EFFECT OF PERINATAL EXPOSURE TO PROGESTERONE AND ESTRADIOL ON ORGANIZATION OF REPRODUCTIVE BEHAVIOR AND NEUROENDOCRINE RESPONSIVENESS IN OPOSSUMS (*MONODELPHIS DOMESTICA*)

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ABSTRACT

The gray short-tailed opossum (*Monodelphis domestica*) is a small (60-150g), pouchless, nocturnal marsupial native to Brazil. Previous studies have shown that when males of this species are castrated as adults and later treated with female hormones (estradiol and progesterone), they will exhibit female receptive behavior and even allow penile intromission. In some eutherian species, such as the laboratory rat, these feminine receptive behavior patterns cannot be activated in castrated adult males because of the defeminizing effects of testosterone, secreted by the male fetus, and the maternal hormone, progesterone, secreted during gestation. The difference between opossums and rats in expression of female receptive behavior might be related to differences in their exposure to progesterone during development. Opossums have a short, 15-day gestation and the critical period for differentiation of sexually dimorphic behavior is 8 days after birth when maternal progesterone is low. By contrast, the critical period for differentiation of sexual behavior in rats is late in the 22-day gestation when progesterone levels are high. The hypothesis tested in this study was that increased exposure to progesterone during the critical postnatal period would defeminize receptive behavior in male short-tailed opossums as well as reduce their ability to exhibit an LH rise. Male and female opossums received on postnatal day 8, either: 1) corn oil alone (i.e., control n=26), or corn oil with: 2) progesterone (n=29); 3) both estradiol and progesterone (E+P, n=29); or 4) a progesterone receptor antagonist,

(mifepristone, n=37). All were gonadectomized (GDX) before the age of puberty (11 weeks of age), and after receiving female hormone replacement therapy as adults (27 weeks of age), they were tested for female sexual behavior by pairing them with stimulus males over a 25-hr period. Blood samples were collected every 3 hours for 16 hours beginning 1 hour before pairing, and the concentrations of estradiol and LH in plasma samples were measured by radioimmunoassay and a Leydig cell bioassay, respectively. Both males and females were sexually receptive to stimulus males, and the hypothesis regarding the defeminizing effect of progesterone on receptive behavior was rejected. Males treated neonatally with both estradiol and progesterone were more likely to copulate than other treatment groups, including control females ($p < 0.015$). This surprising result indicates that estradiol, in combination with progesterone, feminizes sexual behavior in males of this species. Progesterone-treated animals showed more and mifepristone animals showed less fighting ($p < 0.02$) than controls. Mifepristone animals also elicited more scent marking from stimulus males ($p < 0.04$) than animals in other neonatal treatment groups. These results indicate that progesterone has a role in masculinizing fighting behavior in opossums. By increasing progesterone exposure during the critical period of sexual differentiation, aggressive behavior is increased in the adult opossum. The proportion of males and females neonatally treated with estradiol and progesterone that exhibited an LH rise was lower than in control animals. The high level of copulation in E&P-treated males and low incidence of an LH rise in this group suggests differential organization of the GnRH surge mechanism and receptive behavior. Estradiol concentrations revealed that estrous levels of estradiol were necessary for an LH rise, but not copulation. The percentage of control males and females exhibiting LH rises was similar, indicating that a LH rise might be a normal, hormonal response to pairing in male and female opossums.

For my parents, Patty and Denny

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CHAPTER 1

REPRODUCTIVE ENDOCRINOLOGY AND BEHAVIOR OF THE GRAY SHORT-TAILED OPOSSUM: A REVIEW

Introduction

Approximately 30% of the 318 species of extant marsupials (Infraclass Metatheria) live in the New World, primarily in South America, and nearly all (87 of 94 species) belong to a single family, Didelphidae in the order Didelphimorphia (Wilson and Reeder, 2005; Feldhamer et al., 2007). Didelphids are morphologically similar to ancestral marsupials of the Cretaceous and exhibit several primitive or ancestral features including five unfused digits, a dental formula containing 50 teeth, a long rostrum, a well-developed sagittal crest, and a long, relatively hairless, prehensile tail (Feldhamer et al., 2007). Two species: the Virginia opossum (*Didelphis virginiana*) (1-2 kg) and the gray short-tailed opossum (*Monodelphis domestica*) (60-120 g) represent nearly the full near range of body size and geographic distribution for members of this family. The range of the Virginia opossum, the only marsupial in North America, extends into northern United States; whereas *M. domestica* is native to southeastern Brazil and Bolivia where it lives in a dry, rocky scrub forest habitat know as Caatinga (Streilein, 1982).

Metatherians are distinguished from other viviparous mammals (Infraclass Eutheria) based on reproductive anatomy and physiology. Metatherian reproduction is characterized by a short period of gestation followed by a long and complex period of lactation. It is during this

extended period of lactation that most of the growth and development of the young occurs, rather than prior to birth as in eutherians (Tyndale-Biscoe, 2005). The female reproductive tract is the single most important character distinguishing metatherians from eutherians. During metatherian development, the ureters migrate inside (medial) to and above the genital ducts and thus, the oviducts cannot join to form a single vagina, cervix, and uterus as they do in eutherian species (Tyndale-Biscoe, 2005). In metatherian species, birth takes place through a median pseudovaginal canal, allowing for increased ease of delivery (Tyndale-Biscoe, 2005).

The gray short-tailed opossum (*Monodelphis domestica*), hereafter referred to as opossum, is one of 87 species in Didelphidae (Feldhamer, 2007). It inhabits the semi-arid Caatinga region of eastern Brazil (Streilein, 1982). This small (60-150g), pouchless species prefers rocky areas, but has also been known to reside in areas occupied by people. They are solitary and mostly nocturnal animals that exhibit their highest levels of activity during the first several hours of darkness (Streilein, 1982). Breeding in the opossum can take place throughout the year. Sexual maturity occurs at about five months of age (Stonerook and Harder 1992), and in captivity, females remain reproductively active through 30 months of age.

The opossum has proven to be disease resistant and easy to breed in a laboratory environment (Kraus and Fadem, 1987). Because most of body growth and much of fetal development occurs after birth, metatherians are ideal for studies of late fetal development. Also, the pouchless condition allows easier access to young than in other marsupials such as the tammar wallaby (*Macropus eugenii*) or honey possum (*Tarsipes rostratus*) that have a pouch.

Endocrine control of the estrous cycle

The estrous cycle is a sequence of physiological, morphological, and behavioral changes punctuated by recurring periods of estrus and ovulation (Feldhamer, 2007). Most metatherian species exhibit a spontaneous estrous cycle and more than one estrous event in a year (Tyndale-Biscoe and Renfree, 1987; Tyndale Biscoe, 2005). Control of reproductive processes in the female is centered in the hypothalamus, the anterior pituitary, and the ovaries, which interact through positive and negative feedback regulation of their secreted hormones. The control center for secretion of reproductive hormones is the hypothalamus, which acts through secretion of gonadotropin releasing hormone (GnRH) (Senger, 2005). The hypothalamus secretes GnRH in a pulsatile manner into the hypothalamo-hypophyseal portal system which carries the hormone directly to the anterior pituitary. This stimulates the anterior pituitary to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH) into the bloodstream which promotes ovarian follicular development, secretion of ovarian steroids (first estradiol and then progesterone), estrus, and ultimately ovulation.

FSH and LH stimulate theca and granulosa cells of the ovarian follicle to begin the follicular phase, the first of two phases the ovary will experience during the estrous cycle. During this phase, FSH stimulates follicular growth and, with LH, stimulates secretion of estradiol (E). Elevated E inhibits release of GnRH, FSH, and LH secretion by negative feedback. However, an appropriately high level of E provides positive feedback on the secretion of GnRH from the hypothalamus and ultimately results in a surge of LH released from the anterior pituitary. This LH surge triggers ovulation in which the follicular wall ruptures and the oocyte is released into the oviduct.

The granulosa and thecal cells of the ruptured follicle then transform into luteal cells, the follicle develops into a corpus luteum (CL) and the luteal phase of the cycle begins. During this phase, the CL produces progesterone (P) in response to LH stimulation, which also has a negative feedback effect on the hypothalamus, and thus down regulates GnRH, FSH, and LH secretion. P increases the secretory activity of the endometrial lining of the uterus in preparation for implantation of the fertilized oocyte. If implantation and pregnancy do not occur, the CL regresses and P levels decline. Because there is no longer negative feedback upon the hypothalamus by P, GnRH and thus FSH and LH can be produced and released once more, beginning the follicular phase of the next estrous cycle. This sequence of events will continue in spontaneous ovulators unless interrupted by periods of seasonal anestrus or pregnancy and lactation.

Reproductive biology of the gray short-tailed opossum

Due to the solitary nature of gray short-tailed opossums, very little information on their behavior has been gathered for these marsupials in the wild (Streilein, 1982). The current laboratory colonies are descendants of nine individuals brought from their native habitat in Brazil to the National Zoo in 1978 (Fadem et al., 1982). Soon after, it was discovered that the opossum is easily maintained in captivity and able to breed year-round, making it an excellent species to be studied in a laboratory setting (Trupin and Fadem, 1982). Since then, the opossum has been used in a multitude of laboratory studies from genetics and development to behavior and physiology.

Gray short-tailed opossums lack an estrous cycle and estrus is instead induced by male pheromones (Fadem, 1987), a phenomenon that has not often been reported in marsupials.

Estrus in female opossums is induced by a five to eight day exposure to male pheromones on scentmarked objects (Fadem, 1987; Harder et al., 1993; Harder and Jackson, 1998). These male pheromones come from a sexually dimorphic suprasternal scent gland that the males rub on objects in their environment (Fadem and Schwartz, 1986; Fadem and Cole, 1985). Scentmarking behavior in male opossums has been linked to testosterone (Fadem et al., 1989). The female opossum later approaches the scentmarked object and exhibits a behavior termed "nuzzling" to bring the pheromone into her vomeronasal organ which begins her induction into estrus (Poran et al., 1993; Poran, 1998). "Nuzzling" refers to a series of rubbing, tapping, and licking behaviors the female displays on a scentmarked surface (Poran et al., 1993). The estrus-inducing male pheromone is known to be non-volatile (Harder et al., 2008), and therefore, nuzzling is important for transduction of the pheromone. Nuzzling dissolves dry, non-volatile pheromones with nasal and oral secretions and delivers them to the vomeronasal organ (Poran et al., 1993).

Opossums are induced ovulators; i.e., estrous females will not ovulate unless paired with a male (Stonerook and Harder 1992), but ovulation will still occur even if the male is removed before copulation can take place (Baggott et al., 1987). Males and females exhibit a variety of precopulatory behaviors just prior to copulation including genital sniffing, flank grasping, open mouth threats, chasing, circling, and rump dragging (Trupin and Fadem, 1982). Copulation does not occur in the standing position common to many mammals, such as rats, instead it occurs with both animals lying on their sides, often the right, while the male holds the ankles of the female in his back feet and grasps her around the middle with his forelegs and teeth (Trupin and Fadem, 1982; Baggott et al., 1987). Most (86%) copulations occur during the dark phase. An average litter of 7 is born after a 15-day gestation period and weaning occurs eight weeks after birth (Harder et al., 1993). Juvenile females isolated from all male stimuli do not experience puberty

(Stonerook and Harder, 1992), and direct, nuzzling exposure to male scent marks accelerates body growth and the onset of puberty is advanced to 4 months of age (Harder and Jackson, 2003).

Estrus in opossum is characterized by elevated plasma concentrations of E, and estrous cytology in urogenital sinus smears (Fadem, 1987, 1989, Jackson and Harder, 2000) about 8 days following initial exposure to male pheromones. Elevated E is vital for the expression of precopulatory behaviors and for reduction of female threat behavior towards the male (Fadem et al., 1996). Females that have been induced into estrus by exposure to the male pheromone and then paired with a male show a rise in P (Harder et al., 2005) and preovulatory LH rise 11-19 hr after pairing with a male but 9-14 hr before copulation (Jackson et al., 1999). Full receptivity and copulation is dependent elevated P as well as E (Fadem et al., 1996). The precopulatory elevations in P and LH are male-induced and do not occur in estrous females isolated from males (Harder et al. 2005). As such, the LH rise represents a feminine neuroendocrine response to pairing that has also been observed in castrated males following administration of E and P (Harder et al., 2001).

Control of sexual differentiation of behaviors in several eutherian and metatherian species

Although androgens activate masculine behavior, and E and P activate feminine receptive behavior in adulthood, masculine organization of the brain in some mammals is dependent upon estrogen rather than testosterone (Nelson, 2000). The aromatization of testosterone to estradiol in neurons of the forebrain is believed to be responsible for the organization of many masculine behaviors, including sexual behaviors, which are activated by testosterone in adulthood. Studies in the laboratory rat (*Rattus norvegicus*) have determined that circulating estrogens are kept from

masculinizing female fetuses by high circulating levels of α-fetoprotein that binds E and prevents it from crossing the blood brain barrier (Nelson, 2000). However, an androgen-binding globulin is not present in concentrations sufficient to bind circulating testosterone. Thus, testosterone produced by fetal testes is able to reach the brain, where it is aromatized to estradiol and masculinizes neural organization of the forebrain (Nelson, 2000).

Ovarian hormones activate receptive behavior in intact females, and also restore feminine receptive behavior in ovariectomized (OVX) females. In OVX rats, restoration of receptive behaviors depends on the timing and dose of the ovarian hormones E and P (Fadem et al., 1979). Feminine receptive behaviors can be activated with exogenous E and P in castrated adult males of some eutherian species, such as hamsters (Tiefer, 1970). However, in most eutherian species, including laboratory rats, the brain is fully defeminized during normal development such that feminine receptive behaviors cannot be activated in castrated males with exogenous E and P (Goy and McEwen, 1980).

Witcher and Clemens (1987) demonstrated that exposure of fetal rats to maternal ovarian hormones (E&P) during gestation defeminizes adult sexual behavior. Because hamsters have a 16-day gestation and rats have a 22-day gestation, it is possible that the species difference in the behavior of castrated, steroid replaced adult males is due to rats being exposed to maternal hormones for a longer period of time than hamsters. The maternal hormones that act in utero to defeminize sexual behavior expressed in adulthood have less time during the shorter hamster gestation to completely defeminize male behavior. Therefore, when adult males are castrated and given female hormones, they will exhibit female receptive behaviors. If this effect of limited exposure to P during development is able to be generalized, then it should be apparent in marsupials due to their brief periods of gestation (Tyndale-Biscoe, 2005).

Most of body growth and much of development in marsupials occurs after birth (Tyndale-Biscoe, 2005). If defeminization of receptive behaviors in males is diminished with decreased length of gestation, then male opossums would be expected to show high levels of feminine receptive behaviors when castrated and given ovarian hormones in adulthood. This hypothesis is supported by the results of a study done by Fadem and Erianne (1997), in which castrated male opossums were just as likely as females to show receptive behaviors and allow penile intromission when given an E and P treatment previously shown to fully restore reproductive behaviors in OVX females (Fadem et al., 1996). Penile intromission is possible in the male opossums because of the single, cloaca-like opening in the genital region of males that is very similar to what is seen in females (Fadem et al., 1997).

The critical period for organization of sexually dimorphic behavior in opossums has been identified in several studies involving the exposure of neonates to gonadal hormones. Treatment of opossums with the estrogen antagonist tamoxifen during the third week of postnatal life defeminized threat behavior in females, and when followed by pubertal gonadectomy (GDX) and estradiol treatment in adulthood, it also reduced male-typical behavior (Fadem, 1995). This treatment also had a masculinizing effect on body weight in this species. Scentmarking behavior and phallus length however, were not significantly affected in males (Fadem, 1995). Also, opossums treated during the first week of life with E were masculinized with regard to scentmarking behavior, phallus length, and body weight and demasculinized with tamoxifen treatment. Subsequent studies concluded that tamoxifen treatment has antiestrogenic effects in the first week, mixed estrogenic and antiestrogenic effects in the third week, and estrogenic or masculinizing effects when given in the fifth week of postnatal life (Fadem, 2001). Changes in the effects of tamoxifen on male sexual differentiation, paired with high estradiol and androgen

levels on postnatal day eight, as well as the large increase in brain aromatase activity from postnatal day four to postnatal day eight leads to the conclusion that the critical period of sexual differentiation occurs on day eight (Fadem and Harder, 1992).

The existence of a postnatal period of sexual differentiation in opossums makes them useful for further studies of the role of maternal hormones in the organization of sexual behavior in adult males. Hormone exposure can easily be manipulated during neonatal life, and receptive behaviors then monitored once the young reach adulthood. This study was done to examine the importance of maternal hormones, particularly P, during the critical period of sexual differentiation for defeminization of receptive behaviors in male short-tailed opossums.

CHAPTER 2

EFFECT OF PERINATAL EXPOSURE TO PROGESTERONE AND ESTRADIOL ON ORGANIZATION OF REPRODUCTIVE BEHAVIOR AND THE LH RISE IN OPOSSUMS (*MONODELPHIS DOMESTICA*)

INTRODUCTION

The gray short-tailed opossum (*Monodelphis domestica*), hereinafter referred to as opossum, is a small (60-150g) marsupial native to the Caatinga region of Brazil (Streilein, 1982). Females lack an estrous cycle and are instead induced into estrus by exposure to a pheromone present in the male's scentmark (Fadem, 1985; Fadem and Cole, 1985; Fadem, 1987; Harder et al., 1993; Harder and Jackson, 1998). Sexual receptivity is determined by a series of precopulatory behaviors including genital sniffing, flank grasping, open mouth threats, chasing, circling, and rump dragging (Trupin and Fadem, 1982). Contact with a male, but not necessarily copulation, is required for ovulation to occur (Baggott et al., 1987; Stonerook and Harder, 1992). Gestation lasts 15 days and this pouchless marsupial nurses an average litter of 7 young for 8 weeks (Harder et al., 1993).

Male opossums are known to exhibit female receptive behaviors and even allow penile intromission when castrated and treated with female hormones in adulthood (Fadem and Erianne, 1997). This feminine behavior is not typically seen in males of eutherian species, such as the laboratory rat (*Rattus norvegicus*), when castrated adult males are treated with female hormones (Goy and McEwen, 1980). The difference between these two species is believed to be due to

their difference in gestation length and relative critical point of sexual differentiation (Fadem and Erianne, 1997). Gestation length in this comparison relates to the amount of time young are exposed to maternal hormones, particularly progesterone. Rats are exposed to maternal progesterone for a longer period of time during their 22-day gestation than are opossums during their 15-day gestation. In addition, opossums are born in a state that is typical of a marsupial, quite underdeveloped with extensive growth and development occurring after birth (Tyndale-Biscoe, 2005). The critical period of organization of sexual behavior and sexually dimorphic behavior in opossums is 4-16 days post partum (Fadem, 1995; Fadem, 2000; Fadem, 2001). By contrast, the equivalent period of differentiation in rats is day 18-20 of gestation (Wagner, 1998). Exposure to maternal progesterone for a longer time during the organizational period of sexual differentiation, as in rats, is associated with the activation of feminine receptive behaviors in castrated males by female hormones in adulthood. A shorter gestation length, such as that experienced in opossums, is associated with males that are, when castrated, highly responsive to female hormones in adulthood (Fadem and Erianne, 1997).

In rats, maternal progesterone (P) has an important role in sexual differentiation of the brain. Progesterone receptor (PR) expression is much higher in the medial preoptic nucleus (MPN) of males than females (Wagner et al. 1998). The MPN is an area in the hypothalamus that controls multiple sexually differentiated behaviors. PR receptor expression also coincides with a rise in testosterone that characterizes the critical period for sexual differentiation in the rat, suggesting that progesterone influences the effects of testosterone in sexual differentiation of the brain (Wagner et al., 1998). Quadros et al. (2002) reported PR expression in the MPN was not affected by prenatal treatment with androgens or an androgen receptor antagonist, suggesting that estradiol-induced expression of PR may be involved in sexual differentiation of the brain. If

the degree of defeminization of males is positively correlated with the amount of exposure they have to maternal P during sexual differentiation (Wagner et al., 1998; Wagner et al., 2001; Wagner et al., 2004; Quadros et al., 2002), then altering the amount of progesterone during development should have predictable results on male sexual behavior in adulthood**.** Defeminization of the male opossum is not measured by the lordosis quotient, as in rats, but by the lack of feminine behaviors such as screeching, genital sniffing, circling, and rump dragging and by the absence of copulation when paired with a stimulus male (Fadem and Erianne, 1997).

Ovulation is induced in female opossums by male stimuli prior to copulation. A rise in P and the LH surge occur 11-19 hours after pairing with a male but 9-14 hours before copulation (Jackson and Harder, 1999). This feminine neuroendocrine response has also been observed in some (2 of 6) males that were gonadectomized prepubertally and treated with estradiol in adulthood (Harder et al., 2001). Exposure to progesterone during sexual differentiation is expected to defeminize the neuroendocrine response shown by males in adulthood when treated with female hormones, E and P.

This study tested the effects, on adult sexual behavior, of 4 treatments: 1) an oil control, 2) progesterone, 3) progesterone with estradiol, and 4) the progesterone antagonist mifepristone injected on postnatal day 8, a critical period for organization of sexually dimorphic behavior in opossum. The objectives were to test the effects of exposure of neonates to E and P on: 1) expression of feminine sexual behavior as adults when treated with E and P and paired with a stimulus male, and 2) plasma LH concentrations when, as adults, they are treated with E and P and paired with a stimulus male. Hypotheses relating to the effects of each neonatal treatment on feminine sexual behaviors and the LH rise are shown in table 2.1.

Table 2.1. Hypothesized effects of neonatal treatment (on postnatal day 8) on feminine receptive behavior and LH rise in response to pairing with a male in GDX + EP adult opossums.

Neonatal Treatment	Expected Adult Feminine Sexual Behavior		Expected Adult Feminine LH Rise	
	Female	Male	Female	Male
Control (oil)	Unchanged	Unchanged	Unchanged	Unchanged
Progesterone (10 μg)	Unchanged	Decreased	Unchanged	Decreased
Estradiol (1 μg) and Progesterone (10 μg)	Decreased	Decreased	Decreased	Decreased
Mifepristone (25 μg)	Unchanged	Increased	Unchanged	Increased

MATERIALS AND METHODS

Animals

The animals used in this experiment were held in an animal room (approved by the American Association for Accreditation of Laboratory Animal Care) in Aronoff Laboratory at The Ohio State University. The room was maintained at 25-28 ºC. on a 14:10 light:dark cycle with the lights out at 2400. Opossums were housed in individual plastic cages (30x23x15 cm) in a mixed-sex colony with male and female cages on separate shelving racks. Food (Fox Reproduction Food, Milk Specialties Co.) and acidified water (pH=3.8) were provided ad libitum. Litters were obtained from 15 mothers; each mother contributing an average of 2 litters to the study. A total of 121 animals were treated and tested: 1) control (n=26; 14 females, 12 males), 2) progesterone (n=29; 10 females, 19 males), 3) progesterone and estradiol (n=29; 14 females, 15 males), and 4) mifepristone (n=37; 18 females, 19 males). Litters were randomly assigned to a treatment group and all the young in each litter were injected on postnatal day 8 with 5 µl of either corn oil alone (control), or corn oil mixed with 10 µg progesterone, 1 µg estradiol and 10 µg progesterone, or 25 µg mifepristone. The opossums were gonadectomized before puberty at 11 weeks of age and tested at 27 weeks of age. All experimental procedures were conducted according to an ILACUC approved protocol.

Behavioral Testing and Timing of Blood Sample Collection

At 27 weeks of age, 7 mm silastic implants filled with crystallized estradiol were inserted through a small incision in the skin on the back of the neck; the incision was then sutured closed. Isoflourane anesthesia was used for this procedure. The implants were prepared by loading a crystallized mixture of 95% cholesterol and 5% estradiol (E) into 9 mm pieces of silastic tubing to a length of 7 mm, which were then sealed with silastic adhesive. The implants were then rinsed in 100% ethanol and soaked for 16 hours in sterile saline at 37ºC. At 0400 h on the fourth day following implantation and 4 hr prior to pairing with a stimulus male at 0800 h, each test animal received a subcutaneous injection of 375 µg of progesterone dissolved in 200 µl sterile corn oil.

Blood samples were collected from the lateral tail vein of treated animals, which were held in a cylindrical rat restrainer. Samples were drawn into heparinized syringes or 70 µl hematocrit tubes at the following times: immediately prior to insertion of the E implant (400 µl), the day before pairing with a stimulus male (400 µl), 3 hr after the progesterone injection (and 1 hr prior to paring, 60 µl), 1 hr after pairing (400 µl), and at 3-hr intervals (60 µl), thereafter (1200, 1500, 1800, 2100, 2400 hr). Plasma was separated by centrifugation and stored at -20 ^{o}C until assayed for hormone concentrations.

For observation of sexual behavior, experimental animals were placed in the home cage (46x24x20 cm plastic rat cage) of a stimulus male. Three to six pairs of animals were observed on any given day; stimulus males were randomly selected for pairing from a pool of 10 males, although sire-offspring pairing was avoided. Each pair was individually monitored for 10 min, during which time the observer recorded the following behaviors: scentmarking, open mouth threats, screeches, biting, tumbling, chasing, aggressive grabs, body and genital sniffs, sex grabs,

following, and circling. The occurrence of mounting or attempted mounting, copulation, and intromission were also recorded, along with the time they occurred. After the 10-minute behavioral test, paired animals were videorecorded for the occurrence of copulation over a 25-hour period. After this 25-hour period, animals were returned to their home cages, and euthanized within 24 hours; brains were collected following transcardial perfusion for immunohistochemical analysis in a related study.

Hormone Assays

LH Bio-radioimmunoassay:

Concentration of LH in plasma was measured with a Leydig bioassay modified for use in the opossum (Debertin and Pomerantz, 1992). Testes from 7-9 week old mice were minced in Dulbecco's Modified Eagle's Medium (DMEM) containing 0.5% heat-inactivated fetal bovine serum (FBS). Testicular cells were filtered using 70-80 μm silk mesh and preincubated for 1 hour at 34ºC. Incubated cells were centrifuged and resuspended in DMEM + FBS to a final concentration of 2.5 x 10^6 cells/ml. Preincubation and resuspension of cells in fresh DMEM removed androgens produced by endogenous LH. Ovine LH (NIDDK-oLH-I-3) serially diluted from 500 to 0.49 pg/well was used as a reference preparation. Plasma (5 μl) from a prepubertal female opossum pool was added to wells containing LH standards including several 0.0 pg oLH wells. All standards and samples (5 μl) were assayed in triplicate. Forskolin and isobutylmethylxanthine in 50 μl of medium were added for final concentrations of 1.5 μM and 0.1 mM, respectively, and 100 μl of cells was added to a final incubation volume of 200 μl. The 48-well plates were then incubated in 95% air, 5% CO_2 at 34ºC for 4 hours, placed in a 58ºC water bath for 30 min to kill the cells, and then frozen at -20ºC until assayed for testosterone.

A testosterone radioimmunoassay validated for opossum plasma (Fadem and Harder, 1992a) was used to determine androgen concentrations in 25 µl of unextracted bioassay medium. The antibody used (a gift from C.E. Rexroad, Jr., Animal Science Institute, USDA, Beltsville, MD) was raised against testosterone-3-carboxymethyloxime-BSA and exhibits the following cross-reactivities: 39.5% with 5α-dihydrotestosterone; 25.3% with 5α-androstane-3α, 17β-diol; 11.0% with dihydrotestosterone; 5.8% with 5β-androstane-3β, 17β-diol; 14.0% with androstenedione; and less that 1% with progesterone, cortisol, corticosterone, and estradiol-17β. Data are reported as androgen rather than testosterone because of the antiserum cross-reactivity. Wells with 0 pg of LH were included in each assay; they averaged 5.65% with a standard deviation of 1.38. Assay sensitivity ranged from 0.08 ng LH/ml of plasma to 1.83 ng LH/ml with an average of 0.72 ng LH/ml. The concentration of LH in plasma samples was calculated using the log-transformed equation for the LH standard curve based on the square root of the percent of maximum androgen concentrations. Androgen concentrations were expressed as the percent of maximum androgen due to variability in cell concentration and responsiveness, and therefore, basal androgen production, between assays.

Estradiol Radioimmunoassay:

Estradiol (E) was extracted from duplicate 75-µl aliquots of plasma by vortex mixing (2 x for 60 sec) with diethyl ether. The extracts were dried in assay tubes and concentrations were measured with an RIA previously validated for opossum plasma (Fadem and Harder, 1992a). Assay sensitivity is 2 pg/ 200 µl; intra- and interassay coefficients of variation averaged 9% and 16%, respectively. Concentrations of E in extracts were corrected for plasma blank (charcoal stripped plasma) but not procedural loss from extraction (9%).

Statistical Analyses

Preliminary analysis showed that the distribution of 2 measures of threat, screech and open mouth, as well as their sum, labeled threat, and precopulatory behavior were skewed. Those scores were $\log_{10}$ transformed to achieve normal distributions before analysis. The number of fights and scent marking were also skewed, but those scores could not be normalized by either $\log_{10}$ or square root transformations, and those data were dichotomized (0 vs. any fighting or any marking). Group comparisons of binary outcomes were evaluated with the $\chi 2$ statistic, stratified by sex; significant omnibus tests were followed by logistic regression analyses to test for the interaction of group and sex, and to identify groups that differed from the control. Group comparisons of continuous variables were evaluated with 2-way ANOVA, sex by treatment group, followed by post-hoc LSD tests. Significance indicates a type 1 error rate of less than 5%.

Estradiol concentrations were evaluated by sex using a one way GLM ANOVA test of log transformed data. Three data points were determined to be outliers because they had a residual with absolute value greater than 2; these points were removed from the data set prior to ANOVA test.

A neuroendocrine response of treated animals to P injection and pairing with a stimulus male was recognized as the elevation of LH concentration in 1 or more serial plasma samples above a baseline. Baseline LH concentrations were estimated for each individual using an iterative process (Bradshaw et al., 2004; North and Harder, 2008). The mean LH concentration of all (6-8) samples per animal was calculated and values exceeding the mean plus 1.75 standard deviations (SD) were removed temporarily from the data set for calculation of a new mean and SD. This process was repeated until no values in the culled data set were greater than 1.75 SD

above the mean. The remaining values were used to calculate the mean baseline LH concentration for individual profiles, and an LH rise was identified in 1 or more sample values that exceeded the mean baseline concentration + 1.75 SD. The percentage of animals in each treatment group that exhibited a rise was compared to that in the control group using the $\chi 2$ statistic, stratified by sex. Significance indicates a type 1 error rate of less than 5%. All mean values are expressed ± standard error of the mean (SEM).

RESULTS

Sexual Behavior

Both males and females treated with E implants and P prior to pairing showed receptive behavior to stimulus males. Six of 14 (43%) of control females copulated with a stimulus male while 25% of 12 control males copulated. Twelve of 15 (80%) males treated with estradiol and progesterone (E&P) copulated with the stimulus male, a much higher proportion than in any other treatment group (Fig. 2.1) and significantly ($p < 0.015$) higher than the copulation rate for the male control group. In addition, the average number of times copulation occurred in each pair was higher for E&P males (2.5) than for any other treatment group, but the differences were not significant ($p > 0.05$).

The frequency of fighting behaviors exhibited by treated opossums did not differ by sex, and so the data were analyzed for females and males combined by treatment (Fig. 2.2). P-treated animals exhibited a higher frequency of fighting behavior during the first 10 minutes of pairing (8.9 ± 2.2) than mifepristone-treated animals (3.7 ± 1.8) or control animals (2.96 ± 0.89) ($p < 0.02$).

Stimulus males were more likely to exhibit scentmarking behavior when paired with a female than when paired with a male. Stimulus males showed highest scentmarking (42% of the time) when paired with a mifepristone male, significantly ($p < 0.04$) higher than when paired with males in the other 3 treatment groups (Fig. 2.3). Stimulus males were least likely to scentmark when paired with an E&P-treated male (0%).

Plasma Concentrations of Estradiol and LH

Estradiol:

Plasma E concentrations in treated animals sampled 1 hr after pairing were variable, but most (71%) were within the normal physiological range for estrous animals (14-90 pg/ml); 15% of the animals had anestrous-type plasma E concentrations (< 14pg/ml), and super-physiological E concentrations (100-300 pg/ml) were measured in 14% of the opossums. Variation within and between groups was large in some cases, but with the exception of P-treated males, mean values were within the normal physiological range for estrous animals (Table .2.2). The mean plasma E concentrations for each group however, were not significantly different ($p < 0.05$) due to the large amount of variation between groups. Receptive behavior was observed in animals representing a wide range of plasma E concentrations, including animals with anestrous as well as super physiological concentrations of E. The average plasma concentration of E in copulating animals (59.8 p g/ml ±10.3) did not differ from that in noncopulating animals (53.0 pg/ml ±11.1) ($p > 0.05$)

Plasma LH:

An LH rise (>1.75 SD above baseline) was identified in only 28% of the treated opossums, and in most cases the rise was small, 1.75 to 3 times baseline (Figs. 2.4, 2.5). Baseline LH concentrations per animal varied within and between treatment groups but were generally in the range of 0.4 to 2 ng/ml plasma. One or more animals in all 4 treatment groups showed a neuroendocrine response with an LH rise following treatment with E and P and pairing with a stimulus male. Mean peak values per group varied from 0.5 ng/ml in E&P-treated females to 91.1 ng/ml in mifepristone-treated females (Table 2.3). However, the latter value was skewed by a very high concentration (341.77ng/ml) in one of the 4 samples. A higher percentage

of control females showed an LH rise than females in the other 3 treatment groups (Fig. 2.4). Occurrence of an LH rise in E&P males (18.2%), was lower ($p < 0.0003$) than in control males (41.6 %) (Fig. 2.5). Rise percentages for mifepristone treated males and P treated males did not differ significantly from those for the control males.

The LH rise was largely restricted to animals with normal estrous plasma concentrations of E. An LH rise was observed in 27 animals, and 25 of those had estrous concentrations of E while only 2 had anestrous concentrations and none had super physiological E concentrations.

Table 2.2. Mean estradiol concentration for neonatally treated adult male and female opossums (GDX + EP) during behavioral testing with an intact stimulus male.

Neonatal Treatment	Mean (± SEM) Estradiol Concentration (pg/ml)	
	Female	Male
Control (10 females, 12 males)	14.7 ± 2.9	38.8 ± 9.2
Progesterone (8 females, 14 males)	59.8 ± 34.4	118.0 ± 35.1
Estradiol and Progesterone (13 females, 12 males)	77.4 ± 23.5	76.9 ± 18.6
Mifepristone (13 females, 14 males)	24.7 ± 6.6	29.3 ± 5.2

Table 2.3. Mean LH rise concentration for neonatally treated adult male and female opossums (GDX + EP) during behavioral testing with an intact stimulus male.

Neonatal Treatment	Mean (± SEM) LH Rise Concentration (ng/ml)	
	Female	Male
Control (5 females, 5 males)	17.57 ± 16.24	1.20 ± 0.23
Progesterone (1 female, 6 males)	1.21 ± 0	2.30 ± 1.14
Estradiol and Progesterone (1 female, 2 males)	0.50 ± 0	1.38 ± 0.58
Mifepristone (4 females, 8 males)	91.07 ± 83.62	2.74 ± 0.91

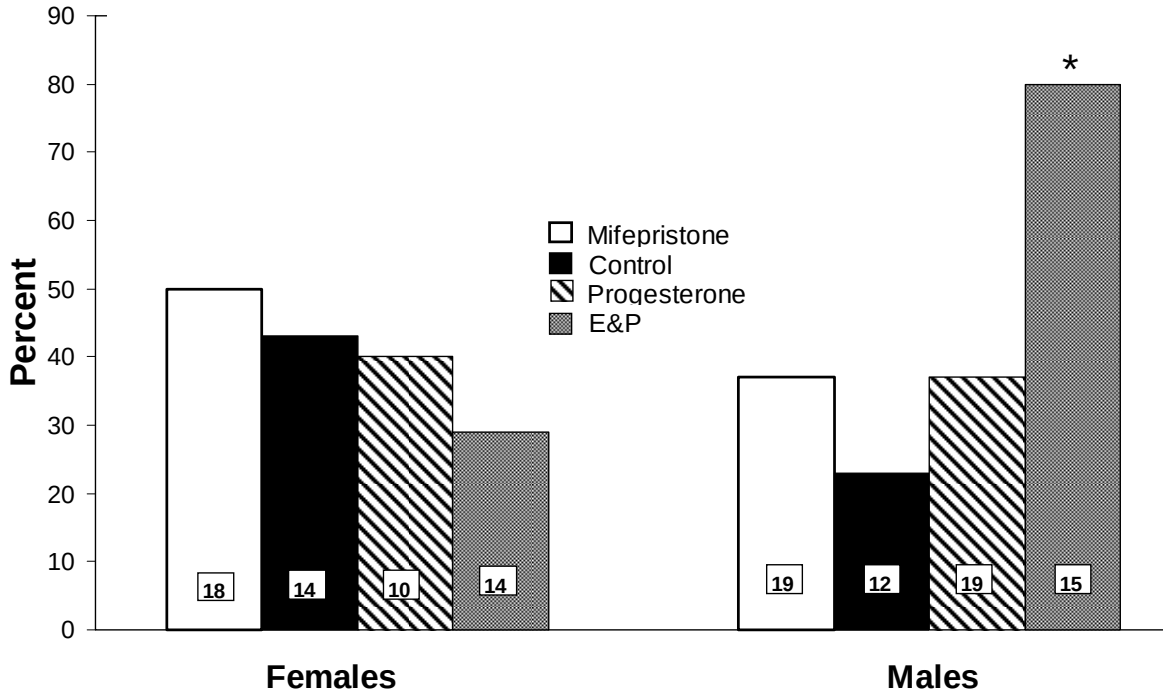

Fig. 2.1. The percentage of animals in each treatment group that copulated with a stimulus male. Test animals were given 1 of 4 hormone treatments on postnatal day 8 (Table 2.1) and GDX at 11 weeks of age (prepubertal). In adulthood (27 weeks of age), they were given E and P to activate feminine receptive behavior and paired with a stimulus male for a 25-h period of observation. Asterisks indicate a significant difference ($p < 0.05$) from control females.

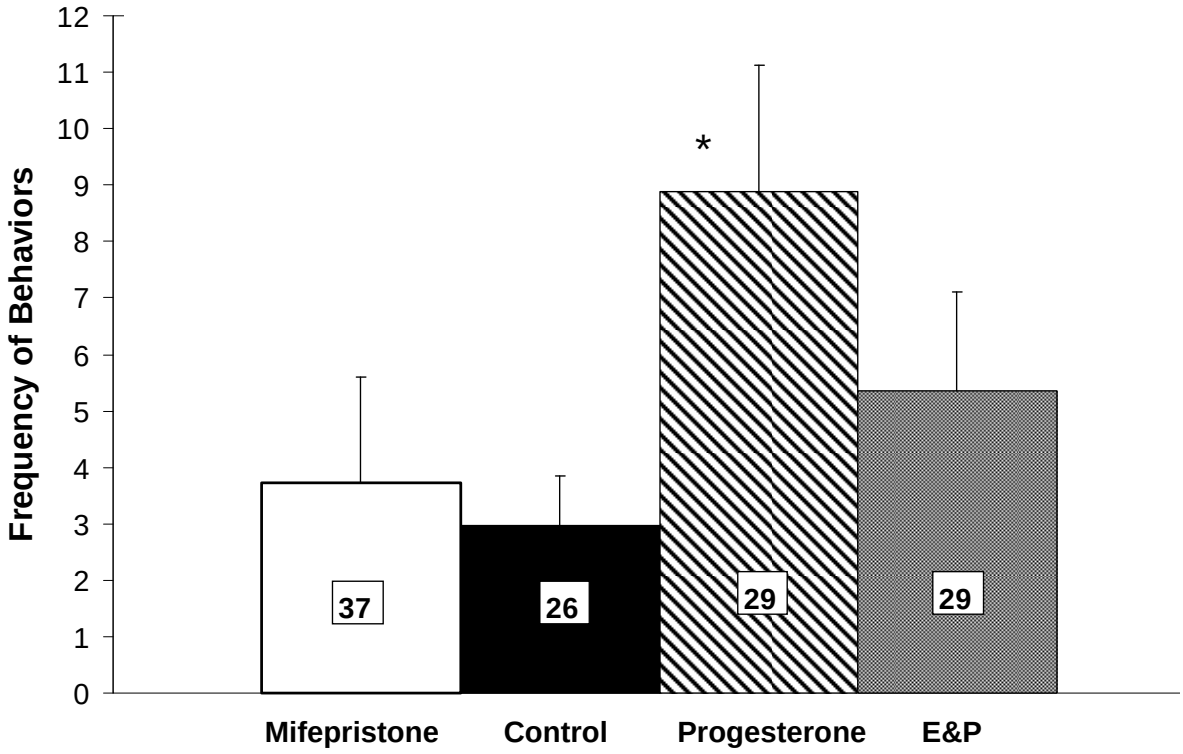

Fig. 2.2. Frequency of fighting behaviors (mean ± SEM) shown by male and female opossums. Test animals were given 1 of 4 hormone treatments on postnatal day 8 (Table 2.1) and GDX at 11 weeks of age (prepubertal). In adulthood (27 weeks of age), they were given E and P to activate feminine receptive behavior and paired with a stimulus male. Observations were taken for the first 10 minutes of a 25-hour pair test period. Recorded fighting behaviors include: aggressive grab, chase, bite, and tumble. Asterisks indicate a significant difference ($p < 0.05$) from control animals.

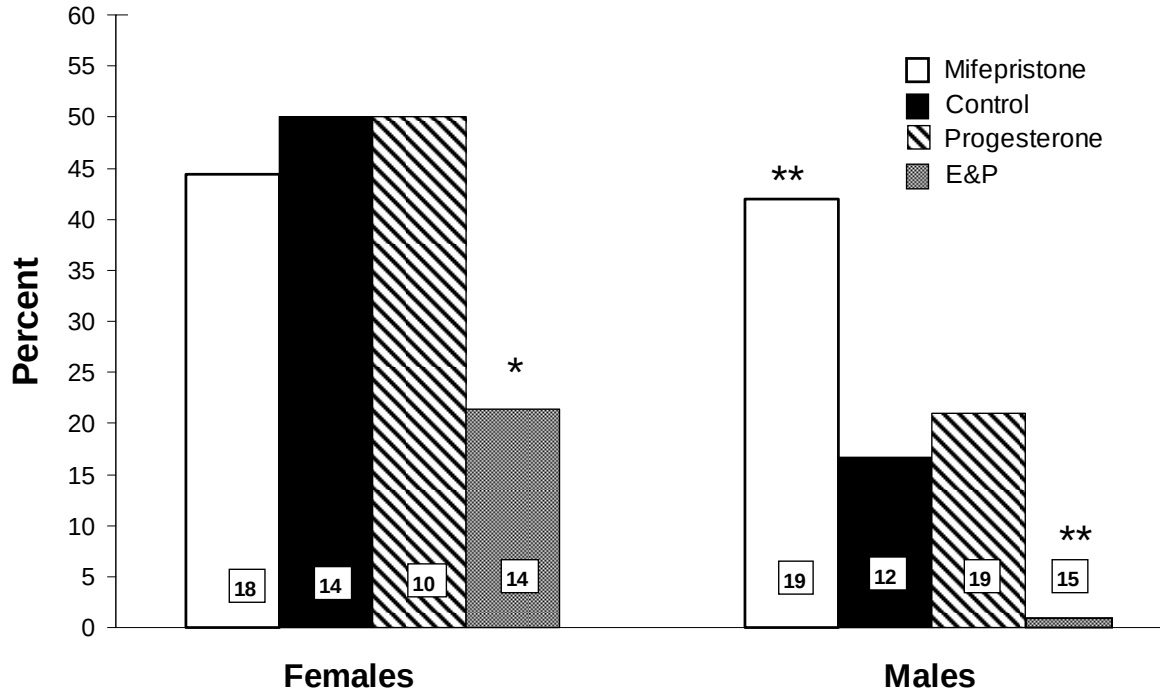

Fig. 2.3. Percent of stimulus males that showed scentmarking behavior when paired with test animals given 1 of 4 hormone treatments on postnatal day 8 (Table 2.1) and GDX at 11 weeks of age (prepubertal). In adulthood (27 weeks of age), they were given E and P to activate feminine receptive behavior and paired with a stimulus male. Observations were taken for the first 10 minutes of a 25-hour pair test period. One asterisk indicates significant difference ($p < 0.05$) from the female control group, two asterisks indicates significant difference ($p < 0.05$) from the male control group.

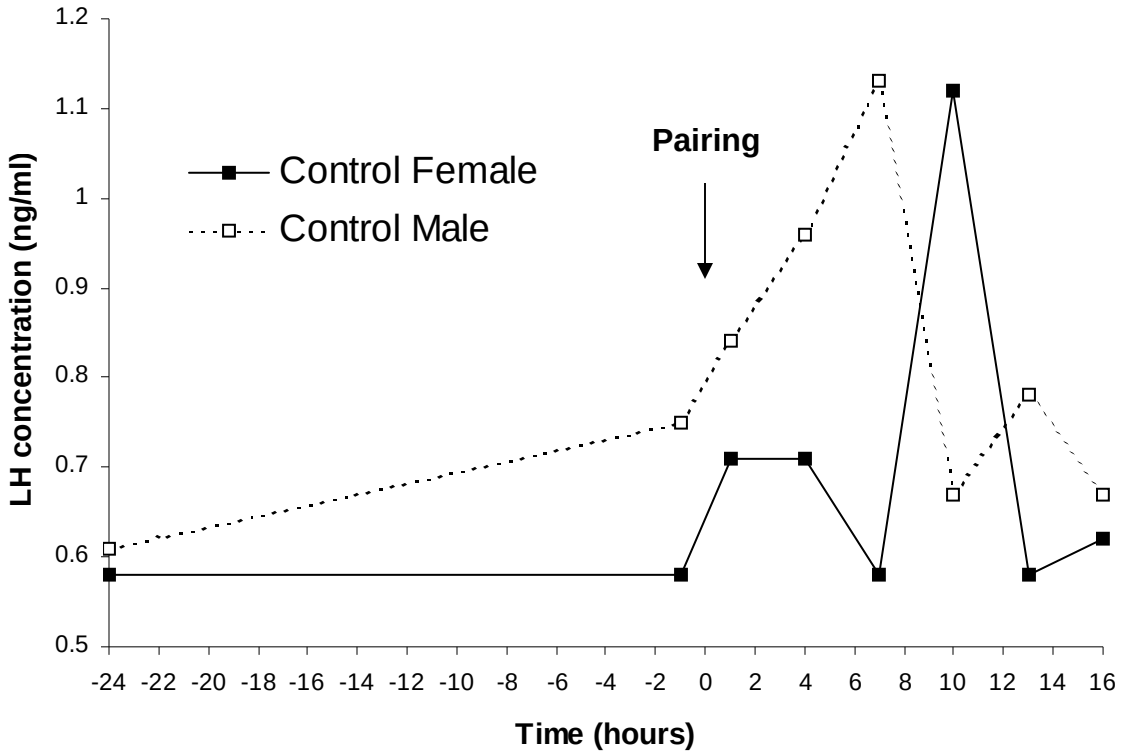

Fig. 2.4. Concentration (ng/ml) of LH for a control female and a control male opossum that were gonadectomized prepubertally, given female E and P in adulthood, and paired with a stimulus male at hour 0. The baseline concentration of LH is 0.61ng/ml for the female and 0.75ng/ml for the male.

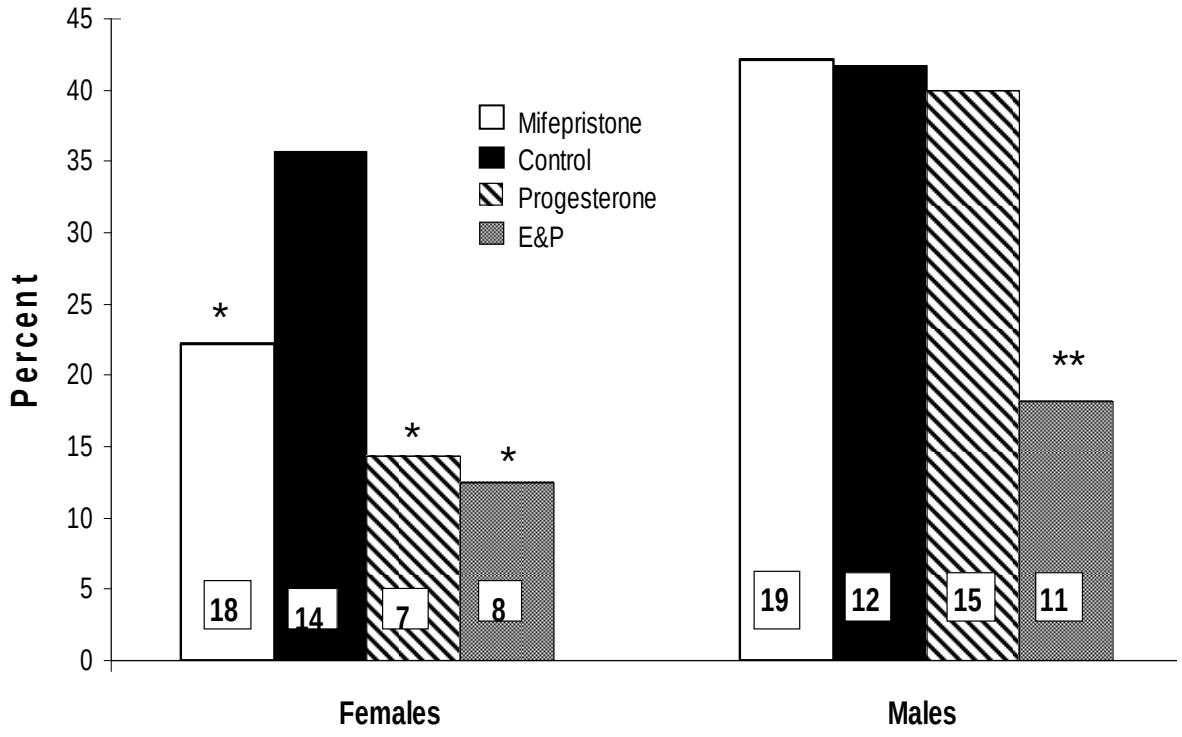

Fig. 2.5. Percent of neonatally treated opossums that exhibited a LH rise when gonadectomized prepubertally, given female hormones (E&P) in adulthood, and paired with a stimulus male. One asterisk indicates significant difference from the female control group; 2 asterisks indicate significant difference from the male control group.

DISCUSSION

Behavioral Responses

This study demonstrates that treatment with progesterone (P) and estradiol (E) or P alone during the critical period of sexual differentiation does not defeminize sexual behavior in the male opossum. The percentage of P-treated animals that copulated was not different from that in control animals. Also contrary to the hypothesis of this study, the high copulation rate (80%) of E&P males suggests a possible organizational role for P acting in concert with E in feminization of sexual behavior in this species. Neonatal treatment with E&P had the opposite effect on females. Consistent with hypothesized defeminizing effects of E&P, the copulation rate in E&P females tended to be lower than in control females, but the difference was not significant ($p > 0.05$). This sex difference suggests that testicular steroids, which were present prior to GDX at 11 weeks of age, could be responsible for the difference between near defeminization in E&P females and the feminization that occurred in E&P males. However, it is unlikely that the androgenic actions of circulating testosterone produce the contrasting effects of E&P treatment because sex differences in androgen levels do not exist before adulthood in this species (Fadem and Harder, 1992b). It does remain possible that aromatization of testosterone to estradiol or local conversion of testosterone to dihydrotestosterone within the brain interact with the exogenous E&P in the male.

The copulation rate observed in control females in this study (43%) is lower than the copulation percentage obtained in a previous study (88%) done by Fadem et al. (1996) that gave

E and P to ovariectomized females to restore feminine receptive behavior. When compared to this earlier study, a lower percentage of control females that copulated could be due to a disparity between the studies in the time during the light cycle in which the animals were paired. Pair tests were done in the first 1-5 hours of the dark phase of the light/dark cycle in the previous study, while this study initially paired animals in the last two hours of the dark phase of the cycle. Harder et al. (1993) found that 86% of copulations occur during the dark phase and 68% of these copulations occur in the first 4 hours after the lights are off. The animals in the current study remained paired throughout the following light and next dark phase, but being paired towards the end of the active period (Streilein, 1982), rather than the at the beginning of the active period could have reduced the probability that the animals would copulate at all in the duration of their pair test.

The elevation of fighting behavior in P-treated males and females suggests that P plays a role in the masculinization of dimorphic, nonsexual behaviors in this species. Aggressiveness in males is activated by androgens, and thus, fighting may be considered a nonsexual behavior that is characteristic of males, similar to scentmarking (Fadem and Corbett, 1993). The differential effect of P alone on fighting behaviors (masculinizing) and the occurrence of copulation (no significant effect) indicates P plays an important role in the organization of nonsexual, dimorphic behaviors, but does not contribute to differentiation of sexual behaviors.

Males treated with mifepristone to block P action during the critical period of sexual differentiation elicited significantly more scentmarking behavior from stimulus males than did control males. The percentage of stimulus males that scentmarked with mifepristone treated males (42%) was similar ($p > 0.05$) to that for stimulus males paired with control females (50%), suggesting similar attractiveness of mifepristone-treated males and control females to stimulus

males. The copulation percentage for mifepristone males (37%) was not significantly higher ($p > 0.05$) than that for control females (43%). This and the similarity in scentmarking behavior elicited by mifepristone males and control females suggests blocking neonatal, neural exposure to P reduced defeminization of proceptive behaviors expressed in adulthood.

Neonatal treatment with E&P and P alone reduced scentmarking behavior elicited from stimulus males by females and males in adulthood, just the opposite of the effect of mifepristone. While this result is congruent with the defeminization hypothesis of this study for females treated neonatally with E&P or P, a similar outcome for E&P males is surprising based on the high percentage of copulations and apparent feminization that occurred in the latter group. A possible explanation for this result relates to the high levels of feminine receptive and copulatory behavior exhibited by E&P males. Perhaps stimulus males were not prompted to scentmark, but instead to initiate copulation very soon after being paired.

Neuroendocrine Responses

The elevation of LH observed in animals paired with a stimulus male in this study differed from that previously observed in intact estrous females in which LH rose from basal levels of 1 ng/ml to peak levels of 3-20 ng/ml, reaching an average peak of 8.0 ng/ml 16 h after pairing. (Jackson et al., 1999; Jackson, 2001). This preovulatory surge preceded copulation and was coincident with a rise in P. Because increases in LH concentration observed in this study were lower and variable in temporal distribution relative to the time of pairing, the term LH rise, rather than LH surge, is used is used to characterize this hormone dynamic.

Neonatal treatment with E&P reduced ($P < 0.05$) the percentage of males and females that exhibited an LH rise. This was in accord with the hypotheses, which predicted a defeminizing effect of increased neonatal exposure to E&P or P on neuroendocrine

responsiveness to male stimuli in adult opossums activated with E and P. A higher percentage of control females showed an LH rise than the other treatment groups, which suggests that endogenous P has a role in organizing female neuroendocrine responsiveness and that any change in exposure to neonatal P, up or down, impairs the ability of the hypothalamus to stimulate the anterior pituitary to release a rise in LH in response to hormonal or social signals.

Female neonatal treatment groups did not differ from the control group in copulation rate, but all 3 treatment groups differed from the control group with respect to the LH rise. This differential response of the LH rise and copulation indicates that the LH rise is not dependent upon copulation, which is also the case in normal estrous female (Baggot et al., 1987). This suggests that the organization of these 2 responses involves distinct neural pathways that are affected differently by hormone exposure during the critical period of organization. This is supported by the significantly lower LH rise percentage but a high copulation rate in E&P males. Adding or blocking P alone in neonatal males does not result in a neuroendocrine response that differs from the response of control males, indicating that P only contributes to defeminization of the LH surge mechanism when it is present in combination with excess E.

LH rise percentages were very similar for control males and females, possibly signifying that LH rises are a normal response to pairing in both males and females. LH levels have not been investigated in intact males in response to pairing, but a rise was recorded in 2 of 6 GDX males treated with E and P in adulthood (Harder et al., 2001). So, the neuroendocrine response to pairing might require priming with ovarian steroids, but it is not sexually differentiated in this species.

With few exceptions, the LH rise was observed only in animals with normal estrous levels of E. However, copulation was observed in animals with a full range of plasma E

concentrations. Thus, in some animals, copulation occurred in the absence of estrous levels of E or the LH rise. The differences in circulating concentration of E within and between groups could be due to variable release of E from the implants, or from variations in metabolism or clearance of E stemming from neonatal hormone treatment.

CONCLUSIONS

Sexual behavior was not defeminized in opossums treated neonatally with P and estradiol E or P alone during the critical period of differentiation, and, thus, the major hypothesis of this study was rejected. This study did reveal a role for P in masculinizing nonsexual, dimorphic fighting behaviors. Also in accord with the hypothesis, blocking P during a critical period of sexual differentiation reduced defeminization of proceptive behaviors observed in adulthood, as inferred from the level of scentmarking elicited from stimulus males. E implants elevated plasma E concentrations to levels typical of intact estrous females in most (71%) but not all treated animals. Estrous levels of E were necessary for an LH rise, but not copulation.

The prediction that treatment with E&P during neural sexual organization would decrease the occurrence of the LH rise was supported by a significantly lower percentage of E&P treated males and P treated and E&P treated female opossums showing a LH rise than control animals. The high level of copulation in E&P-treated males and low occurrence of the LH rise in this group suggests differential organization of the neuroendocrine and behavioral aspects of reproduction in this species. The results showing that control males and females had similar LH rise percentages suggests that a LH rise might be a normal response to pairing in both the female and the male of this species.

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