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THE EFFECTS OF ESTRADIOL BENZOATE, PROGESTERONE, AND GONADOTROPINS ON THE ABILITY OF FEMALE HOUSE MOUSE URINE TO ELICIT MALE ULTRASOUNDS

by
David Mark Zakeski

A Thesis
Presented to the Graduate Committee of Lehigh University in Candidacy for the Degree of Master of Science

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9/12/79
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Professor in Charge

Chairman of Department
ACKNOWLEDGMENTS

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Abstract

Two experiments were conducted to examine how certain gonadal and pituitary hormones affect the ability of adult female house mouse urine to elicit 70 kHz ultrasonic vocalizations from adult male house mice (Mus musculus). Testosterone has been reported elsewhere to eliminate ultrasound elicitation characteristics of female mouse urine (Nyby, Wysocki, Whitney, Dizinno, and Schneider, 1979). In Experiment 1, estradiol benzoate (EB), progesterone (P), and the oil (O) vehicle for these hormones were separately administered to adult ovariectomized females, and the ultrasound elicitation ability of the females' urine assessed. P and EB were chosen because progesterone is an immediate precursor and estradiol an immediate byproduct of testosterone. Relative to the urine from the oil-treated ovariectomized females, the ability of ovariectomized female urine to elicit ultrasounds was totally suppressed by EB and partially suppressed by P. Because these steroid hormones are known to have inhibitory effects upon certain pituitary hormones, these results are consistent with pituitary control of the female ultrasound eliciting cue.

Further suggesting a role for pituitary hormones, hypophysectomy of adult females elsewhere (Nyby et al.,
1979) had been found to also eliminate the ability of their urine to elicit male ultrasounds. In Experiment 2, an attempt was made to restore ultrasound eliciting abilities to hypophysectomized females by administering to them a mixture containing Pregnant Mare Serum (PMS) which has primarily follicle-stimulating hormone (FSH) properties, and Human Chorionic Gonadotropic Hormone (HCG) which has primarily luteinizing hormone (LH) properties. Hormones with FSH and LH properties were chosen because these are the two "sex hormones" of the pituitary gland, are inhibited by gonadal steroid hormones such as testosterone and estradiol, and have the male and female gonads as their primary target glands. As in previous research, hypophysectomy eliminated the ultrasound elicitation ability of female mouse urine. However, replacement of the two gonadotropins to hypophysectomized females did not restore the ability of their urine to elicit ultrasounds from males. Results for both experiments were discussed within the context of possible sexually dimorphic metabolites and/or excretory or secretory products of steroid and pituitary hormones.
CHAPTER 1

Gender Advertisement in Courtship Behavior

A sexually reproducing animal must often devote substantial time and energy to attracting a mate. One of the ways the occurrence of mating is facilitated is by the advertisement of species identity and gender to conspecifics. While the advertisement strategies evolved by the two sexes for minimizing time and energy expenditures may be somewhat different, such advertisement clearly occurs in both sexes (e.g. Barash, 1977).

In most mammalian species, males initiate sex behavior, and to advertise their presence to the females have often evolved courtship signals perceptible at a distance. Long distance courtship signals can include loud sounds, gaudy appearance, and/or eye-catching behavior (Barash, 1977).

Although males tend to initiate sex behavior, the actual occurrence of mating is often controlled by the less aggressive but more discriminating female. The higher selectivity usually characteristic of females is understandable considering the females' greater energy investment in reproduction in most species. Although the female energy investment in reproduction is often higher than that of the male, the same cannot usually be said for the female investment in
advertisement. As compared to males, female advertisement tends to be over a shorter range, and often involves less conspicuous courtship signals (Bermant and Davidson, 1974).

Because of the importance of vision to humans, animal gender adornments such as manes, tusks, and horns are quite perceptible to us, but olfactory adornments are less so (Stoddart, 1974). The fact is, however, that chemical signals from either sex can act as sex attractants for the opposite sex among insects, arthropods, fishes, snakes, reptiles, and many mammals (e.g. Wilson, 1968; Butler, 1970; Doty, 1976; Muller-Schwarze and Mozell, 1976; Shorey, 1976). Within mammals, male olfactory preference for the urine of estrous females has been reported in rats, cats, dogs, stallions, bulls, and rams (Schein and Hale, 1965; Michael and Keverne, 1968; Geyer and Barfield, 1978). As might be expected, anosmia in either sex often has quite severe inhibitory effects on sexual behavior (Bronson, 1976). Indeed, relationships between olfaction and reproductive function have been recorded for rhesus monkeys, cats, pigs, rabbits, guinea pigs, hamsters, and mice (Signoret and Mauleon, 1962; Franck, 1966; Michael and Keverne, 1970; Stoddart, 1974; Powers and Winans, 1975; Beauchamp, Magnus,
Shmunes, and Durham, 1977).

For rodents, much social information is transmitted through the chemosensory modalities (Stoddart, 1974; Muller-Schwarze, and Mozell, 1976). The mouse in particular has been heavily utilized in socio-olfactory research. Anosmia is known to reduce the sex behavior and social interactions of mice (Rowe and Edwards, 1972; Thompson and Edwards, 1972; Edwards, 1974). In male mice, complete removal of the olfactory bulbs eliminates all sexual behavior (Rowe and Edwards, 1972), while in female mice, bulbectomy virtually eliminates the display of sexual receptivity (Thompson and Edwards, 1972). Chemoreception of odors would therefore appear necessary for the display of sex behavior in mice. However, the exact nature of the chemosensory systems involved in the detection of social/sexual odors is not well understood for the mouse.

Based upon other work with hamsters, the primary olfactory and vomeronasal chemosensory systems have been postulated to interact with each other in the mediation of sexual behavior (Powers and Winans, 1975). However, of the two systems the vomeronasal may be the more important since selective destruction of only the olfactory epithelium by application of zinc sulfate did not affect mouse mating behavior (Rowe and Smith, 1972).
Recently, the suggestion has been made that the primary olfactory pathway may be involved in the onset of sexual arousal while the vomeronasal system facilitates adequate sexual performance (Keverne, 1978). However, conclusive statements about the relative importance of the primary olfactory epithelium, the vomeronasal system, and the trigeminal system in the detection of social odors requires further study.

**Advertisement of Female Mouse Gender**

Despite their likely existence, female chemical signals acting as sex attractants have begun to be studied only recently. In this regard, female mouse odors are now known to have both behavioral and physiological effects upon males in a variety of contexts. Dixon and Mackintosh (1975) present evidence for the presence of two sex signalling cues in female mouse urine. Production of the first signal, which is thought to stimulate male sex behavior, depends upon female ovarian hormones. The second signal serves to reduce male aggression and appears not to depend upon ovarian hormones (See also Mugford and Nowell, 1970; 1971; Haag, Jerhoff, and Kirkpatrick, 1974 for another point of view concerning hormonal control of the aggression inhibiting substance). A detailed investigation conducted by Evans, Mackintosh, Kennedy, and Robertson (1978) revealed that aggression
reducing and sexually stimulating olfactory cues of female mice were present in bladder urine, relatively non-volatile, resistant to bacterial or oxidative breakdown, and resistant to change in pH.

In addition to having overt behavioral effects, chemical signals from female mice may induce less observable physiological changes in males. Macrides, Bartke, and Dalterio (1975) reported an increase in serum testosterone in male mice following exposure to strange females. The nature of the female cue was not determined. Maruniak and Bronson (1976) found male mouse gonadotropin secretion to be stimulated by a factor in the urine of female mice. A rapid release of luteinizing hormone (LH) and to a lesser extent follicle-stimulating hormone (FSH) occurred in response to female urine. The males' responsiveness appeared to be independent of their sexual experience and the ovarian state of the female urine donors.

Regardless of whether gonadal steroids mediate signal production, an aggression inhibiting and a sex promoting property clearly exist in the urine of female mice. To increase the possibility for successful reproduction, it is advantageous for females not to behave agonistically toward other females, and for males to court rather than attack females. Chemical signals in
the urine of female mice apparently facilitate proper relationships among conspecifics.

**Ultrasonic Communication in Mice**

In the present thesis, the sex signalling advertisements of female mice were examined in yet another way. Male mice emit high frequency vocalizations around 70 kHz when performing behaviors that indicate a high level of sexual arousal (Sales, 1972; Whitney, Coble, Stockton, and Tilson, 1973). Ultrasounds are emitted during investigation, mounting, and copulation with females. The ultrasounds generally decline across introductions and cease by the time of ejaculation (Sales, 1972). Males seldom produce ultrasounds in response to other males, while females rarely produce ultrasounds in response to males or other females. That the male ultrasounds are related to male sexual motivation is further suggested by evidence that this behavior is modulated by the very same hormones that regulate male sexual behavior (Dizinno and Whitney, 1977; Nyby, Dizinno, and Whitney, 1977a; Nunez, Nyby, and Whitney, 1978).

Whitney, Alpern, Dizinno, and Horowitz (1974) have speculated that these ultrasounds serve the courtship function of reducing female aggression and withdrawal, and thereby facilitate mating. However, for the purposes of the present thesis, the ultrasounds emitted by males in response to females are used primarily as a behavior-
al assay for the presence of female gender advertise-
ments.

**Chemosensory Elicitation of Ultrasounds**

Several experiments have indicated that male mice will emit ultrasounds not only to females but also to female chemosensory cues. Cage shavings taken from female-occupied cages were found to elicit ultrasounds from males even if the males were prevented from making tactual contact with the shavings (Whitney et al., 1974), suggesting that the female cues may have some degree of volatility. High levels of male ultrasound were also emitted to a female in a completely dark room (Whitney et al., 1974), or to an anesthetized female (Nyby, Wysocki, Whitney, and Dizinno, 1977b), providing evidence that vision and female behavior are not necessary for the elicitation of male ultrasounds. All these findings are consistent with the earlier discussion of the importance of chemoreception for mouse sex behavior (Rowe and Edwards, 1972).

**Source of the Female Ultrasound Eliciting Cue**

Since female soiled cage shavings are effective elicitors of ultrasounds, one or more female secretory or excretory products may be the chemical cue responsible. Nyby et al. (1977b) tried to experimentally locate the source of the cue. A variety of different photographic and anesthetization experiments suggested that the cues
are located all over the female body. For example, female urinary, vaginal or facial substances all effectively elicited male ultrasounds while corresponding male and control substances did not. Thus, strong statements about the source of the cue were not possible from these data.

The uncertainty as to the origin of the ultrasound eliciting cue(s) allows several possible explanations. A single, localized cue might exist which is spread to other parts of the female's body by grooming and contact with soiled cage shavings. Another possibility would be a metabolic byproduct, such as a sebaceous gland secretion, which is secreted from various parts of her body. Still another possibility is that all or some combination of various substances produced in different locations on the female's body signal femaleness and hence elicit ultrasounds from males.

Emphasis in the published research and in this thesis has been on urine as a cue for ultrasound elicitation. The reasons for this emphasis are because urine is a common medium of chemical communication among mammals (Bronson, 1976), urine provides a clear comparison between male and female cues, and urine is much easier to obtain, quantify, and control than facial, somatic, and vaginal cues.
Characteristics and Control of the Urinary Ultrasound Eliciting Cue

Based on earlier evidence (Nyby et al., 1977b) that female mouse urine is a much more potent stimulus for eliciting ultrasounds than male urine, a series of experiments (Nyby et al., 1979) were conducted to determine the physical characteristics of the female cue as well as to determine the hormonal underpinnings for the sexual dimorphism in cue production. Because of the direct relevance of this work to the thesis experiments reported here, this work will be described.

One of the questions addressed by Nyby et al. (1979) was whether the high levels of ultrasound emitted in response to females and the low levels of response to males were caused by excitatory and inhibitory substances in female and male urine respectively. In this regard, an examination was made of the ultrasonic response of male mice to female urine, male urine, and a mixture of equal volumes of female and male urine. The mixture of male and female urine was as potent as female urine alone in eliciting ultrasounds. This finding indicated that female urine contains some substance that elicits ultrasounds while male urine appears to be neutral for ultrasound elicitation (when presented in a 1:1 mixture).
Given that female urine is excitatory, dilution with distilled water was performed to see how potency might be reduced. Dilution by a factor of 100 greatly reduced, and dilution by a factor of 1000 eliminated the ultrasound eliciting capability of female urine. The suggestion was made that the female ultrasound eliciting factor may exist in female urine in concentrations very close to those necessary for normal levels of ultrasound elicitation.

The volatility of the ultrasound eliciting cue in female urine was tested by allowing urine to evaporate at room temperature on a watchglass. Reconstitution of the remaining residue with distilled water, followed by comparison of this mixture with normal female urine and then with reconstituted male urine for ability to elicit ultrasounds, revealed the reconstituted female urine to be a potent stimulus for ultrasound elicitation. The ultrasound eliciting cue thus appeared to be relatively nonvolatile.

An open test tube containing female urine was placed in a boiling water bath to see if heat destruction (inactivation) or volatilizing of the cue would occur. After one hour of boiling, female urine appeared to be as potent as normal female urine in eliciting ultrasounds. To further test the heat
stability of the ultrasound eliciting cue, female urine was subjected to the more extreme heat of an autoclave. The ultrasound eliciting property was destroyed, suggesting that the ultrasound factor is composed of a relatively heat-resistant, organic substance.

While the female ultrasound eliciting factor is clearly present in the urine of adult females, the factor was not present in the urine of either young females or young males. Production of the ultrasound eliciting cue thus appears to be turned on during female maturation. Surprisingly, female gonadal hormones do not appear to be of major importance to the initiation or maintenance of ultrasound eliciting cue production. Female urine samples taken from different stages of the estrus cycle were not significantly different in ultrasound eliciting potency, with urine from even the least effective estrus stage eliciting a substantial amount of ultrasound. Further evidence against the contribution of ovarian hormones to the regulation of cue production involved giving groups of adult and prepubertal females ovariectomies. In most cases, the potency of ovariectomized adult female urine to elicit ultrasounds did not vary significantly from that of intact control animals, and the conclusion was reached that during ontogeny, the ovaries are not
of paramount importance for the turning on of the ultrasound eliciting cue.

To examine the possibility that the ultrasound eliciting cue is maintained by steroid hormones from the adrenal cortex in the absence of ovarian steroids, females were both ovariectomized and adrenalectomized. No additional reduction in the ability of ovariectomized female urine to elicit ultrasounds was detected following adrenalectomy, indicating that hormones from the adrenal glands are not responsible for the production of the female urinary ultrasound eliciting cue. Attempts to demonstrate a neonatal role for ovarian and adrenal hormones were also unsuccessful. Administration of testosterone propionate (TP) for the first five days after birth did not prevent females from producing the ultrasound eliciting cue in adulthood. Likewise, castration of males on the day of birth did not cause them to produce the ultrasound eliciting factor in adulthood. Thus, neonatal androgens apparently do not affect the sexual differentiation of the physiological conditions underlying ultrasound eliciting cue production.

Having determined that female gonadal hormones are not of much importance in the initiation or maintenance of ultrasound eliciting cues, the effects
of the male gonadal steroid hormone, TP, on the ability of adult ovariectomized female urine to elicit ultrasounds were examined. Somewhat surprisingly, the potency of the female ultrasound eliciting cue was greatly reduced by exogenous androgen.

Since exogenous androgen is known to inhibit the release of gonadotropins (Campbell and Schwartz, 1978), ultrasound eliciting cue production might be turned off or prevented by TP inhibition of the release of hormones from the pituitary gland. Hypophysectomies were therefore performed on female mice. Urine from hypophysectomized female mice was found to be a much less potent elicitor of ultrasounds than urine from normal females. Results were the same when controls were made for a possible dilution effect from the high urine output following hypophysectomy-induced diabetes insipidus. The significant reduction in the male mouse ultrasonic response to hypophysectomized as compared to normal female urine thus supports a role for pituitary factors in regulating production of the cue.

The findings that both hypophysectomy and the addition of testosterone propionate decrease the ability of adult female urine to elicit male ultrasounds led to the work reported in this thesis.

-15-
In the experiments to be reported, metabolic characteristics of the pituitary dependent cue were further examined by administering (1) estradiol and progesterone to ovariectomized females, and (2) gonadotropins to hypophysectomized females. Progesterone and estradiol were chosen because progesterone is an immediate precursor and estradiol an immediate byproduct of testosterone. Gonadotropic hormones with FSH and LH properties were chosen because these are the two "sex hormones" of the pituitary gland, are inhibited by gonadal steroid hormones such as testosterone and estradiol, and have the male and female gonads as their primary target glands.
CHAPTER 2

General Method

Because of similarities in the two experiments of this thesis, the general methods employed in both experiments will be described. Deviations from the general methods will be described in the individual experiments.

Animals

Three groups of animals were required: subjects, social experience animals, and urine donors. Subjects were experimentally naive DBA/2J adult male mice whose ultrasonic vocalizations were measured in response to urine. Social experience animals were experimentally naive adult C57BL/6J and CD-1 females and experimentally naive adult C57BL/6J and CD-1 males that were systematically placed in the subjects' cages before the subjects were used in an experiment. Urine donors were adult C57BL/6J and CD-1 females. All DBA/2J and C57BL/6J animals were purchased from the Jackson Laboratories (Bar Harbor, Maine). CD-1 social experience animals and CD-1 urine donors were purchased from the Charles River Mouse Farm (St. Louis, Missouri). All animals arrived at our laboratory group housed by sex at 50± days of age.
**Apparatus**

Subjects were individually housed in 12½ cm x 17 cm x 28 cm white translucent plastic cages containing wood shavings bedding. Social experience animals were individually housed in 18 cm x 18 cm x 24 cm metal mesh cages. Urine donors were group housed by treatment in 12½ cm x 17 cm x 28 cm white translucent plastic cages containing wood shavings bedding.

Urine was collected in three Maryland Plastics metabolic cages (E110 Metabolism Units), and drawn from collection vials by 1.0 ml plastic syringes. Six inch long cotton-tipped applicators (swabs) were used to present urine to subjects. 20 x 150 mm glass test tubes were used to contain and present the swabs.

A QMC bat detector set to 68 kHz was used to detect ultrasounds. The bat detector transforms ultrasonic vocalizations into low frequency audible sounds (Sales and Pye, 1974). The microphone of the bat detector was located approximately 25 cm above the floor of the subject's home cage.

**Procedure**

All animals were maintained on a 12 hour-12 hour light-dark cycle with food and water available ad libitum.

Subjects began receiving eight consecutive days
of social experience ten days before the first behavioral trial. Social experience consisted of sequential presentations for three minutes of one social experience male and one social experience female to each subject. The order of presenting male and female social experience animals was alternated each day. Male-male pairings were discontinued before three minutes in cases of extreme aggression.

Two days before the first trial, an initial three minute screening of all subjects with normal females was conducted to determine that the males would emit ultrasounds to an appropriate stimulus (i.e. a female). Of a possible 36 blocks in which ultrasound could have been emitted, only ultrasound screening scores greater than twelve qualified animals to be used as subjects.

Urine was collected the night preceding its use from urine donors group housed by treatment in three metabolic cages. After ten hours in metabolic cages, urine donors were returned to their home cages. Extraction of urine from collection vials using syringes was performed three hours before testing. Metabolic cages were cleaned between urine collections.

To maximize ultrasound detection, food and water were removed from each subject's cage top, and the cage top turned upside down immediately before
testing. Removal of food and water decreased the likelihood of ultrasound wave attenuation, and the inverted cage top eliminated an otherwise partitioned environment. Trials were preceded by a one minute habituation period for each subject. If subjects emitted no ultrasounds during the habituation period, the urine stimulus was presented. If any ultrasounds were emitted during habituation, two full minutes were allowed to elapse following the final ultrasound before the stimulus was presented.

After the habituation period, a three minute trial was conducted in which a cotton swab with 0.1 ml urine was presented to the subject. Urine was injected onto a swab which was then broken off into a test tube such that the experimenter did not touch any part of the swab contained in the test tube. The contents of the tube were spilled into the subject's cage at the start of a trial. Another experimenter unaware of stimulus identity quantified the amount of ultrasound by recording how many of the 36 five-second blocks during the trial contained ultrasound. For the male subjects, data consisted of the number of five-second blocks containing ultrasound.
Experimental Design and Statistics

Testing was done every 48 hours for three days using a repeated measures design. All 3! or six possible sequences of administration of the three different treatments were used an equal number of times as allowed by the number of subjects (e.g. for an N of twenty, four sequences would be used three times, and two sequences would be used four times). Repetition of sequences did not occur until all sequences were used an equal number of times. Balance was thus achieved and an examination of an order effect made possible. Since the assumptions of the analysis of variance (normality, homogeneity of variance) were not met, non-parametric statistics, the Friedman and Wilcoxon tests, were used to analyze the data.
Various gonadal steroids, including testosterone and estradiol, are known to be basically interchangeable with each other in a number of different behavioral and physiological responses. For example, testosterone can substitute for estradiol in activating female rodent sex behavior (Pfaff, 1969), while estradiol can substitute for testosterone in differentiating and activating brain areas controlling male rodent sex behavior (Larsson, Soderston, and Beyer, 1973; Wallis and Luttge, 1975; Attardi and Ohno, 1976). Progesterone is generally less effective than testosterone and estradiol in stimulating these responses. More relevant to the present experiment, testosterone and estradiol also have similar feedback effects on various hypothalamic/pituitary factors. Gay and Hauger (1977) found that testosterone propionate, estradiol benzoate, and other inhibitory gonadal secretions (including progesterone) may be interchanged without detectably changing the post-castration increase in serum gonadotropins in the female rat. Other similar effects of estradiol benzoate and testosterone propionate in rats include increasing prolactin synthesis and release functions, increasing growth hormone release, and decreasing growth hormone
synthesis (Yamamoto, Kasai, and Ieiri, 1975).

Based on the Nyby et al. (1979) finding that testosterone eliminates the ultrasound eliciting cue in female mouse urine, progesterone, a direct metabolic precursor of testosterone, and estradiol benzoate, a direct metabolic byproduct of testosterone, were administered to adult ovariectomized females. The degree to which these two additional gonadal steroids are effective in eliminating the ultrasound eliciting characteristic(s) of female mouse urine should provide further information on the biochemical specificity sufficient for cue inactivation to occur.

Method

Animals

Subjects were eighteen DBA/2J male mice that were 85±5 days of age on the first trial. Social experience animals were seven male and seven female C57BL/6J mice, all 85±5 days of age. Urine donors were ten female C57BL/6J mice, also 85±5 days of age on the first trial.

Procedure

At approximately 55 days of age, all urine donors were bilaterally ovariectomized under Equithesin anesthesia in our laboratory. Nine days after ovariectomy, urine donors began receiving 0.05 ml subcutaneous injections every other day until every urine donor received
fourteen injections. The three treatment conditions were: (1) ovariectomy + estradiol benzoate (300μg/0.05 ml peanut oil), (EB, N = 4) (2) ovariectomy + progesterone (300μg/0.05 ml peanut oil), (P, N = 4) (3) ovariectomy + peanut oil (0.05 ml peanut oil), (0, N = 2). Concentrations of EB and P were well above the minimally active physiological levels (Kalra, Fawcett, Krulich, and McCann, 1973; Gay and Hauger, 1977). Testing began the day after all urine donors received their twelfth injection. Social experience, screening, urine collection, stimulus presentation, and ultrasound monitoring procedures were all performed in the manner already described.

Results

The effects of the Experiment 1 injection treatments on ultrasound production are seen in Figure 1.

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Insert Fig. 1 about here

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Overall differences for the three treatment conditions were significant (Friedman $X^2_2 = 11.467, p < .005$). Contributing to the group differences was the substantial amount of response to the ovariectomy-oil (control) group versus the low response to the groups receiving estradiol and progesterone treatments. The average of the two experimental groups' scores compared to the
Figure 1: Amount of male ultrasound (mean ± standard error) emitted in response to the urine of adult ovariectomized (ovx) females treated with either estradiol benzoate (EB), progesterone (P), or the oil vehicle (O).
control group's score was significant (Wilcoxon, Z = -2.831, p < .005). A comparison of the progesterone group to the estradiol group was also significant (Wilcoxon, Z = -1.988, p < .05). Estradiol benzoate, like testosterone, almost completely suppressed the ultrasound elicitation capability of female mouse urine. The ultrasound elicitation capability of female mouse urine was not as strongly suppressed by progesterone as it was by estradiol benzoate. Although the assumptions of the Analysis of Variance are not satisfied by these data, an Analysis of Variance performed in addition to visual inspection dismissed the possibility of an order effect.
One of the ways in which testosterone and estradiol were recognized earlier as having similar physiological effects was in their feedback effects on gonadotropin levels. Serum levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) have been found by numerous investigators to decline following the addition of estradiol or testosterone to female rats (Taleisnik, Caligaris, and Astrada, 1971; Ajika, Krulich, Fawcett, and McCann, 1972; Kalra et al., 1973; Chobsieng, 1976; Gay and Hauger, 1977; Campbell and Schwartz, 1978). Testosterone (Nyby et al., 1979), and progesterone and estrogen (Experiment 1) could conceivably exert their negative effect on ultrasound eliciting cue production in female mouse urine by considerably reducing serum levels of gonadotropins (and other pituitary factors), hence mimicking the effects of hypophysectomy (Nyby et al., 1979).

In Experiment 2, pituitary hormone replacement therapy to hypophysectomized female mice was performed, and urine ultrasound elicitation ability was assessed. Since pituitary hormone(s) responsible for a urinary sex difference in ultrasound elicitation ability were sought, restoration therapy used gonadotropic hormones
(LH and FSH) which 1) have the male and female gonads as their primary target glands, and 2) are known to be altered by feedback from steroid hormones. Because of the unavailability of pure mouse LH and FSH, Pregnant Mare Serum (PMS) which has primarily FSH properties, and Human Chorionic Gonadotropic Hormone (HCG) which has primarily LH properties, were used. Both of these hormone substitutes are known to have biological activity in the mouse (Zarrow and Wilson, 1961; Zarrow, Christenson, and Eleftheriou, 1971).

Method

Animals

Subjects were twenty DBA/2J male mice that were 80±5 days of age on the first trial. Social experience animals were ten male and ten female CD-1 mice, all 80±5 days of age. Urine donors were nine female C57BL/6J mice, also 80±5 days of age on the first trial.

Procedure

At approximately 40 days of age, six urine donors were hypophysectomized, and three urine donors were sham hypophysectomized. All operations were performed at the Charles River Laboratories. 27 days after the operation, all urine donors began receiving 0.1 ml subcutaneous injections every day until every urine donor
received fourteen injections. Hormone concentrations used were triple a level that is known to have biological activity (Personal communication with Joel Maruniak). The three treatment conditions were: (1) hypophysectomy + HCG (9 IU/0.1 ml) PMS (4.5 IU/0.1 ml) mixture, (HG, N = 3) (2) hypophysectomy + distilled water, (HO, N = 3) (3) sham hypophysectomy + distilled water, (10, N = 3). Testing began on the eleventh day of injections. Social experience, screening, urine collection, stimulus presentation, and ultrasound monitoring procedures were all performed in a fashion similar to Experiment 1.

Results

The effects of the Experiment 2 injection treatments on ultrasound production are seen in Figure 2.

The overall difference for the three treatment groups was significant (Friedman $X^2_2 = 19.225$, $p < .005$). There was a significant difference between the high response to the sham hypophysectomized group versus the low response to the two hypophysectomized groups (Wilcoxon, $Z = -3.883$, $p < .001$). Hypophysectomies almost totally eliminated ultrasound elicitation abilities of female
Figure 2: Top. Amount of male ultrasound (mean ± standard error) emitted in response to the urine of hypophysectomized females treated with gonadotropins (hypox + GTH) or the water solvent (hypox + H₂O), and to the urine of sham hypophysectomized females treated with the water solvent (sham hypox + H₂O).

Bottom. Effects of the three female treatments upon uterine weight in milligrams (mean ± standard error).
mice. Differences between the two hypophysectomized groups were not significant. Gonadotropin therapy was thus ineffective in re-establishing the ultrasound elicitation ability of hypophysectomized female mouse urine.

Overall differences in uterine weights as seen in Figure 2 were significant ($F (2,6) = 11.691, p < .01$), with uteri from the two hypophysectomized groups weighing well below those of the IO group ($F (1,6) = 23.371, p < .01$). The uterine weights of the two hypophysectomized groups were not significantly different. Gonadotropin therapy was thus ineffective in physically restoring the reproductive tracts of hypophysectomized females.
CHAPTER 5
General Discussion

Gonadal Hormone Inhibition of the Ultrasound Eliciting Cue

Earlier work (Nyby et al., 1979) had shown that testosterone administration to females eliminates the ability of their urine to elicit ultrasounds from males. The finding in Experiment 1 that progesterone partially suppressed, while estradiol totally suppressed the ultrasound elicitation ability of female urine suggests similarity of function or possible interconversions of these three structurally similar steroids. Perhaps some shared aspect of the biochemical structures of testosterone, estradiol, and progesterone enables them to reduce the effectiveness of the pituitary dependent ultrasound elicitation cue. In this regard, testosterone, estradiol, and progesterone all have a cyclopentanophenanthrene "nucleus", with the most outstanding structural difference between the three being a ketone functional group at the carbon 17 position of progesterone, where testosterone and estradiol both have hydroxyl groups.

It is interesting to note that the results of Experiment 1 are not only consistent with the Nyby
et al. (1979) report that the ovaries are not directly responsible for the turning on of the ultrasound elicitation cue, but further suggest that ovarian products can actually inhibit expression of the cue. Since estradiol eliminates the female ultrasound elicitation cue (Experiment 1), it is no longer surprising that the injection of castrated male mice with estradiol (Nyby et al., 1979) failed to increase the ability of male urine to elicit ultrasounds.

General Metabolic and Exocrine Glandular Actions of Gonadal Steroids

Since the ultrasound eliciting cue is found only in females, it seems possible that the cue may be produced in some female specific tissue. Consequently, a brief survey of some of the more likely sites of production and the effects of steroid hormones on these tissues are presented.

Since testosterone generally increases the size of the sebaceous glands and stimulates sebum production, it seems unlikely that the ultrasound eliciting substance originates in sebaceous tissue. However, a possibility exists that the testosterone-induced sebum rather than the testosterone per se is responsible for cue destruction or inactivation. Conversely, estradiol exerts the opposite effect of
depressing sebaceous secretion (Muller-Schwarze and Mozell, 1977). Since both testosterone (Nyby et al., 1979) and estradiol (Experiment 1) eliminate the ultrasound elicitation ability of female mouse urine while the two have very different effects on sebaceous secretion, sebaceous products are unlikely to comprise the ultrasound eliciting cue.

The female preputial gland, which is a modified sebaceous gland, may be the site of gonadal steroid influenced cue production. Investigation time of male rats was much higher when they were presented with odors of female preputial glands than control odors (Orsulak and Gawienowski, 1972). A significant attraction was demonstrated by male rats for the lipid but not fatty acid extract of the female preputial gland (Gawienowski, 1975). However, size, lipid content, and synthesis of wax, alkyl acetates, and alkyl glycerols were all increased in female mouse preputial glands by testosterone injections (Spener, Mangold, Sansone, and Hamilton, 1969; Sansone-Bazzano, Bazzano, Reisner, and Hamilton, 1972). Such hormonal regulation seems inconsistent with the preputial gland producing the cue, although one or more of these preputial gland products might be responsible for the cue inactivation or destruction that occurred
after testosterone treatment (Nyby et al., 1979). Unfortunately, the physiological effects of estradiol injections on the preputial glands have not been reported.

Little is known about gonadal steroid effects on other female glands. The endometrial glands, for example, form a prominent part of the uterus of rodents but little is known about the factors controlling their secretion. Furthermore, the extent of their secretion is difficult to quantify because it is not possible to collect (Finn and Martin, 1976).

Although testosterone, estradiol, and progesterone may remove the female mouse ultrasound eliciting cue through glandular effects, the fact is that most of the metabolic and morphologic effects of gonadal steroids are non-glandular. Estrogens stimulate uterine growth, a process that includes retention of water, sodium, calcium, nitrogen, and phosphorus while increasing protein synthesis. In rats, estrogens increase glycogen and mucopolysaccharides in vaginal epithelium, and increase oviduct growth, activity, and secretion (Dohler and Wuttke, 1975). In all age groups of C57BL/6J mice, estradiol causes significant increases in uterine weight, glycogen, alkaline phosphatase, protein, and intraluminal fluid content.
(Holinka, Hetland, and Finch, 1977). Although testosterone does not play a central role in normal female physiology, it is also protein anabolic, increases glycogen, and facilitates the retention of nitrogen and sodium. Overlapping physiological effects such as these may be linked to the ability of both gonadal steroids to eliminate the ultrasound eliciting properties of female mouse urine. Alternatively, these two hormones may of course eliminate the cue in very different ways. It seems reasonable to suggest, however, that the two structurally similar gonadal steroids are either having essentially the same destructive effect on cue production or maintenance, or else are masking the detection of the usual cue perhaps by causing the production of yet another chemosensory cue.

Effects of Steroid Hormones on Gonadotropin Secretion

The observed effects of testosterone (Nyby et al., 1979), and estradiol and progesterone (Experiment 1) on cue production might also be expected due to the known negative feedback these hormones have on pituitary factors, especially FSH and LH (Taleisnik, Caligaris, and Astrada, 1971; Ajika, Krulich, Fawcett, and McCann, 1972; Kalra, Fawcett, Krulich, and McCann, 1973; Chobsieng, 1976; Peluso, Steger, and Hafez, 1977; Gay and Hauger, 1977). Extensive studies by Kalra et al.
(1973), and Gay and Hauger (1977), however, indicated that LH and FSH are affected differently by ovariectomy and gonadal hormone replacement. Serum FSH levels increased nearly fourfold following ovariectomy while serum LH was maintained at precastration levels (Gay and Hauger, 1977). Replacement therapy to ovariectomized females with progesterone alone had variable (neutral to slight reduction) effects on the release of LH and FSH. Both estradiol and testosterone, however, partially lowered plasma FSH levels, and strongly inhibited the release of LH in ovariectomized female rats (Kalra et al., 1973; Campbell and Schwartz, 1978). The suppression of LH more than FSH has also been shown after injections of dihydrotestosterone (DHT) which is closely related to testosterone (Gay, 1973). Unlike testosterone, however, DHT does not undergo conversion to estradiol, indicating that an androgen does not have to be converted to an estrogen to inhibit the gonadotropins.

The levels of LH and/or FSH that might be needed to initiate cue production are not known. Since estrogen and testosterone restrict serum LH levels more severely than FSH levels, LH is more likely than FSH to regulate cue production. The neutral or slight reduction effects of progesterone on gonadotropin levels
might be consistent with its partial suppression of urinary ultrasound elicitation in Experiment 1 if gonadotropins underlie cue production or maintenance. It is possible that the long-term (fourteen days) injections of progesterone in Experiment 1 provided enough time and hormone for some conversion of progesterone to other gonadotropin inhibiting gonadal steroids.

Effects of Hypophysectomy and Pituitary Hormone Replacements

The significant depression of urinary ultrasound eliciting potency due to hypophysectomy (Experiment 2) confirms the report by Nyby et al. (1979) that the ultrasound elicitation characteristics of female mouse urine are eliminated by hypophysectomy. Since hypophysectomy eliminates the ability of female urine to elicit male ultrasounds, then one or more pituitary products or byproducts may comprise the ultrasound eliciting cue.

Some sex differences must exist in normal hypothalamic-pituitary actions to account for the fact that urine from an intact adult female elicits ultrasounds, while urine from an intact adult male or hypophysectomized adult female does not (Nyby et al., 1979). The sex differences that exist in hypothalamic-pituitary hormone activities may be limited to different amounts or patterns of release, and not be utilization or
degradation differences. The same gonadotropins, for example, are present in the pituitary glands of both sexes, but the hypothalamus is sexually dimorphic, and this dimorphism results in different patterns of pituitary gonadotropin release (Turner and Bagnara, 1971).

Higher levels of LH and FSH occur in female than in male rats between days 9 and 23, and between birth to day 17 respectively (Dohler and Wuttke, 1974). If similar gonadotropin secretion occurs in the mouse, then this surge early in life of LH and FSH may have long-term effects on characteristics that would signal femaleness to a male.

While gonadotropins appear to be likely candidates for mediating production of the ultrasound eliciting cue, attempts in Experiment 2 to restore the ultrasound eliciting ability of hypophysectomized females with gonadotropins were not successful. However, there may have been methodological reasons why the replacement therapy did not work. Some target tissues are known to become increasingly refractory to their controlling hormones in the absence of those hormones (e.g. the response of sebaceous glands to testosterone) (Muller-Schwarze and Mozell, 1977). In Experiment 2, hypophysectomized urine donors began receiving hormone injections approximately four weeks after having their
pituitary glands removed. In order to maintain the responsiveness of the gonadotropin target tissues, perhaps it is necessary to begin replacement therapy earlier than was done here. Species differences in tissue responsiveness might be another explanation for the ineffectiveness of the hormone replacement since PMS and HCG were used instead of pure mouse FSH and LH. Other possible problems include the dosage of the hormone mixture and the length of time of administration of the hormones. One or both of these factors might not have allowed for normal physiological activity leading to cue production. In fact, measurements of uterine weight suggested that the hormones were not having certain expected physiological effects. Hysterectomies and uterine weighings performed on all urine donors at the end of the third behavioral trial in Experiment 2 revealed the intact group to have uteri that weighed nearly twice those of the two hypophysectomized groups. Gonadotropin replacement as performed here was therefore ineffective in physically restoring the reproductive tracts of the hypophysectomized females. If the physical restoration of the hypophysectomized female mouse uterus is necessary for the appearance of the ultrasound eliciting cue, then gonadotropic activity cannot be ruled out as underlying cue production.
Possible Involvement of Prolactin

The inability to restore cue production in hypophysectomized females with gonadotropins suggests that testosterone (Nyby et al., 1979) and estradiol and progesterone (Experiment 1) might inhibit cue production by affecting pituitary factors other than FSH and LH. For example, testosterone and estradiol also affect the anterior pituitary hormone prolactin (Prl). However, rather than decreasing Prl, as would be predicted if Prl were important in cue production, the two gonadal steroids actually stimulate the synthesis and release of Prl in castrated female rats when administered separately or combined (Nicoll and Meites, 1962; Chen and Meites, 1970; Kalra et al., 1973; Yamamoto et al., 1975). Such an effect would suggest that Prl does not stimulate cue production but might mask it. Other research, however, supports the notion that Prl does not affect the ultrasound elicitation ability of female mouse urine. For example, Prl is similar in male and female rats, and serum levels coincide in the two sexes (Koch, Chow, and Meites, 1971; Dohler and Wuttke, 1974). Castration of female rats at day 22 was followed by a marked decline in pituitary Prl in adulthood (Ojeda, Vazquez, and Jameson, 1977). Nyby et al. (1979) reported, however, that the urine of female mice castrated at approximately the same age as the rats in the Ojeda...
experiment had relatively normal ultrasound elicitation ability. Ojeda et al. (1977) also reported that androgen sterilization of female rats prevents the development of the hypothalamic structures that cause the cyclic release of Prl. If these results are generalizable to mice, then androgenized female mice would not release Prl normally. However, administration of testosterone for the first five days after birth did not prevent females from producing the ultrasound eliciting cue as adults (Nyby et al., 1979).

Possible Involvement of Other Pituitary Hormones

The hypophysectomies that were performed prior to the Experiment 2 injection treatments removed all possible pituitary contributors to cue production. Pituitary factors other than, or at least in addition to, the gonadotropins or Prl may be necessary for the ultrasound eliciting cue to be present in female mouse urine. For example, somatotropic (growth) hormone (STH or GH) and thyroid stimulating hormone (TSH) are anterior pituitary hormones that have considerable effects on mammalian physiology.

Growth hormone is functionally related to Prl, enhances the effectiveness of the gonadotropins, and plays an important role in the metabolism of proteins, fats, and carbohydrates where sex differences may
exist (Turner and Bagnara, 1971). If growth hormone does contribute to female ultrasound elicitation ability, it may be through sex-specific metabolic activities rather than circulating levels, because no significant sex differences were found in growth hormone concentrations in rats from birth to 60 days of age (Walker, Dussault, Urbina, and Dupont, 1977).

TSH stimulates thyroid hormone (TH) activity at the thyroid gland. No sex difference has been found in rats in TSH or TH metabolism and clearance (Nisula, Galton, and Ingbar, 1977). Thus, if a contribution is made by TH to female mouse ultrasound elicitation ability, the sex difference might more likely be in terms of amounts or patterns of release rather than sex-specific activities.

Recent evidence suggests that TH may have a direct effect on the uterus which regulates the responsiveness of the organ to estradiol (Gardner, Kirkland, Ireland, and Stancel, 1978). Importantly, exogenous TH restores diminished uterine responses to estradiol in ovariec-tomized or hypophysectomized rats. The uterine effect of TH is therefore not dependent on direct mediation by the pituitary gland. The apparent synergistic role of TH for a female reproductive tract response suggests its use in conjunction with other hormones in searching
for the hormonal mechanisms regulating the urinary ultrasound eliciting cue. Present research in our laboratory is including TSH in replacement therapy.

The pituitary gland also secretes a newly discovered hormone called the "feminizing factor" in female rats which maintains a female pattern of steroid metabolism in the liver (Gustaffson, Eneroth, Hokfelt, and Skett, 1978). Pituitary secretion of the feminizing factor is under negative control from the hypothalamus in male rats, and this negative control is absent or reduced in females (Gustaffson et al., 1978). Hypophysectomy at 25 days of age prevented the development of the characteristically female steroid metabolism (Denef, 1974), and hypophysectomy at 60 days of age led to an overall masculinization of hepatic steroid metabolism in female rats (Gustaffson and Stenberg, 1974). If mice have a similar sexually dimorphic pattern of steroid metabolism, then characteristically female excretory products of hepatic steroid metabolism might have been eliminated by the Experiment 2 hypophysectomies which were performed at approximately 40 days of age. Furthermore, the failure of the gonadotropin replacement therapy in Experiment 2 to re-establish the pituitary dependent cue is consistent with the Denef (1974) report that the sexually dimorphic hepatic
steroid metabolism requires the presence of the pituitary gland in situ. Production of the pituitary dependent ultrasound eliciting cue might require direct regulation by the hypothalamus that was not afforded following the hypophysectomies in Experiment 2.

**Exocrine Glandular Effects of Pituitary Hormones**

Unlike the possible gonadal steroid mediated cue destruction discussed earlier, the glandular effects of pituitary hormonal activity would more likely lead to ultrasound eliciting cue production since removal of the pituitary gland eliminates cue production (Nyby et al., 1979; Experiment 2). Although little supporting research has been conducted, hormones of the pituitary gland are believed to be able to affect maturation and/or function of the skin and skin glands indirectly by way of another endocrine organ or directly by direct action at the skin target (Muller-Schwarze and Mozell, 1977). Pituitary hormones that have been reported to stimulate the sebaceous glands of the rat include the gonadotropins (Shuster and Thody, 1974) and TSH (Ebling, 1974). The elimination of female mouse ultrasound elicitation ability following hypophysectomy in Experiment 2 might be expected if one or more pituitary hormones underlie cue production of excretory or secretory products that signal femaleness to a male mouse.
Summary

From the foregoing discussion, some hormonal mechanisms regulating production of the female ultrasound eliciting cue emerge as more likely than others. Surprisingly, EB and P, which are regarded as "female hormones" inhibit expression of the cue. Although gonadotropin replacement in Experiment 2 was ineffective in re-establishing cue production, the negative feedback effects that TP, EB, and perhaps P have on FSH and LH remain a possible explanation for cue elimination in view of possible methodological problems. Other pituitary factors which have to be considered as possibly underlying cue production include Prl, GH, TSH, and the "feminizing factor". However, several arguments can be made against Prl involvement in cue production.

The site or sites of production of the female ultrasound eliciting cue are also unclear. Internal sexually dimorphic tissues whose products eventually are expressed externally seem to be the most likely sites of production. Sebaceous and preputial glands, although sexually dimorphic, do not appear to be likely sources of the ultrasound eliciting cue since the known effects of TP, EB, or hypophysectomy on glandular activities appear to be opposite to their effects on cue
production. It is recognized, however, that preputial gland products whose quantities are increased by gonadal steroid administration might be inactivating the cue. The uterus and the liver, two additional sexually dimorphic tissues, are also possibilities. Finally, the pituitary gland itself which also exhibits sexual dimorphism in the rate and types of hormone released must remain a suspected site of cue production.

Concluding Remarks

It seems fairly clear that the female pituitary gland is involved in regulating the production of the female ultrasound eliciting cue. Future research should be directed at attempting to determine which pituitary factors are involved in cue regulation. In this regard, attempts should be made to obtain mouse pituitary hormones if possible, and to deal with some of the possible methodological problems already discussed. Identification of the appropriate hormones would be an important step in understanding this chemocommunication system of potentially direct reproductive relevance.
REFERENCES


-50-


APPENDIX 1: STATISTICS

Experiment 1

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\[ T_j \]

\[ \bar{X} = 6.87 \quad \sigma = 0.78 \quad 14.44 \]

\[ \Delta x = 105.321 \quad \Delta y = 2.062 \quad \Delta z = 138.136 \]

Friedman Test:

\[ X^2_F = \frac{12}{KJ(J+1)} \left[ \sum_j T_j^2 \right] - 3K(J+1) \]

degrees of freedom = \( J - 1 \)

\[ X^2_2 = 11.467 \quad p < .005 \]
Wilcoxon Test: \( Z = \frac{T - E(T)}{A_T} \)

where \( E(T) = \frac{N(N+1)}{4} \)

and \( A_T^2 = \frac{N(N+1)(2N+1)}{24} \)

for P-E vs 0, \( Z = -2.831 \) therefore \( p < .005 \) (two-tailed)

for P vs E, \( Z = -1.988 \) therefore \( p < .05 \) (two-tailed)

Experiment 2

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-57-
Friedman and Wilcoxon Tests calculated the same way as in Experiment 1.

Friedman $X^2 = 19.225$  $p < .005$

Wilcoxon HG, Ho vs Io $z = -3.883$  $p < .001$ (two-tailed)

AoV Summary Table for Uterine Weights

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*p < .01
VITA

The author was born in Bethlehem, PA on January 16, 1955 to Michael and Jennie Zakeski. After graduating from Salisbury High School in Allentown, PA in June of 1972, he entered Muhlenberg College where he majored in natural sciences for two years. In September of 1974, the author transferred to the University Park campus of the Pennsylvania State University where he enrolled as a double major in the Colleges of Science and Liberal Arts. On March 5, 1977, the author graduated with distinction from Penn State, receiving Bachelor of Science degrees in Biology and Psychology. In September of 1977, the author accepted a Teaching Assistantship which he presently holds in the Ph.D. program in Psychology at Lehigh University. The author has twice been the teaching assistant for the Physiological Psychology Laboratory course at Lehigh, and has also aided in teaching the Industrial Psychology and Human Memory courses.