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GnRH expression and cell proliferation are associated with seasonal breeding and food hoarding in Mongolian gerbils (*Meriones unguiculatus*)

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ABSTRACT

Seasonal brain plasticity contributes to a variety of physiological and behavioral processes. We hypothesized that variations in GnRH expression and cell proliferation facilitated seasonal breeding and food hoarding. Here, we reported seasonal changes in sexual and social behavior, GnRH expression and brain cell proliferation, and the role of photoperiod in inducing seasonal breeding and brain plasticity in Mongolian gerbils (*Meriones unguiculatus*). The gerbils captured in April and July had more mature sexual development, higher exploratory behavior, and preferred novelty much more than those captured in September. Male gerbils captured in April and July had consistently higher GnRH expression than those captured in September. GnRH expression was also found to be suppressed by food-induced hoarding behavior in the breeding season. Both subadult and adult gerbils from April and July had higher cell proliferation in SVZ, hypothalamus and amygdala compared to those in September. However, adult gerbils captured in September preferred familiar objects, and no seasonal differences were found in cell proliferation in hippocampal dentate gyrus among the three seasons. The laboratory study showed that photoperiod alone did not alter reproductive traits, behavior, cell proliferation or cell survival in the detected brain regions. These findings suggest that the structural variations in GnRH expression in hypothalamus and cell proliferation in hypothalamus, amygdala and hippocampus are associated with seasonal breeding and food hoarding in gerbils. It gives a new insight into the proximate physiological and neural basis for these seasonal life-history traits of breeding and food hoarding in small mammals.

1. Introduction

Mammals inhabiting high-latitude regions face critical seasonal fluctuations in climatic conditions and food availability at both the pre- and post-natal life stages. Seasonality is an important factor shaping seasonal ecological adaptations and evolution of life histories in small mammals (Roff, 1992; Stearns, 2000). Seasonal regimes such as photoperiod, temperature and food availability, as well as their combination, generally limit small mammals breeding to more favorable seasons and develop food-hoarding behavior before the coming of harsh winter for maximizing individual's fitness (reproductive success and survival) (Bartke and Steger, 1992; Ebling, 1994). For instance, the effect of photoperiod on seasonal reproduction has been confirmed in wild rodent species. Laboratory studies show that short day (SD) acclimation for 8 to 10 weeks leads to reproductive suppression with decreases in the concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone, as well as testis regression in Syrian hamsters (*Mesocricetus auratus*), Siberian hamsters (*Phodopus sungorus*) and white-footed mice (*Peromyscus leucopus*) (Gaston and Menaker, 1967; Greives et al., 2007; Pyter et al., 2005; Revel et al., 2006). Desert hamsters (*Phodopus roborovskii*) also reduced testes mass in males and the percentage of vaginal opening in females during the 3-month SD acclimation (Zhang et al., 2015).

As a potent stimulator, hypothalamic gonadotropin-releasing hormone (GnRH) neurons secrete GnRH into the pituitary to regulate gonadotropin secretion and sexual behavior (Karsch et al., 1997; Herbison, 2006). The seasonal variations in GnRH expression link brain plasticity and fertility. In recent years, studies both in laboratory and wild species show that neurogenesis in some specific brain regions are essential in regulating reproduction and social communication (Hill et al., 2008; Yoon et al., 2005). The studies in laboratory mice and rats show that new cells generated in the subventricular zone (SVZ) lining

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the lateral ventricles could migrate to olfactory bulb (OB) and influence social recognition between males and females (Feierstein, 2012), the Bruce effect in females after mating (Peretto et al., 2014), and the interactions between a mother and her pups (Furuta and Bridges, 2005; Shingo et al., 2003). Other studies in laboratory and wild rodents and wild chickadees suggest that adult neurogenesis in hippocampal dentate gyrus (DG) and in amygdala (Amy) is associated with learning, memory, social communication and pair bonding (Berson et al., 2002; Freas et al., 2013; Lieberwirth et al., 2012; Snyder et al., 2009). However, there is only a small amount of relevant work with controversial results in ewes (*Ovis aries*) and meadow voles (*Microtus pennsylvanicus*) (Galea and Mcewen, 1999; Migaud et al., 2011; Ormerod and Galea, 2003). Further evidence is required to associate adult neurogenesis with seasonal breeding of small mammals.

Mongolian gerbils (*Meriones unguiculatus*), a social rodent species, are widely distributed in the typical steppe, desert steppe and desert habitats of northern China, Mongolia, and Trans-Baikal region of Russia. In wild, the recorded maximum lifespan (apparent longevity) was 29.5 months for female Mongolian gerbils and 22.2 months for males, and the average longevity of male and female gerbils is 4.6 months (standard deviation [SD] = 3.93) and 5.3 months (SD = 4.95), respectively (Xia et al., 1982; Liu et al., 2009, 2017). Field studies also report that the minimum age at maturity or first breeding of gerbils is 2–3 months. The reproduction and recruitment occur primarily from March to August and the gerbils hoard food with a breeding lull in September and October; however, a minority of gerbils could breed during December to February (Liu et al., 2007, 2017). Accordingly, gerbils could be usually divided into three seasonal cohorts based on the month of their birth. The winter cohort consisted of a mix of winter-born and summer-born individuals; the spring cohort consisted of young born from March to May, which became independent during April and early June; and the summer cohort consisted of gerbils born from June to August, which left the nest between the end of June and breeding season of the year (Liu et al., 2017). The previous studies have reported seasonal cohort-specific life-history patterns in the apparent longevity, age of maturity, potential reproductive lifespan and dispersal in a natural enclosure. For example, the gerbils born in winter and spring displayed an earlier age of sexual maturity compared to those born in summer, while the summer cohort gerbils commonly delayed their reproduction to the following year (Liu et al., 2017). Although seasonal life-history patterns are apparent, the neural basis for seasonal reproduction and food hoarding in wild rodents is still under scrutiny. We hypothesized that the variations in GnRH expression and cell proliferation facilitated life-history traits of seasonal breeding and food hoarding. To test this hypothesis, we took seasonal samplings in April, July, and September in natural populations, manipulated food-induced hoarding in the breeding season, and tested the role of photoperiod in inducing seasonal reproduction and brain plasticity in Mongolian gerbils.

2. Materials and methods

2.1. Subjects

In the field study, subjects were captured in different seasons in Suniteyouqi County, Inner Mongolia, China (E111°08′, N41°55′). Some animals were also raised in Taipusiqi County, Inner Mongolia, China (E115°17′, N41°58′).

In the laboratory study, subjects were from laboratory breeding colonies raised in the Institute of Zoology, the Chinese Academy of Sciences in Beijing, China. Subjects were housed in same-sex groups (2–3) in plastic cages (30 × 15 × 20 cm) that contained sawdust bedding and were fed with standard rat food and water ad libitum. All animals were kept under a 16L:8D photoperiod with lights on at 0400 h and at 23 ± 1 °C. All animal procedures were in agreement with the permission of the Animal Care and Use Committee of Institute of Zoology, the Chinese Academy of Sciences.

2.2. Behavioral tests

Open field test was conducted in an opaque acrylic box (60 × 60 × 50 cm) to assess the exploratory behavior. The central area (15 × 15 cm) and corners of the box were distinguished with visual lines. After the animals were acclimated to the new environment for 5 min, the distance and duration in the center or corners and the frequency of center entries were recorded for 10-minute period by a video camera. The box was wiped with 75% ethanol between tests.

Novelty recognition examination consisted of a 10-minute training phase and a 5-minute testing phase at 1-hour intervals. Two plastic toys with different shapes (one is a cube, the other is a pyramid) were used as the familiar object and novel object, respectively. During 10-minute training period, the gerbils were kept with the cube toy and familiar with it (defined as the familiar object) in a plastic cage (42 × 27 × 18 cm) with a hyaline lid. One hour later, they were kept with the cube and pyramid toys (the latter was defined as novel subject) together in the cage for 5-minute test. Novelty and familiar preferences (i.e., the duration and frequency of investigating familiar or novel object) during 5-minute test were recorded. The toys and cage were wiped with 75% ethanol after each training and test.

Elevated plus maze was used to test the anxiety-like behavior. The maze contained two opposite open arms (30 × 6 cm) crossing two closed arms (30 × 6 × 30 cm) with a central platform (6 × 6 cm) elevated 75 cm above the floor. Subjects were placed onto the central platform facing one closed arm, and the duration and frequency in the closed and open arms were recorded during the 5-minute test. The maze was wiped with 75% ethanol between tests.

Y-maze consisted of three closed arms (40 × 8 × 30 cm) at a 120° angle. The novel arm, start arm and food arm were defined artificially with different visual signs (one square, triangle or round card was stuck to the wall, respectively). During the 5-minute training period, subjects which have been fasted for 24 h were kept in the apparatus and trained to form memories using food with the start arm and food arm open. One hour later, the subjects were placed in the apparatus with three arms open but no food supply. Short-term memory was determined by the duration and frequency in the food arm during the 10-minute test period.

The apparatus for behavior tests was cleaned thoroughly with 75% ethanol between uses to remove odor cues from previous individuals. All the behavioral data were analyzed with EthoVision Image Tracking System 20.0 (Noldus, Holland).

2.3. Tissue collection

After post-fixation in 4% paraformaldehyde and dehydration with 15% and 30% sucrose at 4 °C, brains were cut into 40 μm coronal frozen sections (CM1950, Leica, Germany). One set of brain sections (with a 240-μm interval) was used for BrdU, Ki67 and GnRH IHC, respectively. Testes and ovaries were cut into 7 μm horizontal paraffin sections (RM2235, Leica, Germany) for H&E staining.

2.4. Immunohistochemistry (IHC)

IHC for BrdU, Ki-67 and GnRH were processed on a modified protocol (Lieberwirth et al., 2012). Briefly, sections were incubated in 0.3% hydrogen peroxide and 10% methanol in 0.1 M PBS for 15 min (this step was not necessary for GnRH immunofluorescence, IF). Denaturation with 2 N HCl in 0.1 M PBS at 60 °C for 30 min was required for BrdU IHC, but not for Ki-67 and GnRH IHC. After 1-h of incubation with 10% normal goat serum (NGS) containing 0.5% Triton-X 100 in 0.1 M PBS (BrdU IHC) or 10% NGS in TBS (2% BSA 2% NGS 0.5% Triton-X 100 in 0.1 M PBS, Ki67 and GnRH IHC), mice-anti-BrdU (1:2000, G3G4, DSHB, Iowa, USA), rabbit-anti-Ki67 (1:2000, ab15580, Abcam, Cambridge, UK) and rabbit-anti-GnRH (1:2000 and 1:1000, ab5617, Abcam, Cambridge, UK) were used as primary antibodies, respectively, for 2 h at room temperature and then 4 °C overnight. Biotin-conjugated goat-anti-mice (1:600, 115–065-146, Jackson, PA, USA), goat-anti-rabbit (1:600, W1002, Vector, CA, USA), Alexa flour 594-conjugated goat-anti-rabbit (1:300, 111–585-003, Jackson, PA, USA), and Alexa flour 488-conjugated goat-anti-rabbit (1:300, 111–545-003, Jackson, PA, USA) were used as second antibodies, respectively (IF was processed in dark from the step of incubating with second antibody), at room temperature for 2 h. For biotin-conjugated antibodies, sections were placed into ABC complex (PK-6100, Vector, CA, USA) for 90 min, and positive cells and fibers were visualized with DAB (SK-4100, Vector, CA, USA). GnRH IF was counterstained with DAPI (H1200, Vector, CA, USA). The negative controls processing the secondary antibody with the omission of the primary were performed to verify the specificity of the label.

2.5. H&E staining

H&E staining of the testis and ovary was done as previously described (Seminara et al., 2004). Ten coronal sections of the seminiferous tubule with mature sperm in the same spermatogenic cycle in each subject were quantified, while serial sections of the ovary in each subject were quantified entirely by Image J software.

2.6. Sexual hormone radioimmunoassay

Serum testosterone was measured by ^{125}I-Rabbit-Testosterone Radioimmunoassay Kit (Beijing North Institute of Biological Technology, Beijing, China). Fetal bovine serum was used as the intraplate control. The intra-assay coefficient of variation (CV) was < 10% and inter-assay CV was < 15%. The lower and upper limits of the testosterone assay kit were 0.1 ng/mL and 20 ng/mL. Serum testosterone was determined in a single test and expressed as ng/mL.

2.7. Data quantification and statistical analyses

Images were acquired using a Nikon eclipse 80i microscope and SPOT advanced software for IHC sections and a Zeiss LSM 710 confocal microscope and ZEN 2010 software for IF sections, and were quantified with Image J software and Image Pro Plus 6.0 software, respectively. Cell numbers and optical density were measured by one student blindly to the treatment group. Different experimental groups were processed at the same threshold parameter and in parallel to account for variation in the labeling process. The stained cells and fibers were identified and quantified bilaterally for the specific brain regions. At least 3 sections were counted bilaterally for each brain region. Brain regions were identified and defined using the rat brain atlas (Paxinos and Watson, 1998).

All data analyses were performed in SPSS 20.0 software. Kolmogorov-Smirnov and Levene tests were used to examine the normality and homogeneity of variance respectively. The data that were not normally distributed and the percent data were performed with Arcsin or Lg_{10} transformation before analysis. Qualitative data (i.e., MVG, testicular descent index and vaginal opening) were analyzed using χ^2-test, and quantitative data were analyzed using two-way ANOVA (sex × season in Experiment I and sex × photoperiod in Experiment III), one-way ANOVA, and independent-samples *t*-test. The data in young subjects were analyzed by one-way ANOVA since there was only one young male in September. The effect size estimates (partial Eta-Squared, η_p^2 for ANOVA) was calculated by SPSS. All data were expressed as means ± SE, and the significance level was set at $\alpha = 0.05$.

2.8. Experimental designs

2.8.1. Experiment I

In order to reveal seasonal changes in sexual maturation, behavior, and brain plasticity, we captured 123 gerbils from the Suniteyouqi County, Inner Mongolia, China (E111°08′, N41°55′), in April, July, and September in 2015 (44 in April, 38 in July and 41 in September). Body mass and sex were recorded, and gerbils were considered young if their body mass was ≤30 g, subadult with body mass of 30–50 g, and adult with body mass ≥ 50 g (Liu et al., 2007, 2017). Sexual maturation was determined by the testicular descent index in males, vaginal opening in females, and the midventral gland (MVG) in both sexes. The open field test and novelty recognition test were conducted to measure exploratory behavior and recognition memory, respectively. A thymidine analogue, 5-bromo-2′-deoxyuridine (BrdU) which can be incorporated into DNA during the S-phase of the cell cycle was used to detect brain cell proliferation (Lieberwirth et al., 2012; Pan et al., 2013). Considering the difficulty of recapture in the field, the gerbils were intraperitoneally injected with BrdU (300 mg/kg body mass, Sigma, CA, USA) for only one time. Two hours after BrdU injection, subjects were anesthetized by intraperitoneal injection with an overdose of pentobarbital (50 mg/kg) and perfused with 0.9% saline followed by 4% paraformaldehyde solution. The brains were separated, post-fixed overnight, and then stored in 30% sucrose in 0.1 M PBS. The structure of sexual organs and the sexual hormones in serum were examined. We measured hypothalamic GnRH expression and cell proliferation in several brain regions. For measurement of cell survival, the animals need to be recaptured and sacrificed 3–6 weeks after BrdU injection (Lieberwirth et al., 2012; Pan et al., 2013). We did not test cell survival due to the low success rate of capture-recapture in the field.

2.8.2. Experiment II

To investigate the relationship between reproduction and food hoarding, we induced the gerbils to hoard food by supplementing wheat seeds during breeding season. Forty adult male gerbils captured in July 2015 in Taipusiqi County, Inner Mongolia, China (E115°17′, N41°58′) were raised in 8 enclosures (10 × 10 m, natural grass is rich in all enclosures) randomly, and 4 enclosures were supplemented with wheat seeds to induce food-hoarding behavior (inducing group) for 4 weeks (40 g wheat in a dish, 3 dishes at one time, twice per week) while others served as the control group (without supplemental wheat seeds, non-inducing group) (Liu et al., 2009). After the 4-week acclimation, 1–2 gerbils from each enclosure were sacrificed (inducing group, n = 6; non-inducing group, n = 7) by perfusion for GnRH measurement.

2.8.3. Experiment III

To verify the role of photoperiods in inducing seasonal breeding and brain plasticity, photoperiodic acclimation was conducted in the laboratory gerbils. Adult gerbils (70–90 days of age, 38 males and 29 females) from the laboratory breeding colony were singly housed at 23 ± 1 °C with light-controlled conditions of 16L:8D (lights on at 04:00). They were divided into one of three groups randomly: LD, in 16L:8D photoperiod for 10 weeks; SD, in 8L:16D photoperiod for 10 weeks; SL, in SD for 6 weeks to recover their photosensitivity and then transferred back to LD condition for the next 4 weeks. Reproductive activity was determined by both the weight and structure of sexual organs and the levels of serum sexual hormones. Open field test, elevated plus maze, and Y-maze tests were conducted in the last two weeks. In the 6th week, the gerbils were intraperitoneally injected with BrdU (150 mg/kg, once every day) for 7 days. Four weeks after BrdU injection, all gerbils were perfused for tests of cell survival by BrdU IHC. One set of brain sections was used to test cell proliferation by Ki67 (an endogenous marker of cell proliferation) IHC (Lieberwirth et al., 2012; Pan et al., 2013).

Table 1
Body mass in Mongolian gerbils captured in different seasons.

Body mass (g)	April		July		September		P		η_p^2	
	Male	Female	Male	Female	Male	Female	Season	Sex	Season	Sex
Young	15.80 ± 3.21	20.44 ± 4.11	21.95 ± 1.60	21.56 ± 2.01	–	25.26 ± 1.84	0.069	–	0.16	–
Subadult	37.88 ± 1.96	39.46 ± 2.11	43.60 ± 3.41	42.08 ± 4.48	38.13 ± 2.16	38.27 ± 1.21	0.206	0.828	0.069	0.001
Adult	62.96 ± 1.12	56.69 ± 1.61	62.34 ± 2.60	58.04 ± 1.39	61.75 ± 3.57	56.49 ± 1.48	0.847	0.001	0.007	0.204

Results are presented as mean ± SE.

3. Results

3.1. Seasonal differences in development and sexual maturation

Significant seasonal effects on sexual development were observed both in subadult and adult subjects, but not in young subjects. No seasonal differences were found in the body mass of gerbils captured in the three seasons (Young, $F_{2,22} = 3.03$, $P = 0.069$, $\eta_p^2 = 0.216$; Subadult, $F_{2,44} = 1.64$, $P = 0.206$, $\eta_p^2 = 0.069$; Adult, $F_{2,46} = 0.17$, $P = 0.847$, $\eta_p^2 = 0.007$; Table 1), but subadults and adults captured in April and July had a higher MVG score than those in September (Subadult, $P = 0.019$; Adult male, $P = 0.009$ and Adult female, $P = 0.007$; Fig. 1A–D). Males were deemed to have attained sexual maturity if the testicular descent index had reached 2. Seasonal differences in sexual maturity were found in adult males ($P = 0.05$; Fig. 1F) but not in subadult males ($P = 0.074$; Fig. 1E), and more adult males captured in April and July attained sexual maturity than those captured in September. In addition, a larger number of mature sperm were observed in adult males ($P = 0.013$; Fig. 1J) but not in subadult males ($P = 0.064$; Fig. 1I) captured in April and July when compared with those captured in September. Adult males captured in September had consistently lower serum testosterone levels than those captured in other seasons ($F_{2,19} = 4.51$, $P = 0.025$, $\eta_p^2 = 0.322$; Fig. 1K). In contrast, the percentage of subadult and adult females with open vaginas captured in different seasons was similar (Subadult, $P = 0.137$; Adult, $P = 0.374$; Fig. 1G and H), and no seasonal variation was observed in the structure of the ovary (Fig. 1L). Serum estradiol levels of females were not considered because of the estrous cycle. Sexual differences were discovered in adults, where adult males had higher body mass ($F_{1,46} = 11.81$, $P = 0.001$, $\eta_p^2 = 0.204$; Table 1) and MVG scores ($P = 0.002$) than adult females.

3.2. Seasonal differences in behavior

Seasonal effects were found in exploratory behavior and novelty recognition (Fig. 2). Gerbils captured in April and July had longer total distance (Young, $F_{2,21} = 5.56$, $P = 0.012$, $\eta_p^2 = 0.346$; Subadult, $F_{2,44} = 7.59$, $P = 0.001$, $\eta_p^2 = 0.256$; Adult, $F_{2,46} = 5.25$, $P = 0.009$, $\eta_p^2 = 0.186$; Fig. 2A, D and G) and higher frequency of center entries (Young, $F_{2,21} = 5.22$, $P = 0.014$, $\eta_p^2 = 0.332$; Subadult, $F_{2,44} = 5.37$, $P = 0.008$, $\eta_p^2 = 0.196$; Adult, $F_{2,46} = 3.83$, $P = 0.029$, $\eta_p^2 = 0.143$; Fig. 2C, F and I). Subadults during this period moved more at the center ($F_{2,44} = 7.31$, $P = 0.002$, $\eta_p^2 = 0.249$; Fig. 2E). Moreover, gerbils at different ages showed different novelty recognition behavior. Subadults captured in April and July preferred to investigate the novel object ($F_{2,44} = 3.86$, $P = 0.029$, $\eta_p^2 = 0.149$; Fig. 2J) whereas adults captured in July and September were likely to investigate the familiar object ($F_{2,46} = 2.97$, $P = 0.061$, $\eta_p^2 = 0.114$; Fig. 2K).

3.3. Seasonal changes in brain plasticity

GnRH-ir cells and fibers were mainly discovered in the medial preoptic area (MPOA), hypothalamic arcuate nucleus (Arc), and median eminence (ME) (Fig. 3). Seasonal comparisons were only conducted in males because of the estrous cycle in females, and significant differences were found both in subadult and adult subjects. Compared with those captured in July and September, subadult and adult gerbils in April expressed more GnRH in MPOA ($F_{2,12} = 6.07$, $P = 0.015$, $\eta_p^2 = 0.503$) and ME ($F_{2,10} = 4,75$, $P = 0.036$, $\eta_p^2 = 0.487$), respectively (Fig. 3A and B).

In comparison to the control (non-hoarding group), gerbils with food-induced hoarding behavior in breeding season had lower GnRH expression in Arc ($t = 2.45$, $df = 11$, $P = 0.032$, $\eta_p^2 = 0.353$), but not in MPOA ($t = 1.09$, $df = 11$, $P = 0.300$, $\eta_p^2 = 0.097$) or ME ($t = 1.17$, $df = 7$, $P = 0.281$, $\eta_p^2 = 0.163$; Fig. 3C).

A large number of new cells labeled by BrdU were examined in several brain regions, including SVZ, DG, Arc and Amy (Fig. 4). Seasonal effects on cell proliferation in these regions were mainly found in subadult and adult subjects, but not in young subjects, while sexual differences were just found in adult subjects (Fig. 5). More cell proliferation was observed in the SVZ, DG, paraventricular nucleus (PVN), ventromedial hypothalamic nucleus (VMH), Arc, and Amy of subadult gerbils captured in April and July when compared to those captured in September (SVZ, $F_{2,37} = 30.07$, $P < 0.01$, $\eta_p^2 = 0.619$; DG, $F_{2,37} = 2.82$, $P = 0.072$, $\eta_p^2 = 0.132$; PVN, $F_{2,37} = 3.98$, $P = 0.027$, $\eta_p^2 = 0.177$; VMH, $F_{2,37} = 6.42$, $P = 0.004$, $\eta_p^2 = 0.258$; Arc, $F_{2,37} = 31.14$, $P < 0.01$, $\eta_p^2 = 0.627$; Amy, $F_{2,37} = 13.98$, $P < 0.01$, $\eta_p^2 = 0.430$; Fig. 5A, C). The similar seasonal differences also existed in the adult stage. Adult gerbils captured in April and July had a higher level of cell proliferation in most brain regions, except in DG, than those captured in September (SVZ, $F_{2,37} = 7.16$, $P = 0.002$, $\eta_p^2 = 0.279$; CA, $F_{2,37} = 3.55$, $P = 0.039$, $\eta_p^2 = 0.161$; PVN, $F_{2,37} = 2.77$, $P = 0.075$, $\eta_p^2 = 0.130$; LH, $F_{2,37} = 5.97$, $P = 0.006$, $\eta_p^2 = 0.244$; VMH, $F_{2,37} = 8.20$, $P = 0.001$, $\eta_p^2 = 0.307$; Arc, $F_{2,37} = 5.19$, $P = 0.010$, $\eta_p^2 = 0.219$; Amy, $F_{2,37} = 7.88$, $P = 0.001$, $\eta_p^2 = 0.299$; DG, $F_{2,37} = 1.24$, $P = 0.302$, $\eta_p^2 = 0.063$; Fig. 5D–H). Moreover, adult females had more cell proliferation in the SVZ, DG, Arc, and Amy than adult males (SVZ, $F_{1,37} = 4.16$, $P = 0.048$, $\eta_p^2 = 0.101$; DG, $F_{1,37} = 5.43$, $P = 0.025$, $\eta_p^2 = 0.128$; Arc, $F_{1,37} = 4.65$, $P = 0.038$, $\eta_p^2 = 0.112$; Amy, $F_{1,37} = 4.42$, $P = 0.042$, $\eta_p^2 = 0.107$; Fig. 5D–G).

3.4. The effect of photoperiod on sexual development and behavior

The weight and structure of testes, as well as the testosterone level in serum in male gerbils were not influenced by photoperiod (Table 2). The structure of ovaries, assessed by the number of different follicle cells/mm^2 showed no photoperiodic difference (Table 2).

There was no photoperiodic difference in exploratory behavior in the open field test, short-term memory in the Y-maze test, and anxiety-like behavior in the elevated plus maze among the three groups (Table 3). Sexual differences were only found in the anxiety-like behavior, where males preferred entering the open arms (Table 3).

3.5. The effect of photoperiod on brain plasticity

Cell proliferation in several brain regions was labeled by Ki67. In all detected brain regions, sexual differences were only found in VMH ($F_{1,26} = 14.15$, $P = 0.001$, $\eta_p^2 = 0.352$). Photoperiodic effects were found in VMH and Arc, where gerbils acclimated in LD condition had a higher level of cell proliferation (VMH, $F_{2,26} = 5.20$, $P = 0.013$,

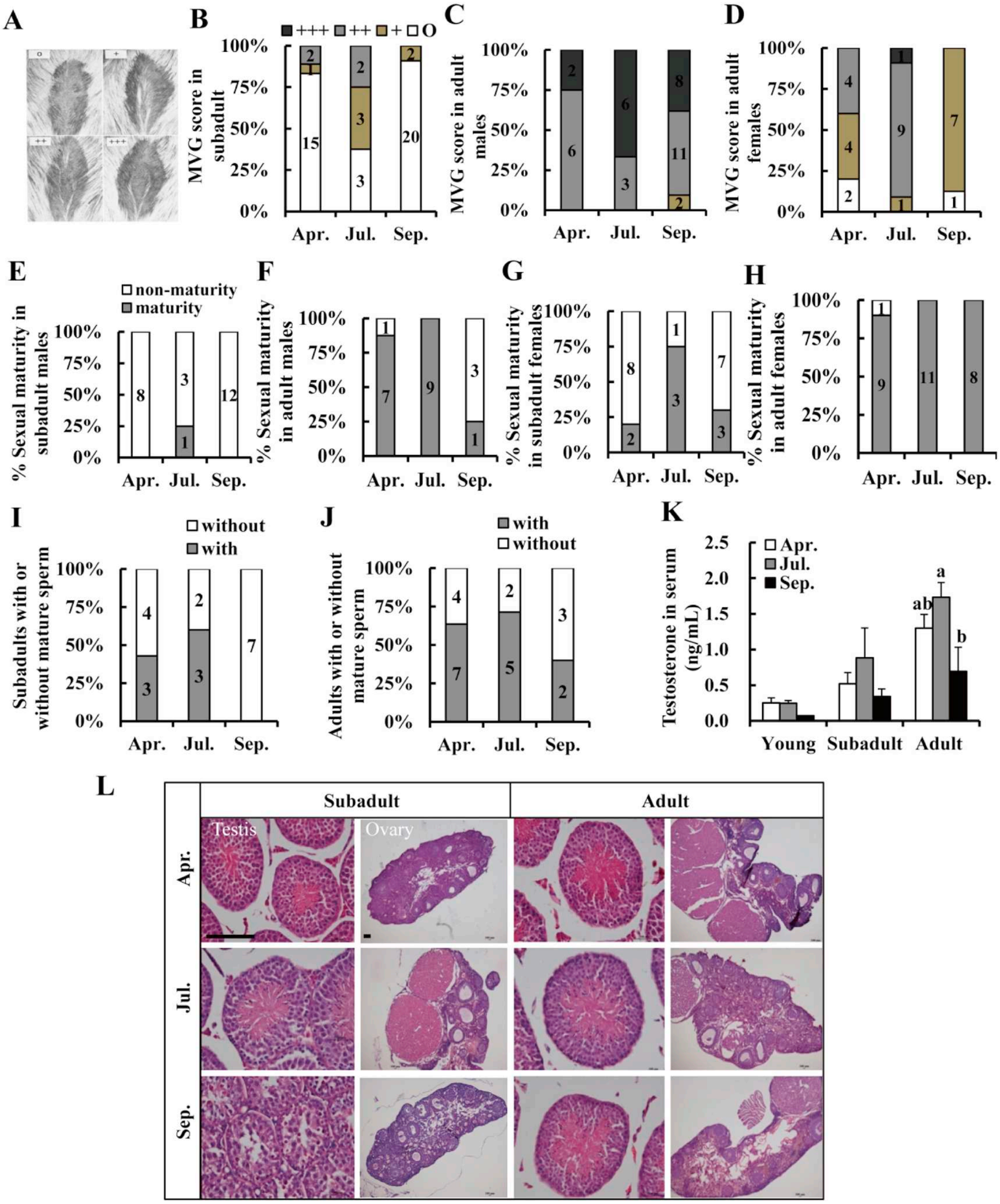

Fig. 1. Seasonal changes in sexual development of gerbils. The categorization of midventral gland (MVG) in gerbils (A). Higher MVG scores were observed in subadult (B) and adult (C and D) gerbils captured in April (Apr.) and July (Jul.) when compared to those captured in September (Sep.). Adult males (C) had higher MVG scores than adult females (D). The different numbers in the bar represent sample size. No seasonal differences were found in the percentage of individuals with sexual maturity in subadult males (E). Adult males from April and July had higher percentages of individuals with sexual maturity compared to those from September (F). The percentage of subadult (G) and adult (H) female gerbils with sexual maturity was not influenced by season. Adult (J), but not the suadult gerbils (I) in April and July had more mature sperm than those in September. Serum testosterone levels of gerbils were higher in breeding season (K). Photomicrographs illustrating the structure of testes and ovaries stained by H&E (L). Results are presented as mean ± SE, and significant differences are indicated by different superscripts if $P \leq 0.05$. Scale bar = 200 μm in testis and 300 μm in ovary.

$\eta_p^2 = 0.286$; Arc, $F_{2,26} = 4.46$, $P = 0.022$, $\eta_p^2 = 0.255$; Fig. 6A). Cell survival was labeled by BrdU, and there were no photoperiodic effects ($P > 0.05$) or sexual differences ($P > 0.05$) in all detected brain regions (SVZ, CA, DG, PVN, VMH, Arc, LH and Amy, Fig. 6B).

4. Discussion

Small mammals usually breed in the most appropriate seasons to optimize their fitness. Both males and females show better reproductive performance in spring and summer than in autumn (Liu et al., 2007; Smaï-Hamdidouche et al., 2013; Kheddache et al., 2017). Moreover, the females are more likely to suppress their fertility during harsh time due to more reproductive investments than males (Bronson, 1989; Hammond and Diamond, 1992). For instance, the onset of seasonal infertility in Syrian hamsters occurs earlier in females than males (Beery et al., 2007). The seasonal pattern of breeding in Mongolian gerbils is typical both in agricultural croplands and semi-natural condition (Xia et al., 1982; Liu et al., 2007). In the present study, we found

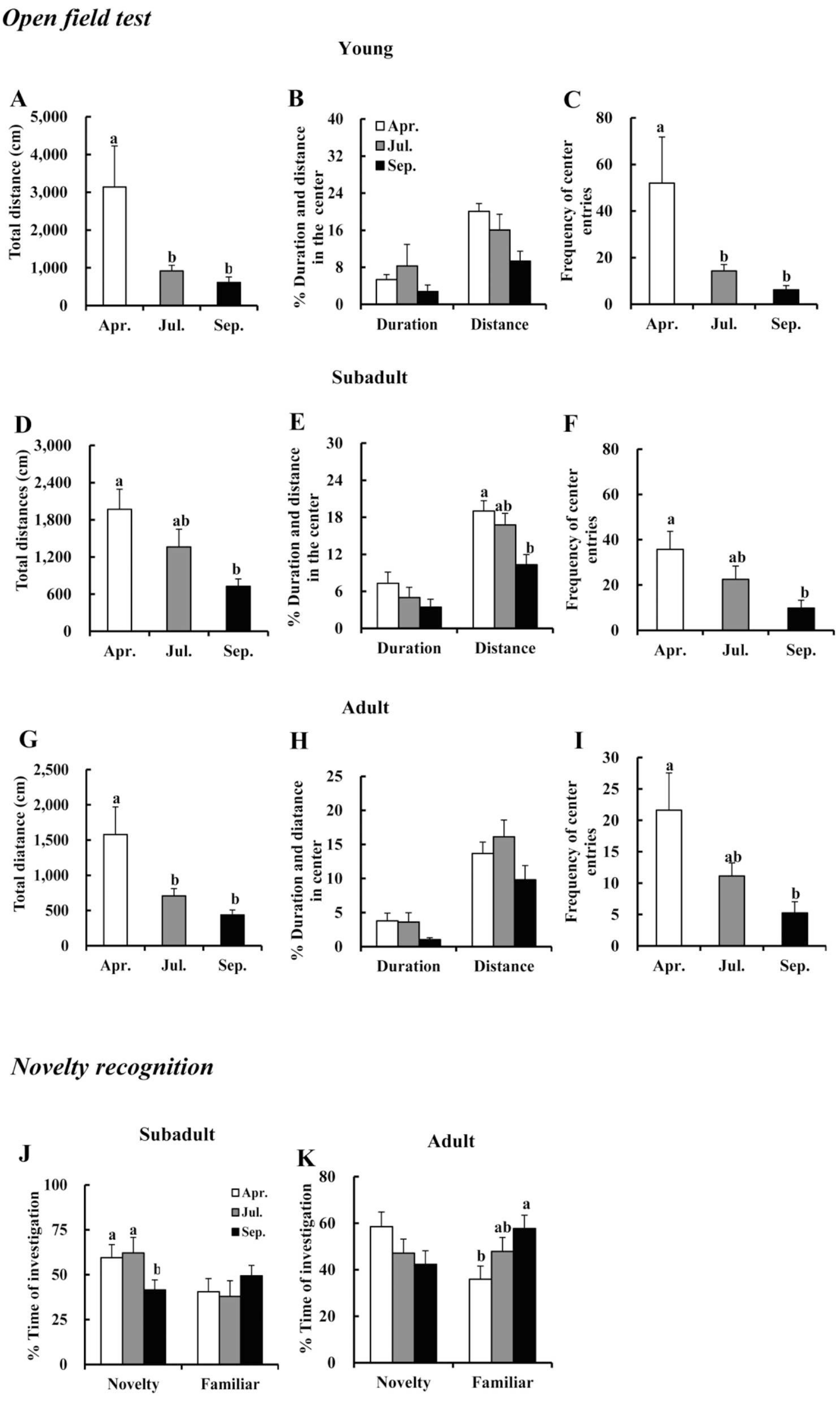

Fig. 2. Seasonal changes in exploratory behavior (A–I) and novelty recognition (J, K) in gerbils. Gerbils of all ages captured in April (Apr.) and July (Jul.) had longer moving distance (Young, A; subadult, D; adult, G) and a higher frequency of center entries (Young, C; subadult, F; adult, I). Compared to those captured in September (Sep.), subadult gerbils captured in April and July had a higher percentage of distance at the center (E). Subadult gerbils captured in April and July showed novelty preference (J), while adult gerbils captured in July and September were more likely to investigate familiar objects (K). Results are presented as mean ± SE, and significant difference are indicated by different superscripts if $P \leq 0.05$.

that the gerbils captured in field showed seasonal variations in sexual development, with accentuation in April and July and attenuation in September, and such seasonal variations are significant in males, but not in females including the percentage of open vaginas and ovary structure. The female breeding ratio was 5/20 in April, 5/15 in August and 0/18 in September in all subadult and adult gerbils we captured. The females that were in pregnancy or lactation were released to the field and only the non-reproductive females were included in our study. A common idea in rodent behavioral ecology is that young females show delayed sexual maturation or reproductive suppression when reared with conspecific females (Wasser and Barash, 1983; Solomon and French, 1997). Therefore, the female subjects in present study may not totally represent the reproductive status of population due to the defect in samples. There is also another possibility of the individual

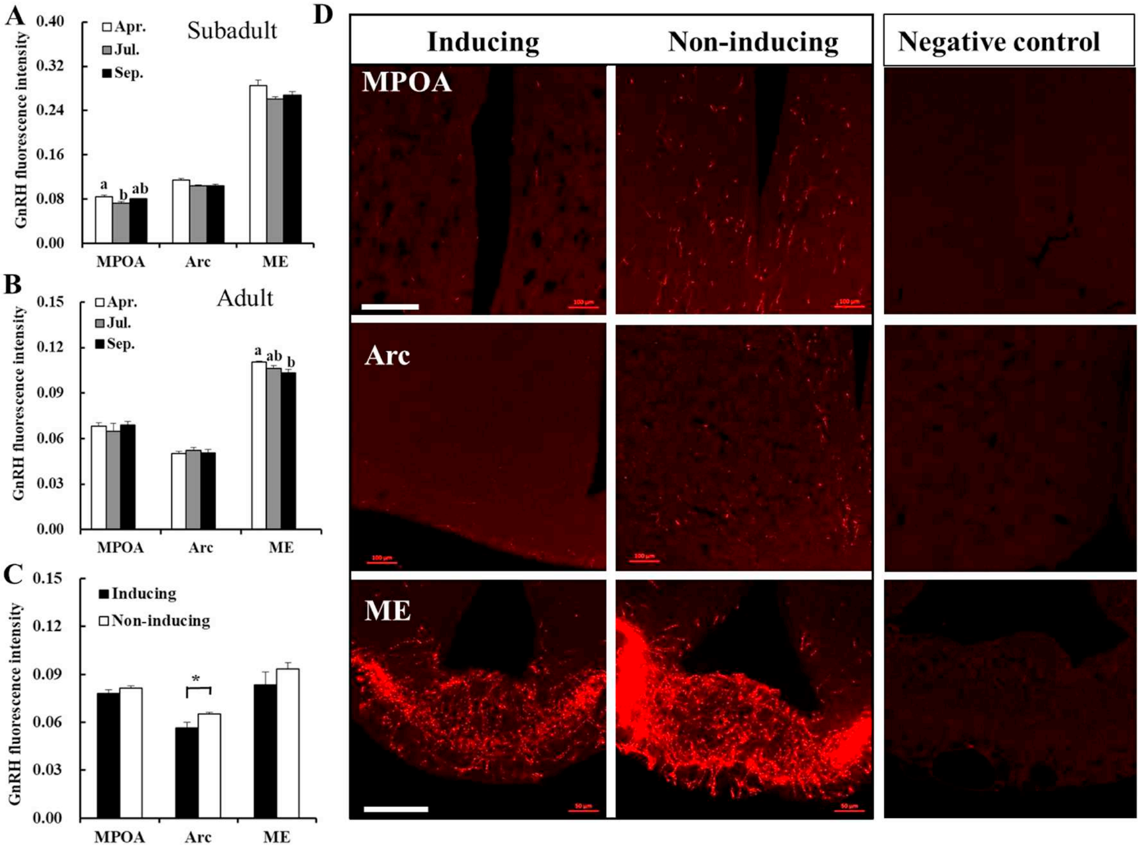

Fig. 3. Seasonal changes and food hoarding-induced changes in GnRH expression. A higher GnRH fluorescence intensity was observed in the medial preoptic area (MPOA) of subadult gerbils (A) and in the median eminence (ME) of adult gerbils (B) in April (Apr.) compared to July (Jul.) and September (Sep.). Inducing food hoarding inhibited the expression of GnRH in the arcuate nucleus (Arc) (C). Photomicrographs illustrating GnRH-ir cells and fibers in MPOA, Arc and ME, and the negative control with the omission of the primary antibody (D). White scale bar = 200 μm in MPOA and Arc and 100 μm in ME. Results are presented as mean ± SE, and significant differences are indicated by different superscripts if $P \le 0.05$.

difference in reproductive development in females. The previous study report that some female gerbils breed during December to February and about 17.1% of new recruitments are born in winter (Liu et al., 2007, 2017), which may offer a perspective on the undetected change in female fertility in gerbils.

In breeding season, small mammals increase dispersal and social communication to maximize their opportunity to mate. In the present study, we found that gerbils in April showed more locomotion and exploratory behavior in the open field test. Moreover, subadult gerbils in April and July were more likely to investigate novel objects than those in September. These characteristics, including novelty-preference and risk-taking in subadults, may promote their communication with potential mates (Somerville, 2013; Spear, 2000). However, adult gerbils in September were more likely to investigate familiar objects, suggesting that they gradually developed a better recognition memory in anticipation of hoarding season. This was supported by previous studies, which show that birds and small mammals have better memory in hoarding season than in other seasons (Barnea and Nottebohm, 1996; LaDage et al., 2010). These seasonal variations in social behavior and recognition memory may facilitate seasonal life-history traits of breeding and food hoarding.

GnRH is a critical internal signal from the hypothalamus that regulates reproductive function in all mammalian species (Herbison, 2006). As predicted, GnRH expression in gerbils increased during breeding season compared to hoarding season. Interestingly, the highest level of GnRH was found in April instead of July. Gerbils captured in April are mostly individuals born in early spring or over winter. Studies show that previous harsh experience may promote the onset of reproduction (Wingfield, 1994). Therefore, the highest expression of GnRH would be crucial to trigger the firing of breeding in early spring. In addition, we found that food-induced hoarding gerbils decreased GnRH expression compared to non-hoarding gerbils even in breeding season. Based on our present and previous observation of the video in the field, the gerbils would continuously hoard all the supplemental seeds once one individual in the family found them. In addition to differences in hoarding behavior, the two groups also differed in the amount of food. As the food was necessary to induce hoarding behavior, we cannot find an alternate way to control for this variable. Therefore, we cannot exclude the possibility that differences in the amount of food may attribute, directly or indirectly, to the observed differences in GnRH expression. Our laboratory study showed that the activity increased during hoarding tests, and daily energy expenditure decreased after hoarding tests in the high-hoarding gerbils compared to non-hoarding gerbils, suggesting that the animals would suppress some processes to compensate for the cost of food hoarding (Unpublished data). These data imply that the energy investment for food hoarding may tradeoff with energy investment for reproduction in favor of processes necessary for individual survival. Metabolic fuel hypothesis explores the mechanism of infertility seen in many situations such as famine, cold exposure and excessive exercise on the neuroendocrine perspective (Wade and Jones, 2004). Changes of glycogen and fatty acids can be perceived by some special cell groups in the posterior polar region of the brain, and the activity of GnRH neurons is suppressed through the forebrain when the body is in negative energy balance (Wade et al., 1996; Wade and Jones, 2004). In addition, stress-induced infertility has been confirmed (Ferin, 1999; Whirledge and Cidlowski, 2017). For example, the rats with restraint stress up-regulate the expression of RFamid-related peptide (RFRP), which is the upstream

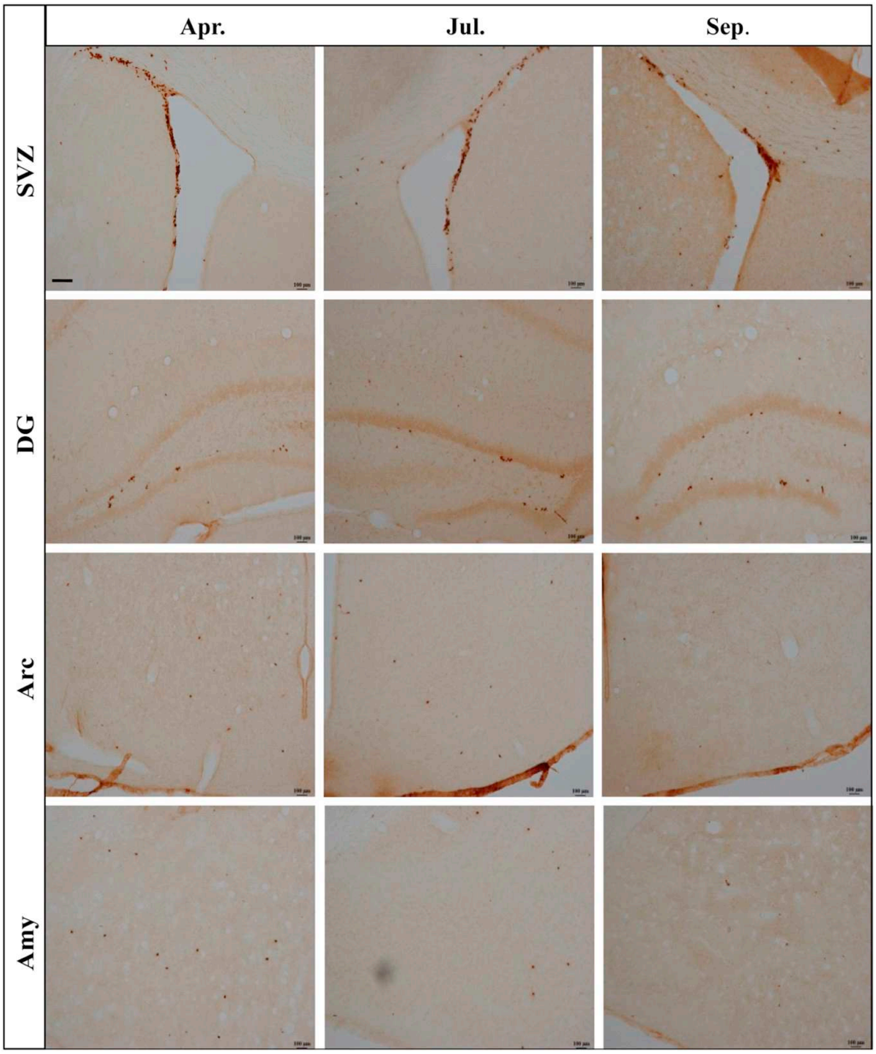

Fig. 4. Photomicrographs illustrating BrdU-ir cells in the subventricular zone (SVZ), hippocampal dentate gyrus (DG), arcuate nucleus (Arc), and amygdala (Amy) in gerbils captured in April (Apr.), July (Jul.), and September (Sep.). Scale bar = 200 μm.

inhibitory transmitter of GnRH neurons (Kirby et al., 2009). The stress in the process of hoarding food of gerbils may affect their breeding. This recession of fertility in hoarding season may be a result of evolution that ensures that the hoarding process runs smoothly for winter survival.

Seasonal brain plasticity is a fundamental feature in birds and small mammals that inhabit seasonal environments (Tramontin and Brenowitz, 2000). Activation of adult-born neurons may contribute to recognition memory and adaptation to changing environments (Kempermann et al., 2004). New cells have been documented in several brain regions, including SVZ/OB, DG (Cameron and Mckay, 2001; Lledo and Saghatelyan, 2005), and other non-traditional neurogenic niches, such as the hypothalamus (Lee et al., 2012; Pencea et al., 2001; Kokoeva et al., 2005). Likewise, cell proliferation was also labeled in these brain regions of gerbils. Moreover, the level of cell proliferation in gerbils was higher in breeding season than in the anestrus period. Similar variation is also found in previous studies of ewes, which show that the number of new cells in hypothalamus doubles during the breeding season (Migaud et al., 2011). However, some studies in meadow voles (*Microtus pennsylvanicus*) show inconsistent results in which the cell proliferation in the hippocampus is suppressed during breeding season (Galea and Mcewen, 1999; Spritzer et al., 2017). Alternatively, the survival of new cells, rather than the proliferation, is influenced by reproductive status in adult male meadow voles (Ormerod and Galea, 2003). These controversial data may reflect species-specific and brain region-specific responses to different seasons. Interestingly, the seasonal difference of cell proliferation in the DG area became undetectable in adults. Many previous studies in rats, Siberian chipmunks (*Tamias sibiricus*) and black-capped chickadees (*Poecile atricapillus*) have shown that the adult hippocampal cell proliferation increases in hoarding season in comparison to other seasons (Barnea and Nottebohm, 1994; Pan et al., 2013). The hoarding season for Mongolian gerbils is from September to the end of October. The gerbils have just started to take part in hoarding food when they were captured. This may be the reason why we did not find increased cell proliferation in the hippocampus in September compared to other seasons. The laboratory study shows that high-hoarding gerbils have more cell proliferation in the hippocampus and hypothalamus than non-hoarding gerbils (Unpublished data). Taken together, these data demonstrate that seasonal plasticity of cell proliferation in the adult brain may be involved in mediating seasonal breeding and food hoarding.

Some studies show that testosterone may be involved in regulating cell proliferation and its stimulating effect has been proved in castrated rats (Wainwright et al., 2011), but not in mice and meadow voles (Ormerod and Galea, 2003; Benice and Raber, 2010). However, the seasonal difference in cell proliferation of gerbils emerged in subadults and adults, whereas the seasonal variation in testosterone didn't appear until adulthood. It seems that seasonal changes in brain cell

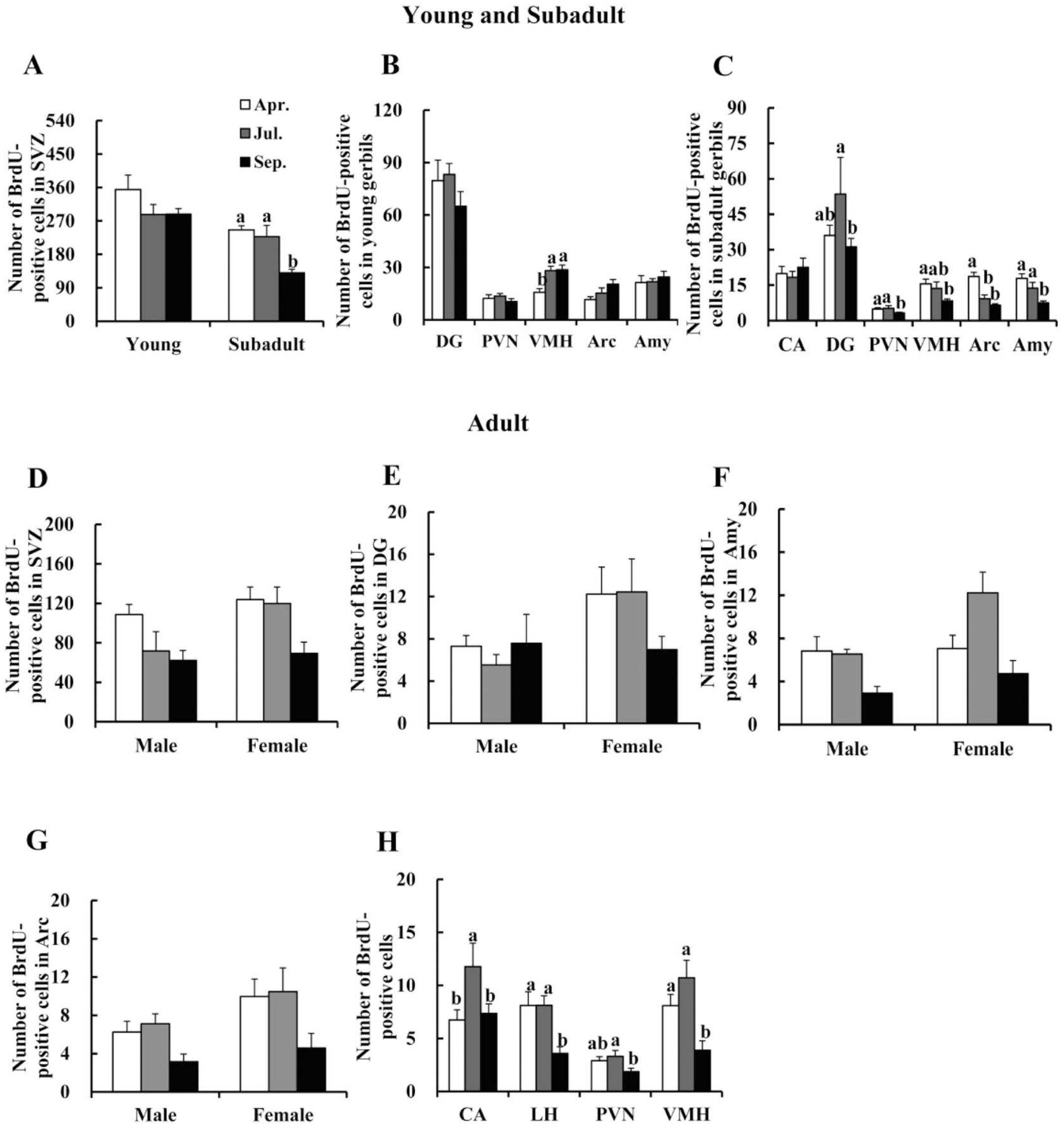

Fig. 5. Cell proliferation in several brain regions of gerbils captured in April (Apr.), July (Jul.) and September (Sep.). Seasonal effects on cell proliferation were mainly found in subadult and adult stages, but not in the young stage, and a larger number of BrdU-ir cells were observed in gerbils from April and July compared to those from September. Sexual differences were only found in adults, where adult females had more cell proliferation in the subventricular zone (SVZ), hippocampal dentate gyrus (DG), arcuate nucleus (Arc), and amygdala (Amy) than males. PVN, paraventricular nucleus; VMH, ventromedial hypothalamic nucleus; LH, lateral hypothalamic area; CA, hippocampal CA area. Results are presented as mean ± SE, and significant differences are indicated by different superscripts if $P \leq 0.05$.

Table 2
The photoperiodic effects on the sexual development of gerbils.

Parameter	LD	SD	SL	P	η_p^2
Testes (g)	0.80 ± 0.04	0.73 ± 0.06	0.75 ± 0.04	0.542	0.035
Epididymis (g)	0.32 ± 0.03	0.28 ± 0.03	0.27 ± 0.03	0.420	0.050
Seminal vesicle (g)	0.28 ± 0.04	0.30 ± 0.0.05	0.28 ± 0.07	0.670	0.023
Epigonadal fat in males (g)	0.98 ± 0.10	0.90 ± 0.11	1.01 ± 0.16	0.810	0.012
% of spermatid	41.21 ± 2.26	35.91 ± 2.98	40.99 ± 3.15	0.355	0.158
% of sperm	38. 38 ± 2.35	37.43 ± 2.89	38.84 ± 2.19	0.927	0.013
Size of seminiferous tubule (mm^2)	0.15 ± 0.01	0.14 ± 0.01	0.16 ± 0.01	0.583	0.086
Testosterone in serum (ng/mL)	0.55 ± 0.12	0.47 ± 0.10	0.59 ± 0.15	0.787	0.013
Ovaries (g)	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.616	0.038
Uterus (g)	0.05 ± 0.01	0.05 ± 0.00	0.06 ± 0.01	0.427	0.066
Epigonadal fat in females (g)	0.07 ± 0.01	0.08 ± 0.03	0.07 ± 0.03	0.512	0.052
Number of secondary follicle/mm^3	0.37 ± 0.05	0.37 ± 0.03	0.31 ± 0.03	0.535	0.099
Number of tertiary follicle/mm^3	0.21 ± 0.02	0.14 ± 0.03	0.22 ± 0.03	0.110	0.307

Results are presented as mean ± SE.

Table 3
The photoperiodic effects on behavior of gerbils.

		LD	SD	SL	P		η_p^2	
					Photoperiod	Sex	Photoperiod	Sex
Open field test	Distance in center (m)	13.06 ± 0.88	15.19 ± 1.02	13.36 ± 1.04	0.251	0.417	0.041	0.010
	Distance in corner (m)	48.09 ± 1.73	43.10 ± 1.73	44.58 ± 2.68	0.232	0.735	0.043	0.002
	Frequency of center entries	47.54 ± 3.14	52.26 ± 3.01	47.48 ± 3.66	0.501	0.717	0.021	0.002
Y-maze	% Duration in the food arm	47.60 ± 2.83	48.59 ± 2.63	46.02 ± 2.23	0.786	0.748	0.008	0.002
	Latency to the food arm (s)	13.24 ± 2.77	7.35 ± 1.69	12.39 ± 3.48	0.333	0.575	0.035	0.005
Elevated plus maze	% Entries in open arms	51.17 ± 3.53	57.80 ± 3.86	55.39 ± 4.20	0.454	0.077	0.027	0.053
	% Time spent on open arms	61.63 ± 2.78	55.84 ± 2.52	61.71 ± 2.52	0.185	< 0.001	0.057	0.336

Results are presented as mean ± SE. LD, long day; SD, short day; SL, transformation from short day to long day.

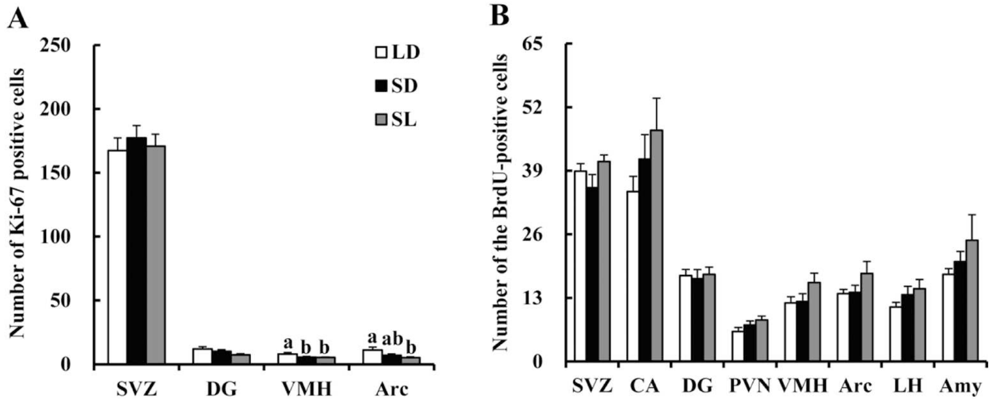

Fig. 6. Photoperiodic effects on cell proliferation and cell survival in several brain regions. No photoperiodic differences were found in the number of Ki67-positive cells in most detected regions except the ventromedial hypothalamic nucleus (VMH) and arcuate nucleus (Arc) (A). Cell survival was not affected by photoperiod (B). SVZ, subventricular zone; DG, hippocampal dentate gyrus; CA, hippocampal CA area; PVN, paraventricular nucleus; VMH, ventromedial hypothalamic nucleus; LH, lateral hypothalamic area; Amy, amygdala nucleus. LD, long day; SD, short day; SL, transformation from short day to long day. Results are mean ± SE. $**P < 0.01$.

proliferation are not likely attributable to plasma testosterone. It is well-known that locomotion (exercise) and exploration increase cell proliferation and neurogenesis in hippocampus in mice and wild rodents (van Praag et al., 1999; Lieberwirth et al., 2016; Ramírez-Rodríguez et al., 2018). In addition, a larger social network can lead to better memory function and reduced neuroinflammation in aged mice (Smith et al., 2018). Therefore, it seems reasonable to speculate on the possible causal connection between the seasonal increases in locomotion and exploration and the seasonal increases in brain cell proliferation. The present study also showed that the cell proliferation in some specific brain regions, including SVZ, DG, Arc, and Amy, revealed sex differences, where adult females generated more new cells than males. Similar sexual dimorphism is also observed in adult rats and meadow voles (Galea and Mcewen, 1999; Spritzer et al., 2017), but not in grey squirrels (*Sciurus carolinensis*) (Lavenex et al., 2000). The sex hormones may be involved in this sexual dimorphism (Galea et al., 2006), and the stimulating effect of estradiol on hippocampal cell proliferation has been confirmed in female rodents (Tanapat et al., 1999; MeFrick and Kim, 2018). The sexual dimorphism in cell proliferation in specific brain regions may have some implications for sex differences in reproductive behavior or other behavior.

Short-day length is a reliable cue for predicting winter and its suppressing effects on reproduction have been confirmed in majority of small mammals, including white-footed mice (*Peromyscus leucopus*) (Pyter et al., 2005), Syrian hamsters (Revel et al., 2006) and Siberian hamsters (Greives et al., 2008). Several previous studies examining the photoperiodic response of reproduction in Mongolian gerbils demonstrated that 10-week SD acclimation (< 10 hour light) induced testicular regression in adult males in comparison with LD control (Petterborg et al., 1984; Karakaş and Gündüz, 2002). However, gerbils treated with 10-week SD acclimation did not shrink sexual organs, decrease sexual hormones, or change exploratory behavior, anxiety-like behavior, or short-term memory in the present study. Moreover, the transformation of the photoperiod still had no effects on the recovery of photoperiodic sensitivity. Recently, the view of "cyclical histogenesis" has received more and more attention (Hazlerigg and Lincoln, 2011), and the annual cycle from anestrus to reproduction may be a recapitulation of early brain developmental mechanisms (Murphy and Ebling, 2011). Hence, we examined cell proliferation and survival in several brain regions. No photoperiodic effects were found in the two main regions (SVZ and DG). However, gerbils treated with LD had more cell proliferation in VMH and Arc than SD gerbils. Less is known about the role of these new cells and why this photoperiodic difference became undetectable in cell survival. Previous studies indicate that Mongolian gerbils can maintain stable energy balance in different light cycles (Karakaş and Gündüz, 2002; Li et al., 2003; Li and Wang, 2005; Yao et al., 2018). In consistence with our results, SD acclimation for 10 weeks in 14L-born prepubertal gerbils did not induce testicular regression except under 0 L, 2 L and 24 L (Karakaş and Gündüz, 2002). Snell strain house mice (*Mus musculus*) acclimated under SD also had unchanged reproductive organ weights compared to LD control (Petterborg et al., 1984). Different photoperiod experiences in early life may affect testis development and different species may have diverse responses to photoperiod. Food restriction is critical to reduce body weight and inhibit reproduction in Mongolian gerbils (Karakaş et al., 2005) and Abert's Towhees *Melozone aberti* (Davies et al., 2015). These data indicate that the interaction of photoperiod and food shortage or low temperature, but not photoperiod alone, induced seasonal breeding. This photoperiodic refractoriness in gerbils may explain the adaptive mechanism for their wide distribution from west to east (> 1500 km) stretching over desert, desert steppe and typical steppe of northern China (Zhou et al., 2001), and also contribute to stepping over the barrier for winter reproduction in the context of enough food availability.

5. Conclusion

The wild study reports seasonal variations in reproductive physiology, behavior and brain plasticity at different age stages in wild-caught Mongolian gerbils. The gerbils in breeding seasons have more

Table 4
Summary for the seasonal and sexual differences in physiology and behavior at different age stages in wild-caught Mongolian gerbils.

Parameters	Young	Subadult		Adult	
	Season	Season	Sex	Season	Sex
Body weight	ns	ns	ns	ns	Male > Female
Midventral gland	–	Jul. > Apr. and Sep.	ns	Apr. and Jul. > Sep.	Male > Female
Male sexual development	–	ns	–	Apr. and Jul. > Sep.	–
Female sexual development	–	ns	–	ns	–
Exploratory behavior	Apr. > Jul. and Sep.	Apr. > Sep.	ns	Apr. > Sep.	ns
Novelty preference	ns	Apr. and Jul. > Sep.	Male > Female	ns	ns
Familiar preference	ns	ns	ns	Apr. < Sep.	ns
GnRH expression	–	Apr. > Jul. or Sep.	–	Apr. > Sep.	–
Brain cell proliferation	ns	Apr. and Jul. > Sep.	ns	Apr. and Jul. > Sep. (except in DG)	Male < Female

ns, not significant.

mature sexual development, higher exploratory behavior, higher novelty preference, higher GnRH expression in hypothalamus and higher cell proliferation in hypothalamus, amygdala and hippocampus than those in non-breeding (hoarding) season (Table 4). The laboratory study shows that photoperiod alone could not alter reproductive traits, behavior, cell proliferation or cell survival in the detected brain regions. All these data demonstrate that structural plasticity in GnRH expression and brain cell proliferation is associated with seasonal reproduction and food hoarding in gerbils, but a single photoperiod cue may not be critical to induce seasonal life-history traits and brain structural plasticity. The refractoriness to photoperiod in reproduction and neurogenesis may partly support the phenomenon of winter reproduction in Mongolian gerbils. These findings provide the proximate physiological and neural basis for these seasonal life-history traits of breeding and food hoarding in small mammals.

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