

## Acute Exposure to Molinate Alters Neuroendocrine Control of Ovulation in the Rat

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Received October 5, 2004; accepted December 10, 2004

Molinate, a thiocarbamate herbicide, has been reported to impair reproductive capability in the male rat and alter pregnancy outcome in a two-generation study. Published data are lacking on the effects of acute exposure to molinate in the female. Based on this work and our previous observations with related dithiocarbamate compounds, we hypothesized that a single exposure to molinate during the critical window for the neural trigger of ovulation on the day of proestrus (PRO) would block the luteinizing hormone (LH) surge and delay ovulation. To examine the effect of molinate on the LH surge, ovariectomized (OVX) rats were implanted with Silastic capsules containing estradiol benzoate to mimic physiological levels on proestrus. Doses of 25 and 50 mg/kg molinate significantly suppressed LH and prolactin secretion. Intact regularly cycling females gavaged with 0, 25, or 50 mg/kg molinate at 1300 h on PRO were examined on estrus or estrus + 1 day for the presence of oocytes in the oviduct. All control females had oocytes in the oviduct on estrus. Molinate doses of 6.25 to 50 mg/kg delayed ovulation for 24 h. Estrous cyclicity was examined after daily exposure to 50 mg/kg (21 days). Estrous cyclicity was irregular in the molinate group, showing extended days in estrus. Two experiments were conducted to determine whether molinate blocked the LH surge via a central nervous system (CNS) mode of action or via an alteration in pituitary response. In the first experiment, we evaluated the release of LH in control and molinate-treated rats after a bolus dose of exogenous GnRH. Luteinizing hormone release was comparable in the two groups, suggesting that the effect of molinate is centrally mediated. To further examine the potential role of the CNS, we examined the pulsatile release of LH present in the long-term OVX females. In this model, the pulsatile pattern of LH secretion is directly correlated with GnRH release from the hypothalamus. A significant decrease in the LH pulse frequency was observed in molinate-treated females. These results indicate that molinate is able to delay ovulation by suppressing the LH surge on the day of proestrus and that the brain is the primary target site for the effects on pituitary hormone secretion.

**Key Words:** molinate; luteinizing hormone surge; gonadotropin releasing hormone; ovulation; prolactin; estrous cyclicity.

### INTRODUCTION

Molinate (S-ethyl hexahydro-1H-azepine-1-carbothioate) is a selective, preemergence thiocarbamate herbicide used for weed control in rice fields. In 1997, 1.3 million pounds of molinate were applied by aerial spraying over the 6-week spring period in California. The principal type of exposure would be short term and seasonal. Since the 1970s, a number of rodent studies with molinate have led to concern for human reproductive health. In male rodents, molinate exposure led to abnormal sperm morphology, testicular degeneration, and decreased fertility (Ellis *et al.*, 1998). These effects were attributed to the effect of the molinate sulfoxide metabolite, which was shown to induce inhibition of the enzyme neutral cholesterol ester hydrolase (nCEH). This inhibition results in interference of cholesterol mobilization and subsequently decreased steroidogenesis within the Leydig cells. In accordance with this mechanism of action, molinate caused a marked decrease in circulating and testicular testosterone (Ellis *et al.*, 1998). Molinate sulfoxide is a primary metabolite in rodents, whereas it is a minor metabolite in humans (Wilkes *et al.*, 1993). From these studies, the short-term oral NOEL was set at 11.5 mg/kg-day for reduced fertility in rats (Cochran *et al.*, 1997).

Molinate has also been found to be a reproductive toxicant in the female rat. Although there have been relatively few studies that have examined the toxic effects of short-term or acute exposures to molinate in the female, a two-generation reproduction study (Gilles and Richter, 1989) reported ovarian interstitial tissue vacuolization and cystic follicles in the ovaries of SD rats. The NOEL set for this effect was 1.9 mg/kg-day (Cochran *et al.*, 1997). These researchers also noted significant reductions in the number of implantation sites and a decreased litter size in the F1s at 33.3 mg/kg-day, which did not appear to correlate with the ovarian histopathology.

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The pregnancy failures in the two-generation study are similar to toxicant-induced changes in pregnancy outcome that we have observed in our laboratory after acute pre-conceptional (day of vaginal proestrus) exposure to a variety of compounds such as chlordimeform and dithiocarbamates (Cooper *et al.*, 1994; Stoker *et al.*, 1996). In these studies, there was a reduction in litter size, an increase in the number of resorptions, and intrauterine growth retardation after delayed ovulation. Also, the vacuolization and cystic follicles after extended dosing with molinate (Gilles and Richter, 1989) may indicate that these females were in persistent estrus (Everett, 1989), which can be induced by compounds that interfere with the neuroendocrine control of ovulation (Walker *et al.*, 1980). Thus, the effects of molinate exposure on pregnancy outcome led us to hypothesize that molinate may suppress the LH surge and delay ovulation, thereby altering pregnancy outcome.

In our previous work, we have examined the effect of environmental toxicants on the neuroendocrine regulation of the ovulatory surge of LH (Cooper *et al.*, 1999; Stoker *et al.*, 2001, for review). For example, the formamidine pesticides chlordimeform and amitraz, which block norepinephrine (NE) binding to the  $\alpha_2$  receptor (Costa *et al.*, 1988), have been shown to inhibit the ovulatory surge of LH if administered during the critical period of vaginal proestrus (Goldman *et al.*, 1991). Similarly, agents that impair NE synthesis, such as the dithiocarbamates (*e.g.*, metham sodium and thiram [Goldman, 1994; Stoker *et al.*, 1993; Stoker *et al.*, 2003]) or chlorotriazines (*e.g.*, atrazine [Cooper *et al.*, 1996, 2000]) will also suppress the ovulatory surge of LH.

The objectives of the present study were (1) to examine the effect of an acute exposure to molinate on the generation of the LH surge and ovulation in the rat; (2) to examine whether a longer exposure to molinate alters estrous cyclicity; and (3) to investigate the possibility that molinate has a mechanism of action that involves a disruption of the GnRH hypothalamic-pituitary regulation of LH. To do this, we examined the ability of the pituitary to respond to exogenous GnRH after molinate exposure. First, we characterized the effect of molinate on central nervous system (CNS) control of pituitary hormone release. We then examined GnRH-stimulated LH secretion in the ovariectomized female (to preclude steroidal feedback). Next, we measured hypothalamic dopamine and norepinephrine concentrations, as these catecholamines are known to be involved in the regulation of GnRH. Finally, because carbamates are also known to inhibit acetylcholinesterase and such an inhibition may alter GnRH, we examined cholinesterase activity in the brains and serum of the molinate-treated rats.

## METHODS

**Animals.** All animal procedures were approved in advance by the NHEERL Institutional Animal Care and Use Committee. Ninety-day-old female Long-Evans hooded rats were obtained from Charles River Laboratories (Raleigh, NC) and housed in pairs in an AAALAC accredited facility at 22°C on a 14:10 h light-dark cycle (lights on 0600 h). All animals were provided with food and

water *ad libitum*. After a 3-week period of estrous cycle monitoring, only those females exhibiting at least 5 consecutive 4-day estrous cycles were selected for use.

**Dosing.** Molinate (S-ethyl hexahydro-1H-azepine-1-carbothioate) was purchased from ChemService (West Chester, PA, PS-501) at a purity of 99.2%. The doses employed in these studies were based on previous findings in reproductive studies. Doses were prepared in corn oil to dose by gavage in a volume of 0.2 ml per 100 g BW. The doses used for each experimental group are described below. Note: None of the females showed clinical signs of general toxicity or neurotoxicity in any of the experiments.

**Ovariectomy and estrogen implants.** In those studies evaluating the effect of molinate on estrogen-induced LH and prolactin surges, regularly cycling females were anesthetized with a mixture of ketamine (87 mg/kg *i.p.*) and xylazine (13 mg/kg *i.p.*) and bilaterally ovariectomized on day 0. At the time of ovariectomy, an 8-mm Silastic capsule (Scientific Products medical grade silicone tubing 1.57 mm ID  $\times$  3.18 mm OD) containing estradiol benzoate (4 mg/ml in sesame oil) was implanted subcutaneously in the right flank region. All surgeries were performed between 1200 and 1300 h. Previous work in our laboratory has demonstrated that these capsule dimensions and estradiol concentration maintained blood estradiol levels (80–120 pg/ml) comparable to those seen in the intact proestrous animal (Goldman and Cooper, 1993). After 3 days, the implant produces a daily, late-afternoon surge of LH and prolactin (Cooper *et al.*, 1980) and thus provides a means of obtaining a synchronized cohort of animals in which the timing and amplitude of each hormone can be evaluated under different doses of molinate. Also, with this model, any potential confounding effects of molinate on ovarian hormone secretion or concentration are eliminated. In these experiments, the LH surge was characterized on day 3 (72 h) after the capsule was implanted.

### *Experiment 1: Time and Dose-Response Effects of Molinate on LH and Prolactin Secretion*

**Experiment 1a: Characterization of the LH surge in individual females.** This experiment was performed to examine the LH surge in individual females after an acute dose of molinate on day 3 (72 h) after an estradiol capsule was implanted. Females were dosed with corn oil (controls) or molinate at doses of 25 and 50 mg/kg by gavage at 1300 h rat time ( $n = 6$  per group). Serial tail bleeds (300  $\mu$ l of blood/ time point) were conducted 0, 2, 4, and 6 h after the acute exposure to molinate (1300, 1500, 1700 and 1900 h). Blood was collected in Microtainer tubes (Becton Dickinson, Franklin Lakes, NJ), and after centrifugation (1260  $\times$  g at 4°C for 30 min), serum was stored at -80°C until assayed for LH. This allowed us to examine the LH surges of individual females exposed to molinate.

**Experiment 1b: Characterization of serum and pituitary hormones.** To evaluate the immediate effects of molinate on LH and prolactin (PRL), and to collect pituitaries and brains for subsequent analyses, another group of females was ovariectomized and received an estradiol implant on day 0 as described in Experiment 1. On day 3, groups of females ( $n = 8$ ) were gavaged one time with 0, 25, or 50 mg/kg of molinate at 1300 h. This dosing regimen was selected based on our previous work, which determined the most effective time and dose of a dithiocarbamate to interfere with the neural trigger of the LH surge and result in a 24 h delay of ovulation (Stoker *et al.*, 1993). In this experiment, eight females receiving each dose of vehicle or molinate were killed by decapitation at 0 (1300 h), 1 (1400 h), 3 (1600 h), or 6 (1900 h) h after the dose (*e.g.*, 0, 1, 3, or 6 h post-gavage). Trunk blood was collected in 16  $\times$  125-mm serum-separation tubes (Becton Dickinson, Rutherford, NJ) and centrifuged (1260  $\times$  g at 4°C for 30 min.). The serum was subsequently stored at -80°C until assayed for LH and prolactin. The pituitaries were removed and the neural lobe was dissected free and discarded. The remaining anterior lobe was weighed, placed in 1 ml of HEPES-buffered (10 mM, pH 7.4) medium 199 containing 0.3% BSA (Sigma, fraction V), sonicated (Fisher Sonic Dismembrator, Model 300), and frozen immediately at -80°C until assayed for pituitary LH and PRL. The brains were removed and frozen at -80°C, until further measurement of catecholamine content and acetylcholinesterase activity.

### Experiment 2: Effect of Molinate on Ovulation

Because molinate was shown to suppress the LH surge in the OVX/E2 females at doses of 25 and 50 mg/kg after a single dose at 1300 h, the effects of molinate on ovulation were examined in intact regularly cycling Long-Evans Hooded rat (LE) females. These females were gavaged with control vehicle (corn oil), 1.56, 3.125, 6.25, 12.5, 25, or 50 mg/kg molinate in a volume of 0.2 ml per 100 g BW at 1300 h on the day of vaginal proestrus.

To assess ovulation in the females dosed on proestrus, mature oocytes were collected and quantified according to the procedure described by Perreault and Mattson (1993) approximately 16 or 40 h after the expected LH surge (1700 h on proestrus) ( $n = 8$  for each dose at each time point for collection). At 0900 h after vaginal cytology, the females were euthanized by an overdose of ketamine/xylazine with confirmed heart palpitation. Briefly, the oviducts were dissected and flushed with phosphate buffered saline (PBS, Gibco, Gaithersburg, MD) containing 0.1% bovine serum albumin (BSA; fraction V, Sigma Chemical Co.). The presence or absence of a cumulus mass was noted, and the dish was examined for the presence of cumulus-free oocytes. The cumulus mass, when present, was transferred to 0.3% hyaluronidase. Once the cumulus mass dispersed, the individual oocytes were counted and examined to confirm maturational stage as described by Stoker *et al.* (2003).

### Experiment 3: 21-Day Molinate Exposure

**Experiment 3a: effect of subchronic exposure on estrous cyclicity.** Based on the suppression of the LH surge and delayed ovulation, ovarian function was monitored by daily vaginal lavage after exposure to the highest dose (50 mg/kg) of molinate for 21 days. Intact regularly cycling female rats (showing at least 5 consecutive 4-day cycles prior to dosing) were gavaged at 1300 h daily with corn oil ( $n = 20$ ) or 50 mg/kg molinate ( $n = 13$ ) for 21 days. A dose-response test on ovarian cyclicity was not conducted because of the high cost of molinate. Vaginal cytology and daily body weights were assessed in these females on a daily basis during the exposure period.

**Experiment 3b: Effect of subchronic exposure on LH and PRL secretion.** After the 21-day exposure to 50 mg/kg of molinate, the same females were ovariectomized and implanted with subcutaneous estradiol implants, as described in the LH surge studies above (day 22 of exposure). Females continued to receive daily oral doses of control vehicle or 50 mg/kg molinate at 1300 h. Then on day 25 of molinate dosing (72 h after the implant), groups of females from the control group and the 50 mg/kg group were decapitated at 0 (1300 h), 1 (1400 h), 3 (1600 h), or 6 (1900 h) h after administration of the 25th dose of molinate (*e.g.*, 0, 1, 3, or 6 h post-gavage) and blood and pituitaries were collected as described above. Serum and pituitary LH and PRL levels were then determined by radioimmunoassay.

### Experiment 4: Examining the Site of Action of Molinate

**Experiment 4a: LH secretion after exogenous GnRH administration.** To determine the target site of the effect of molinate on the hypothalamic-pituitary axis, the response to exogenous gonadotropin releasing hormone (GnRH) was examined in 50 mg/kg molinate-treated ovariectomized females bearing indwelling cardiac catheters. If the pituitary is able to respond normally to GnRH stimulation 1 h after exposure to molinate, then the toxicant is not affecting the pituitary secretion of peptide hormone and the effects on LH secretion are deduced to be mediated in the brain. This study was performed in rats that had been ovariectomized 3 weeks prior to the experiment. This allowed us to study the pituitary response to GnRH without the influence of gonadal steroid feedback. First, the ovariectomized females were surgically fitted with in-dwelling cardiac catheters under halothane anesthesia as described elsewhere (Goldman and Cooper, 1993; Harms and Ojeda, 1974). Placement of the catheters was performed within 5 min with the animals under anesthesia, and they returned to consciousness immediately after the procedure. Two hours after placement of the catheters, the females were gavaged with either corn oil or 50 mg/kg of molinate. One hour after dosing, three baseline bleeds were taken 10 min apart and replaced with 100  $\mu$ l of heparinized saline (ICN Biomedical, Costa Mesa, CA;

10 IU heparin per ml saline). Immediately after the third baseline collection, 50 ng/kg GnRH (Bachem, San Carlos, CA) was administered through the catheter. Additional blood samples were collected at 5, 10, 15, 20, 30, 40, 50, and 60 min after the bolus administration of GnRH. The blood was centrifuged and serum was stored at  $-80^{\circ}\text{C}$  until assayed for LH in the DELPHIA assay as described below.

**Experiment 4b: LH pulses in long-term ovariectomized females.** LE female normal cycling rats were ovariectomized and allowed to acclimate for 3 weeks before being fitted surgically with indwelling cardiac catheters under halothane anesthesia as described above. This animal model allowed us to examine pulses of LH that correlate with GnRH stimulation of the pituitary, with no gonadal steroid feedback. This experiment was done using the highest dose of molinate that was shown to suppress LH. Two hours after placement of the catheters, a baseline bleed of 200  $\mu$ l was taken and replaced with 100  $\mu$ l of heparinized saline (ICN Biomedical; 10 IU heparin per ml saline). Every 10 min baseline bleeds were obtained for a total of 1 h or seven 10-min samplings. After the last baseline sample was drawn, the females were dosed with control or 50 mg/kg molinate by gavage ( $n = 6$  per group). Two hours after this administration of molinate to the animal, another seven 10-min samples were obtained. The blood was centrifuged and serum stored at  $-80^{\circ}\text{C}$  until being assayed for LH as described below.

**Experiment 4c: hypothalamic catecholamine concentrations after molinate exposure.** To determine whether alterations in hypothalamic neurotransmitter concentrations may account for the changes observed in the secretion of LH and prolactin following molinate exposure, norepinephrine and dopamine were measured in the brains of the Experiment 1b females at the 3 h post-exposure time point. For catecholamine determinations, the hypothalamus of the control, 25 and 50 mg/kg females were dissected and divided into the anterior (AH) and posterior hypothalamus (PH) and catecholamines were separated by high performance liquid chromatography (HPLC) for measurement of concentrations of norepinephrine (NE) and dopamine (DA) by electrochemical detection. These procedures were done as described in detail in Goldman *et al.* (1994).

**Experiment 4d: serum and brain cholinesterase activity after molinate exposure.** To determine if the suppression of the LH surge could be the result of alterations in cholinesterase activity, serum and brain cholinesterase activity was measured in the 3 h post-exposure time samples collected in Experiment 1b (above). Acetylcholinesterase (AChE) activity was measured in the serum and brains of the control and 50 mg/kg females by means of an acetylcholinesterase radiometric assay (as described by Johnson and Russell, 1975). Briefly, immediately after removal from the freezer, the brain was sliced in half longitudinally with a single-edge blade. One half was refrozen at  $-80^{\circ}\text{C}$ , and the other half was homogenized in a Polytron (Brinkmann Instruments, Westbury, NY) for 20 s (setting 5) on ice at a ratio of 1:3 (w/v; *e.g.*, 1 g of brain homogenized in 2 ml of buffer) in 0.1 M sodium phosphate buffer (pH 8.0) with 1% Triton X-100. The serum required no dilution.

The radiometric assay was run with a total reaction volume of 100  $\mu$ l. The substrate (0.6 mM acetylcholine iodide and 0.1  $\mu$ Ci of 3H-acetylcholine iodide [82 mCi/mmol, NEN Life Sciences Products, Boston, MA] per 20  $\mu$ l) had a final substrate concentration of 1.2 mM. Preliminary assays were performed to determine conditions of tissue concentration and incubation times that yielded linear rates of hydrolysis. The assay was conducted at  $26^{\circ}\text{C}$  with 10  $\mu$ l of 1:3 brain homogenate and 40  $\mu$ l of undiluted serum, with each requiring 30 s of incubation. After the reaction was stopped and scintillant was added, the activity was counted in a 2200CA Tri-Carb Scintillation Counter (Packard Instruments, Downers Grove, IL) within 24 h. Counting efficiency, as determined by an external quench standard, was approximately 57%.

**Hormone assays.** Serum estradiol levels were measured using a Coat-a-Count radioimmunoassay (RIA) kit obtained from Diagnostic Products Corporation (Los Angeles, CA). Serum and pituitary LH and PRL were measured by radioimmunoassay. These pituitary peptide RIAs were performed with the following materials, supplied by the National Hormone and Pituitary Agency for LH and PRL, respectively: iodination preparation I-9 and I-6; reference preparation RP-3, RP-3; and antisera S-11, S-9. Iodination material was radiolabeled

with  $^{125}\text{I}$  (Dupont/New England Nuclear) by a modification of the chloramine-T method of Greenwood *et al.* (1963). Labeled antigen was separated from unreacted iodide by gel filtration chromatography as described elsewhere (Goldman *et al.*, 1986). Sample serum and pituitary homogenate were pipetted with appropriate dilutions to a final assay volume of 500  $\mu\text{l}$  with 100 mM phosphate buffer containing 1% BSA. Standard reference preparations were serially diluted for the standard curves. Next, 200  $\mu\text{l}$  of primary antisera in 100 mM potassium phosphate, 76.8 mM EDTA, 1% BSA, and 3% normal rabbit serum (pH 7.4) were pipetted into each assay tube, vortexed, and incubated at 5°C for 24 h. Then, 100  $\mu\text{l}$  of the iodinated hormone was added to each tube, and the tube was vortexed and incubated for 24 h. A second antibody (goat anti-rabbit gamma globulin (Calbiochem, San Diego, CA) at a dilution of 1 U/100  $\mu\text{l}$ ) was then added, vortexed, and incubated for 24 h. The samples were centrifuged at 1260  $\times$  g for 30 min, the supernate aspirated, and the sample tube, with pellet, was counted on a gamma counter. Intra-assay coefficients of variation for the LH and PRL assays were 1.1% and 0.9%, respectively.

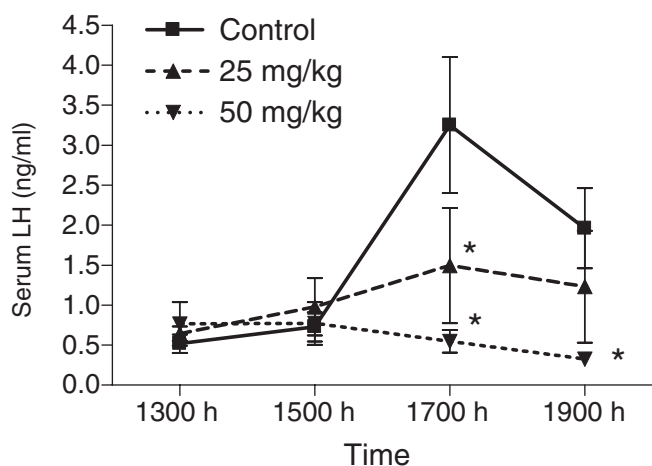
Because of the small volume of sample, serum LH levels from the blood samples of the catheterized females were quantified using the rat LH dissociation enhanced lanthanide fluorometric immunoassay (DELFLIA), which was designed by Haavisto *et al.* (1993) and was used in this study as modified according to methods described by Bielmeier *et al.* (2004).

**Statistics.** All data were analyzed for treatment effects by analysis of variance (ANOVA) with the General Linear Model (GLM) procedures (SAS, version 8.1; SAS Institute, Inc., Cary, NC), and for homogeneity of variance, with Bartlett's test (GraphPad InStat, GraphPad Software, San Diego, CA). When significant treatment effects ( $p < 0.05$ ) were indicated by GLM, the Dunnett's *t*-test was used to compare each treatment group with the control. The occurrence of ovulation, estrous cyclicity, and LH pulse data were analyzed using the chi squared and the Fisher exact tests of probability.

## RESULTS

### Experiment 1: Time and Dose-Response Effects of Molinate on LH and Prolactin Secretion

**Experiment 1a. LH surge in individual females.** Figure 1 depicts the LH surge in the ovariectomized estradiol benzoate



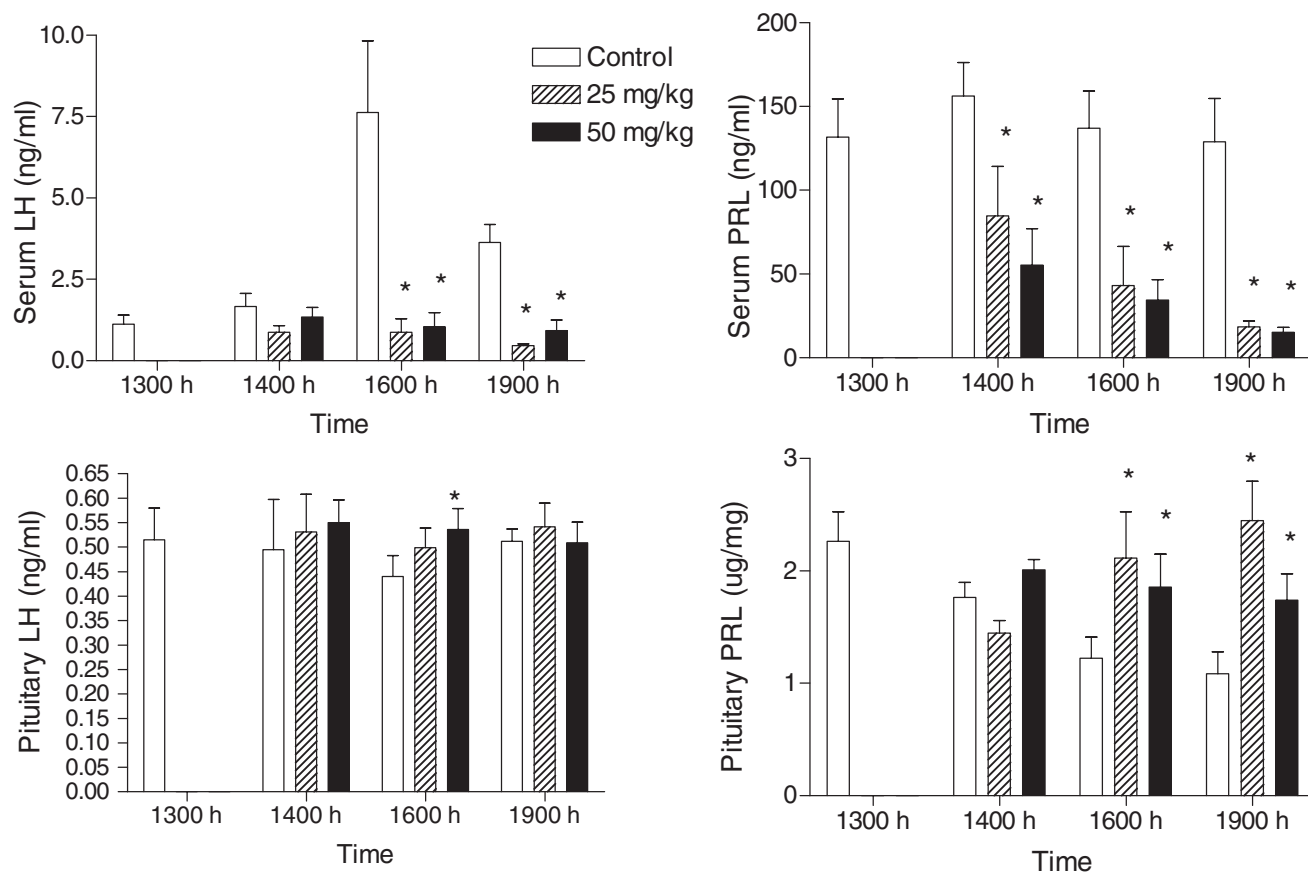
**FIG. 1.** Mean serum LH concentrations (ng/ml)  $\pm$  the S.E. in blood sampled over the afternoon of the photoperiod in OVX/EB rats receiving 0, 25, or 50 mg/kg molinate given at 1300 h (4 h prior to the anticipated LH surge). \* $p < 0.05$  when compared to controls at the same time point.

(EB)-treated females after individual tail bleeds. While the control females displayed an LH surge at 1700 h (Fig. 1), the concentration of this hormone was significantly decreased at 1700 h by administration of both 25 mg/kg and 50 mg/kg molinate. At 1900 h, only the LH concentration of the 50 mg/kg dose was still significantly decreased as compared to the control.

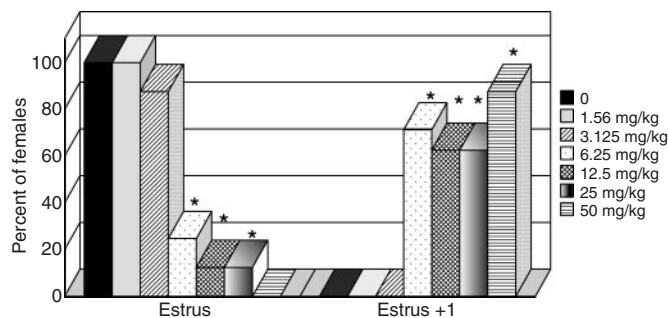
**Experiment 1b. LH surge in serial necropsies.** Both the 25 and 50 mg/kg doses of molinate significantly suppressed serum LH at 1600 h and 1900 h as compared to the control mean at each time point (Fig. 2), similar to the results in the first experiment with serial bleeds. Pituitary LH concentration was lowest at 1600 h (at the time of the peak serum LH) in controls. The pituitary LH in the 50 mg/kg group was significantly greater than controls at 1600 h. In addition, serum prolactin was significantly decreased at 1400 h, 1600 h, and 1900 h by 25 and 50 mg/kg molinate as compared to the control mean at each time point (Fig. 2). Pituitary prolactin concentration showed a decline over time in the controls (from 1400 h to 1900 h), but a similar decline was not observed in the 25 and 50 mg/kg dose groups at 1600 h and 1900 h. The mean concentration was significantly increased at 1600 h and 1900 h as compared to the control mean at each time point (Fig. 2).

**Experiment 2: effect of molinate on ovulation.** Examination of the oviducts on vaginal estrus (0900 h) revealed that 100% of the control and 1.56 mg/kg group and 87.5% of the 3.125 mg/kg molinate females had ovulated. In contrast, none of the 50 mg/kg group had ovulated, and only 12.5% of the 12.5 and 25 mg/kg molinate groups and 25% of the 6.25 mg/kg group had ovulated (Fig. 3). When females were euthanized on estrus + 1 day (the first day of diestrus for the controls), none of the control females had oocytes in the oviduct, indicating that the oocytes present 24 h earlier had passed through the oviduct into the uterus. In the molinate-treated females, 77.7% of the 50 mg/kg group, 60.0% of the 25 mg/kg group, 62.5% of the 12.5 mg/kg group, and 75.0% of the 6.25 mg/kg group had oocytes in cumulus present in the oviduct on estrus + 1 (Fig. 3). These females were in estrus for the second day, when the oocytes were recovered from the oviduct. Therefore, ovulation was delayed for 24 h in the 6.25 to 50 mg/kg molinate-dosed females. Interestingly, in the 1.56 and 3.125 mg/kg groups on estrus + 1 day, 87.5% of the oviducts contained oocytes which were not in cumulus, even though the vaginal smears were diestrus, not estrus. Taken together with results on estrus, this observation is consistent with ovulation having occurred on estrus with atypical retention of the oocytes in the oviduct.

As only two molinate-treatment groups had ovulated on the day of vaginal estrus, a comparison of oocyte number could only be made for the day of estrus with the 1.56 and 3.125 mg/kg group and the controls. There was no difference in the number of oocytes in cumulus between controls ( $16.6 \pm 0.57$ ) and the 1.56 mg/kg group ( $16.3 \pm 0.65$ ) or the 3.125 mg/kg group ( $15.6 \pm 1.07$ ) (Table 1). The number of control oocytes on estrus



**FIG. 2.** The effect of a single exposure to molinate (0, 25 or 50 mg/kg) on the estrogen-induced LH surge, serum PRL, and pituitary LH and PRL. Females were dosed at 1300 h and killed 0, 1, 3, or 6 h later. Bars represent the mean concentration of hormone  $\pm$  S.E. \* $p < 0.05$  compared to the control mean at that time point (*i.e.*, 1300 h, 1400 h, etc.).



**FIG. 3.** Percent of females with cumulus-enclosed oocytes in the oviduct on Estrus or Estrus + 1. The percentage of females ovulating after treatment with 0, 1.56, 3.125, 6.25, 12.5, 25, or 50 mg/kg molinate is represented by vertical columns. \* $p < 0.05$  when compared with controls.

**TABLE 1**

**Mean Number of Oocytes in Oviduct on Estrus or Estrus + 1**

Treatment	Estrus	Estrus + 1
Control (mg/kg)	16.5 $\pm$ 0.57	N/A
1.56	16.4 $\pm$ 0.65	16.4 $\pm$ 0.61 NC
3.125	15.6 $\pm$ 1.07	14.6 $\pm$ 0.95 NC
6.25	N/A	15.2 $\pm$ 2.15
12.5	N/A	14.6 $\pm$ 2.29
25	N/A	12.0 $\pm$ 1.92*
50	N/A	13.7 $\pm$ 1.41

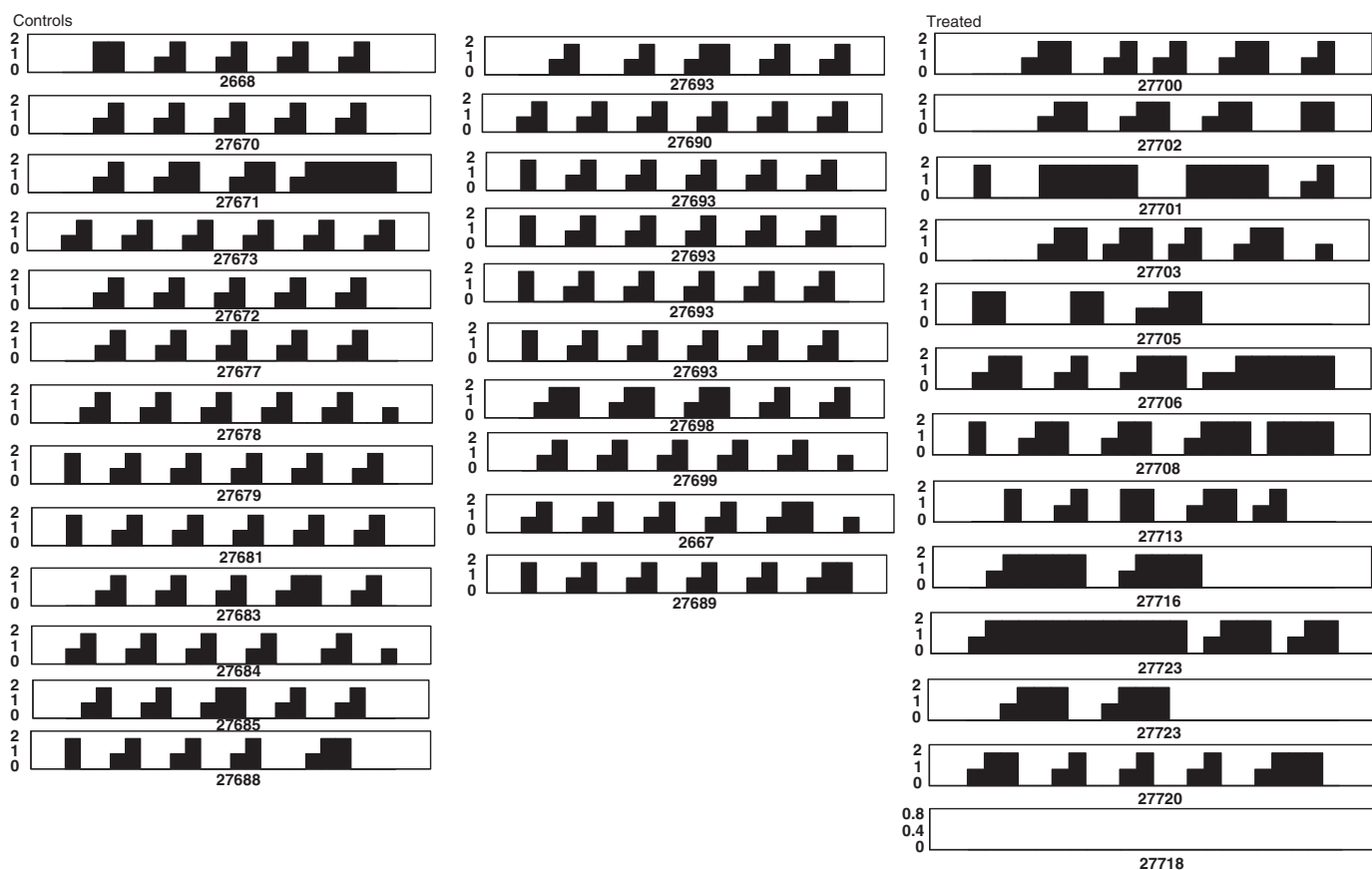
*Note.* All oocytes in oviduct were enclosed in cumulus mass, unless designated by NC for 'no cumulus.' Mean number of cumulus enclosed oocytes  $\pm$  S.E.

\* $p < 0.05$  when compared to control oocytes on Estrus day. N/A = non-applicable because no comparison could be made (*i.e.*, no oocytes were observed on Estrus or Estrus + 1 or only one female had oocytes).

was also compared to the delayed molinate treatment groups on estrus + 1 (second day of estrus). From that comparison, it was clear that the number of oocytes present in the controls (16.6  $\pm$  0.57) was significantly greater than the 25 mg/kg group (12  $\pm$  1.92), which ovulated on the second day. However, there was no difference between the number of control oocytes on

estrus and the 6.25, 12.5, or 50 mg/kg groups (15.2  $\pm$  2.15, 14.6  $\pm$  2.29, 13.7  $\pm$  1.41) on estrus + 1 (Table 1).

The oocytes recovered from the delayed molinate-treated females appeared normal; *i.e.*, they had metaphase II chromosomes and a single polar body.



**FIG. 4.** The effect of molinate exposure on estrous cyclicity over a 21-day exposure period in individual females. All females displayed regular 4-day estrous cycles prior to the exposure period. Panel a displays the control group and panel b displays the molinate-treated group.

### Experiment 3: 21-Day Exposure to Molinate

**Experiment 3a: Effect of subchronic exposure on estrous cyclicity.** As shown in Figure 4a (controls) and 4b (molinate-treated), continued exposure to 50 mg/kg molinate for 21 days induced a significant change in the ovarian cycle of females, as indicated by vaginal cytology. Females selected had five repetitive 4-day cycles prior to the treatment period (data not shown). Molinate-exposed females had more irregular cycles than the controls (Table 2). There was a significant difference in the mean number of days in estrus during the 21-day period in the molinate group as compared to the controls ( $6.00 \pm 0.32$  in controls vs.  $8.54 \pm 1.19$  in treated mice). There was also a significant difference in the estrous ratio, defined as the number of days in estrus divided by the number of cycles in the specified dosing period ( $1.19 \pm 0.09$  in controls vs.  $2.62 \pm 0.42$  in the 50 mg/kg molinate group). Body weight in the molinate and control groups was not different over the 21 days of exposure (data not shown).

**Experiment 3b: effect of subchronic exposure on LH and PRL secretion.** Following the 21 days of exposure to 50 mg/kg molinate or control vehicle, the females were ovariectomized and implanted with EB Silastic capsules to compare the secretion of the LH and PRL surges with the previous measurements.

**TABLE 2**

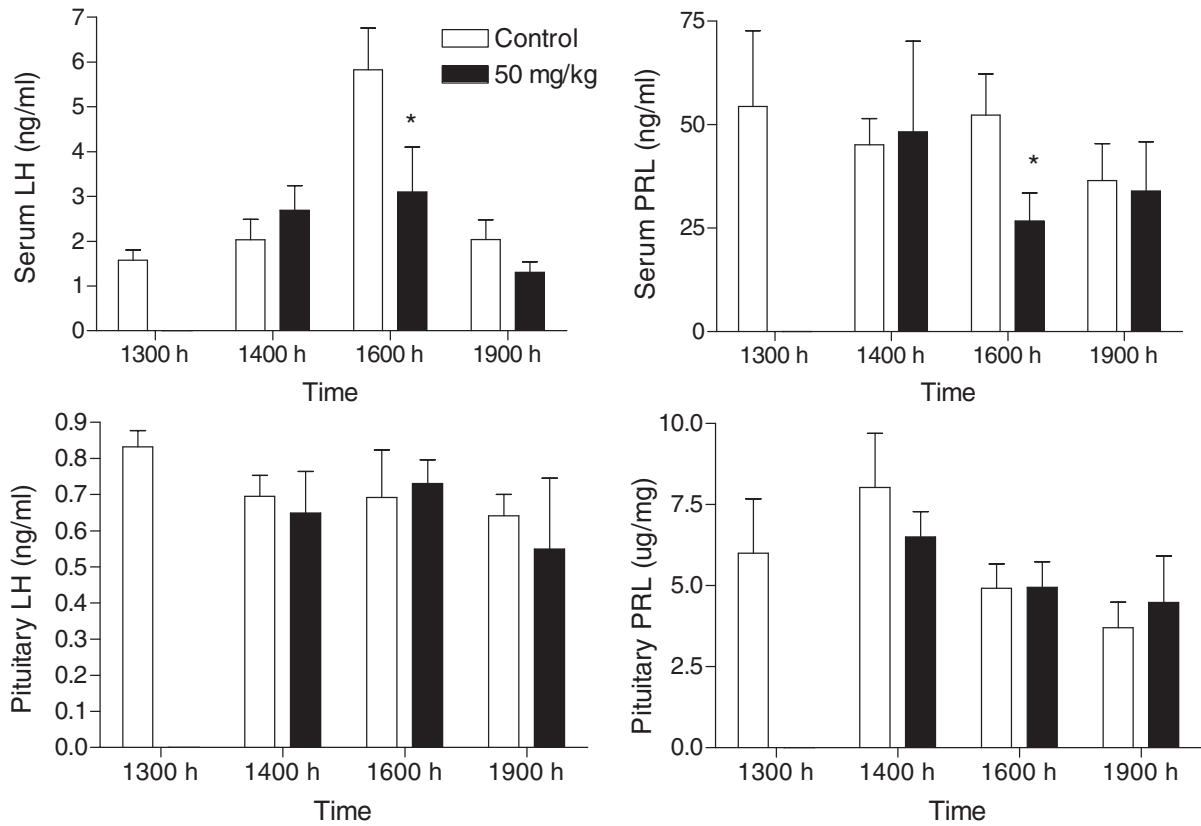
**Estrous Cyclicity Data for Control and Molinate 21 Day Exposed Adult Females ( $n = 20$  For Controls and  $n = 13$  for Molinate)**

Estrous Cycle Parameters	Controls	50 mg/kg Molinate
Percent cycling	100.0%	69.2%*
Percent irregular cycle	5.0%	61.5%*
Percent with Increased days in estrus	5.0%	69.2%*
Mean number days in estrus	$6.0 \pm 0.32$	$8.54 \pm 1.19^*$
Mean E ratio (no. E/no. cycles)	$1.19 \pm 0.09$	$2.62 \pm 0.42^*$
Percent with increased days in diestrus	0	30.8%*
Mean number days in diestrus	$10.9 \pm 0.28$	$9.85 \pm 1.18$
Mean number of cycles	$4.95 \pm 0.09$	$3.38 \pm 0.40^*$

Mean  $\pm$  S.E.

\* $p < 0.05$  as compared to controls.

On the 25th day of dosing, or 72 h after the surgery, 50 mg/kg of molinate again significantly suppressed the mean serum LH and PRL concentrations at 1600 h as compared to the control mean hormone concentrations (Fig. 5). However, there were no significant differences in the concentration of pituitary LH or PRL at any of the time points.



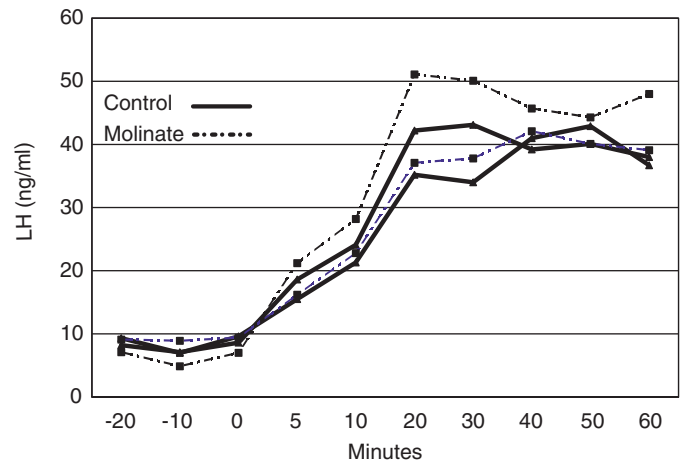
**FIG. 5.** The effect of a 21-day exposure to molinate (0, 25, or 50 mg/kg) on the estrogen-induced LH surge, serum PRL concentrations, and pituitary LH and PRL. Females were dosed at 1300 h and killed 0, 1, 3, or 6 h later. Bars represent the mean concentration of LH or PRL  $\pm$  S.E. \* $p < 0.05$  compared to the control at that time point.

#### Experiment 4: Examining the Site of Action of Molinate

##### Experiment 4a: LH secretion following exogenous GnRH.

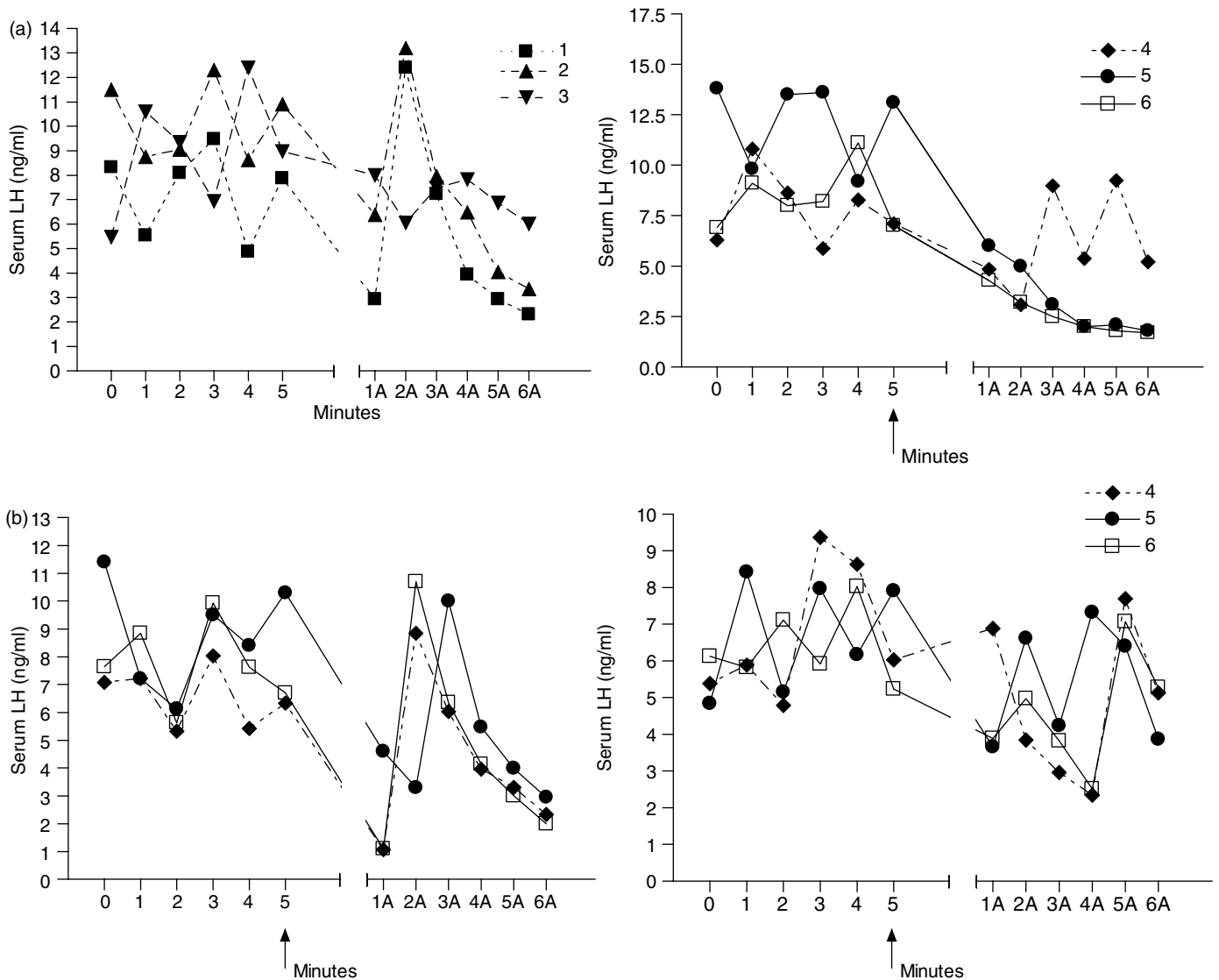
To determine whether the suppression of the estrogen-induced LH surge observed after a single exposure to molinate could be reversed by a bolus of intravenous GnRH, LH secretion was examined in molinate-treated (50 mg/kg) ovariectomized females bearing indwelling cardiac catheters 1 h after exposure to molinate. When the females were injected intravenously with 50 ng of GnRH, the serum LH secretory response in the molinate-exposed females was comparable to the control females (Fig. 6;  $n = 2$  per group with individual response shown).

**Experiment 4b: LH pulses in long-term ovariectomized females.** By measuring LH pulses in the long-term (3 weeks) ovariectomized females, the pulse frequency/amplitude of LH secretion can be correlated with the direct stimulation of the pituitary by GnRH. Luteinizing hormone pulse frequency (determined by graphing the 10 min serum LH concentrations and calculating frequency of pulses over the 1 h period) was consistent between the control and baseline testing periods, with 2.40 and 2.67 mean pulses per hour in the two blocks of females. However, 1 and 2 h after a single exposure to molinate (50 mg/kg), the mean pulses were slower than during the



**FIG. 6.** The effect of an intracardiac injection of 50 ng/kg GnRH on LH secretion in females dosed with molinate 1 h before the bolus. The peak concentrations observed in the molinate-exposed females was not different than that observed in the control females.

baseline period. For example, in the first group of females the baseline mean number of pulses per hour was  $2.40 \pm 0.18$ , whereas 1 h after the single exposure to molinate, the mean number of pulses per hour was  $1.00 \pm 0.31$  (Fig. 7a; individual



**FIG. 7.** The effect of a single administration of molinate (50 mg/kg) on LH pulsatility in long-term ovariectomized female rats implanted with an indwelling cardiac catheter. Two graphs are shown for each group (1 h post-dose in graph a and 2 h post-dose in panel b) to distinguish individual female pulses. Baseline pulses are seen from 0 to 5 (10-min samplings) on the left of each graph, and LH pulses on the right were taken after either 1 h (graph a) or 2 h (graph b) post-dose (1A–6A, *i.e.*, sample 1 after dose = 1A).

females in this group were plotted in two graphs to show individual pulsatility). In the second group of females, the mean number of LH pulses in the baseline sampling hour was  $2.50 \pm 0.28$ , while the pulses decreased to  $1.67 \pm 0.33$  per hour 2 h after molinate exposure (Fig. 7b, individual females in this group were plotted in two graphs to show individual pulsatility). Therefore, it appears that the suppression of GnRH pulsatility by molinate occurs approximately 1 h after exposure. The amplitude was correspondingly larger for each pulse observed in the molinate females at 1 and 2 h post-dose (Fig. 7a and 7b).

**Experiment 4c: hypothalamic catecholamine concentrations after molinate exposure.** Although there was no difference in catecholamine NE in the AH between the 25 mg/kg group

and the control at 3 h post-dose, there was a significant increase in the 50 mg/kg group at 3 h post-dose (Table 3). In the posterior hypothalamus, there was no significant difference between the mean control NE or DA concentrations and the molinate dose groups at 3 hours post dose.

**Experiment 4d: serum and brain cholinesterase activity following molinate exposure.** Because molinate has been shown to cause decreased cholinesterase activity after extended dosing, we investigated the effects of a single administration of 50 mg/kg molinate on cholinesterase in the brain and blood 3 h after exposure to molinate. No significant differences between control and treated groups were observed in cholinesterase activity, suggesting that the inhibition of this enzyme is not

**TABLE 3**  
**The Effect of Molinate on Neurotransmitter Concentrations**  
**in the Anterior (AH) and Posterior Hypothalamus (PH) 3 h**  
**Post-Dose in the Ovx/E2 Study**

Molinate (mg/kg)	Hours (post-dose)	AH	PH	
		NE (pg/ $\mu$ g)	NE (pg/ $\mu$ g)	DA (pg/ $\mu$ g)
Control	0	16.55 $\pm$ 1.6	14.21 $\pm$ 1.9	1.50 $\pm$ 0.22
	3	16.10 $\pm$ 1.7	15.81 $\pm$ 1.3	2.17 $\pm$ 0.23
25 mg/kg	3	16.58 $\pm$ 1.1	18.63 $\pm$ 1.5	2.32 $\pm$ 0.21
50 mg/kg	3	19.04 $\pm$ 0.6*	19.54 $\pm$ 2.9	2.60 $\pm$ 0.36

Mean  $\pm$  S.E.

\* $p < 0.05$  compared to the control mean;  $n = 7$ /group.

the cause of the suppression of LH or PRL or the observed delay in ovulation (data not shown).

### DISCUSSION

The data from these experiments indicate that molinate disrupts the neuroendocrine control of ovarian function in the rat. Molinate delayed ovulation for 24 h after a single exposure to 6.25 to 50 mg/kg on the afternoon of proestrus, and this delay was associated with suppression of the LH surge (documented at 25 and 50 mg/kg). The data also suggest that the brain, and not the pituitary, is the primary target site of the effect on pituitary luteinizing hormone secretion. A single exposure to molinate did not alter the GnRH-induced response of the pituitary but did decrease LH inter-pulse frequency in the long-term ovariectomized model. In addition, this study demonstrated that a 21-day exposure to molinate results in a significant disruption in estrous cyclicity, as seen with irregular cyclicity and increased days in estrus.

Central nervous system mechanisms involved in the control of the LH surge are linked to the circadian rhythm, and when the LH surge is blocked by centrally acting compounds, it recurs 24 h later. Such delays in ovulation, which retain the oocyte in its follicle for an extra day, can affect the ability of the released ovum to be fertilized normally. Several studies have shown that a delay in ovulation will alter pregnancy outcome, with a decrease in survivability of the ensuing embryo or fetus (Butcher *et al.*, 1969a; Butcher and Fugo, 1967; Butcher *et al.*, 1969b; Butcher, 1976; Stoker *et al.*, 1996; Cooper *et al.*, 1994). Oocytes retained in the follicle as a consequence of a blocked LH surge exhibit impaired cortical granule release and subsequent polyspermy (Peluso and Butcher, 1974; Stoker *et al.*, 2003). Therefore, although the molinate-exposed females ovulated a normal complement of oocytes after the 24 h delay of ovulation, it is reasonable to predict that they would not undergo normal fertilization and development. Such an effect would be consistent with most of the outcomes reported in the multigenerational study, including decreased numbers of implantations, increased

resorptions, and reduced litter size (Gilles and Richter, 1989). Similar alterations in pregnancy outcome have been associated with a delay in ovulation by a variety of environmental toxicants (Cooper *et al.*, 1996; Stoker *et al.*, 1996).

The data from these experiments also show that the mode of action of the effects of acute exposure to molinate on LH involves a disruption of the CNS control of pituitary function, not the synthesis of the hormones in the pituitary or altered gonadal feedback to the hypothalamus. The experiments that examined the effect of molinate on the LH surge were performed in females that had been ovariectomized and treated with a constant amount of estradiol benzoate. The fact that serum prolactin was decreased within 1 h of treatment and LH secretion within 3 h would also argue against a change in steroid regulation. Furthermore, the finding that the drop in pituitary LH and PRL concentration observed in controls was not observed in molinate-treated females also suggests that the appropriate CNS signals were altered. Finally, the molinate-induced blockade of LH release was reversed in response to a bolus injection of synthetic GnRH. This indicated that the gonadotrophs of the exposed females were responsive to the releasing peptide, which may indicate that the lack of LH secretion in the molinate-exposed females is due to an attenuation or alteration of GnRH release. However, the possibility still remains that such a large bolus of GnRH may have overcome subtle changes in responsiveness of the pituitary.

The mechanism through which molinate affects GnRH control of luteinizing hormone remains undetermined. In previous studies, we reported that dithiocarbamates (metham sodium and thiram) and chlorotriazines (atrazine) induce a suppression of both LH and PRL that is similar to that observed after molinate exposure. In these studies, the decrease in PRL was associated with a decrease in dopamine (DA) turnover within the hypothalamus, as indicated by an increased DA concentration within the medial basal hypothalamus and an altered DA/dihydroxyphenyl-acetic acid (DOPAC) ratio (Langdale *et al.*, 2004). However, changes in DA concentrations were not noted in this study. We also examined the effect of molinate on NE neuronal activity. Again, other work in our lab has shown that the suppression of the LH surge by both dithiocarbamates and chlorotriazines was associated with a decrease in NE synthesis. The dithiocarbamates are known to decrease dopamine beta-hydroxylase (DBH) activity, an enzyme that converts DA to NE (Lippman and Lloyd, 1971). However, in this study NE concentrations in the anterior hypothalamus were significantly increased when compared to controls at 3 h post molinate exposure, suggesting that molinate decreased the release of NE, and subsequently the NE-induced release of GnRH. Further studies are needed to determine the precise mechanism whereby molinate is able to affect the hypothalamic control of GnRH release.

Because it has been reported that AChE inhibition can also alter GnRH functioning (Kaur and Kaur, 2001; Kalra and Kalra, 1983), AChE activity was measured in the brains and serum

collected from rats 3 h after molinate exposure. However, the AChE activity in the treated and control rats was not different. Our data would agree with other subchronic studies which caused a decrease in brain and AChE activity in red blood cell and brain, but only after a 16-day exposure (NOEL of 25 mg/kg) (Horner, 1994).

The disruption of estrous cyclicity by a 21-day exposure to molinate is likely due to the blockade of LH, leading to persistent follicles. Molinate brings about changes in ovarian function, similar to what has been shown prior to the onset of reproductive senescence in the rat (Cooper *et al.*, 1986). In aging rats, the loss of ovarian cycles develops around 1 year of age and is characterized by the appearance of persistent or constant estrus, a condition in which the vaginal epithelial cells remain cornified and the ovaries become polyfollicular and lack corpora lutea (Cooper *et al.*, 1986; Everett, 1989). There is a general understanding that the neuroendocrine events responsible for this loss of ovarian cycling results from changes within the CNS that lead to a decreased amplitude and a delay in the onset of the pro-estrous LH surge (Cooper *et al.*, 1980; van der Schoot, 1976). These alterations in the pre-ovulatory surge of LH are believed to occur after an age-dependent reduction in the GnRH pulse frequency (Scarborough and Wise, 1990). We found that molinate reduces the frequency of GnRH pulses (by examining LH over time in the long-term ovariectomized rat model), similar to the reduction observed with aging. This observation introduces the possibility that exposures to molinate could be associated with an early onset of reproductive senescence and/or earlier mammary tumor development in rats, as in studies that found similar effects after atrazine exposure (Eldridge *et al.*, 1994).

The mechanism of the effect of molinate on male reproduction in rodents, namely, inhibition of the enzyme neutral cholesteryl ester hydrolase (nCEH) and the subsequent disruption of testosterone mobilization (Ellis *et al.*, 1998), is not likely relevant to the present effects on the neuroendocrine control LH and PRL secretion. As mentioned, our second experiment used the ovariectomized female implanted with estradiol capsules, and our fourth experiment examined LH pulsatility in ovariectomized females, demonstrating that these effects occurred independently of steroid synthesis. In addition, acute doses of molinate in the intact female inhibit the LH surge and ovulation within hours of administration, an effect too rapid for changes in steroidogenesis to have affected GnRH stimulation of LH release. Further studies are needed to determine whether the parent compound or one of the metabolites of molinate, such as molinate sulfoxide or hydroxymolinate, may be responsible for these effects in the female.

In this study, an acute exposure to 3.125 mg/kg of molinate is the no-effect level for the 24 h delay in ovulation. Even though this low dose and the dose of 1.56 mg/kg had no effect on the timing of ovulation, these dose levels did appear to prevent or delay transport of the mature oocyte from the oviduct late on the day of estrus, as oocytes were still present in the uterus at 48 h. The mechanism of this effect is unknown. The timing of the

passage of the embryo into the endometrial environment is an essential step for the establishment of implantation, and oviductal contraction and secretion are regulated by many factors, such as prostaglandins and gonadal steroids (Spilman and Harper, 1975). Recent work in the cow has also suggested that the preovulatory LH surge can stimulate the maximum oviductal production of prostaglandins and endothelin-1 (Wijayagunawardane *et al.*, 2001). Therefore, it is possible that low concentrations of prostaglandins, steroids, or LH may have contributed to the delayed oviduct transport observed here. A study is now in progress in our laboratory to determine the dose response and mechanism of this effect.

In summary, these experiments demonstrate a clear effect of molinate on the estrogen-induced LH and prolactin surge. The data from these experiments demonstrate that molinate has a dramatic effect on the neuroendocrine control of ovarian function in the rat. A single dose of molinate inhibited the estrogen-induced LH and prolactin surge and delayed ovulation for 24 h, an effect commensurate with a suppression of the LH surge. Because molinate altered the pulsatility of LH in the long-term ovariectomized animal, and because the females exposed to molinate showed a normal response to the GnRH challenge and did not affect the response of the pituitary to GnRH, it appears that molinate may interfere with the hypothalamic control of GnRH. The specific mechanism of action by which molinate disrupts this control of GnRH remains to be determined.

#### ACKNOWLEDGMENTS

The authors are grateful to the National Hormone and Pituitary Agency for the gift of the radioimmunoassay materials. They also thank Keith McElroy and Susan Jeffay for their technical assistance with some of these experiments.

The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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