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Dynamic expressions of hypothalamic genes regulate seasonal breeding in a natural rodent population

Dawei Wang[1] | Ning Li[1] | Lin Tian[1] | Fei Ren[1] | Zhengguang Li[1] | Yan Chen[1,2] | Lan Liu[1,2] | Xiangfa Hu[1] | Xuechang Zhang[1] | Ying Song[1] | Roelof A. Hut[3] | Xiao-Hui Liu[1]

[1]State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China

[2]College of Life Sciences, Sichuan University, Chengdu, China

[3]Chronobiology Unit, Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, The Netherlands

Correspondence
Xiao-Hui Liu and Ying Song, State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, 2 Yuanmingyuanxi Road, Beijing, 100193, China.
Emails: liuxiaohui@caas.cn; ysong@ippcaas.cn

Roelof A. Hut, Chronobiology Unit, Groningen Institute for Evolutionary Life Sciences, University of Groningen, PO box 11103, 9700CC Groningen, The Netherlands.
Email: r.a.hut@rug.nl

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Abstract

Seasonal breeding is a universal reproductive strategy in many animals. Hypothalamic genes, especially type 2 and 3 iodothyronine deiodinases (*Dio2/3*), RFamide-related peptide 3 (*Rfrp-3*), kisspeptin (*Kiss-1*) and gonadotropin-releasing hormone (*GnRH*), are involved in a photoperiodic pathway that encodes seasonal signals from day length in many vertebrate species. However, the seasonal expression patterns of these genes in wild mammals are less studied. Here, we present a four-year field investigation to reveal seasonal rhythm and age-dependent reproductive activity in male Brandt's voles (*Lasiopodomys brandtii*) and to detect relationships among seasonal expression profiles of hypothalamic genes, testicular activity, age and annual day length. From breeding season (April) to nonbreeding season (October), adult male voles displayed a synchronous peak in gonadal activity with annual day length around summer solstice, which was jointly caused by age structure shifts and age-dependent gonadal development patterns. Overwintered males maintained reproductive activity until late in the breeding season, whereas most newborn males terminated gonadal development completely, except for a minority of males born early in spring. Consistently, the synchronous and opposite expression profiles of *Dio2/3* suggest their central function to decode photoperiodic signals and to predict the onset of the nonbreeding season. Moreover, changes in *Dio2/3* signals may guide the actions of *Kiss-1* and *Rfrp-3* to regulate the age-dependent divergence of reproductive strategy in wild Brandt's vole. Our results provide evidence on how hypothalamic photoperiod genes regulate seasonal breeding in a natural rodent population.

KEYWORDS
age-dependent gonadal development, Brandt's vole, hypothalamic genes, photoperiod, seasonal breeding

1 | INTRODUCTION

Seasonal breeding is a universal reproductive strategy in many animals, especially in high latitude regions (Baker, 1938; Bronson & Heideman, 1994). In seasonal breeding animals, active reproductive status is limited within a specific period in an annual cycle for the delivery of offspring in spring and summer with maximum survival rates (Prendergast, 2005). According to the photoperiod (day length) during the breeding season, seasonal breeders can be divided into long photoperiod (LP; day length > 12 hr, spring and summer) and

Dawei Wang and Ning Li contributed equally to this work.

short photoperiod (SP; day length < 12 hr, autumn and winter) breeders. Most of the small rodents in northern temperate zones belong to LP breeders. During the annual cycle of day length, their gonadal glands are generally activated in spring and summer (LP condition) and atrophied in autumn and winter (SP condition; Baker, 1938; Bronson & Heideman, 1994). Moreover, an abundance of laboratory evidence confirms that gonadal gland activity of rodents is inhibited when exposed to SP conditions but is promoted in LP conditions (Ansel et al., 2011; Bronson & Heideman, 1994; Prendergast, 2005; Stevenson & Prendergast, 2013). Thus, photoperiod is considered as the most predictable environmental signal to regulate seasonal breeding.

Day length is the signal used by seasonal breeders to set the timing of their internal annual calendar in the brain, which is known as "circannual clock" that anticipate seasonal shifts and synchronize reproductive activity with the seasons (Goldman, 2001). The pars tuberalis (PT) in the pituitary and the tanycytes in hypothalamus play central roles in annual timing of mammals (Hau et al., 2017; Hut, Dardente, & Riede, 2014; Lewis & Ebling, 2017; Wood et al., 2015). Light information is perceived by the retina and transmitted into the pineal gland via the suprachiasmatic nucleus to control the secretion of nocturnal melatonin from the pineal gland (Hazlerigg & Simonneaux, 2015; Hut & Beersma, 2011; Ono et al., 2008). The melatonin signal is received by abundant melatonin receptors in thyrotrophs of the PT, which subsequently regulate the production of thyroid-stimulating hormone (TSH) (Hanon et al., 2008; Morgan, Barrett, Howell, & Helliwell, 1994; Ono et al., 2008; Sáenz de Miera, Sage-Ciocca, Simonneaux, Pévet, & Monecke, 2018). Then, TSH binds to receptors in tanycytes at the base of the 3rd ventricle in the hypothalamus to regulate a set of "photoperiodic genes", which adjust the seasonal activity of gonadotropin-releasing hormone (GnRH) (Revel, Masson-Pévet, Pévet, Mikkelsen, & Simonneaux, 2009).

Type 2 and 3 iodothyronine deiodinases (*Dio2* and *Dio3*) respond directly to TSH signal in the hypothalamus. Upregulation of *Dio2* expression and downregulation of *Dio3* have been observed in hypothalamic tanycytes of Siberian hamster or photoperiod-sensitive rats when treated with TSH (Helfer, Ross, & Morgan, 2013; Klosen, Sébert, Rasri, Laran-Chich, & Simonneaux, 2013). The two deiodinases then regulate local thyroid hormone levels in hypothalamic tanycytes via opposite actions. DIO2 converts prohormone thyroxine (T_4) into bioactive triiodothyronine (T_3), whereas DIO3 inactivates both T_4 and T_3 (Prendergast, Pyter, Kampf-Lassin, Patel, & Stevenson, 2013; Yasuo et al., 2005). Atrophic testes of Siberian hamster under SP condition can be restored by infusion of exogenous TSH and T_3, indicating their important role in regulation of reproductive activity (Banks, Delibegovic, & Stevenson, 2016; Barrett et al., 2007; Freeman, Teubner, Smith, & Prendergast, 2007; Klosen et al., 2013). Therefore, it is consensus that *Dio2/3* translates the light information by affecting hypothalamic T_3 level which further regulates activity of the hypothalamic–pituitary–gonadal (HPG) axis.

Hypothalamic *Dio2* and *Dio3* are sensitive to day length or photoperiod. Under the SP condition, a significant decrease in *Dio2* expression has been observed in male Syrian hamsters and melatonin-proficient mice (Herwig, Petri, & Barrett, 2012; Milesi, Simonneaux, & Klosen, 2017; Ono et al., 2008; Revel, Saboureau, Pevet, Mikkelsen, & Simonneaux, 2006; Sáenz de Miera et al., 2014; Yasuo, Yoshimura, Ebihara, & Korf, 2007). Decreased *Dio2* was also detected in Siberian hamster in one study (Stevenson, 2017) but not in others (Barrett et al., 2007; Kampf-Lassin & Prendergast, 2013; Prendergast et al., 2013). The upregulation of *Dio3* has been observed under the SP condition in all above research studies. Opposite expression patterns of *Dio2* and *Dio3* have been observed in Siberian hamster housed outdoors to expose individuals to the natural photoperiod of the boreal region (Petri et al., 2016). Studies on female common vole (*Microtus arvalis*) suggest opposing seasonal expression patterns between *Dio2* or *TSHβ* (thyrotropin subunit beta) and *Dio3*, as well as a positive correlation between *TSHβ* and *Dio2* (Król et al., 2012). The TSH-deiodinase pathway, acting as a seasonal timer in response to change in photoperiod or day length, is conserved in a range of animals from fish to mammals (Hanon et al., 2008; Nakane & Yoshimura, 2014).

The proteins Kisspeptin (Kp, encoded by *Kiss-1*) in the arcuate (Arc) and the anteroventral periventricular (AVPV) nucleus and RFamide-related peptide 3 (RFRP3, encoded by *Rfrp-3*) in the dorsomedial hypothalamus (DMH) are involved in regulation of photoperiodic response of the gonadal gland in rodents (Simonneaux, Ancel, Poirel, & Gauer, 2013). Research suggests that they are a link between the TSH-deiodinase pathway and gonadotropin-releasing hormone (*GnRH*), which controls the secretion of gonadotropin in the pituitary, that is luteinizing (LH) and follicle-stimulating (FSH) hormones. The Kp protein promotes the activity of GnRH neurons in rat (Irwig et al., 2004) and recovery of atrophic testes in Syrian hamster (Ansel et al., 2011; Revel, Saboureau, Masson-Pevet, et al., 2006). The RFRP-3 protein affects the activity of the HPG axis, but has contrasting functions in response to LP or SP exposure. Plasma LH levels of male Siberian hamsters are rapidly inhibited by central injection of RFRP-3 under the LP condition, but levels are dramatically stimulated in the SP condition (Ubuka et al., 2012). These results were supported by the study demonstrating restored testicular activity due to SP inhibition via a chronic central infusion of RFRP-3 in male Syrian hamsters (Ancel et al., 2012).

The expression of both *Kiss-1* and *Rfrp-3* can respond to changes in photoperiod and thyroid hormone. The *Kiss-1* expression in the Arc is regulated by both melatonin and testosterone feedback, depending on which force is stronger, and thus, the photoperiodic response varied among species. *Kiss-1* in the AVPV is upregulated by testosterone, but not affected by melatonin in both male Syrian and Siberian hamsters (Ansel et al., 2010; Rasri-Klosen, Simonneaux, & Klosen, 2017). *Rfrp-3* is always inhibited by SP exposure and melatonin (Rasri-Klosen et al., 2017; Revel, Saboureau, Pevet, Simonneaux, & Mikkelsen, 2008; Ubuka et al., 2012). Administration of exogenous T_3 or TSH reverses the expression of both genes from the SP to LP response pattern in hamsters (Henson, Carter, & Freeman, 2013; Klosen et al., 2013). Peripheral T_3 injection increases testes mass and reduces *Rfrp-3* expression in the whole hypothalamus (Banks et al., 2016). Thus, all these studies suggest that thyroid hormone mediates the response of *Kiss-1* and *Rfrp-3* to photoperiod. However, all evidence to date has primarily been based on laboratory experiments using laboratory animals, and thus, we lack evidence from free-living mammals.

Brandt's vole (*Lasiopodomys brandtii*, Figure S1a) is a small, non-hibernating, herbivorous and social rodent that is mainly distributed in the steppes in China, the Republic of Mongolia and the Baikal Lake region of Russia (Liu, Yue, Wang, Li, & Cong, 2013; Wang et al., 2011; Yue, Wang, Huang, & Liu, 2009; Zhong, Wang, Zhou, & Wang, 2007; Zhong, Wang, & Wan, 1998). Following overt seasonal changes in its habitat (Figure S1b), Brandt's vole shows strong seasonal breeding and its population size increases dramatically from spring (early March) to autumn (late August) (Zhong et al., 1998; Zhong et al., 2007; Li, Hou, Wan, & Zhang, 2016; Li, Yin, et al., 2016; Figure S1c,d). Mass of gonadal glands of adult voles is approximately 20-fold heavier in June than in October (Liu & Sun, 1993; Wang, Cong, Wang, & Liu, 2010). Due to a short lifespan (<14 months; Figure S1c), the age groups in a population shift naturally from overwintered voles in spring to newborn ones after summer (Liu & Sun, 1993; Shi, Hai, Guo, & Lv, 1999; Shi, Gao, Ren, & Wang, 2004; Figure S1e). Results from recent study on Brandt's vole in half-hectare enclosures in the field provided similar observations of population changes in age groups (Chen et al., 2019). Overwintered males display higher testosterone levels than newborn ones throughout the breeding season, and only a minority of newborn ones grow into sexual maturity in their year of birth, indicating age-dependent gonadal development patterns. Unfortunately, the molecular mechanisms governing them are still unclear in Brandt's vole.

In the present study, by characterizing the seasonal expression patterns of hypothalamic genes and their relationships with changes in day length and physiological parameters, we explored the role of hypothalamic photoperiodic genes (*Dio2*, *Dio3*, *Rfrp-3*, *Kiss-1* and *GnRH*) in seasonal breeding in a natural rodent population. We described the process of annual periodic (seasonal) transition in testicular activity. Synchronized but opposite expression patterns in *Dio2* and *Dio3* with the changes of day length and testicular activity indicated their potential functions in transmission of photoperiodic signals in wild rodent. Dramatically different expression of *Kiss-1* and *Rfrp-3* among seasons and age groups implied their key roles in regulation of age-dependent gonadal development. These results revealed interdependent and interactional roles of hypothalamic genes in regulation of seasonal breeding in a wild rodent.

2 | MATERIAL AND METHODS

2.1 | Study site and animal capture

Voles were captured using live traps made by wire mesh (Figure S2, left) in grasslands around East Ujimqin Banner (45°30′N, 116°58′E; Inner Mongolia, China) from 2010 to 2012 and in 2014. All captured voles were brought to a laboratory in Maodong pasture of Xilinhot (44°11′N, 116°27′E; Li, Hou, et al., 2016) for tissue sampling. Age classification was based on body mass and then pelage colour. Our previous survey on physical characters of voles suggested the body mass of adult voles with sperm in the epididymis could be as low as 20 g, which may overlap with weights of some juvenile voles. Thus, for those voles with body mass of in the range of 15–25 g, we further separated them according to their fur colour and hair morphology. The pelage colour was whitish, light sand-yellow in overwintered (OW) voles, and the colour was sand-yellow and grey-yellow in non-overwintered adult (NA) and young (NY) ones (Liu & Sun, 1993; Wang et al., 2010; Figure S2). The hair of NY is finer and shorter and feels softer than hair of NA voles. However, of the voles collected in 2010 and 2011, we simply categorized the voles as adults and young because there was no record of fur colour, so the adults might be a mixture of OW and NA voles from May to July of those years. Exact trapping locations were determined by GPS (Map60CSx; Garmin International Inc.), and day length was recorded for each trapping date. All the study procedures conformed to the institutional guidelines for animal use and care from the Institute of Plant Protection at the Chinese Academy of Agricultural Sciences.

2.2 | Experimental design

2.2.1 | Seasonal rhythms of testicular activity

The change in testicular activity was investigated from breeding to nonbreeding seasons in 2010 and 2011. Adult males (body mass > 20 g) were chosen from 6 to 10 vole family groups once or twice each month from April to September in 2010 and to October in 2011. Approximately 2 ml of fresh faeces was collected from each male vole within 2–3 hr of defecation and then stored at −20°C until the testosterone extraction process occurred. Subsequently, voles were sacrificed, weighed and dissected. Mass of testes and epididymides, as well as faecal testosterone levels, were recorded or measured. In total, 81 and 170 adult males were collected in 2010 and 2011, respectively.

2.2.2 | Age differentiation of seasonal pattern in testicular activity

Due to our observations of a wide divergence in testicular activity in June among individuals (see Results and Figure 1), we suspected that there were different developmental patterns among age groups. Thus, males from 10 to 80 family groups were divided into OW, NA and NY groups and sacrificed each month from April to September in 2012. To calculate the body mass proportions of each age group of all voles, body mass of each vole was measured. All OW and some of the newborn voles were dissected to weigh their body and testicular mass. A total of 465 males were collected to analyse body mass and age structure, and 187 of them were dissected to weigh and analyse testicular activity.

2.2.3 | Seasonality and age differentiation of hypothalamic photoperiodic genes

To investigate the seasonal change in hypothalamic gene (*Dio2*, *Dio3*, *Rfrp-3*, *Kiss-1* and *GnRH*) expression, males were captured in four stages in 2014: in April (the beginning of breeding season), June (the main breeding season), August (the end of breeding season)

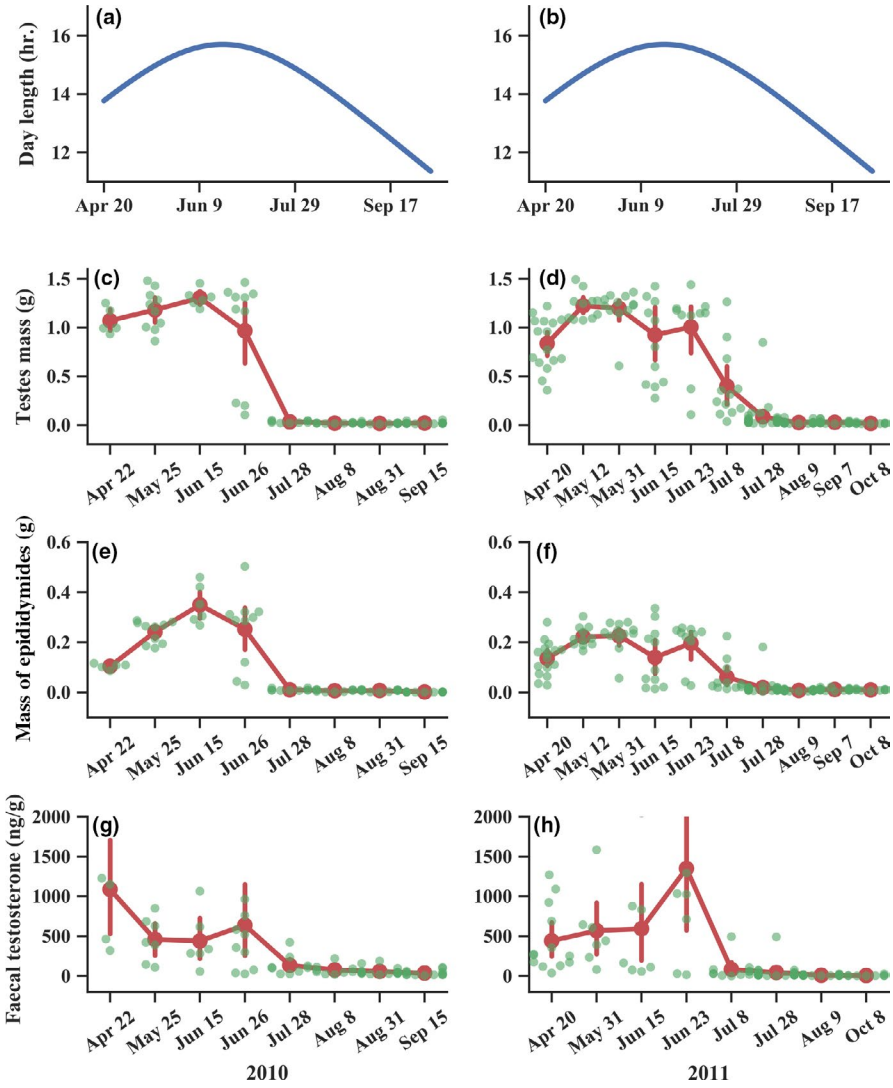

FIGURE 1 Seasonal variation (mean ± *SEM*) of day length (a and b), testes mass (c and d), mass of epididymides (e and f) and faecal testosterone levels (g and h) in wild adult male Brandt's voles in two successive years. Gonadal activity displayed corresponding peaks with day length around summer solstice in each annual cycle. Red line represents the seasonal change in the mean values and *SEM* and green dots represent each sample value

and October (the nonbreeding season). After capture in the morning, body mass and testicular mass were recorded. Brains of voles were removed via rapid decapitation, and the hypothalamus was removed quickly, the location of which was determined according to Prendergast et al. (2013): the optic chiasm at the anterior border, the mammillary bodies at the posterior border and laterally at the hypothalamic sulci. Hypothalamus samples were stored at −80°C for further RNA isolation after soaking in RNAlater® Solution (Applied Biosystems) at 4°C overnight. In total, 260 male voles were sampled; among them, 182 males (captured in June) were used for analysis of age differentiation in gene expression.

2.3 | Hormone extraction and measurement

Testosterone was extracted and measured following the method of a previous study (Wang, Wang, Zhang, Zhang, & Zhang, 2009). First, 100 mg freeze-dried faecal sample was extracted with 1 ml of water and 2 ml of ethyl acetate for 5 min using a vortex mixer (Haimen Kylin-Bell). Then, the sample was fragmented for 1 min using sonification (45 kHz; KQ-300DE; Kunshan) and shaken vigorously on a motorized shaker for 1 hr. After centrifugation at 10,621 *g* for 3 min, 1 ml of the liquid supernatant ethyl acetate layer was transferred to a polypropylene microcentrifuge tube and fully evaporated in pure N_2. The dried samples were each dissolved overnight with 1 ml of phosphate buffer solution (0.1 M, pH 7.0), and then, the faecal extraction was quantified in a single radioimmunoassay (RIA) using a ^{125}I RIA kit (Kemei [Beimian] Institute of Biotechnology). The human antiserum used was highly specific for the testosterone tested. Cross-reactivity with other steroid hormones was <0.01%, and the intra-assay variability was <10% for all samples. The detectable range was 2–2,000 ng/dl. The formula used to calculate faecal hormone levels is the following:

$$\text{Faecal hormone content (ng/g)} = \text{hormone level (ng/dl)} \times 0.1\,\text{(dl)} \times 2/0.1\,\text{(g)}$$

2.4 | RNA isolation and reverse transcription-PCR

Total RNA was isolated using RNAprep Pure Tissue Kit (Tiangen Biotech [Beijing] Co.), and RNA concentration was measured using the NanoDrop 2000 (Thermo Fisher Scientific). All RNA samples had acceptable 260/280 ratios between 1.8 and 2.0. cDNAs were

generated with 400 ng RNA in a 20 μl volume using the FastQuant RT Kit (Tiangen Biotech Co.).

2.5 | Quantitative real-time PCR (qRT–PCR) procedure

Gene fragments were cloned by designed primers based on conserved regions among known gene sequences from multiple rodent species. The fragment of *β-actin* gene (MK301451) and the full-length cDNA of *Dio2* (KX856007), *Dio3* (KX889114), *Kiss-1* (KX833248), *Rfrp-3* (KY038930) and *GnRH* (KY038929) were obtained as described previously (Chen et al., 2017; Liu et al., 2017, 2018; Ren, 2014). The specific primers for qRT–PCR were designed based on the known sequences mentioned above (Table 1).

To assess the expression levels of *Dio2*, *Kiss-1*, *Rfrp-3* and *GnRH*, we used the BioMark™ HD System (Fluidigm Sciences Inc.) following Guo et al.'s (2016) method with slight modifications. Three housekeeping genes, *β-actin*, *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) and *Hprt1* (hypoxanthine phosphoribosyltransferase 1), were included in our preliminary qPCR experiments. The *β-actin* outperformed the other two genes in stability and was chosen as the reference gene in later experiments and analysis. Pre-amplification of cDNA was conducted with 14 cycles in a 5 μl volume using TaqMan PreAmp Master Mix (Applied Biosystems) including pooled primers of each primer at 50 nM concentrations. Then, unincorporated primers were removed by a cleanup step with exonuclease I, and the final products were diluted 1:14 before use in qPCRs. We used SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Inc.) with the Fluidigm Biomark HD system for quantifying the cDNA levels after loading samples and assays into the Dynamic Array IFC (Fluidigm Sciences Inc.). *Dio3* relative expression was determined using SYBR Green PCR mix (Applied Biosystems) in an Applied Biosystems 7500 (Applied Biosystems) because it could not be detected by the BioMark™ HD System. The thermal cycler conditions were 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 40 s and then 72°C for 10 min. Each gene sample was run triplicately and normalized to *β-actin*. The $2^{-\Delta\Delta CT}$ method was used to analyse the relative changes in gene expression.

2.6 | Statistical analysis

Seasonal variation of body mass, gonadal mass and logarithmic testosterone level was tested by one-way ANOVA for overall difference and Fisher's least significant difference (LSD) test for the difference between any two points. Seasonal variations of gene expression were tested by Kruskal–Wallis test for overall difference and Mann–Whitney *U* test for the difference between any two points, time points or age groups. Spearman's correlation was used between gene expression and day length, body mass or testes mass, as well as among them. All statistical analyses were performed using SPSS 20.0. Figures were made using GRAPH PRISM 6.0 and PYTHON 3.7. The significance threshold was set at $\alpha = .05$.

3 | RESULTS

3.1 | Seasonal rhythms of testicular activity

During the sampling period, annual day length displayed sinusoidal fluctuation, which reached a maximum of ~15.7 hr on summer solstice and dropped to a minimum of 12.6 hr in September 2010 and 11.4 hr in October 2011 (Figure 1a,b). Analysis of 251 adult male voles in two successive years showed that their gonadal mass displayed synchronous seasonal changes with annual day length; that is, the heaviest testes and epididymides were observed in May and June, and then quickly decreased to an annual nadir at the end of July, in spite of LPs at that time (all seasonal variation of gonadal mass was analysed by one-way ANOVA, $p < .001$; Figure 1c–f). Similarly, faecal testosterone of 201 males also showed significant declines after the summer solstice in both years (one-way ANOVA, $p < .001$; Figure 1g,h). In addition, all data of gonadal mass and faecal testosterone showed higher standard deviations in June than in

TABLE 1 Information on gene primers of Brandt's vole

Genes	Length	Annealing temperature	Primer sequence	Reference
Dio2	103 bp	62°C	F: TGCCTACAAACAGGTTAAATTGGGT R: GGCTGTCTTCTTCAAGGCATAA	Liu et al. (2018)
Dio3	136 bp	62°C	F: TCAACAGTGAAGGCGAGGAGGT R: TCGTGGGCCTGCTTGAAGAAAT	Liu et al. (2018)
Rfrp-3	124 bp	62°C	F: GACAAATATCTCCAGCCTAGAGG R: GGGCTGGACTCATCTTAATAACAT	Ren (2014)
Kiss-1	143 bp	62°C	F: CACTGGCTTCTTGGCAGCTACTG R: GCCCTTTTCCCAGGCATTGA	Chen et al. (2017)
GnRH	124 bp	62°C	F: CGATTCTTTCCAAGAGATGGG R: CATCAGACTTTCCAGAGCTCCT	Liu et al. (2017)
β-action	213 bp	62°C	F: GCTCTCTTCCAGCCTTCCTTCCTG R: GTGTTGGCGTACAGGTCCTTGCGG	Ren (2014)

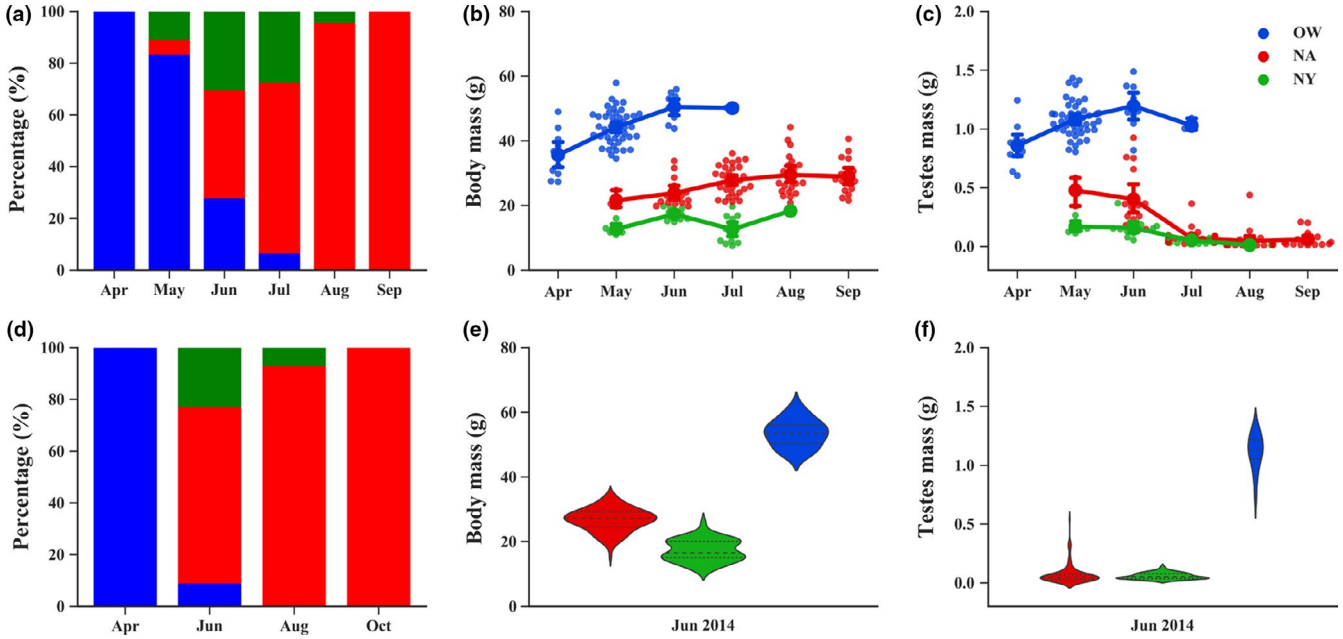

FIGURE 2 Seasonal variation and age differentiation of testis development. (a) and (d) show the age structure in 2012 and 2014, respectively; (b) and (c) show the monthly variations and age differentiation of body and testes mass in 2012, and the lines represent the monthly change in the mean ± *SEM*; (e) and (f) show the age differentiation of body and testes mass in violin plots for 2014. Testes mass (f) displayed a positive distribution in NA (skewness: 3.3676, $n = 124$) and NY groups (skewness: 0.8278, $n = 47$), indicating the existence of rapidly developed testis in a fraction of newborn males. Distinct gaps in body mass among the three age groups and a nearly indistinct gap in testes mass between NA and NY groups indicate that gonadal development in most newborn males was inhibited. NA, non-overwintered adult; NY, non-overwintered young; OW, overwintered adult

other sampling dates (Figure 1c–h), suggesting stronger divergence in gonadal growth status of adult males at that time of year.

3.2 | Age-dependent seasonal patterns of testicular development

Because of the higher standard deviations observed in gonadal mass in May and June, we hypothesized that age-dependent gonadal development was under the influence of photoperiodic history. Thus, we classified 187 male voles into OW, NA and NY groups from voles captured in April to September in 2012. The whole population consisted of primarily OW voles in April and was renewed by newborn ones in September (Figure 2a). The turning point occurred in June, when the proportion of NA males was over 50% and OW was lower than 30%. Similar seasonal shifts of age structure were also observed in 2014 with a much lower proportion of OW (~10%) in June (Figure 2d). Body mass significantly increased along with seasonal shifting in all three age groups (one-way ANOVA: all $p < .01$), and OW males displayed the heaviest body mass from May to July in 2012 (LSD, all $p < .001$). A conspicuous gap in body mass was found between OW (minimum: 43.7 g) and NA males (maximum: 38.9 g; Figure 2b). Overall, testes mass displayed different seasonal development patterns among age groups. In OW males, testes mass significantly increased from April to June (one-way ANOVA: $p < .001$; LSD, April vs. May, April vs. June, all $p < .05$) and sustained this high level until July, whereas the highest testes mass of newborn males occurred in May and June and then prominently decreased in July, and finally maintained the low level until October (LSD, each point from April to June vs. each point from July to September, all $p < .05$). As expected, the difference in testis growth was observed in June between OW and NA groups (Figure 2c). Within the NA group, we observed the largest variance in testes mass: the biggest testes mass of NA voles approximated the lowest value of the OW males, but the mean value was one-third of that of OW males (means ± *SE*: OW, 1.1965 ± 0.0597 g, CV [coefficient of variation] = 0.0499, $n = 10$; NA, 0.4024 ± 0.0641 g, CV = 0.1593, $n = 15$; Figure 2c).

With a greater sample size in 2014, similar results of a gap in body weight and divergence in gonadal data between OW and newborn groups were observed (Figure 2e,f). A wider gap in body mass, approximately 12 g, was determined between OW (minimum: 46.9 g) and NA males (maximum: 34.4 g) in 2014 than in 2012 (Figure 2e). Testes mass of OW males was significantly heavier than that of newborn males in all seasons (means ± *SE*: OW, 1.1123 ± 0.0390 g, $n = 16$; NA, 0.0713 ± 0.0071, $n = 124$; NY, 0.0580 ± 0.0040 g, $n = 42$; one-way ANOVA: $df = 2$, $F = 81.909$, $p < .01$; LSD: all $p < .01$). The violin plot shows age-dependent testicular development patterns among the three age groups in June (Figure 2f). A slightly negative distribution (skewness: −1.0479, $n = 16$) indicated the occurrence of relatively inhibited testicular activity in some individuals of the OW group. Compared with NY males (skewness: 0.8278, $n = 47$), a strong positive distribution (skewness: 3.3676, $n = 124$) indicated the occurrence of rapidly developed testes in a fraction of NA males, and their modes indicated that most of the NA males had similar testes mass

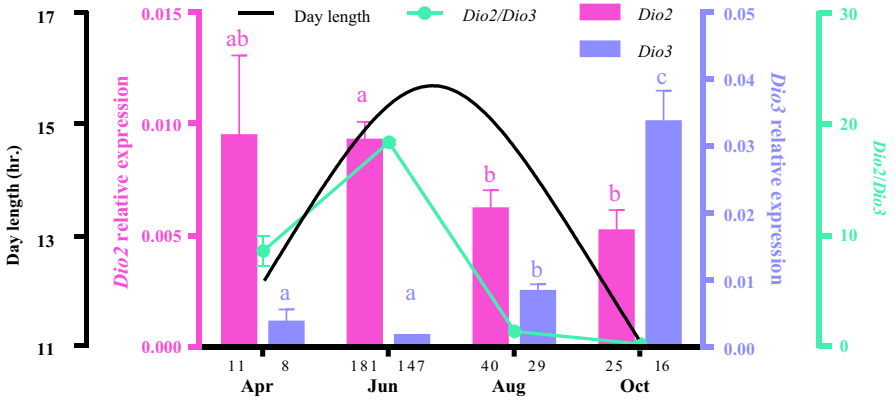

FIGURE 3 Seasonal changes in day length (black line and left *Y* axis), expression of hypothalamic *Dio2* (pink bars and left *Y* axis) and *Dio3* (blue-purple bars and right Y axis), and the ratio of *Dio2* expression to *Dio3* expression (*Dio2*/*Dio3*, cyan line and right *Y* axis) in all male Brandt's vole sampling in 2014. Similar fluctuations were observed among day length, *Dio2* expression (Kruskal–Wallis test: $\chi^2 = 8.746$, $df = 3$, $p = .033$), and the ratio of *Dio2*/*Dio3* ($\chi^2 = 62.540$, $df = 3$, $p < .001$), but *Dio3* displayed a contrary seasonal pattern to *Dio2* ($\chi^2 = 84.645$, $df = 3$, $p < .001$). After June, *Dio2* expression significantly decreases, while *Dio3* expression displayed significantly higher expression. Correlation of day length was positive with *Dio2* expression ($r = .159$, $p = .011$, $n = 257$), but negative with *Dio3* expression ($r = -.490$, $p < .001$, $n = 200$). The numbers under bars indicate the sample sizes. Different letters above bars indicate significant differences between groups, and pink or blue-purple colours indicate *Dio2* or *Dio3*, respectively

to that of the NY males. Taken together, results from both years indicate distinct age-dependent gonadal developmental patterns in June, the inflection point of annual photoperiod.

3.3 | Seasonal and age-dependent expression of hypothalamic photoperiodic genes

3.3.1 | Seasonal expression of hypothalamic *Dio2* and *Dio3* and correlation with day length

Dio2 and *Dio3* genes are reported to play an important role in the hypothalamus in response to photoperiodic signals. We analysed the seasonal dynamic expression of both genes, the ratio of *Dio2* to *Dio3* expression, and the correlations between gene expression and day length in all 260 males collected in 2014. The expression of both genes displayed significant seasonal variation from April to October (Kruskal–Wallis test: *Dio2*, $\chi^2 = 8.746$, $df = 3$, $p = .033$; *Dio3*, $\chi^2 = 84.645$, $df = 3$, $p < .001$, Figure 3). The expression of *Dio2* increased from April to an annual peak in June and subsequently decreased (one-way ANOVA, LSD, $p < .05$) to a low level from August to October (Table S1). In contrast, the expression of *Dio3* decreased in April and reached the lowest level in June and then significantly increased ($p < .001$) from August to October (Table S1). Furthermore, the expression of the two genes was significantly correlated with day length, either positively in *Dio2* or negatively in *Dio3* for the whole population (Spearman's correlation: *Dio2*, $r = .159$, $p = .011$, $n = 257$; *Dio3*, $r = -.490$, $p < .001$, $n = 200$), as well as for either the NA (*Dio2*, $r = .154$, $p = .036$, $n = 185$; *Dio3*, $r = -.538$, $p < .001$, $n = 143$) or the NY (*Dio2*, $r = .306$, $p = .043$, $n = 44$; *Dio3*, $r = -.463$, $p = .004$, $n = 36$; Table S3) group. The ratio of *Dio2*/*Dio3* also displayed significant seasonal variation ($\chi^2 = 62.540$, $df = 3$, $p < .001$) and showed positive correlations with day length ($r = .337$, $p < .001$, $n = 199$) and testes mass ($r = .388$, $p < .001$, $n = 199$). The expression of the two genes was not significantly differentiated among the three age groups (Table S2). These results indicate that both hypothalamic *Dio2* and *Dio3* are likely closely associated with change in annual day length regardless of age and gonad activity.

3.3.2 | Seasonal and age-dependent expression of *Rfrp-3*, *Kiss-1* and *GnRH*

Rfrp-3 and *Kiss-1* are considered to associate with photoperiod, age and reproductive activity, while GnRH is the terminal neuropeptide that determines gonadal activity. We analysed the expression patterns of *Rfrp-3*, *Kiss-1* and *GnRH* in the whole population and among the three age groups in June. The expression of *Rfrp-3* displayed significant seasonal changes (Kruskal–Wallis test: $\chi^2 = 79.967$, $df = 3$, $p < .001$; Figure 4a) which continually increased through the breeding season from April to August and sharply decreased in October (Mann–Whitney *U* test: all $p < .001$ between any two months; Table S1). *Rfrp-3* expression significantly increased in OW males from April to June ($Z = -4.342$, $p < .001$) and in NA groups from June to August, and expression significantly decreased in NA groups from August to October (Mann–Whitney *U* test: all $p < .01$ between any two months; Table S1). A significant difference in *Rfrp-3* expression was detected among the three age groups in June (Kruskal–Wallis test: $\chi^2 = 24.424$, $df = 2$, $p < .001$) with the highest *Rfrp-3* expression in the NA groups and the lowest in the NY group (Mann–Whitney *U* test: both $p < .01$), indicating age-dependent expression patterns in *Rfrp-3*.

The expression of *Kiss-1* also displayed prominent seasonal changes. It significantly increased in June and maintained a high level until October (Kruskal–Wallis test: $\chi^2 = 9.900$, $df = 3$, $p = .019$; all $p < .05$ between April and each of the other months; Figure 4e). Among adult males, a significant increase in expression of *Kiss-1* was observed in the

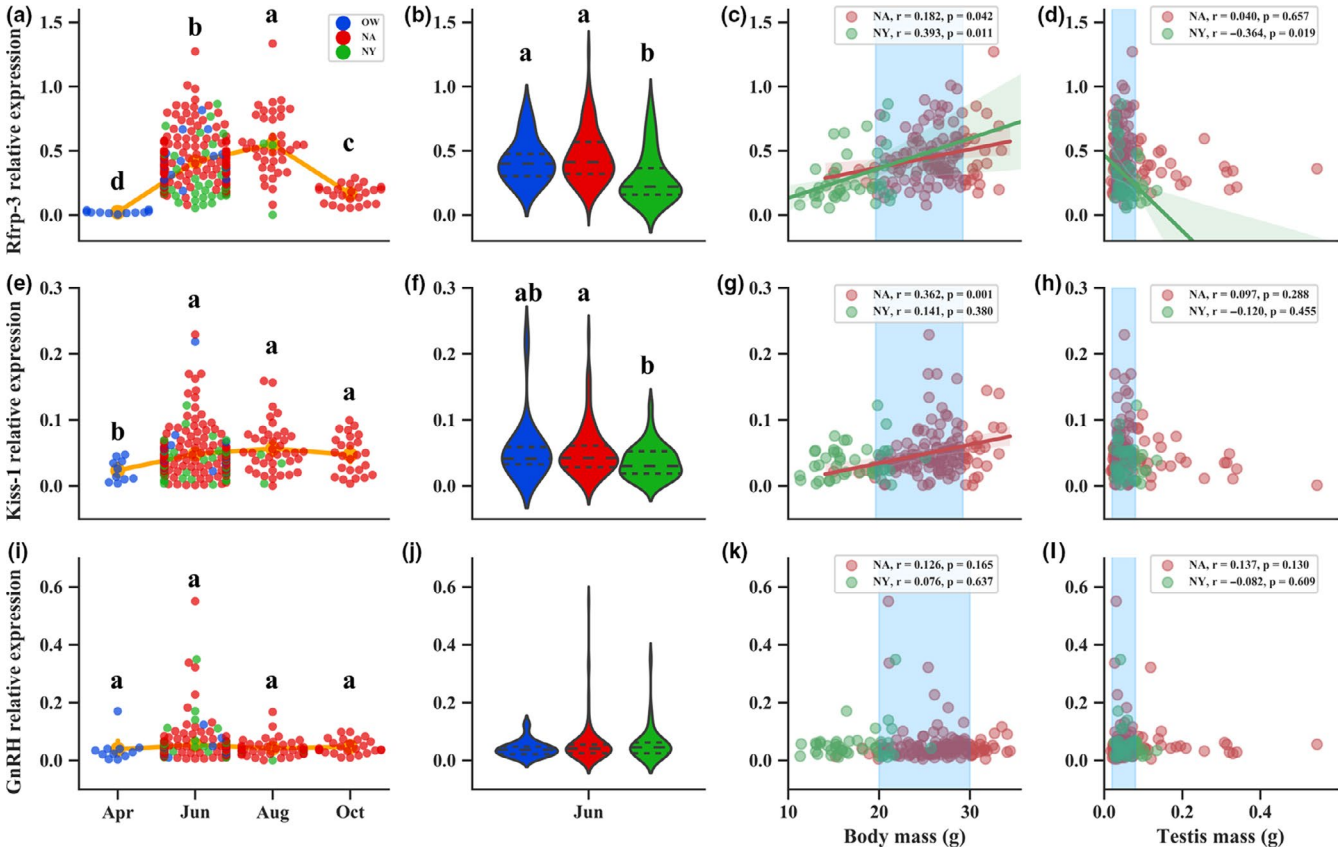

FIGURE 4 Seasonal variations in gonadal mass and relative expression of hypothalamic genes of Brandt's vole in 2014. (a–d) *Rfrp-3*; (e–h) *Kiss-1*; (i–l) *GnRH*. *Rfrp-3* displayed significant monthly variation (a), while *Kiss-1* significantly increased from April to June and then maintained high levels (e). Adult males had higher *Rfrp-3* and *Kiss-1* expression in June (b and f). Positive correlations in June were found between body mass and *Rfrp-3* and *Kiss-1* (c and g). *Rfrp-3* displayed a negative correlation with testes mass in the NY group (d). No seasonal or age differences and correlations with body or testes mass were found in *GnRH* (i and j). Extreme expression levels of the three genes were primarily observed in pubescent males of the mid-body mass range (20–30 g, c, g, k, blue-shaded area) and having small testes (<0.1 g, d, h, l, blue-shaded area) in June. NA, non-overwintered adult; NY, non-overwintered young; OW, overwintered adult; orange lines represent the mean values of all samples in each month and different letters represent the significant differences in the population among months or age groups in the two left columns of panels; within each violin plot, the three dotted lines from top to bottom represent the 25th percentile, median and 75th percentile of data

OW group from April to June (p = .013), and the NA group maintained a similar level from June to October, although its highest value was detected in June. When the three age groups coexisted in June, both OW and NA groups displayed significant higher *Kiss-1* expression than that of the NY group (Mann–Whitney *U* test: OW & NY, p = .085; NA & NY, p = .018; Figure 4). These results also indicate age-dependent expression patterns in *Kiss-1*, like *Rfrp-3*. In contrast, *GnRH* expression did not show any significant seasonal or age differences (Figure 4i,j).

We analysed the correlations among gene expression, developmental stage (body mass) and gonadal activity (testes mass) using data collected in June 2014. *Rfrp-3* expression was positively correlated with body mass of both NA and NY males rather than of OW males, and NY males displayed a stronger correlation (NA, r = .182, p = .043, n = 124; NY, r = .393, p = .011, n = 41; Figure 4c). *Rfrp-3* expression negatively correlated with testes mass only of NY males and not with that of the OW and NA groups (r = −.364, p = .019, n = 41; Figure 4d, Table S2). *Kiss-1* expression was positively correlated with body mass of NA males (r = .362, p < .001, n = 122; Figure 4g) but not with body mass of both OW and NY groups; it was also not correlated with testes mass in either group (Figure 4h; Table S2). These results indicate the strong correlation between *Rfrp-3* and *Kiss-1* expression and postnatal testicular development in newborns, especially in earlier stages of testicular development. The expression of *GnRH* was not significantly correlated with body or testes mass (Figure 4k,l; Table S2).

We analysed the correlation between gene expression and day length. A significant correlation was found between day length and *Rfrp-3* at the population level (Spearman's correlation: r = .241, p < .001, n = 258), and the OW group displayed a stronger correlation than the NA group (OW: r = .898, p < .001, n = 28; NA: r = .226, p = .002, n = 186). A significant correlation between *Kiss-1* and day length was detected just in the OW group (r = .410, p = .034, n = 27), and a weak correlation between *GnRH* and day length was detected only at the population level (r = .145, p = .020, n = 258; Table S3). These results indicate that *Rfrp-3* and *Kiss-1* may respond to day length primarily in adult males, especially adults in the OW group.

TABLE 2 The seasonal correlation analysis between the expression of two hypothalamic genes in the three age groups of male Brandt's vole

	April	June			August	October
	OW	OW	NA	NY	NA	NA
Dio2/Dio3	**r = .738***	r = −.192	r = .091	r = −.295	r = .041	r = −.018
	p = .037	p = .529	p = .365	p = .096	p = .844	p = .948
	n = 8	n = 13	n = 101	n = 33	n = 26	n = 16
Dio2/Rfrp-3	r = −.200	r = −.209	r = −.065	r = .286	r = .140	r = .322
	p = .555	p = .438	p = .478	p = .070	p = .410	p = .125
	n = 11	n = 16	n = 123	n = 41	n = 37	n = 24
Dio2/Kiss-1	r = −.552	r = −.038	r = −.056	r = −.280	r = −.110	**r = .479***
	p = .098	p = .888	p = .540	p = .076	p = .510	**p = .018**
	n = 10	n = 16	n = 122	n = 41	n = 38	**n = 24**
Dio2/GnRH	r = −.218	r = −.268	r = −.004	r = −.180	r = −.158	r = .117
	p = .519	p = .316	p = .969	p = .259	p = .342	p = .585
	n = 11	n = 16	n = 122	n = 41	n = 38	n = 24
Dio3/Rfrp-3	**r = −.857****	r = −.407	r = .164	r = −.093	r = .305	r = .444
	p = .007	p = .168	p = .102	p = .607	p = .139	p = .085
	n = 8	n = 13	n = 101	n = 33	n = 25	n = 16
Dio3/Kiss-1	r = −.464	r = −.236	r = .034	r = −.176	r = .214	r = .347
	p = .294	p = .437	p = .738	p = .327	p = .293	p = .188
	n = 7	n = 13	n = 100	n = 33	n = 26	n = 16
Dio3/GnRH	r = −.524	r = −.418	r = .170	r = −.080	r = .203	r = .250
	p = .183	p = .156	p = .091	p = .656	p = .319	p = .350
	n = 8	n = 13	n = 100	n = 33	n = 26	n = 16
Rfrp-3/Kiss-1	r = .103	r = .488	**r = .498*****	**r = .596*****	**r = .623*****	**r = .537****
	p = .777	p = .055	**p < .001**	**p < .001**	**p < .001**	**p = .007**
	n = 10	n = 16	**n = 123**	**n = 41**	**n = 38**	**n = 24**
Rfrp-3/GnRH	r = .564	**r = .497***	**r = .394*****	**r = .431****	**r = .447****	r = .271
	p = .071	**p = .050**	**p < .001**	**p = .005**	**p = .005**	p = .200
	n = 11	**n = 16**	**n = 123**	**n = 41**	**n = 38**	n = 24
Kiss-1/GnRH	r = −.091	**r = .562***	**r = .362*****	**r = .385***	**r = .625*****	r = .152
	p = .803	**p = .024**	**p < .001**	**p = .013**	**p < .001**	p = .478
	n = 10	**n = 16**	**n = 122**	**n = 41**	**n = 39**	n = 24

Note: Spearman's correlation; bold types represent significant correlations.
Abbreviations: NA, non-overwintered adult; NY, non-overwintered young; OW, overwintered adult.
*$p < .05$; **$p < .01$; ***$p < .001$.

3.3.3 | Correlation analysis of expression among the five hypothalamic genes

To explore the possible interactions among hypothalamic genes, we analysed the correlations of gene expression among the three age groups in April, June, August and October (Table 2). *Dio2* and *Dio3* showed few significant correlations with the other three genes. In contrast, positive correlations were mainly detected among *Rfrp-3*, *Kiss-1* and *GnRH*. Significant positive correlations between *Rfrp-3* and *Kiss-1* were found only among the NA and NY groups from June to October. Significant positive correlations of *Rfrp-3* or *Kiss-1* and *GnRH* were detected among all three age groups in June and August, but not in October. These results indicate their relations changed along with the change in seasons, especially in newborn voles.

4 | DISCUSSION

4.1 | The potential role of hypothalamic *Dio2/3* in anticipation of seasonal shifts

The prominent result in the current study shows a synchronization between day length and gonadal activity in adult males from 2010 to 2012, and peaks of gonadal activity in OW and NA males were found from May to June that rapidly declined after the summer solstice. Many laboratory studies suggest that hypothalamic *Dio2* and *Dio3*

genes in rodents respond to photoperiod (Herwig et al., 2012; Król et al., 2012; Ono et al., 2008; Revel, Saboureau, Pevet, et al., 2006; Sáenz de Miera, Bothorel, Jaeger, Simonneaux, & Hazlerigg, 2017), but there is still no direct evidence in wild animals. Our investigation indicates strong associations among multiple factors: hypothalamic *Dio2/3*, day length and testes mass. *Dio2* expression increased with increasing day length and decreased with decreasing day length, and the opposite was observed in *Dio3* expression. Strong correlations were displayed between expression of each of the two deiodinases and day length, as well as between their ratio (*Dio2*/*Dio3*) and testes mass. Their synergetic function on thyroid hormones implies the potential role of hypothalamic *Dio2/3* is in anticipation of seasonal change. Our results are consistent with photoperiodic response experiments conducted on mice, hamsters and voles showing that LP stimulated *Dio2* expression and SP elevated *Dio3* expression (Herwig et al., 2012; Król et al., 2012; Ono et al., 2008). A study on Siberian hamsters grown outdoors under natural photoperiodic conditions (Hannover, Germany; ~52°N latitude) also showed a peak of *Dio2* expression in June and *Dio3* expression in October in an annual cycle. Our results also revealed that wild Brandt's voles regulated the seasonality of HPG activities via the opposite function of *Dio2/3*, which synchronously enhance or weaken T_3 levels in the hypothalamus, especially in tanycytes, depending on day length. Taken together, like other seasonal breeding rodent species (Banks et al., 2016; Barrett et al., 2007; Freeman et al., 2007; Prendergast et al., 2013), our results imply that hypothalamic *Dio2* and *Dio3* play key roles in regulation of gonadal activity in wild Brandt's vole by synergistically regulating local T_3 levels in response to seasonal changes in day length.

4.2 | Age-dependent seasonality of gonadal activity and the possible role of hypothalamic deiodinase

Another distinct result in our present study is an overt age-dependent gonadal development pattern that occurred in June. Similar results from both 2012 and 2014 demonstrated that gonadal development had been inhibited in the majority of NA males in this period, while OW males still maintained high testes mass. Our recent field investigation on Brandt's voles also showed that OW males had delayed inhibition of testosterone secretion from August to October, one month later than the newborn voles whose reproductive activity were suppressed almost completely in early July (Chen et al., 2019). Similar age-dependent patterns were also found in montane vole (*Microtus montanus*; Negus, Berger, & Brown, 1986) and bank vole (*Clethrionomys glareolus*; Tkadlec & Zejda, 1998). Moreover, observation of a positive-skewed distribution of testes mass values in this study indicated the occurrence of rapidly developed testes in some individuals of NA males who were likely born in spring. However, the most striking feature is the inhibited testicular development in the majority of newborn males, especially in those males born after mid-summer, the peak of annual day length (Figure 4). Thus, gonadal developmental pattern is both annual day length- and age-dependent in this species. This feature is in accord with the variable lifespan of Brandt's vole, which is <14 months, and no wild vole has been recorded to survive over two winters. Generally, offspring born in early spring rarely survive the winter, but those born late in the breeding season will comprise the majority of the population of the overwintered voles in the next year (Shi et al., 1999; Figure S1c). Thus, OW males should breed as many offspring as possible, while newborn voles should conserve their energy for survival during the severe winter. The different reproductive expectations on an age group appear to determine individuals' reproductive developmental strategies in the breeding season.

Divergence of postnatal development pattern is a normal phenomenon in short-lived rodents and is affected by early-life events as early as the foetal period (Horton, 2005). Much efforts have been put forth to reveal their molecular mechanisms. Sáenz de Miera et al. (2017) found that male offspring of Siberian hamsters exposed to LP during gestation displayed higher *TSHβ* and *Dio2* expression than those exposed to SP, but this difference was absent two weeks later and higher *TSHr* and *Dio3* expression was found in the SP-exposed offspring. Moreover, offspring born in LP or SP conditions displayed significantly different *Dio2* and *Dio3* expression patterns. These studies indicate that maternal photoperiodic history determines the postnatal gonadal development patterns through the TSH-deiodinase axis of offspring. This mechanism could explain the rapidly developed gonads observed in those Brandt's voles born in spring. It is possible that some proteins, such as monocarboxylate transporter 8 (*Mct8*), a transporter of thyroid hormones in tanycytes, may also influence the effects of deiodinase on gonad activity by mediating the balance of hypothalamus T_3 levels. Petri et al. (2016) reported a peak of expression of *Mct8* in early September between the peaks of *Dio2* and *Dio3* expression and was accompanied by testes inhibition, which suggests that the increase in *Mct8* may help sustain the level of T_3 in tanycytes and maintain gonadal activity when *Dio2* decreases from June and thereafter. We speculate that *Mct8* may play an antagonistic role to the TSH-deiodinase axis to maintain gonadal activity in OW males of Brandt's vole after summer solstice.

4.3 | The possible roles of hypothalamic *Kiss-1* and *Rfrp-3*

In this study, both *Kiss-1* and *Rfrp-3* displayed significant age-dependent expression patterns (Figure 4). In June, the NA group displayed the highest expression of both genes and the maximum values occurred in those individuals with small testes mass. These results indicate that, directed by the TSH-deiodinase axis, *Kiss-1* and *Rfrp-3* appear to be more active in newborn males, especially when they are nearing puberty. Similarly, other studies also showed that both *Kiss-1* and *Rfrp-3* reached peaks of expression before or around puberty and then decreased or maintained their expression levels in adult male mice (Poling & Kauffman, 2015), rats (Iwasa et al., 2012; Walker, Kirson, Perez, & Gore, 2012) and Siberian hamsters (Prendergast et al., 2013).

The effect of *Kiss-1* on the HPG axis is positive according to previous reports. The increase in *Kiss-1* during puberty induces sexual maturation (Funes et al., 2003) and high *Kiss-1* expression levels facilitated adult males to maintain their sexually mature condition (Chen et al., 2017; Geraghty, Muroy, Kriegsfeld, Bentley, & Kaufer, 2016; Semaan & Kauffman, 2015; Terasawa, Guerriero, & Plant, 2013). An unexpected result of *Kiss-1* is that its expression remained elevated from June to October, even in voles in the nonbreeding condition having fully regressed gonads, which contradicts its positive effect on HPG activity. Prominent kp neurons locate in the Arc nucleus of male mice, rats and hamsters (Kauffman et al., 2007; Revel et al., 2007; Smith et al., 2005); our data which sampled the entire hypothalamus likely represented the changes in *Kiss-1* mainly from the Arc nucleus in voles. One explanation of stable *Kiss-1* expression from summer to autumn is that SP decreased testosterone levels and weakened its negative feedback on *Kiss-1* neurons in the Arc (Ansel et al., 2010; Rasri-Klosen et al., 2017; Sáenz de Miera et al., 2014; Smith et al., 2005). Because limitation of food availability can suppress *Kiss-1* expression in rat and mouse, our second explanation is that more abundant food availability from summer to autumn possibly helped maintain the high expression of *Kiss-1* for metabolic processes rather than reproductive processes (Castellano et al., 2005; Luque, Kineman, & Tena-Sempere, 2007). In addition, the amount or sensitivity of its receptor, *GPR54*, which also displays seasonal shifts, may weaken the effect of high levels of *Kiss-1* in autumn. For example, *GPR54* expression in the male striped hamster (*Cricetulus barabensis*), a seasonal breeder, is lower in winter than it is in spring and autumn (Xu, Xue, Li, Xu, & Chen, 2017). It is possible that low expression of *GPR54* can weaken the positive effects of *Kiss-1* on the HPG axis in the nonbreeding season; however, this speculation needs further verification.

Previous studies claimed that *Rfrp-3* expression is inhibited by SP conditions and melatonin in hamsters (Henningsen, Poirel, et al., 2016; Rasri-Klosen et al., 2017; Revel et al., 2008; Ubuka et al., 2012). Similarly, we also found a strong correlation between *Rfrp-3* and day length: a significant increase in *Rfrp-3* expression with increasing day length (from April to August) and a decline in *Rfrp-3* expression with the decreasing phase of day length (from August in October). Our results agree with those reports on the effect of *Rfrp-3* on HPG being possibly opposite in different photoperiod (Henningsen, Ancel, Mikkelsen, Gauer, & Simonneaux, 2017; Ubuka et al., 2012). The expression of *Rfrp-3* was higher in summer than in autumn, higher in adults than in juveniles, and positively correlated with the expression of *Kiss-1* and *GnRH*, implying its positive effects on the HPG axis. However, evidence of its negative effect suggests that an increase in the expression of *Rfrp-3* in August is associated with the full inhibition of testes activity, which is further supported by the negative correlation of *Rfrp-3* expression with testes mass in NY males, as well as its high expression around puberty. Exogenous dose administration experiments should be further designed to verify the specific effects of *Rfrp-3* on the HPG axis of Brandt's vole in different photoperiods.

4.3.1 | Strong correlations among *Rfrp-3*, *Kiss-1* and *GnRH* imply tight interactions

Our data revealed that significant correlations among *Rfrp-3*, *Kiss-1* and *GnRH* were observed from June to October but not in April, indicating that their interactions were dependent upon the photoperiodic background. The RFRP neuropeptide is considered as the critical switch between hypothalamic T_3 and GnRH activities in mammalian seasonal breeders (Henningsen, Gauer, & Simonneaux, 2016). Fibres of RFRP-3 are located near GnRH and Kp neurons, and its receptors are also expressed on both neurons. Studies have shown that GnRH neurons also express RFRP and Kp receptors in rodent species (Irwig et al., 2004; Rizwan et al., 2012; Rizwan et al., 2012; Ubuka et al., 2012). The overall research thus implies a high probability that the three genes, *Rfrp-3*, *Kiss-1* and *GnRH*, closely interact with each other to regulate the activity of the HPG axis during the breeding season in Brandt's vole.

Notably, the expression levels of *Kiss-1* and *Rfrp-3* genes also dramatically varied in postnatal development stages. Similar phenomena were found in other rodents. For example, the expression of *Kiss-1* gradually increased until the eighth to ninth postnatal weeks in male rats and mice (Clarkson & Herbison, 2006; Walker et al., 2012); the expression of *Rfrp* and its receptor reached their peaks at sixth postnatal week, and then significantly decreased at the 7-week age in rat (Iwasa et al., 2012); and GnRH mRNA and protein also increased during puberty and sustained high levels into adulthood (Gore, Roberts, & Gibson, 1999). Our observation of some extreme expression values of *Kiss-1*, *Rfrp-3* and *GnRH* in males with mid-levels of body mass (20–30 g) and small testes (<0.1 g) in June 2014 indicates that the simultaneous strong expression of the three genes during puberty may results in strong correlations among them in the breeding season (Stevenson, Hahn, MacDougall-Shackleton, & Ball, 2012). However, the positive correlation between *Kiss-1* and *Rfrp-3* in a nonbreeding season also indicated that their interaction might occur when gonadal activity was fully inhibited. *GnRH* expression did not show any seasonal variation and age differentiation from breeding to nonbreeding seasons, as well as any correlation with day length, indicating the low plasticity of GnRH neurons in Brandt's vole, similar to previous reports (Gorman & Yellon, 1996; Messina et al., 2011; Stevenson et al., 2012; Urbanski, Doan, & Pierce, 1991).

5 | CONCLUSION

Our current study outlines annual periodic and synchronous variation of reproductive activity and molecular neuroendocrine characteristics in response to the change in seasons in free-living Brandt's voles. The synchronous variation of *Dio2/3* with the change in day length suggests their key roles in decoding photoperiodic signals. Possibly guided by changes in *Dio2/3* signals, *Kiss-1* and *Rfrp-3* also play important roles in regulation of age-dependent gonadal development in Brandt's vole. Moreover, there may be an interaction

mechanism between *Rfrp-3* and *Kiss-1* in influencing the activity of the HPG system. Overall, our current results provide ample evidence on how hypothalamic photoperiod genes regulate seasonal breeding in a natural rodent population.

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AUTHOR CONTRIBUTIONS

The research was designed by D.W. and X-H.L. Ecological and physiological research was conducted by D.W. Molecular research was performed by N.L. Fieldwork and laboratory work were conducted by L.T., F.R., Z.L., Y.C., L.L., X.H. and X.Z. Data were analysed and the manuscript was written by D.W., N.L., Y.S., X.L., and R.A.H.

DATA AVAILBILITY STATEMENT

Data for figures and tables are available in the DRYAD archives under accession https://doi.org/10.5061/dryad.4913978. The cDNA sequences of genes have been uploaded to the GenBank database with the Accession nos as follows: *β-actin*, MK301451; *Dio2*, KX856007; *Dio3*, KX889114; *Rfrp-3*, KY038930; *Kiss-1*, KX833248; and *GnRH*: KY038929.

ORCID

Xiao-Hui Liu https://orcid.org/0000-0002-8674-1787

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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