

**Effects of Photoperiod on Novelty Preference in Preadolescent
Siberian Hamster (*Phodopus sungorus*) Pups**

Honors Thesis
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II. Abstract

Photoperiod (day length) has been shown to have significant effects on the physical and behavioral development of seasonally breeding mammals and to modulate the timing of their dispersal from the natal burrow or den. Behaviors associated with dispersal, such as novelty preference and exploratory behaviors, have been shown to be modulated by anti-Müllerian hormone (AMH) in mice. Whereas photoperiodic modulation of serum AMH concentrations in female Siberian hamsters (*Phodopus sungorus*) has been demonstrated, novelty preference and exploratory behaviors have not been previously investigated in this species. Through the manipulation of day length (DL) in Siberian hamsters, this study sought to determine if photoperiod-induced differences in serum AMH concentration correlate with behaviors that are associated with dispersal. Although AMH was significantly different in males and females, and day length had a significant effect on AMH in males, our evaluations of novelty preference and exploratory behaviors revealed no effects of sex or photoperiod when evaluated at 21 days of age. The information acquired from this study will contribute to the understanding and characterization of the associations among DL, AMH, and the behaviors associated with the dispersal of adolescent mammals. Utilizing this study's results, future research is required to determine the age at which sex differences in novelty preference and exploratory behavior are first apparent in Siberian hamsters and to evaluate variations in AMH associated with age, sex, and photoperiod.

III. Acknowledgments

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IV. Table of Contents

	Page Number
Title Page.....	1
Abstract.....	2
Acknowledgments.....	3
Table of Contents.....	4
Abbreviations.....	5
Introduction.....	6
Literature Review.....	8
Materials and Methods.....	11
Results.....	15
Discussion.....	19
Literature Cited.....	22
Appendix.....	24

IV a. Abbreviations

AMH: anti-Müllerian hormone

DL: day length

SD: short day (8 hours of light per day)

LD: long day (16 hours of light per day)

LD/M: long-day male

SD/M: short-day male

LD/F: long-day female

SD/F: short-day female

V. Introduction

Dispersal away from the natal site, such as a den or burrow, is generally more common in male than in female mammals (Palanza et al. 2001). The exploratory and novelty seeking behaviors associated with dispersal (e.g. increased locomotor and investigational activity) are also more commonly exhibited by males than females (Morgan et al. 2011, Palanza et al. 2001). Photoperiod, or day length (DL), has been shown to have significant effects on the physical and behavioral development of seasonally breeding mammals and to modulate their dispersal (Goldman 1999, Palanza et al. 2001). For instance, male rodents born late in the breeding season (e.g., after the summer solstice when day length decreases) tend to postpone dispersal until the following spring (Smale et al. 1997). Conversely, males born early in the breeding season (e.g., throughout the spring when day length increases) tend to disperse shortly after weaning. Interestingly, recent studies utilizing knockout mice have shown that anti-Müllerian hormone (AMH) modulates behaviors associated with dispersal such as novelty preference and exploratory behaviors in young male mice (Morgan et al. 2011, Palanza et al. 2001). Building on previous findings that demonstrated that photoperiod also modulates serum AMH concentrations in female Siberian hamsters at 10 weeks of age (Kabithé & Place 2008), the present study was designed to determine if AMH differs by sex and photoperiod in hamsters at a younger age (3 weeks) and if any differences in AMH correlate with differences in novelty preference and exploratory behavior.

As its name indicates, anti-Müllerian hormone, also known as Müllerian inhibitory substance, is produced by developing testes during sexual differentiation in fetal males and leads to the regression of the Müllerian ducts, which become the uterus, oviducts, and upper third of the vagina in females (Jost 1947). Recent studies have shown that AMH also participates in

sexually dimorphic brain development and consequent sex-specific behaviors (Wang et al. 2009, Wittmann & McLennan 2013). Studies using juvenile AMH knockout and wild-type mice demonstrated the essential effects of AMH on novelty preference and exploratory behaviors in young animals (Morgan et al. 2011, Palanza et al. 2001). Specifically, the elimination of AMH or its receptor reduced the level of novelty preference and exploratory behavior in males, which resulted in the loss of sexual dimorphism in these behaviors.

Mice are not particularly photosensitive, and therefore do not show distinct responses to varying day lengths. Because gonadal development is substantially delayed when Siberian hamsters (*Phodopus sungorus*) are reared in short days (SD), this species was a logical choice to investigate the potential associations among AMH, DL, and the behaviors associated with dispersal (Goldman 1999, Place et al. 2004, Pyter & Nelson 2006, Timonin et al. 2006). The objectives of this study were to (1) assess the effects of DL and sex on novelty preference in juvenile Siberian hamsters, (2) to evaluate the effects of DL and sex on circulating concentrations of AMH, and (3) to determine if circulating concentrations of AMH and novelty preference are correlated.

VI. Literature Review

Research conducted by Lambin (1994), Morgan et al. (2011), Palanza et al. (2001), and Smale et al. (1997) has demonstrated that rodents, especially seasonally breeding species (e.g., Townsend's voles and Belding's ground squirrels), disperse away from their natal sites during specific times of year, depending on their season of birth (e.g., spring vs. summer). It has been suggested that by moving away, rodents avoid inbreeding, population saturation, and resource depletion within a given territory. Lambin (1994) and various other field studies (Guaffre et al. 2009, Nunes et al. 1998) have demonstrated that males are more likely to disperse and display exploratory and novelty seeking behavior than females. Males disperse away from their natal burrows to establish territories and mating opportunities, whereas females remain relatively close to their natal burrow throughout their lives. Although little is known about the natural history of Siberian hamsters in the wild, specifically at what ages males disperse when born into increasing or decreasing photoperiod, it is assumed that their life history resembles that of voles and other photosensitive rodents for which dispersal has been studied in free-ranging animals (Lambin 1994).

In addition to dispersal and novelty seeking behaviors, photoperiod has been shown to modulate serum AMH concentrations in 10-week-old female Siberian hamsters (Kabithe & Place 2008). Anti-Müllerian hormone, also known as Müllerian inhibitory substance, is produced by developing testes during sexual differentiation in fetal males and leads to the regression of the Müllerian ducts, which become the uterus, oviducts, and upper third of the vagina in females (Jost 1947). Recent studies utilizing AMH knockout mice have shown that AMH also modulates novelty preference and exploratory behaviors in young male mice (Morgan et al. 2011, Palanza

et al. 2001) and that AMH participates in sexually dimorphic brain development and consequent sex-specific behaviors (Wang et al. 2009, Wittmann & McLennan 2013).

Previous studies have demonstrated the essential effects of AMH on novelty preference in mice (Morgan et al. 2011). Both Morgan et al. (2011) and Wang et al. (2009) utilized AMH or AMH receptor knockout and wild-type mice to demonstrate that AMH influences sexually dimorphic behaviors and that an inherent lack of AMH or its receptor results in the loss of the sex differences in novelty preference and exploratory behavior. Behavioral trials performed by Morgan et al. (2011) consisted of habituation, familiarization, and novelty portions in which focal animals were serially presented with a familiar object, two identical familiar objects, and a novel and familiar object, respectively. The Morgan et al. (2011) study and its protocols were the basis for the determination of novelty preference in the present study. Results reported by Morgan et al. (2011) included the observation of a novelty preference in wild-type male mice and a significant difference in the object preference of AMH^{+/+} and AMH^{-/-} male mice. Neither of the experimental female groups nor the knockout male group demonstrated a significant preference for the novel or familiar object, i.e., their investigation of objects was essentially random.

The exploratory behavior of AMH knockout and wild-type mice observed by Palanza et al. (2001) and Wang et al. (2009) was defined by the amount of time an animal spent in a novel vs. a familiar compartment and the number of times an animal reared onto its hindlimbs, respectively. The present study also utilized a proxy value for exploratory behavior. The more time a focal animal spent at the periphery of the test arena, the less time it will have spent exploring the interior of the arena, and thus the less exploratory it was considered to be. Wang et

al. (2009) noted that AMH^{-/-} male mice were less exploratory than wild-type males, and AMH^{-/-} males were more similar to females in this regard.

The absence of AMH throughout development in AMH^{-/-} mice is in sharp contrast to the present study's wild type hamsters that were raised in different photoperiods. Although the knockout mouse studies have determined AMH's essential effects on novelty preference and exploratory behaviors, especially with regard to males, the result may not be broadly applicable due to the artificial genetic modification of the mice. Through the elimination of AMH or its receptor these studies may have bypassed physiologically crucial mechanisms involving AMH. For example, Wittmann & McLennan (2013) recently determined the importance of AMH in regulating the number of calbindin-positive neurons in the sexually dimorphic nucleus of the preoptic area of male mice. They propose AMH's effects on this area of the brain are important for sexual dimorphic differentiation during and after fetal development.

Whereas photoperiod has been shown to modulate serum AMH concentrations in female Siberian hamsters (Kabithe & Place 2008), novelty preference and exploratory behavior have not been previously investigated in this species. For these reasons, the present study was designed to investigate the effects of DL and sex on the novelty preference and exploratory behavior of preadolescent Siberian hamsters through the natural modulation of circulating AMH concentrations via the manipulation of photoperiod. A central purpose of this study was to determine if novelty preference and exploratory behavior are sexually dimorphic in Siberian hamsters and if the sexual dimorphism is modulated by DL and/or AMH.

VII. Materials and Methods

Experimental Animals

The Siberian hamster breeding colony used to complete this study was derived from wild-bred stock obtained from Dr. K. Wynne-Edwards (Department of Comparative Endocrinology, University of Calgary). Colony animals were housed at the Cornell University College of Veterinary Medicine East Campus Research Facility in polypropylene cages with food (Teklad Mouse Breeder Diet 8626) and water available *ad libitum* at 21 ± 5 °C (ambient temperature) and $50\pm 10\%$ humidity. Prior to the study, colony animals were held in 14 hours of light per day (14L). Mature male and female Siberian hamsters selected as breeding pairs were then housed in either long days (LD, 16 hours of light per day) or short days (SD, 8 hours of light per day). Resulting litters remained in LD or SD throughout the study and pups were weaned on postnatal day 18. Same sex siblings were housed together in their natal photoperiod until postnatal day 21, at which time a male and female preadolescent pup from each litter were used to perform a series of behavioral trials (see Appendix Fig. 5). Each of the four experimental groups (LD males, SD males, LD females, and SD females) contained ten animals.

Behavioral Trials

The methods and materials used to complete the behavioral trials were modeled after studies in mice described by Morgan et al. (2011), Palanza et al. (2001), and Wang et al. (2009). Whereas the movements of the mice used in the Palanza et al. (2001) and Wang et al. (2009) studies were tracked using infrared beam disturbances and computer programs, the present study defined hamster exploratory behavior as the inverse of time spent at the periphery of the test arena. Behavioral trials were composed of three experimental subdivisions: habituation,

familiarization, and novelty. Every habituation series consisted of eight 5-minute habituation trials, each separated by 2-minute rest intervals. The habituation trials were followed by a 2-minute rest interval, a single 5-minute familiarization trial, another 2-minute rest interval, and finally a 5-minute novelty trial (see Appendix Fig. 6). During each trial, the focal hamster pup was placed in a uniform test arena (44 x 31 x 12 cm translucent plastic basin) and returned to its home cage for the 2-minute rest intervals. The habituation trials presented the hamster pup with opportunities to investigate the testing arena and an object (25mL glass Erlenmeyer flask) to which it was initially unfamiliar. Familiarization involved exposure of the animal to an additional identical object (another flask). Finally, the novelty trial introduced the pup to a different unfamiliar object (glass cover to a Coplin jar). All behavioral observations occurred under dim red light, during the hamster's active period, 1-2 hours after lights-off. The arena and objects were cleaned with 50% ethanol between subjects. A trained assistant who was blind to the experimental groups scored the video recordings of the novelty trials for "time at the novel and familiar objects" and "time at periphery of test arena" (see Appendix for details). The computer program (*Event Coder*) used to score the video recordings was developed and kindly provided by Dr. Michael Goldstein (Department of Psychology, Cornell University).

Blood Sample Collection

On the day following the behavioral trials (postnatal day 22), blood samples were collected from the retroorbital sinus of experimental animals under Isoflurane anesthesia. Some animals that were used in the behavioral trials had been previously committed to other studies, which precluded the collection of blood from those animals. Because blood samples could not be collected from all behaviorally tested animals, additional blood samples were collected from other animals of the proper age, sex, and DL. Serum samples from non-tested hamsters

improved group sample sizes so that the effects of day length and sex on AMH could be evaluated. Blood samples were clotted on ice for one hour and then centrifuged at 4°C and 1300g for 20 minutes. Serum was drawn off from each sample and stored at -80°C until assayed for AMH. Experimental procedures were conducted with the approval of Cornell University's Institutional Animal Care and Use Committee and in accordance with the NRC Guide for the Care and Use of Laboratory Animals.

AMH Assay

Serum AMH concentrations (ng/mL) were quantified using the *Ansh Labs* Rat and Mouse AMH ELISA (AL-113, Webster, TX), which had been previously validated for use in Siberian hamsters by demonstrating that a serial dilution of hamster serum was parallel to the assay's standard curve. Serum samples were diluted according to the assay instructions and run in duplicate to determine AMH concentrations. Samples with a concentration less than the detection limit of the assay (2.30 ng/mL) were assigned that value for statistical purposes. All samples were run in a single assay and the intra-assay coefficient of variation was 5.1%.

Statistical Analysis

The commercial statistical program *JMP*[®] *Pro* version 10.0.2 (SAS Institute Inc., Cary, NC) was used for all statistical analyses. The Shapiro-Wilk test was used to determine if data were normally distributed and the Levene's test was used to assess for unequal variances.

The "object preference" score for each hamster was calculated using the data collected from *Event Coder* using the following formula:

$$\frac{(\text{time spent at novel object}) - (\text{time spent at familiar object})}{(\text{total time spent at both objects})}$$

“Preference” scores were normally distributed and analyzed by a one-sample *t*-test for each experimental group. Preference scores range from -1 to 1. A preference score of zero = random, or no preference. Scores closer to -1 or 1 indicate preference for the familiar or novel object, respectively.

The “proportion of time at novel” score for each hamster was calculated using the following formula:

$$\frac{\text{(time spent at novel)}}{\text{(total time spent at both objects)}}$$

Mean “proportion of time at novel” scores were analyzed by a two-factor analysis of variance (ANOVA) for the main effects of DL and sex.

The “proportion of time at periphery” score for each hamster was calculated using the following formula:

$$\frac{\text{(time spent at periphery)}}{\text{(duration of novelty trial = 300 seconds)}}$$

Mean “proportion of time at periphery” scores were analyzed by a two-factor ANOVA for the main effects of DL and sex.

Mean serum AMH concentrations (ng/mL) were not normally distributed and thus analyzed with a non-parametric ANOVA (van der Waerden test) to determine differences among the experimental groups. Post-hoc non-parametric pairwise comparisons were performed using the Steel Dwass method.

Pearson product-moment correlations were calculated to determine if serum AMH concentration correlated with any of the behavioral measures.

VIII. Results

Behavioral Trials

Novel vs. Familiar Object Preference:

None of the experimental groups exhibited a preference for the novel or familiar object, that is, none of the preference scores were significantly different from zero, which represents a random pattern of behavior (Fig. 1). There was no significant effect of DL or sex on object preference scores.

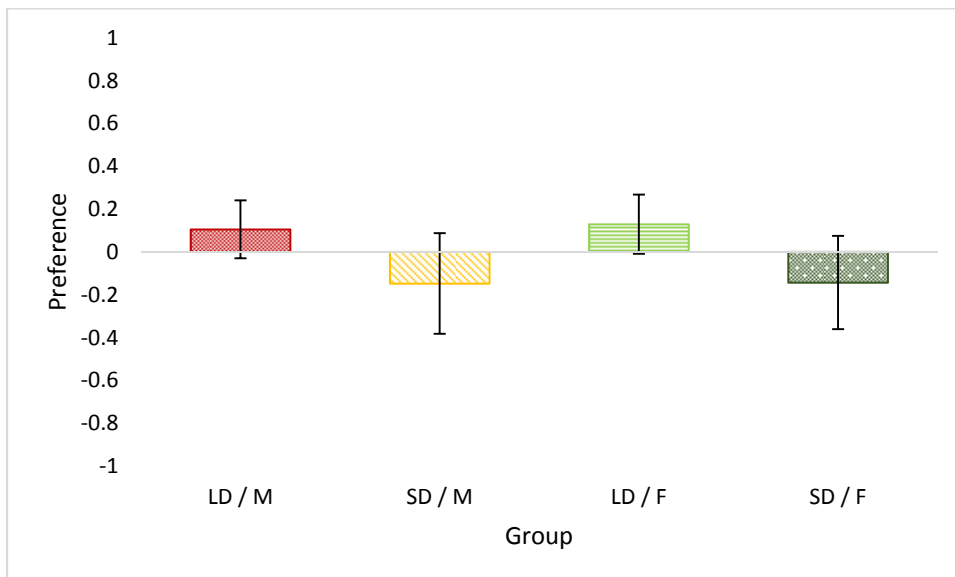


Figure 1: Object preference scores (mean \pm SEM) during “novelty” portion of behavioral trials. Trial subjects were presented with a familiar object (25mL glass Erlenmeyer flask) and a novel object (glass cover to a Coplin jar). Video recordings of the “novelty” portion of the behavioral trials were scored for preference and used to generate the data values displayed. A preference score of 1 indicates maximum preference for the novel object. A preference score of -1 indicates maximum preference for the familiar object. A preference score of 0 indicates a random preference. $n=10$ for all groups. LD/M = LD-males, SD/M = SD-males, LD/F = LD-females, and SD/F = SD-females.

Proportion of Time at Novel:

Each group spent approximately 50% of their object inspection time during the “novelty” section of the behavioral trials investigating the novel object (Fig. 2). There was no statistically significant difference in the “proportion of time spent at novel object” among the groups, no effect of DL or sex, and no interaction effect of DL and sex.

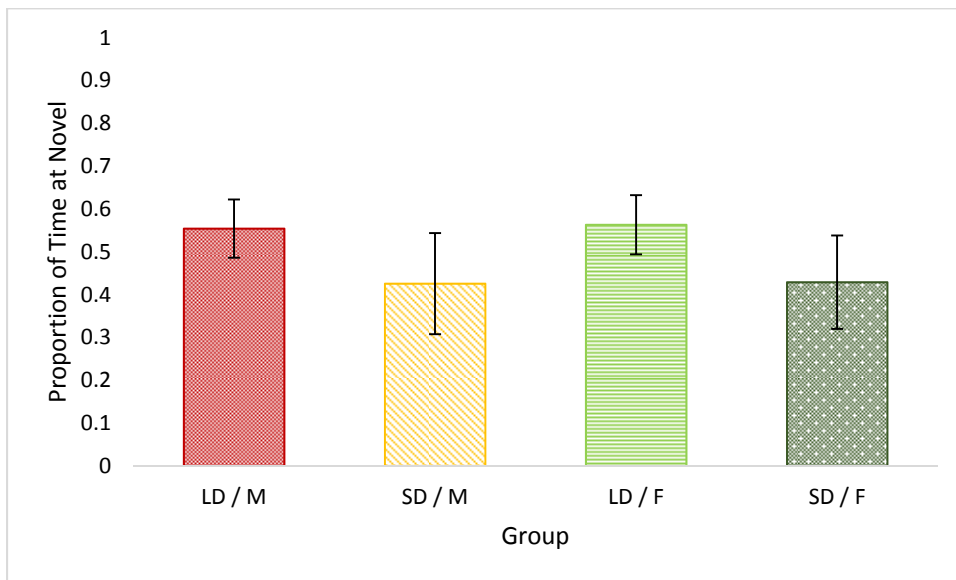


Figure 2: Proportion of time spent at novel object during “novelty” portion of behavioral trials (mean \pm SEM). Trial subjects were presented with a familiar object (25mL glass Erlenmeyer flask) and a novel object (glass cover to a Coplin jar). $n=10$ for all groups. LD/M = LD-males, SD/M = SD-males, LD/F = LD-females, and SD/F = SD-females.

Proportion of Time at Periphery:

All groups spent a majority of their time at the test arena’s edge (Fig. 3). There was no statistically significant difference in the “proportion of time at periphery” among the groups, no effect of DL or sex, and no interaction effect of DL and sex.

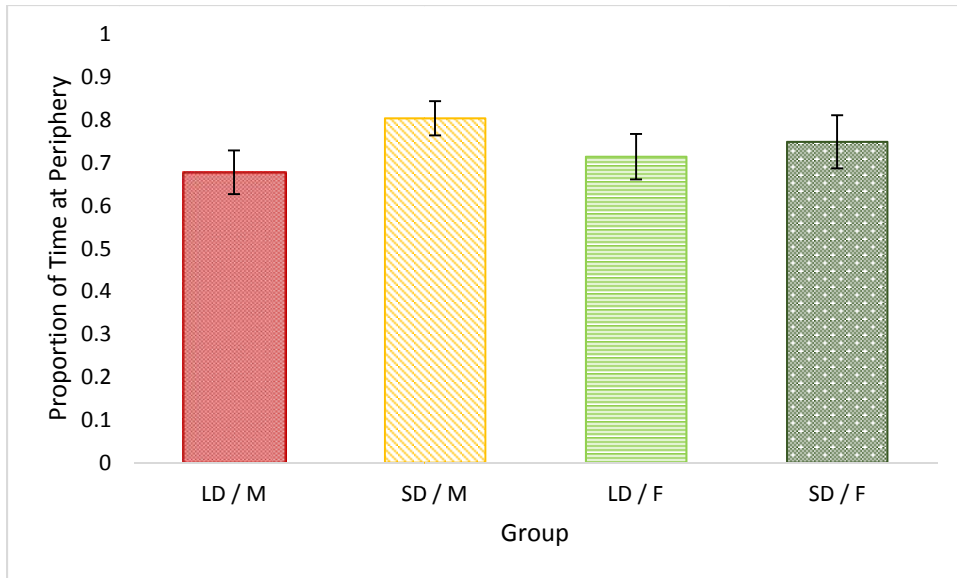


Figure 3: Proportion of time spent at arena periphery during “novelty” portion of behavioral trials (mean \pm SEM). Trial subjects were placed in an arena with which they had been previously habituated and presented with familiar and novel objects. Video recordings of the “novelty” portion of the behavioral trials were scored for time at periphery and used to generate the data values displayed. LD/M = LD-males (n=9), SD/M = SD-males (n=10), LD/F = LD-females (n=9), and SD/F = SD-females (n=10).

Summary – Behavioral Trials:

Each experimental group spent approximately the same amount of time at the novel and familiar trial object and the majority of their time at the periphery of the test arena. Statistical analyses revealed no effects of DL or sex on “object preference”, “proportion of time at novel object”, or “proportion of time at periphery”.

AMH Assay

Mean AMH (ng/mL) vs. Group

Mean serum AMH concentrations ranged from <math><2.30 - 101.86\text{ ng/mL}</math> (Fig. 4). Male and female AMH values were significantly different from one another, i.e. generally higher in females than in males. There was a statistically significant difference in AMH concentrations between LD/M and SD/M, which indicates DL had a significant effect on AMH in males. There was no statistically significant difference in mean serum AMH concentration between LD/F and SD/F.

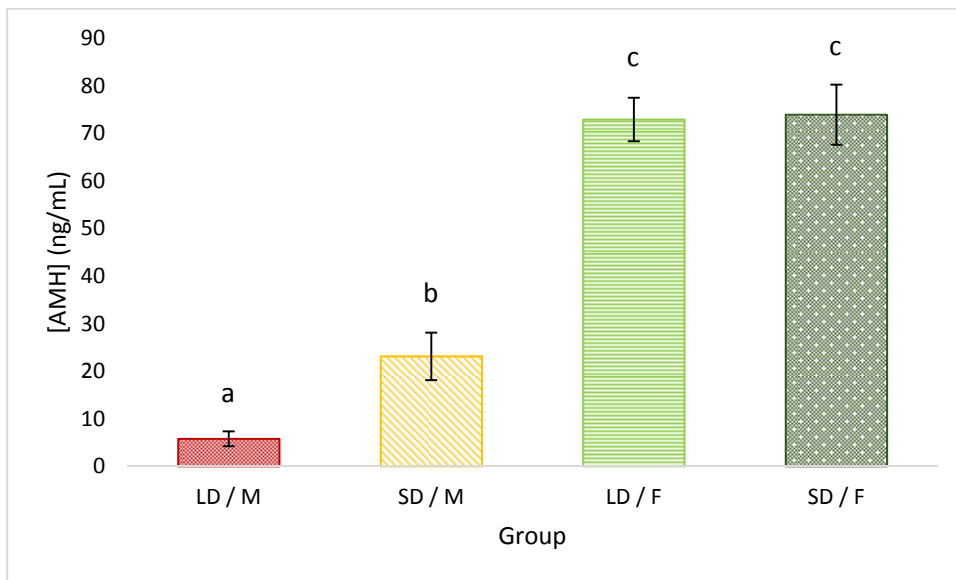


Figure 4: Mean (\pm SEM) serum AMH concentrations (ng/mL) of 22-day-old male and female hamsters held in long day (LD) or short day (SD). Different letters denote significantly different AMH concentrations between groups. LD/M = LD-males (n=12), SD/M = SD-males (n=12), LD/F = LD-females (n=11), and SD/F = SD-females (n=5).

Summary – AMH Assay

There was an effect of DL and sex on circulating AMH concentrations as indicated by the significant difference between LD/M and SD/M and the male and female groups, respectively.

There was no difference in circulating AMH concentrations between LD/F and SD/F.

Summary – Behaviors and AMH

For the subset of hamsters that were evaluated for both AMH and behaviors, there was no significant correlation between serum AMH concentrations and any of the behavioral measures (“object preference”, “proportion of time at novel object”, and “proportion of time at periphery”).

IX. Discussion

Whereas the present study demonstrated significant effects of sex and photoperiod on serum AMH concentrations in Siberian hamsters, these factors did not appear to influence novelty preference or exploratory behavior. This lack of effect on behaviors associated with dispersal is in contrast to the sex differences described in wild-type mice that were utilized in the AMH knockout studies (Morgan et al. 2011, Palanza et al. 2001). Even though mice and hamsters were evaluated at the same age (postnatal day 21), a possible explanation for the difference between species is the fact that gestation is shorter in hamsters than in mice (18 days vs. 21 days). Therefore, hamsters may be less developed at postnatal day 21 than mice and sexual dimorphisms in novelty preference and exploratory behavior may not become apparent in hamsters until an older age. Based on the results of the present study, future research is required to evaluate the relationship between age and the sex difference in dispersal-related behaviors in Siberian hamsters.

Serum AMH concentrations were higher in female than in male hamsters, and the effect of sex was independent of DL. This result is consistent with previous studies that found sex-specific changes in AMH concentrations as individuals progress through postnatal development

(Al-Attar et al. 1997, Hudson et al. 1990, Lee et al. 1996). Specifically, in mice and human, AMH decreases in males and increases in females as they age. Therefore, at some point during the time between pre-natal and postnatal development, the sex difference in serum AMH concentrations may disappear and then reverse. Al-Attar et al. (1997) and Wittmann & McLennan (2013) found AMH levels drop markedly in male mice immediately before weaning on postnatal day 21 and that testes have already extensively matured by this age. Recalling the developmental differences in mice and hamsters, future investigations are warranted to determine if serum AMH concentrations may be higher in male than in female hamsters at younger ages.

An effect of DL on serum AMH concentration was apparent in male but not in female Siberian hamsters. Park et al. (2014) noted that female ovarian histology is very similar at 3 weeks of age in female Siberian hamsters that were held in SD and LD, and the same follicle classes that produce AMH were present in both SD and LD ovaries. Only 14 genes (not including *Amh*) were differentially expressed in ovaries from LD and SD females (Park et al. 2014) at 3 weeks of age. Although Park et al. (2014) found SD ovaries weighed significantly less than LD ovaries at 3 weeks, the present study suggests that reduced ovarian mass in SD does not result in decreased serum AMH concentration. Conversely, Kabithe & Place (2008) determined that serum AMH concentration was lower in SD than in LD females at 10 weeks of age. In that study, SD ovaries also weighed significantly less than LD ovaries, but the histology of SD and LD ovaries was strikingly different. Specifically, the SD ovaries lacked small antral follicles, which may be largely responsible for the release of AMH into the circulation (Broekmans et al. 2008).

Gonadal mass was also reduced in SD males as compared to LD males at 3 weeks of age (Park et al. 2003), due to the profound delay of testicular development in SD relative to LD in

Siberian hamsters (Timonin et al. 2006). To our knowledge, LD vs. SD histological comparisons of testes have not been reported in Siberian hamsters at 3 weeks of age, nor at younger ages. Additional studies will be needed to elucidate a mechanism to explain the serum AMH difference in LD and SD males at 3 weeks of age. Research would include comparisons of testicular histology, gene expression, and AMH production between LD and SD males at 3 weeks of age and perhaps at younger ages.

Based on the present study, future investigations utilizing the Siberian hamster as a model could contribute to the understanding and characterization of the links among DL, hormones, and the behaviors associated with the dispersal of adolescent animals. A particularly fruitful area of study would be the direct observation of dispersal and the associated behaviors in free-ranging Siberian hamsters. This would be logistically challenging, however, due to the habitat in which they live. Future directions include the utilization of the present study's results to determine the age at which sexually dimorphic differences in novelty preference and exploratory behavior are present and to evaluate variations in AMH associated with age, sex, and photoperiod.

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Appendix

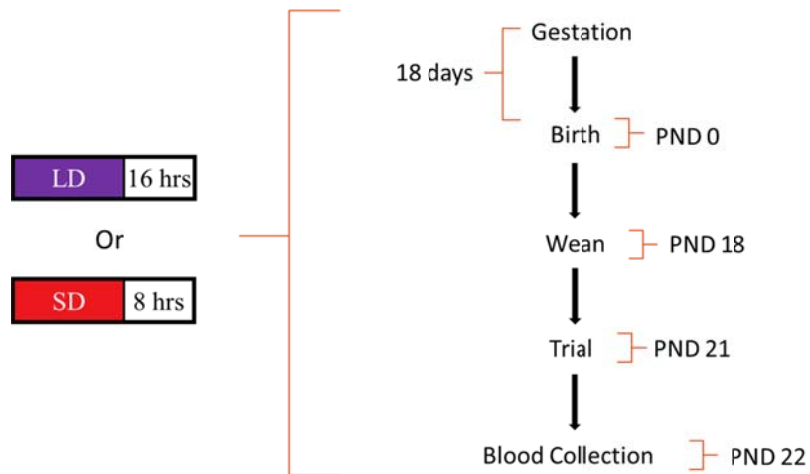


Figure 5: Study methods. Prior to the study, colony animals were held in 14 hours of light per day (14L). Breeding pairs used to generate experimental animals were transferred to either long days (LD, 16 hours of light per day) or short days (SD, 8 hours of light per day). Resulting litters remained in their respective day lengths throughout the study and pups were weaned on postnatal day 18. Same sex siblings were housed together in their natal photoperiod until postnatal day 21, at which time a male and female preadolescent pup from each litter were used to perform a series of behavioral trials. Blood samples were collected the day after behavioral studies were completed (postnatal day 22).

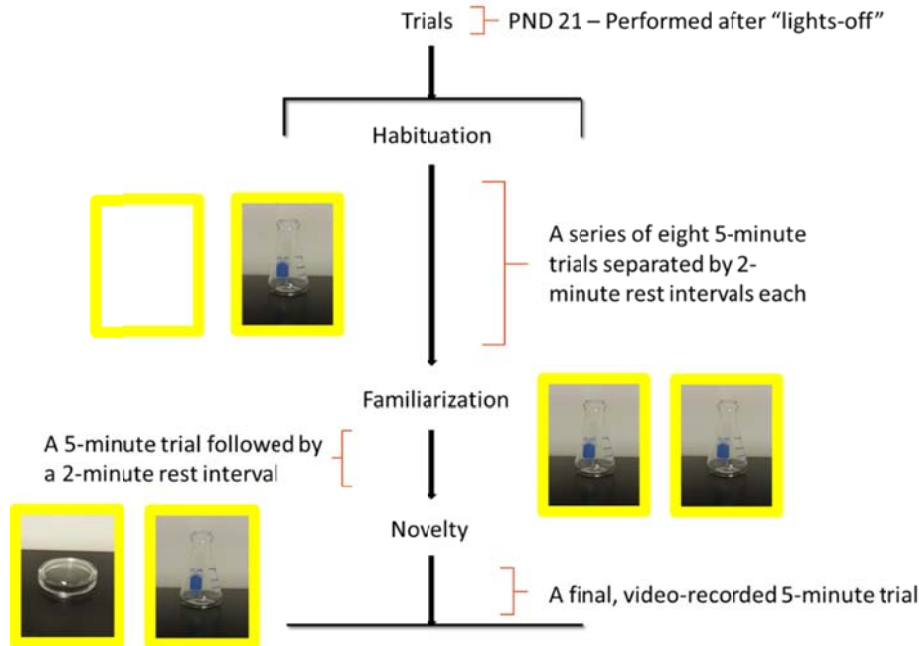


Figure 6: Behavioral trial methods. Behavioral trials were composed of three experimental subdivisions: habituation, familiarization, and novelty. Every habituation series consisted of eight 5-minute habituation trials, each separated by 2-minute rest intervals. The habituation trials were followed by a 2-minute rest interval, a single 5-minute familiarization trial, another 2-minute rest interval, and finally a 5-minute novelty trial. During each trial, the focal hamster pup was placed in a uniform trial arena (44 x 31 x 12 cm translucent plastic basin) and returned to its home cage for the 2-minute rest intervals. The habituation trials presented the hamster pup with opportunities to investigate the testing arena and a particular unfamiliar object (25mL glass Erlenmeyer flask). Familiarization involved exposure of the animal to an additional identical object (another flask). Finally, the novelty trial introduced the pup to a different unfamiliar item (glass cover to a Coplin jar).

Method Details:

Time at Objects: scored when focal hamsters were within the 6x6 cm area surrounding either the familiar or novel object and facing or actively investigating either.

Time at Periphery of Trial Arena: scored when focal hamsters were both static and dynamic within one body width of the trial arena’s periphery.