Behavioral Responses of Rats Exposed to Long-Term Dietary Vinclozolin

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Vinclozolin is a fungicide used on food crops with human exposure estimated at ~2 μg/kg/day from ingestion; occupational exposure, however, may be greater. The metabolites of vinclozolin have been reported to act as antiandrogens and have adverse effects on reproductive physiology and behavior in animals. Here, pregnant rats were fed soy-free diets containing 0, 10, 150, or 750 ppm of vinclozolin (approximately 0, 0.8, 12, and 60 mg/kg/day for an adult) beginning on gestational day 7, and offspring were continued on these diets through sacrifice at postnatal day 77. Male and female offspring were assessed for changes in several nonreproductive sexually dimorphic behaviors: open field and running wheel locomotor activity, play behavior, and consumption of saccharin- and sodium chloride-flavored solutions. There was a significant interaction of sex with vinclozolin exposure on running wheel activity, which indicated that females in the high-dose exposure group were hypoactive compared to same-sex controls. There was a significant overall effect of vinclozolin exposure on fluid consumption, and high-dose animals showed increased intake of the saccharin solution and decreased intake of plain water while saccharin was available. Effects were more pronounced in females, which drank 40.8% more saccharin than control females, whereas males drank 6.2% more than control males. There were no effects of vinclozolin treatment on play behavior or sodium solution intake. Gestational duration, total and live pups per litter, litter sex ratios, and birth weight were also not significantly affected, nor were body weight and food intake for dams and offspring. These results indicate that long-term dietary exposure to vinclozolin does not have severe toxicological consequences on the nonreproductive behaviors measured here. However, exposure may cause subtle alterations in locomotor activity and consumption of saccharin-flavored solution.

Keywords: *Fungicide; endocrine disrupter; androgen*

INTRODUCTION

Vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione], a dicarboximide fungicide used on fruits and vegetables in the United States and Europe, was designed to inhibit the production of a fungal steroid, ergosterol. Human vinclozolin exposure is limited and comes primarily from the consumption of commercial produce, which has been shown to contain <20% of the U.S. Environmental Protection Agency's (EPA) reference dose of 12 μg/kg/day (*1*). Occupational exposure in production workers, although estimated at 10 times this amount, has been reported to result in minimal hormonal alterations (*2*).

Vinclozolin itself has little affinity for the androgen receptor (K_i > 700), but its two major metabolites, M1 and M2, are both effective antagonists, with K_i values of 92 and 9.7 μM, respectively (*3*). Both metabolites have been detected on the leaves of plants treated with vinclozolin and in vivo following animal exposure to vinclozolin (*3*). Vinclozolin is purely androgenic as neither it nor its metabolites interact with the estrogen receptor, and binding to the progesterone receptor has been reported to occur only in vitro (*4*).

Rodent studies have established that perinatal exposure to vinclozolin, at the lowest dose administered, 3.125 mg/kg/day, resulted in demasculinization of reproductive systems in male offspring (*5*). In a separate study, male sexual behavior was disrupted by perinatal vinclozolin at the lowest administered dose of 100 mg/kg/day (*6*). Peripubertal exposure, at the low dose of 30 mg/kg/day, delayed puberty and retarded the growth of sex accessory glands in male rats (*7*). Females similarly treated have shown only minimal effects. Data are currently not available with regard to vinclozolin effects on nonreproductive behaviors.

A variety of adverse effects in humans and wildlife may be related to exposure to environmental compounds with hormonal activity (see ref *8* for review). Attention has focused on estrogenic and/or androgenic compounds because treatment with exogenous steroid hormones, especially in the neonate, may influence several hormone-dependent, organizational patterns of development. In addition to physiological changes, certain sexually dimorphic behaviors can be altered following manipula-

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tion of estrogen and/or androgen. These include the expected reproductive or mating behaviors and other less obvious behaviors that are also sexually dimorphic and hormone-sensitive, including rodent play and consumption of flavored solutions (*9*, *10*).

The evidence of vinclozolin-induced effects on male reproductive physiology and behavior, along with the evidence of alterations in nonreproductive behaviors following androgen treatment, justify the current investigation of potential vinclozolin effects on nonreproductive behaviors. Because behavior represents the sum outcome of events at the molecular, cellular, and organ levels, recent workshops on endocrine disrupter research needs have recommended including such assessments (*11*). The behaviors assessed here were chosen because they are sexually dimorphic and subject to disruption by steroid hormones, making them sensitive to potential vinclozolin-induced effects. The chosen behaviors are also easily assessed, making them suitable for a preliminary study such as this one. Although the significance of some behaviors may not always be obvious, the behaviors chosen here reflect exposure to developmental and/or adult levels of circulating steroid hormones and as such may offer a valuable method to assess such exposure.

Beginning on gestational day (GD) 7, vinclozolin was mixed into the food of pregnant rats to give intake levels of approximately 0, 0.8, 12, or 60 mg/kg/day; the lowest dose is ~10 times the human allowable daily intake (*12*), and the highest dose is in the range of those previously shown to cause reproductive tract toxicity in rodents. Offspring continued on the same diets until sacrifice at postnatal day (PND) 77. Several litter measures were taken at parturition and, between PND 22 and 77, male and female offspring were assessed for open field and running wheel activity, play behavior, and intake of sodium- and saccharin-flavored solutions.

MATERIALS AND METHODS

Subjects and Vinclozolin Treatment. Forty-eight datemated primiparous Sprague−Dawley rats were obtained from the National Center for Toxicological Research (NCTR) breeding colony (plug date = gestational day 0). Each dam was housed individually in a standard polycarbonate cage lined with wood chip bedding. The housing room was maintained on a normal 12/12 h light/dark cycle; temperature was maintained at 23 ± 3 °C and humidity at 50 ± 10%.

Food and water were provided ad libitum. Two weeks prior to mating, dams were shifted from the standard autoclaved NIH-31 pellet diet to an irradiated soy- and alfalfa-free diet (5K96, purchased from Purina Mills, St. Louis, MO). This diet is based on the NIH-31 formula, except that casein replaces the protein contributed by soy and alfalfa, soy oil is replaced with corn oil, and the vitamin mix is adjusted for irradiation. Beginning on GD 7 and continuing through weaning of offspring on PND 22, dams consumed 5K96 chow containing 0 (n = 12), 10 (n = 12), 150 (n = 12), or 750 (n = 12) ppm of vinclozolin (purified from Ronilan WP, BASF Corp., Research Triangle Park, NC, by Battelle, Columbus, OH). For a 250 g rat consuming 20 g of chow per day, these doses are approximately equivalent to 0, 0.8, 10, and 60 mg of vinclozolin/kg/day, respectively. Vinclozolin purity was >99% as assessed by the Division of Chemistry at NCTR using HPLC analytical methods, mass spectrometry, and ^{1}H NMR. Vinclozolin was mixed into the standard 5K96 feed by the Diet Preparation Staff, The Bionetics Corp., at NCTR, and selected batches of feed were analyzed by the Division of Chemistry.

The day of birth was designated PND 1. On PND 2, litters were randomly culled to eight pups, four males and four females, and the pups were tattooed on the dorsal surface of the paw for identification purposes. Litters remained with their biological dam whenever possible; cross-fostering of a pup with another dam to maintain litter size and sex distribution was rare and done only within treatment groups. Offspring were weaned on PND 22 and housed two per cage with a same-sex sibling. Weaned pups continued on the dosed 5K96 diet until sacrifice on PND 77.

All animal procedures were approved by the NCTR Institutional Animal Care and Use Committee (IACUC).

Physical Measures. Body weight and food intake were measured weekly for each dam on GD 1, 7, 14, and 21 and on postparturitional days (PPD) 7 and 14.

Gestational duration and reproductive outcomes (total and live pups/litter, total and live sex ratios, and average weight/live pup) were assessed on the day of birth. Offspring body weights were measured weekly on PND 2, 8, 15, 21, 28, 42, 56, 70, and 77. Offspring food intake (grams) was measured weekly beginning on PND 28.

Behavioral Measures. All animals were randomly assigned to behavioral tests, and all nonautomated assessments were conducted by testers blind to the experimental treatment. Animals remained on a standard 12/12 light−dark cycle throughout, with lights on at 7:00 a.m. and all tests performed within 6 h of this time. The assessments described here are very similar to those reported previously (*13*−*15*).

Maternal Nursing Behavior. Each dam was assessed for the presence of nursing on the mornings of PND 3, 7, 10, 14, 17, and 21 and on the afternoons of PND 3, 7, 10, 14, and 17 by observation of posture in the home cage. If the dam was in the crouched position with at least one pup nursing, a score of nursing was recorded; if the dam was in any other position, a score of not nursing was recorded.

Open Field Activity. One male and one female from each of the 48 litters were tested before puberty (PNDs 22−24), a different pair at approximately the time of puberty (PNDs 43−45), and a third pair as young adults (PNDs 65−67). Activity was measured for individual animals in a Plexiglas cube (46.5 × 46.5 cm) bisected by photobeams, as previously described (*16*). Three consecutive daily sessions of 60 min each were run under normal room lighting. Activity was recorded as the number of photobeam breaks per 3 min period (total of 20 3-min periods/test session).

Play Behavior. Play behavior was assessed in 96 pairs of prepubertal animals (PND 35) using methodology previously described (*17*). On PND 34, two males and two females from each of the 48 litters were individually housed in clean cages. Twenty-four hours later, the animals were reunited with their same-sex sibling in a clean cage and, after a 3 min acclimation period, the total number of pins exhibited during the subsequent 5 min test period was recorded. A pin was defined as one animal having its dorsal surface to the ground while the other animal was on top (*18*).

Residential Running Wheel Activity. From PND 63 to 77, one male and one female from each litter were individually housed in a residential running wheel as previously described (*16*). Each apparatus was a standard housing cage, with all environmental conditions as described above for normal housing, but equipped with a running wheel (34.3 cm diameter). Number of wheel revolutions per normal 12 h dark period and 12 h light period were recorded for each of 14 consecutive days.

Intake of Flavored Solutions. For 3 consecutive days over the course of 1 week (PND 69−75), intake of two flavored solutions was determined in one male and one female from each litter, as previously described (*14*, *15*). These subjects were siblings of animals concurrently assessed for running wheel behavior and were individually housed in their home vivarium. Intake of a sweet solution containing 0.03% saccharin (ICN Biochemicals Inc., Aurora, OH) in water was measured on PND 69−71 by placing two bottles on each animal's cage, one containing plain water and the other containing the saccharin solution. Intake of a salt solution containing 3.0% sodium chloride (ICN Biochemicals Inc.) in water was measured in the same animals on PND 73−75 by placing two bottles on each animal's cage, one containing plain water and the other containing the salt solution. All bottles

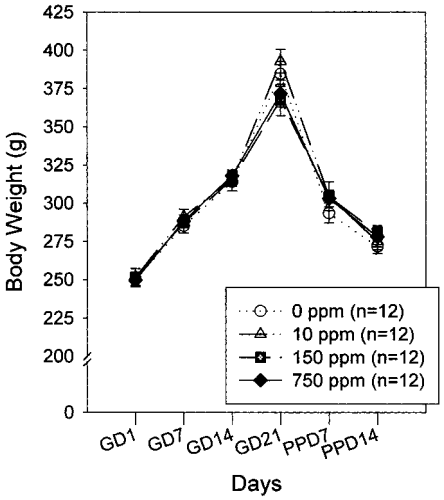

Figure 1. Dam body weight (mean ± SEM). Dams were weighed on GD 1, 7, 14, and 21 and on PPD 7 and 14. There were no effects of vinclozolin treatment on dam body weight.

were weighed once daily and the amount consumed in milliliters per day was divided by PND 70 body weight to yield milliliters consumed/day/kilogram.

Statistical Analyses. All analyses were conducted using the litter as the unit of analysis. Analyses of variance (ANOVA) were used to determine treatment effects using vinclozolin dose and sex as between-group variables. Several analyses involved repeated measures over days and were done with multivariate techniques, which were implemented using a mixed model. Homogeneity of variance was tested using a likelihood ratio test based on the negative log likelihoods from two different models, a mixed autoregressive and a mixed heterogeneous autoregressive model. If chi square yielded $p < 0.05$, the heterogeneous variance model was rejected. Post-hoc tests (two-sided Dunnett's or Student-Newman-Keuls) were applied only if analysis of variance attained significance at or below the 0.05 level. Maternal nursing behavior data were assessed via separate chi square analyses for each date and time.

RESULTS

Physical Measures. There were no significant effects of vinclozolin treatment or treatment by date interactions on dam food intake (data not shown) or body weight, and all dams showed normal weight gain over the gestational period [$F(5,277) = 365.54$ ($p < 0.0001$)] (Figure 1). There were no significant effects of vinclozolin treatment or treatment by date interactions on offspring food intake or body weight from PND 1 to

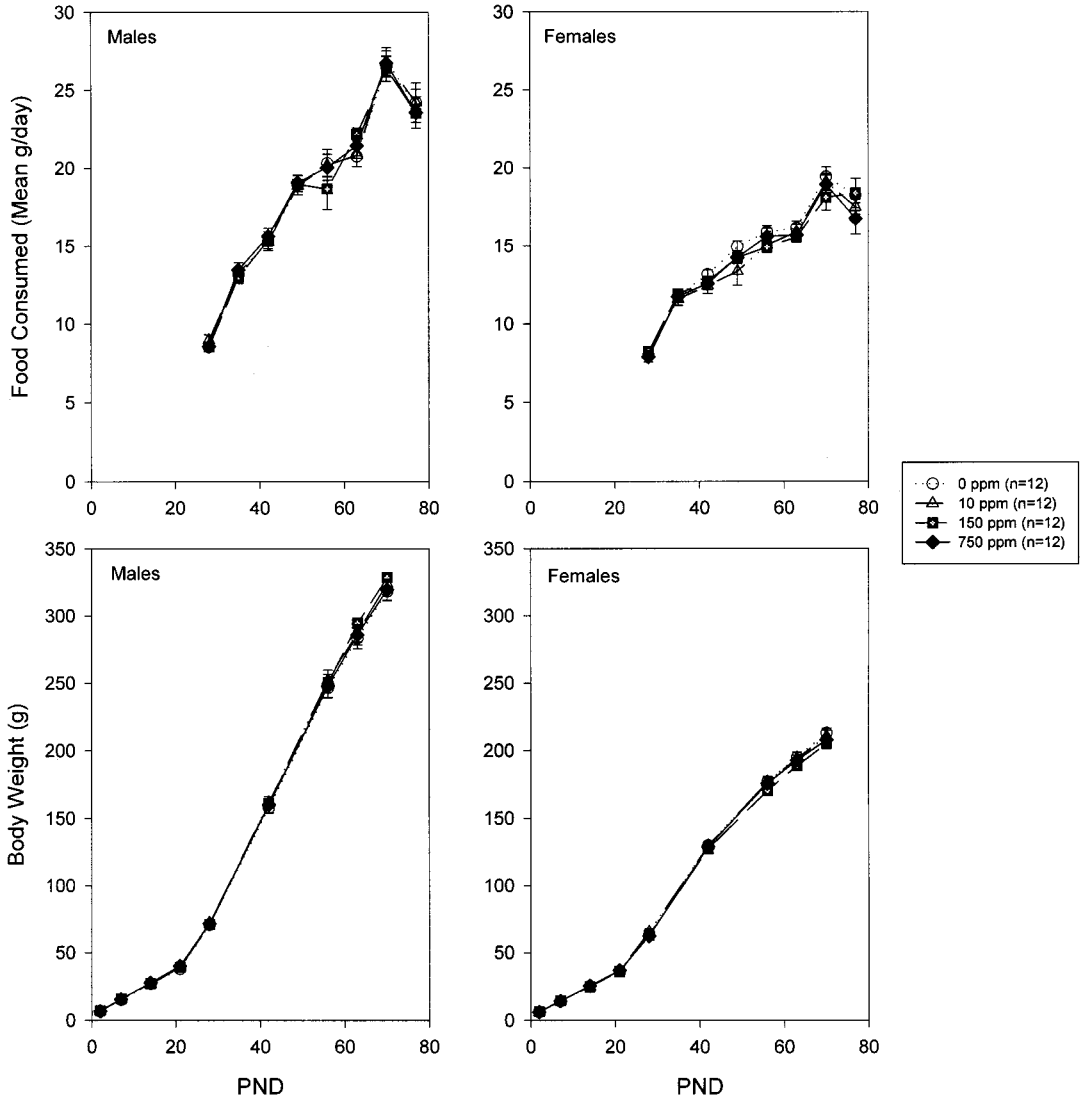

Figure 2. Male and female offspring food consumption (top) and body weight (bottom) (mean ± SEM). Feeders were weighed on PND 28, 35, 42, 49, 56, 63, 70, and 77. Offspring were weighed on PND 2, 8, 15, 21, 28, 42, 56, 70, and 77. Weaning occurred on PND 22, and pups were pair-housed until PND 65. There were no effects of vinclozolin treatment on offspring food consumption or body weight.

Table 1. Physical and Litter Measures

	0 ppm	10 ppm	150 ppm	750 ppm
av dam food intake (g/day) (GD 7−PPD 21)	31.53 ± 1.53	31.54 ± 1.57	32.48 ± 1.52	31.84 ± 1.76
gestational duration (days)	22.5 ± 0.2	22.6 ± 0.2	22.6 ± 0.2	22.2 ± 0.2
total pups/litter	14.0 ± 0.58	13.4 ± 0.62	11.6 ± 1.23	13.7 ± 0.47
live pups/litter	13.5 ± 0.6	13.0 ± 0.64	11.4 ± 1.26	13.3 ± 0.45
total sex ratio (m/f)	0.85 ± 0.11	1.56 ± 0.26	1.29 ± 0.26	1.27 ± 0.20
live sex ratio (m/f)	0.85 ± 0.11	1.56 ± 0.26	1.30 ± 0.26	1.03 ± 0.20
av wt/live pup (g)	6.12 ± 0.13	6.36 ± 0.17	6.57 ± 0.22	6.08 ± 0.18

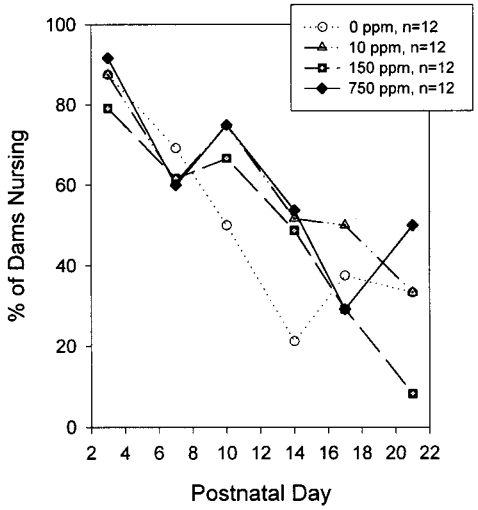

Figure 3. Percent of dams nursing on each of 6 days during the preweaning period (PND 3−21). Each dam was assessed twice per day, once in the morning and once in the afternoon; each point represents the mean of the two assessments. There were no significant effects of vinclozolin treatment on any day.

77, but there was a significant sex by date interaction on both measures, with males in all treatment groups eating more and weighing more with increasing age as compared to females [$F(7,587) = 408.88$ ($p < 0.0001$) and $F(10,706) = 3633.21$ ($p < 0.0001$) for food consumption and body weight, respectively] (Figure 2). There were no significant effects of vinclozolin treatment on gestational duration, total and live pups per litter, total and live sex ratios, or average live birth weight per pup (Table 1).

Behavioral Measures. *Maternal Nursing Behavior.* Percent of dams nursing on any day (mean of morning and afternoon observations) during the 21 day preweaning period ranged from 8.3% on PND 21 for the 150 ppm group to 91.7% on PND 3 for the 750 ppm group (Figure 3). Chi square analyses at each day showed no significant differences in percent of dams nursing among treatment groups.

Open Field Activity. There were significant sex by treatment interactions on the mean number of photobeam breaks on PNDs 22−24 [$F(3,213) = 3.09$ ($p < 0.03$)] and PNDs 63−65 [$F(3,236) = 6.23$ ($p < 0.0005$)] (Figure 4); however, no treatment group was significantly different from same-sex controls at any age. There was a significant effect of day at all three ages, indicating that animals were more active on the first test session than the subsequent two sessions (data not shown).

Play Behavior. Number of pins per 5 min test session ranged from 5.25 to 6.83 in 0 ppm females and males, respectively, and from to 3.08 to 6.33 in 750 ppm females and males, respectively. There were no significant treatment effects (Figure 5).

Residential Running Wheel Activity. There was a significant sex by treatment interaction [$F(3,1181) = 4.73$ ($p < 0.003$)] on night period activity. Post-hoc analyses indicated decreased activity in 750 ppm females compared to same-sex controls ($p < 0.003$) (Figure 6, bottom right). There were no significant day by treatment or sex by day by treatment interactions. There was a significant day effect with animals running more in later days ($p < 0.0002$) and a significant sex effect with females running more than males ($p < 0.0001$).

There were no significant treatment effects on light period running wheel activity (data not shown). There was a significant day effect with animals running less in later days ($p < 0.0002$) and a significant sex effect with females running more than males ($p < 0.02$).

Intake of Flavored Solutions. There was a significant overall effect of vinclozolin treatment on intake of the saccharin-flavored solution [$F(3,15) = 3.86$ ($p < 0.04$)] (Figure 7a). Post-hoc analyses indicated increased intake in the 750 ppm group compared to the 0 ppm group

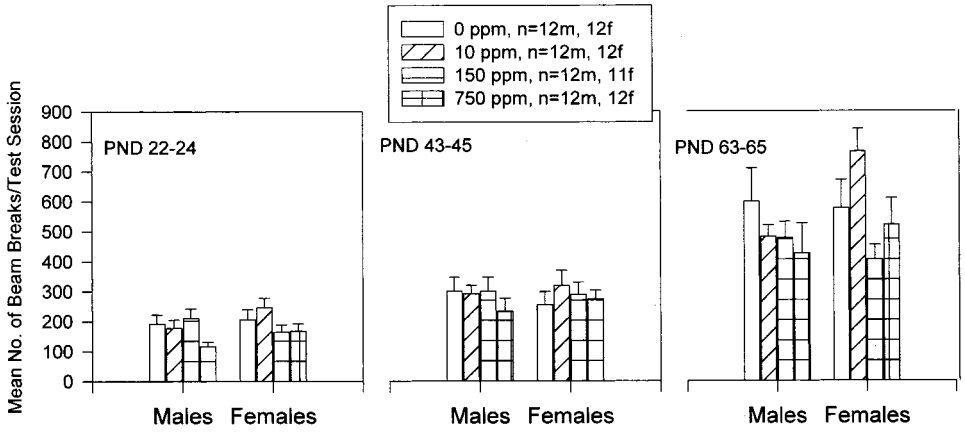

Figure 4. Mean number of photobeam breaks (± SEM) per 1 h test session on PND 22−24, 43−45, and 63−65. A different male and female from each litter were tested at each PND period. There was a significant sex by treatment interaction at PND 22−24 and 63−65, but no treatment group was significantly different from same-sex controls at any age.

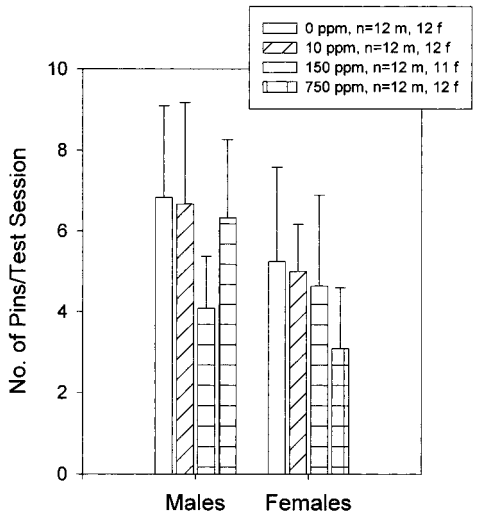

Figure 5. Mean number of pins (± SEM) per same-sex sibling pair. One male pair and one female pair from each litter were individually housed for 24 h before a 3 min acclimation followed by a 5 min play behavior test session on PND 35. There were no significant effects of vinclozolin treatment on number of pins.

($p < 0.05$). There was a significant day effect on saccharin intake, with animals drinking less on later days ($p < 0.0002$) (data not shown), and a significant sex effect with females drinking more than males ($p < 0.04$). There was a significant overall effect of vinclozolin treatment on intake of plain water when saccharin-flavored water was available [$F(3,15) = 4.44$ ($p < 0.03$)] (Figure 7b). Post-hoc analyses indicated decreased intake of plain water in the 750 ppm group compared to the 0 ppm group ($p < 0.03$). There was a significant sex effect on plain water intake while saccharin-flavored water was available, with females drinking more than males ($p < 0.03$).

There were no significant overall effects of vinclozolin treatment on intake of a 3.0% sodium chloride-flavored solution [$F(3,15) = 0.70$ ($p < 0.6$)] (Figure 8a). There was a significant day effect, with animals drinking less on later days ($p < 0.001$) (data not shown), and a significant sex effect, with females drinking more than males ($p < 0.0008$). There were no significant treatment effects on intake of plain water while sodium-flavored water was available (Figure 8b). There was a significant day effect, with animals drinking less on later days ($p < 0.0005$) (data not shown), and a significant sex effect, with females drinking more than males ($p < 0.0002$).

DISCUSSION

Developmental and lifelong exposure to vinclozolin resulted in minimal alterations in the behaviors assessed here. Female rats, however, appeared more sensitive to vinclozolin exposure than males. High-dose females were significantly less active in running wheel assessments than same-sex controls, whereas the activity of males was relatively unaffected. In addition, intake of a saccharin-flavored solution was significantly increased in high-dose rats, with females 41% above same-sex controls and males only 6% above controls. There were no signs of vinclozolin-related maternal toxicity and no effects on body weight, litter measures, play, open field activity, or sodium solution consumption. These preliminary data indicate that developmental and lifelong consumption of vinclozolin, at doses >1000 times estimated human exposure, results in only mild alterations in some steroid-sensitive behaviors in

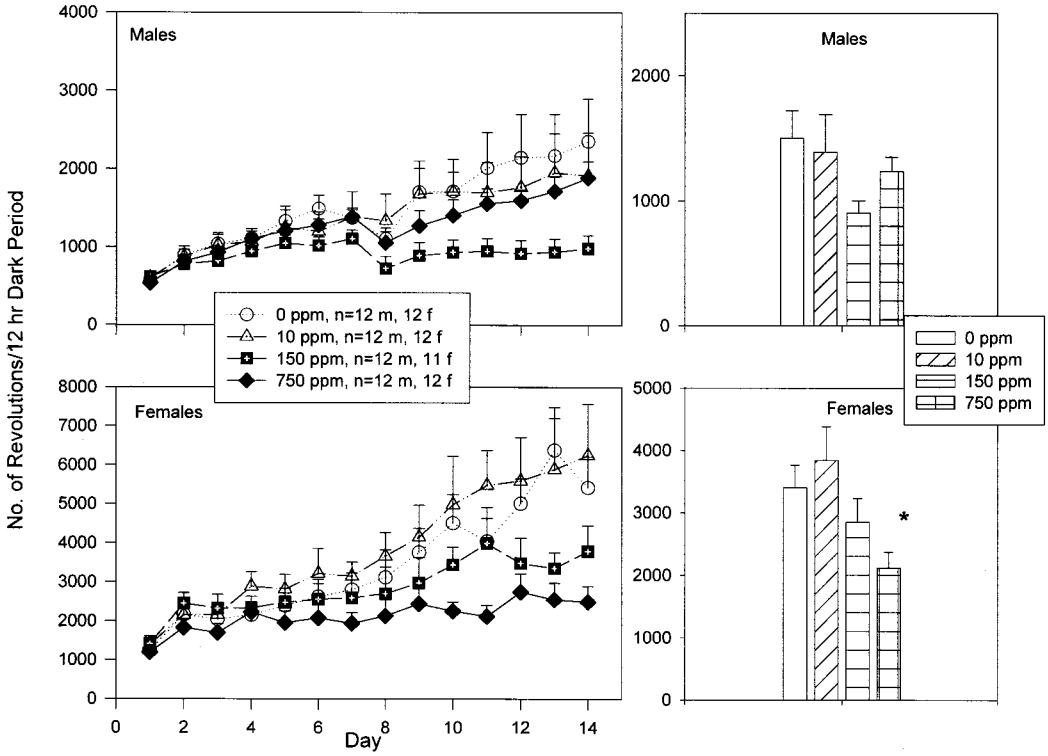

Figure 6. (Left panels) Number of dark period running wheel revolutions (± SEM) on each of 14 nights for one male and one female per litter. There was a significant night effect with all animals running more in later nights, but no significant night by treatment interactions. (Right panels) Mean number of dark period running wheel revolutions (± SEM) over 14 nights (PND 63−77) for one male and one female per litter. A sex by treatment interaction indicated 750 ppm females were less active than control females (*, $p < 0.003$).

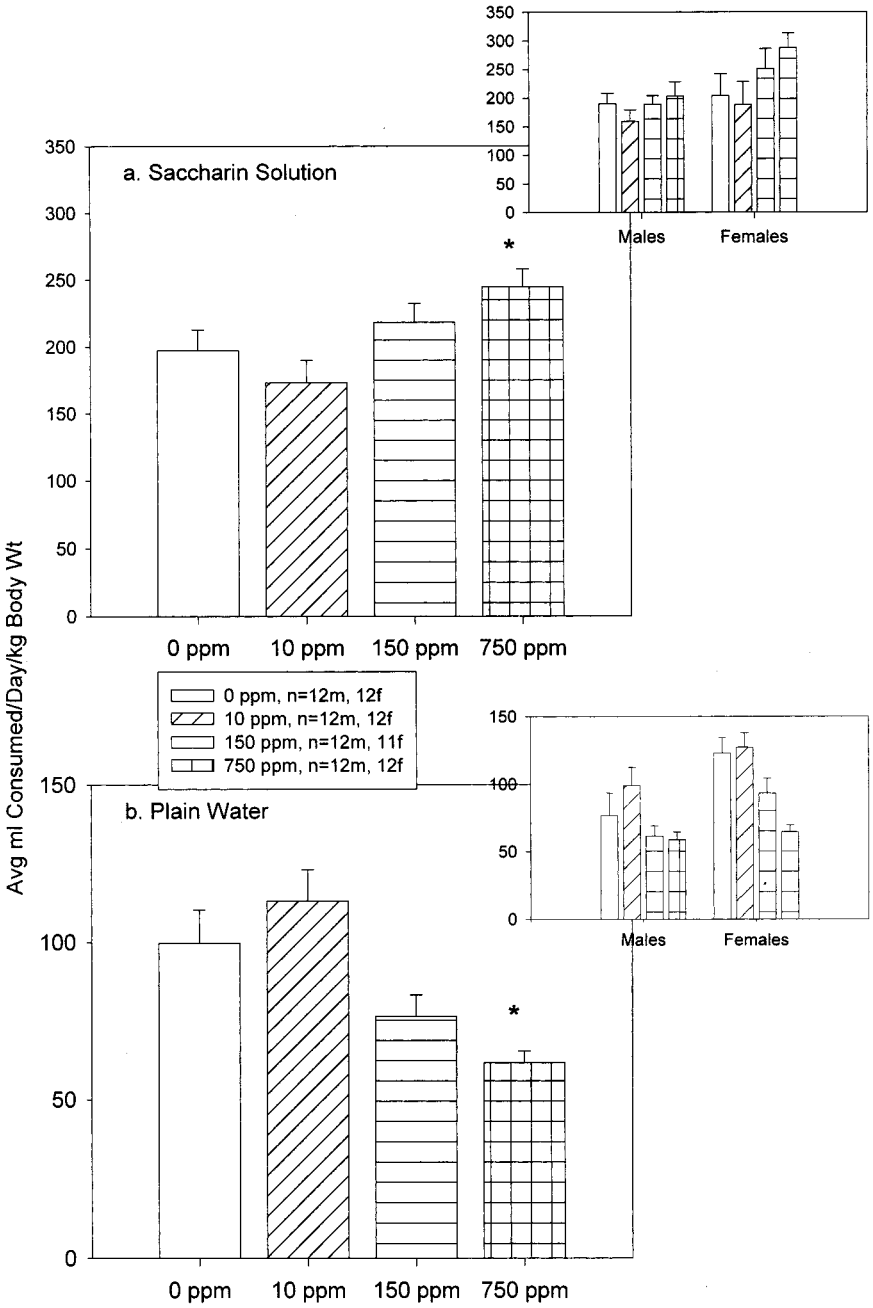

Figure 7. (a) Consumption of 0.03% saccharin solution (mL/day/kg) in one male and one female per litter on PND 69−71 (mean ± SEM). A significant effect of vinclozolin treatment indicated that the 750 ppm group consumed more than controls (*, $p < 0.05$). (Inset) There was not a significant sex by treatment interaction, although high-dose females consumed 41% more than same-sex controls. (b) Consumption of plain water when saccharin water was available (mL/kg/day) in one male and one female per litter on PND 69−71 (mean ± SEM). There was a significant effect of vinclozolin treatment with the high-dose group drinking less than controls (*, $p < 0.03$). (Inset) There was not a significant sex by treatment interaction.

female rodents. A separate but concurrent study is evaluating reproductive system physiology and will be reported at a later date.

Running wheel activity is a sexually dimorphic behavior after puberty, with female rats running more than males (*19*), confirmed here by the overall effect of sex (see Results). In females, neonatal treatment with testosterone ameliorated the pubertal increase and decreased adult running following exogenous estrogen (*20*, *21*). The decreased running in females exposed to the antiandrogen vinclozolin that is reported here appears to be a contradiction; if neonatal testosterone treatment caused decreased running, then neonatal treatment with an antitestosterone might be expected to increase running. Our protocol, however, included both neonatal and adult vinclozolin exposure, making adult effects on this behavior also possible. There are reports of increased running activity (related to overall androgen-induced increases in activity) in ovariectomized females (but not males) treated with androgen as adults (*22*). This suggests that although neonatal androgen will masculinize (i.e., decrease) running behavior in adult females, adult androgen exposure may hyperfeminize (i.e., increase) running behavior. The current results, a masculinization of running in females (but not males), suggest that *adult* vinclozolin exposure affects running behavior through its action as an antiandrogen. Because we do not report a dose-related trend, it appears that the threshold for a vinclozolin-induced decrease in running activity would be between

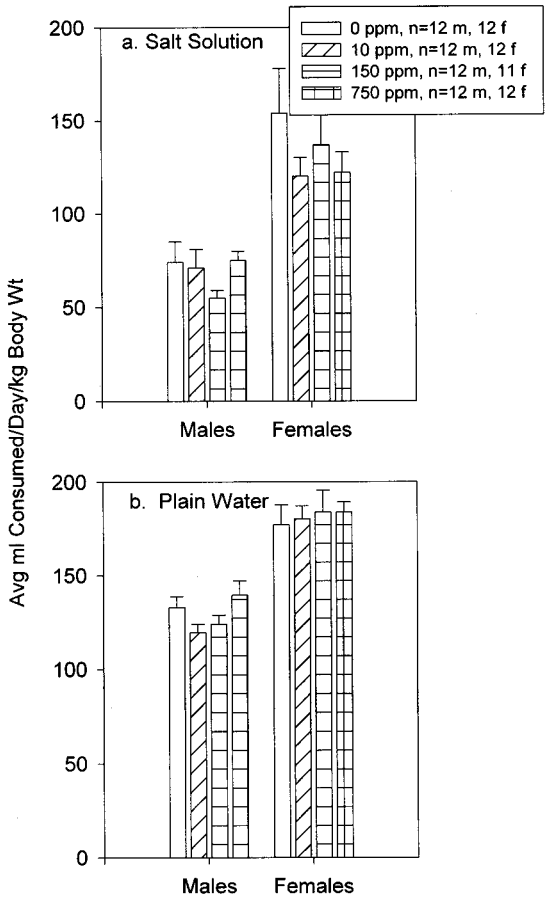

Figure 8. (a) Consumption of 3.0% sodium chloride solution (mL/day/kg) in one male and one female per litter on PND 73−75 (mean ± SEM). There were no significant treatment effects. (b) Consumption of plain water when saltwater was available (mL/day/kg) in one male and one female per litter on PND 73−75 (mean ± SEM). There were no significant treatment effects.

our middle dose of 12 mg/kg/day and our highest dose of 60 mg/kg/day.

The intake of saccharin-flavored solutions per kilogram of body weight is greater in female rats beginning at puberty (*23*), as confirmed here by the overall effect of sex (see Results). Several laboratories have reported sexually dimorphic effects on saccharin intake when corrections are made for body weight (*24*, *25*). Organizational actions of androgens are thought to mediate this behavior as neonatal testosterone exposure has been shown to decrease saccharin intake in adult females (*26*). Thus, an absence of androgenic stimulation appears to be required for the development of a female taste preference (i.e., high saccharin intake). Here, we report increased saccharin intake in high-dose vinclozolin animals that did not interact with sex (although females drank 41% more than same-sex controls, whereas males drank only 6% more than their controls). It is likely that the *neonatal* exposure to the antiandrogenic vinclozolin resulted in the observed increase. Our experimental design cannot distinguish effects of neonatal versus acute exposure, yet earlier reports show effects of neonatal testosterone treatment and no effects of adult treatment on saccharin intake (*27*, *28*). Thus, the typically female pattern of high intake of a saccharin-flavored solution may be susceptible to disruption, with females becoming hyperfeminized, following neonatal exposure to antiandrogenic compounds. As with running behavior, the lack of a dose-related trend suggests that the threshold for this effect lies between 12 and 60 mg/kg/day.

Our results suggest no vinclozolin-induced alterations in several of the assessed behaviors: open field activity, play behavior, and sodium solution intake. Body weight, food intake, and litter measures were likewise not severely altered in rats exposed to vinclozolin as assessed here. Because the sexual dimorphism in open field and play activity reported here is mild, it is possible that these methods were insensitive to potential vinclozolin-induced changes. However, sexual dimorphism in open field activity does not peak until PND 60 (*29*), the age at our last measurement, and sex differences in rodent play behavior are not always apparent even when there has been a period of social deprivation (*30*). Thus, although females had 6% more beam breaks and males pinned 33% more, indicating mild sexual dimorphism, more detailed assessments of these behaviors may indicate subtle vinclozolin-induced effects. The strong sex difference in sodium solution intake ($p < 0.0008$) reported here and the use of identical methodology that identified increased intake after exposure to several exogenous estrogens (*14*, *15*) strongly support the conclusion that the antiandrogenic vinclozolin has no effects on sodium solution intake.

In summary, these data indicate behavioral changes in adult females (i.e., decreased running wheel activity and increased saccharin intake) exposed to high doses of vinclozolin. There are also possibilities, not addressed in this study, that vinclozolin-induced effects on behavior will be magnified after multiple generations of exposure, that vinclozolin effects will occur primarily on reproductive behaviors, and/or that vinclozolin will act in a synergistic way with exposure to other hormone disrupters. Our data do support previous findings that androgens can modulate sexually dimorphic behaviors in the female rodent by interaction with the androgen receptor (*31*, *32*); they further suggest that some nonreproductive sexually dimorphic behaviors may be affected by environmental hormone mimics and that such behaviors may be more sensitive to disruption in females than in males.

SAFETY

Vinclozolin is a white, crystalline solid with a molecular weight of 286.11. As characterized by the manufacturer, Batelle (Columbus, OH), vinclozolin is stable under normal temperatures and pressures. The U.S. Environmental Protection Agency has classified vinclozolin as a Group C chemical, a possible human carcinogen.

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