onset of Puberty

5.3.1 Introduction

In mammals, puberty marks the onset of the reproductive life stage of an animal and after the onset of puberty; animals are physiologically capable of reproduction. In females, the first estrus cycle signals the onset of puberty. Female mice will typically experience their first cycle somewhere between postnatal days 35 and 45. The onset of puberty has been shown to be a sensitive marker for developmental exposure to estrogens in general (Gray, 1992) and bisphenol A specifically (Honma et al., 2002; Howdeshell et al., 1999). Puberty is an easily measured endpoint, and the puberty data collected will allow me to compare the relative sensitivity of the animals in this study to animals in the previously mentioned studies.
5.3.2 Methods

The mice were housed and treated as described earlier. After the mice were weaned, 21 individuals were checked daily and a vaginal smear was taken. A vaginal smear consists of taking a small volume of saline in a pipette tip, carefully injecting it into the vaginal canal and recollecting it within the pipette. Saline handled in this way will collect sloughed vaginal cells which can then be examined under a light microscope. If vaginal smears are taken daily from an adult animal, the degree of cornification present in the vaginal cells will cycle in synchrony with the estrus cycle. For the purpose of this experiment, the first day of puberty was defined as the day on which cornified cells were first detected in the smear. Vaginal smears were taken for at least two days after the first cornification to verify that the animal was indeed cycling.

5.3.3 Results

Developmental treatment had a significant main effect on the onset of puberty (p < 0.001). A post-hoc analysis showed that animals in the EE 5 and BPA 200 groups reached puberty at a significantly earlier age than the stripped-oil control (Figure 5.6). The animals exposed to the low dose BPA 2 group did not have an accelerated age of first puberty. Within this small sample, the treatment had no effect on the body weight at puberty (p = 0.5146), or body weight at weaning (p = 0.5764) (data not shown).
Figure 5.6: The effect of developmental exposure to EE or BPA on the onset of puberty in the mouse as measured by the first cornified smear

Mean ± the standard error

** p = 0.0017 vs. the stripped oil control

*** p < 0.0001 vs. stripped oil control

Both BPA and EE were successful in stimulating puberty in female mice.
5.3.4 Discussion

Both BPA and EE were capable of accelerating puberty in female mice. This was not surprising, as estrogens previously have been shown to advance puberty (Honma et al., 2002; Howdeshell et al., 1999). However, Howdeshell et al. (1999) found that developmental exposure to 2.4 µg/kg/day BPA advanced puberty, whereas I (and Honma et al., 2002) found no stimulation of puberty at a dose of 2 µg/kg/day. There are a number of possible explanations for this difference. The sample size in this experiment was relatively small. Even a large sample size, however, would most likely not have resulted in a significant difference between the SO and the BPA 2 groups if the means remained less than one day apart. I also used a different mouse strain (C57-Bl/6 used here vs. CF-1 used by Howdeshell et al., 1999), however, the mouse strain used in this experiment was carefully chosen in part because it has been shown to be highly sensitive to estrogens (Spearow et al., 1999).

I defined puberty differently in this experiment than did Howdeshell et al. (1999). As described earlier, I used the age at which cornified cells first appeared in the vaginal smear as a marker for the onset of puberty. Howdeshell et al. looked at the time elapsed between vaginal opening and first estrus as their definition of puberty. Their definition is unusual and is not typically used as a marker for puberty in the mouse, as vaginal opening has been shown to correlate weakly with the onset of puberty in the mouse (Cooper et al., 1993; Nelson et al., 1990; Safranski et al., 1993). It is unclear from their published data if their treated mice would have shown accelerated puberty using my definition. It is clear from their data, however, that their control animals reached puberty at a later age than my animals (approximately 41 days vs. 38.5 days for my control females). This raises the possibility that my control animals reached puberty precocially for some reason and masked any potential effects of a low dose of BPA. One final
point to consider is that Howdeshell *et al.* found a significant difference in body weight at weaning in the same treatment groups that showed an accelerated onset of puberty. I found no body weight differences between any of my groups and this raises the possibility that the onset of puberty they reported may have been caused in part by a difference in body weight.

Honma *et al.* (2002) measured both vaginal opening and first estrus in their paper and found no effect with a dose of 2 µg/kg/day BPA. This study used yet another strain of mouse (ICR/Jcl) and instead of oral administration, used subcutaneous injection. In addition, the control animals in this study achieved first estrus at a very young age (28 days), possibility masking low dose effects.

Due to the methodological differences and the confounding factors within each experiment, it is difficult to directly compare these studies. Taken together, however, it is clear that BPA does accelerate the onset of puberty in mice. It is possible that this acceleration may occur at doses considered environmentally relevant (i.e. 2 µg/kg/day), however the data from this experiment do not support such a claim.
6. ANXIETY-RELATED BEHAVIORS

6.1 General Introduction

Anxiety is a common term in rodent behavioral literature, with publications dating back to at least the 1950s (McCleary, 1954 is the earliest PubMed reference to anxiety in rodents). Anxiety is most closely associated with human emotion, the general scientific consensus however, considers anxiety to be wariness of threatening or novel aspects of the environment which results in autonomic responses as well as avoidance and fear (Gordon and Hen, 2004; Lesch et al., 2003).

In rodents, anxiety-like behaviors have been shown to be sensitive to the developmental estrogen environment in general (Leret et al., 1994), and to BPA and EE specifically (Dugard et al., 2001; Farabollini et al., 1999). Anxiety-like behaviors, therefore, are a natural choice for study in this project, as my results can be easily placed in context with the earlier research. It is important to note that I did not simply repeat these studies, but in fact expanded upon them. The previous work has been completed on rats not mice, and this current study investigated lower dose ranges than previously reported.

Two general types of anxiety tests are commonly used. The first requires the animal to associate a neutral stimulus, like a light or a tone, with a naturally unpleasant stimulus such as a foot shock. Once the association is established, behavior in the presence of just the neutral stimulus can be measured. Using this classical Pavlovian paradigm, anxiety can be quantified by measuring how quickly the animal acquires the task, and how quickly the anxious-like behavior extinguishes after the aversive stimulus is removed. Fear conditioning using a foot-shock is
commonly used in rodent research to measure anxiety-like behavior in this fashion (Weiss et al., 2000).

The second type of anxiety tests take advantage of rodents’ natural desire to explore and their fear of open and exposed areas. Placing an animal in a novel but uncomfortable physical environment and measuring the balance between exploration and fearful behavior allows for a general quantification of anxiety. Less anxious animals spend a higher percentage of time exploring; more anxious animals spend a higher percentage of the time frozen or hiding. The elevated-plus maze, the light/dark preference chamber, and open-field activity are all commonly used anxiety assays that utilize this balance between exploration and novelty.

I used the second type of anxiety assay in this project, specifically the elevated-plus maze and the light/dark preference chamber. Both of these assays are simple to run and are relatively high-throughput. They are also fairly easy to score and require only basic equipment (as opposed to fear conditioning which would require some method of delivering an electrical shock paired with a stimulus).

I chose to use two different anxiety assays in this project. Confounding factors (like activity level, for example) can make results on any one assay difficult to interpret. Using two different assays allows me to make measures of anxious behavior in two different situations, increasing the likelihood of identifying factors which may skew results in one of the assays. Consistent results between the two assays would enhance my ability to draw conclusions as to the effect of environmental estrogens on anxiety-like behavior.
6.2 Elevated-Plus Maze

6.2.1 Introduction

The elevated-plus maze has been used as a simple and reliable measure of general anxiety levels in rodents for 20 years (dating back at least to Pellow et al., 1985). This assay simultaneously creates aversive as well as comfortable environments by having open and enclosed runways that the animal can explore. Mice prefer the closed runways, but will naturally want to explore the entire apparatus. Anxiety therefore can be quantified by measuring the amount of time spent in open vs. enclosed arms. Highly anxious animals will spend more time in the enclosed arms.

Activity level can potentially be a confounding factor in this assay. An animal that is hyperactive will visit more areas of the maze than a sedentary animal, and this could be independent of anxiety level. This factor cannot be controlled, but it can be measured quite easily by tracking the number of transitions between different areas of the maze. This assay also allows for an activity-independent measure of anxiety. In addition to moving within the apparatus, animals will explore by looking outside and around the maze. These explorations are easily measured and usually referred to as “unprotected head-dips” because the animal will typically extend its head outside and below the level of the runways to investigate the surrounding area. Head dips obviously require very little movement on the part of the animal but are explorations of novel space, and are therefore an activity-independent measure of anxiety in this assay. By measuring transitions and head-dips, the possible confounding factor of activity can be assessed.
Other confounding factors exist as well, but are easily controlled. The maze runways are constructed of Plexiglas and are cleaned in-between each trial to prevent the presence of odors. All the animals were run with the maze in the same position in the same room under only red-light illumination with the experimenter observing from outside the room. This was done in an attempt to make the maze environment identical for each mouse and thereby lower variability.

6.2.2 Methods

The mice were dosed and ovariectomized as described earlier. Fifty-six mice (fourteen per treatment group) were used in this experiment. The elevated-plus maze consists of four arms (4 cm x 30 cm) mounted at ninety degree angles to each other so that they intersect and form a plus shape. Two opposing arms are surrounded with Plexiglas walls to form a hallway effect, while the other two arms are left open and exposed (Figure 6.1).

![Figure 6.1: Schematic of the elevated-plus maze as viewed from above](image)
This plus is then elevated four feet off the ground. This elevation discourages the mice from spontaneously jumping from the maze, but is low enough so that the mice are not injured if they fall. During the 15 minute trial the mouse is placed into the center of the maze under red-light and allowed to freely explore the four arms. Mice were run in this apparatus once during the beginning of their dark cycle and the amount of time spent in the enclosed arms, open arms and center chamber were recorded. In addition, unprotected head dips, defined as the head of the mouse extending out over the edge and below the level of the arm, were recorded as a rough measure of locomotion-independent, exploratory behavior. The total number of transitions among the arms was also recorded. All observations were made via a closed circuit television connected to a camera mounted directly over the maze. This allowed for real-time observation of the animals without an experimenter having to be in the room.

6.2.3 Results

The developmental treatment had a significant main effect on the time spent in the open arms of the maze (p = 0.0047). A post-hoc analysis showed that the EE 5 group spent significantly less time in the open arms compared to the control animals (p = 0.0015). The BPA 200 group also spent less time in the open arms than controls, although this difference did not quite reach significance (p = 0.0603) (Figure 6.2). The developmental treatment had no significant effect on the number of transitions between the closed arms (p = 0.2399) (Figure 6.3). The developmental treatment also had no significant effect on unprotected head dips (p = 0.1860), although the post-hoc analysis indicated that the EE 5 treated animals did complete fewer head dips than the control animals (p = 0.0308) (Figure 6.4).
Figure 6.2: Time spent in the open arms of an elevated-plus maze during a fifteen minute trial in mice developmentally exposed to an environmental estrogen

Mean ± the standard error

+ p = 0.0603

** p = 0.0015
Figure 6.3: The number of transitions between closed arms in an elevated-plus maze during a fifteen minute trial in mice developmentally exposed to an environmental estrogen

Mean ± the standard error.

No group different significantly from the stripped oil control.
Figure 6.4: Number of unprotected head dips during a fifteen minute trial on an elevated-plus maze

Mean ± the standard error.

# p = 0.0308

There was no main effect for treatment in this analysis, but the EE treated animals did show fewer head dips than control animals.
6.2.4 Discussion

Animals exposed to EE and to the high dose of BPA showed altered behavior in the elevated-plus maze consistent with an increased level of anxiety. These results seem independent of activity level, as there was no difference between the groups in the number of transitions between the closed arms of the maze. In addition, the pattern of unprotected head-dips was similar to the time spent in each arm. Based on these results, it is apparent that EE and high doses of BPA increase anxiety-like behavior in the elevated-plus maze.

The results from this experiment differ somewhat from previous published work. Farabollini et al. (1999) also measured the effects of developmental exposure to BPA on elevated-plus behavior. They found no clear effects in female animals, in contrast to my study. Any number of factors could contribute to the differences in results, their high dose of BPA was greater than mine (400 vs. 200 µg/kg/day) and only given during the neonatal period. In addition, Farabollini et al. (1999) used intact adult rats, as opposed to my ovariectomized mice. These factors could have made their assay less sensitive to the effects of BPA than was my assay.

Dugard et al. (2001) also used an elevated-plus maze to measure the developmental effects of exposure to EE on adult behavior. Despite some methodological differences between our two studies, the results are quite similar, with EE exposed animals spending more time in the closed arms of the maze. The synchrony in results between Dugard et al. and my study lends confidence to my conclusion that developmental exposure to environmental estrogens can alter adult anxiety-like behavior as measured in an elevated-plus maze.
6.3 Light/Dark Preference Chamber

6.3.1 Introduction

The light/dark preference chamber is not as commonly used to measure anxiety as the elevated plus maze, but it has been successfully used in mice (Crawley, 1999). Like the elevated plus maze, this apparatus also creates aversive and comfortable environments. In this case however the aversive stimulus is a bright open area and the comforting stimulus is a dark enclosed area. Mice will feel safer in the enclosed half of the box, but will naturally want to explore the lighted area as well. Anxiety therefore can be quantified by measuring the amount of time spent in the lighted vs. dark sections of the box. Highly anxious animals will spend more time in the dark half of the chamber.

Activity levels can potentially be a confounding factor in this assay, perhaps even more so than in the elevated plus maze. This cannot be controlled, but it can be measured by tracking the number of transitions between the two halves of the box. A more active animal will make more transitions.

In an attempt to make the assay identical for every mouse, the box was cleaned with alcohol in between every trial. The same maze, with the same light source was used for every mouse, and every trial was run in the same room at the beginning of the dark cycle. Once the mouse was placed in the apparatus, the experimenter left the room, allowing the mouse to explore freely.
6.3.2 Methods

Fifty-six mice (fourteen per treatment group) were dosed and ovariectomized as described earlier and run in this assay. The light/dark preference chamber consists of two small (40 cm x 20 cm) Plexiglas enclosures connected by a mouse-sized PVC tube (4 cm in diameter). These enclosures were identical with the exception that one was completely covered with dark plastic, while the other enclosure was left open. The open chamber was illuminated with a bright, incandescent light and the floors of both chambers were covered with paper towels during use. To begin the trial, the mouse was placed within the PVC tube and positioned in the opening between the light and dark chamber, facing the light chamber. Once placed in the apparatus, the mouse will immediately dart from the PVC tube into the light box. The mouse will then freeze and slowly start to investigate the surroundings before walking back through the tube to the dark half of the box. The latency to enter the dark box was recorded and from that point, the mouse was allowed to freely explore both chambers of the apparatus for 15 minutes.

The amount of time spent in each half of the box was recorded, as is the number of transitions between each half. All observations were made via a closed circuit television connected to a camera mounted directly over the light/dark chamber. This allowed for real time observation without an experimenter having to be present in the room. All mice were run in this apparatus once during the beginning of the dark cycle.

6.3.3 Results

The developmental treatment had a significant main effect on the amount of time spent in the lighted half of the box (p = 0.0002). Post-hoc analyses showed that both the EE 5 treated animals (p < 0.0001) and the BPA 200 animals (p = 0.0023) spent less time in the lighted section
than did the control animals (Figure 6.5). There was no treatment effect on the number of transitions between the two sections (p = 0.5527) (Figure 6.6). There was also no treatment effect on the latency to first enter the dark half of the chamber (p = 0.0995), but a post-hoc analysis did show that the EE 5 treated animals had a longer latency (p = 0.0233) (Figure 6.7).
Figure 6.5: The amount of time spent in the light chamber (as opposed to the dark chamber) during a fifteen minute trial

Mean ± the standard error

** p = 0.0023

*** p < 0.0001
Figure 6.6: The number of transitions between the light and dark chamber in a fifteen minute trial

Mean ± standard error

No group different significantly from the stripped oil control.
Figure 6.7: The latency before the first transition from the light to the dark chamber

Mean ± the standard error

# p = 0.0233

There was no main effect of treatment, but the EE exposed animals did show a longer latency before entering the dark chamber.
6.3.4 Discussion

Animals exposed to EE and to the high dose of BPA showed altered behavior in the light/dark preference chamber consistent with an increased level of anxiety. These results seem independent of activity level, as there was no difference between the groups in the number of transitions between the two halves of the chamber. In addition, mice exposed developmentally to EE showed an increased latency before entering the dark chamber, again indicating that they are more anxious than control animals. Based on these results, it is apparent that EE and high doses of BPA increase anxiety-like behavior in the light/dark preference chamber.

The results in this experiment can again be compared to the work of Dugard et al. (2001). In addition to an elevated-plus maze, Dugard et al. (2001) measured the latency to enter a lighted chamber from a dark one. They did not attempt to measure the total amount of time spent in each chamber, as I did, and they started the animals in the dark half of the chamber, but their results are again consistent with mine, showing that developmental exposure to EE increases latency to enter a novel chamber. This again lends confidence to my conclusion that developmental exposure to environmental estrogens can alter adult anxiety-like behavior as measured in a light/dark preference chamber.

6.4 General Discussion on Anxiety-Like Behaviors

The results in these two anxiety assays are quite congruent. On both assays, animals exposed developmentally to the high dose of BPA or to EE show altered behavior consistent with an increase in anxiety. These results are in general consensus with other published work of a similar nature, although very few papers have been published on the effects of developmental exposure to environmental estrogens on adult anxiety. It is also important to note that the
effective doses shown in this series of experiments are lower than what has been previously published.

In these experiments, the same mice were run in both anxiety assays, potentially altering the effects on the second assay run (in this case the light/dark preference chamber). A small follow-up study (data not shown) indicated that animals run in just the light/dark preference chamber showed similar performance to the animals run in this experiment. In conclusion, the results of these experiments serve as a strong indication that the environmental estrogens used here are altering the development of the nervous system and impacting adult behavior.
7. SHORT TERM SPATIAL MEMORY

7.1 General Introduction

Much of the current research on estrogen focuses on its effects on cognition. Estrogen treatments have been shown to improve some forms of verbal and spatial memory in post-menopausal women. Estrogen is also thought to act as a protective factor against Alzheimer’s disease as well as injuries associated with strokes or stress (Duff and Hampson, 2000; Robinson et al., 1994; Wise et al., 2000).

These findings in humans were based in part on work completed in rodent models. The influences of estrogen on memory in rodents have been extensively investigated. In these studies, a dose of estrogen is administered to female rodents, which are generally ovariectomized prior to the experiment. The rodents are then subjected to a test that measures spatial memory. Commonly used spatial memory tests are the Morris water maze, the radial arm maze and the T-maze. In general, estrogens have been found to enhance rodents’ performance on these assays, indicating that estrogen is facilitating cognitive function in some fashion (Williams, 1998).

Most of this work linking estrogen with cognition has focused on adult animals. Far less research has investigated the prenatal effect of estrogen on cognition. A few studies, however, do show that developmental exposure to a strong estrogen does increase adult spatial ability in female rats. It is thought that developmental estrogen exposure masculinizes the female nervous system, thereby increasing spatial ability (male rats are superior to female rats in many spatial tasks) (Isgor and Sengelaub, 1998; Williams et al., 1990).

A number of possible mechanisms may account for estrogen’s modulation of memory. Estrogen acts at the cellular level to increase potassium conductance and hyperpolarize neurons.
These effects seem to occur via modulation of protein kinase A. Estrogen also has been shown to increase neuron spine density in the rat hippocampus (Wooley, 1998).

The hippocampus is a vital brain structure for many types of memory and the hippocampus of mice has been shown to express both the $\alpha$ and the $\beta$ estrogen receptor (Shughrue et al., 1997). Estrogen enhances the expression of brain derived neurotrophic factor in the hippocampus of female rats (Gibbs, 1999) and appears to decrease apoptosis, factors which could contribute to the increased spine density seen in the hippocampus. Estrogen also appears to act as a neuroprotective factor against ischemic injury (Wise et al., 2000), which again, could contribute to neuron survival.

In addition, estrogen appears to interact with cholinergic systems as the memory-enhancing effects of estrogen are blocked by administration of the acetylcholine muscarinic receptor agonist scopolamine (Packard, 1998). Furthermore, chronic estrogen treatment increases levels of norepinephrine, dopamine, serotonin and GABA in various brain regions (Luine et al., 1998). The interaction of some or all of these effects may explain how estrogen can improve memory. To date, however, no clear mechanism has been conclusively identified.

Memory can be tested in many fashions but the majority of studies utilize a maze task. Two of the most commonly used mazes are the Morris water maze and the radial arm maze. The Morris water maze, a test of spatial reference memory, has produced conflicting results. Estrogen treatments have been shown to improve (Packard, 1998; Rissanen et al., 1999), hinder or have no effect (Daniel et al., 1999) on the rodents' performance in this maze. Neonatal BPA treatment has been investigated with the Morris water maze, again giving confusing results (Carr et al., 2003). Based on the conflicting results generated by this maze, I have chosen not to use it in this project.
The radial-arm maze, a test of spatial working memory, has given much clearer results. In the vast majority of tests, estrogen has been shown to improve rodents' performance in the maze (Daniel et al., 1997; Daniel et al., 1999; Luine et al., 1998; Fader et al., 1999). It appears that the dose of estrogen used also plays a factor, with low doses improving memory and high levels of estrogen hindering spatial memory. Performance in the radial arm maze appears to be consistently estrogen dependent and it is an excellent choice for use in this project.

As with the anxiety-related assays, I also used two distinct tests of spatial memory. This allows me to identify potential confounding factors as well as strengthens my ability to draw conclusions. The Barnes maze is not as commonly used as the radial arm maze or the Morris water maze, but it has been used successfully in the same strain of mice that I am using (Holmes et al., 2002; Koopmans et al., 2003). It is also a more complex maze than a T or Y maze which is desirable as estrogens appear to play a more beneficial effect on memory when spatial demands are high (Bimonte and Denenberg, 1999; Luine et al., 1998). Despite the fact that the Barnes maze has not previously been used in estrogen research, the characteristics of the maze make it a good choice for the second maze in this project.

Estrogen appears to improve short-term (working) spatial memory more so than long-term (reference) spatial memory (Luine et al., 1998; Wilson et al., 1999). For this reason, I focused on short-term memory in this project. In mice, working memory relates to performance within a trial, as opposed to between trials or days. Testing a mouse repeatedly within a matter of minutes on the location of a reward would measure working memory. Testing a mouse repeatedly over a matter of days on the location of a reward would measure reference memory.

It is important that these assays focus specifically on spatial memory. The mazes themselves are featureless, therefore it is important that the mazes be constructed to allow the
mice the ability to see, and orient themselves by, features in the room. As a result, the radial arm maze was constructed of clear Plexiglas. The Barnes maze used in this project was a flat tabletop with no rim or surrounding edge to obstruct vision. By constructing the mazes with particular characteristics and using them to test memory over a short period of time, I was able to build assays that specifically tested spatial working memory.

7.2 Radial-Arm Maze

7.2.1 Introduction

The radial arm maze is a commonly used test of spatial memory and has been used to test memory in rats for decades. More recently, the maze has been modified for use in mice. Many variations of the maze exist, but all possess a number of identical arms radiating out from a central chamber. Each arm contains a reward or negative reinforcer, and the rodent must learn and remember which arms to visit. To measure short term spatial memory, rewards (typically food) are placed at the end of each arm and the animal is allowed multiple arm choices. Within a trial, each new arm visited results in positive reinforcement, each old arm revisited results in negative reinforcement. The mice quickly learn to visit only novel arms, at which point the main factor determining performance is remembering which arms have been visited (i.e. short-term spatial memory).

Pilot studies indicate that mice will learn to eat a food reward from cups in the maze and avoid previously visited arms within the first two trials. I decided that ten total trials would be sufficient to measure working memory in this experiment.
7.2.2 Methods

Sixty-four mice (16 per treatment group) were dosed and ovariectomized as described earlier and run in this assay. A fully baited eight armed radial maze was used to measure short-term spatial working memory. The maze was constructed of clear Plexiglas and consisted of eight arms 10 cm wide by 50 cm long radiating out from a central octagonal chamber. This central chamber could be isolated from the rest of the maze by manually lowering a set of doors via a pulley system attached to the ceiling. A small (~50 mg) piece of soft bacon flavored rodent pellet (BioServ F3580) was used as a reward and was placed in a small cup at the end of each arm (Figure 7.1). These pellets were also placed outside the end of each arm, adjacent to small holes drilled into the Plexiglas. This was done to prevent the mice from discerning which arms were baited simply by food odor.
To begin a trial, a mouse was placed in the central octagonal chamber and was given thirty seconds to acclimate. After thirty seconds, a tone sounded and two seconds later, the doors were raised, allowing the mouse to travel down any arm to collect and eat food. This tone was always sounded two seconds before the doors were raised. While the mouse was exploring the arm, the doors were lowered again, containing the mouse within its chosen arm, encouraging exploration and food collection. This also prevented the mouse from carrying food back to the central chamber before eating. Once the reward pellet was consumed, the doors were reopened.
and the mouse was allowed to return to the central chamber. Due to the design of the maze, the mouse must return to the central chamber before it can choose a new arm to explore.

It has been noted that mice often rely on sequencing patterns and not memory to collect food in a radial maze (EF Rissman, personal communication). To control for this, the doors surrounding the central chamber were closed as soon as the mouse returned to the central chamber. The mouse was then subjected to a 10 second delay before being allowed to travel down a new arm. This delay prevented the mice from completing the maze in a sequenced pattern. To further delay the mice, a small Plexiglas hurdle (4 cm high) was placed at the beginning of each arm. This hurdle prevented the mice from sprinting back and forth between arms before the central barrier could be closed. The hurdle also served as the decision point for the mice. Once all four paws were over the hurdle and inside the arm, the barrier doors were closed and the mouse was isolated from the central chamber. Once the mouse consumed the food in this arm, the doors were again raised and the process repeated.

If a mouse chose a previously visited arm, it would receive no reward, having already consumed the food in that arm. In addition, the mouse was isolated in the arm for thirty seconds. In this way, the mice quickly learned to choose arms not already visited.

The mice were allowed to make eight total arm choices. A memory error was recorded if a mouse traveled down a previously explored arm. With eight total choices, a mouse could receive a reward after every choice, but any incorrect choice decreased the amount of reward pellets the animal received in the trial. The total number of correct arm choices was recorded as well as the number of correct choices before the first error.
The mice were run in this maze twice a day during the beginning and middle of the light cycle for five consecutive days, resulting in ten total trials. The mice were placed on a 23 hour food deprivation schedule and monitored for excess weight loss throughout testing.

7.2.3 Results

The data were analyzed by trial. The main effect of treatment significantly altered the mean number of errors committed in the maze in trials six, seven, eight, nine and ten (p < 0.002 in each case). In those five trials, post-hoc analyses showed that the EE 5 treated animals made significantly fewer errors than the SO control animals (Figure 7.2). Treatment also had an effect on the latency until the first error in trials five, seven, nine and ten (p < 0.03 in each case) (Figure 7.3). In those trials, post hoc analyses showed that EE treated animals had a significantly greater latency until the first error when compared to control animals.

Within each treatment group, I also measured the rate of improvement. To do this, I compared the performance (total number of errors) on each trial, using trial one as the baseline. There was a significant main effect of trial on the total number of errors in the EE (p < 0.0001), BPA 200 (p < 0.0001) and BPA 2 (p = 0.0048) treatment groups. There was no main effect of trial on performance in the SO group (p = 0.2462). Post hoc analyses revealed that the EE treated animals committed significantly fewer errors from trial three onward. The BPA 200 group committed significantly fewer errors from trial two onward, and the BPA 2 group committed significantly fewer errors from trial eight onward.

I made a similar comparison with the first error committed per trial. There was a significant main effect of trial on the first error in the EE (p < 0.0001) and the BPA 200 (p = 0.0092) groups. There was no significant main effect of trial on the first error committed in the
BPA 2 and the SO groups. Post hoc analyses revealed that the EE group committed the first error significantly later from trial three onward. The BPA 200 group committed their first error significantly later from trial eight onward.
**Figure 7.2: Total number of errors made per trial in the radial arm maze**

Mean ± the standard error (only shown in SO and EE 5 groups)

** * p < 0.001 vs. stripped oil control on trials 6, 7, 8, 9 and 10

The animals exposed developmentally to 5 µg/kg/day EE consistently commit less errors in the radial arm maze in the second half of the trials.
Figure 7.3: The arm choice on which the first error was made in the radial arm maze

Mean ± the standard error (only shown in the SO and EE groups)

* p < 0.05  on trails 5, 7, 9 and 10

The EE 5 group consistently made more correct choices before their first error.
7.2.4 Discussion

The results from this experiment clearly indicate that mice developmentally exposed to EE show improved performance in the radial arm maze as adults. The mice exposed to this compound made fewer errors and took longer to make their first error than control mice.

Developmental exposure to BPA had no obvious effects on adult radial maze performance. Animals in both the BPA 2 and BPA 200 doses were not significantly different from control animals in the number of errors made or the latency to the first error on any of the ten trials. When the data were sorted by treatment however, some minor differences did emerge. Animals in the EE 5 and both BPA dose groups showed improvement over the course of the ten trials. The animals in the control group showed no improvement over the ten trials. This improvement appeared to be dose dependent, with the EE 5 and BPA 200 animals showing improvement on an earlier trial than the BPA 2 animals.

This is the first time that animals developmentally exposed to environmental estrogens have been tested in a radial arm maze. The results clearly indicate that animals exposed to EE during development show improved performance on this maze as adults. There is also some evidence to suggest that developmental exposure to BPA can also influence radial maze performance.
7.3 Barnes Maze

7.3.1 Introduction

The Barnes maze was first used by Carol Barnes to measure spatial memory in senescent rats (Barnes, 1979). Since then, it has been used to measure memory in a variety of experimental designs utilizing both rats and mice. The Barnes maze is constructed of a large, elevated circular platform. This elevated, exposed surface serves as a negative reinforcer and motivates the rodent to escape. Once the animal learns how to escape, remembering where the escape is located (i.e. spatial memory) determines how well the animal performs.

Like the radial arm maze, this maze can be used to measure short-term spatial memory. In contrast to the radial maze however, the Barnes maze utilizes negative reinforcement as motivation. This make the Barnes maze a good complement to the radial arm maze used in this project.

Pilot studies indicate that mice will learn to escape from the platform and repeatedly find the location of the escape within ten trials. Based on this, it was decided to run ten trials per day for five consecutive days.

7.3.2 Methods

Sixty-four mice (16 per treatment group) were dosed and ovariectomized as described earlier and run in this assay. The Barnes maze consists of a circular platform three feet in diameter raised four feet from the ground. The platform is made of solid pine covered with a clear polyethylene coat. Twenty holes (4 cm in diameter) were bored through the platform
equidistant from each other and three centimeters from the outer edge. These holes are large enough for a mouse to comfortably fit through (Figure 7.4).

**Figure 7.4: Schematic of the Barnes maze**

Small hooks were screwed into the bottom of the platform to allow a plastic escape box (9 cm x 15 cm x 8 cm) to be positioned under any one of the holes. From this position, the escape box cannot be seen from the top of the platform. This box is large enough for a mouse to comfortably sit within. A large, bright light is positioned directly over the center of the platform and a spray bottle is used to moisten the top of the platform with water. The water and the light serve as negative reinforcers to motivate the mouse to escape the platform through one of the twenty holes into the escape box below.
Each mouse started in the center of the platform covered by two nested mouse cages placed open end down over the mouse. When a trial began, the outer, opaque cage box was removed, leaving the mouse still contained within a smaller, clear cage box. After a five second delay, this cage box was removed, allowing the mouse to explore. Using the inner, clear cage box allowed for a five second period of orientation during which the movement of the mouse is still restricted. Pilot trials indicated that mice will tend to dart from the center of the platform when first released, and then begin exploring. The inner, clear box allowed a mouse to orient and determine a direction in which to dart, thus reducing the variability in the assay.

Mice ran once per day in this modified Barnes maze, during the middle of the light cycle. Each day consisted of 10 trials. Within these ten trials, the location of the escape box was kept constant. A mouse was allowed to freely explore the top of the maze until it found and entered the escape box. Once the mouse entered the box, it was given a one minute rest period and then placed back onto the center of the maze covered by the two nested cages. The maze was then rotated a random number of degrees and the escape box was moved so that it occupied the same spatial location within the room. After a thirty second delay, the cages were again removed and another run began. For each run, the number of holes investigated was recorded, as well as any refusals to enter the reward box or refusals to remain in the reward box.

After the ten trials were completed, the mouse was returned to its home cage. On the following day’s trial, the position of the escape box was changed to another random location, so that the mouse was forced to re-learn the placement of the reward. This ensured that only short-term spatial memory was being tested and not long-term or reference memory. Each mouse was run in this assay for five consecutive days, resulting in fifty total trials.
7.3.3 Results

The data were analyzed by trial. Because this assay generated fifty separate trials with four treatment groups in each trial, a large number of statistical analyses were performed, increasing the likelihood of a falsely significant result on any one of the trials. Therefore, it is important to look at the overall trend in the data as well as the specific results of the analyses. Out of the fifty trials, there was a main effect of the treatment \( (p < 0.05) \) on the number of errors made in nine separate trials. On all nine of these trials, post hoc analyses revealed that the EE 5 treated animals made significantly \( (p < 0.05) \) fewer errors than control animals. In addition, there were nine additional trials in which there was a marginal treatment effect \( (0.06 < p < 0.08) \). On all of these nine trials, post hoc analyses revealed that the EE treated animals made marginally fewer errors than the SO treated animals \( (p < 0.06) \). No other treatment group differed significantly from the SO treated animals on more than one trial. These trials are split into five days and shown in Figures 7.5-7.9.

The data were also analyzed by day. This required pooling the data from the ten trials on each day. The pooled data from the five days were then analyzed for treatment effects. There was a significant main effect of treatment \( (p < 0.02) \) on the number of errors made on each of the five days. Post hoc analyses revealed that the EE treated animals made significantly fewer errors \( (p < 0.01) \) than control animals on all five days. In addition, the BPA 200 treated animals made significantly \( (p = 0.0379) \) fewer errors than the control animals on day one.

With these pooled data, I also measured the rate of improvement within each treatment group. I compared the number of errors on each day, using day one as the baseline. There was a significant main effect of the day \( (p < 0.001) \) on the number of errors made. Post hoc analyses
revealed that every treatment group made significantly ($p < 0.01$) fewer errors by either day two (EE and SO treated animals) or day three (BPA 2 and BPA 200 treated animals).

Lastly, I analyzed the number of errors made by which of the twenty holes the escape box was located under. There was no main effect of escape location on the number of errors made ($p = 0.3632$).
**Figure 7.5: Performance on the Barnes maze, as measured by the number of errors made per trial, on Day 1**

Mean ± the standard error

# There was no main effect of treatment on any trial in day one, but post-hoc analyses showed that the EE 5 treated animals made marginally fewer errors than control animals on trials 6 and 8
Figure 7.6: Performance on the Barnes maze, as measured by the number of errors made per trial, on Day 2

Mean ± the standard error

# There was no main effect of treatment on these trials, but post-hoc analyses showed that the EE 5 treated animals made marginally (p < 0.06) fewer errors than control animals on trials 13 and 17

* p < 0.05 for the EE 5 animals compared to control animals on trials 15, 16, 19 and 20
Figure 7.7: Performance on the Barnes maze, as measured by the number of errors made per trial, on Day 3

Mean ± the standard error

# There was no main effect of treatment on these trials, but post-hoc analyses showed that the EE 5 treated animals made marginally (p < 0.06) fewer errors than control animals on trials 24 and 26

* p < 0.05 for the EE 5 animals compared to control animals on trials 27 and 30
Figure 7.8: Performance on the Barnes maze, as measured by the number of errors made per trial, on Day 4

Mean ± the standard error

# There was no main effect of treatment on any trial in day one, but post-hoc analyses showed that the EE 5 treated animals made marginally fewer errors than control animals on trials 36, 37 and 39.
Figure 7.9: Performance on the Barnes maze, as measured by the number of errors made per trial, on Day 5

Mean ± the standard error

* p < 0.05 for the EE 5 animals compared to control animals on trials 43, 49 and 50.
7.3.4 Discussion

The results from this experiment clearly indicate that mice developmentally exposed to EE show improved performance in the Barnes maze as adults. The mice exposed to this compound made fewer errors before escaping from the maze. This difference was not seen in every trial, but was consistently seen throughout the five days of the experiment.

Developmental exposure to BPA had no effect on adult Barnes maze performance. Animals in both the BPA 2 and BPA 200 doses were not significantly different from control animals in the number of errors made. These results are most likely due to differences in spatial memory, as all treatment groups showed improvement over time, indicating that the mice did learn how to escape from the maze.

This is the first time that animals developmentally exposed to environmental estrogens have been tested in a Barnes maze. The results clearly indicate that animals exposed to EE during development show improved performance on this maze as adults. There is no evidence to suggest that developmental exposure to BPA can also influence Barnes maze performance.

7.4 Spatial Memory Discussion

The results from both the radial arm maze and the Barnes maze are quite congruent. In both cases, animals exposed developmentally to EE showed improved performance as adults. Animals exposed developmentally to BPA were similar to control animals in most measures.

This is the first study to show developmental effects of environmental estrogens on adult spatial memory. Carr et al. (2003) investigated BPA exposure using a Morris water maze, but found few positive results.
These results show that estrogenic endocrine disruption can cause permanent changes in the nervous system. These results are also consistent with the theory that estrogens masculinize the rodent nervous system, as the animals exposed to EE developmentally showed improved (i.e. more masculine) spatial memory as adults.

In these experiments, the same mice were run in both spatial memory assays, potentially skewing the effects on the second maze run (in this case the radial arm maze). A small follow-up study (data not shown) indicated that animals run in just the radial arm maze showed similar performance to the animals run in this experiment. Overall, I have shown that spatial memory tasks are fairly complex, yet are sensitive enough to identify animals that have been masculinized by developmental exposure to environmental estrogens.
8. GENERAL METHODOLOGY - RAT STUDIES

To strengthen any conclusions on the general risk environmental estrogens pose to humans as well as wildlife, I studied two different estrogens, and two different model organisms, the mouse and the rat. All rat studies were conducted at the Environmental Protection Agency in Research Triangle Park, North Carolina and every procedure described here was reviewed and approved by the institutional animal care and use committee at the Environmental Protection Agency.

The rats were housed in standard cages, as described earlier. Pine shavings were used as bedding, to complement previous studies in the lab. Time pregnant rats were purchased from Charles River Laboratories and received on gestational day two (GD-2). These pregnant rats were housed individually. On GD-7, each rat was ranked according to weight and placed into a treatment group semi-randomly such that each treatment group had roughly the same average weight. Pregnant dams were dosed via oral gavage from GD-7 through PND-18 with the appropriate chemical dissolved in corn oil.

The rat groups received ethinyl estradiol (EE) doses of 0.05, 0.5, 5 and 50 µg/kg per day to allow parallels to be made with the current EE literature (e.g. Sawaki et al., 2003) and overlap with the dose used in the mouse study. The doses of bisphenol A (BPA) were 2, 20, and 200 µg/kg per day. These doses complement those used in the mouse portions of this project. Not all doses were used in each individual study.

Rats were weaned on PND-23. From this point, female rats were housed individually. All animals were allowed to reach adulthood (at least PND 50) before being tested in any assay.
Females that were ovariectomized were done so after PND 40 and given at least two weeks to recover from surgery prior to any behavioral testing.
9. INVESTIGATION OF THE VEHICLE CONTROL - RATS

9.1 Introduction

Corn oil is commonly used as a vehicle for many studies at the EPA. Corn products have previously been shown to contain a potential mitogen active on estrogen-sensitive tissues (Markaverich et al., 2002). In addition, corn oil specifically was shown to be estrogenic in a mouse uterotrophic assay earlier in this project. The potential endocrine disrupting action of corn oil needed to be studied in rats before it was used as a vehicle for my experiments.

The immature rat uterotrophic assay is a quick and simple test for estrogenic activity. After weaning (postnatal day 23), young female animals were orally dosed with the compound of interest for three days. On the fourth day, the animals were killed and the uteri were weighed. The uterus in a prepubescent animal will be small; however in the presence of an estrogen, the uterus will enlarge and swell. Simply weighing the uterus will give an indication of estrogen exposure.

9.2 Methods

Twenty-four 23 day old Sprague Dawley rats were split into four treatment groups:

- Tocopherol-Stripped Corn Oil
- Mazola Brand Corn Oil
- Sigma Corn Oil
- Nothing
Each rat received 0.2mL of the appropriate solution via oral gavage for three days. On the fourth day, the animals were weighed, killed and the uterus was removed as described in the mouse uterotrophic assay. The body weight and uterine weight were recorded and a uterine index was calculated.

9.3 Results

There was no main effect of treatment on the uterine weight ($p = 0.9387$) (Figure 9.1) or the uterine index ($p = 0.9452$) (Figure 9.2).
Figure 9.1: Uterine Weight in 27 day old rats after three days of exposure to corn oil

Mean ± the standard error

No group differed significantly from the stripped oil control.
Figure 9.2: Uterine Index (Uterine weight/body weight x 100) in 27 day old rats after three days of exposure to corn oil

Mean ± the standard error

No group differed significantly from the stripped oil control.
9.4 Discussion

Unlike mice, the rats appear insensitive to any compounds that may be present in corn oil. The bottle of Mazola corn oil used in this experiment was the same bottle that proved to be uterotrophic in the mouse. This precludes the possibility of individual bottle differences causing the results seen here. For this reason, it was decided to use Sigma brand corn oil for all rat experiments in this project. This remains consistent with other studies that have been completed at the EPA.
10. GENERAL REPRODUCTIVE ENDPOINTS - RATS

10.1 General Introduction

As with the studies in mice, it is important to complement any behavioral findings with general mortality and reproductive data. These data are fairly easy to collect and will allow me to place this study in context with other work which has also used similar endpoints (e.g. Sawaki et al., 2003) as well as compare this rat study to my earlier findings in mice. The endpoints I chose to measure are commonly used within the reproductive toxicology field, namely puberty, anogenital distance, mortality and areola number.

10.2 AGD, Mortality, Areolas

10.2.1 Introduction

Anogenital distance (AGD) is commonly measured in rats, although there is some indication that the AGD in rats isn’t as sensitive to the prenatal hormonal environment as the AGD is in mice (Ryan and Vandenbergh, 2002). Nevertheless, with the high sample size used in this experiment, even a small change in AGD could be significant. There is a good possibility that a dose of a strong estrogen (like EE) will alter the development of the genitalia in female rats by partially stimulating this androgen sensitive tissue.

Much like the genitalia, nipple number in rats is sensitive to prenatal exposure to androgens or anti-androgens (Hotchkiss et al., 2004). Female rats normally have twelve nipples whereas adult male rats typically have none. Male rats exposed to antiandrogens prenatally, however, develop a greater number of nipples than unexposed male rats. Conversely, female rats
exposed to androgens prenatally develop a fewer number of nipples than unexposed female rats (Gray et al., 1999; Mylchreest et al., 2000; Wolf et al., 2000).

Adult nipple number is strongly correlated with neonatal areola number in rats (Hotchkiss, 2001). Therefore, it is relatively simple to determine if nipple development is disrupted in rats exposed to a strong estrogen during development by counting the number of areolas in neonatal rats. This measure takes mere seconds to complete and does not require lengthy restraint or shaving of the belly like it would in adult rats. Once again, estrogens do not directly influence this pathway, but high doses of a strong estrogen, like EE, may alter this pathway by weakly stimulating the androgen receptor (Bigsby et al., 1999).

One last, easily measured general endpoint is mortality. Litter size offers a rough estimation of pup mortality, as pup death will decrease the mean size of the litters. However, natural variability in litter size occurs in completely healthy animals. A more precise measure is to compare the number of pups born with the number of pups implanted. Implantation results in a dark scar in the uterine tissue. After a litter has been weaned, the dam’s uterus can be examined for these scars to obtain an accurate estimation of the number of fetuses that were originally implanted in the uterus. This number can be compared to the number of pups actually born or weaned. This method of measuring pup mortality is precise because it controls for the natural variation in litter size seen in rodents.

10.2.2 Methods

Rats were exposed to the appropriate compounds as described earlier. On PND 2, each pup was sexed, weighed and measured for AGD. To measure AGD, each pup was carefully restrained on its back underneath a dissecting microscope. An ocular micrometer was then used
to measure the distance between the anterior end of the anus and the posterior end of the genital papilla.

On PND 14, the pups were again sexed and weighed. At this time-point, areolas are beginning to develop and are visible on the skin. In addition, the body hair is not yet dense enough to obscure them from view. Each rat is gently restrained belly-up, placing any areolas in plain view. The areolas are counted and any abnormal appearance is noted.

After weaning, the previously pregnant dams are euthanized. The uterus is removed intact from each dam and examined on a light-box for the presence of uterine scars. The number of uterine scars is a good estimation of the number of implanted fetuses and this value can be compared to actual litter size to determine pup mortality.

The data in this experiment were collected over four blocks. Unfortunately, due to the organization of the experiment, blocks one and two did not include any animals from the EE 50 treatment group.

10.2.3 Results - Mortality

There was a significant (p < 0.003) main effect of treatment on the number of uterine scars, the litter size at PND 2, the mortality at PND 2 and the litter size at weaning. In contrast, there was no main effect of treatment on the mortality at weaning (p = 0.2059). Post hoc analyses revealed that the EE 50 group had significantly (p < 0.01) fewer uterine scars (Figure 10.1), a smaller litter size (Figure 10.2) and higher mortality at PND 2 and a smaller litter size at weaning (Figure 10.3). No other groups differed significantly from the control.
Figure 10.1: Pup Implants as Indicated by Uterine Scars Present During a Post-Pregnancy Necropsy

Mean ± the standard error

** p = 0.0087

The high dose EE group showed fewer implantation scars. This is most likely due to absorption of the embryos soon after treatment began on gestational day 7.
**Figure 10.2: Litter Size at Postnatal Day 2**

Mean ± the standard error

*** p < 0.0001

The litter size of the high dose EE group is already significantly lower than the control at PND-2, indicating that pup loss most likely occurs prenatally.
Figure 10.3: Litter Size at Weaning (Postnatal Day 23)

Mean ± the standard error

*** p = 0.0009 compared to the control

The litter size at weaning remained small in the EE 50 group. Most of this pup mortality occurred earlier in development as evidenced by Figures 1 and 2.
10.2.4 Results – Areolas

All males measured in this experiment had the expected areola count of zero, giving strong indication that there was no treatment effect on male areola development. The female data were a bit more complex. It was observed that the experimenter counting areolas became more proficient after the first block of animals was measured. This can be seen by looking at the areola count in the control animals across the four blocks (Figure 10.4). As a result, there is a main effect of block on areola count in control animals (p = 0.0024), with block one counts significantly lower than the other three blocks (p < 0.008). This trend remained consistent through all treatment groups. When the data were analyzed by blocks, there was no significant main effect of treatment on areola counts in female rats.
Figure 10.4: Areola count in control female rats across the four blocks

Mean ± the standard error

*** p < 0.001 compared to Block 1

** p < 0.01 compared to Block 1
10.2.5 Results – AGD

In female rats, treatment had a significant main effect on the AGD ($p = 0.0005$) and the AGDI ($p < 0.0001$) but not pup weight at PND 2. Post hoc analyses revealed a significantly longer AGD in the EE 5 ($p = 0.0024$) and EE 50 ($p = 0.0116$) treatment groups when compared to the oil controls (Figure 10.5). The AGDI was also significantly larger in the EE 50 treated animals ($p = 0.0018$) but the AGDI in the EE 5 treated animals only approached significance ($p = 0.0707$) (Figure 10.6).

In male rats, there was no main effect of treatment on AGD ($p = 0.4055$). There was a main effect of treatment on pup weight at PND 2 ($p < 0.0001$) and on AGDI ($p = 0.0059$). Post hoc analyses revealed that male pups from the EE 0.05 treatment group were significantly heavier than the oil controls ($p = 0.0082$) (Figure 10.7). The AGDI was significantly smaller in the EE 0.05 males ($p = 0.0042$) and the EE 0.5 males ($p = 0.0322$) (Figure 10.8).

There was a significant block effect on AGD, weight and AGDI ($p < 0.05$) across most of the treatment groups and in both sexes. Post hoc analyses revealed that animals from block three had significantly longer AGDs and higher weights ($p < 0.05$). An appropriate treatment by block interaction could not be calculated because the EE 50 treatment group was not present in every block. However, the trends seen in AGD and AGDI in the overall analysis remained consistent through all of the blocks.
Figure 10.5: AGD on PND-2 in female rats exposed developmentally to EE

Mean ± the standard error

** p = 0.0024 when compared to the control

* p = 0.0116 when compared to the control
Figure 10.6: AGDI (anogenital distance/body weight) at PND-2 in female rats exposed developmentally to EE

Mean ± the standard error

+ p = 0.0707 when compared to the control

** p = 0.0018 when compared to the control
**Figure 10.7:** Weight at PND-2 in male rats exposed developmentally to EE

Mean ± the standard error

** p = 0.0082 when compared to the control
Figure 10.8: AGDI (anogenital distance/body weight) at PND-2 in male rats exposed developmentally to EE

Mean ± the standard error

** p = 0.0042 when compared to the control

* p = 0.0322 when compared to the control
10.2.6 Discussion

Developmental exposure to BPA had no effect on any measure of mortality. Developmental exposure to EE however, impacted a number of the general endpoints related to mortality. Dams exposed to the highest dose of EE had significantly smaller litters both at PND 2 and at weaning. This finding was expected and is in agreement with earlier research (Sawaki et al., 2003). Mortality at weaning however was not sensitive to EE exposure. This was unexpected as mortality should be a less variable measure than litter size. These results most likely indicate that the uterine scars in the high dose EE group were unusually faint. This conclusion is supported by the results shown in Figure 1. Animals in the EE 50 exposure group had significantly fewer implants than controls, despite the dosing regimen not starting until after pup implantation. It is likely that exposure to the high dose of EE caused early pup loss. The pups that were lost early in pregnancy presumably left less of a uterine scar, and this scar had more time to heal, making it more difficult to identify in the post-weaning necropsy. These disappearing uterine scars actually made mortality a more variable measure than litter size.

Areola development was not impacted by EE exposure. The data however, did vary by block, and this variance could have been misconstrued as a treatment effect. The experimenter measuring the areolas became more proficient after block one, so as a result areola counts increased in blocks two, three and four. The EE 50 treatment group was only present in blocks three and four. Analyzing the data without accounting for the block effect showed a significantly higher areola count in the EE 50 group. This was not due to the treatment however, but rather the block effect and the unbalanced design of the experiment.

The two highest doses of EE lengthened AGD and increased AGDI in female rats. This was somewhat surprising, as these measures are sensitive to androgen exposure. These results
indicate that a strong estrogen, like EE, can stimulate these androgen-sensitive tissues potentially by weakly interacting with the androgen receptor.

10.3 Onset of Puberty

10.3.1 Introduction

Developmental exposure to a strong estrogen, like EE, may stimulate the development of the ovaries and thereby cause precocial puberty. Weak estrogens can stimulate puberty as well. Earlier in this project, BPA was shown to stimulate puberty in mice. Measuring puberty in the rat allowed me to compare the sensitivity of mice and rats to estrogenic endocrine disruption. Unlike the earlier mouse experiment, I used vaginal opening as an indicator of puberty in the rat. Vaginal opening has been shown to correlate strongly with the onset of puberty in the rat (Ojeda et al., 1976).

10.3.2 Methods

After weaning on postnatal day 25, rats were checked every other day for vaginal opening. There was no minimum size requirement for the opening, as long as it clearly opened into the body. Animals in the EE 50 treatment group were excluded from this study because their malformed genitalia made it impossible to discern exactly when the vagina opened. The animals in this experiment were split into three blocks. Due to the timing of births in block 2, a different experimenter measured vaginal opening than did in blocks 1 and 3. In all cases, however, the experimenter was blind to the treatment group of the animals. In addition, all of the
BPA exposed animals were in Block 3. This experimental design is not ideal, but could not be avoided due to the timing and organization necessary for other experiments in this project.

### 10.3.3 Results

There was a main effect of treatment \( (p < 0.0001) \) on the age of vaginal opening. Post hoc analyses revealed that EE 5 treated animals had a significantly \( (p < 0.0001) \) earlier age of vaginal opening when compared to the oil treated control animals (Figure 10.9). These animals were also significantly lighter than control animals on the day of vaginal opening \( (p = 0.0051) \). No other treatments differed significantly from the control.

These data were collected over three separate blocks. Analysis of the data by block showed that there was a significant main effect of block \( (p < 0.0002) \) on the age of vaginal opening in the control animals. As a result, it was necessary to reanalyze all of the data by blocks. This reanalysis showed a significant main effect for treatment \( (p < 0.01) \) on vaginal opening in each block. Post hoc analyses showed that the EE 5 treated animals had a significantly earlier age of vaginal opening \( (p < 0.04) \) when compared to control animals in blocks 1 and 3. However, in block 2, the EE 5 treated animals had a significantly later age of vaginal opening \( (p = 0.0107) \) when compared to control animals. This analysis by blocks also showed that the EE 0.5 treated animals had a significantly earlier age of vaginal opening \( (p = 0.0068) \) in Block 3.
Figure 10.9: The Effect of Developmental Exposure to EE or BPA on the Onset of Puberty in the Rat as Measured by Vaginal Opening

Mean ± the standard error.

*** p < 0.0001 when compared to the control
10.3.4 Discussion

Developmental exposure to EE caused earlier puberty in rats. This was expected as a similar trend was seen in mice earlier in this project. This result cannot be explained by an increase in body weight, as these animals were actually lighter at puberty than control animals.

There was a significant block effect in this experiment, with animals from block two actually showing the completely opposite trend, with EE exposed animals reaching puberty later. There is no obvious explanation for this result, as the animals were treated identically in each block. A different experimenter measured vaginal opening in block two, and it is possible that this may have had some effect on the data. Control animals did not differ in vaginal opening in block three, however.

BPA had no effect on the onset of puberty in the rat. This is in contrast to the findings in mice and may indicate that mice are more sensitive to the developmental effects of BPA than rats.
11. GENERAL BACKGROUND – RAT BEHAVIORAL STUDIES

The results in the previous sections show that some physiological endpoints are sensitive to endocrine disruption in rats. It is important to extend this research to investigate behavioral endpoints. Behavioral endpoints may be more sensitive to disruption than physiological endpoints because of the number of different body systems involved in behavior. Slight alterations in any one system might impact only a few physiological endpoints, but could potentially impact many behaviors. Studying behavior therefore, increases the likelihood of finding significant results. In addition, the masculinizing effect of estrogen on the nervous system contrasts its feminizing effect on the reproductive system. Since the majority of endocrine disruptor research has focused on reproductive endpoints, studying behavior could potentially reveal new and unexpected consequences of endocrine disruption.

I focused this study on sexually dimorphic behaviors. Developmental estrogen exposure masculinizes the rodent nervous system and therefore there was a good probability that sexually dimorphic behaviors would be sensitive to the type of endocrine disruption I studied. A number of sexually dimorphic behaviors are present in rodents. I focused on three behaviors: activity, saccharin preference and lordosis. Activity and lordosis are both sensitive to adult estrogen levels and therefore these behaviors can be used to measure adult sensitivity to estrogen. An animal masculinized by developmental estrogen exposure should show a decreased sensitivity to estrogen. This adult sensitivity has not previously been studied with regard to endocrine disruption.
12. LORDOSIS

12.1 Introduction

Lordosis is the body positioning of a female rodent in response to a male mount. Lordosis is characterized by an arched back and neck which exposes the vagina for easier intromission by the male. When circulating estrogens are high (during the estrus period of the cycle) females will reflexively lordose when mounted by a male, or even when pressure is applied to the flank.

Ovariectomized females have low circulating estrogen levels, and will therefore not lordose, even if mounted vigorously. Estrogen/progesterone replacement can mimic the natural estrous cycle and will result in recovery of lordosis behavior in a dose-dependent fashion (Albert et al., 1991). Since lordosis is dose-dependent, it can be used as a behavioral biomarker for estrogen sensitivity.

Lordosis was used in this fashion in two separate experiments. The first experiment compared the estrogen sensitivity of two different rat strains. The second experiment investigated the estrogen sensitivity of animals exposed prenatally to environmental estrogens.

12.2 Pilot Lordosis Study - Strain Sensitivity/Dose Response

12.2.1 Introduction

As mentioned earlier, different mouse strains show differing sensitivities to estrogen (Spearow et al., 1999). Some evidence indicates that rat strains may also differ in their sensitivity to estrogens, including BPA and EE (Diel et al., 2004; Long et al., 2000; Steinmetz et
This experiment determined whether two commonly used strains of rat, the Long Evans and the Sprague-Dawley, differ in their sensitivity to estrogen. This experiment also generated a comprehensive dose response curve for both strains. The information in this curve was necessary to be able to use lordosis as a biomarker for estrogen sensitivity later in the project.

12.2.2 Methods

Adult, ovariectomized Sprague-Dawley and Long Evans female rats as well as adult, intact stud Sprague-Dawley males were purchased from Charles River Laboratories. Rats were received and housed two per cage in a room with a reversed light cycle (lights on from 9pm-11am). The animals were given two weeks to recover from surgery and acclimate to the light cycle before any experimentation took place.

Each animal was randomly assigned to one treatment group. The treatment groups consisted of EE dissolved in corn oil in concentrations ranging from 0 to 250 µg/kg. Animals received the solution via oral gavage.

This lordosis assay followed a three day protocol.

Day One – All females were dosed orally with the appropriate solution prior to 10am.

Day Two – All females were dosed orally with the appropriate solution prior to 10am. All manipulations were done blind to the treatment group from this point on.

Day Three Morning – All females were subcutaneously injected with a 0.5mg of progesterone dissolved in 0.1 ml of corn oil at 7:30am. All females, as well as the stud males, were moved into the behavioral observation room and allowed to acclimate. While in this room,
rats have normal access to food and water. This room has an identical light cycle to that of the animal room.

Day Three Afternoon – Animal testing began at 1:30pm (3.5 hours after lights-out). Each stud male was placed into a clear cage, free of bedding and allowed to acclimate for at least 5 minutes. The cage was placed on a specially made cart with a clear shelf and an angled mirror to facilitate observation of mountings. The cage was lit from the bottom by a fiber light. Each female was placed in this cage and the male was allowed to mount her five times. A mount was defined as a male placing his front paws onto the flank of the female and thrusting into the hindquarters. Mounts into the side or head of the female were not counted. For each mount, the female had her lordosis response scored from zero to three as follows:

0 – no lordosis
1 – slight lordosis including slight back arch and very brief or no freezing
2 – moderate lordosis including obvious back arch and brief freezing (less than 0.5 sec)
3 – full lordosis including extreme back arch, resulting in the head facing upwards, and clear freezing (more than 0.5 sec)

If two minutes elapsed without any attempted mount, the female was removed from the cage and placed immediately into the cage of another male. In this experiment, male refusals were rare, and the combination of two males always resulted in five mounts.

The stud males were rotated to prevent exhaustion. Rotating four males was sufficient to stimulate up to thirty females without any male showing signs of fatigue. Once the afternoon session was complete, the rats were returned to their home room. The males could be used as studs the next day; the females were given one week to allow circulating estrogen levels to drop
before being used again. All lordosis testing took place in the same room and the same
experimenter, blind to the treatment groups, scored every trial.

Following this protocol, a large number of females were behaviorally assessed in a fairly
short period of time. In addition, repeated measures can and were taken on the same females.

12.2.3 Results

As expected, the dose of EE had a significant main effect on both the number of lordoses
per five mounts (p < 0.0001) and the total score per five mounts (p < 0.0001). There was no main
effect of the rat strain on either lordoses (p = 0.1737) (Figure 12.1) or score (p = 0.1939) (Figure
12.2), nor was there a significant interaction between the dose of EE and the strain (p = 0.4749
for lordoses and p = 0.7194 for the score).

Because it was determined that no difference existed in lordosis behavior between the
two strains, I completed an expanded lordosis dose-response curve in only the Long Evans strain
(Figures 12.3 and 12.4). This expanded dose-response curve is important for the later
experiment investigating adult sensitivity to estrogen as measured by lordosis in animals
developmentally exposed to EE or BPA. Using this expanded dose response curve, it was
determined that both the number of lordoses and the score decreased drastically below an adult
EE exposure of 65µg/kg.
There was no significant main effect of strain on lordoses but a post hoc analysis showed a significant difference of $p = 0.015$ at 19µg/kg EE.
Figure 12.2: Lordosis Score Dose Response to Ethinyl Estradiol in Sprague-Dawley (SD) and Long Evans (LE) Rats Exposed to Ethinyl Estradiol Orally for Two Days

Mean ± the standard error

All lordoses were scored on a scale of 0-3.

There were no significant differences between the two strains at any dose.
Note the steady drop off in lordoses below 65 µg/kg.

Figure 12.3: Expanded Lordosis Dose Response to Ethinyl Estradiol of Long Evans Rats Exposed to Ethinyl Estradiol Orally for Two Days

Mean ± the standard error

(n > 5 for every dose)
Figure 12.4: Expanded Lordosis Score Dose Response to Ethinyl Estradiol in Long Evans Rats Exposed to Ethinyl Estradiol Orally for Two Days

Mean ± the standard error

All lordoses were scored on a scale of 0-3.

Note the steep drop off in scores below 65 µg/kg.
12.2.4 Discussion

There was no difference in strain sensitivity to estrogen. Only one significant difference in response to EE was found between the strains at one dose in one measurement (lordoses at 19 µg/kg). This difference disappeared when the lordoses were transformed into scores. One significant difference out of twenty total measurements is not sufficient to conclude that any sensitivity difference exists between the two strains tested, especially with no main effect of strain. Based on this finding, it was decided to use Long Evans rats for the rest of this project. This strain is the preferred choice for the motor activity assay, as it will allow for some comparison to historical data generated in this laboratory.

In addition, future experiments in this study using lordosis as a biomarker for estrogen sensitivity will use a reference dose of 65 µg/kg. This dose was the lowest to elicit a strong behavioral response. A shifted dose-response curve to estrogen as a result of developmental masculinization will theoretically show the largest difference at this dose (Figure 12.5).
12.3 Main Lordosis Study - Developmental Exposure to Ethinyl Estradiol

12.3.1 Introduction

Exposure to strong estrogens, like EE, during development masculinizes the nervous system. Since male rats will typically not lordose, it is expected that females masculinized by developmental EE exposure will show impaired lordosis behavior as adults. No reports of measuring endocrine disruption in this fashion have been published to date.

12.3.2 Methods

Female rats were exposed developmentally to EE and ovariectomized as described earlier. As adults, these rats were then exposed to 65 µg/kg of EE orally for two consecutive
days and 0.5 mg of progesterone subcutaneously on the morning of testing. They were then run through the exact same lordosis protocol as was earlier described.

12.3.3 Results

There was a significant main effect of the developmental treatment on the number of mounts per trial (p = 0.0003) (Figure 12.6), the number of lordoses per trial (p < 0.0001) (Figure 12.7), the score (p < 0.0001) (Figure 12.8) and the number of refusals (p < 0.0001) (Figure 12.9). Post hoc analyses revealed that developmental exposure to any of the three doses tested (EE 0.05, EE 0.5 or EE 5) significantly altered performance in this assay on all of the measures mentioned above.

Due to the unexpectedly high number of refusals in these data, separate analyses were performed on the subset of animals that were mounted the maximum five times. Even with the number of mounts normalized in this fashion, there was still a significant main effect of the developmental treatment on the number of lordoses per trial (p = 0.0159) (Figure 12.10), the score (p = 0.0167) (Figure 12.11) and the number of refusals per trial (p = 0.0059).

Animals run in this experiment were exposed developmentally to environmental estrogens in one of two blocks. Reanalyzing the data including blocks showed that this block had a significant main effect on mounts, lordoses and score (p < 0.002). There was no significant block by treatment interaction on any of these measures (p > 0.08). Reanalyzing the data by blocks yielded no differences in the overall results. This experiment also used multiple animals (two or three) from the same litter. When the data was organized into litter means and reanalyzed, no different results were seen.
Figure 12.6: The number of times a hormonally primed, ovariectomized female was mounted by a stud male

Mean ± the standard error

*** p < 0.001 when compared to the control
Figure 12.7: The number of lordoses per trial (with a maximum of five mounts)

Mean ± the standard error

*** p < 0.001 when compared to the control
Figure 12.8: Lordosis score per trial. A maximum of five lordoses scored on a scale of 0-3 for a total possible score of 15

Mean ± the standard error

*** p < 0.001 when compared to the control
Figure 12.9: The number of refusals to mount per trial. A maximum of two refusals were allowed before the female was removed from testing.

Mean ± the standard error

*** p < 0.001 when compared to the control
Figure 12.10: The number of lordoses in the subset of animals that were mounted five times

Mean ± the standard error

+ p = 0.075 when compared to the control

* p = 0.020 when compared to the control

** p = 0.004 when compared to the control
Figure 12.11: Total score in the subset of animals that were mounted five times

Mean ± the standard error

+ p = 0.080 when compared to the control

* p = 0.022 when compared to the control

** p = 0.004 when compared to the control
12.3.4 Discussion

Animals developmentally exposed to ethinyl estradiol showed severely disrupted lordosis in adulthood. This indicates that these animals had been behaviorally masculinized and were less sensitive to the adult effects of estrogen. This disruption occurred at every dose used in this experiment. The persistence of the disruption even at very low doses such as 0.5 and 0.05 µg/kg was surprising. Using a similar experimental design and physiological endpoints, Sawaki et al. (2003) found no effects of EE at doses below 50 µg/kg. Earlier results from this project found no effects at doses below 5 µg/kg. Finding clear masculinization at a dose three orders of magnitude below the previously published low dose was unexpected.

This disruption in lordosis persisted even when the number of mounts was controlled, and when the data were analyzed in this fashion, a clear dose response emerged. Disruptions in the number of times mounted as well as lordosis response when mounted, suggest that female rats developmentally exposed to EE are both less attractive to males and have a compromised ability to appropriately respond to mounts. Attractiveness, as indicated by the number of male refusals to mount, showed no clear dose response.

Adult behavioral sensitivity to estrogen, as measured by lordosis behavior, is exquisitely sensitive to developmental disruption via exposure to estrogens. In hindsight, it would have been interesting to test the endocrine disrupting effects of BPA in this assay.
13. SACCHARIN PREFERENCE

13.1 Introduction

Rodents show a clear sexual dimorphism in their preference for sweet tasting solutions. When given a choice, female rats will drink a greater volume of saccharin solution than will males. This phenomenon was first observed in the 1960’s (Valenstein et al., 1967) and has since become a commonly used assay for sexual dimorphism. Female animals show relatively constant saccharin preference over time as adults, so it is commonly thought that this preference is not sensitive to adult hormone levels. In addition prenatal disruption of development can alter adult saccharin preference, indicating that this sexually dimorphic behavior is organized prenatally (McGivern et al., 1984). It is therefore expected that female rats exposed developmentally to EE will show a masculinized pattern of saccharin preference, whereas female rats exposed to EE only as adults will not differ from controls. Prior to testing this hypothesis however, I first confirmed that saccharin preference is indeed sexually dimorphic.

13.2 Pilot Study – Saccharin Preference

13.2.1 Methods

Fifteen adult male, fifteen adult female intact and fifteen adult female ovariectomized Long Evans rats were purchased from Charles River Laboratories, North Carolina. All animals were given two weeks to acclimate after arrival in the laboratory. After acclimation, all three groups of animals were tested for saccharin preference. This test consisted of exposing each rat to two separate ad-lib sources of water. One bottle of water contained deionized water, while the
other bottle contained a 0.25% saccharin solution. Each bottle was weighed daily for five consecutive days to measure fluid intake from each solution. The position of the bottles within the cage was switched on a daily basis to control for any position biases. This pilot study was conducted to confirm sexual dimorphism of saccharin preference in adult rats.

To gauge the volume of fluid consumption, I weighed the two water bottles daily. However, the density of the two solutions was not quite the same. Deionized water has a density of one, so each liter of deionized water weighs 1000 grams. A 0.25% saccharin solution has 2.5 grams of saccharin added to each liter of deionized water, yielding a weight of 1002.5 grams per liter of solution. Having a density difference between the two solutions could potentially skew fluid consumption calculations based on weight. In this case however, the small change in the density of the solutions does not alter the overall calculations. For example, if a rat drinks 100 grams of the saccharin solution and 2 grams of deionized water, the preference calculations would be as follows:

Weight of saccharin solution consumed (100) divided by total weight of liquid consumed (102) equals 0.98.

Correcting the weight of the saccharin solution to truly reflect the volume, the calculation would be as follows:

Corrected weight of the saccharin solution consumed (99.75) divided by the corrected total weight of liquid consumed (101.75) equals 0.98.
These calculations show that the slight difference in density between the two solutions does not affect the final calculation. For simplicity, it was decided to use the weight of the two solutions in all calculations.

In addition, a subset of the two female groups was run through a second saccharin preference test. In this test half the animals received a choice between deionized water and a 0.25% saccharin solution, while the other half received a choice between deionized water and a 0.5% saccharin solution. This pilot study was conducted to confirm the optimal strength saccharin solution for these tests. The vast majority of the saccharin preference literature uses a 0.25% saccharin solution. Concentrations higher than 0.5% could not be tested as the saccharin would not completely dissolve in the water.

13.2.2 Results

There was a significant main effect of sex on saccharin preference (p < 0.0001). There was no significant effect of the concentration of saccharin in the solution (p = 0.9288), nor was there any interaction between sex and the concentration of saccharin in the solution (p = 0.9513) (Figure 13.1).

When comparing the saccharin preference of ovariectomized females to ovariectomized females with estradiol replacement over a period of five days, there was a significant main effect of estradiol replacement on saccharin preference (p = 0.0050). There was no main effect of the different days (one through five) on preference (p = 0.1140) but there was a significant interaction between the estradiol replacement and the day saccharin preference was measured (p < 0.0001), indicating that saccharin preference was differentially impacted by estradiol treatment over time (Figure 13.2).
Figure 13.1: Saccharin preference in male and female rats using either a 0.25% or a 0.5% saccharin solution

Mean ± the standard error

*** $p < 0.001$ when compared to females

** $p = 0.009$ when compared to females
Figure 13.2: Saccharin Preference in ovariectomized females and ovariectomized females given 125 µg/kg EE

Mean ± the standard error

* p < 0.01 when compared to ovariectomized females
13.2.3 Discussion

As expected, males and females show sexually dimorphic saccharin preference, with females preferring to drink greater quantities of the saccharin solution. Changing the concentration of the solution from 0.25% to 0.5% had no impact of this preference. Therefore, it was decided to use the 0.25% solution in all future tests. Saccharin in the 0.25% solution dissolves more rapidly and completely. In addition, using a 0.25% solution will use less saccharin over the course of the experiment. Most importantly, the vast majority of published studies use a 0.25% saccharin solution.

Ovariectomized female rats did show an altered pattern of saccharin preference over the course of five days when compared to ovariectomized females given estradiol. This contrasts previous literature in this field. It was still decided to use intact animals for the developmental exposure study due to time constraints. Future studies may want to consider investigating this effect of adult estrogen on preference in more detail. It was unexpected to find that saccharin preference changes over a period of five days. It will be important to analyze all future data by day, as saccharin preference can clearly change over time.

13.3 Main Study – Saccharin Preference

13.3.1 Introduction

The pilot studies confirmed that saccharin preference is indeed sexually dimorphic in rats, as expected. The pilot study also showed an effect of adult estradiol exposure on saccharin
Due to time and space constraints, it was decided to only study intact adult female animals in this main study.

### 13.3.2 Methods

The rats were dosed and tested as described earlier. All testing took place in the animals’ home cages. The saccharin preference test requires the use of two separate water bottles being placed into the cage. The rats are normally only given one water bottle. To control for any novelty associated with the presence of two water bottles, each rat was given two water bottles (filled only with water) for at least three consecutive days prior to the start of the saccharin preference test. All water bottles in this experiment used a ball-bearing style of sipper tube, as these leak less than open ended tubes. Care was also taken to observe the bedding in the cages every time the bottles were weighed to determine if any leakage had occurred.

It is simple to weigh the water bottles without disturbing the animal, so the rats were not removed from their home cage throughout the duration of the testing with the exception of normal bedding changes that occur. The animal care staff at the EPA was informed whenever a saccharin preference test was ongoing, and knew to be careful with the water bottles when changing cages so as to not spill any of the contents. As a further precaution, each bottle was individually identifiable to help avoid misplacement by the staff.

### 13.3.3 Results

An initial analysis of the data showed that there was a main effect on saccharin preference for both the developmental treatment ($p < 0.0001$) and the day of testing ($p < 0.0001$). There was no interaction between day and treatment ($p = 0.8080$). Post hoc analyses of this
entire data set showed that animals in the BPA 20, EE 5 and EE 50 exposure groups all had significantly lower saccharin preference than control animals (p < 0.002). Animals in the BPA 200 group had an apparently greater saccharin preference than controls that was close to significant (p = 0.0579) (Figure 13.3).

As a result of the main effect of day, the data were then analyzed by day. As seen in the pilot study, there was a main effect of day on saccharin preference in control animals (p = 0.0101). Post hoc analysis showed that saccharin preference consistently decreased from day one through day five. The same main effect was seen in all of the treatment groups (p < 0.0005) with the same trend in lessening of the saccharin preference over time.

The data were then organized into days and tested for a treatment effect. When analyzed in this fashion, the developmental exposure had a significant main effect on saccharin preference on Day 3 (p = 0.0010), Day 4, (p = 0.0267) and Day 5 (p = 0.0177). Post hoc analyses on these days showed that animals in the EE 50 group had significantly lower saccharin preference on Day 5. Animals in the EE 5 group had significantly lower saccharin preference on Days 4 and 5. Animals in the EE 0.5 group had significantly lower saccharin preference on Day 3. Animals in the BPA 20 group had significantly lower saccharin preference on Days 3 and 4 (Figures 13.4 and 13.5). No other groups differed significantly from the controls.

The animals in this experiment were exposed developmentally to environmental estrogens in one of two blocks. The block had no effect on the saccharin preference when the entire data set was analyzed together (p = 0.9988) or when the data set was analyzed by day (p > 0.2).

This experiment used multiple animals (two or three) from the same litter. When the data were organized into litter means and reanalyzed, no different results were seen, other than
making the animals in the BPA 20 group only marginally different ($p = 0.0788$) from controls in the overall analysis.

Figure 13.3: Saccharin preference in female rats developmentally exposed to EE or BPA

Mean ± the standard error

$p < 0.004$ when compared to the control

$+ p = 0.0579$ when compared to the control
Figure 13.4: Saccharin preference by day in females developmentally exposed to EE

Mean ± the standard error

* p < 0.05 when compared to the control on that day

** p < 0.01 when compared to the control on that day

Animals exposed to 5 and 50µg/kg/day EE show a clearly masculinized saccharin preference compared to the vehicle control on Days 3, 4 and 5.
Figure 13.5: Saccharin preference by day in rats developmentally exposed to bisphenol A

Mean ± the Standard Error

* p < 0.05 when compared to the control on that day

** p < 0.01 when compared to the control on that day

Animals exposed to 20μg/kg/day BPA show a masculinized saccharin preference compared to the vehicle control on Days 3 and 4.
13.3.4 Discussion

Saccharin preference in rats was shown to be sensitive to developmental exposure to either 5 or 50 µg/kg/day of EE. These doses clearly masculinized preference on the last three days of testing. In contrast, developmental exposure to BPA had no clear effect on adult saccharin preference. The middle dose of BPA (20 µg/kg/day) did masculinize saccharin preference on two days of testing. There is no clear reason as to why the middle dose of BPA would be the most active, as this wasn’t seen with any other assay.

The pattern of saccharin preference seen in this project was unexpected, with all animals showing a greater preference for saccharin during the first few days of the assay. It seems likely that the presence of a saccharin solution served as a novelty for these rats and enticed a large amount of saccharin consumption during the first day or two of the assay. After the novelty wears off, all of the animals consumed less saccharin, with the developmentally masculinized animals consuming significantly less than control animals. This consumption pattern did not alter the overall results, as there was no interaction with the treatment groups. It did force me to analyze the data by day, which could have resulted in some loss of statistical power. The variability in this assay was relatively low however, which compensated for any loss of power due to splitting the data by day.
14. MOTOR ACTIVITY

14.1 Introduction

Estrogens can influence activity patterns. Female rats will typically show higher baseline levels of activity than males (McCarthy et al., 1993). It was therefore expected that females exposed to EE developmentally will show male levels of activity (i.e. lower levels of activity). However, estrogens will alter activity in an activational-type manner (Colvin and Sawyer, 1969). Therefore, it was necessary to use ovariectomized rats exposed to estrogens during development, to determine their response only to the organizational effects of estrogens. After the activity in ovariectomized rats was measured, these same rats were then dosed with EE as adults to measure sensitivity to the compound as related to developmental exposure.

14.2 Methods

All rats were dosed and ovariectomized as described earlier. Motor activity was measured using a figure-8 maze. This maze consists of interconnected alleys (10 cm wide) fashioned to form the shape of an “8” with two dead-end hallways extending from the center (Figure 1).
The mazes are constructed of stainless steel and acrylic plastic. There are sixteen mazes total, all contained within one room. While in the mazes, the movement of each rat can be tracked continuously with photobeams. This eliminates the need for experimenters entering the room and disturbing the rats, as activity level is very sensitive to changes in the external environment. The maze is covered with a heavy acrylic top, which fits onto screws and prevents the rat from escaping. The rats were provided with water via a bottle placed into a hole located in the center of the acrylic top. In addition, 5 pellets of food were placed in the center portion of the maze. The mazes have a steel mesh bottom and a galvanized pan filled with bedding.
material to collect urine and feces and the entire mazes apparatus was cleaned thoroughly between trials.

On the night of testing, the rats were transferred from the animal room to the testing room and placed into small transfer cages. The rats were allowed to acclimate to the room for five minutes. After the five minute acclimation time, the rats were quickly transferred into the sixteen figure-8 mazes.

The rats were allowed to freely explore the maze for 10 hours in the dark. The activity trial started approximately an hour before the dark cycle began. Rats are more active during the beginning and end of the dark cycle, and timing the activity assay in this manner will allow measurement during these high activity periods.

14.3 Pilot Study – Motor Activity

14.3.1 Introduction

The figure 8 maze utilized in this study is not typically used for long, overnight, measures of activity. It was therefore necessary to complete two separate pilot studies. The first pilot study measured overnight activity levels in male, female and ovariectomized female rats. If activity is indeed sexually dimorphic, then female rats should have higher activity levels than male rats. In addition, if this activity difference is controlled by adult estrogen levels, ovariectomized female rats should show lower levels of activity than intact females. In addition, if ovariectomized female rats do indeed show low activity levels, exposing these females to estrogen should restore the high activity levels. Determining the dose of estradiol, as
well as the duration of exposure, needed to restore activity is vital for the experimental assays on developmentally exposed animals.

14.3.2 Methods

All animals were run in the figure-8 maze as previously described. Adult male, adult female intact, and adult female ovariectomized Long-Evans rats were purchased from Charles River Laboratories, North Carolina. All animals were first run in the maze to determine the effects of sex and ovarian status on overnight motor activity. The ovariectomized females were then exposed to either 175 µg/kg/day or 275 µg/kg/day EE for fourteen days and rerun in the maze.

14.3.3 Results

There was a significant main effect of sex and ovarian status on the total activity in the maze (p <0.0001). A post hoc analysis showed that intact females had significantly higher activity levels (p < 0.001) than either males or ovariectomized females (Figure 14.2).

In the follow-up pilot study investigating the effect of estradiol replacement on activity levels, there was a significant main effect of estradiol level on total activity (p = 0.0148). Post hoc analyses showed that ovariectomized female rats receiving 275 µg/kg EE for fourteen days had an activity level that was close to significantly higher than the negative control (p = 0.075), although their activity was still significantly lower than intact females (p = 0.025). This difference between the negative control and the EE replacement rats most likely would have reached significance had the sample size been larger. Rats receiving 175 µg/kg EE for fourteen days did not show a recovery in activity levels (p = 0.2228) (Figure 14.3).
** Figure 14.2: Motor activity levels in adult male, adult female, and adult ovariectomized female rats 

Mean ± the standard error

** *p < 0.01 when compared to males or ovariectomized females

Note that the sexual dimorphism present in this behavior is completely abolished by removing the ovaries.
Figure 14.3: Motor activity in adult female ovariectomized rats given ethinyl estradiol for 14 days

Mean ± the standard error

*+ p = 0.0029 vs. intact positive control and p = 0.075 vs. vehicle negative control.

Note that 275µg/kg/day treatment appears to partially restore activity levels. This p value would most likely have reached significance with a larger sample size.
14.3.4 Discussion

These pilot studies clearly show that activity level is sexually dimorphic and at least partially dependent on adult estrogen levels. As such, motor activity is an appropriate behavior for use to study the effects of prenatal endocrine disruptor action on adult behavioral sensitivity to estrogen. This sensitivity can be tested by exposing ovariectomized females to a known estrogen level and measuring the increase in activity. This assay is not ideal, however, because the sexually dimorphic activity cannot be fully restored in ovariectomized animals. With this submaximal activity restoration, it will be difficult to find significant changes in estrogen sensitivity between treatment groups unless the variability is quite low. Variability in the pilot studies was higher than expected, however this particular assay is the only motor activity set-up available.

Based on these pilot data, it was decided to use 275 µg/kg/day EE for 14 days to restore activity levels and test developmentally exposed animals’ sensitivity to estrogen. This dose did not fully restore activity levels, but previous work with running wheels indicates that full restoration of activity may not be possible in ovariectomized rats (LE Gray, personal communication).

14.4 Main Study – Motor Activity

14.4.1 Introduction

The pilot studies indicate that overnight motor activity in the figure-8 maze is indeed sexually dimorphic and partially dependent on adult estrogen levels. These characteristics make this assay ideal for measuring adult sensitivity to estrogen in developmentally masculinized
female rats. This requires ovariectomizing the developmentally dosed animals and exposing them to a known concentration of EE. It was determined from the pilot studies that 275 µg/kg/day for two weeks would sufficiently (although not maximally) restore motor activity levels and would serve as a good reference dose for testing sensitivity to estrogen.

14.4.2 Methods

Developmental exposure and adult testing was conducted in Long Evans rats as described earlier. All animals were ovariectomized prior to testing. In addition, two or three females from every litter were run as adults.

As adults, all animals were run through the maze once. These animals were then given 275 µg/kg EE via oral gavage for fourteen days with the exception of the vehicle control animals. The rats exposed developmentally to the vehicle control were split into two groups as adults. One half did receive the 275 µg/kg EE for fourteen days and served as the positive control in this experiment (0 in Figures 14.5 and 14.6). The remaining vehicle control animals only received corn oil as adults (0 + OIL in figures 14.5 and 14.6) and served as a negative control.

14.4.3 Results

During the data analysis, one trial had to be removed from these results due to experimenter error during the data collection.

I first organized the data by treatment group and analyzed total activity both before and after adult estradiol exposure. Comparing the activity before estradiol exposure revealed a significant main effect of treatment (p = 0.0453). Post hoc analyses showed that animals in the
EE 50 (p = 0.0036) and BPA 2 (p = 0.0225) exposure groups had baseline activities higher than controls (Figure 14.4).

After fourteen days of estradiol exposure, all of the treatment groups had similar activity levels, with no group differing from the controls exposed to estradiol. Control animals exposed to oil for fourteen days did differ from the control exposed to estradiol (p = 0.0327), showing that the adult estradiol exposure was sufficient to elevate activity levels in control animals (Figure 14.5).

To determine the effect of the developmental treatment on adult sensitivity to estrogen, I calculated the difference in activity after estradiol exposure for each animal. These values were then used in an ANOVA. This analysis revealed no main effect of treatment on sensitivity to estrogen (p = 0.0953). Despite the lack of significance, the results do follow the expected pattern, with animals in the EE 50 treatment group showing the lowest sensitivity to estrogen (Figure 14.6).

I ran an additional ANOVA which compared the means within each treatment before and after estradiol exposure without calculating means for each individual animal. This analysis yielded a similar pattern of results. Adult estradiol treatment significantly increased activity in control animals (p = 0.0075). Adult estradiol exposure clearly did not raise activity levels in the EE 50 animals (p = 0.6946) or the BPA 200 animals (p = 0.1300). Without exception, every other treatment group showed evidence of an increase in activity; however the values never reached significance (p values were between 0.06 and 0.09 for all other treatment groups).

The animals run in this experiment were from one of two developmental exposure blocks. When analyzed for block, it was shown that the two blocks did influence baseline activity levels (p = 0.0025). When block and treatments were analyzed together, both had a significant effect
on baseline activity levels ($p < 0.05$) with no block by treatment interaction ($p = 0.7199$). This re-analysis of the data had no impact on the results; the EE 50 and BPA 2 animals still showed higher baseline activity than the controls ($p < 0.05$).

Adult sensitivity to estrogen was also influenced by block ($p = 0.0468$), again with no treatment by block interaction ($p = 0.9741$). Re-analyzing the data by blocks did not change any of the results.

This experiment also used multiple animals (two or three) from the same litter. When the data were organized into litter means and reanalyzed, the main effect of treatment on estrogen sensitivity did reach significance ($p = 0.0437$). Post hoc analyses revealed that all treatment groups were significantly less sensitive to estrogen than the control animals ($p < 0.02$) with the exception of the EE 0.5 ($p = 0.0894$) and the BPA 20 ($p = 0.0984$) animals (Figure 14.7).
Figure 14.4: Motor Activity in Adult, Ovariectomized Female Rats Developmentally Exposed to an Environmental Estrogen

Mean ± the standard error.

** p = 0.0036 when compared to the control

* p = 0.0225 when compared to the control.

The EE 50 and BPA 2 animals have a higher baseline activity level when compared to the other treatment groups.
Figure 14.5: Motor Activity in Adult, Ovariectomized Female Rats Developmentally Exposed to an Environmental Estrogen and then Given Estrogen for 14 Days as an Adult

Mean ± the standard error.

* p = 0.0327 when compared to the positive control (0).

No treatment group differed from the positive control (0) except for the negative control (0 + OIL).
Figure 14.6: Change in total activity after 14 days of adult ethinyl estradiol treatment

Mean ± the standard error

The main effect of treatment did not reach significance (p = 0.0953)

These values were determined by subtracting the activity from each animal after estradiol exposure from the activity before estradiol exposure to give an indicator of adult sensitivity to estrogen.
Figure 14.7: Change in total activity, analyzed by litter means, after 14 days of adult ethinyl estradiol treatment

Mean ± the standard error

* p < 0.02 when compared to the positive control (0)
14.4.4 Discussion

Developmental exposure to a high dose of a strong estrogen (i.e. EE 50) increased baseline activity in female rats. This result may arise from these animals being more anxious or by some other unknown mechanism. Surprisingly, exposure to the lowest dose of BPA had the same effect. There is no clear explanation for why this would happen. As expected, adult exposure to estradiol increased activity in all of the animals with the exception of the two previously mentioned groups with the elevated baseline activity.

All of the animals developmentally exposed to an environmental estrogen whether it be EE or BPA showed a decreased sensitivity to estrogen as measured by the increase in activity after adult estradiol exposure. This indicates that the developmental exposure to the environmental estrogen masculinized the female rats and made them less sensitive to estrogens as adults. Masculinization of these animals was expected; however, the degree of masculinization was striking. In addition, this masculinization occurred in the BPA 2 group. This dose group showed no masculinization in any other assays (including both mice and rats) in this project. This indicates that adult behavioral sensitivity to estrogen is very sensitive to developmental disruption.

Despite the apparent sensitivity of this assay, the data were quite variable. Analyzing the data using individual means resulted in few significant findings. Taking litter means, however, controlled for some of this individual variability and resulted in significant differences in almost all of the treatment groups. In this case, lowering the sample size (and potentially reducing the power of my statistical analysis) by calculating litter means actually resulting in increasing the statistical power by reducing the variability within each treatment.
15. GENERAL DISCUSSION

In this project, I have found that developmental exposure to either bisphenol A (BPA) or ethinyl estradiol (EE) can masculinize female mice and rats. Of the two compounds tested here, EE was the stronger endocrine disruptor, impacting every behavioral assay used in this project. BPA was a weaker endocrine disruptor, but did impact selected behaviors, namely anxiety in the mouse and motor activity in the rat.

In this study I also measured traditional reproductive endpoints used in toxicology. I found that both EE and BPA can accelerate the onset of puberty. This was expected and parallels earlier research, however I was not able to replicate the low-dose puberty accelerating effect of BPA reported by Howdeshell et al. (1999). EE, as a strong endocrine disruptor, was able to lengthen the AGD in female as well as decrease litter size at high doses in the rat. BPA had no obvious impact on either of these endpoints.

Every behavioral assay employed in this project was successful in identifying the developmental effects of the two endocrine disruptors studied, although with varying degrees of sensitivity. The assays of short-term spatial memory, the Barnes maze and the radial-arm maze, identified masculinization in mice exposed to 5 µg/kg day EE, but did not consistently detect any masculinization in animals exposed to any dose BPA. As such, these assays were less sensitive to prenatal masculinization than some of the other behavioral assays. In addition, both tests of spatial memory are very time-consuming and low-throughput. For these reasons, I would not enthusiastically recommend using these assays in future studies on environmental estrogens. There are other tests of spatial memory, such as the Morris water maze and various T-maze
paradigms. These assays are as time-consuming as the two mazes I used, and it is unlikely that they would be more sensitive to the effects of estrogenic endocrine disruption.

The assays of anxious behaviors were quite sensitive to the effects of the compounds I studied. Developmental exposure to either EE or BPA altered adult behavior in both the light/dark preference chamber and the elevated-plus maze. In this project, only mice were tested in these assays. However, estrogenic compounds do alter rat performance in these assays as well, both with adult administration (Lund et al., 2005; Patisaul et al., 2005) and developmental administrations (Arabo et al., 2005; Farrabolini et al., 1999). There is a sufficient history to merit a study on the effects of developmental exposure to environmentally relevant levels of environmental estrogens in the rat using my protocol. I think it is likely that the results of such a study would parallel the findings I am reporting here in the mouse.

These anxiety assays are somewhat unique. The results seen in all other assays used in this project can be attributed to masculinization of the female rodent nervous system. This is not necessarily true with the anxiety assays. The female mice exposed to EE showed increased anxiety, and male mice do not generally show high levels of anxiety. I am confident in the finding of my anxiety study, as they closely parallel previous research (Arabo et al., 2005; Farrabolini et al., 1999). It is possible that gonadectomized male rodents would have high anxiety levels, and that this anxiety is masked by adult androgen levels. This theory would match my results, as I prenatally masculinized and then ovariectomized female mice, in a sense creating gonadectomized male mice. There is some indirect evidence supporting this theory in rats (Svennson et al., 2000), but there is also evidence refuting it (Zimmerberg and Farley, 1993).

Saccharin preference in rats was clearly disrupted by developmental exposure to EE, but was not clearly disrupted by BPA. The saccharin preference seen in female rats developmentally
exposed to both 5 and 50 µg/kg/day EE indicated that these animals were indeed masculinized. The pattern of saccharin preference was unusual, with all animals showing a greater preference for saccharin during the first few days of the assay. To the best of my knowledge, this pattern has not been reported previously. It seems likely that the presence of a saccharin solution served as a novel stimulus for these rats and enticed a large amount of saccharin consumption during the first day or two of the assay.

This pattern did not negatively affect the data, as developmental treatment seemed to have no impact on this trend. When the data were analyzed by day, the decrease in saccharin preference in masculinized animals clearly emerged. If I had measured saccharin preference for only two or three days, however, there is a good chance I would have missed this pattern and drawn the incorrect conclusion that EE had no impact on preference. It is not known why this previously unreported pattern appeared in this experiment, but future projects studying endocrine disruption on saccharin preference should measure the preference over a period of multiple days.

A relatively new endpoint studied in this project was adult behavioral sensitivity to estrogen. Estrogen serves vastly different roles in male and female mammals; therefore masculinized females should be less sensitive to the behavioral effects of estrogen as adults. I measured this sensitivity with two separate assays, motor activity and lordosis. In both of these assays, masculinized animals showed a decreased sensitivity to estrogen, as expected. The extreme sensitivity of these assays to masculinization was unexpected, however. Animals exposed to any of the doses of EE used in this project (down to 0.05 µg/kg/day) showed severely impaired lordosis behavior as adults. Unfortunately, I was unable to measure the masculinizing effects of BPA in this assay. With the apparent sensitivity of this assay, there is a very good chance that animals developmentally exposed to BPA would show disrupted lordosis behavior.
I did measure the masculinizing effects of EE and BPA in the motor activity assay. Like lordosis behavior, adult sensitivity to estrogen as measured by activity was exquisitely sensitive to developmental masculinization. Animals exposed to the lowest dose of BPA (2 µg/kg/day) showed decreased sensitivity to estrogen in this assay, as did exposure to 5 µg/kg/day EE. Unlike the lordosis behavior, individual sensitivity in this assay was quite high, and these effects did not reach significance unless they were analyzed with litter means as opposed to individual means. Even with this analysis, two of the treatment groups still did not quite reach significance.

Both of these assays were very sensitive, and are therefore good potential assays for future behavioral work in endocrine disruption. The lordosis behavior may be the more powerful of the two assays, however. The results of the lordosis experiment were much less variable than the results of the activity assay. Plus, the lordosis assay can be easily completed with one week’s work, whereas the motor activity assay required two full weeks of dosing. In addition, running a large number of animals in figure-8 mazes requires a sophisticated computerized arrangement. The lordosis assay is easy to run and requires no specialized equipment. It is high throughput, as I was easily able to test thirty animals in an afternoon. I also found that there was very low variability in the lordosis behavior. The only negatives are that it requires the developmentally exposed females to be surgically ovariectomized (as would any measure of adult sensitivity to estrogen). It also requires some pre-training of the males to ensure that they mount all control females with equal zeal. Overall, however, I believe the positive aspects of the assay far outweigh the negatives and I would strongly consider using this assay in any future studies on estrogenic endocrine disruption.

This lordosis assay, as well as the motor activity assay, detected masculinization at developmental dose ranges far below what was detected with the general reproductive endpoints.
I found an accelerated onset of puberty at a dose of 200 µg/kg/day BPA in the mouse and found no effect of BPA on any measured reproductive endpoint in the rat. With EE, I found an increased onset of puberty in both the mouse and the rat at 5 µg/kg/day. I found no effect on any of the reproductive endpoints or mortality at any lower dose. In contrast, I found behavioral effects at the lowest doses tested for both EE and BPA in the behavioral assays. The results of this project show the importance of including behavioral assays in endocrine disruptor research. Previously published research on the developmental effects of EE on reproductive endpoints in the rat found no abnormalities below an oral dose of 50 µg/kg/day (Sawaki et al., 2003). Another study using behavioral endpoints in rats found effects at injected doses of 15 µg/kg/day (Dugard et al., 2001). By using carefully designed behavioral assays, I found developmental effects of EE at oral doses down to 0.05 µg/kg/day.

Previously published work on the developmental effects of BPA have found some reproductive endpoints to be sensitive even at low doses of 2 µg/kg/day (Howdeshell et al., 1999; Welshons et al., 1999). I was also able to see effects at this low dose in rats using the motor activity assay. I was not able to reproduce the effects seen by Howdeshell et al. (1999) on puberty in mice, however.

It is important to note that 2 µg/kg/day BPA and 0.05 µg/kg/day EE were the lowest doses used in this project and I found both of these doses to be sufficient to cause masculinization. It is possible that even lower doses of these two compounds may also have been active. The masculinizing potential of these compounds at such low levels was surprising. Future behavioral studies with these compounds should attempt to find the lowest observable effect level.
Ethynyl estradiol is generally considered a strong estrogen whereas BPA is relatively weak. My project supports this conclusion with regard to the reproductive endpoints. I observed acceleration of puberty and pup mortality in the rat with EE but not BPA. Similarly, I found that 5 µg/kg/day EE was sufficient to accelerate puberty in the mouse, but 200 µg/kg/day of BPA was required to accelerate puberty.

The behavioral assays mirrored this trend as well. In mice, masculinization by EE was clear in both the anxiety and memory assays, whereas BPA was only able to disrupt behavior in the anxiety assays. In the rat, BPA had no effect on saccharin preference at any dose, whereas EE did alter preference at higher doses. Dose responsivity in the motor activity assay was less clear, most likely due to the high individual variability. This makes it difficult to definitively state which compound was stronger.

Both mice and rats were sensitive to the effects of EE. A developmental treatment of 5 µg/kg/day was able to disrupt both physiology and behavior in both species. As stated earlier, rat behavior was more sensitive to EE than was physiology. In mice, it is impossible to make such a claim as only one dose of EE was tested. Species sensitivity to BPA was a bit more complex. Mice seemed equally sensitive to BPA with both behavioral and physiological endpoints. 200 µg/kg/day BPA accelerated the onset of puberty as well as disrupted anxiety behavior in mice. The lower dose of 2 µg/kg/day had no consistent effect on any of the endpoints measured in mice.

Rats were physiologically insensitive to BPA. None of the doses altered any of the physiological endpoints measured. In contrast, rat behavior was quite sensitive to disruption by BPA. Sensitivity to estrogen as measured by motor activity was disrupted at the lowest BPA dose used. In general, mouse physiology and behavior appeared equally sensitive to disruption
by both BPA and EE. Rat physiology was less sensitive to disruption than was behavior for both BPA and EE. Stated another way, rat behavior was the more sensitive set of endpoints measured in this project, and rat physiology was less sensitive. Mouse behavior and physiology were both intermediate in sensitivity.

It is widely accepted that humans are exposed to BPA at levels equivalent to 2 µg/kg/day (Goodson et al., 2004; Olea et al., 1996) from a variety of sources. Wildlife exposure to BPA is less well established, but measurements of surface water indicate potential exposure levels well above the 2 µg/kg/day level (Furhacker et al., 2000). I have found that exposure to this dose of BPA can alter development of the nervous system and consequently adult behavior in the rat.

Human exposure to EE is less well defined; however, clinically relevant doses of EE fall in the range of 0.4 – 0.8 ug/kg/day (Timms et al., 2005) and EE is present in surface water as well (Desbrow et al. 1998; Larsson et al., 1999; Routledge et al., 1998; Ternes et al., 1999). Based on this, it is probable that both humans and wildlife are exposed to doses higher than the active dose of 0.05 µg/kg/day that I found in this project.

I think it is highly likely that sensitive populations of both humans and wildlife are exposed to both BPA and EE at doses shown to be active in this project. Other estrogens are commonly found in the environment as well. Developmental exposure to phytoestrogens has been shown to alter adult physiology and behavior in rodents (Kouki et al., 2005; Wisniewski et al., 2005), as can BPA and EE. In addition, developmental exposure to antiandrogens can alter the development of accessory sex glands in male rodents (Gray et al., 2000) as can BPA and EE (Timms et al., 2005). Humans and wildlife are exposed not only to BPA and EE, but also phytoestrogens and antiandrogens. This complex endocrine disruptor environment is likely to magnify the effects seen in any one of the compounds alone. Future studies on endocrine
disruptor action should focus on the combinational effects of these types of mixtures to more closely represent the environment in which both humans and wildlife live.
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