Prepubertal ovarian inhibition of Light/Dark Box exploration and novelty object investigation in juvenile Siberian hamsters

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Abstract

The overwhelming majority of research on the role of gonadal hormones in behavioral development has focused on perinatal, pubertal, or adult life stages. The juvenile period has been overlooked because it is thought to be a time of gonadal quiescence. In the present study, we tested whether prepubertal gonadectomy impacts the behavior of male and female juvenile hamsters on the Light/Dark Box, Novel Object, and Social Approach tests (Experiment 1) and compared these findings to those obtained after adult gonadectomy (Experiment 2). Prepubertal ovariectomy increased exploration (i.e. time spent in the light zone of the Light/Dark Box) and novel object investigation of juveniles indicating an inhibitory role for the juvenile ovary; social approach was unaffected. In contrast, adult ovariectomy and castration (both prepubertal and adult) had no effect on any behavioral measure. Experiment 3 tested whether rearing hamsters in a short day length (SD), which delays puberty in this species, extends the interval of juvenile ovarian inhibition on exploration and novelty seeking. We also tested whether provision of estradiol reverses the effects of prepubertal ovariectomy. Hormonal manipulations and behavioral tests of Experiment 3 were conducted at ages when long day-reared hamsters are adult (as in Experiment 2), but SD-reared hamsters remain reproductively immature. Ovariectomy again increased exploration in the SD-reared juveniles despite the older age of surgery and testing. Estradiol treatment had no effect. These findings reveal a novel role for the juvenile ovary in exploration and novelty seeking that is unlikely to be mediated exclusively by estradiol.

Keywords: juvenile, adult, gonadectomy, prepubertal ovary, affective behavior, novelty seeking, social approach, estradiol

Introduction

Gonadal hormones play critical and far-reaching roles in behavioral development. Effects of gonadal hormones are typically characterized as either long-term, "organizational" actions that persist long after hormonal exposure or short-term, "activational" actions that wane shortly after the hormone is removed (Arnold, 2017; De Vries et al., 2014; McCarthy et al., 2018; Schulz and Sisk, 2016). Most research in behavioral endocrinology has focused on perinatal, pubertal, and adult periods. Organizational actions of gonadal steroids are thought to organize neural circuits during the perinatal and pubertal periods, which are later 'activated' when gonadal steroid secretion increases at puberty and into adulthood. The juvenile period is typically overlooked because it is considered a time of gonadal quiescence. However, the gonads of juveniles secrete measurable amounts of hormones in many species including rats, mice, Siberian hamsters, Syrian hamsters, rhesus monkeys, and humans (Courant et al., 2010; Dionyssiou-Asteriou and Zachari, 1992; Döhler and Wuttke, 1975; Janfaza et al., 2006; Mannan and O'Shaughnessy, 1991; Phalen et al., 2010; Sisk and Turek, 1983; Vesper et al., 2015; Winter et al., 1987; Yellon and Goldman, 1984). Furthermore, juvenile steroids have physiological actions as they provide negative feedback to the hypothalamic-pituitary-gonadal axis even during the juvenile period (Andrews and Ojeda, 1981; Dubois et al., 2016; Meijs-Roelofs and Kramer, 1979; Plant, 1986; Ramirez and Mccann, 1965; Sisk and Turek, 1983; Winter and Faiman, 1972). Hence, it is reasonable to ask whether juvenile gonadal hormones impact behavior.

There is a small, but growing body of literature implicating juvenile gonadal hormones in the regulation of behavior. Ages of developmental stages vary across species, sexes and environmental conditions, but a general timeline for many rodents approximates the following: perinatal and neonatal periods = embryonic day 18 to postnatal day [P]10; juvenile period = P14 to P30; pubertal period = P30 to P55; young adulthood > P55. Neonatal and prepubertal ovariectomy diminish sex differences in several adult reproductive and non-reproductive traits

(Hendricks, 1992; Fitch and Denenberg, 1998). Because measures were taken in adulthood, however, it is difficult to determine whether these effects are due to the absence of ovarian hormones during the juvenile or pubertal period. Nevertheless, behavioral effects of prepubertal ovariectomy can be greater when surgery is performed before versus after the juvenile period (Field et al., 2004; Gerall et al., 1973). Furthermore, deficits in female sex behavior of aromatase knockout female mice can be ameliorated by daily estradiol injections administered during the juvenile period (Brock et al., 2011). Collectively, these findings suggest that the ovaries potentiate feminization of the brain and behavior through organizational actions of estradiol during the juvenile period (Bakker and Brock, 2010). Analogous mechanisms may be in place for juvenile males. Male Syrian hamsters remain sensitive to the organizational actions of adult levels of testosterone administered during the juvenile period (Schulz et al., 2009), but it is not known whether endogenous, prepubertal levels of testicular hormones can similarly impact behavior. We have recently found that the gonads of juveniles also support more immediate, likely activational actions on juvenile behavior. Gonadectomy at 15 days of age increases social play behavior in male and female juvenile Siberian hamsters indicating an inhibitory role for the juvenile gonads on play in this species (Paul et al., 2018). These findings counter the notion of quiescent juvenile gonads, and raise the question as to the extent to which gonadal hormones influence behavior during the juvenile period. To begin to address this question, the present study assessed the role of the juvenile gonads in tests of affective, novelty-seeking, and social approach behaviors.

Siberian hamsters provide unique opportunities to test the role of gonadal hormones across development because the timing of their puberty is plastic. Siberian hamsters use day length to coordinate reproductive maturation with summer breeding conditions (Paul et al., 2008; Stevenson et al., 2017). Hamsters reared in long, summer-like day lengths (LDs) undergo rapid pubertal development that begins around 20 days of age for males and between 35-50 days of

age for females; adulthood is reached by 60 to 80 days of age (Adam et al., 2000; Yellon and Goldman, 1984). When reared in short, winter-like day lengths (SDs), however, puberty is delayed by several months in order to prevent breeding during the winter. Under these conditions, reproductive development is initiated around 100 days of age or later (Adam et al., 2000; Hoffmann, 1978; Paul et al., 2006). Hence, with this model gonadal manipulations can be performed on animals that are the same age, but in different pubertal phases – around 80-100 days of age, when LD-reared hamsters are adult, but SD-reared hamsters remain reproductively immature. This provides a model to test whether developmental changes in the role of gonadal hormones across adolescence are due to pubertal status or some other age-related process (reviewed in Walker et al., 2017).

The present study used the Siberian hamster model to test the impact of prepubertal (Experiment 1) and adult (Experiment 2) gonadectomy on exploration/anxiety-like behavior (Light/Dark Box Test), novelty seeking (Novel Object Test), and social approach (Social Approach Test) of juvenile and adult LD-reared hamsters. These experiments uncovered an inhibitory role for the juvenile ovary in exploration and novelty seeking that was not present in adult females; castration did not affect behavioral measures of juvenile or adult males in these experiments. Hence, in Experiment 3 we tested the impact of prepubertal ovariectomy on 80 day-old “juvenile” SD-reared hamsters to test whether the loss of juvenile ovarian behavioral inhibition is due to age or pubertal status. We further tested whether provision of estradiol implants would reverse the effects of gonadectomy in SD-reared, ovariectomized juvenile hamsters. As for LD-reared juveniles, ovariectomy increased exploratory behaviors in SD-reared juvenile females, but this effect was not reversed by estradiol implants. Collectively, these findings reveal a novel role for the prepubertal ovary in the regulation of affective and/or novelty-seeking behaviors that is unlikely to be regulated exclusively by estradiol.

Materials and Methods

Animals and Housing Conditions

Siberian hamsters (*Phodopus sungorus*) were obtained from our breeding colony, which was derived from animals provided by Dr. Brian Prendergast, University of Chicago. Hamsters were kept in well-ventilated, light-proof environmental housing units that provided either a long day photoperiod (LD; 15:9-hr light:dark cycle) or short day photoperiod (SD; 10:14-hr light:dark cycle); dim red light was present during the dark phase. Within these units, hamsters were housed in clear, polysulfone cages (18.4cm x 29.2cm x 12.7cm) furnished with lab grade shredded aspen bedding (LADS Pet Supplies). All hamsters were weaned on postnatal day (P)18, at which point hamsters were housed in same-sex groups of 2-3 hamsters per cage. Tap water and rodent chow (2016 Teklad global 16% protein rodent diet, Envigo; isoflavone content undetectable to 20 mg/kg) were available *ad libitum*. Ambient temperature was maintained at 22 ± 2°C. Hamsters were fitted with ear tags for individual identification at surgery (Experiment 1) or at weaning (Experiments 2 and 3). All procedures were approved by the University at Buffalo, SUNY Institutional Animal Care and Use Committee and were in accordance with the *Guide for Care and Use of Laboratory Animals*.

Experiment Timelines

Experiment 1, LD prepubertal gonadectomy. Forty-eight male and 43 female hamsters were gestated and reared in an LD. Hamsters underwent gonadectomy or sham-operation procedures on P15 ± 1, and behavioral tests were conducted between P29-P32. To assess whether early life surgery impacts behaviors measured in this study, another group of hamsters was not operated upon and served as non-surgical controls (NSCs). NSCs were otherwise treated identically to gonadectomized (GNX) and sham-operated (Sham) hamsters. Within 2 days of behavioral testing, hamsters were sacrificed, at which point uterine weights, testes weights, and body mass were recorded. One female GNX hamster was excluded from

analyses because of extremely low body mass at the time of testing (11g). Two female Sham hamsters were excluded because genetic malformations were noted in siblings within the same litter.

Experiment 2, LD adult gonadectomy. Thirty-three male and 28 female hamsters were gestated and reared in an LD. Hamsters underwent gonadectomy or sham-operation procedures between P81-P89, and behavioral tests were conducted between P102-P111. Within 2 days of behavioral testing, hamsters were sacrificed, at which point uterine weights, testes weights, and body mass were recorded. Two male GNX and 2 female Sham hamsters were excluded from analyses because of post-surgical complications.

Experiment 3, SD prepubertal ovariectomy: age versus pubertal status. Fifty-seven female hamsters were gestated and reared in an SD; males were not tested in this experiment because castration did not impact behavior in experiments 1 and 2. Hamsters underwent ovariectomy or sham-operation procedures between P80-P85 and behavioral testing between P101-P111. At surgery, ovariectomized (OVX) hamsters received an estradiol implant (E2; estradiol diluted in cholesterol), a cholesterol implant (Ch; vehicle control), or a blank implant (B; empty control). All Sham hamsters received a B implant. The effect of ovariectomy was tested by comparing the behavior of OVX+B hamsters to that of Sham+B hamsters. The effect of estradiol was tested by comparing the behavior of OVX+E2 hamsters to that of OVX+Ch and OVX+B hamsters. Within 2 days of behavioral testing, hamsters were sacrificed, at which point uterine weights and body mass were recorded. Vaginal opening is often used as a marker of pubertal onset in several rodent species, including Siberian hamsters (Haigh et al., 1988; Place et al., 2004; Place and Cruickshank, 2009). To ensure prepubertal status at the time of hormone manipulations, vaginal patency was assessed at surgery. Hamsters that had undergone vaginal

opening were excluded from analyses; number of animals excluded within each group was 1 Sham+B, 1 OVX+Ch, and 3 OVX+E2.

Surgical Procedures.

For all surgical procedures, hamsters were administered Metacam (0.5mg/kg, SC) prior to the start of surgery. Hamsters were anesthetized with isoflurane vapors, and body temperature was maintained using a heating pad. After surgery, hamsters were administered sterile saline (1ml for juveniles, 2.5ml for adults, SC) and placed under a heat lamp to aid thermoregulation until they were ambulatory. Metacam was administered (0.5mg/kg, SC) daily for 2 days following surgery as a postoperative analgesic.

Castrations. The lower ventrum was shaved and then disinfected with soap, alcohol, and Betadine solution. A single incision was made through the skin and abdominal wall, and one testis and epididymis were externalized using forceps. The testicular vein was ligated with sterile vicryl sutures, and the testis and epididymis were removed by cutting the tissue just above the suture. Remaining tissue was replaced inside the animal. The contralateral testis and epididymis were then externalized and removed through the same incision. The abdominal wall and skin were then closed sequentially using sterile vicryl sutures. Sham castrations were conducted in the same manner except that the testicular vein was not ligated or cut, and the testes and epididymides were replaced inside the animal following externalization.

Ovariectomy. The dorsal left and right flanks were shaved and disinfected with soap, alcohol, and Betadine solution. An incision was made through the skin and abdominal wall on one flank, and the ipsilateral ovary was externalized using forceps. The ovarian vein was ligated with sterile vicryl sutures, and the ovary was removed by cutting the tissue just above the suture. Remaining tissue was then replaced inside the animal. The abdominal wall was closed using

sterile vicryl sutures, and the skin closed using surgical wound clips or sutures. The contralateral ovary was then removed using the same procedures. Sham ovariectomies were conducted in a similar manner except that the ovarian vein was not ligated or cut, and the ovaries were replaced inside the animal following externalization.

Subcutaneous capsule preparation and implantation. For estradiol implants, crystalline estradiol benzoate (catalog #E8875-1G, MilliporeSigma, St. Louis, MO) was diluted with crystalline cholesterol (catalog #C8667-5G, MilliporeSigma, St. Louis, MO) to provide a 10% (wt/wt) final concentration of estradiol. For cholesterol implants, only the crystalline cholesterol was used. The estradiol:cholesterol mixture or cholesterol was then packed into Silastic tubing (catalog #508-009, internal diameter = 1.98mm; outside diameter = 3.18mm, Dow Corning, Midland, MI) to a length of 4mm and sealed with ~3mm of sealant on both sides. This capsule length and estradiol:cholesterol ratio have previously been shown to provide adult-like levels of estradiol in Siberian hamsters (Bartness, 1995). A 4mm space was left empty for the blank capsules. Each end was sealed with GE Silicone 2+ Clear caulk. Caulk was given a minimum of 24 hours to cure before the sealed ends were trimmed to precisely 3mm and stored at -20°C. Prior to the surgery, capsules were sterilized in a bath of Wavicide (Medical Chemical Corporation, Torrance, CA) for 4-8 hours and then washed in sterile saline. Capsules were then submerged in sterile saline at 37°C for 24 hours before surgery to allow hormone release to equilibrate.

Capsule implantations were conducted in Experiment 3 during ovariectomy or sham-surgery procedures. The upper dorsal surface was shaved and disinfected with soap, alcohol, and Betadine solution. An SC incision was made just below the nape, and the sterile capsule was inserted. The incision was then closed with surgical wound clips.

Behavioral Testing:

Behavioral testing occurred during the mid-light phase (7.5 and 5 ± 1.5 h after lights-on for LD and SD, respectively) to minimize circadian differences across experiments conducted in different photoperiods (as in Prendergast and Nelson, 2005). Hamsters were subjected to a Light/Dark Box Test, Novel Object Test, and Social Approach Test. Behavioral tests were conducted sequentially in the above-mentioned order with Novel Object and Social Approach tests beginning immediately upon the completion of the prior test.

Light/Dark Box Test. The hamster was placed inside a dark box (38.9cm x 12.7cm x 15.2cm) with a single entrance to an illuminated open arena (40.0cm x 39.9cm x 31.2cm). The entrance was initially blocked by a metal door. At the start of the test, the metal door was removed, and the hamster was allowed to explore the light and dark zones of the apparatus for 10 minutes. The amount of time spent in the light zone was used as a measure of anxiety/exploratory drive.

Novel Object Test. The Light/Dark Box test served as an acclimation phase for the Novel Object Test, which was conducted in the same apparatus. Immediately following the Light/Dark Box Test, the hamster was removed, and a novel, empty, polycarbonate cage (14.6cm x 11.2cm x 17.8cm) was placed inside the illuminated open field against the wall opposite the dark chamber. The walls of the cage were constructed of plastic bars that allowed the subject to look into, but not enter the cage. The hamster was again placed in the dark box. At the start of the test, the metal door was removed, and the hamster was allowed to explore the apparatus for 5 minutes. The amount of time spent in the investigation zone surrounding the empty cage was used as a measure of novel object investigation.

Social Approach Test. Immediately following the Novel Object Test, the hamster was removed, and the novel cage was replaced with an identical polycarbonate cage containing a novel same-

sex, same-age conspecific. The test hamster was again placed in the dark box, the metal door removed, and the test hamster allowed to explore the apparatus for 5 minutes. The subject and stimulus hamsters were able to interact by touching noses, but could not pass through the bars to enter or leave the cage. The amount of time spent in the investigation zone surrounding the caged conspecific was used as a measure of social approach.

Behavior was recorded by a camera mounted above the arena using Media Recorder 4 software (Noldus Information Technology Inc., Wageningen, The Netherlands). Time spent in the light zone, novel object investigation, and social approach were scored automatically using EthoVision XT10 software (Noldus Information Technology Inc., Wageningen, The Netherlands).

Reproductive Measures

In Experiment 3, vaginal opening was recorded at surgery to confirm prepubertal status and uterine weight measures were recorded at sacrifice to confirm effectiveness of hormone treatments. At sacrifice, hamsters were perfused intracardially with physiological saline followed by 4% paraformaldehyde, and brains were removed for other experiments. Following perfusion, the uterus was removed and weighed on a digital balance (Mettler Toledo™ NewClassic ML 104 /03). Because ovariectomy cuts the upper portion of the uterine horns, a modified uterine weight was used in which the 1st cm from the base of the uterus was dissected out and weighed. If the uterus was less than 1 cm in length, the entire uterus was weighed and the length was recorded. A correction factor was then applied to provide the weight/1 cm. This uterine weight measure was also recorded for a subset of LD-reared, Sham adult female hamsters from Experiment 2 to provide a reference for adult uterine weights using this method. Estrous cycle was not monitored, and therefore UWs of Sham animals were not collected at the same stage of the estrous cycle.

Statistical Analyses

In Experiment 1, the effect of early life surgery was assessed by comparing the behavior of Sham and NSC hamsters using a t-Test. Effects of gonadectomy and sex in Experiments 1 and 2 were assessed using ANOVA. In Experiment 3, the effect of ovariectomy was assessed by comparing the behavior of Sham+B and OVX+B groups using a t-Test, whereas the effect of hormone treatment was assessed by comparing the behavior of OVX+B, OVX+Ch, and OVX+E2 using one-way ANOVA. Differences in uterine weight measures of all groups in Experiment 3 plus the subset LD adult Sham females were assessed using a one-way ANOVA. Where significant main effects or interactions were detected in the overall ANOVA, post hoc comparisons were conducted using Fisher's PLSD. Significance was assumed when $P<0.05$. All statistical analyses were conducted using SPSS Statistics Version 23 (IBM, Armonk, NY).

Results

Experiment 1. Prepubertal gonadal influences on exploration, novelty seeking, and social approach

Effects of Early Life Surgery. Early life sham surgery did not impact any behavioral measure of female juvenile hamsters (female Sham vs. female NSC t-Tests: time in light zone of the Light/Dark Box, $t_{(24)}=0.80$, $P=0.43$; novel object investigation, $t_{(24)}=0.37$, $P=0.72$; social approach, $t_{(23)}=1.41$, $P=0.17$). For males, early life sham surgery increased novel object investigation ($t_{(26.6)}=2.50$, $P<0.05$, Cohen's $d=0.86$, male Sham vs. male NSC, t-Test), but did not alter other behavioral measures (male Sham vs. male NSC t-Tests: time in light zone of the Light/Dark Box, $t_{(31)}=0.66$, $P=0.51$; social approach, $t_{(27)}=1.36$, $P=0.19$). Hence, for Light/Dark Box and Social Approach tests, NSC and Sham juvenile hamsters were combined into a single Intact group, and subsequent analyses were conducted using a 2 x 2 ANOVA with Sex (male/female) and Gonadal Status (Intact/GNX) as independent variables. For the Novel Object

Test, only female NSC and Sham groups were combined into an Intact group. Given that this resulted in unequal numbers of groups between the sexes, male and female novel object data were analyzed in separate one-way ANOVAs with Gonadal Status (Intact/GNX) as the independent variable for females and Surgery (NSC/Sham/GNX) as the independent variable for males.

Light/Dark Box Test. Prepubertal GNX had a sex-specific effect on performance in the Light/Dark Box Test (Fig. 1A). There was a significant interaction between Sex and Gonadal Status on the time juvenile hamsters spent in the light ($F_{(1,84)}$=4.02, P<0.05, partial eta^2=0.05, two-way ANOVA). Prepubertal GNX increased time spent in the light for female juveniles (P<0.05, Cohen's d=0.82, female GNX vs. female Intact, Fisher's PLSD), but had no effect on male juveniles (P=0.61, male GNX vs. male Intact, Fisher's PLSD).

Novel Object Test. As seen for the Light/Dark Box Test, prepubertal GNX affected novel object investigation of female, but not male, juveniles (Fig. 1B). Prepubertal GNX increased the time female juveniles spent investigating the novel object ($F_{(1,38)}$=4.16, P<0.05, partial eta^2=0.10, one-way ANOVA). For juvenile males, there was a main effect of Surgery ($F_{(1,44)}$=3.94, P<0.03, partial eta^2=0.15, one-way ANOVA) due to the early life surgery effect stated above (P<0.01, Cohen's d=0.86, male NSC vs. male Sham, Fisher's PLSD). There were no significant differences in the time spent investigating the novel object between GNX and Sham (P=0.24, Fisher's PLSD) or GNX and NSC (P=0.14, Fisher's PLSD) male juveniles.

Social Approach Test. Neither Sex nor prepubertal GNX impacted social approach (Fig. 1C; $F_{(1,76)}$=0.714, P=0.40, main effect of Sex; $F_{(1,76)}$=1.87, P=0.18, main effect of Gonadal Status; $F_{(1,76)}$=0.52, P=0.47, interaction, two-way ANOVA).

Experiment 2. Absence of postpubertal gonadal influences on exploration, novelty seeking, and social approach

Adult GNX did not impact any behavioral measure (Fig. 2; main effect of Surgery and the interaction, two-way ANOVA statistics: time in the light zone of the Light/Dark Box, $F_{(1,53)}<0.56$, $P>0.45$; novel object investigation, $F_{(1,52)}<0.59$, $P>0.44$; social approach, $F_{(1,48)}<1.91$, $P>0.17$). The main effect of Sex approached significance for social approach ($F_{(1,48)}=3.80$, $P=0.06$, two-way ANOVA), but not for time in the light zone of the Light/Dark Box ($F_{(1,48)}=0.21$, $P=0.64$, two-way ANOVA) or novel object investigation ($F_{(1,48)}=0.14$, $P=0.71$, two-way ANOVA).

Experiment 3. Developmental loss of ovarian inhibition on exploration and novelty seeking: age versus pubertal status

Light/Dark Box Test. As seen for LD-reared female *juveniles*, prepubertal OVX increased time spent in the light zone for SD-reared female juveniles even though surgery and testing occurred at ~P85 and ~P106, respectively (Fig. 3A; $t_{(19)}=2.70$, $P<0.02$, Cohen's $d=1.21$, Sham+B vs. OVX+B, t-Test). There were no significant effects of estradiol treatment on time spent in the light zone of OVX females (Fig. 3B; $F_{(2,34)}=1.18$, $P=0.32$, one-way ANOVA).

Novel Object and Social Approach Tests. Unlike time in the light zone of the Light/Dark Box, prepubertal OVX did not significantly alter novel object investigation (Fig. 3C; $t_{(21)}=1.55$, $P=0.14$, Sham+B vs. OVX+B, t-Test) or social approach (Fig. 3E; $t_{(20)}=0.75$, $P=0.46$, Sham+B vs. OVX+B, t-Test) of SD-reared female juveniles. In addition, estradiol treatment did not alter novel object investigation (Fig. 3D; $F_{(2,34)}=1.61$, $P=0.22$, one-way ANOVA) or social approach (Fig. 3F; $F_{(2,35)}=1.51$, $P=0.24$, one-way ANOVA) of OVX SD-reared females.

Verification of Estradiol Capsules. The overall ANOVA indicated significant differences in 1cm uterine weight measures (1cm UWs) between groups (Fig. 4; $F_{(4,56)}=56.51$, $P<0.001$, partial

eta^2=0.82, one-way ANOVA). Variability was high in the LD-Sham adult females, likely due to varying estrous cycle stage in these animals. Mean 1cm UWs of SD-OVX+E2 females was significantly greater than that of LD-Sham adult females (P<0.001, Cohen's d=1.45, Fisher's PLSD), because values of all SD-OVX+E2 animals were in the upper range of LD-Sham adults. Mean 1cm UWs of SD-OVX+E2 was also greater than those of all other SD-reared groups (P<0.001, Cohen's d>5.08, Fisher's PLSD). Mean 1cm UWs of SD-Sham+B, SD-OVX+B, and SD-OVX+Ch were significantly lower than that of LD-Sham adult females (P<0.001, Cohen's d>1.28, Fisher's PLSD) and did not differ from each other (P>0.19, Fisher's PLSD). One SD-Sham+B female and 1 SD-OVX+B female had 1cm UWs that were outliers (1.5 times the interquartile range, SPSS Box and Whiskers Plot). These animals were included in behavioral analyses above because they met the criteria of absence of vaginal opening at the time of surgical and hormonal manipulations (surgery/hormone manipulations at P80-P85, 1cm UWs recorded at P102-P113). Inclusion of these 2 animals did not affect the outcome of any statistical comparison.

Discussion

The present findings argue for an active role for the ovary in the regulation of juvenile behavior. Prepubertal ovariectomy increased time spent in the light zone of the Light/Dark Box Test and novel object investigation in the Novel Object Test in juvenile female hamsters. To our knowledge, this is the first demonstration that the ovary inhibits Light/Dark Box 'exploration' or novelty seeking during the juvenile period. Inclusion of non-surgical controls in LD-reared juvenile hamsters allowed us to rule out potential procedural confounds of surgery (e.g., anesthesia, early life surgical stress). These data support previous studies implicating the juvenile ovary in both organizational and activational actions on behavior (see Introduction). Juvenile gonadal hormones also contribute to physiological regulation of the hypothalamic-pituitary-gonadal axes (i.e. negative feedback on pituitary gonadotropin secretion; Andrews and

Ojeda, 1981; Dubois et al., 2016; Meijs-Roelofs and Kramer, 1979; Plant, 1986; Ramirez and Mccann, 1965; Sisk and Turek, 1983; Winter and Faiman, 1972). Clearly, the juvenile gonads should not be considered functionally quiescent, neither physiologically nor behaviorally.

There was no effect of ovariectomy in adulthood, suggesting that the influence of the ovary on Light/Dark Box exploration and novelty object investigation changes across adolescence with the prepubertal ovary having a greater influence than that of the adult ovary. Notably, the response to ovariectomy of SD-reared female hamsters was more similar to LD-reared juveniles than LD-reared adults, even though SD-reared hamsters underwent surgery and testing at the same ages as the latter. Ovariectomy increased Light/Dark Box exploration in SD-reared hamsters; differences in novel object investigation were not significant (P=0.14), but were in the same direction as that seen in LD-reared juveniles (OVX > Sham). These data indicate that SD-rearing extends the period during which the ovary inhibits Light/Dark Box exploration and perhaps novel object investigation. Hence, an age-specific process is unlikely to be responsible for the loss of ovarian inhibition across adolescence. Instead, given that SD-reared females were reproductively immature at the time of surgery and testing, the present findings suggest that activation of the reproductive axis at puberty may be responsible for the developmental decrease in ovarian inhibition of Light/Dark Box exploration. The finding that ovariectomy increases Light/Dark Box exploration in both LD-reared, P30 juveniles and SD-reared, P105 'juveniles' is remarkable given that these two juvenile states are not equivalent. While SD-rearing delays reproductive maturation of the ovary (e.g., emergence of antral follicles, corpora lutea, and elevated gonadal steroids), it does not simply pause development (Park et al., 2014). Rather, a distinct developmental path is taken that is characterized by changes in gene expression and the emergence of hypertrophied granulosa cells that are likely capable of producing both steroid and peptide hormones (Kabithe and Place, 2008; Park et al., 2014; Van den Hurk et al., 2002). In addition, SD-reared juveniles exhibit several winter adaptations that

are absent in LD-reared juveniles (Paul et al., 2008; Stevenson et al., 2017). Hence investigations into the commonalities and differences between these two 'juvenile' phases may reveal the underlying mechanism through which the ovary influences juvenile behavior.

Castration in males did not impact Light/Dark Box exploration or novel object investigation in juveniles or adults, suggesting a sex-specific role for gonadal hormones in juvenile exploration and novelty seeking. Caution is warranted, however, as the timing of gonadectomy in the present study may not have been optimal for males. Pubertal development appears to begin earlier in male than female Siberian hamsters. Increases in testes weights and circulating gonadotropins occur as early as 20 days of age in LD-reared males (Yellon and Goldman, 1984), whereas in LD-reared females, uterine weights increase around 45 days of age (Adam et al., 2000). Hence, behavioral testing was conducted prior to puberty in females, but likely during early puberty in males. If the effect of gonadectomy is restricted to the juvenile phase, earlier time points may be needed to reveal an effect of castration. Notably, suppression of testicular hormones using a GnRH antagonist decreases preference for novelty in mid-pubertal male rats (Cyrenne and Brown, 2011). At present, it is not clear whether the different findings in these studies are due to the species tested (hamsters versus rats), age of subjects (early versus mid puberty), testing procedures (novel object investigation versus novel object recognition), or method of gonadal hormone suppression (castration versus GnRH antagonist). Other studies have also implicated a role for the peripubertal testis in behavioral development. In a design similar to the present study, prepubertal castration increased social play in 30-day-old LD-reared male and female hamsters (Paul et al., 2018). Experiments in Syrian hamsters have shown that the brain remains sensitive to the organizational actions of testosterone on adult reproductive behavior during the juvenile and pubertal periods (Schulz et al., 2009). Furthermore, 19 days of testosterone treatment during the juvenile period increased volumes of several sexually dimorphic brain regions to adult male-typical levels (Schulz et al., 2009). More

studies are needed to assess behaviors impacted by juvenile and early adolescent gonadal hormones as well as potential sex differences and windows of sensitivity for these actions.

Early life surgery can impact adult behavior, including decreases in novelty seeking of rats (Vetter-O'Hagen and Spear, 2012). In the present experiment, early life surgery also impacted novel seeking of juvenile hamsters, but in the opposite direction and only in males. The difference in the direction of the effect may be due to species or procedural differences – e.g., timing of surgery, timing of testing, or behavioral testing paradigm. Early life surgery effects are likely due to the stress of surgery during sensitive periods early in postnatal development (Boersma et al., 2014; Horovitz et al., 2012; Varlinskaya et al., 2013). Similar to the present experiment, early life stress often impacts males and females to different degrees (Bilbo and Schwarz, 2012; Nelson and Lenz, 2017), and male-specific effects of early life surgery have also been reported for ethanol intake (Vetter-O'Hagen and Spear, 2011). These findings emphasize the importance of studying both sexes and including non-surgical controls in studies using surgical manipulations during early postnatal development.

The physiological mechanism through which the ovary modulates juvenile behavior is not clear. Findings from previous studies suggest estradiol as a prime candidate. Circulating estradiol is elevated from around P10 to P25 in rats (Döhler and Wuttke, 1975; Konkle and McCarthy, 2011; Walker et al., 2012), with some studies reporting higher values during these ages than in adulthood (Döhler and Wuttke, 1975; Walker et al., 2012). Furthermore, studies in aromatase knockout mice suggest that estradiol has long-term, organizational actions during the juvenile period in females. Aromatase knockout mice, which cannot produce estrogens, exhibit deficits in female sex behavior, even when hormonally primed with exogenous estradiol and progesterone prior to behavioral testing (Bakker et al., 2002). These deficits are ameliorated by daily estradiol injections administered from P15 to P25 (Brock et al., 2011). In the present

study, we tested whether prepubertal estradiol also has more immediate actions on juvenile behavior. Counter to our hypothesis, however, provision of estradiol to SD-reared, ovariectomized prepubertal hamsters did not affect their Light/Dark Box exploration or novel object investigation. Hence, the prepubertal ovary does not appear to act through estradiol alone to inhibit juvenile exploration and novelty seeking. Other ovarian hormones may act alone or in concert with estradiol to mediate these effects. Developmental hormone profiles in rats point toward progesterone and testosterone as possible candidates. Circulating progesterone begins to increase around the third week of life, and circulating testosterone exhibits a transient increase around 15 days of age in female rats (Döhler and Wuttke, 1975; Walker et al., 2012). Although a detailed developmental profile of gonadotropins and androgens is available for male Siberian hamsters (Yellon and Goldman, 1984), a similar detailed profile is not available for female Siberian hamsters or for estrogens and progestins in either sex.

While our data do not support a role for estradiol in juvenile Light/Dark Box exploration and novel object investigation, there are a few caveats to this conclusion. It is possible that low to moderate, rather than high, estradiol levels are needed to modulate juvenile behavior, as has been proposed for estradiol's organizational actions on female sex behavior (Döhler et al., 1984). In the present study, we implanted estradiol capsules that have previously been shown to mimic adult levels of estradiol (Bartness, 1995). Uterine weight measures in the current experiment further indicated that estradiol levels of hormone-treated hamsters were in the upper-adult range. While this hormone treatment may mimic elevated estradiol levels seen in juvenile female rats, it is not known whether Siberian hamsters exhibit a similar juvenile elevation in circulating estradiol. Future studies are needed to characterize the developmental profile of estradiol in hamsters and to determine whether differing "doses" of estradiol have different effects on juvenile exploration and novelty seeking. Polycarbonate cages can contain bisphenols, including bisphenol A, which is a weak estrogen receptor agonist (Patisaul, 2019).

Hence, bisphenol contamination is a possible confound in the present experiments. Another caveat is that estradiol was only administered to SD-reared females. In addition to the ovarian and seasonal differences discussed above, SD-housing decreases estradiol secretion and alters the sensitivity of the brain to circulating steroids in adult Syrian and Siberian hamsters (Bittman et al., 1996; Ellis and Turek, 1983; Rendon et al., 2017; Tamarkin et al., 1976). Whether similar actions of photoperiod occur in juveniles is not known, but this raises the possibility that different results may be obtained if estradiol is administered to ovariectomized, LD-reared juveniles.

Gonadal steroids have been shown to impact anxiety, novelty seeking, and social behavior in rodents (Adkins-Regan, 2005; Cyrenne and Brown, 2011; Walf and Frye, 2006). Hence, it is surprising that gonadectomy had no impact on behavioral measures of adult hamsters in the present study. Although species differences may be responsible, a close inspection of the literature suggests other possibilities as well. Steroid manipulations impact anxiety-like behavior in several affective behavioral tests (e.g. Aikey et al., 2002; Frye and Seliga, 2001; Mora et al., 1996; Morgan and Pfaff, 2002), but null results are occasionally reported (Hodosy et al., 2012; Nomikos and Spyraki, 1988), including for the Light/Dark Box Test (Domonkos et al., 2017). Time of behavioral testing can modulate steroid influences on Light/Dark Box behavior. Male Tfm mice that have a mutation in the androgen receptor exhibit decreased exploration in the Light/Dark Box test compared to wild type mice when tested in the dark phase of the light/dark cycle but not when tested during the light phase (Chen et al., 2014); present experiments were conducted during the light phase. Steroid influences on novelty seeking may depend upon testing procedures. Testosterone promotes novelty seeking in the novel object recognition test (Cyrenne and Brown, 2011). In a test that lacks a learning or memory component, however, Vetter-O'Hagen and Spear (2012) failed to find effects of castration or ovariectomy in the Novel Object Test. Gonadal steroids modulate many aspects of social behavior (Choleris et al., 2009;

Ervin et al., 2015), but few studies test their impact on social approach. Castration decreases social interactions in male rats, but this effect manifests between 2 and 4 weeks post-surgery (Primus and Kellogg, 1990). Hence, the 3-week interval between surgery and behavioral testing in the present experiment may not have been long enough to detect effects of gonadectomy on social behavior of adults.

It is difficult to tease apart contributions of anxiety, exploratory drive, learning, memory, and locomotor activity to behavioral measures in rodent affective and novelty-seeking tests. The Light/Dark Box Test is typically used to measure anxiety, but the paradigm is also based on natural motivation of rodents to explore novel environments (Bourin et al., 2007; Bourin and Hascoët, 2003). In the present experiment, the Novel Object Test included the dark refuge of the Light/Dark Box Test. Hence, rodents' natural anxiety toward light may have influenced the amount of time they investigated the novel object in the well-lit portion of the testing arena. Novel Object Recognition Tests are often used as a test of learning and memory (Antunes and Biala, 2012). In these tests, the procedure includes two phases: 1) a sampling phase in which the animal is exposed to an object and 2) a testing phase in which the animal is provided with the now familiar object along with a novel object (or the familiar object in a new location). Because we did not include a sampling phase in our Novel Object Test, effects of recognition learning and memory were minimized. Nevertheless, hamsters were exposed to the apparatus during the Light/Dark Box Test just prior to the Novel Object Test. Hence other forms of learning (e.g., acclimation/habituation to the testing apparatus) could have impacted performance in the present study. Changes in locomotor activity, which are modulated by gonadal steroids in adults (Ellis and Turek, 1983; Morgan and Pfaff, 2002), can also impact behavioral measures in affective and novelty-seeking tests. Because activity was not recorded in the dark zone of the arena, it is difficult to assess potential contributions from locomotor activity in the present tests. However, given that steroids typically increase general locomotor

activity (Ellis and Turek, 1983; Morgan and Pfaff, 2002), it is unlikely that this mechanism is responsible for the increase in Light/Dark Box exploration and novelty seeking seen in the present study after prepubertal ovariectomy. Regardless of mechanism, the present findings demonstrate that the juvenile ovary modulates Light/Dark Box exploration and novelty object investigation. Whether this is due to ovarian regulation of anxiety, motivation to explore novelty, learning, memory, and/or locomotor activity remains to be elucidated.

Conclusions

Previous findings in rats, mice, and hamsters suggest that the juvenile gonads can have long-term organizational actions on female sex behaviors as well as immediate, likely activational, actions on juvenile play behavior (Brock et al., 2011; Field et al., 2004; Gerall et al., 1973; Paul et al., 2018). The present experiments extend the category of behaviors impacted to exploration and novelty seeking in female juveniles. These findings suggest that juvenile gonadal hormones regulate a wide-range of social, emotional, and reward-associated behaviors. Although the present effects are likely activational in nature, they could have long-term consequences by affecting the developmental trajectory of an individual. Circulating steroid levels are low, not absent, in prepubertal boys and girls, with sex differences also present prior to puberty (Courant et al., 2010; Janfaza et al., 2006). Hence, similar behavioral actions are possible in humans. If so, it will be essential to determine whether juvenile gonadal hormones contribute to behavioral disorders that arise before puberty. Future studies are needed to assess possible mechanisms, species differences, and sex differences. This research will provide a better understanding of the extent to which juvenile gonads are active regulators of behavioral development.

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Declarations of Interest

None

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Figure Legends

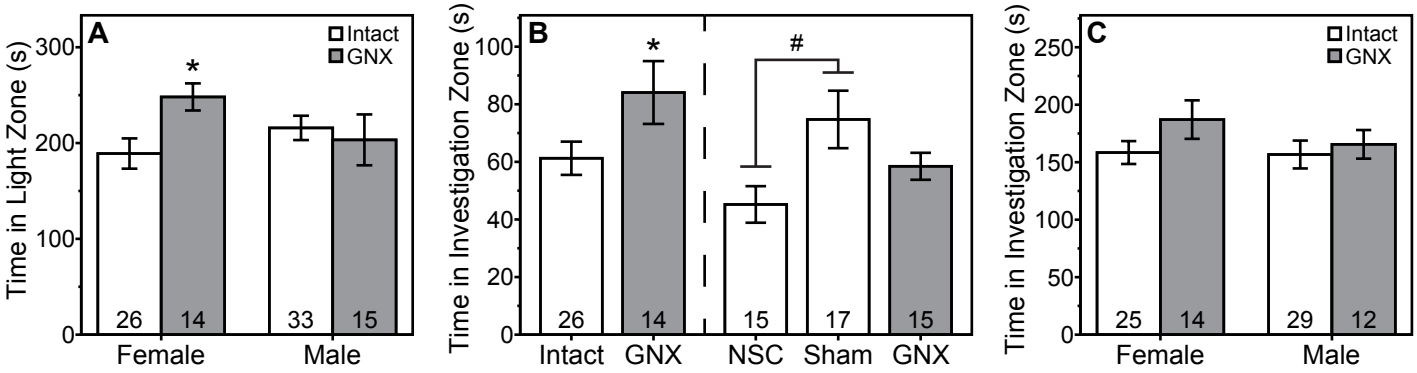
Figure 1. Prepubertal ovariectomy increases exploration and novelty seeking. Amount of time juvenile hamsters spent investigating the light zone (A), novel empty cage (B), and novel same-sex conspecific (C) during Light/Dark Box, Novel Object, and Social Approach tests, respectively. Hamsters were gonadectomized (GNX), sham-operated (Sham), or left un-operated (non-surgical controls; NSC) at ~P15 and tested at ~P30. NSC and Sham measures only differed for novel object investigation of males, t-Test, $P<0.05$; denoted by #. For all other measures, NSC and Sham groups were combined into a single gonadal intact group (Intact). *Indicates significant difference between GNX and Intact groups (Fisher's PLSD [Light/Dark Box test] or t-Test [Novel Object test], $P<0.05$). Sample sizes indicated within bars.

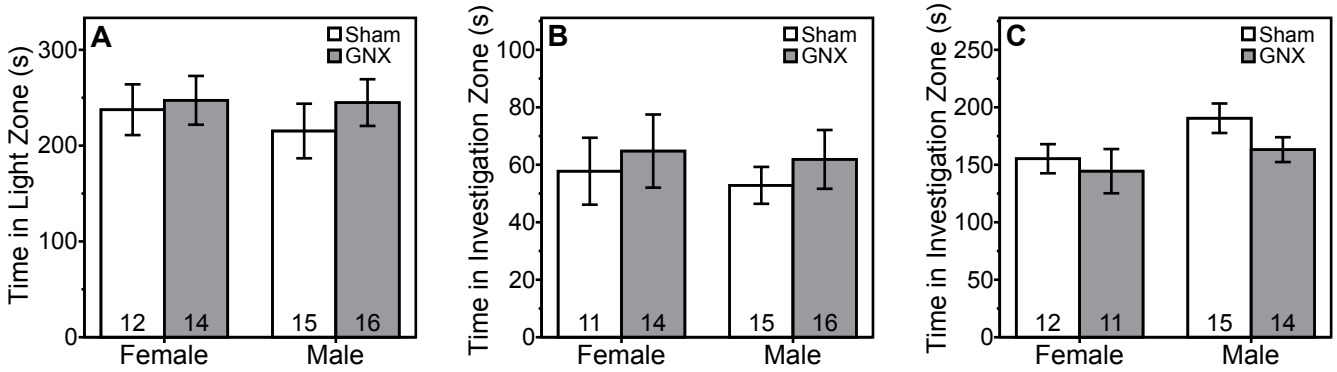
Figure 2. Postpubertal gonadectomy does not impact exploration, novelty seeking, or social approach. Amount of time adult hamsters spent investigating the light zone (A), novel empty cage (B), and novel same-sex conspecific (C) during Light/Dark Box, Novel Object, and Social Approach tests, respectively. Hamsters were gonadectomized (GNX) or sham-operated (Sham) at ~P85 and tested at ~P106. Sample sizes indicated within bars.

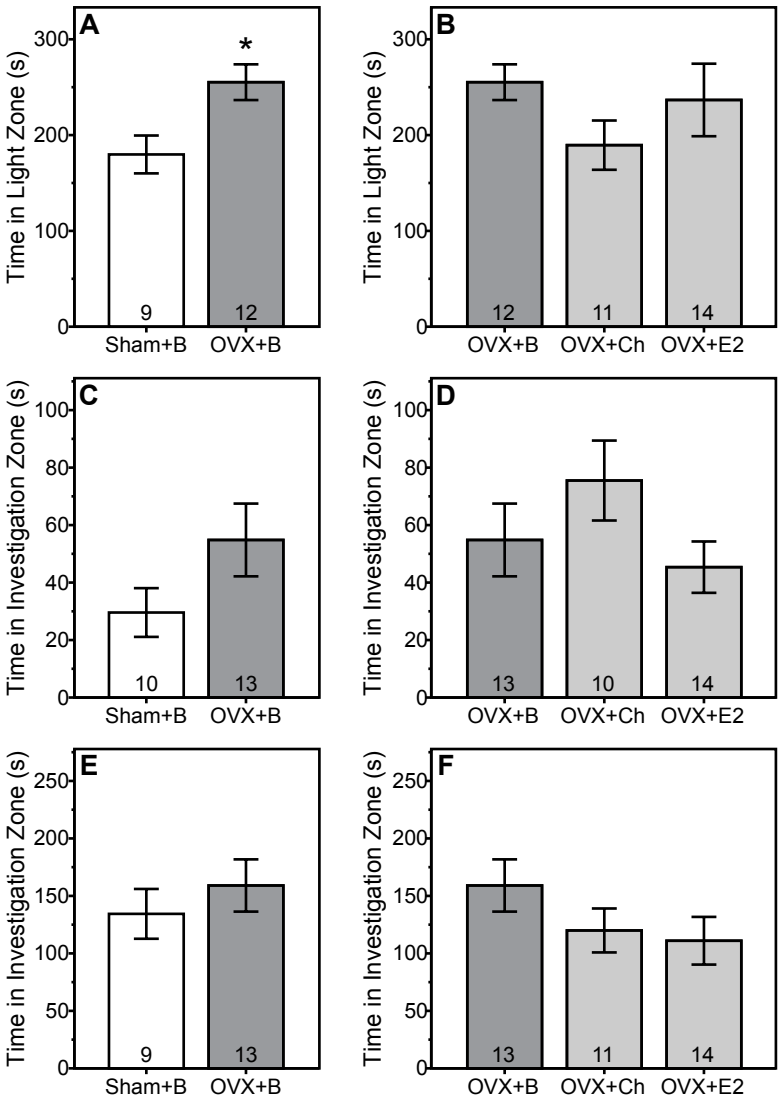
Figure 3. SD-rearing extends behavioral sensitivity of exploration to prepubertal ovariectomy. Amount of time SD-reared juvenile female hamsters spent investigating the light zone (A), novel empty cage (B), and novel same-sex conspecific (C) during Light/Dark Box, Novel Object, and Social Approach tests, respectively. Hamsters were sham-operated and implanted with a blank capsule (Sham+B) or ovariectomized and implanted with a blank (OVX+B), cholesterol-filled (OVX+Ch), or estradiol-filled (OVX+E) capsule at ~P83. Behavioral tests were conducted at ~P106. Note that puberty begins later than P105 in SD-reared female Siberian hamsters (Adam et al., 2000). *Indicates significant difference between Sham+B and GNX+B groups (Fisher's PLSD, $P<0.05$). Sample sizes indicated within bars.

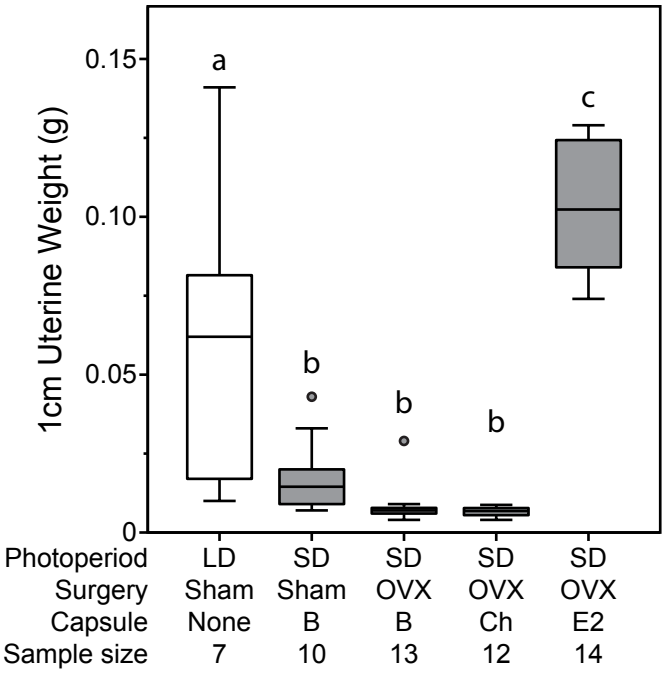
Figure 4. Estradiol capsules increased uterine weights to the upper range of adult LD-reared hamsters. Box and Whiskers plot showing the median (horizontal bar within each box), 1.5-interquartile range (ends of each box), and full range (whiskers) for 1cm uterine weight measures (1cm UWs) of SD-reared hamsters in Experiment 3 as well as a subset of LD-reared Sham adult females from Experiment 2. SD-reared hamsters were sham-operated and implanted with a blank capsule (SD-Sham+B) or ovariectomized and implanted with a blank (SD-OVX+B), cholesterol-filled (SD-OVX+Ch), or estradiol-filled (SD-OVX+E2) capsule at ~P83. LD-reared hamsters were sham-operated at ~P86, but did not receive a capsule implant (LD-Sham). 1cm UWs were recorded at sacrifice at ~P108. Differences in mean 1cm UWs are

indicated by letters above each box; groups with differing letters differ significantly from each other ($P<0.001$, Fisher's PLSD).


A
Intact
GNX
Time in Light Zone (s)
300
200
100
0
*
26
14
33
15
Female
Male
B
Time in Investigation Zone (s)
100
80
60
40
20
0
*
#
26
14
15
17
15
Intact
GNX
NSC
Sham
GNX
C
Intact
GNX
Time in Investigation Zone (s)
250
200
150
100
50
0
25
14
29
12
Female
Male


A
Sham
GNX
Time in Light Zone (s)
300
200
100
0
12
14
15
16
Female
Male
B
Sham
GNX
Time in Investigation Zone (s)
100
80
60
40
20
0
11
14
15
16
Female
Male
C
Sham
GNX
Time in Investigation Zone (s)
250
200
150
100
50
0
12
11
15
14
Female
Male


A
B
C
D
E
F
Time in Light Zone (s)
Time in Investigation Zone (s)
0
100
200
300
20
40
60
80
150
250
50
*
9
12
11
13
10
14
Sham+B
OVX+B
OVX+Ch
OVX+E2


1cm Uterine Weight (g)
0.15
0.10
0.05
0
a
b
b
b
c
Photoperiod LD SD SD SD SD
Surgery Sham Sham OVX OVX OVX
Capsule None B B Ch E2
Sample size 7 10 13 12 14