THE RELATIONSHIP BETWEEN SEXUAL BEHAVIOR AND FEMALE HORMONE LEVELS DURING THE MENSTRUAL CYCLE, AND THE EFFECT OF AN ANTIANDROGEN, IN LABORATORY RHESUS MONKEYS

by

Deanne F. Johnson

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(Chairman, Graduate Council)

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INTRODUCTION

Ovarian hormones exert less stringent control over the expression of sexual behavior in female monkeys and humans than they do in other species. Unlike non-primate mammals and prosimians, in which mating behavior is highly correlated with the rapid follicular growth preceding ovulation, female monkeys may copulate at any stage of the menstrual cycle and even during pregnancy (Rowell, 1972; Loy, 1971). While some studies have failed to find any correlation between mating and the menstrual cycle (in Macaca arctoides, Slob, Goy, Wiegand, and Scheffler, 1975; in Macaca fuscata, Hanby, Robertson, and Phoenix, 1971; and in Macaca sylvana, MacRoberts and MacRoberts, 1966), more have reported rhythmic changes in sexual interaction during the female's cycle. For species in which the females exhibit a sexual skin swelling in response to their changing endocrine status, the peak in copulation frequency usually coincides with the maximal midcycle swelling presumed to correspond to the fertile phase of the menstrual cycle: in Macaca nemestrina (Tokuda, Simons, and Jensen, 1968; Goldfoot, 1971; Bullock, Paris, and Goy, 1972; Eaton, 1973), in Macaca nigra (Dixson, 1977), in Papio anubis (Rowell, 1969), in Papio hamadryas (Kummer, 1968), in Papio ursinus (Saayman, 1968), in Cercocebus albigenea (Rowell and Chalmers, 1970), in Miopithicus talapoin (Scruton and Herbert, 1970), in Pan troglodytes (Young and Orbison, 1944; van Lawick-Goodall, 1968), and in Gorilla gorilla (Hess, 1973; Nadler, 1976). Jay (1965) estimates that periods of sexual interaction in langurs occur during the ovulatory portion of

the female cycle, but generally field observations of species in which females have no external signs of their ovarian state other than menstruation (often impossible to detect in the wild) have not led to any conclusion regarding the timing of mating within the menstrual cycle (Gartlan, 1969). For rhesus monkeys, reports from the field indicate cyclicity in sexual interactions (Carpenter, 1942b; Conoway and Koford, 1964; Kaufmann, 1965; Southwick, Beg, and Siddiqi, 1965; Lindburg, 1971), however, the peaks of behavior do not always occur at midcycle (Loy, 1970, 1971). Rhesus monkeys in the laboratory have been reported to show a midcycle peak in copulatory interaction (Phoenix, Goy, Resko, and Koering, 1968; Michael and Zumpe, 1970a; Goy and Resko, 1972). Cyclicity in human sexual behavior has been reported, but some studies have found a midcycle peak (Udry and Morris, 1968), while others report a peak after menstruation (Spitz, Gold, and Adams, 1975).

Often it is the male's behavior rather than the female's which has been found to be maximal at midcycle. Eaton and Resko (1974) found more ejaculations in the follicular than the luteal phase of the menstrual cycle in laboratory pigtailed macaques, but female behavior failed to show any cyclicity. Similarly, in the rhesus macaque, Michael, Herbert, and Wellegalla (1967) reported rhythmic changes in the behavior of three males which corresponded to the female's menstrual cycle; but female presenting to the male and accepting male mount attempts have not been found to vary reliably with the menstrual cycle (Michael and Wellegalla, 1968; Czaja and Bielert, 1975). Certain other rhesus female sexual soliciting behaviors have been reported to peak at midcycle, for example, time spent sitting near a male (Czaja and Bielert, 1975) and pressing a

lever to gain access to a male (Keverne, 1976; Michael and Bonsall, 1977).

In order to help provide a systematic analysis of the way a monkey pair interacts, and to fully elucidate the manner in which ovarian hormones may affect the pair's sexual interaction, it has proven useful to differentiate three aspects of female sexuality (Beach, 1976). Attractiveness is defined as the sexual stimulus value of females to males and is measured by the intensity of male sexual behavior (mounting attempts, mounts, intromissions, ejaculations). Female proceptivity is defined as interest in initiating sexual interaction with a male and is indicated by the female approaching and maintaining proximity to the male and by those responses which solicit male sexual behavior. Female receptivity is defined as willingness to receive the male in copulation and is measured by proportion of times the female accepts the male's mount attempt by assuming the appropriate "present" posture. Studies with ovariectomized rhesus have shown that the ovarian hormones estradiol (E_2), testosterone (T), and progesterone (P^4) have the potential to differentially affect these facets of female sexuality and thereby modify the sexual interaction of rhesus monkeys.

Estradiol promotes sexual interaction by enhancing both attractiveness and proceptivity. Reports by many authors in different laboratories have shown that all aspects of male sexual behavior--e.g. mounts, intromissions, ejaculations, and acceptance of female solicitations--are more frequent when ovariectomized females receive estradiol benzoate (EB) than when they do not (Michael et al., 1967; Trimble and Herbert, 1968; Johnson and Phoenix, 1976). Rates of female sexual solicitation (indicating proceptivity) are also greater when the spayed females are

treated with EB (Michael and Wellegalla, 1968; Zumpe and Michael, 1970; Johnson and Phoenix, 1976). Female receptivity, in contrast, does not appear to depend on E_2. Ovariectomized rhesus receiving no exogenous hormones have high levels of acceptance of male mount initiations (near 100%), and treatment with EB does not cause an increase in female acceptance (Johnson and Phoenix, 1976; Baum, Everitt, Herbert, and Keverne, 1977).

Like E_2, T enhances both attractiveness and proceptivity, but does not alter receptivity, in spayed rhesus. Treatment of ovariectomized females with testosterone propionate (TP) increases male sexual performance (Johnson and Phoenix, 1976; Wallen and Goy, 1977), and suppression of adrenal androgen output may reduce female attractiveness even in EB-treated females (Johnson and Phoenix, 1976). Female soliciting behavior is also augmented by TP in spayed females (Trimble and Herbert, 1968; Everitt and Herbert, 1975; Johnson and Phoenix, 1976), and some investigators would assign T the primary hormonal role in regulating female proceptivity (Baum et al., 1977a). The effects of T may depend at least partially on its conversion to E_2 or another metabolite since the non-aromatizable androgen, dihydrotestosterone propionate (DHTP), does not affect spayed females in the same way as does T. Rather, DHTP fails to alter attractiveness or proceptivity; but reduces female receptivity (Wallen and Goy, 1977).

Progesterone has received less study than E_2 or T in ovariectomized rhesus. Ball (1941) first reported an inhibitory effect of P^4 on rhesus sexual activity, and Michael, Saayman, and Zumpe (1968) found female receptivity reduced by large (pharmacological) doses of P^4. However a recent report now indicates that P^4 in physiological amounts reduces

sexual interaction by suppressing female attractiveness, but not receptivity or proceptivity (Baum, Keverne, Everitt, Herbert and de Greef, 1977). Female proceptive behavior may even be augmented in spayed females receiving EB and P^4 compared to females receiving EB alone. Progesterone given alone, however, did not increase proceptivity over the level found in untreated females. Since intravaginal application of P^4 had similar behavior results to IM injection (but did not elevate plasma P^4 concentration), and since female proceptivity and receptivity were not diminished by P^4, these investigators conclude that P^4 probably reduced attractiveness via a peripheral, vaginal action.

Although experiments with ovariectomized animals permit manipulation of hormone levels which allow potential hormonal effects on behavior to be seen, their results do not indicate the significance of these potential controls in intact animals. The concentrations of E_2, T, and P^4 all have been found to vary systematically across the female menstrual cycle (Hess and Resko, 1973), but the physiological range of hormone levels is normally more restricted than that artificially imposed by an experimenter, and the normal fluctuations during the menstrual cycle may not be of sufficient magnitude to alter behavior in the same manner as does exogenous hormone treatment. Additionally, we must recognize that the ovary produces more hormones than those considered here, and these other products may also contribute to the sexual condition of the intact female rhesus.

Field and laboratory work with intact rhesus have led to the belief that sexual behavior is seasonal and, within the mating season, peaks during the ovulatory portion of the female menstrual cycle. Though the

existence of annual breeding and nonbreeding seasons has been repeatedly documented (but see Loy, 1971), the data for the relationship of sexual behavior to the menstrual cycle are less convincing. In these studies portions of the menstrual cycle have been designated follicular, ovulatory, or luteal by observing when the menses occur. Because of variability in cycle length and possible anovulatory cycles, menstruation is an inaccurate reference for estimating stages of the ovarian cycle and related hormone levels; and conclusions about hormonal correlates of behavior change must be tentative. Furthermore, since the menstrual cycle involves simultaneous fluctuations in several hormones, the particular contribution each may make to variations in sexual interaction is confounded.

In the present study, hormone concentratons in the female's peripheral blood serum are used to determine which stage of the ovarian cycle the female is in at any particular time. Direct laproscopic observation of the ovaries would yield even more accurate information as to the timing of ovulation, but such a procedure would be incompatible with behavioral testing. Behaviors occurring during the different parts of the cycle are compared, and direct correlations of changes in sexual behavior with varying hormone levels are determined for each individual. In addition, since T has been accorded a crucial role in modulating female sexuality, the effects of administration of an antiandrogen to the females throughout one menstrual cycle are reported.

MATERIALS AND METHODS

Subjects: Ten intact female and ten vasectomized male rhesus monkeys were used. All were adult and had been at the Oregon Primate

Center for several years. The males were sexually experienced and the females had served as breeders before use in this study. Eight females and eight males were wild caught, the others were born and reared at the Primate Center. All were housed in individual cages at constant day length and temperature and maintained on Purina Monkey Chow supplemented with fresh fruit.

Apparatus: The testing cage (pictured in Figure 1) has been described previously (Eaton, 1973) and was divided into four holding compartments which opened via vertically sliding doors into a runway which served as the test arena. The doors were operated manually by a system of overhead pulleys. To the right of each door in the runway was an illuminated switch which darkened when pressed. The apparatus was constructed with clear Plexiglas so that an animal in the runway could see into each of the holding compartments. Several months before testing with a male, the females were taught to press the illuminated switches to gain access to food reward in the associated holding compartment.

Testing Procedure: During a test one or two observers sat immediately in front of the runway in full view of the animals. A male was placed in one of the holding compartments, and a female released in the runway. All switches were dark, and the behavior of both animals was recorded. After 3 min the switches were illuminated, and if the female pressed one, the door next to it was opened. In this way the female could release the male into the runway. If the female did not release the male in 5 min, the test was terminated. If the male was released, the social and sexual behavior of the pair was recorded for 10 min. If no intromissions occurred during this time, the test was terminated. If

the male achieved an intromission during this period, but did not ejaculate, an additional 5 min of interaction was allowed before the test was terminated. If the male ejaculated at any time, the test was terminated 10 min after ejaculation.

Each female was randomly assigned a male partner with whom she was tested throughout the experiment. Each pair was tested in the morning once each Monday, Wednesday, and Friday, or each Tuesday, Thursday, and Saturday throughout the female's menstrual cycle. Testing began on the second or third day of menstrual bleeding for each female, and continued for three consecutive menstrual cycles. Tests were conducted from February through May, 1976.

The following behaviors were recorded:

I. Before the male is released--

A. Female

1. Prox--approaching and sitting within one foot of the holding compartment containing the male.
2. Present--assuming the normal receptive posture, perineum oriented toward the male.
3. Invite--while seated near the male's compartment, making a hand slap, head duck, or head bob (Michael and Zumpe, 1970b).
4. Grimace--retracting the lips and corners of the mouth to bare the clenched teeth. A submissive expression.
5. Release male--pressing the switch next to the male's compartment.

6. Press other--pressing a switch adjacent to a compartment other than that containing the male.

7. Move--moving all four feet from the area adjacent to one door to the area adjacent to another door in the runway. A measure of activity.

B. Male

1. Lipsmack--smacking the lips together. The tongue may move rapidly in and out of the mouth.

2. Erection--having penile erection.

3. Threat--making a threatening expression or gesture (open-mouthed stare, ear flip, lunge) toward the female.

C. Male and Female

1. Display--vigorously shaking the cage.

2. Yawn--opening the mouth wide, exposing the teeth.

3. Threat away--making a threatening expression or gesture directed away from the partner (Ball and Hartman, 1935; Altmann, 1962; Zumpe and Michael, 1970).

II. After the male is released--

A. Female

1. Present--same posture as I.A.2.

2. Invite--same gestures as I.A.3.

3. Accept male--assuming the present posture after being contacted by the male.

4. Grimace--same expression as I.A.4.

5. Reach/look back--reaching back to touch or grasp the male or turning the head to look at him during a mount.

B. Male

1. Approach--approaching the female, lipsmacking.

2. Contact--placing one or both hands on the female's hips or lower back before she presents or invites.

3. Accept female--contacting the female within 5 sec after she presents or invites.

4. Mount--grasping the female's hips with the hands and clasping her ankles or calves with the feet.

5. Erection--same as I.B.2.

6. Intromission--inserting the erect penis into the vagina.

7. Pelvic thrusts--making rhythmic pelvic movements during intromission.

8. Ejaculation--pausing after rapid pelvic thrusting with accompanying spasmodic muscular contraction in the thighs, vertical jerking movements of the tail, and seminal emission.

9. Sex explore--making a visual, olfactory, or manual examination of the female's genital region.

C. Male and Female

1. Prox--approaching and sitting within one foot of the seated partner.

2. Groom solicit--exposing part of the body to be groomed and averting the eyes from the partner.
3. Groom--manually picking through the fur of the partner.
4. Rejecting jerk--spasmodically jerking the upper body. An annoyance response.
5. Threat--same as I.B.3. May be accompanied by hitting or grabbing at the partner.
6. Aggression--vigorously biting the partner.
7. Yawn--same as I.C.2.
8. Threat away--same as I.C.3.

Behavior was recorded on checksheets divided into 30 sec intervals. Most behaviors were scored as total frequency of occurrence, and the scores later converted to rates (occurrences/min) depending on the length of the test. For grooming however, the duration of the behavior was tallied by counting the number of 30 sec intervals in which the behavior occurred at least once; this score was then converted into the proportion of the total time spent grooming. The latency to the occurrence of four behaviors (release of the male, first mount, first intromission and ejaculation) was computed to the nearest 30 sec.

Hormone Analysis: A 3 ml blood sample was drawn from the saphenous vein of each female every morning at approximately 8:30 a.m. (prior to any behavioral tests). The samples were allowed to clot at 4°C and the serum removed and stored frozen at -20°C in 0.25 ml aliquots used for steroid analysis. E_2, T, and P^4 were quantified by radioimmunoassay.

A brief description of the general principles of radioimmunoassay, followed by a detailed description of the procedure followed and the reagents used in performing radioimmunoassays in this study, is given in Appendix I. These assay procedures have been previously validated in this laboratory (Resko, 1971; Resko, Norman, Niswender, and Spies, 1974).

Serum samples were analyzed for two menstrual cycles (one control cycle and one experimental cycle) for each female. Initially samples from every other day were assayed, and then if necessary blood from additional days was analyzed to pinpoint the day of E_2 peak. In one female, no evidence of ovulation (no E_2 peak, no luteal P^4 rise) was found in the control cycle analyzed, so her other control cycle was also analyzed. For the initial analyses, each female's blood samples were all processed at the same time so as to avoid inter-assay variation. In cases where certain samples, or entire assays, appeared to be contaminated, repeat analyses were performed.

Drug Preparation and Administration: During cycles 1 and 2 females received control injections of .5 ml propylene glycol at the time of daily bleeding. During cycle 3 they received injections of flutamide as described below.

Powdered flutamide (Schering), 2-2-2-triflouro-2-methyl-4'-nitro-m-propronotoluidide, was first dissolved in a small amount of methanol. This solution was allowed to sit overnight to evaporate the methanol, and the flutamide crystals were then dissolved in propylene glycol at a concentration of 90.9 mg/ml. This solution was warmed under hot tap water and shaken before each use to prevent recrystalization of the

drug. Females received 20 mg/kg/day in a divided dose, half in the morning before behavior testing (at the time of bleeding) and half in the afternoon. Injections were given IM in alternate legs. Since the females weighed between 4.6 and 8.0 kg, they received between .51 and .88 ml at each injection. After 1 week it was noted that some females were developing soreness in the limbs receiving the IM injection, so injections were given subcutaneously at multiple sites over the body. After 2 weeks some females developed infections around the injection sites, so the concentration of the flutamide solution was reduced to 66.7 mg/ml. This weaker solution was well-tolerated by all females, even though the amount injected was increased to between .66 and 1.20 ml in order to maintain a daily flutamide dose of 20 mg/kg.

Statistical Analyses: For menstrual cycles in which an E_2 peak was found (for nine females in control cycles and for six females in flutamide cycles), the day of E_2 peak was called day 0 and the days following the peak called day 1, day 2, etc., up to the day before the next menstruation. The days preceding the peak were called day -1 (the day before the peak), day -2 (2 days before the peak), etc., back to and including the first day of menstruation. In the rhesus female, ovulation occurs about 48 hr after the E_2 peak (Weick, Dierschke, Karsch, Hotchkiss, and Knobil, 1973), thus day 2 would be the day of ovulation in this scheme. Since behavior tests were given (and hormone determinations made) only every other day, for analysis the data were grouped for days 1 and 2, days 3 and 4, days 5 and 6, etc., and for days -1 and -2, days -3 and -4, etc. Day 0 was examined alone.

Performance during control and flutamide cycles was compared with a paired t-test on means for each day group for the six females showing an E_2 peak in both cycles.

For each behavior the mean score of all pairs for each day group was computed, and the occurrence of behavior during the different parts of the cycle was compared. T-tests were performed on mean behavior scores computed for each pair for the follicular phase (days -14 to 2) vs. the luteal phase (days 3 to 16) of each cycle. In some cases comparisons were also made between the midportion of the cycle, when E_2 and T levels were high, (days -4 to 0) and the remaining days of the cycle.

Since individual differences in pairs' behavior may have been overshadowed when looking at group means, the data for each pair were also analyzed individually. The correlation between scores for each behavior and serum levels of each hormone was determined in each cycle with the Spearman rank-order correlation coefficient (rho).

For all statistical analyses, the .05 level of probability for a two-tailed test was chosen as the critical level of statistical significance.

RESULTS

Consistent, predictable changes in sexual interaction related to the stage of the female's menstrual cycle or to her hormone levels were not found in this group of 20 rhesus monkeys. Variations between pairs, and from cycle to cycle, were more remarkable than were similarities. However, both males and females sometimes showed significant changes in behavior correlated with the female's hormonal state.

A. Menstrual cycles and hormone determinations

The lengths of menstrual cycles were compared with t-tests for control and flutamide cycles of the 10 females and no differences were found. The mean cycle length was 28.8 days. The three consecutive cycles observed in this study were compared with those occurring in these females during the same months of the previous year, and in the 2 months prior to this study, and no differences in length were significant. This indicates that the procedures of testing and bleeding the females in this study did not disrupt their usual patterns of menstruation.

The mean serum concentrations of E_2, T, and P^4 in the control cycles are shown in Figure 2. The data from nine females were included in computing the means (one female [7169] showed no E_2 peak in either control cycle) with the daily values aligned around the day of E_2 peak, day 0, and mean values determined for 2 day blocks on either side of this day.

Figures 3, 4, and 5 show the mean hormone levels of E_2, T and P^4 for the six females showing an E_2 peak when treated with flutamide. The mean hormone values for these six females in their control cycle are also shown for comparison. Statistical tests comparing the hormone levels of the control and flutamide cycles showed that although the midcycle peak of E_2 was not prevented (a chi-square test showed no significant difference between the number of females having an E_2 peak in the control [nine females] and flutamide [six females] cycle [$X^2 = 1.067$, df = 1]), mean levels of E_2 and T in the six females having an E_2 peak in both cycles were significantly lower during the flutamide

cycle (t = 2.638 and 5.800, respectively, df = 11). There were no significant differences in mean P^4 levels.

Complete hormone data for all females is given in Appendix II.

B. Behavioral variation during the menstrual cycle

The number of pairs contributing data to the various behavioral analyses varies with respect to the cycle under consideration (nine possible pairs in the control cycle, six in the flutamide cycle) and the portion of the behavior test considered (one pair had no post-release pair test data since the female [2740] never released the male; and one female [6253] did not release the male in the luteal phase of the control cycle, thus eliminating this pair from any comparisons of pair test behavior in the follicular and luteal phase of that cycle).

Male sexual behavior did not vary consistently with the female menstrual cycle. Mean mounting rate was the only behavior to vary significantly between the follicular and luteal phases in both the control and flutamide cycle (Table 1). Males mounted more often during the follicular phase than the luteal phase (control: t = 2.999, df = 6; flutamide: t = 3.768, df = 4). Four other male behaviors varied significantly during the flutamide cycle: rates of contacting and intromitting, and frequency of ejaculations, were higher, and mean mount latency was shorter, in the follicular phase than the luteal phase (t = 4.024, 3.199, 2.808, and 3.494, respectively, df = 4). In the control cycle, however, neither male contacts, intromissions, nor ejaculations showed any systematic change. As can be seen in Figures 6, 7, and 8, the

luteal phase decline in these male behaviors found during the flutamide cycle was not seen in the control cycle.

When the behaviors of each male were correlated with his partner's hormone levels, significant correlations were few and non-systematic. Significant correlations between at least one category of male sexual behavior and female E_2 were found for only two males; with P^4 for five males; and with T for three males. Furthermore, a significant correlation between behavior and hormones found in one cycle was seldom repeated in the other cycle of the same pair. Thus, knowledge of a female's precise hormonal state would not allow reliable predictions about her partner's sexual performance.

Female sexual behavior showed a similar lack of consistent correspondence to the menstrual cycle or to individual hormone levels. The only indications of cyclic alterations in female interest in the male appeared before the male was released. Females pressed the unilluminated, inoperative switch next to the male's compartment ("press male") more often in the follicular than the luteal phase of both cycles ($t = 2.894$, $df = 8$ [control], 3.713 $df = 5$ [flutamide]) (Figure 9). Two other measures, "prox" (sitting by the confined male) and "open other" (opening a compartment not containing the male) showed evidence of changes with the cycle, (Figures 10 and 11), although the differences between the follicular and luteal phases were not significant. When the days of highest mean E_2 and T concentrations, -4 through 0, were compared to the rest of the cycle, the females proxed the confined male more often, and opened fewer other compartments during these midcycle days ($t = 3.602$ and 2.317, respectively, $df = 8$) in the control cycle. These patterns were not

significant in the flutamide cycle. Though the latency to release the male appeared to be shortest during the middle of the cycle, the differences were not statistically significant (Figure 12).

Once the male had been released, female proceptive behavior did not change significantly during the menstrual cycle. Female presents, invitations and proxs were not significantly more frequent in the follicular than the luteal phase; and when the midcycle days were compared to the rest of the cycle, again the differences in mean rates of these female solicitations were not significant. Female rates of "threatening away" (another form of solicitation) also failed to vary significantly across the cycle except during that part of the pair test after the male ejaculated. In the control cycle, post-ejaculatory threatening away occurred significantly more frequently during the follicular than the luteal phase ($t = 3.058$, $df = 5$) (Table 2).

Female receptivity also failed to change with the cycle. The mean female acceptance ratio remained near 1 throughout this study (Figure 13).

As with male sex behavior, correlating individual females' behavior with their own hormone levels revealed no consistent relationships. Three females had at least one positive correlation between a proceptive behavior and E_2 levels; three others had negative correlations. For T there were five females with a positively correlated proceptive behavior and three with negative correlations. Similarly, P^4 was negatively correlated with a proceptive behavior in four females and positively correlated in two females. The measure of receptivity, the female acceptance ratio, was significantly correlated with hormone levels

(positively with E_2) in only one female. As with the males, most correlations were significant in only one cycle of an individual.

C. Effect of flutamide on behavior

When the behavior displayed during the flutamide cycle was compared with that of the control cycle, few significant differences were found. For the males, the mean latency to intromission was longer, ($t = 2.386$, $df = 13$) and fewer pelvic thrusts were displayed during an intromission ($t = 2.398$, $df = 13$) in the flutamide cycle. Differences in female behavior between the cycles were inconsistent. Female proximity responses to the confined male, and inviting and "reaching back" during the pair test occurred at lower rates in the flutamide cycle ($t = 2.669$, 2.284, 2.239, respectively; $df = 13$). However, presenting to the confined male, grooming and threatening away in the pair test were more frequent in the flutamide cycle ($t = 2.554$, 3.095, 2.859, respectively; $df = 13$) (Table 3). No other social or sexual behaviors showed significant changes from the control in the flutamide cycle.

DISCUSSION

Contrary to previous reports of rhythmicity in rhesus sexual behavior, few reliable correlations between patterns of sexual interaction and female hormone levels during the menstrual cycle were found in this study. The data are thus more reminiscent of those of Slob et al. (1975) for the male stumptailed macaque, and Eaton and Resko (1974) for the female pigtailed macaque, than those reported for laboratory rhesus (Michael et al., 1967; Michael and Zumpe, 1970a; Michael and Bonsall, 1977). The

behavioral variations which are significant in this study are in the same direction as those found previously in rhesus; but more striking is the lack of hormonal influence on the majority of sexual behaviors measured in both sexes.

When male interest did show significant variation during the female menstrual cycle, it was higher in the follicular than the luteal phase, a finding consistent with the generally expected facilitatory effects of E_2 and T and inhibitory effects of P^4 on attractiveness in ovariectomized females.

Since ovarian T is not required for females to be attractive to males (EB-treated spayed rhesus are attractive [Johnson and Phoenix, 1976]), T is probably not primarily responsible for the few cyclic changes in female attractiveness which were found. The maximum and minimum concentrations of E_2 and P^4 are out of phase with each other in the menstrual cycle, so either or both of these hormones may have a regulatory role in determining attractiveness in the intact female. For the four individual males which showed significant correlations of their sexual behavior with female hormone patterns, three had negative correlations with P^4, while none had any correlation with their partners' E_2. This could indicate that it is the increased P^4 levels rather than the decreased E_2 in the luteal phase which detracts from the female's attractiveness. However, one male had positive correlations of his sexual behavior with his female's P^4 levels, and since most males showed no consistent correlation between their behavior and any female hormone measured, the overall conclusion must be that other factors contribute as much or more to the female's sexual attractiveness as does her ovarian

condition. Though the female hormonal state during the luteal phase of the cycle may be less than optimum for sexual interaction with some males, others do not demonstrate a diminished interest in sexual activity at this time.

While these results indicate that the rhesus male's sexual behavior is not necessarily restricted by the female's ovarian condition, they do not demonstrate that the male cannot discriminate between females in different hormonal states. Studies in different laboratories, using different testing procedures, and in the field have found variation with the menstrual cycle in female attractiveness. In some cases (Michael et al., 1967), only a small number of males was studied, a technique which can lead to erroneous conclusions in a species with individual differences as great as are found in macaques; and usually the female hormonal state was only indirectly estimated from time of menstruation. Recently Michael and Bonsall (1977) have quantified ovarian hormones in nine female rhesus while pairing them with seven males, but the only behavioral data reported for the males was ejaculation frequency, which was greatest at midcycle. That a similar peak was not found here may be a function of the longer test duration in Michael and Bonsall's study.

Differences in group size may also affect the degree of rhythmicity found in female attractiveness. In the laboratory pair test the male, given a chance to copulate with one female or none at all, may be less discriminating regarding the female partner's ovarian status than he would be in a troop situation where there might be a choice of partners in various stages of the menstrual cycle. However, group environments also place restrictions on the individuals which complicate the interpre-

tation of behavior. For example, Goldfoot (1971) found a complex interaction between stage of the menstrual cycle and social position of the female sex partners chosen by a male in laboratory groups (one male/three females) of pigtailed macaques; and in larger groups, higher ranking males may restrict the behavior of lower ranking males (Stephenson, 1975).

It is interesting to note that female attractiveness may be a function of the male's hormonal state as well as the female's. In the wild, rhesus are seasonal breeders, with no sexual activity occurring for several months of the year. The factors that control the breeding season are poorly understood, but it is evident that hormones may play important roles. During the non-mating season, male T levels are very low (Gordon, Rose, and Bernstein, 1976) and the females are presumed to be pregnant or anovulatory (although evidence on changes in ovarian function of free-ranging females is limited [Vandenberg and Post, 1976]). Interestingly, it may be the lack of male T rather than female infertility which accounts for the lack of female attractiveness.

Vandenberg (1969) and Vandenberg and Drickamer (1974) have shown that treatment of females with EB in the non-breeding season will stimulate sexual activity and T secretion in the males. More recently, however, Vandenberg and Post (1976) have reported that males and females captured during the non-mating season will begin to copulate when paired if the males are implanted with TP. The increased sexual activity did not cause the females to resume cycling or change their rate of sexual presentation to the males. The change in sexual attractiveness of these females thus was apparently mediated by the hormonal condition of the

males, not the females. In Vandenberg's earlier studies the EB-treated females may have stimulated male T output primarily, and the increased female attractiveness again could have resulted from this male hormone increase rather than directly from the change in female E_2. In any case, it is clear that in the wild as well as in the laboratory, the female's hormonal state is not the only, or even necessarily the primary, factor in determining her sexual attractiveness to males.

Turning now to female behavior, it was not surprising that female receptivity did not vary during the menstrual cycle. This aspect of female sexuality (measured by the female acceptance ratio) has been found least sensitive to changes in female hormone state in spayed animals. Except for one series of tests that showed an enhancing effect of EB on female receptivity, a finding which was not observed in a second experiment with the same females (Johnson and Phoenix, 1976), no facilitating influences of exogenous steroids on this aspect of behavior have been reported. There is a report of an inhibitory effect of P^4 on female receptivity (Michael et al., 1968) in which female refusals increased dramatically when the females received 25 mg P^4; however, only two females served as subjects, and a more physiological dose of P^4 (5 mg) did not affect female refusals. In the present study only one of eight females had a significant correlation between her acceptance ratio and any of the three hormones measured. Thus it seems clear that most females will permit males to mount regardless of their ovarian condition. Wallen and Goy (1977) have noted that there may be other subtle actions taken by the female, e.g., facilitating intromission, which would be indicative of enhanced "receptivity," but these could not be identified in this study.

From work with ovariectomized females, one would expect female proceptive behavior to be most evident at that time of the menstrual cycle when estradiol and testosterone concentrations are greatest, i.e., the late follicular and ovulatory stages. Female behavior before release of the male follows this pattern to some extent in this study: most females sat near the male and pressed the switch near his compartment more often, and opened empty compartments less often when E_2 and T were high than at other times. However, after the male entered the test cage, female proceptive behavior did not show these hormonal effects. Rates of female presenting, inviting, proxing, and grooming did not vary systematically with the menstrual cycle.

The degree of cyclicity found in female proceptive behavior seems to change with the test situation or environment. The more that completion of copulation depends upon the female, the more likely she is to show cyclic variations in proceptivity. Thus, when the female is separated from the male, as in the "pre-release" portion of the tests reported here, proceptivity, measured by sitting near the male and attempting to release him, varies with the cycle. Czaja and Bielert (1975) also found female proximity to a confined male to vary across the menstrual cycle in four females; and recently Michael and Bonsall (1977) have reported a midcycle peak in the speed with which females lever-pressed to release a male partner. That a similar cyclicity in release latency was not found here may be a function of the difficulty of the task: Michael and Bonsall's females had to press 250 times for the male, while my females had only to press once. Eaton and Resko (1974) also failed to find any correlation between release latency and hormone levels in pigtailed

monkeys required to press a lever once to release a male. Other proceptive behaviors that the female could display toward a confined male, e.g., presenting and inviting, are not as reliable in showing cyclicity. Neither Czaja and Bielert (1975) nor I found presenting to vary with the female's hormones; and though those researchers reported cyclic variation in female invitations, I found none.

When the female is with a male in a pair test, proceptive behavior is even less likely to fluctuate reliably with the menstrual cycle. Michael and Wellegalla (1968) found female "invitations" (these included presents and head-ducks) to change rhythmically in 15 of 22 cases, but in 8 "invitations" were high in the follicular phase, while in 7 these behaviors declined at midcycle. Czaja and Bielert (1975) found no cyclicity in female presentations, but reported that invitations (hand slap and body flex) and proximity responses were highest at midcycle during pair tests. In the present study, only female post-ejaculatory threatening-away showed a significant relationship to the menstrual cycle.

In a related species, Macaca nemistrina, Eaton (1973) and Eaton and Resko (1974) found no relationship between female presenting or proximity responses with phase of the menstrual cycle or hormone levels in pair tests, although Bullock et al. (1972) had reported more frequent female presentation in the follicular compared to the luteal phase in these animals. When these same females were tested with males in a more complex social situation, three females with one male, differences related to the menstrual cycle were found in even more female behaviors (e.g., present, prox, follow, groom, sex pout); and the dominance rank

of the female in the group was also a factor in the variation in female sexual behavior (Goldfoot, 1971).

In free-ranging groups of rhesus monkeys where complex social and environmental factors, as well as hormones, influence individuals, there have been reports of females showing periods of "estrus." These are distinguished by behavior such as following a male, sitting near and grooming him, inviting him to mount and cooperating when he attempts to mount (Carpenter, 1942a; Conoway and Koford, 1964; Kaufman, 1965; Loy, 1970, 1971). According to the terminology of this paper, the females are showing increased proceptivity and receptivity. These "estrus periods" may reappear cyclicly during the mating season, leading researchers to speculate that they are facilitated by particular hormonal states in the females, especially the high E_2 levels associated with ovulation. Though it seems advantageous for the females to show the most intense proceptivity near the time of ovulation, Loy (1970) has reported two peaks of "estrous behavior," one midcycle and one perimenstrual, and some females have shown such behavior during pregnancy (Kaufmann, 1965; Zumpe and Michael, 1970; Loy, 1971; Bielert, Czaja, Eisele, Scheffler, Robinson, and Goy, 1976), a situation not readily explainable by reference to hormonal conditions.

Even in the wild it is important to note the range of individual variation in female displays of proceptivity. Though some females may show relatively discrete periods of sexual interaction which reoccur at approximately 28 day intervals, many do not. Reports on the duration of "estrus" range from 1-95 days, and estrous cycle length (from the beginning of one "estrus" to the beginning of the next) has varied from 6-50

days (Kaufman, 1965; Loy, 1971). Thus, even in the free-ranging environment, where the female's proceptive display may be more essential to successful copulation that it is in a laboratory pair test, individuality among animals precludes simple prediction of cyclicity related to hormone levels in the occurrence of this behavior.

The antiandrogenic drug, flutamide, was given to the females to assess the contribution of T to any correlation of sexual behavior with the menstrual cycle. The drug unexpectedly caused a significant depression in serum E_2 and T concentration in the females. In male rats flutamide appears to interfere with T uptake and/or inhibit nuclear binding of the androgen in target tissues (Peets, Henson, and Neri, 1974). Flutamide also interferes with the negative feedback effect of endogenous androgen in intact male rats, producing large elevations in circulating LH and T (Sodersten, Gray, Damassa, Smith, and Davidson, 1975). The decrease in circulating hormones in the rhesus females was thus surprising. Gay and Tomacari (1974) have found that an antibody to T can suppress follicle stimulating hormone (FSH) levels in female rats without interfering with ovulation. Though FSH secretion patterns differ in the rat and monkey, it may be that the anti-androgen given these monkeys suppressed FSH, and that this in turn led to the reduced serum levels of E_2. Since ovarian T is a product of E_2 metabolism (Turner and Bagnara, 1971), reduced E_2 output could result in lower T levels. Alternately, a decline in adrenal androgen output, or increased clearance or metabolic conversion of T, could account for the reduced peripheral concentrations seen. Of course, if flutamide does interfere with T binding in target cells, the serum levels of the hormone tell us nothing about the actual amounts which are

affecting the tissues. One needs some additional measure of T action in the female to clarify the androgen state of a flutamide-treated rhesus female.

Whatever the ultimate physiological action of the drug, sexual behavior was largely unaffected by treatment. The effects on female behavior were equivocal, with some measures of proceptivity increasing and others decreasing. The changes in male behavior, longer latency to intromit and fewer pelvic thrusts per intromission, could be indicative of a peripheral effect of flutamide at the vagina, perhaps a decrease in vaginal lubrication hindering intromission.

In previous work, flutamide has been given to males, not females. These studies have failed to find any change in sexual behavior in male rats treated with flutamide in doses which have profound anti-androgenic effects peripherally and which interfere with central feedback control mechanisms regulating T production (Neri, Florance, Koziol, and van Cleave, 1972; Sodersten et al., 1975). The inference that flutamide does not act on androgen mechanisms controlling behavior in the same manner as on those producing peripheral effects in rats, however, cannot be generalized to primates without experimentation. Testosterone's control of male sexual behavior appears to be via different metabolic routes in rats and monkeys: male monkeys do not require, as rats do, that T be metabolically convertable to estrogen before it can enhance male sexual behavior (Phoenix, 1974). The metabolic pathways involved in androgen regulation of female sexual behavior have received little study; however, Wallen and Goy (1977) have recently reported that the nonaromatizable androgen, DHTP, was not effective in enhancing female proceptivity or attractiveness

in spayed rhesus. Whether flutamide's anti-androgenic properties depend upon the metabolic pathway of androgen action is not known.

In any case, behavioral differences between the control and flutamide cycles were minimal. Cyclic variation in male behaviors occurred primarily in the flutamide cycle, apparently because of a decline in mean rates of male behavior in the luteal phase of that cycle not seen in the control cycle. It seems unlikely that this difference can be explained by reduced levels of E_2 since E_2 levels were lower throughout the flutamide cycle, not just in the luteal phase; neither do alterations in female T metabolism explain the effect since male behavior was almost never correlated with female T levels, and work with ovariectomized females has indicated only small amounts of T are needed to enhance female attractiveness. Female proceptivity, known to be affected by E_2 and T in spayed females, was not systematically altered by the lowered concentrations of E_2 and T, or the possible interference with T action, caused by flutamide in these normally cycling rhesus females. These results lend further support to the overall finding in this study that female ovarian hormones do not have reliable, predictable effects on the sexual interaction of pairs of intact rhesus monkeys.

SUMMARY AND CONCLUSIONS

The interaction of unique pairs of intact female and vasectomized male rhesus monkeys was observed in laboratory tests during two control and one experimental menstrual cycle of each female. During the experimental cycle a non-steroidal antiandrogen, flutamide, was administered to the females. Daily peripheral serum levels of estradiol, testosterone,

and progesterone were determined by radioimmunoassay for each female throughout one control cycle and the experimental cycle.

The data were examined to determine any correlations between hormone levels or stage of the menstrual cycle and behavior, and to determine the effects of the antiandrogen on the sexual interaction of the pairs. The behavior was analyzed with respect to female attractiveness, value as a sexual stimulus for the male; proceptivity, interest in initiating sexual interaction with a male; and receptivity, willingness to receive a male in copulation.

Although a few pairs showed some significant relationships between attractiveness or proceptivity and the menstrual cycle, with these attributes being greatest in the follicular and ovulatory portions of the cycle, none of the three aspects of female sexuality was reliably affected by changing hormone levels during the menstrual cycle or by administration of flutamide. Receptivity remained high in all pairs throughout the study, demonstrating the same freedom from hormonal control which has been reported for this trait in ovariectomized rhesus.

Thus, though hormones have the potential to modify sexual interaction in rhesus monkeys, other factors (e.g. individual development and experience, environment, and social setting) are at least as important as the hormonal variation of the menstrual cycle in determining the behavioral interaction between a male and female rhesus. The cyclic fluctuations in mating behavior which have been reported in rhesus monkeys are apparently the result of an interaction of the social and environmental situation with the changing female hormone levels. Further study may elucidate the parameters of this interaction.

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Table 1. Male behavior scores (mean ± S.E.) during the follicular and luteal phase in the control (n = 7 males) and flutamide (n = 5 males) cycle.

	Cycle			
	Control		Flutamide	
	Phase		Phase	
Behavior	Follicular	Luteal	Follicular	Luteal
Contact per min	1.60 ± .38	1.29 ± .39	1.98 ± .30 *	0.83 ± .17
Mount per min	1.66 ± .35 *	1.30 ± .37	2.05 ± .33 *	0.83 ± .16
Intromission per min	1.32 ± .38	1.18 ± .41	1.72 ± .38 *	0.66 ± .17
Ejaculation per test	0.78 ± .12	0.67 ± .16	0.97 ± .03 *	0.49 ± .11
Mount latency (min)	1.15 ± .30	1.42 ± .32	0.88 ± .17 *	1.41 ± .28

* $p < .05$, t-test, follicular vs. luteal phase

Table 2. Rates (no. per min) of female proceptive behavior (mean ± S.E.) during the follicular and luteal phase in the control (n = 7 females) and flutamide (n = 5 females) cycle.

	Cycle			
	Control		Flutamide	
	Phase		Phase	
Behavior	Follicular	Luteal	Follicular	Luteal
Present	.03 ± .02	.08 ± .04	.05 ± .03	.05 ± .02
Invite	.17 ± .05	.15 ± .06	.11 ± .08	.06 ± .04
Prox	.19 ± .10	.24 ± .11	.26 ± .10	.31 ± .13
Threat-away (pre-ejaculatory)	.58 ± .18	.55 ± .23	.82 ± .30	.56 ± .24
Threat-away (post-ejaculatory)	.30 ± .10 *	.11 ± .06	.37 ± .18	.23 ± .12

* $p < .05$, t-test, follicular vs. luteal phase

Table 3. Behavioral measures (mean ± S.E.) showing significant difference between the control and flutamide cycle ($p < .05$, t-test, all comparisons).

Behavior	Cycle	
	Control	Flutamide
♂ Latency to intromit (min)	1.63 ± .20*	2.53 ± .58°
♂ Thrusts per intromission	7.00 ± .45*	5.59 ± .31°
♀ Prox per min to confined ♂	1.18 ± .25**	.80 ± .22°°
♀ Present per min to confined ♂	.02 ± .01**	.04 ± .01°°
♀ Invite per min	.19 ± .03*	.09 ± .02°
♀ Reach back per min	.27 ± .03*	.23 ± .04°
♀ Groom, proportion of test	.06 ± .02*	.12 ± .03°
♀ Threat-away per min	.54 ± .04*	.73 ± .05°

* n = 8 pairs

° n = 5 pairs

** n = 9 pairs

°° n = 6 pairs

Figure 1. The test cage. The male was placed in one of the rear compartments and the female released in the front runway. The door lights could be illuminated from the switch box shown at the left. When the female pressed one of the switches, the adjacent light went out and the experimentor opened the adjacent door by pulling on the appropriate rope. The door to the compartment second from the left is shown in the open position. The large front doors open to allow cleaning.

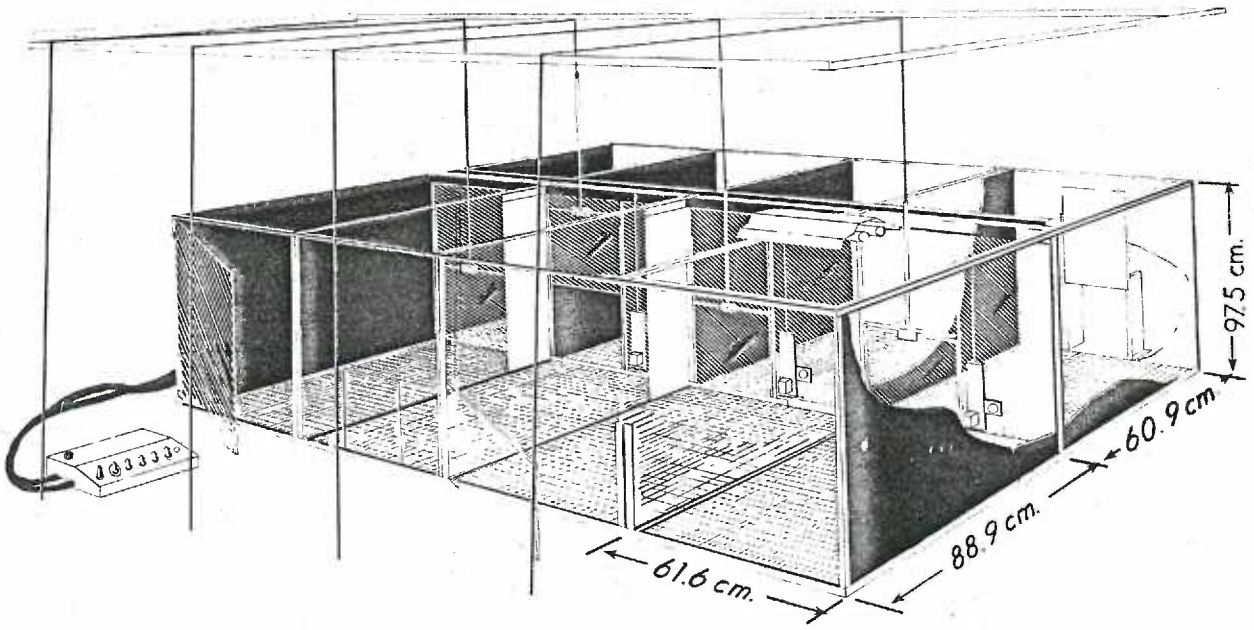

97.5 cm.
60.9 cm.
88.9 cm.
61.6 cm.

Figure 2. Serum concentrations of estradiol, testosterone, and progesterone during a control cycle for nine females. Day 0 is the day of estradiol (E_2) peak. Data are plotted as means $\pm$ S.E. (shaded areas).

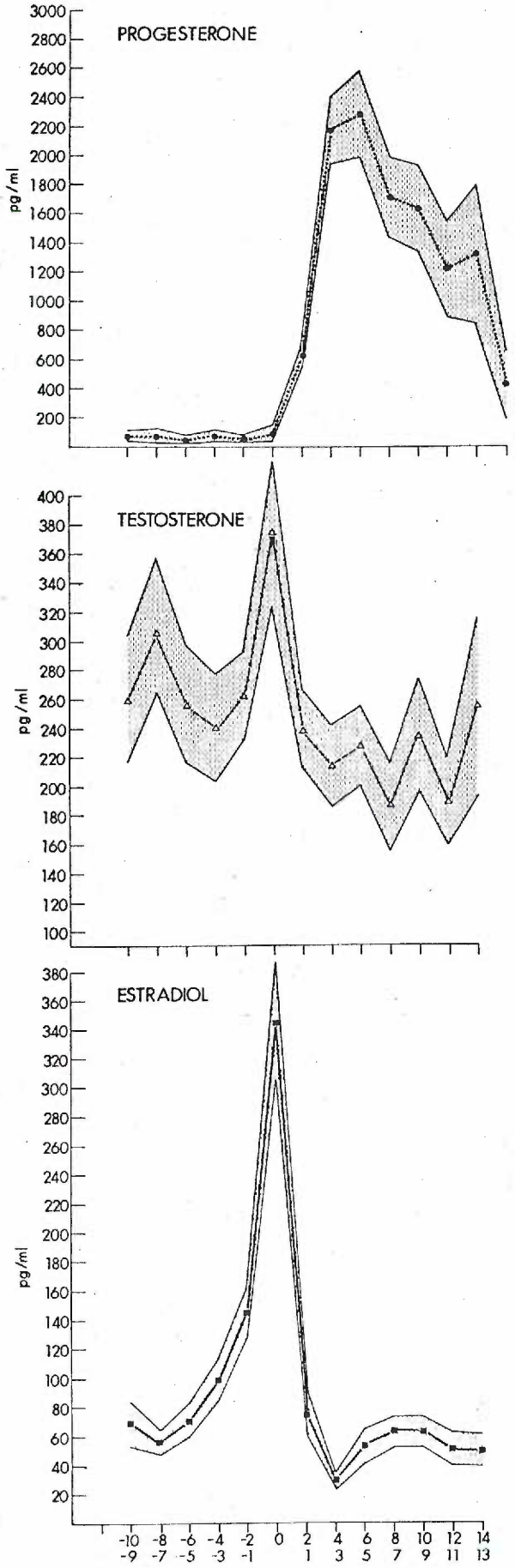

PROGESTERONE
pg/ml
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
TESTOSTERONE
pg/ml
400
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
ESTRADIOL
pg/ml
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
-10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
-9 -7 -5 -3 -1 1 3 5 7 9 11 13

Figure 3. Serum concentrations of estradiol (E_2) during a control and an experimental (flutamide-treatment) menstrual cycle for six females. Day 0 is the day of estradiol (E_2) peak. Data are plotted as means $\pm$ S.E. (shaded areas).

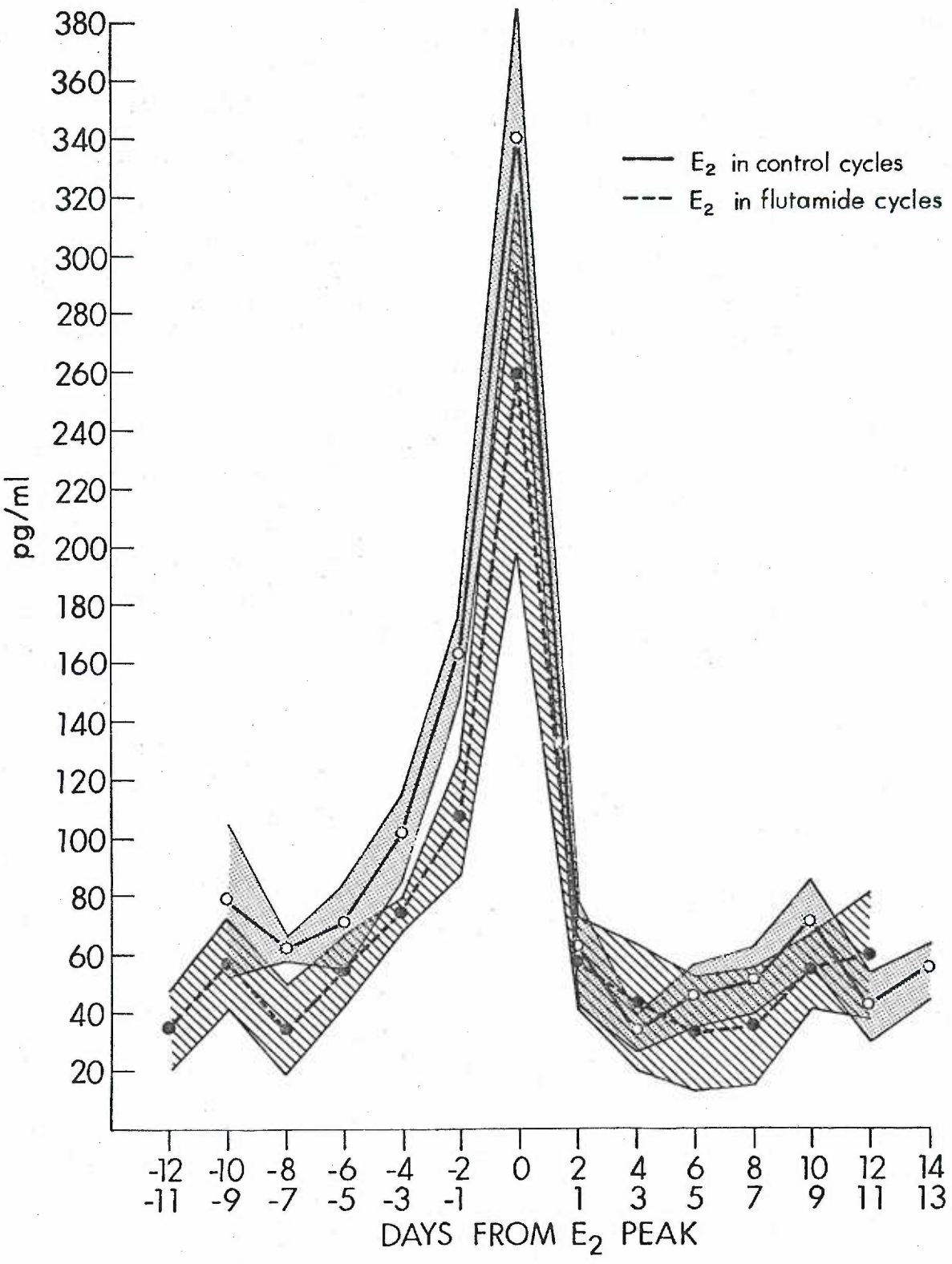

380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
pg/ml
E2 in control cycles
E2 in flutamide cycles
-12 -11
-10 -9
-8 -7
-6 -5
-4 -3
-2 -1
0
2 1
4 3
6 5
8 7
10 9
12 11
14 13
DAYS FROM E2 PEAK

Figure 4. Serum concentration of testosterone (T) during a control and an experimental (flutamide-treatment) menstrual cycle for six females. Day 0 is the day of estradiol (E_2) peak. Data are plotted as means $\pm$ S.E. (shaded areas).

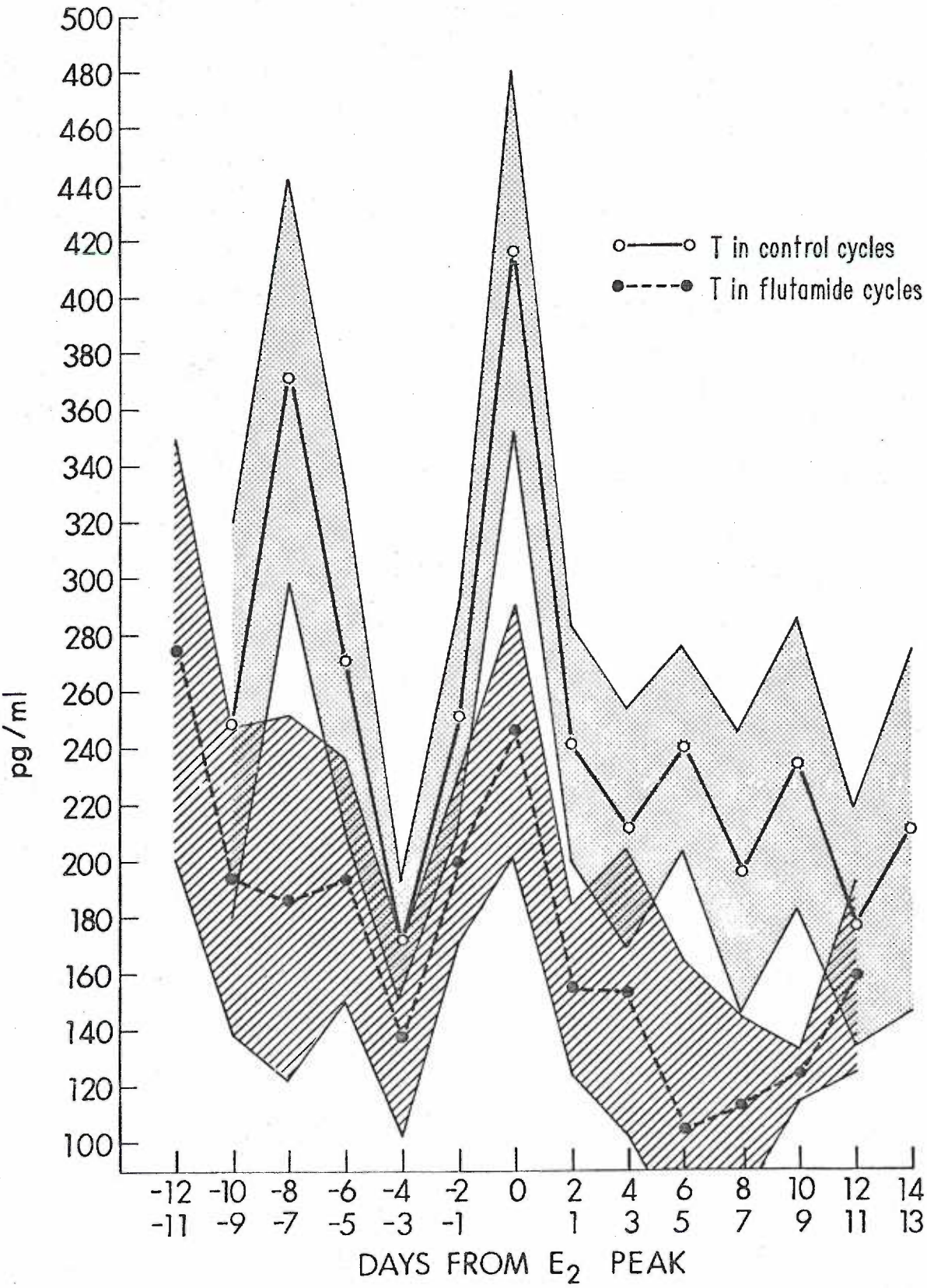

500
480
460
440
420
400
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
pg/ml
T in control cycles
T in flutamide cycles
-12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
-11 -9 -7 -5 -3 -1 1 3 5 7 9 11 13
DAYS FROM E_2 PEAK

Figure 5. Serum concentrations of progesterone (P^4) during a control and an experimental (flutamide-treatment) menstrual cycle for six females. Day 0 is the day of estradiol (E_2) peak. Data are plotted as means $\pm$ S.E. (shaded areas).

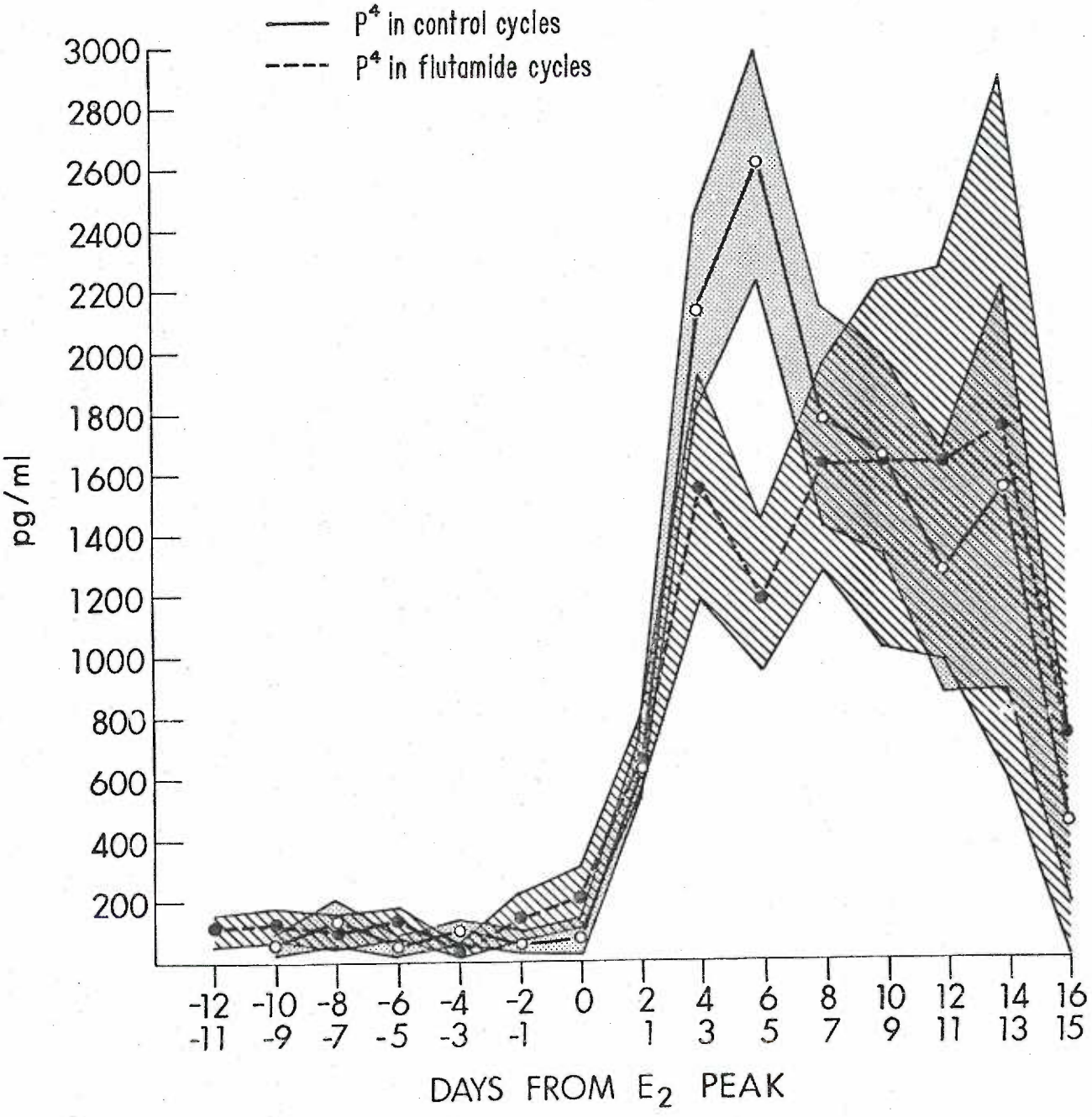

P4 in control cycles
P4 in flutamide cycles
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
pg/ml
-12 -11
-10 -9
-8 -7
-6 -5
-4 -3
-2 -1
0
2 1
4 3
6 5
8 7
10 9
12 11
14 13
16 15
DAYS FROM E2 PEAK

Figure 6. Male contact rate in the control and flutamide cycle. Day 0 is the day of estradiol (E_2) peak. Data are plotted as means $\pm$ S.E. (shaded areas).

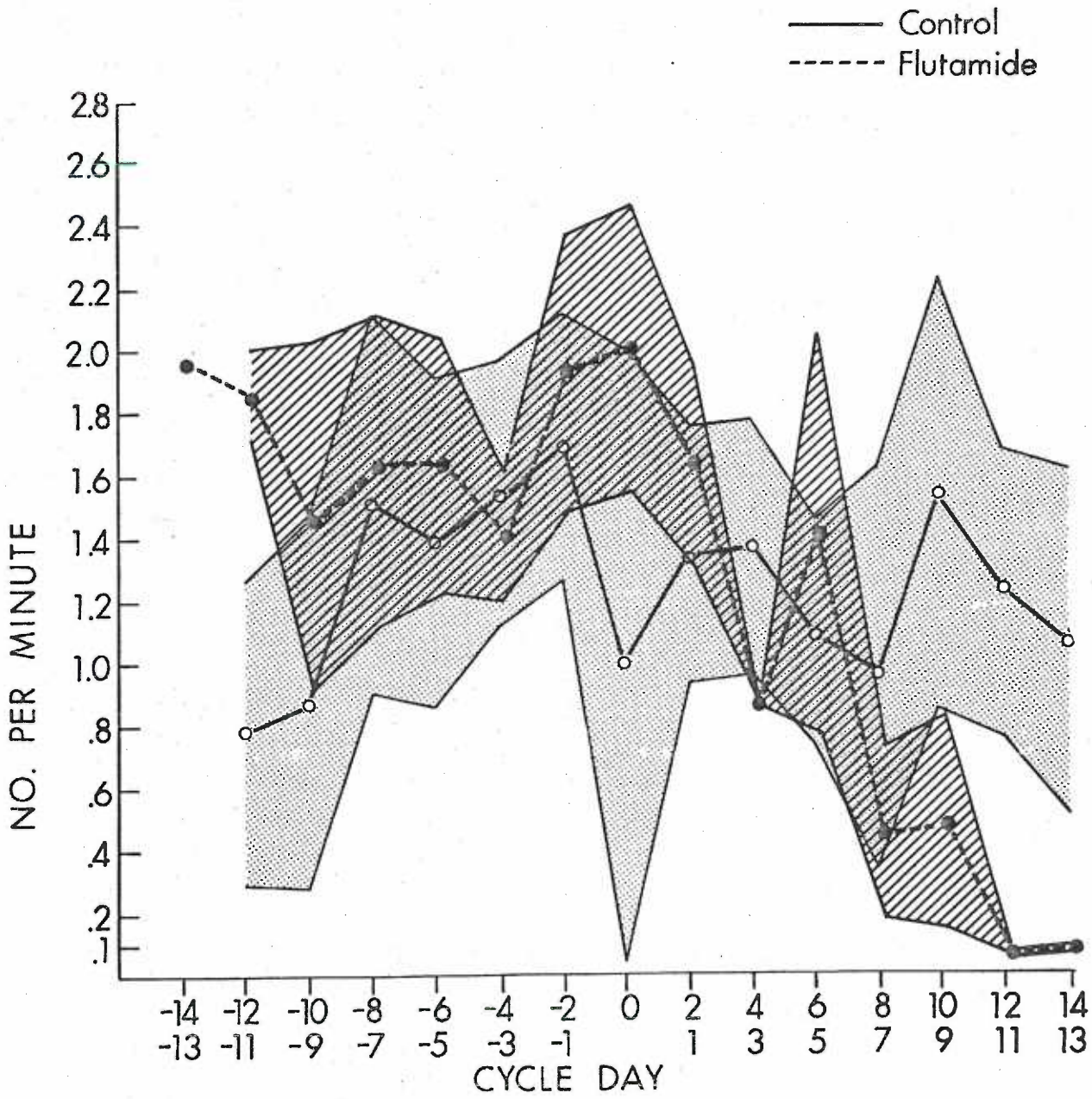

Control
Flutamide
NO. PER MINUTE
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
.1
-14 -12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
-13 -11 -9 -7 -5 -3 -1 1 3 5 7 9 11 13
CYCLE DAY

Figure 7. Male intromission rate in the control and flutamide cycle. Day 0 is the day of estradiol (E_2) peak. Data are plotted as means $\pm$ S.E. (shaded areas).

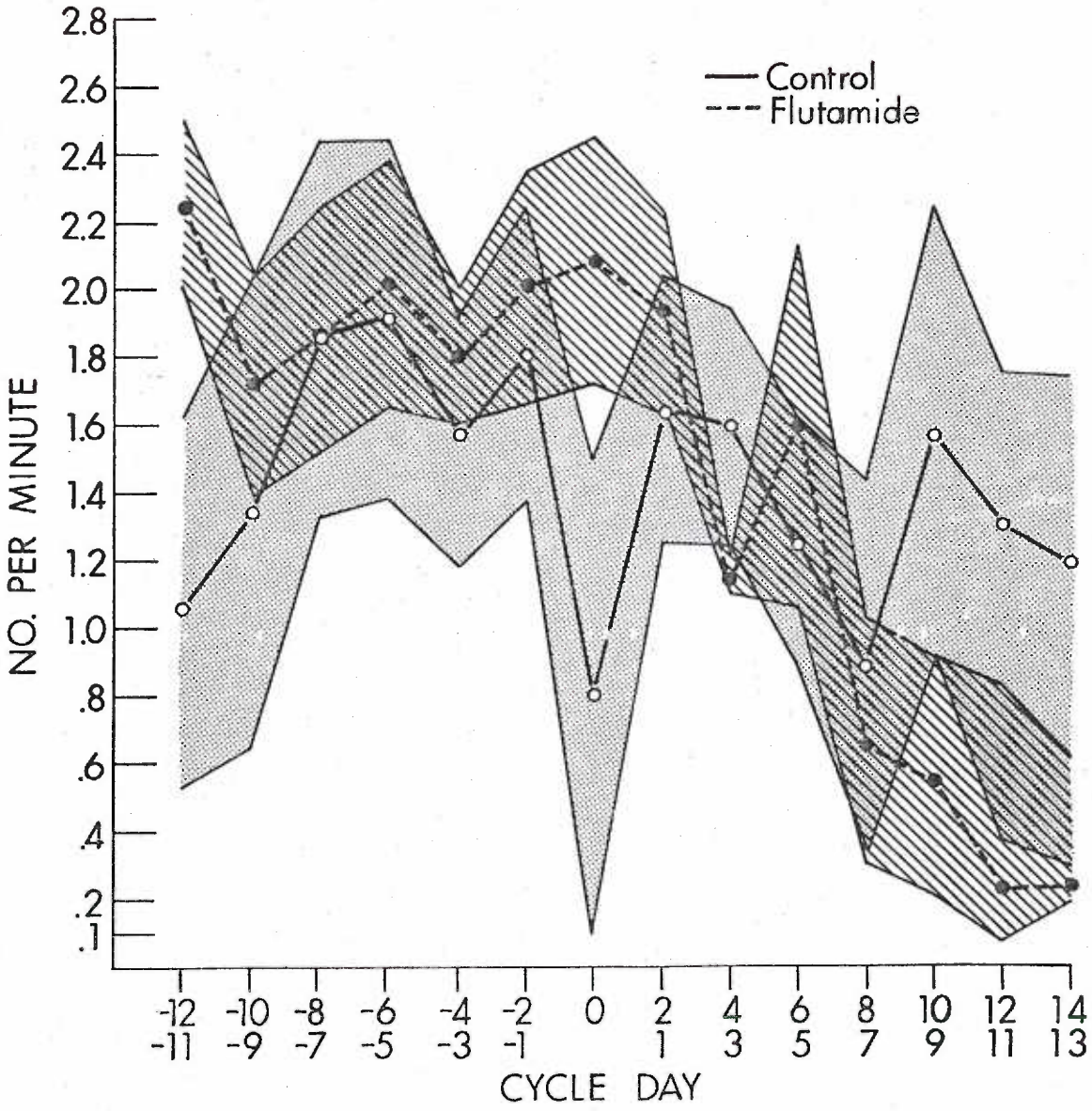

Control
Flutamide
NO. PER MINUTE
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
.1
-12 -11
-10 -9
-8 -7
-6 -5
-4 -3
-2 -1
0
2 1
4 3
6 5
8 7
10 9
12 11
14 13
CYCLE DAY

Figure 8. Number of ejaculations per test in the control and flutamide cycle. Day 0 is the day of estradiol (E_2) peak. Data are plotted as means $\pm$ S.E. (shaded areas).

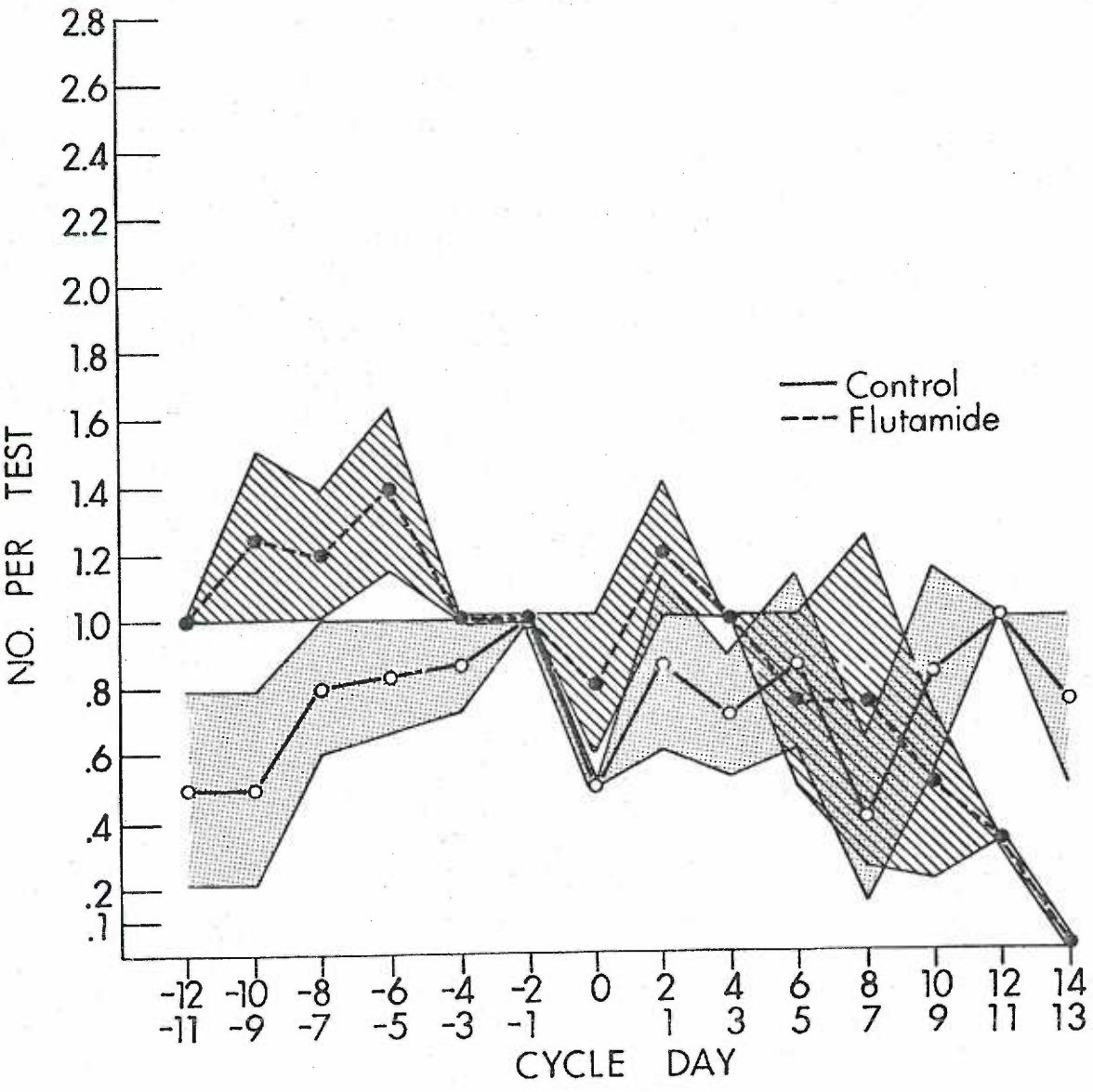

NO. PER TEST
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
.1
Control
Flutamide
-12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
-11 -9 -7 -5 -3 -1 1 3 5 7 9 11 13
CYCLE DAY

Figure 9. Female "press male" rate in the control and flutamide cycle. Day 0 is the day of estradiol (E_2) peak. Data are plotted as means $\pm$ S.E. (shaded areas).

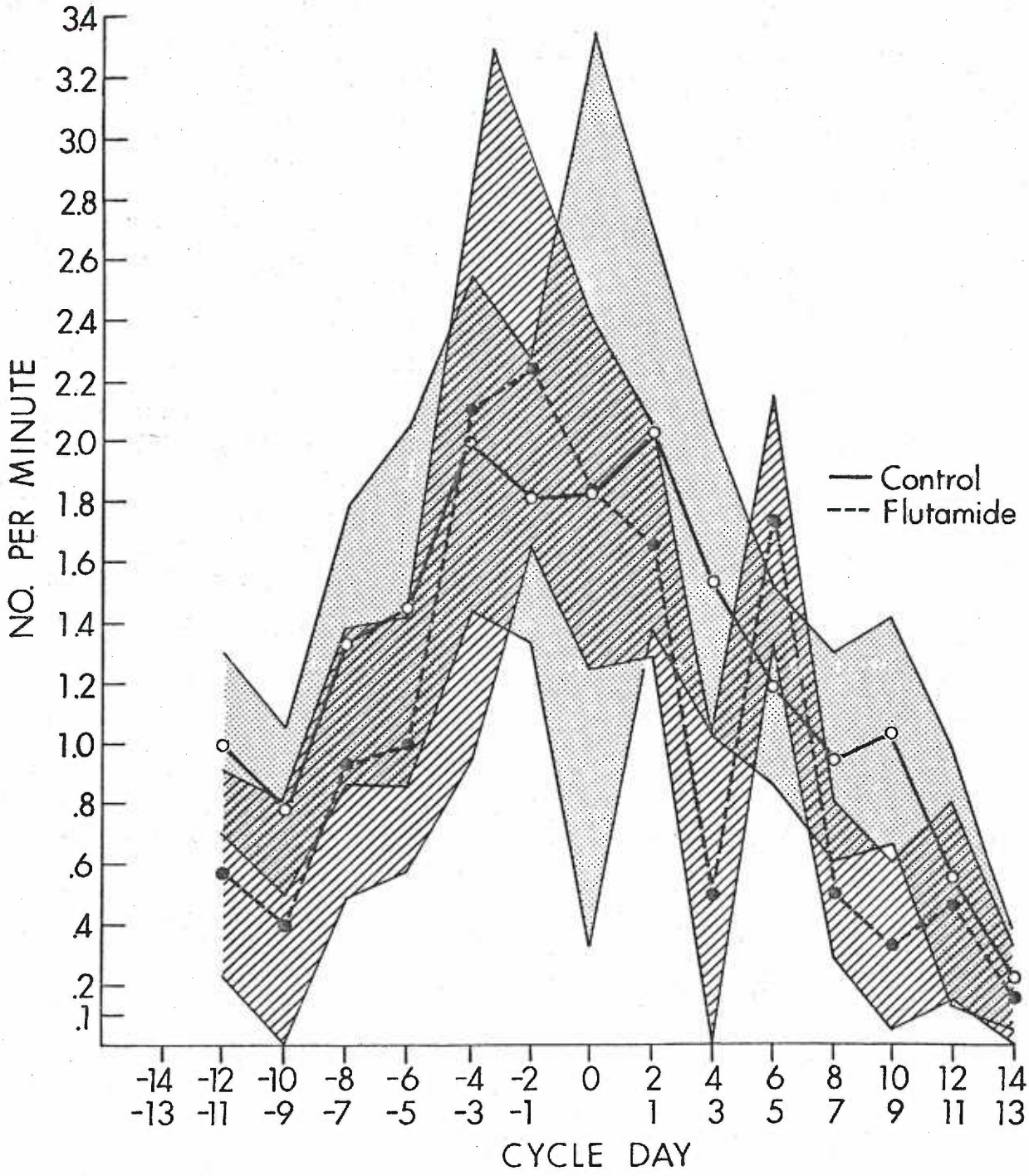

NO. PER MINUTE
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
.1
-14 -12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
-13 -11 -9 -7 -5 -3 -1 1 3 5 7 9 11 13
CYCLE DAY
Control
Flutamide

Figure 10. Female prox rate to a confined male in the control and flutamide cycle. Day 0 is the day of estradiol (E_2) peak. Data are plotted as means $\pm$ S.E. (shaded areas).

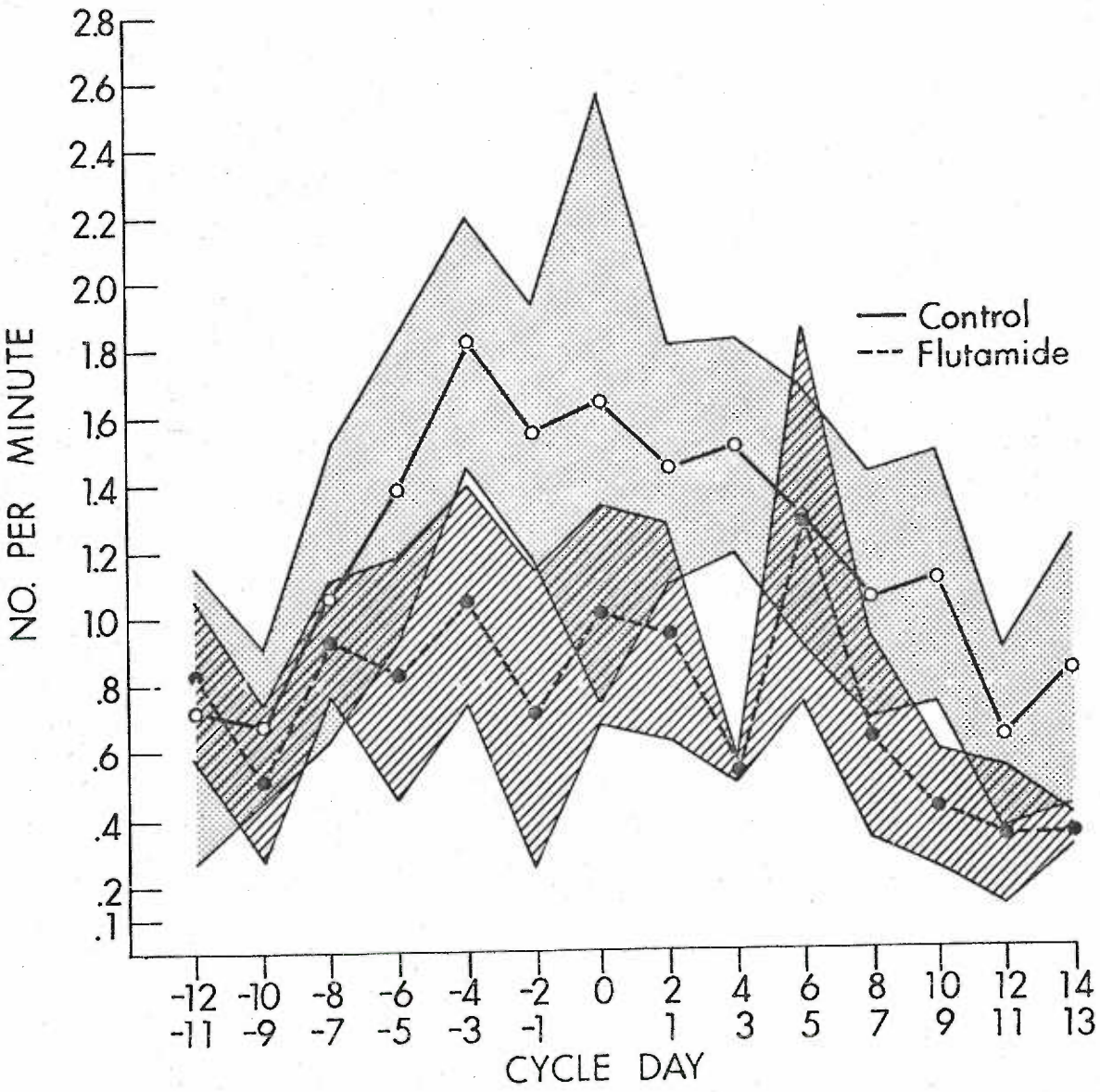

NO. PER MINUTE
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
.1
Control
Flutamide
-12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
-11 -9 -7 -5 -3 -1 1 3 5 7 9 11 13
CYCLE DAY

Figure 11. Number of empty compartments opened per test by females in the control and flutamide cycle. Day 0 is the day of estradiol (E_2) peak. Data are plotted as means $\pm$ S.E. (shaded areas).

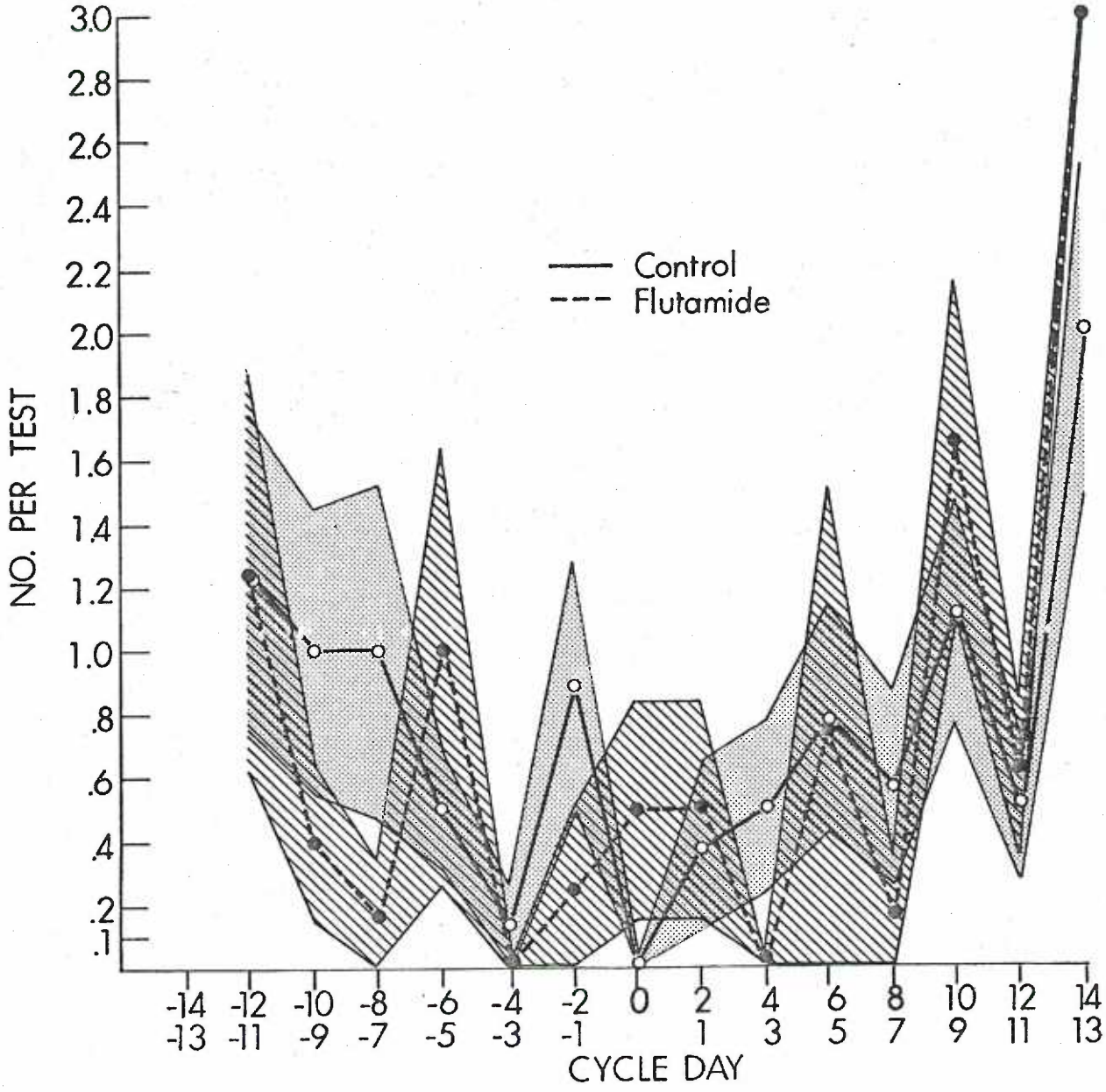

3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
.1
NO. PER TEST
Control
Flutamide
-14 -12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
-13 -11 -9 -7 -5 -3 -1 1 3 5 7 9 11 13
CYCLE DAY

Figure 12. Latency to release the male, in tests in which the male was released, in the control and flutamide cycle. Day 0 is the day of estradiol (E_2) peak. Data are plotted as means $\pm$ S.E. (shaded areas).

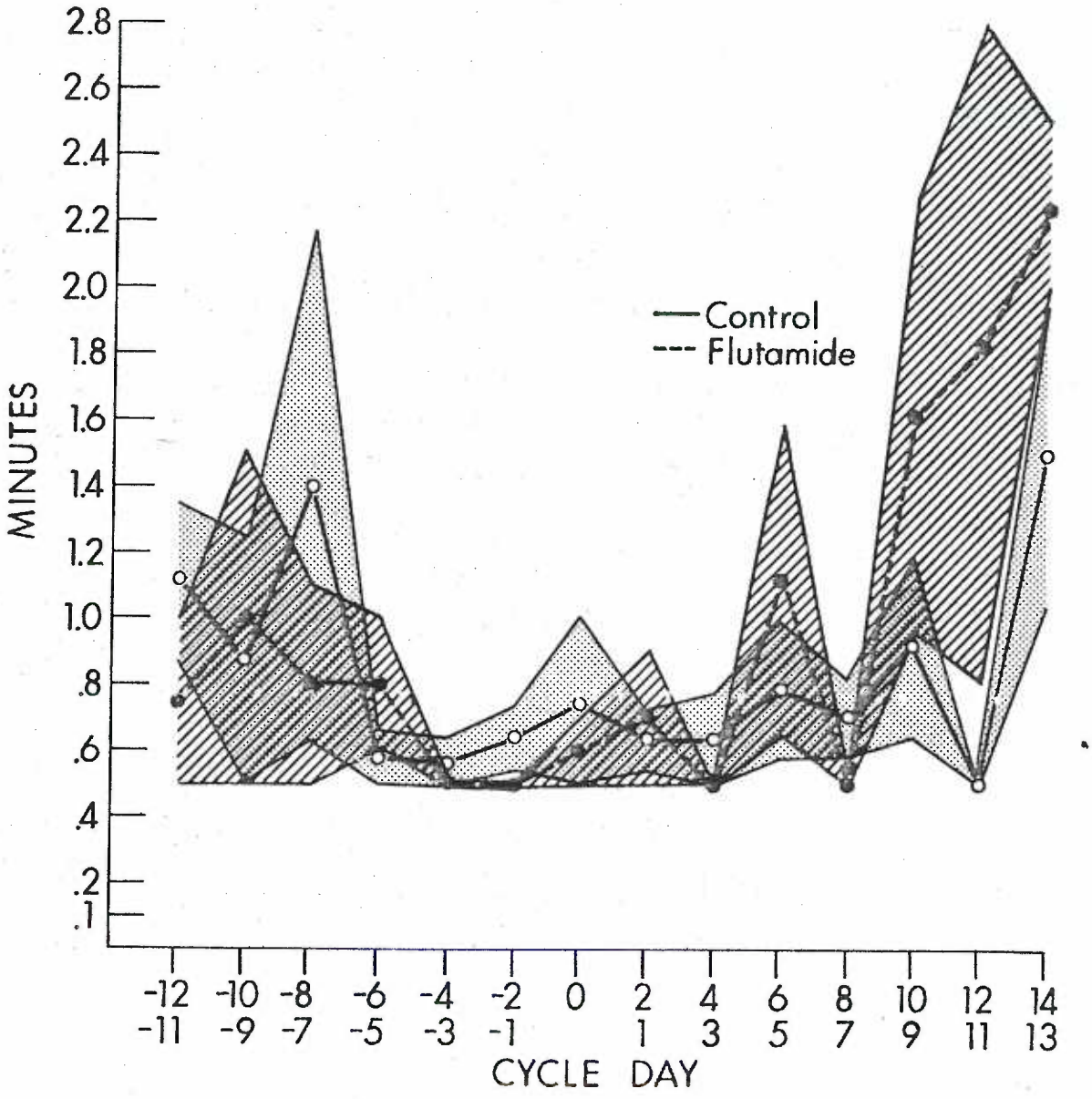
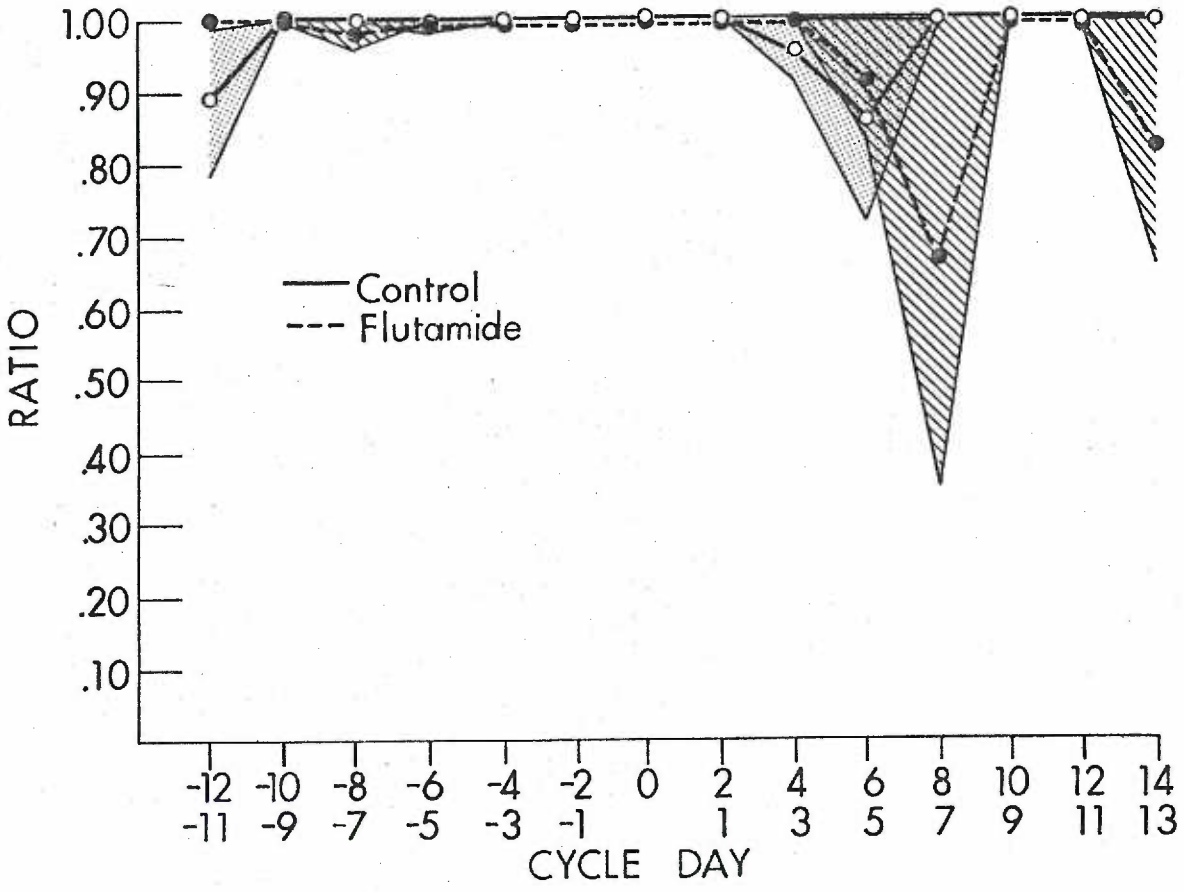

MINUTES
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
.1
Control
Flutamide
-12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
-11 -9 -7 -5 -3 -1 1 3 5 7 9 11 13
CYCLE DAY

Figure 13. Female acceptance ratio in the control and flutamide cycle. Day 0 is the day of estradiol (E_2) peak. Data are plotted as means $\pm$ S.E. (shaded areas).


1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
RATIO
Control
Flutamide
-12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
-11 -9 -7 -5 -3 -1 1 3 5 7 9 11 13
CYCLE DAY

APPENDIX I

Radioimmunoassay

A. General Principals

Radioimmunoassay is one form of displacement or saturation analysis. The original procedures were developed to measure insulin (Yalow and Berson, 1959). In the assay radiolabeled hormone (antigen) molecules (Ag*) compete physiochemically with nonlabeled hormone molecules (Ag), in standard or unknown quantities, for binding sites on antibody molecules (Ab), allowing unknown concentrations of antigen to be determined. As increasing amounts of Ag are added to the assay, the limited binding sites of the antibody are progressively saturated, and the antibody can bind less Ag*. The concentrations of Ab and Ag* are adjusted to allow about 50% of the Ag* to be bound in the absence of unlabeled Ag. A diminished binding of Ag* is evidence for the presence of Ag. After an incubation of Ab, Ag*, and Ag, the antigen-antibody complexes (bound antigen, Ag* and Ag) are separated from the free antigen, and the radioactivity of one or both phases is measured. By comparing the results using unknown amounts of Ag to those using standard, known, amounts of Ag, the unknown Ag (hormone) concentrations can be determined.

The reliability of radioimmunoassays depends upon equal competition between the standard and unknown hormones with the radiolabeled hormone for binding sites on the antibody. The main criteria used to assess reliability are precision, sensitivity, specificity, accuracy, and reproducibility (Midgley, Niswender, and Rebar, 1969). Precision is

defined as the extent to which a set of measurements of the same sample agrees with the mean of that set, i.e., the variation in the estimation of the unlabeled hormone. Precision thus describes the ability of the assay system to distinguish between two hormone concentrations at any point on the standard curve. Sensitivity is a related concept, defined as the smallest quantity of unlabeled hormone which can be distinguished from no hormone. The specificity of an assay is the extent to which the system is free from interference by substances other than the one intended to be measured. There are three types of such interference: a) immunologic cross-reaction of the antibody with other antigenic material, b) heterogeneous antibody, i.e., the absence of a purely specific antibody, and c) differences in the composition of the environment of the assay reaction (e.g., pH, ionic strength, temperature) which may modify the primary antigen-antibody reaction. Accuracy is the extent to which given measurements of a substance agree with the exact amount of the substance present. Reproducibility is defined as the duplicability of measurements within and between assays.

B. Procedure

In each assay, in addition to the serum samples, the following tubes were carried through the procedure in order that corrections could be made for procedural losses and contamination:

1. Four "recovery" tubes each containing 100λ each tritium-labeled E_2, P^4, and T. "Initial" values were obtained by pipetting 100λ of the tritium-labeled solutions directly into 2 scintillation vials each for counting.

2. Four "blanks" each containing 250λ distilled water.

3. Two high- and two low-pool samples containing ovariectomized rhesus monkey plasma with known amounts of steroid added. High pools contained approximately 100 pg E_2 and T, and 1000 pg P^4; low pools contained approximately 20 pg E_2 and T, and 200 pg P^4.

The 250λ serum samples were removed from the freezer and allowed to thaw. An equal volume (250λ) of distilled water was added to each tube and the sample then poured into a 35 ml conical tube which was placed on ice. Each tube which had contained the sample was then filled with 4 ml ice cold ether, poured into the corresponding 35 ml extraction tube, and then was rinsed again with 3 ml of ether, which was added to the extraction tube for a final ether volume of 7 ml. The tubes were stoppered, vortexed 4 min to extract the steroids, centrifuged 5 min at 5.3 g, and the ether fraction removed and placed in clean conical tubes. This fraction was dried under filtered air in a 40°C water bath (this technique was used for drying throughout the procedure) and then concentrated and dried twice with decreasing volumes (2 ml, 1 ml) of ether in the tip of the tube. Samples were stored dissolved in 10 drops of ethanol in the refrigerator.

Chromatographic separation of the hormones to be assayed was carried out on Sephadex LH-20 columns. A column packed with 1.0 gram of Sephadex LH-20 with a solvent system of hexane:benzene:methanol (62:20:13) (column system 1) was used to separate E_2 from the neutral steroids; a column packed with 1.5 grams Sephadex LH-20 with the solvent system hexane:ethyl acetate:methanol (92:2:5) (column system II) was used to separate P^4 and T. The columns were stored under the eluting solvent

solution at all times when not in use. Fresh solvent solutions were made up each day, and the columns were washed with 15 ml fresh solvent each day before use.

The samples were dried and then applied to the columns in system 1 dissolved in 50λ of solvent. The sample tube was rinsed 3 times with an additional 50 ml solvent which was added to the columns. Appropriate volumes of solvent were then added to the columns to allow collection of the effluent fraction containing the desired hormones. The effluent fraction volumes collected for each column system are shown below:

Column System I	Column System II
0-6 ml: P^4 + T	0-6 ml: waste
6-10 ml: waste	6-12.5 ml: P^4
10-15 ml: E_2	12.5-20.5 ml: waste
	20.5-30 ml: T

The first effluent fraction from System I (0-6 ml containing P^4 and T) was collected in the extraction tube, dried, concentrated twice with ether, and applied as the sample for column system II.

The fractions containing the desired hormones were collected in 13 x 100 mm culture tubes, dried, concentrated twice with ether, and stored under 10 drops of ethanol in the refrigerator until assayed. Fractions from recovery samples were collected directly into scintillation vials.

A new standard curve was made up for each radioimmunoassay. Hormone standard solutions were pipetted into 13 x 100 culture tubes in concentra-

tions of 5, 10, 20, 30, 50, 75, 100, and 150 pg for E_2 and T (also 200 pg for T). For P^4, concentrations of 50, 100, 200, 300, 400, 500, 750, 1000, and 1500 pg were used. All standard curves were made up in duplicate, and carried 6 blank tubes (zero steroid).

For the radioimmunoassay, the ethanol in the culture tubes containing the samples and the standard curve was dried. 100λ of the appropriate antibody solution (at room temperature) was added to all tubes, except for 3 of the blanks from the standard curve; the tubes were vortexed for 10 sec and allowed to stand at room temperature for 0.5 to 3.0 hr. After this incubation, 100λ of the appropriate tritium-labeled hormone solution was added to all tubes. The tubes were then vortexed 10 sec, placed on ice and stored in the refrigerator overnight. After this incubation, the tubes still on ice, 100λ of ice cold 0.5% gel/PBS was added to all tubes. 1.0 ml of ice-cold dextran-coated charcoal solution was next added to all tubes except 1 of the remaining blanks. This blank received 1.0 ml ice-cold phosphate buffered saline. The tubes were immediately vortexed, allowed to stand 15 min, and then centrifuged at 4°C for 10 min at 2500 g. After centrifugation the supernatant was poured into scintillation vials containing 10 ml triton solution. Vials were capped, shaken vigorously by hand, and placed in a scintillation counter to determine their radioactivity.

The best fit equation describing the points of the standard curve was computed with a logit transformation (Skelley, Brown, and Besch, 1973) and this was used to convert the counts per minute from the scintillation counter of the samples to picograms of hormone per ml of serum. These values were corrected for procedural losses (percent recovery = mean

"recovery" value/mean "initial" value), and the mean blank value was subtracted to yield the final result.

C. Reagents

1. Phosphate buffered saline (PBS): 0.01 M sodium phosphate monobasic, 0.14 M sodium chloride, and 0.01% sodium azide in distilled water, adjusted to pH 7.0 with HCl and stored at 4°C.

2. Gel solution (gel-PBS): 0.1% or 0.5% Bacto gelatin (Difco Laboratories, Detroit, MI) dissolved in PBS and stored at 4°C.

3. Antisera solutions: antisera developed in rabbits diluted in 0.1% gel-PBS to a working concentration which yielded approximately 50% binding.

4. Tritium-labeled steroid solutions ($^{3}HE_2$, ^{3}HT, and $^{3}HP^{4}$): ^{3}H-estradiol (2, 4, 6, 7-^{3}H) (Amersham Searle), ^{3}H-testosterone (1, 2, 6, 7-^{3}H) (New England Nuclear), and ^{3}H-progesterone (1, 2, 6, 7-^{3}H) (Amersham Searle) made up in 0.1% gel-PBS as follows:

   a. 250 µc/250 µl when received was transferred to a 50 ml amber glass stoppered bottle and dried. 50 ml distilled ice-cold ethanol (ETOH) was added to the bottle. This stock solution contained 5 µc/ml of the label; it was stored at -16°C.

b. A working solution made up in 0.1% gel-PBS contained approximately 4000 cpm for $^{3}HE_{2}$ and ^{3}HT, 6000 cpm for $^{3}HP^{4}$. These were prepared by placing an appropriate amount of stock solution into a 100 ml volumetric flask, drying, adding 0.1% gel-PBS to the 100 ml volume, and storing at 4°C. The working solutions were checked before use the first time since each batch of label received was different.

5. E_2, T, and P^4 standard solutions: 10 mg E_2, 10 mg T, or 10 mg P^4 dissolved in 10 ml ice-cold ETOH; then further diluted with ETOH to yield working concentrations of 1 pg/ml for E_2 and T, 10 pg/ml for P^4; and then stored at 4°C in glass stoppered volumetric flasks.

6. Dextran-coated charcoal: 0.25% charcoal (Norit A, Fisher Scientific Co., Fairlawn, NJ) and 0.025% dextran T-70 (Pharmacia, Uppsala, Sweden) suspended in PBS. This solution was prepared in liter volumes and stored at 4°C. The solution was stirred continuously during use.

7. Ether: freshly distilled 2% ETOH/ether, not over three days old. ETOH was absolute glass distilled, and the ether (anhydrous absolute, Mallinckrodt) was collected over the ETOH, the collecting flask shaken periodically during distillation.

8. Triton solution: a water solubilizer made up of 60 g Omniflor (New England Nuclear), 12 l xylene (Mallinckrodt), and 6 l Triton N-101 (Rohm and Haas, Philadelphia, PA).

REFERENCES

1. Midgley, A.R., Jr., Niswender, G.D., & Rebar, R.W. Principles for the assessment of the reliability of radioimmunoassay methods (precision, accuracy, sensitivity, specificity). Acta Endocrinol., 1969, 63, Suppl. 142, 163-184.

2. Skelley, D.S., Brown, L.P., & Besch, P.K. Radioimmunoassay. Clin. Chem., 1973, 19, 146-186.

3. Yalow, R.S., & Berson, S.A. Assay of plasma insulin in human subjects by immunological methods. Nature, 1959, 184, 1648-1649.

APPENDIX II

Individual Female Hormone Levels

The serum concentrations of estradiol (E_2), testosterone (T), and progesterone (P^4), as measured by radioimmunoassay, during a control and an experimental (flutamide-treatment) menstrual cycle in each of ten females are shown in the following figures, A through J.

Figure A. Cyclic variations in the concentrations of steroids during one control and one experimental (flutamide) menstrual cycle in ♀ 1382. Day 0 is the day of estradiol (E_2) peak, or, if no E_2 peak was detected, the day midway between the start of the menstruation beginning the cycle and the start of the menstruation ending the cycle.

E_2 = estradiol, T = testosterone, P^4 = progesterone

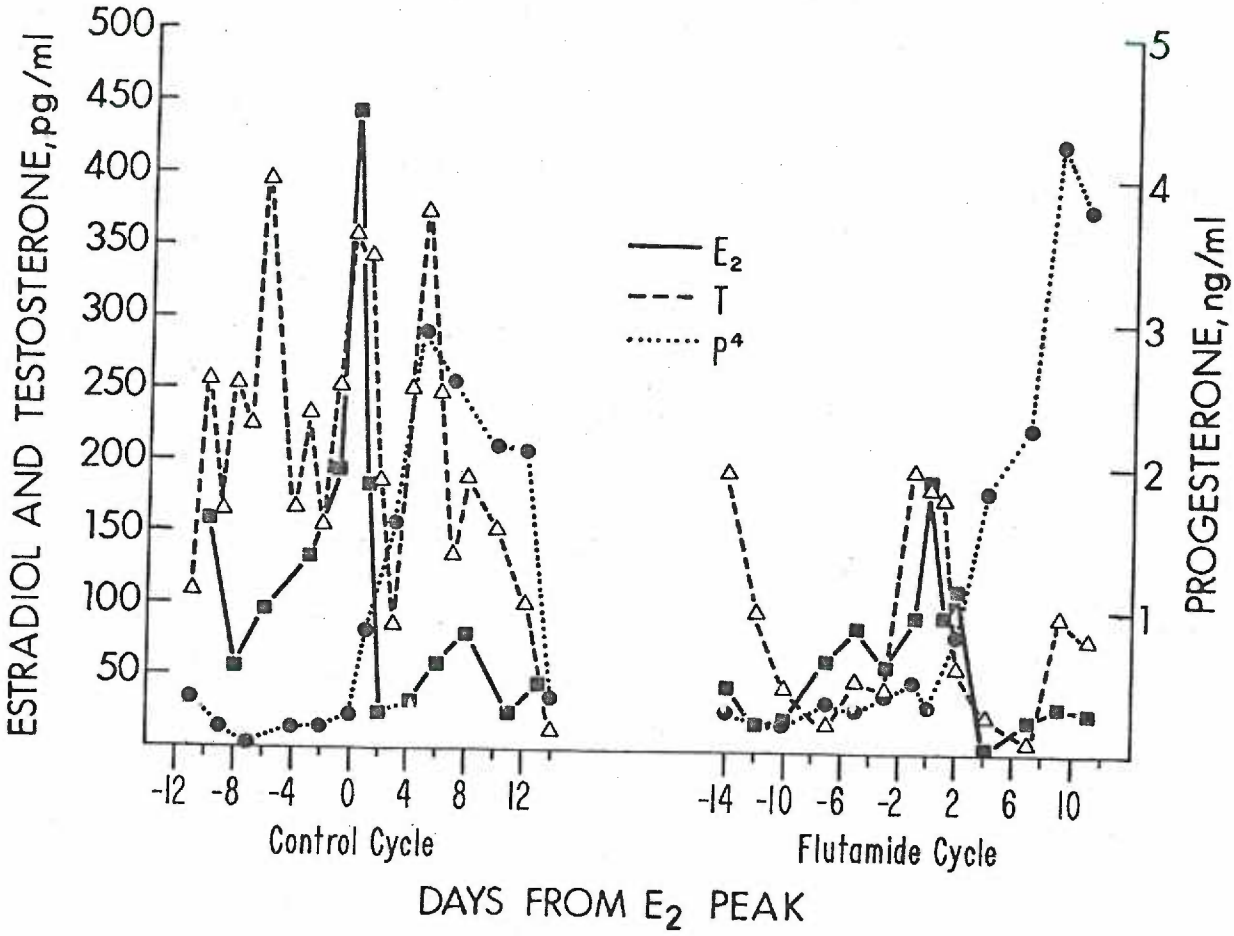

ESTRADIOL AND TESTOSTERONE, pg/ml
500
450
400
350
300
250
200
150
100
50
PROGESTERONE, ng/ml
5
4
3
2
1
E_2
T
P^4
-12 -8 -4 0 4 8 12
Control Cycle
-14 -10 -6 -2 2 6 10
Flutamide Cycle
DAYS FROM E_2 PEAK

Figure B. Cyclic variations in steroid concentrations during one control and one experimental (flutamide) menstrual cycle in ♀ 2740. Abbreviations and labeling of days are as in Figure A.

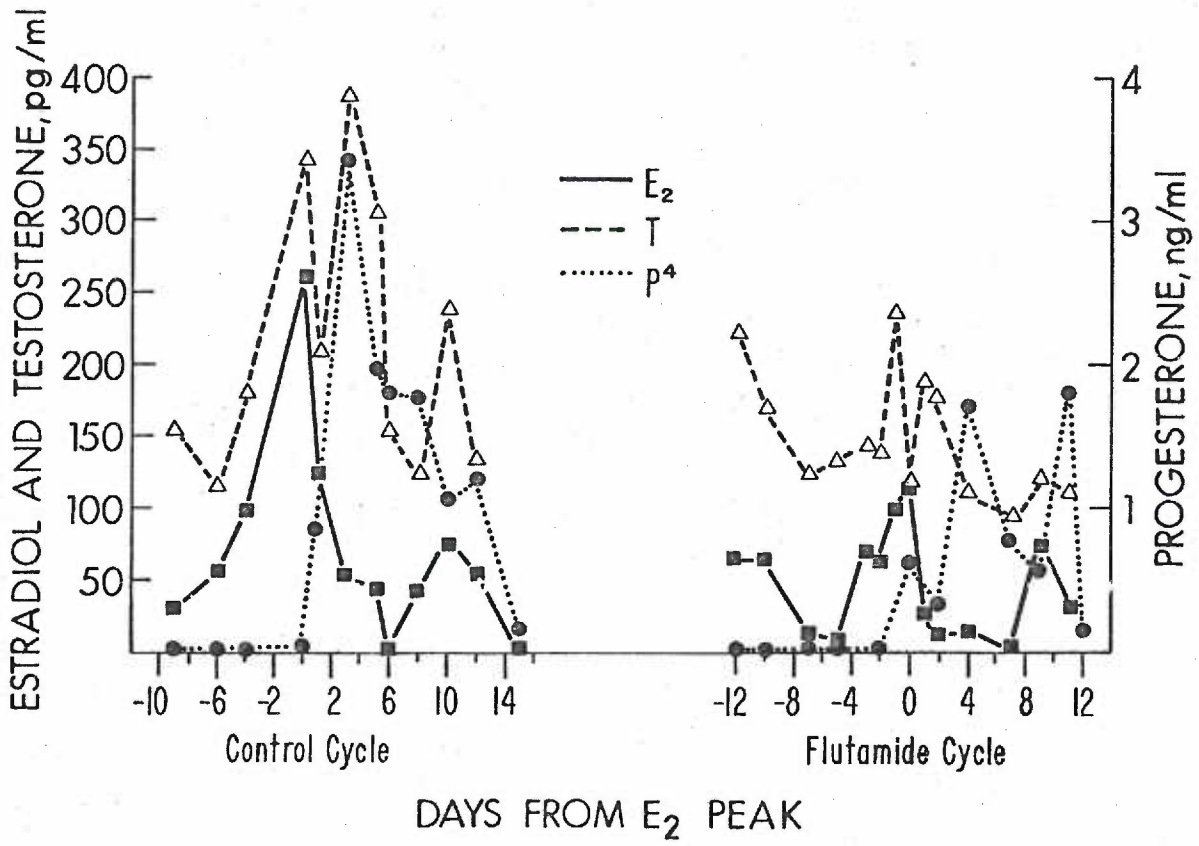

ESTRADIOL AND TESTOSTERONE, pg/ml
400
350
300
250
200
150
100
50
PROGESTERONE, ng/ml
4
3
2
1
E_2
T
P^4
-10 -6 -2 2 6 10 14
Control Cycle
-12 -8 -4 0 4 8 12
Flutamide Cycle
DAYS FROM E_2 PEAK

Figure C. Cyclic variations in steroid concentrations during one control and one experimental (flutamide) menstrual cycle in ♀ 2819. Abbreviations and labeling of days are as in Figure A.

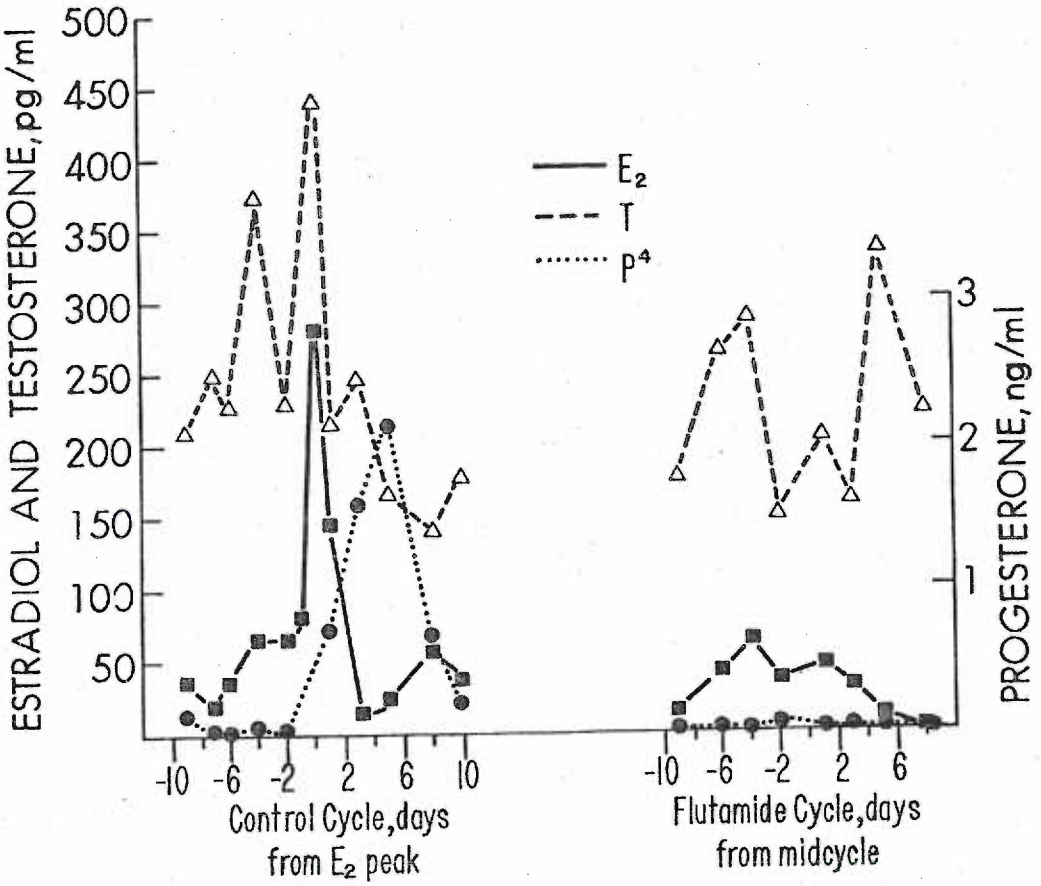

ESTRADIOL AND TESTOSTERONE, pg/ml
500
450
400
350
300
250
200
150
100
50
PROGESTERONE, ng/ml
3
2
1
E2
T
P4
-10 -6 -2 2 6 10
Control Cycle, days
from E2 peak
-10 -6 -2 2 6
Flutamide Cycle, days
from midcycle

Figure D. Cyclic variations in steroid concentrations during one control and one experimental (flutamide) menstrual cycle in ♀ 5680. Abbreviations and labeling of days are as in Figure A.

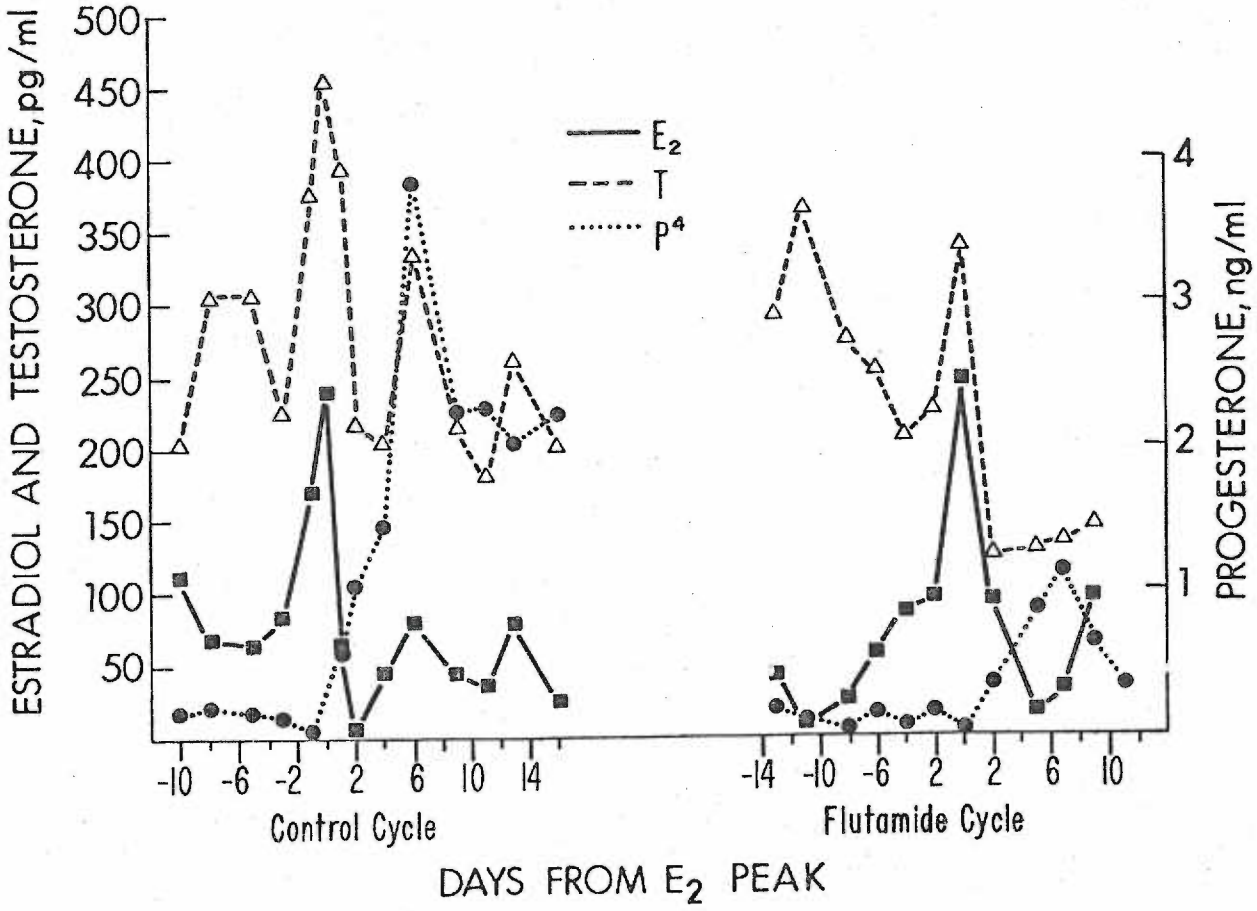

ESTRADIOL AND TESTOSTERONE, pg/ml
500
450
400
350
300
250
200
150
100
50
E_2
T
P^4
4
3
2
1
PROGESTERONE, ng/ml
-10 -6 -2 2 6 10 14
Control Cycle
-14 -10 -6 2 2 6 10
Flutamide Cycle
DAYS FROM E_2 PEAK

Figure E. Cyclic variations in steroid concentrations during one control and one experimental (flutamide) menstrual cycle in ♀ 5746. Abbreviations and labeling of days are as in Figure A.

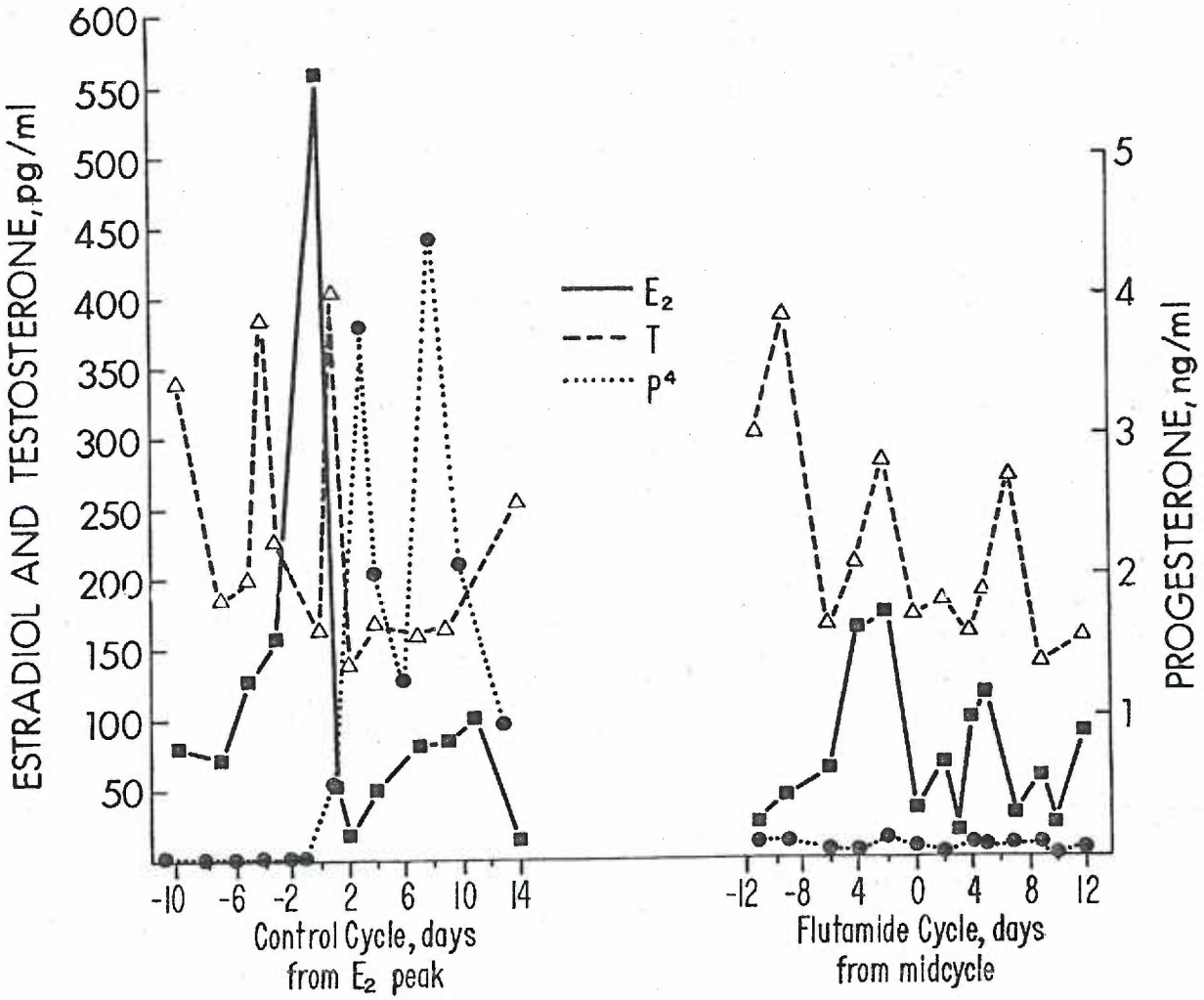

ESTRADIOL AND TESTOSTERONE, pg/ml
600
550
500
450
400
350
300
250
200
150
100
50
PROGESTERONE, ng/ml
5
4
3
2
1
E_2
T
P^4
-10 -6 -2 2 6 10 14
Control Cycle, days
from E_2 peak
-12 -8 4 0 4 8 12
Flutamide Cycle, days
from midcycle

Figure F. Cyclic variations in steroid concentrations during one control and one experimental (flutamide) menstrual cycle in ♀ 6253. Abbreviations and labeling of days are as in Figure A.

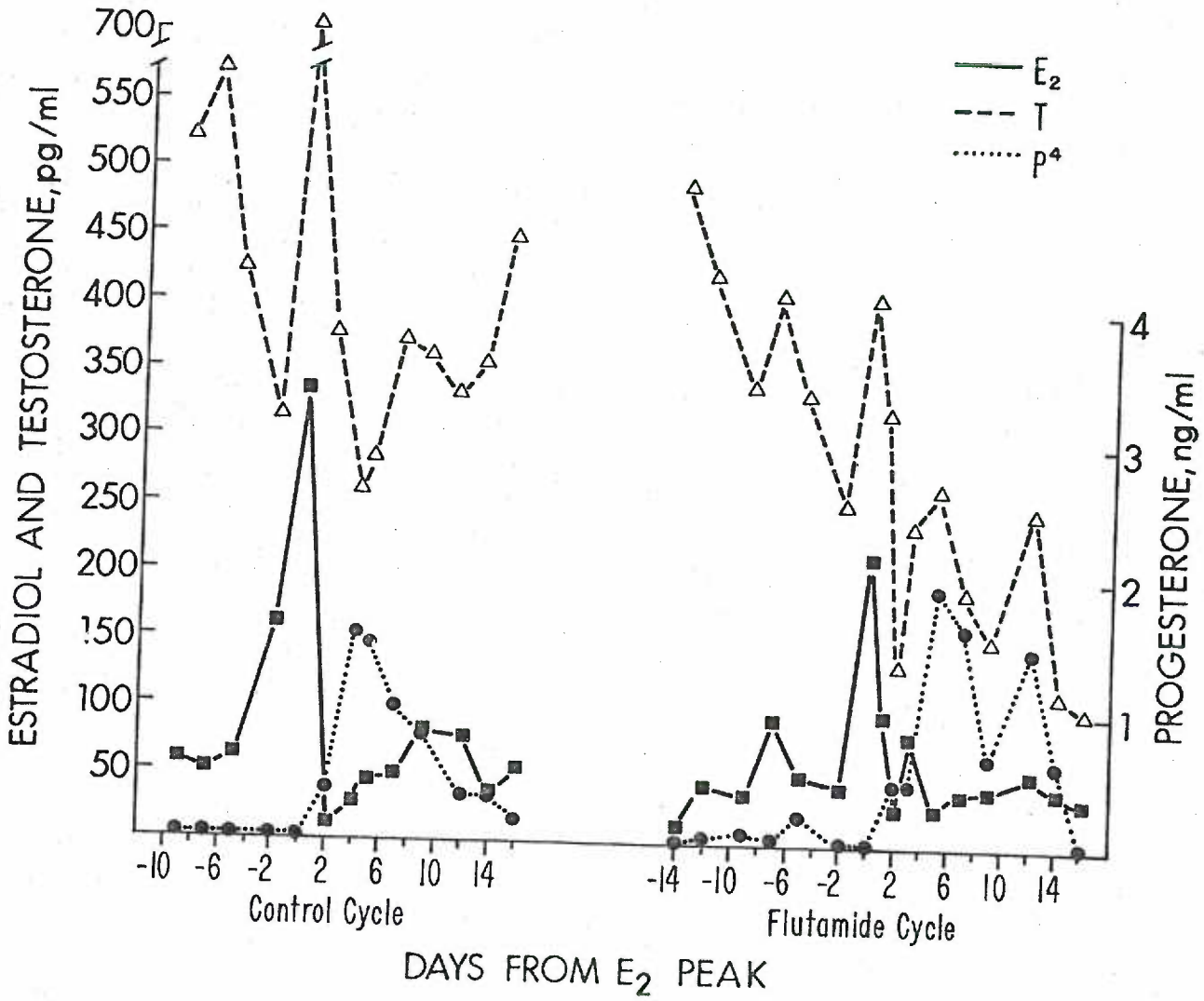

ESTRADIOL AND TESTOSTERONE, pg/ml
700
550
500
450
400
350
300
250
200
150
100
50
PROGESTERONE, ng/ml
4
3
2
1
E_2
T
P^4
-10 -6 -2 2 6 10 14
Control Cycle
-14 -10 -6 -2 2 6 10 14
Flutamide Cycle
DAYS FROM E_2 PEAK

Figure G. Cyclic variations in steroid concentrations during one control and one experimental (flutamide) menstrual cycle in ♀ 6746. Abbreviations and labeling of days are as in Figure A.

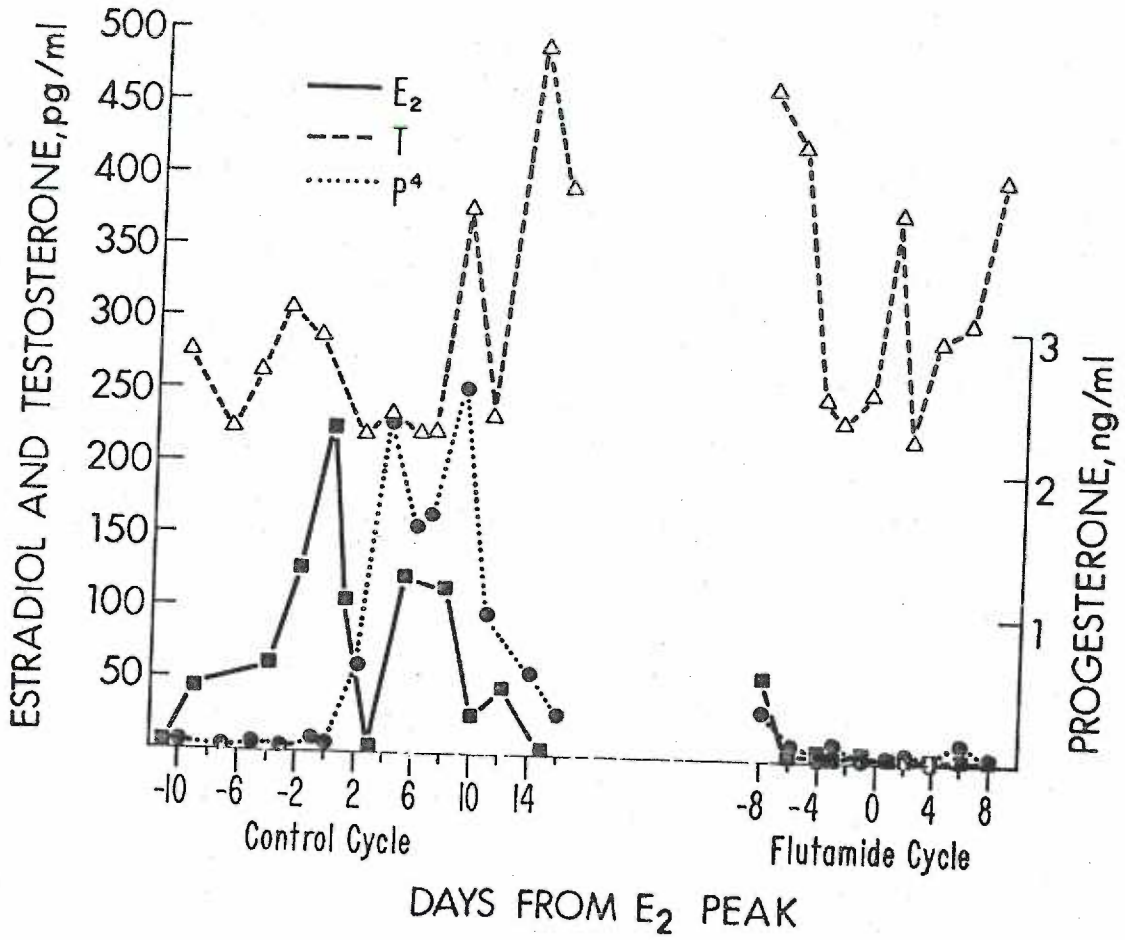

ESTRADIOL AND TESTOSTERONE, pg/ml
500
450
400
350
300
250
200
150
100
50
E_2
T
P^4
PROGESTERONE, ng/ml
3
2
1
-10 -6 -2 2 6 10 14
Control Cycle
-8 -4 0 4 8
Flutamide Cycle
DAYS FROM E_2 PEAK

Figure H. Cyclic variations in steroid concentrations during one control and one experimental (flutamide) menstrual cycle in ♀ 6755. Abbreviations and labeling of days are as in Figure A.

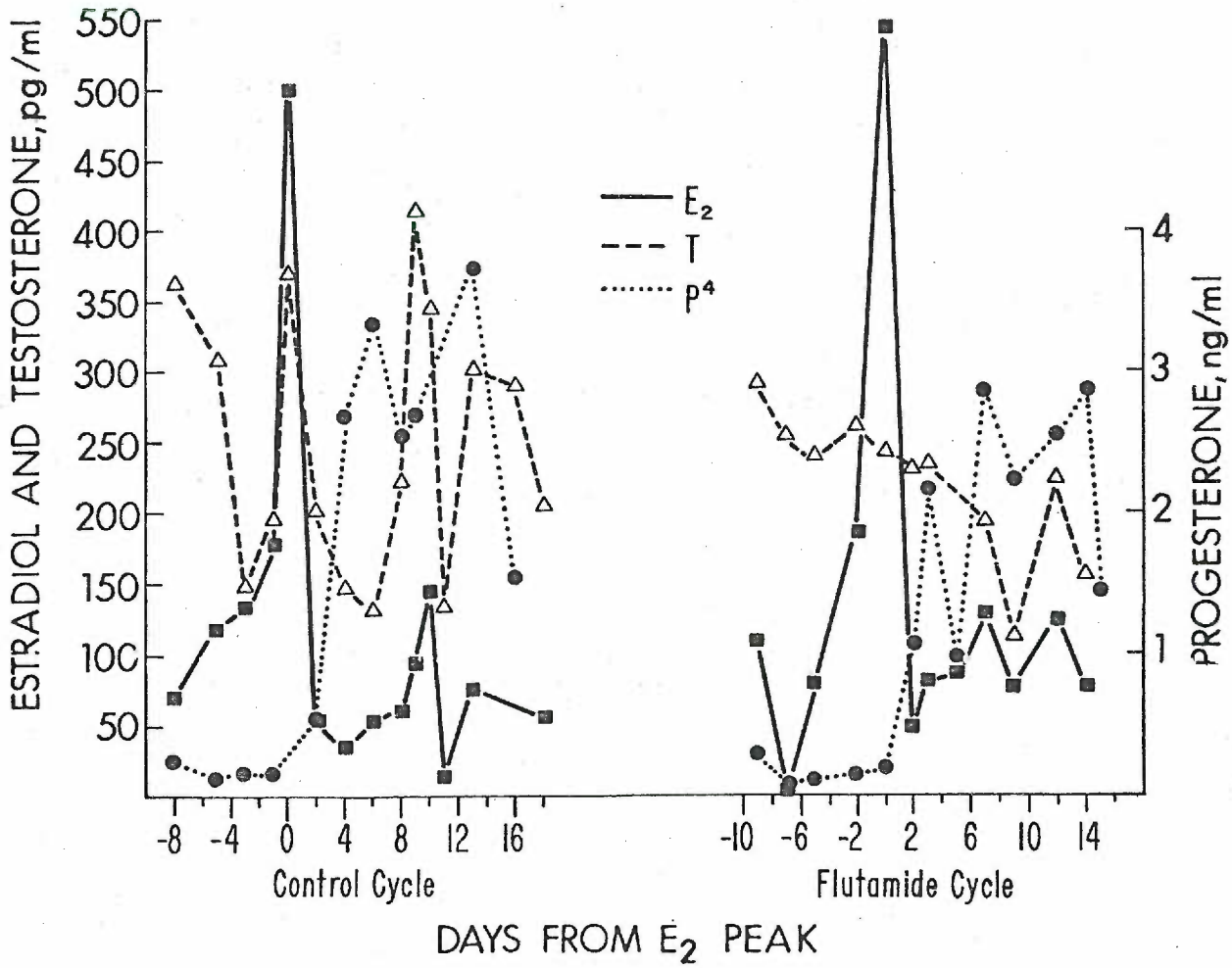

ESTRADIOL AND TESTOSTERONE, pg/ml
550
500
450
400
350
300
250
200
150
100
50
PROGESTERONE, ng/ml
4
3
2
1
E_2
T
P^4
-8 -4 0 4 8 12 16
Control Cycle
-10 -6 -2 2 6 10 14
Flutamide Cycle
DAYS FROM E_2 PEAK

Figure I. Cyclic variations in steroid concentrations during one control and one experimental (flutamide) menstrual cycle in ♀ 6938. Abbreviations and labeling of days are as in Figure A.

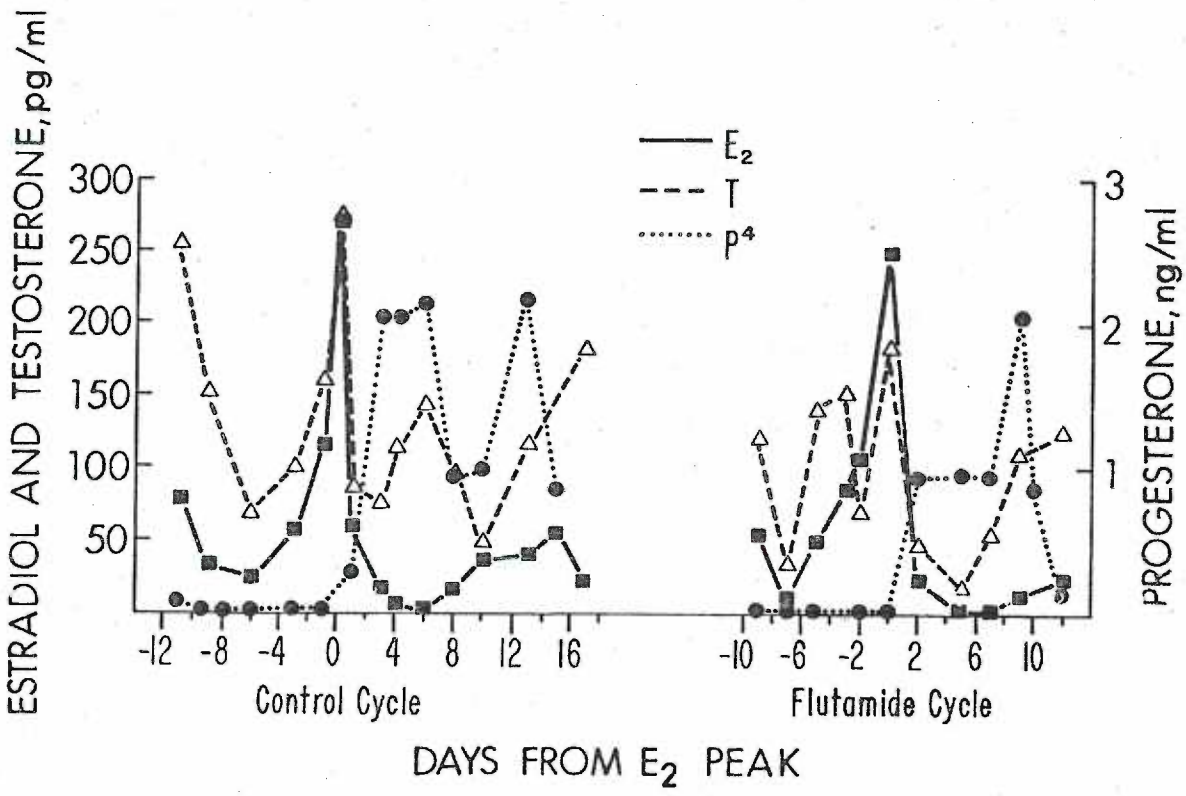
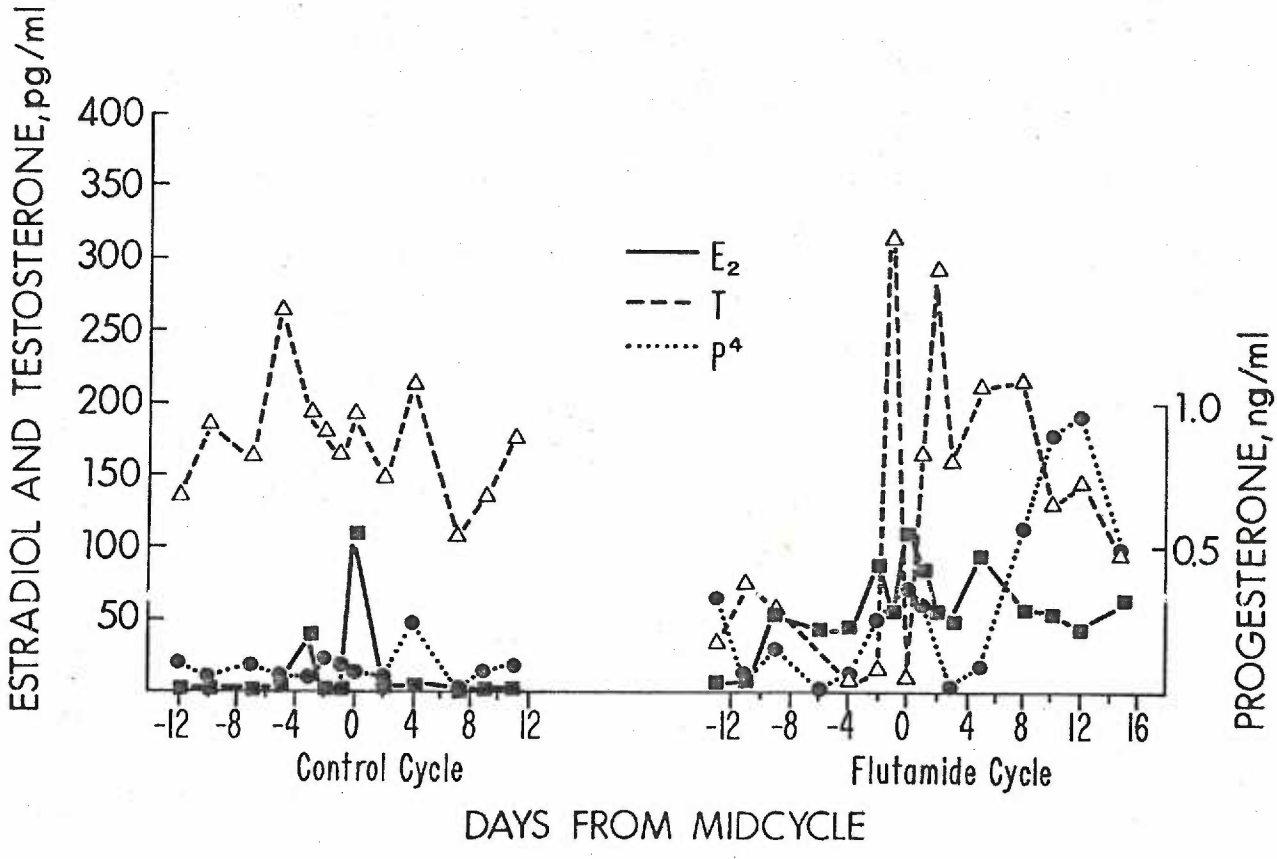

ESTRADIOL AND TESTOSTERONE, pg/ml
PROGESTERONE, ng/ml
E_2
T
P^4
300
250
200
150
100
50
3
2
1
-12 -8 -4 0 4 8 12 16
-10 -6 -2 2 6 10
Control Cycle
Flutamide Cycle
DAYS FROM E_2 PEAK

Figure J. Cyclic variations in steroid concentrations during one control and one experimental (flutamide) menstrual cycle in ♀ 7169. Abbreviations and labeling of days are as in Figure A.


ESTRADIOL AND TESTOSTERONE, pg/ml
400
350
300
250
200
150
100
50
E_2
T
P^4
1.0
0.5
PROGESTERONE, ng/ml
-12 -8 -4 0 4 8 12
Control Cycle
-12 -8 -4 0 4 8 12 16
Flutamide Cycle
DAYS FROM MIDCYCLE