Mate preference for dominant vs. subordinate males in young female Syrian hamsters
(*Mesocricetus auratus*) following chemically-accelerated ovarian follicle depletion

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Abstract

Life history theory predicts that selectivity for mates generally declines as females age. We previously demonstrated this phenomenon in Syrian hamsters (*Mesocricetus auratus*), in that older females showed reduced preference for dominant over subordinate males. To test the hypothesis that decreased reproductive quality due to aging reduces mate preference, we decoupled reproductive and chronological age by treating young female hamsters with 4-vinylcyclohexene diepoxide (VCD), which destroys ovarian follicles and functionally accelerates ovarian follicle depletion without compromising the general health of rodents. In this study, VCD effectively reduced follicle numbers in young Syrian hamsters. VCD-treated and control females were allowed to choose between a dominant and a subordinate male in a Y-maze on the day of proestrus. Both VCD-treated and control females demonstrated preference for the dominant male by leaving a greater proportion of vaginal scent marks near him, which is a behavior females display when soliciting prospective mates. However, there was no effect of treatment on the proportion of vaginal scent marks left for the dominant male. Furthermore, ovarian follicle numbers were not significantly correlated with any behaviors in either group. We conclude that accelerated ovarian follicle depletion does not reduce mate preference in young female hamsters.

Key words: mate preference, hamster, ovary, aging

1. Introduction

In mate selection, females must weigh the benefits of being choosy (e.g. fertility and genetic quality of mate) against the costs (e.g. time, energy, predation risk). Life history theory predicts that females of advanced age should be less choosy than younger females, given the age-associated decline in fertility/fecundity and increased cost of remaining selective. Indeed, old age is associated with reduced selectivity [1-4] and greater variability in mate preference in several species (e.g. guppy, cricket, cockroach, and moth) [1, 4]. While studies on age and mate choice in mammals are limited, recent evidence suggests that old (15 months old) female Syrian hamsters (*Mesocricetus auratus*) have reduced preference for dominant males as compared to young females [5]. Young females of this species generally show strong preference for dominant over subordinate males [6-9].

There are several physiological factors that might explain why older females demonstrate reduced mate selectivity, including the age-related decline in reproductive potential. Moore and Moore [2] found that older female cockroaches (*Nauphoeta cinerea*) are less choosy than young females, and they indicated that this decrease in choosiness was correlated with the age-related decline in reproductive quality, as determined by the number of offspring produced. However, the relative importance of reproductive age in female mate choice has yet to be explicitly tested. Like the cockroach, the age at which female hamsters show reduced mate preference (15 months) is also an age at which fertility has significantly declined [10]. Reproductive aging in female mammals occurs at a more rapid rate than other physiological systems, and it is due in part to a progressive decrease in the size and quality of the ovarian follicular pool [11]. Additionally, the rate of ovarian senescence is variable among individual females depending on their life history (i.e. season of birth, parity) [12] and genetic background [13]. Therefore,

reproductive aging can vary among individuals and it is not always correlated with chronological age.

The purpose of this study was to determine if ovarian follicle depletion contributes to reduced female mate preference and selectivity for a mate, as we previously observed in chronologically old Syrian hamsters [5]. In the previous study we compared mate preferences of 6- and 15-month-old female hamsters, and we found young females left a significant proportion of vaginal scent marks in the vicinity of the dominant male, whereas 15-month-old females did not. For the present study, we decoupled ovarian and chronological age by treating young (8-week-old) female hamsters with 4-vinylcyclohexene diepoxide (VCD), a toxicant that accelerates ovarian follicle loss. VCD is specifically lethal to preantral follicles in rodents, which accelerates ovarian senescence [14] without compromising brain function [15] or other body systems [16, 17]. Accordingly, VCD is often used in rodents as a tool to study the transition period and physiological consequences of age-related reproductive failure in females [18-20], and it is increasingly used to study female behavior associated with ovarian senescence [15, 16, 21, 22]. To determine if ovarian age influences selectivity for potential mates, VCD-treated females were allowed to choose between a dominant and subordinate male on the day of proestrus. We inferred mate preferences from scent marking behavior and the amount of time females spent near each prospective mate [5], and compared the behaviors of VCD- and vehicle-treated females.

2. Materials and methods

2.1 Animals

Eight-week-old Syrian hamsters were purchased from Charles River Laboratories (Wilmington, MA). Upon arrival at our facility, animals were housed individually in polycarbonate cages. Food (Prolab 1000, Syracuse, NY) and water were available *ad libitum*. Hamsters were maintained on 14 h of light/day with lights on between 20:00 and 10:00 EST. Ambient temperature and relative humidity were held constant at 21 ± 5 °C and $50 \pm 10\%$ respectively. Males and females were housed in one room, and individuals were transported to a separate empty room for behavioral observation. Ovaries from five 15-month-old female hamsters were obtained from a breeding colony maintained at Cornell University. All experimental procedures were approved by Cornell University's Institutional Animal Care and Use Committee.

2.2 VCD treatment

Eight-week-old female hamsters were treated with 400 mg/kg VCD (Sigma Aldrich, St. Louis, MO) once daily for 10 days by i.p. injection. This dose was based on a pilot experiment in which 8-week-old hamsters treated with 400 mg/kg had follicle numbers comparable to those counted in the ovaries of 15-month-old females. Control females were given daily vehicle (1:1 mixture of 0.9% saline and DMSO) injections. Injections were administered under isoflurane anesthesia (3%) during the light phase of the light-dark cycle (between 08:00 and 10:00 EST).

2.3 Behavioral tests and observations

Behavioral testing was conducted between 1 and 9 weeks after the final VCD injection, over a period of three days. This included the metestrous, diestrous, and proestrous phases of the

4-day estrous cycle. Mate preference was observed on the day of proestrus, a time at which females actively solicit mates [23]. Females were tested in pairs, which included one VCD-treated and one control female of the same age with synchronous estrous cycles. Behavioral observations were made between 10:15 and 12:00 under dim white light to facilitate video recording. No more than two pairs of females were tested per week. The day before testing began each female was confirmed to be in estrus by placing a non-experimental male into the cage and monitoring for lordosis, evident by the arching of back and tail up posture that is characteristic of receptive females in estrus [23]. The same male was used throughout the experiment, and he was not allowed to mount the females. All animals were sexually naïve at the time of behavioral testing.

Days 1 and 2 (metestrus and diestrus): Establishment of male dominance relationships

The first day of testing occurred on the day of metestrus. Females observed the formation of a dominant-subordinate relationship between two unrelated males in a three-compartment Plexiglas arena, as described previously [5]. Males within a dyad were of the same age (mean age across all dyads: 18.5 ± 0.7 wk) and were matched for body mass ($<7.5\%$ difference within a dyad). Test females (one VCD-treated and one control) were placed in separate enclosures (50 cm x 23 cm x 31 cm) at opposite ends of the arena. Males were placed in the central enclosure (50 cm x 44 cm x 31 cm), where they were separated from the females by coarse wire mesh framed with Plexiglas. Each male was marked by placing a small piece of green or yellow tape on the dorsum. Males were placed in the central compartment of the arena one by one and covered with an open-ended Plexiglas container to isolate them from each other and the females before the observations began. The isolation containers were removed and the males were observed for 5 minutes.

The males were monitored for signs that a dominant-subordinate relationship had been established. Males of this species enter into rolling fights and bite and kick until one has established dominance [8]. Subordinance is easily recognized when a male displays the tail up posture following one of these rolling fights [8, 23].

The first observation period was immediately followed by a 10-minute rest period in which the males were returned to their home cages. Females remained in their enclosures in the arena. The first day of observation concluded with a second 5-minute observation period identical to the first. The tape was removed from each male, and all animals were returned to their home cages. The same procedure was conducted on day two of testing (diestrus). The side of the arena in which the VCD-treated female was placed was alternated with each pair of females tested. The arena was cleaned with 50% ethanol between groups.

Twelve pairs of females were tested. Mean age at the time of behavioral testing was 16.7 ± 0.5 wk. Eleven individual females were exposed to more than one dyad of males because eight dyads failed to establish a clear dominant-subordinate relationship during the first two days of observation. These trials were terminated at the end of testing on day 2. Six females were exposed to a second dyad on the next consecutive estrous cycle. The dominant-subordinate relationships remained stable over the four-day testing period in all dyads that formed such a relationship. Males from dyads that failed to establish a dominant-subordinate relationship on the first two days of testing were re-paired with another male for future testing with a different pair of VCD- and vehicle-treated females.

Day 3 (proestrus): Y-maze tests

The third day of testing (proestrus) began with a 5-minute observation of the male dyad, identical to those performed on days 1 and 2, except that only one of the two females (the focal

female) was placed in her enclosure in the arena. Thereafter, all three animals were then returned to their home cages for a 10-minute rest period.

Female mate preference was observed in a Plexiglas Y maze after the male-male dyad observation. First, the focal female was allowed to explore the empty Y maze for 5 minutes, where she had access to the entire maze except the male enclosures at the distal ends of the arms. The female was then placed in an enclosure at the base of the Y maze while each male was placed in an enclosure at the end of the Y, where they were separated from the female by a coarse wire mesh. The female was released from her enclosure and allowed to move freely throughout the Y maze for 10 minutes. She was able to interact with the males through the wire mesh. The Y-maze test was subject to direct observation and video recording. After 10 minutes, the animals were returned to their home cages, and the Y maze was thoroughly cleaned with 50% ethanol. The males were allowed to rest for 10 minutes, and both the male dyad observation and Y maze were repeated with the remaining female. The order of females tested on day 3 and the positioning of the dominant and subordinate males in the enclosures at the distal ends of the Y maze were alternated with each group.

Day 4 (estrus): Confirmation of estrous cycles

On the day following the Y-maze tests, both females were confirmed to be in estrus by placing the same non-experimental male in their cages and monitoring for lordosis. All females displayed lordosis, indicating that a regular 4-day estrous cycle had been maintained during the testing period and that the Y maze test occurred on the day of proestrus. Each female was then killed by CO₂ inhalation. The uterus and both ovaries were removed, dissected free of fat and connective tissue, and weighed. The right ovary was fixed in 10% buffered formalin overnight.

2.4 Behavior scoring

An observer, who was unaware of the male dominance relationship and of the female treatment, counted the vaginal scent marks the females left in each arm of the Y maze as the test was performed. Vaginal scent marking occurs as females press their genital area against the floor of the Y maze and move forward to deposit streaks of vaginal secretions [23]. This behavior is difficult to detect on video-recordings and therefore it was scored live. Vaginal scent marking is a means of soliciting potential mates, and females often leave a trail of vaginal secretions back to her burrow, and males follow this trail [23]. Young females leave more vaginal scent marks near dominant males and mate with them more often than subordinate males [5, 24].

A different observer, who was also unaware of the male dominance relationship and of the female treatment, scored the number of flank scent marks left in each arm of the Y maze on each video recording using EventCoder 1.0b11, a program written and provided by Michael Goldstein (Cornell University, Ithaca, NY). Flank marking occurs as hamsters rub their dorsal flank along vertical surfaces of the Y maze and leave behind sebaceous secretions [23]. This observer also recorded first approach and the amount of time that each female spent near each prospective mate, i.e., her face and/or front paws were in contact with the coarse wire mesh of the male's enclosure.

2.5 Ovarian follicle counts

Formalin-fixed ovaries were serially dehydrated to 70% ethanol and embedded in paraffin. Each ovary was sectioned at 6 μm and every 10th section was placed on glass slides and stained with hematoxylin and eosin. Ovary sections were viewed under 400x magnification and follicles were classified as primordial, primary, or secondary and then counted. Primordial follicles were characterized by an oocyte surrounded by a single layer of flattened granulosa cells. Primary follicles had a single layer of cuboidal granulosa cells, and secondary follicles had

more than one layer of cuboidal granulosa cells. Only follicles in which a healthy oocyte nucleus could be visualized were counted.

2.6 Statistical analysis

Data were analyzed with JMP Pro statistical software (Version 11.0, SAS Institute, Cary, NC). Pre-treatment and time-of-testing body masses were analyzed by t-tests, and paired ovarian mass, and uterine mass were analyzed by Wilcoxon Rank Sum. Ovarian follicle numbers were compared across vehicle-treated, 15-month-old, and VCD-treated females by Kruskal-Wallis tests followed by Wilcoxon each pair *post hoc* analysis.

Vaginal scent marks, flank scent marks, and time near the dominant male were calculated as proportions (dominant-subordinate/dominant+subordinate). The proportions of vaginal scent marks left near the dominant male were analyzed by t-test, and proportions of flank scent marks and time near the dominant male were analyzed by Wilcoxon rank sum. One sample t-tests or Wilcoxon signed rank tests were used to determine if each of these proportions were significantly different from random, which would have a value of zero. A significantly positive result would indicate preference for the dominant male. The total time spent near males was analyzed by Wilcoxon rank sum. First approach was analyzed by Chi Square. Relationships between follicle numbers and each of the behavioral measures were analyzed by linear regression. Data are reported as means $\pm$ standard error, all statistical tests were two-tailed, and $p < 0.05$ was considered significant.

3. Results

3.1 Effects of VCD treatment

VCD-treated females had significantly fewer primordial, primary, and secondary follicles than vehicle-treated controls (Fig. 1, $p < 0.0001$ all follicle types). The number of primordial and secondary ovarian follicles in VCD-treated females did not differ from those counted in the ovaries of untreated 15-month-old females. VCD-treated females had fewer primary follicles than 15-month-old females. The number of ovarian follicles was not correlated with the mate preference behaviors of VCD-treated or control females.

Body, ovarian, and uterine masses of VCD-treated and control females are given in Table 1. Paired ovarian mass was significantly less in VCD treated females as compared to controls. Uterine mass did not differ between the two groups. Mean body masses at the start of treatment and at the time of behavioral testing were not significantly different.

3.2 Mate preference behavior

Scent marking behavior

The proportion of vaginal scent marks left near the dominant male was significantly different from random (zero) and in favor of the dominant male for both VCD treated ($p = 0.014$) and control ($p = 0.04$) females (Fig. 2A). There was no significant difference between vehicle- and VCD-treated females in the proportion of vaginal scent marks left for the dominant male ($p = 0.49$). The proportion of flank scent marks left in the dominant male's side of the Y maze was positive in both groups of females, but different from random in the VCD-treated females only (Fig. 2B, $p = 0.013$). The proportion of flank marks left for the dominant male did not differ between the two groups ($p = 0.23$).

Time near males

Seventy-five percent of VCD-treated and 66.7% of control females first approached the dominant male in the Y maze. VCD treatment did not have a significant effect on the proportion of females that first approached the dominant male ($p = 0.65$). The proportion of time spent near the dominant male was not different from random in the vehicle-treated (Fig. 2C, $p = 0.23$) and the VCD-treated groups ($p = 0.08$), and the groups were not significantly different ($p = 0.53$) in this regard. Both groups of females spent about 45% of the 10-minute trial near the males (Fig. 2D, $p = 0.41$).

4. Discussion

To our knowledge, this is the first study to specifically evaluate the role of reproductive quality in female mate preference in a mammal. The data suggest that the size of the follicular reserve alone does not influence female mate preference in the hamster. In rodents, ovarian follicle depletion is rapidly accelerated by VCD treatment, which destroys ovarian follicles whereas somatic tissues and longevity are not adversely affected [15-17]. In the present study, VCD treatment effectively depleted the ovaries of follicles in Syrian hamsters. In a previous study [5], female Syrian hamsters showed reduced preference for dominant males at 15 months of age as compared to younger females (6 months old). VCD treatment at 8 weeks of age resulted in follicle numbers comparable to those found in 15-month-old animals. Therefore, we effectively decoupled ovarian and chronological age. This allowed us to specifically test the influence of the ovarian follicular reserve on female mate preference.

Both VCD and vehicle treated females demonstrated preference for the dominant male by leaving proportionally more vaginal scent marks near him in a Y maze. This result is expected for young female Syrian hamsters [5, 24]. Vaginal scent marking is a behavior used by females

to solicit potential mates and lead them to her nest [24]. The frequency of this behavior increases substantially on the day of proestrus as females prepare to mate [23], and it is likely the most important indicator of preference measured in this study, because vaginal scent marking has been correlated with actual mate preference [7]. VCD-treated and control females left the same proportion of vaginal scent marks near the dominant male as controls, indicating that individuals depauperate of ovarian follicles were no less choosy than controls. In contrast to vaginal scent marking, flank scent marking may have a general broadcast and/or agonistic defense function [23]. The proportion of flank scent marks left for the dominant male was in the positive direction for both VCD-treated and control females. Interestingly, VCD-treated but not control females, left a significant (different from zero) proportion of flank scent marks near dominant males. However, the proportion was no different from controls.

The proportion of time spent near the dominant male by both the VCD-treated females and controls was not significantly different from random. This is in contrast to a previous report in which young, virgin females (6 months old) spent significantly more time near the dominant male as compared to the subordinate male in a Y maze [5]. Both VCD-treated and control females actively investigated the males for about 45% of the trial, suggesting that VCD treatment did not affect activity level or overall interest in the prospective mates.

Moore and Moore [2] reported that reduced female choosiness was correlated with female age and reproductive quality in the lobster cockroach (*Nauphoeta cinerea*). These conclusions were based on correlations between female age, fecundity, and choosiness. It is possible that the same relationship exists in Syrian hamsters because reduced mate preference was observed at an age at which fertility is reduced [5, 10]. We did not evaluate the fertility of VCD-treated Syrian hamsters in this study, but in a previous study involving Siberian hamsters

(*Phodopus sungorus*), fertility and fecundity declined following a comparable VCD regime [25]. In the present study we specifically tested the influence of the ovarian reserve on mate selectivity by manipulating follicle number and reproductive potential with VCD. We found no relationship between ovarian age and choosiness in the Syrian hamster using this experimental design.

The conclusions drawn from this experiment are limited by the methods used for behavioral analysis. The Y maze test provides a basic evaluation of mate preference, and in the present study it was used as a consistent follow-up to our previous study, which suggested old female hamsters have reduced mate preference [5]. However, the actual mating events of VCD-treated and control young females should be tested in order to more definitively rule out the influence of ovarian follicle depletion on female mate preference.

There may be several reasons why VCD-treated hamsters were not less selective for dominant males than control females in the present study. In our model we selectively accelerated ovarian follicle depletion, but aging or dysfunction of the hypothalamic-pituitary (HP) axis was unlikely to have been affected by VCD. Natural aging of the HP axis contributes to reproductive aging in many rodents [26, 27], and the hypothalamus (i.e. the ventromedial and medial preoptic nuclei) is known to modulate mating behaviors in Syrian hamsters [28-31]. VCD treatment was also unlikely to have altered ovarian steroid production in hamsters, based on previous studies in rats and mice [16, 32, 33]. Ovarian steroids are known to be important for both vaginal and flank scent marking behaviors [34, 35], and age-related changes may influence mate preferences and behavior of naturally-aged females.

A female's strength of preference in mate choice is also thought to be a function of body condition. Individuals of better quality are expected to be better able to pay the costs of being

choosy, and condition declines in old age as somatic aging progresses [36]. Aging is also associated with a deterioration of sensory ability. This decline may cause females to alter mate preferences or decrease choosiness with age because they are either unable to distinguish between more and less attractive mates, or their perception of the desirable mate has changed [37]. In hamsters this may include visual, olfactory, or auditory function. In female cockroaches, old age was significantly associated with an increase in the amount of time required for females to respond to the male's courtship display [2]. It is possible that this delay was due to deficits in sensory function. Given the complexity of mate choice behavior, and the lack of evidence for an influence of ovarian follicle depletion alone, it is likely that multiple aspects of the aging process are involved.

5. Conclusions

Accelerating ovarian follicle depletion did not influence mate preference behaviors of young female Syrian hamsters in Y-maze tests. We conclude that the status of the ovarian follicular reserve does not influence mate preference, and therefore somatic aging processes are likely to be major contributors to mate preference behavior in aging female hamsters. Additional research will be required to determine which aspects of the aging process influence female mate preference in mammals.

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Figure legends

Figure 1. Mean ($\pm$ SEM) number of follicles counted in every 10th section of the right ovary of VCD-treated (400 mg/kg), 15-month-old, and vehicle-treated hamsters. For each follicle type, bars with different symbols are significantly different from each other ($p < 0.05$).

Figure 2. (a) Proportion of vaginal scent marks left near the dominant male. (b) Proportion of flank scent marks left near the dominant male. (c) Proportion of time spent near the dominant male. Proportions are graphed as mean $\pm$ SEM and * denotes proportions that are significantly different from zero. (d) Total time (sec, mean $\pm$ SEM) spent near the dominant (black bars) and subordinate (white bars) male.

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Table 1. Mean ($\pm$ SEM) paired ovarian mass, uterine mass, and pre-treatment and time-of-testing body masses of vehicle- and VCD-treated female Syrian hamsters.

	Vehicle	VCD	p-value
Paired ovary mass (mg)	38.36 ($\pm$ 1.8)	27.33 ($\pm$ 1.5)	0.0002
Uterus mass (mg)	624.18 ($\pm$ 15.7)	598.45 ($\pm$ 13.4)	0.21
Pre-treatment body mass (g)	135.28 ($\pm$ 3.6)	125.88 ($\pm$ 2.9)	0.054
Body mass at testing (g)	141.96 ($\pm$ 4.3)	137.80 ($\pm$ 2.8)	0.48

FIG1

FIG2