

## **COMPROMISED FERTILITY IN FREE FEEDING OF WILD-CAUGHT NORWAY RATS (RATTUS NORVEGICUS) WITH A LIQUID BAIT CONTAINING 4-VINYLCYCLOHEXENE DIEPOXIDE AND TRIPTOLIDE**

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# COMPROMISED FERTILITY IN FREE FEEDING OF WILD-CAUGHT NORWAY RATS (*RATTUS NORVEGICUS*) WITH A LIQUID BAIT CONTAINING 4-VINYLCYCLOHEXENE DIEPOXIDE AND TRIPTOLIDE

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**Abstract:** Wild rat pests in the environment cause crop and property damage and carry disease. Traditional methods of reducing populations of these pests involve poisons that can cause accidental exposures in other animals and humans. Fertility management with nonlethal chemicals would be an improved method of rat pest population control. Two chemicals known to target ovarian function in female rats are 4-vinylcyclohexene diepoxide (VCD) and triptolide. Additionally, triptolide impairs spermatogenesis in males. A liquid bait containing no active ingredients (control), or containing triptolide (0.001%) and VCD (0.109%; active) was prepared to investigate the potential use of these agents for wild rat pest population control. Liquid bait was made available to male ( $n = 8$  control;  $n = 8$  active) and female ( $n = 8$  control;  $n = 8$  active) Sprague Dawley rats (*Rattus norvegicus*) for oral consumption prior to breeding. Whereas, control bait-treated females produced normal-sized litters ( $10.0 \pm 1.7$  pups/litter), treated females delivered no pups. Wild Norway male ( $n = 20$ ) and female ( $n = 20$ ) rats (*Rattus norvegicus*) were trapped, individually housed, and one group given free access to control bait, one group to active bait. Following three cycles of treatment-matched mating pairs, females consuming control bait (control) produced normal litter sizes ( $9.73 \pm 0.73$  pups/litter). Females who had consumed active bait (treated) produced no litters on breeding cycles one and two; however, 2 of 10 females produced small litters on the third mating cycle. In a fourth breeding cycle, control females were crossmated with treated males, and treated females were crossmated with control males. In both groups, some dams produced litters, while others did not. The differences in response reflect a heterogeneity in return to cyclicity between females. These results suggest a potential approach to integrated pest management by compromising fertility, and could provide a novel alternative to traditional poisons for reducing populations of wild rat pests.

**Keywords:** Fertility control, pest control, population management, *Rattus norvegicus*, wild Norway rat.

## INTRODUCTION

Norway rat (*Rattus norvegicus*) pests cause extensive crop loss, damage infrastructure, and are vectors for several zoonotic diseases.<sup>26</sup> The most common method used to reduce rat pest populations is the use of acute and anticoagulant rodenticides.<sup>25</sup> However, these poisons are non-specific and may kill nontarget animals, such as companion animals, that consume them.<sup>5</sup> They can also disable or kill predators that eat rats killed by poisons, such as hawks and owls.<sup>17</sup> The worst possible scenario is the accidental poison-

ing of humans, which can lead to death.<sup>1</sup> Due to these problems, the U.S. EPA has restricted the retail sale of rodenticides for residential use.<sup>9</sup> Additionally, these poisons do not cause a long-term reduction in rat populations because of a rapid reproductive rebound by the survivors and immigration of nearby rats.<sup>4</sup> Therefore, a more desirable approach to reducing and managing wild rat populations would be a nonlethal method of impairing reproductive capacity.

Repeated daily injection (intraperitoneal) of mice and rats with the occupational chemical 4-vinylcyclohexene diepoxide (VCD) has been well studied and found to cause specific loss of ovarian small (primordial) follicles by accelerating the natural process of atresia.<sup>10,24</sup> Mechanistic studies determined that VCD causes this *selective* follicle loss in rat ovaries by directly inhibiting the downstream signaling pathway regulated by the survival receptor, c-kit, located on the plasma membrane of the oocyte.<sup>19</sup> Targeting of this receptor induces oocyte degeneration in these

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small follicles. Mammalian females are born with a set cohort of oocytes contained in primordial follicles.<sup>14</sup> Because of the nonregenerating nature of the oocyte, loss of these follicles is irreversible, subsequently resulting in ovarian failure. Previous studies in mice demonstrated that VCD-induced follicle loss causes depletion of primordial follicles within 15 days of daily dosing, and an average of complete ovarian failure within 46 days of the cessation of dosing.<sup>20</sup> As a result, following loss of ovarian function, the mouse retains residual ovarian tissue. So this approach was used to generate an animal model for studies related to menopause-associated health risks.<sup>20</sup> VCD has also been shown to cause ovarian failure in rats, following repeated dosing.<sup>12,18,21</sup> Evidence has been provided that daily dosing of mice and rats with VCD, or the parent product 4-vinyl cyclohexene, does not produce generalized toxicity or effects in other tissues.<sup>22,23,27</sup> Therefore, use of VCD as a method of sterilization (ovarian failure) in female rat pests might hold promise as a nontoxic method of population control.

Triptolide is a major bioactive component in the traditional Chinese medicinal herb, *Tripterygium wilfordii* Hook F. It has been shown to cause menstrual cycle disruptions in women who are taking the herb as an anti-inflammatory and antirheumatic drug.<sup>28</sup> Treatment of female rats with triptolide lengthened the interval between estrous cycles and enhanced the amount of apoptosis in ovarian secondary follicles.<sup>15,28</sup> There was no effect on primordial follicles. Two mechanistic studies in cultured rat granulosa cells reported that triptolide in vitro disrupts the cAMP-dependent steroidogenic pathway, specifically by inhibiting expression of estrogen-synthesizing enzymes.<sup>29,30</sup> Additionally, *Tripterygium wilfordii* treatment causes infertility in male rats.<sup>8</sup> This infertility was reversible in 40% of treated males.<sup>11</sup> Thus, using triptolide as a potential for disrupting reproduction in wild female and male rat pests would be a practical approach to population reduction. Because triptolide disrupts gonadal function by targeting the growing and not the primordial population of ovarian follicles, effects of this compound in females would likely be reversible.

In a previous study, female SD rats consumed a liquid bait containing a combination of these chemicals (VCD and triptolide) for 15 days.<sup>2</sup> When mated with nontreated proven male breeders, smaller litter sizes were produced after a first breeding cycle. Thus, there was an immediate reversible infertility (triptolide effect). However,

in a second breeding cycle, litter sizes were restored to normal. Ultimately, continuous longer exposure to active bait would produce subsequent irreversible sterility (VCD effect). Additionally, in males, spermatogenesis would likely also be impaired (triptolide effect). Therefore, the objective of this study was to determine the breeding outcome when males were also exposed to active bait and to assess its effectiveness in wild-caught rats. This will test the feasibility of approaching wild rat pest control by a novel approach of fertility-population management combining triptolide and VCD in a liquid bait for oral consumption as an alternative to poisoning with rodenticides.

## MATERIALS AND METHODS

Liquid fertility control bait was obtained from SenesTech Inc. (Flagstaff, Arizona 86004, USA). 4-Vinylcyclohexene diepoxide was purchased from Sigma-Aldrich Chemical Co. (St. Louis, Missouri 63178, USA). Triptolide (>98% pure by HPLC analysis) was obtained from Stanford Chemicals (Irvine, California 92618, USA; [www.stanfordchem.com](http://www.stanfordchem.com)). Other proprietary materials were added to increase palatability of the liquid formulation.

### Animals

**Inbred rats:** Immature female SD (*Rattus norvegicus*) rats ( $n = 16$ , 21 days old) were purchased from Harlan Laboratories (Indianapolis, Indiana 46250, USA), and housed individually in ventilated cages. Mature SD males ( $n = 16$ ) were purchased from Harlan Laboratories, which were also individually housed in ventilated cages. Males were transferred from a previous protocol in which they had gone through six breeding rounds. Females and males were randomly separated into two groups ( $n = 8/\text{group}$ ) to be exposed daily to liquid emulsion (control bait), or liquid emulsion (active bait) VCD (0.109%) and triptolide, (0.001%) by voluntary oral consumption through a Dyet's feeding tube (35 or 50 ml). The oral dose of VCD was based on previous reports.<sup>2,3,22</sup> Following 21 days of daily exposure, bait was discontinued and individual pairs of treatment-matched males and females were housed for a 15-day breeding cycle. After delivery of litters, pups were counted, and euthanized on postnatal day (pnd) 4, 5, or 6. A second feeding and breeding round was conducted. Following the second breeding cycle, dams were euthanized and ovaries collected. Ovaries were fixed and pro-

cessed for histological evaluation by follicle classification and counting as described below. All experiments were carried out according to a SenesTech Institutional Animal Care and Use Committee approved protocol 01-13.

### Wild-caught rats

For bait consumption and breeding studies, sexually mature, wild-caught male ( $n = 20$ ) and female ( $n = 20$ ) Norway rats, *Rattus norvegicus*, were live-trapped with permission on privately owned property near Fort Collins, Colorado, USA. Rats were doused with Delta Dust to eliminate any ectoparasites before being transported to the National Wildlife Research Center (NWRC) in Fort Collins, Colorado. Upon arrival at NWRC, rats were housed individually in cages and provided with commercial rat chow and apple or carrot slices, and water ad libitum at all times. Rats were also provided with shelters, burlap pieces or shredded paper for nesting, and chew sticks for enrichment. The animal room where the rats were housed was maintained at 72°C, ambient humidity, and 12-hr on-off light cycle. All rats were quarantined for a 3.5-wk period to allow for any females that were pregnant at the time of capture to have their litters.

Males and females were housed individually for a period of 5 days before exposure to liquid bait. The experimental design was as follows. Rats were housed and given daily access (4 PM–9 AM) to control liquid bait ( $n = 10$  male,  $n = 10$  female; control) or active liquid bait ( $n = 10$  male,  $n = 10$  female; VCD, 0.109%; triptolide, 0.001%; treated) for a total of 50 days with breeding cycles, as follows. Males and females were housed individually for 15 consecutive days (days 1–15). On day 16 of access to bait, control females and males, and treated females and males were housed as individual pairs for mating (treatment-paired, randomly assigned, breeding cycle 1) with continued access to bait (days 16–35). Females and males were separated for 2 wk (rest cycle days 36–50) to allow pups to be born and counted. At that time access to bait was withdrawn. Two more breeding and rest cycles were performed (days 51–95; days 96–130). For each breeding cycle, treatment-paired males and females were randomly reassigned to determine fertility of males. For a fourth consecutive breeding cycle, control females were crossbred with treated males, or treated females were crossbred with control males (days 151–172). Males were removed, euthanized by CO<sub>2</sub> followed by cervical dislocation, and testes and

epididymi collected (day 172) for weighing and fixation for transport to SenesTech Inc., Flagstaff, Arizona for histological evaluation. Pup numbers were counted when litters were born (~ days 172–196; ~ days 122–146 after removal of bait). Pups were euthanized with isoflurane exposure on postnatal day 4, 5, or 6. Following delivery, dams were euthanized with CO<sub>2</sub> followed by cervical dislocation, and ovaries and uteri were collected, weighed, and fixed for transport to SenesTech Inc., Flagstaff, Arizona for histological evaluation.

All experiments were carried out according to an NWRC Institutional Animal Care and Use Committee approved study protocol (QA-2151).

### Tissue collection

Following the pairing for the final breeding round, male rats were euthanized and organs were collected. Testes and epididymi were removed. Individual wet weights of testes and epididymi were normalized to individual body weights. To determine testis volume, the long and short axis of the testis was measured, to the nearest 0.1 mm, with dial calipers. Testis volume was estimated using the formula for a prolate spheroid ( $\text{length} \times \text{width}^2 \times 0.523$ ).

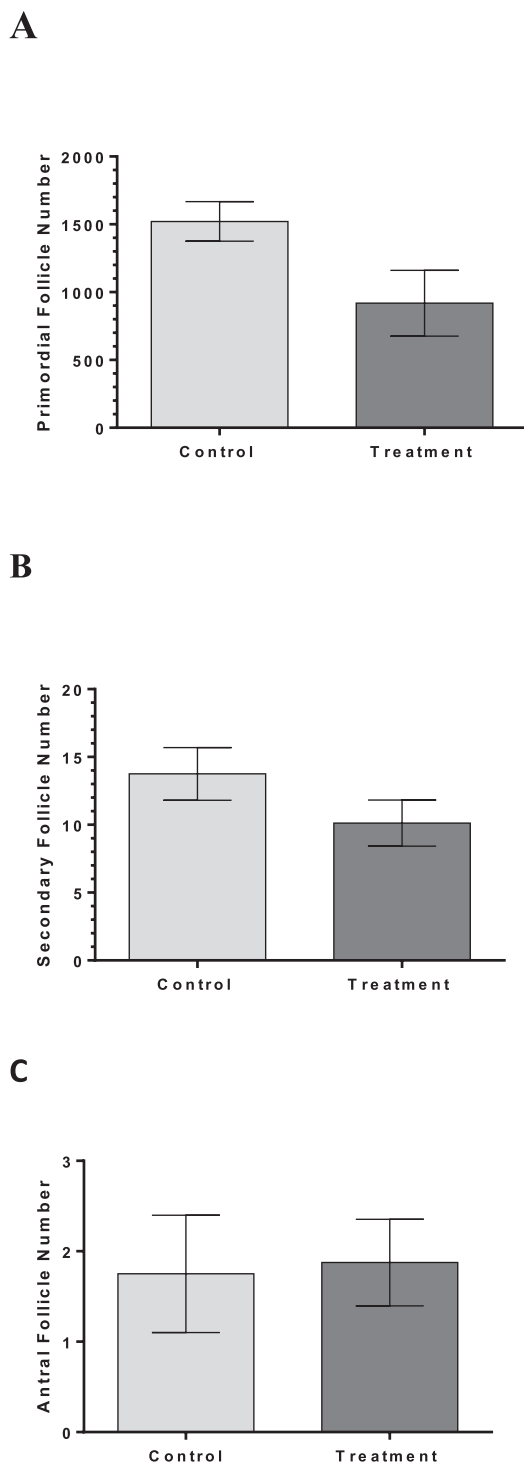
Following parturition of the final litter in the final breeding round, female rats were euthanized and organs were collected. Individual wet weights of the ovaries and uteri were normalized to individual body weights. If females were determined by vaginal cytology to be in estrus at the time of tissue collection, uterine wet weights for those animals were not included in those data.

For both male and female rats, kidneys, adrenal glands, livers, and spleens were removed and trimmed of fat and connective tissue. Wet weights of individual organs from each rat were normalized to body weight.

### Histological evaluation

Testes were fixed in modified Davidson's fluid for 48 hr, stained with hematoxylin and eosin, and sectioned (5  $\mu\text{m}$  thick). Every 10th section was microscopically observed and evaluated for presence of germ cell development and sperm.

Ovaries were fixed in 10% neutral phosphate buffered formalin, stained with hematoxylin and eosin, and sectioned (5  $\mu\text{m}$  thick). Oocyte-containing follicles were classified and counted in every 10th section.<sup>21</sup> Primordial follicles contained an oocyte surrounded by a complete ring of squamous granulosa cells; primary follicles contained



**Figure 1.** Effect of active bait on ovarian follicle numbers in inbred rats. Female SD rats were treated daily with control (light bars) or active (dark bars) bait as described in methods. Following dosing, ovaries were collected for histological evaluation as described

an oocyte surrounded by a complete layer of cuboidal granulosa cells; secondary follicles contained an oocyte surrounded by more than one layer of granulosa cells; and antral follicles contained an oocyte surrounded by multilayered granulosa cells and an antral cavity. Follicles were only counted if the oocyte nucleus was visible. The evaluator was blinded to treatment.

### Circulating testosterone and estrogen

Serum was collected and sent to Idexx Health and Sciences (Columbia, Missouri 65201, USA) for analysis of testosterone and 17 $\beta$ -estradiol.

### Statistical analyses

Comparisons of tissue weights and ovarian follicle counts were conducted using unpaired, two-tailed *t*-tests with  $\alpha$  set at 0.05. Comparison of litter sizes among treatment-matched and cross-bred pairings were conducted using a Kruskal-Wallis test with  $\alpha$  set at 0.05. Analyses were performed using GraphPad Prism (Version 6.05, GraphPad Software, Inc., La Jolla, California 92037, USA). All means are presented with their standard errors.

## RESULTS

### Inbred SD rats

Male and female SD rats (individually housed) were given daily access to control or active bait, then paired for breeding. Following delivery, pups were counted. After two breeding rounds, dams and males given control bait (control) averaged  $10.0 \pm 1.7$  pups/litter, whereas those given active bait (treated) delivered no pups. Compared with ovaries from control animals, there was a nonsignificant ( $P = 0.056$ ) trend for fewer primordial follicles in treated animals (Fig. 1). There were no differences ( $P > 0.05$ ) between groups in numbers of secondary or antral follicles.

### Wild-caught Norway rats

A second experiment was conducted to determine whether wild-caught Norway rats would also voluntarily consume control and/or active bait, and if treatment would impact litter size following mating.

in methods. Follicles were classified as primordial (A), secondary (B), or antral (C). Values are mean numbers  $\pm$  SE.



Individual daily bait consumption was measured over the 2 wk prior to the onset of each of breeding cycles one and two. Mean daily bait consumption was not different ( $P > 0.05$ ) in treated males ( $8.12 \pm 0.21$  ml/day per rat) compared with controls ( $10.63 \pm 0.18$  ml/day per rat). Whereas, mean daily bait consumption was lower ( $P < 0.05$ ) in treated females ( $3.17 \pm 0.15$  ml/day per rat) than in the control group ( $9.21 \pm 0.51$  ml/day per rat). There was no difference ( $P > 0.05$ ) in daily consumption of bait between control males and control females.

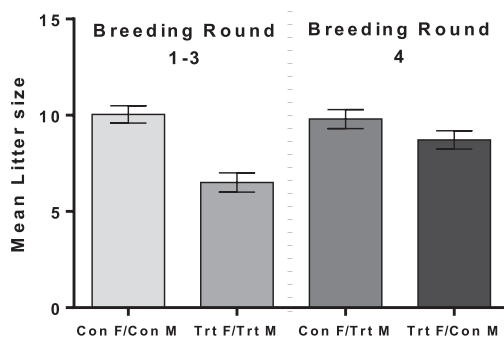
Mean body weight gain over the 50-day baiting period was not different ( $P > 0.05$ ) in males that had consumed control bait ( $12.0 \pm 8.7$  g gain) compared with those consuming active bait ( $14.5 \pm 8.3$  g gain). There was also no difference ( $P > 0.05$ ) between groups during that time in females ( $7.0 \pm 4.0$  g gain, controls;  $-5.0 \pm 5.2$  g loss, treated).

In the treatment-paired mating rounds mean litter size was different ( $P < 0.001$ ) between groups (control,  $9.73 \pm 0.73$  pups/litter; treated,  $0.43 \pm 0.28$  pups/litter). In treated animals, no pups were born in the first two breeding cycles. Two dams from this group gave birth to litters (6 and 7 pups, respectively) on the third breeding cycle (66 days at mating after bait removal).

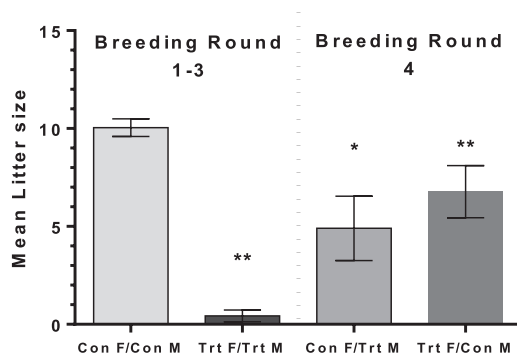
In the crossbreeding round, 5/10 control females had litters when mated with treated males, and 7/10 treated females had litters when mated with control males. However, when analyzing litters born to the 5 dams who had pups in the group of control females mated with treated males, mean litter size was  $9.80 \pm 0.45$  pups/litter. This was not different ( $P > 0.05$ ) from mean litter size in the control-matched group ( $10.04 \pm 0.45$  pups/litter, Fig. 2A). When analyzing litters born to 7 dams who had pups in the treated females mated with control males group, mean litter size was  $8.71 \pm 0.47$  pups/litter which was also not different ( $P > 0.05$ ) from the control group. (Note: in the latter group, 1 dam produced 2 pups, and was omitted from the calculation of individual litter size as an outlier). Taken as a whole group, mean litter sizes were reduced ( $P < 0.05$ ) in the crossbreeding round relative to treatment-paired breeding ( $4.90 \pm 1.64$  pups/litter, control females mated with treated males;  $6.11 \pm 1.41$  pups/litter, treated female mated with control male (Fig. 2B).

Relative to controls, testes weights in treated males were lower ( $P < 0.05$ ; Fig. 3A). Testis volume was also lower ( $P < 0.05$ ) than controls in treated males (Fig. 3B). Likewise, epididymis

## A females with litters

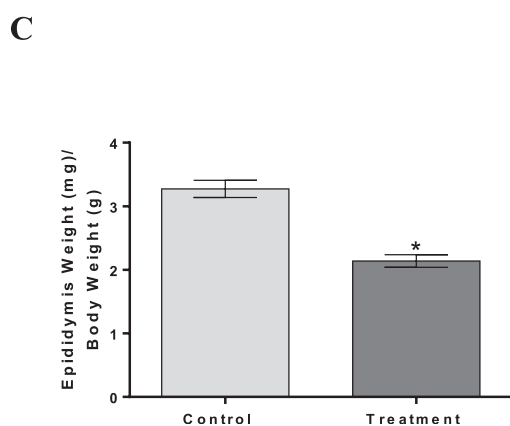
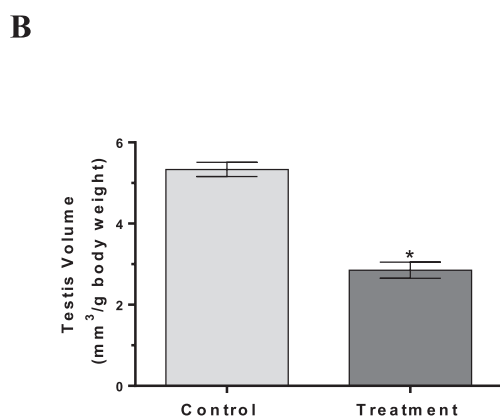
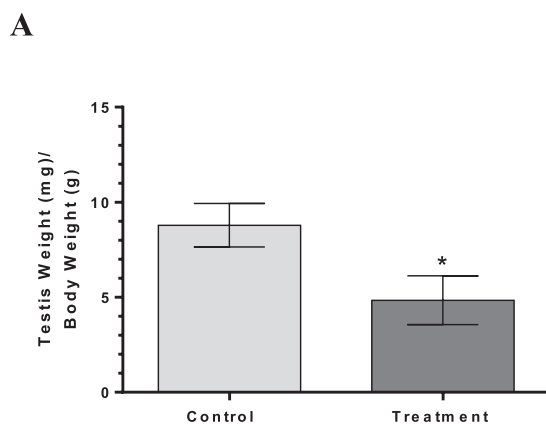


## B all females

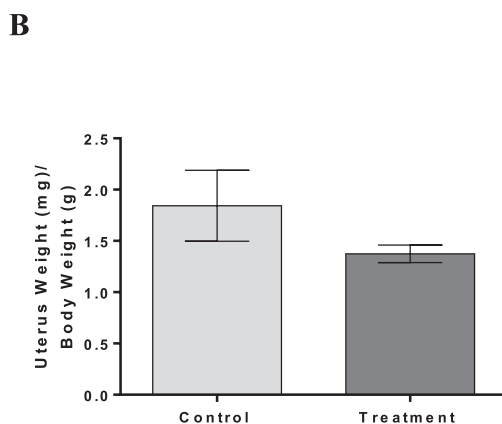
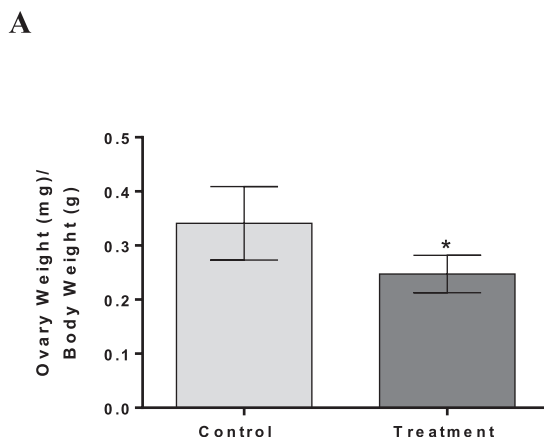


**Figure 2.** Litter sizes in wild-caught Norway rats following consumption of active bait. Wild-caught Norwegian rats were trapped, housed, exposed to bait, and mated as described in methods. Rats were treatment-matched by bait (control female paired with control male, con/con; active bait female paired with active bait male, trt/trt) for three breeding rounds. In the fourth breeding round rats were crossmated (control female paired with treated male; treated female paired with control male). Mean litter size in (A) only those females having pups, (B) all females (representative population). Note: values in the treatment-matched groups are average means from the first three breeding rounds. Values are mean litter size  $\pm$  SE for each group. \* $P < 0.05$ ; \*\* $P < 0.01$  different from con/con.

weights were lower ( $P < 0.05$ ) in treated males, compared with controls (Fig. 3C). Circulating testosterone levels in males at the time of tissue collection were not different ( $P > 0.05$ ) between groups ( $0.83 \pm 0.15$  ng/ml control;  $0.99 \pm 0.19$  ng/ml treated).



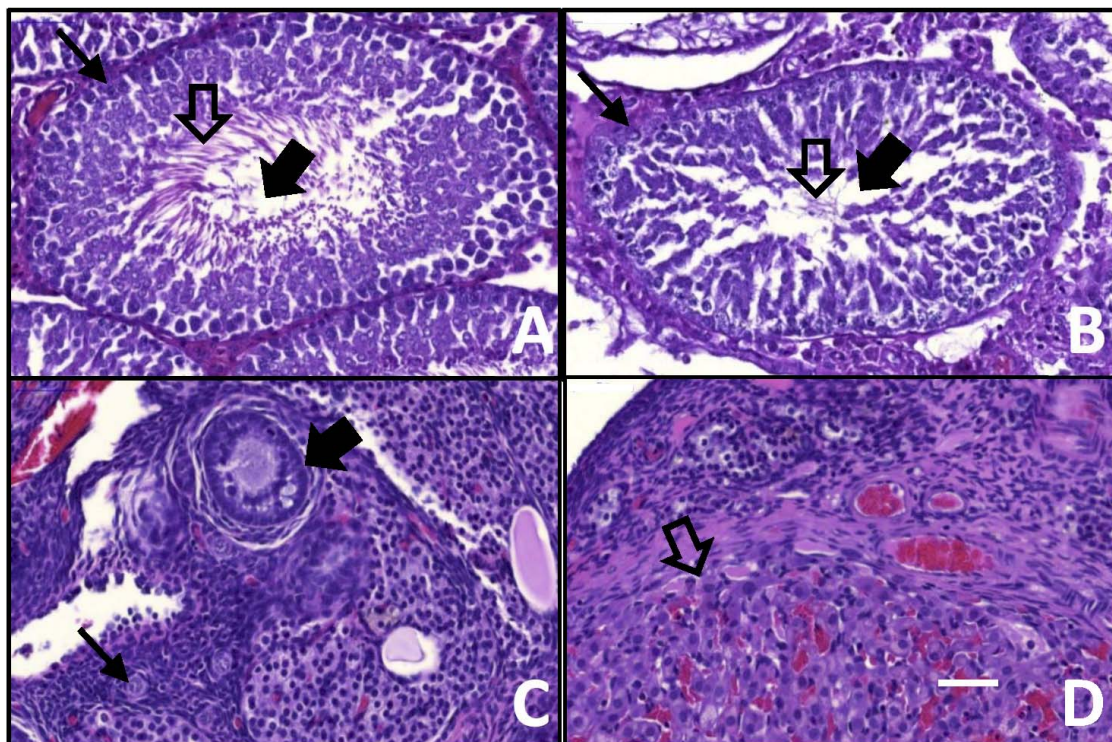
**Figure 3.** Testes and epididymi weights in males consuming active bait. Wild-caught Norway male rats were allowed to consume control (light bars) or active (dark bars) bait as described in methods. **A.** Following four breeding rounds, testes were collected and weighed. **B.** Testicular volume was calculated as



**Figure 4.** Ovarian and uterine weights in females consuming active bait. Wild-caught Norway female rats were allowed to consume control (light bars) or active (dark bars) bait as described in methods. Following four breeding rounds, ovaries (**A**) and uteri (**B**) were collected and weighed. Values are mean mg/gm body weight  $\pm$  SE with ovaries pooled for each animal. \* $P < 0.05$  different from control.

Ovarian weights were lower ( $P < 0.05$ ) in treated females than in controls (Fig. 4A). Uterine wet weights were not different ( $P > 0.05$ ) between groups (Fig. 4B). Circulating  $17\beta$ -estradiol levels were measured in females and there were no differences ( $P > 0.05$ ) between groups: control,  $46.34 \pm 6.02$  pg/ml; treated,  $51.63 \pm 5.14$  pg/ml.

described in methods. **C.** Epididymi were weighed. Values are mean mg/gm body weight  $\pm$  SE with testes and epididymi pooled for each animal. \* $P < 0.05$  different from control.



**Figure 5.** Micrographs of testes and ovaries from control and treated rats. Wild-caught Norway male (A, B) and female (C, D) rats were allowed to consume control (A, C) or active (B, D) bait as described in methods. Following four breeding rounds, testes and ovaries were collected, fixed, sectioned, and stained with hematoxylin and eosin. A, B. Seminiferous tubules: small arrow = spermatogonia; open large arrow = spermatozoa; closed large arrows = adluminal space. C, D. Ovarian sections: small arrow = healthy primordial follicle; closed large arrow = healthy growing follicle; large open arrow = corpus luteum. Magnification,  $\times 40$ . Bar = 50  $\mu\text{m}$ .

Testes from male rats were prepared for histological evaluation. Micrographs of a representative seminiferous tubule from a control and a treated rat are shown in Figure 5. The control rat tubule (Fig. 5A) contains a well-organized layer of spermatogonia lining the basement membrane with numerous spermatozoa filling the adluminal space. Conversely, the tubule from the treated rat (Fig. 5B) shows disorganized placement of germ cells, loss of integrity in the germ cell epithelium, and a greatly reduced number of spermatozoa in the adluminal space.

Ovaries from control and treated females contained numerous corpora lutea and oocyte-containing follicles of all sizes (Fig. 5C, D). Those from treated animals contained atretic follicles with pyknotic nuclei (Fig. 5D). Relative to control females, in treated females there was a reduction ( $P < 0.05$ ) of 53% in primordial, 35% in primary, and 48% in secondary follicles (Fig. 6). There were no differences ( $P > 0.05$ ) in numbers of antral follicles between groups (control,  $3.4 \pm$

$0.73$  follicles counted; treatment,  $3.3 \pm 0.06$  follicles counted).

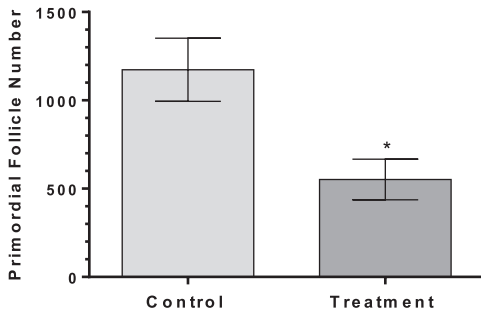
Compared with controls, there was no effect of treatment ( $P > 0.05$ ) on normalized wet weights of adrenals, kidneys, spleen or livers, or between males and females (Table 1).

## DISCUSSION

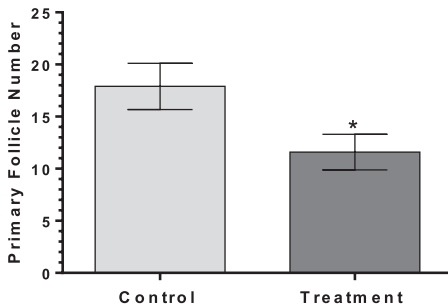
A previous study had observed reduced litter size in female SD rats exposed to the liquid active bait; however, no effect of treatment on males was measured.<sup>2</sup> Therefore, the initial experiment was performed in an inbred SD strain of male and female rats to determine whether consumption by both males and females of the active liquid bait would affect litter size. Litter sizes in control males and females were in a normal, expected range. No pups were born to treated dams that had been mated with treated males. Follicle counts in ovaries collected from treated dams showed a trend ( $P < 0.1$ ) for reduced numbers of primordial follicles as compared with those from



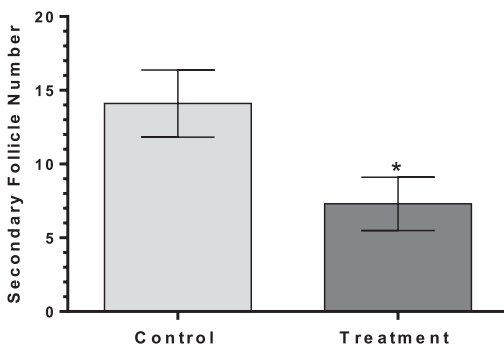
A



B



C



**Figure 6.** Effect of active bait on ovarian follicle numbers. Wild-caught female rats consumed control (light bars) or active (dark bars) bait as described in methods. Following dosing, ovaries were collected for histological evaluation as described in methods. Folli-

cles were classified as primordial (A), primary (B), or secondary (C), and counted. Values are mean numbers  $\pm$  SE. \* $P < 0.05$  different from control.

control dams. There were no differences in numbers of larger follicles between those groups. VCD has a selective effect on primordial follicles, whereas triptolide is known to damage larger ovarian pre-antral and antral follicles.<sup>13,28</sup> Because primordial follicles were trending to be reduced by active bait treatment, VCD appears to have started to have its predicted effect. In addition to its effect in females, triptolide also impairs spermatogenesis in males.<sup>8</sup> The surprising result of no litters born in treated rats suggests that this is the result of triptolide affecting males and females. This might be due to mating avoidance on the part of males, females, or both. Alternatively, it could be because of direct gonadal effects in the rats. There was no difference ( $P > 0.05$ ) in numbers of larger follicles in ovaries from control versus treated dams. This supports that the triptolide effect was solely on males. However, in the time between active bait exposure and collection of ovaries (45–56 days), remaining primordial follicles could have been recruited to repopulate the larger growing pool. Further investigation is required to distinguish between these possibilities.

With the demonstration that active bait is effective at curbing reproduction in the inbred rat strain, an experiment was designed to determine whether the same outcome would result in wild-caught rats. Control and treatment rats voluntarily consumed control or active bait, respectively, over a daily 17-hr period of time for 50 days. There was no difference in consumption of control versus active bait in males, although more control bait was consumed by females compared with active bait. The reason for this difference in females is unknown; however, this finding demonstrates that wild rats will voluntarily consume active bait. Body weight gain during the bait-consuming time was similar between males and females, and in both treatment groups.

Throughout three breeding cycles of treatment-paired males and females, there was an expected litter size of ~9–10 pups in control animals. Interestingly, in animals that had only consumed active bait during the first breeding cycle, no litters were born in the first two breeding cycles, and two smaller litters were born in the third breeding cycle. The lack of pups born in the first two cycles is the same as the results obtained with

cles were classified as primordial (A), primary (B), or secondary (C), and counted. Values are mean numbers  $\pm$  SE. \* $P < 0.05$  different from control.

**Table 1.** Body and tissue weights in wild-caught Norway rats following consumption of control or active bait. Values are mean gm  $\pm$  SE, normalized to body weight. Adrenals from each animal were pooled.

Treatment	Sex	Body weight (g)	Adrenal	Kidney	Spleen	Liver
Control bait	Male	388.5 $\pm$ 14.16	0.3008 $\pm$ 0.02188	6.843 $\pm$ 0.3110	2.510 $\pm$ 0.2252	36.99 $\pm$ 1.243
	Female	309 $\pm$ 10.43	0.3539 $\pm$ 0.01396	7.433 $\pm$ 0.1669	2.856 $\pm$ 0.2362	36.72 $\pm$ 1.396
Active bait	Male	397.5 $\pm$ 10.94	0.2691 $\pm$ 0.01410	7.072 $\pm$ 0.2891	2.584 $\pm$ 0.2015	37.11 $\pm$ 0.6858
	Female	311.0 $\pm$ 12.99	0.3653 $\pm$ 0.02761	6.851 $\pm$ 0.3474	3.217 $\pm$ 0.4687	37.80 $\pm$ 2.249

SD rats. The results of a few small litters in the third cycle likely reflect that the initial triptolide effect had become reversed since active bait had been withdrawn, and male and female fertility had begun to return.

The fourth cycle was a crossbreeding between control females and treated males, as well as treated females and control males. Whereas some of the dams in both groups gave birth to relatively normal-sized litters (8–11 pups), others did not have any pups. This observation suggests that triptolide caused infertility in both females and males, and the effect had reversed in some females and males and not in others. In analyzing the means of each of the two crossbred groups as a representative population of wild rats, mean pup numbers were reduced compared with treatment-paired control animals and greater than that in treatment-paired treated animals. From this experiment, it appears that fertility had returned more readily in treated females (7/10 litters) than in treated males (5/10 litters). It should also be noted that the time since removal of active bait from the treated animals had been 115–122 days by the fourth breeding cycle. Thus, it is not surprising that this reversible effect was not permanent, and sterility had not occurred in females or in males. Whereas this might be desirable if the goal is to provide a reversible form of fertility control to be effective at reducing wild rat populations, more frequent or continuous exposure to active bait would be required to cause persistent infertility. However, it is also of note that in an unrestricted environment, even a reduction in litter numbers and litter sizes in response to limited bait consumption would provide a beneficial effect on population control.

Weights of testes and epididymi taken from treated males were lower than in controls. This would result from a direct effect of triptolide targeting epididymal sperm viability as reported in other studies.<sup>8</sup> In a longer-term study, triptolide was shown to also directly target testicular spermatogenesis.<sup>11</sup> Histological evaluation of tes-

tes from treated males revealed numerous unhealthy-appearing seminiferous tubules with visibly reduced numbers of spermatozoa, compared with those from control males. This provided morphological evidence of reduced fertility in treated males. Circulating testosterone levels were not different between the two groups. Testosterone levels represent the point in time of sample collection, rather than an ongoing effect on spermatogenesis. Therefore, reversibility of the triptolide effect may have restored testicular function. Conversely, high levels of testosterone in the testes and reproductive tract are required to support spermatogenesis.<sup>6</sup> Therefore, circulating levels may not be affected as markedly as those concentrated within the testes and subsequently the epididymi. Further testing is required to distinguish between these possibilities.

Weights of ovaries taken from treated females were lower than those in controls, however, uterine weights were not different between groups. Reduced ovarian weight would be the result of reduced large follicle development, a known effect of triptolide.<sup>28</sup> Uterine weights are directly affected by the trophic effects of 17 $\beta$ -estradiol produced in ovarian large antral follicles.<sup>16</sup> There were no differences in circulating 17 $\beta$ -estradiol levels between groups of females. Thus, as with males, the lack of apparent steroid (estrogen) effects on the reproductive tract (uterus) may reflect the reversibility of triptolide and return of follicle development.

Histological evaluation of ovaries demonstrated a reduction in numbers of primordial follicles in wild-caught treated females compared with controls. This is evidence of destruction of primordial follicles by VCD.<sup>13</sup> The reduction in primary and secondary follicles demonstrates less recruitment of those populations from the reduced primordial follicle pool.<sup>13</sup> Clearly, VCD destruction of primordial follicles had not been complete during the exposure time frame. In order for sterility to result in a female, destruction of the entire primordial follicle pool needs to be complete.<sup>20,21</sup> If not, females will continue to

undergo estrous cycles for a protracted period of time.<sup>21</sup>

There was no difference between groups in numbers of antral follicles. As with uterine weights, this would result if triptolide effects on larger follicles had become reversed. Taken together, the findings support that triptolide effects on males and females caused the initial reduction in fertility, which had started to reverse once exposure to active bait had ceased. Further, VCD had begun to affect primordial follicles in females, however, sterility (irreversible) had not yet been achieved. Future studies are being planned to determine the optimal exposure time and concentration of active ingredients that will be sufficient to cause initial infertility in males and females, with ultimate sterility in females. Under those conditions, dramatic reductions in populations of wild rat pests could be expected.

There were no effects of active bait on normalized adrenal, kidney, spleen, or liver weights. Likewise, there was no difference in those weights between males and females. These results demonstrate that there are no gross effects of VCD or triptolide on those tissues, and are in support of selective effects of those chemicals on the reproductive system.

In summary, the results presented here demonstrate that voluntary consumption of active bait inhibits fertility in male and female rats for up to 122 days following cessation of a 50-day exposure. Furthermore, crossbreeding demonstrates that active bait consumption affects fertility in both females and males. Therefore, the approach of using active liquid bait for fertility-population control in environments infested with wild rats may prove to be a feasible alternative to the use of poisonous rodenticides.

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