Effect of photoperiod and 6-methoxybenzoxazolinone (6-MBOA) on the reproduction
of male Brandt's voles (*Lasiopodomys brandtii*)

Xin Dai^{a, b} · Jia Shi^a · Mei Han^a · Ai Qin Wang^a · Wan Hong Wei^{a, b} · Sheng Mei Yang^{a, b*}

^a College of Bioscience and Biotechnology, Yangzhou University, 48 East Wenhui Road, Yangzhou 225009, P.R. China

^b Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University, Yangzhou 225009, P.R. China

Abstract

Plant secondary metabolite 6-methoxybenzoxazolinone (6-MBOA) has been suggested to stimulate animal reproduction. 6-MBOA is detected in *Leymus chinensis*, a main diet of Brandt's vole (*Lasiopodomys brandtii*). We have previously reported a stimulatory effect of 6-MBOA on reproduction of male Brandt's voles under a short-day photoperiod. The goal of this study was to investigate the effect of 6-MBOA on reproductive physiology of male Brandt's voles under a long-day photoperiod and examine if 6-MBOA under this photoperiodic regime altered the reproductive status of male Brandt's voles differently than the short-day photoperiod. Under the long-day photoperiod, a high dose of 6-MBOA decreased *KiSS-1* mRNA in the arcuate nucleus (ARC), and we also saw a decrease in circulating levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone (T). Steroidogenic acute regulatory protein (*StAR*) and cytochrome P450_{11 α 1} (*CYP11 α 1*) in the testes, and relative testis weight also decreased with 6-MBOA administration. Compared to the short-day photoperiod, animals under the long-day photoperiod exhibited increased body weight as well as all other reproductive parameters. Our results showed that 6-MBOA inhibited the reproduction of male Brandt's vole under a long-day photoperiod, a stark contrast from its stimulatory effects under a short-day photoperiod. The paradoxical effects of 6-MBOA suggest it may act as a partial agonist of melatonin. These results provide insight into the complex interactions between environmental factors such as photoperiod and diet in the control of Brandt's

* Corresponding author. E-mail address: smyang@yzu.edu.cn.

vole reproduction.

Key words: Brandt's vole, 6-MBOA, Photoperiod, Reproductive activity, *KiSS-1/GPR54* system

1. Introduction

Seasonal changes have been considered a naturally occurring mechanism to turn on and off the reproductive system in adult mammals to ensure the offspring are born under conditions optimal for survival (Lincoln and Short, 1980; Karsch et al., 1984; Scherbarth and Steinlechner, 2010; Kriegsfeld et al., 2015). In seasonally reproducing mammals, the length of day or photoperiod is the most powerful environmental signal responsible for the timing of reproductive transitions (Lincoln and Short, 1980; Kriegsfeld et al., 2015). The photoperiodic signal is transduced to central and peripheral tissues by melatonin secreted from the pineal gland (Reiter, 1980; Karsch et al., 1984; Malpoux et al., 1997; Revel et al., 2007). Photoperiod changes in all temperate regions with astronomical precision from year to year. However, despite this precision in photoperiod, other environmental parameters, such as temperature, rainfall etc., vary markedly from year to year. Animals distributed in this type of habitat have developed the ability to respond to these local cues in addition to photoperiodic changes (Both and Visser, 2001; Both et al., 2005; Visser and Both, 2005; Dardente et al., 2016).

In some coevolved ecological systems, plant secondary metabolites (PSMs) transmit valuable environmental information by providing chemical cues that fine-tune the timing and degree of reproduction in herbivores. 6-methoxybenzoinone (6-MBOA), a phenolic PSM known to be present mainly in freshly growing plants of family Gramineae (Niemeyer, 1988; Gianoli and Niemeyer, 1997; Argandoña et al., 1981), was reported to stimulate the onset of breeding in the montane vole, *Microtus montanus* (Berger et al., 1981). The stimulatory effect of 6-MBOA on gonads has also been observed in other rodent species, but some mammals are relatively insensitive or unresponsive to 6-MBOA (Willard et al., 2006; Diedrich et al., 2014; Dai et al., 2016). How 6-MBOA exerts its effects is currently unclear, but it may act through one or

more of the following mechanisms: 1) 6-MBOA, structurally similar to melatonin (Yuwiler and Winters, 1985; Sanders et al., 1981), competes with melatonin for receptor sites (Sweat and Berger, 1988; Ellis, 1972; Peat and Kinson, 1971) and may stimulate the gonads by antagonizing melatonin actions (Gower and Berger, 1990); 2). 6-MBOA may enhance melatonin synthesis and desensitize melatonin receptors (Yuwiler and Winters, 1985; Daya et al., 1990), leading to the reversal of gonadal atrophy (Reiter et al., 1975, 1976); and 3) very high concentrations of 6-MBOA may inhibit melatonin synthesis, thereby alleviating the inhibitory effect of melatonin on reproduction (Yuwiler and Winters, 1985).

The Brandt's vole (*Lasiopodomys brandtii*) is a seasonally reproductive mammal (reproductively active from March to September) native to the grasslands of Inner Mongolia, China (Xie et al., 1994; Shi et al., 1999; Wan et al., 2002). The time of reproductive onset in this vole varies annually, resulting in tremendous fluctuations in its population. Such variations suggest the photoperiodic cue is not the only information used by this species to time its seasonal reproduction (Wan et al., 2002; Zhang et al., 2003). In a previous study, we successfully detected 6-MBOA in *Leymus chinensis* (Gramineae family) (Dai et al., 2014), the dominant plant in the grasslands of Inner Mongolia and the main diet of Brandt's vole (Wang et al., 1992). In this regard, Brandt's vole represents not only an ideal model for studying the effect and mechanisms of 6-MBOA, but also how a dietary chemical interacts with photoperiodic cues to control reproduction.

We previously reported that 6-MBOA upregulated *KiSS-1* / *GPR54* expression in Brandt's vole kept under a short-day photoperiod, leading to the stimulation of the hypothalamus-pituitary-gonadal (HPG) axis (Dai et al., 2016). In this study, we investigated the effect of 6-MBOA on the same reproductive parameters in Brandt's vole kept under a long-day photoperiod. Our aim was to compare the physiological response of male Brandt's vole to different photoperiods and examine how 6-MBOA interacted with different photoperiodic regimens. The results from this study should provide important insight into environmental factors driving the onset of reproduction and the co-evolution between Brandt's vole and dietary plant chemicals. Further, our

results may allow helpful predictions on the fluctuation of Brandt's vole population under the global climate change that alters plant growth and chemical production.

2. Material and methods

2.1. Animals and treatments

Brandt's voles, captured from the grasslands of Inner Mongolia, were bred in the animal vivarium of Yangzhou University, Jiangsu Province, China under controlled environmental conditions of 22 ± 1 °C, relative humidity of $50 \pm 5\%$, and a long-day photoperiod of 16 h light: 8 h dark (light on at 6:00 am and off at 10:00 pm). Voles were weaned at 21 days of age and housed individually in polypropylene cages for 25 days under the long-day photoperiod. Thereafter, 12 male voles were acclimated to a short-day photoperiod of 8 h light: 16 h dark for 8 weeks (light on at 8:00 am and off at 4:00 pm), while the other 50 male voles remained under a long-day photoperiod for 8 weeks. All voles were provided filtered tap water and standard rodent chow *ad libitum* before and throughout the experimental period. Following acclimation, a cohort of 32 voles from long-day photoperiod was randomly and equally assigned to one of the four groups: LPW (long-day photoperiod without 6-MBOA treatment), and low-, middle-, and high-dose 6-MBOA groups. 8 voles from short-day photoperiod was assigned to SPW (short-day photoperiod without MBOA treatment) group. All procedures were approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine of Yangzhou University.

A stock solution (250 µg/mL) of 6-MBOA (95% purity; Wu-Jing Company, Shanghai, China) was prepared as previously described (Dai et al., 2016). On the day of administration, the stock solution was brought to room temperature and diluted 2- or 20-fold with 5% propylene glycol for the middle and low dose of 6-MBOA, respectively. The injection was carried out as previously described (Dai et al., 2016). Each vole received one 0.4 mL injection per day of prepared 6-MBOA solution (low dose group of 5 µg 6-MBOA per day, middle dose group of 50 µg 6-MBOA per day, or high dose group of 100 µg 6-MBOA per day). The injections were administered intraperitoneally between 8:30 and 9:30 am for 12 consecutive days and commenced

115 immediately after the 8-week acclimation under the long- or short-day photoperiod.
116 The selected doses of 5–100 µg per day were based on the full range of observed
117 responses described by Sanders et al. (1981), Gower and Berger (1990), and Martin et
118 al. (2008). The LPW and SPW groups both received 0.4 mL 5% propylene glycol
119 vehicle.

120 Voles were weighed on days 1, 4, and 8 of injection. On day 13, 24 h after the last
121 injection, all animals were weighed and decapitated after anesthetizing with ether.
122 Serum samples were collected as previously described (Dai et al., 2016). Paired testes
123 were collected, weighed, immersed in a sample protector for RNA (TaKaRa, Dalian,
124 China), and stored at -20 °C. Brains were removed and dissected on ice to extract the
125 two fragments of hypothalamus in a mouse brain matrix. The two fragments of
126 hypothalamus were obtained by making three coronal cuts, with the first cut in the
127 middle of the second and third cut as previously described (Dai et al., 2016). These
128 hypothalamic fragments were stored individually in a sample protector for RNA
129 (TaKaRa) at -20 °C. The fragment between the first and second cut included the
130 anteroventral periventricular nucleus (AVPV) region of the hypothalamus, and that
131 between the first and third cut contained the arcuate nucleus (ARC) region. The
132 mRNA levels of *Kiss-1* were determined in individual fragments, whereas the two
133 fragments of mRNA were pooled to measure the expression of *GPR54* and *GnRH* in
134 the entire hypothalamus.

136 2.2. Enzyme-linked immunosorbent assay (ELISA) for serum hormones

137 Serum hormones, namely luteinizing hormone (LH), follicle stimulating hormone
138 (FSH) and testosterone (T), were quantified in duplicates using an ELISA kit (Jingke
139 Chemical Technology Limited Company, Shanghai, China) according to the
140 manufacturers' instructions and as previously reported (Dai et al., 2016). The purity
141 of the LH, FSH, and T standard preparations were all > 95%. The intra- and
142 inter-assay coefficients of variation were < 9 and < 13%, respectively, for all three
143 hormones.

2.3. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted and stored using the procedure established in our previous study (Dai et al., 2016). RNA samples of 1 µg were reverse-transcribed using PrimeScript 1st strand cDNA synthesis kit (TaKaRa) following the manufacturer's instructions. Gene expression was measured by qPCR. The sequences of qPCR primers for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were obtained from RTPrimerDB (ID: 2921; Lefever et al., 2009), those for *β-actin* from previous studies (Hegab et al., 2014), and those for *KiSS-1*, *GPR54*, gonadotropin-releasing hormone (*GnRH*), steroidogenic acute regulatory protein (*StAR*), and cytochrome P450 11a1 (*CYP11a1*) from our previous study (Dai et al., 2016) (Table 1). PCR reactions were conducted in a real-time PCR system (Applied Biosystems, Foster City, California, USA) using SYBR Premix EX Taq II (TaKaRa) as previously described (Dai et al., 2016). Standard curves were constructed for each gene via serial 5-fold dilutions of cDNA. Amplification efficiency ranged between 0.9 and 1.0, confirming the validity of the comparative quantification method. No amplification was detected in the absence of template or non-RT control. The fold change of gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001), using *β-actin* (O'shaughnessy et al., 2002) and *GAPDH* (Xi et al., 2011) as housekeeping genes for testis and hypothalamus gene expression, respectively.

2.4. Statistical analysis

All variables were tested for normality and homogeneity by Shapiro–Wilk and Levene test, and were transformed by $\log_{10}$ when necessary. The effect of 6-MBOA doses on body mass of voles under long-day photoperiod was studied using repeated measures analysis, where body weight on day 1 was the covariate, followed by the Tukey post hoc test. The effect of photoperiod on body mass was studied between SPW and LPW groups using repeated measures analysis. The effect of 6-MBOA

doses and photoperiod on gene expression in the hypothalamus, relative testis weight, and serum hormones were determined using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Paired samples *t*-test was used to compare the differences in the mRNA levels of *KiSS-I* between the AVPV and ARC regions of the hypothalamus. Statistical significance was determined at $P < 0.05$. All analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Differences in body and testis weight

The body weight of voles did not differ significantly among long-day photoperiod groups, while the body weight between the two photoperiods was significantly different ($F_{1, 14} = 8.452$, $P = 0.011$) (Table 2). Relative testis weight differed significantly among the 5 treatment groups ($F_{4, 35} = 29.677$, $P < 0.001$). Relative testis weight was higher in the LPW group compared with the middle, high 6-MBOA dose groups, and SPW group ($P = 0.008$, $P = 0.024$, and $P < 0.001$, respectively; Fig. 1A), and it was lower in the SPW group compared with the low, middle, and high dose groups ($P < 0.001$, for all comparisons).

3.2. Differences in serum hormones

The concentration of serum LH differed significantly among the 5 treatment groups ($F_{4, 35} = 27.628$, $P < 0.001$), with the high 6-MBOA dose group having lower serum LH than the LPW group ($P = 0.044$; Fig. 1B) and the SPW group having lower serum LH than all remaining groups ($P < 0.001$, for all comparisons). The concentration of serum FSH followed the same trend and also differed significantly among the 5 treatment groups ($F_{4, 35} = 22.275$, $P < 0.001$), with the high 6-MBOA dose group having lower serum FSH than the LPW group ($P = 0.042$; Fig. 1C) and the SPW group also having lower serum FSH than all remaining groups ($P < 0.001$, for all comparisons). Similarly, the concentration of serum T differed significantly among the 5 treatment groups ($F_{4, 35} = 25.848$, $P < 0.001$), with the LPW group with

higher than high dose and SPW groups of serum T ($P = 0.005$ and $P < 0.001$, respectively; Fig. 1D) and the SPW group with lower serum T than all remaining groups ($P < 0.001$, for all comparisons).

3.3. Expression of sex hormone synthesis genes in the testis

The mRNA level of *StAR* differed significantly among the 5 treatment groups ($F_{4, 35} = 31.439$, $P < 0.001$), with the LPW group having higher *StAR* mRNA than low dose group ($P = 0.002$) and the remaining groups ($P < 0.001$, for all comparisons; Fig. 2A), and the low, middle and high dose groups having higher *StAR* mRNA than SPW group ($P < 0.001$, $P = 0.001$, and $P < 0.001$, respectively). The mRNA level of *CYP11a1* also differed significantly among the 5 groups ($F_{4, 35} = 20.109$, $P < 0.001$), with the LPW group having higher *CYP11a1* mRNA than the low dose group ($P = 0.009$) and all remaining groups ($P < 0.001$, for all comparisons; Fig. 2B) and the low dose group having higher than the high and SPW groups ($P = 0.019$ and $P = 0.001$, respectively). Further, *CYP11a1* expression the middle group was higher than the SPW group ($P = 0.046$).

3.4. *KiSS-1/GPR54* system response in the hypothalamus

The mRNA level of *KiSS-1* in the AVPV differed significantly among the 5 treatment groups ($F_{4, 35} = 3.316$, $P = 0.021$), with the SPW group having lower *KiSS-1* mRNA than the middle dose group ($P = 0.013$). The mRNA level of *KiSS-1* in the ARC also differed significantly among the 5 treatment groups ($F_{4, 35} = 6.410$, $P = 0.001$), with the LPW group having higher *KiSS-1* mRNA than the high dose and SPW groups ($P = 0.028$ and $P < 0.001$, respectively; Fig. 3B). The mRNA level of *GPR54* in the hypothalamus did not differ among the 5 treatment groups ($F_{4, 35} = 1.748$, $P = 0.162$; Fig. 3C). The mRNA level of *GnRH* in the hypothalamus differed significantly among the 5 treatment groups ($F_{4, 35} = 39.500$, $P < 0.001$), with the SPW group having lower *GnRH* mRNA than the remaining groups ($P < 0.001$, for all comparisons; Fig. 3D). In addition, the mRNA levels of *KiSS-1* in the ARC were higher than those in the AVPV ($P < 0.001$; Fig. 4).

4. Discussion

Our current results demonstrate that the reproductive effects of 6-MBOA on male Brandt's voles kept under a long-day photoperiod contrast markedly with its effects reported for animals kept under a short-day photoperiod (Dai et al., 2016), which was performed during the same time period with our current study. Although, MBOA was inhibitory to reproductive parameters examined in this study, it did not affect body weight as it did in animals kept under the short-day photoperiod (Dai et al., 2016). This particular finding suggests 6-MBOA consistently targets the reproductive axis but may have a more minor role in the regulation of body mass (Diedrich et al., 2014).

Photoperiod alone can significantly affect both the body mass and the reproductive parameters of some seasonally breeding rodents such as the montane vole (Gower and Berger, 1990), Syrian hamsters (*Mesocricetus auratus*) (Klosen et al., 2013; Revel et al., 2006; Revel et al., 2007) and Siberian hamsters (*Phodopus sungorus*) (Klosen et al., 2013; Ebling and Barrett, 2008). The affected reproductive parameters include a diverse array of reproductive gene expression as well as circulating gonadotropins and testosterone, and relative testis weight. In this study, the majority of Brandt's vole's reproductive parameters measured were significantly higher under long-day photoperiod than short-day photoperiod, a finding consistent with a previous study (Zhao and Wang, 2006) and confirms Brandt's vole as a long-day breeder. Interestingly, although 6-MBOA had stimulatory effects under short-day photoperiod, its effects were far less potent than those of the long-day photoperiod. These suggest the photoperiod is still the primary cue in the seasonal control of Brandt's vole's reproduction, and 6-MBOA may serve as a modulatory signal of the primary photoperiodic cue.

Under a long-day photoperiod, 6-MBOA decreased some reproductive gene expression and circulating gonadotropin levels, which resulted in the decline of circulating testosterone and relative testis weight. These inhibitory effects were consistent with its long-term and acute effects on the white-footed mice and prepubertal male rats, respectively (Martin et al., 2008; Vaughan et al., 1988). However, our current findings contradicted the stimulatory effects of 6-MBOA on the

reproductive parameters of several rodent species, including Brandt's voles kept under the short-day photoperiod (Dai et al., 2016; Berger et al., 1981; Sanders et al., 1981; Negus and Berger, 1977). These paradoxical effects of 6-MBOA suggest the mechanism underlying the action of 6-MBOA may be relatively complex (discussed in a later paragraph). It is known that melatonin can provide an endocrine representation of photoperiod and plays an important role in the seasonal control of reproduction (Goldman, 2001; Henningsen et al., 2016; Dardente et al., 2016). Because of the structural similarity between 6-MBOA to melatonin (Yuwiler and Winters, 1985; Sanders et al., 1981), we posit that 6-MBOA may act through the melatonin pathway. However, in male Syrian hamsters exposed to short-day or long-day photoperiod, 6-MBOA had no effect on their reproduction axis (Urbanski et al., 1990). This discrepancy may be caused by a difference in the treatment regimen in that 6-MBOA was subcutaneously implanted into Syrian hamsters but injected into Brandt's vole, leading to a difference in the effective dose of 6-MBOA available to the animal. In addition, a difference in species may contribute to this discrepancy. For example, the testes of Syrian hamsters underwent a greater degree of regression than the Brandt's voles when placed under a short-day photoperiod (Urbanski et al., 1990; Dai et al., 2016). The species difference could also account for the differences in response to 6-MBOA between Brandt's vole and Djungarian hamsters (Diedrich et al., 2014).

The mechanisms underlying the effect of 6-MBOA on reproduction are complicated and remain unclear. One hypothesis suggests that 6-MBOA competes with melatonin for the same tissue receptor sites (Sweat and Berger, 1988; Ellis, 1972; Peat and Kinson, 1971) and is probably capable of antagonizing melatonin action to promote reproduction (Gower and Berger, 1990). In the present study, we found that circulating gonadotropins and testosterone, and relative testis weight declined after 6-MBOA treatment. Based on the paradoxical effect of 6-MBOA under short-day and long-day photoperiod and its structural similarity to melatonin (Yuwiler and Winters, 1985; Sanders et al., 1981), we proposed that 6-MBOA can have either agonistic or antagonistic effects on the melatonin pathway depending on the endogenous

melatonergic tone. In other words, 6-MBOA may act as a partial melatonin signaling agonist. Partial agonists occupy the receptor but only partially activate the receptor compared to a full agonist and display the properties of both agonists and antagonists depending on the state of receptor occupancy (Childress and O'Brien, 2000). Some isoflavone metabolites were also found to be partial agonists of estrogen signaling and could exert estrogenic effects in a low-estrogen environment, and anti-estrogenic effects in a high estrogen environment (Hwang et al., 2006). It is well known that in short-day photoperiod rodents have a longer duration of enhanced melatonin secretion (Yellon et al., 1982; Niklowitz et al., 1994), so in our study Brandt's voles kept in short-day are assumed to have more melatonin amounts than voles in the long-day photoperiod. Under the short-day photoperiod when melatonin levels are high, 6-MBOA could compete with endogenous melatonin for receptor binding and alleviate the inhibitory effect of melatonin. Under the long-day photoperiod when melatonin levels are low, 6-MBOA could be binding to melatonin receptors and acting as a weak agonist to inhibit reproduction. This hypothesis was supported by the report that 6-MBOA can partially prevent the inhibitory effects of melatonin on growth and sexual maturation of juvenile male montane vole (Gower and Berger, 1990).

In this study, regardless of the photoperiod and 6-MBOA treatment, the mRNA levels of *KiSS-1* in the ARC were higher than the AVPV, a result consistent with our previous observations on these voles (Dai et al., 2016) and the dominant presence of *KiSS-1* neurons in the ARC reported for several male rodents (Clarkson et al., 2006; Gottsch et al., 2004; Smith et al., 2006; Semaan and Kauffman, 2010). In our previous (Dai et al., 2016) and present studies, 6-MBOA did not alter the expression of *KiSS-1* in AVPV. The AVPV *KiSS-1* neuronal population is much smaller in males and plays a less critical function in male reproduction (Semaan and Kauffman, 2010). Thus it stands to reason that this population was not targeted by inhibitory factors such as 6-MBOA, which might support the hypothesis that *KiSS-1* in the ARC could be involved in the melatonin-mediated photoperiod transduction (Henningsen et al., 2016; Dardente et al., 2016). As mentioned, some parameters in the upstream HPG-axis of

male Brandt's vole did not undergo a noticeable response to the 6-MBOA treatment except the *Kiss-1* expression in the ARC and circulating gonadotropins declined only in the high dose group. In contrast, the expressions of *StAR* and *CYP11a1*, which encode for important enzymes involved in the synthesis of testosterone in the testis (Mukherjee and Haldar, 2014), were reduced sharply in response to 6-MBOA treatment. Further, the expressions of *StAR* and *CYP11a1* changed more dramatically than other parameters measured previously after 6-MBOA treatment (Dai et al., 2016). Since melatonin receptors are located in pars tuberalis of anterior pituitary (Henningsen et al., 2016; Dardente et al., 2016) and testis (Izzo et al., 2010; Mukherjee and Haldar, 2014), we propose that 6-MBOA may act as a partial melatonin agonist that could directly act on both pituitary and testis. Therefore, steroidogenic genes (*StAR* and *CYP11a1*) were directly regulated by both upstream HPG-axis and 6-MBOA, which would amplify the negative effect on testis and inhibit steroidogenic genes resulting in the decline of circulating testosterone and relative testis weight. Król et al. (2012) suggested that the dose and duration of 6-MBOA treatment might be important for its effectiveness. In our previous study, we discovered the dose-dependent effect of 6-MBOA (Dai et al., 2016). The present study consistently shows that there is also a dose-dependent effect of 6-MBOA in the majority of the parameters measured, with a strong inhibitory effect in the high dose 6-MBOA group. The reason GPR54 expression did not differ among groups is not clear and, thus, needs further exploration. Although GnRH expression did not vary significantly in response to 6-MBOA treatment, its expression shows a decreasing trend with the increasing 6-MBOA dose, which may result in the decline of circulating gonadotropins. Negative feedback to the hypothalamus from steroid hormones could be a contributing factor for the smaller variation in the most upstream region of the HPG-axis.

According to our previous study (Dai et al., 2014), the young sprouts of *L. chinensis* have high contents of 6-MBOA (100 µg/g). Assuming Brandt's voles can eat at least 1g *L. chinensis* sprout in the early spring, each wild vole could consume up to 100µg 6-MBOA a day. In this regard, 6-MBOA dosages used in this study

(5-100 μ g 6-MBOA injection per day) are ecologically relevant. In the early spring, a period of Brandt's voles reproductive recrudescence, 6-MBOA in Brandt's vole habitat should be more abundant according to a recent study reporting higher 6-MBOA content in younger *L. chinensis* (Dai et al., 2014). This period also corresponds to a higher endogenous level of melatonin in Brandt's vole compared to the summer months. As such, dietary 6-MBOA could act as an environment cue to facilitate reproductive recrudescence. As summertime approaches and the day length increases, 6-MBOA becomes less abundant in aged *L. chinensis* (Dai et al., 2014), thus lessening its negative impact on vole reproduction under the long-day photoperiod. However, there is no evidence for 6-MBOA directly acting through the melatonin receptor, so the precise target of 6-MBOA need further deeper investigations.

In summary, our results demonstrated that the reproductive status of male Brandt's vole was significantly impacted by photoperiod and 6-MBOA. Long-day photoperiod robustly stimulated the reproductive status of male Brandt's vole compared to the short-day photoperiod, and 6-MBOA could act via both *KiSS-1/GPR54* system and testis of HPG-axis to inhibit the reproductive activity of male Brandt's vole kept under a long-day photoperiod. 6-MBOA may exert its effect by acting as a partial agonist of melatonin to modulate the HPG-axis. Our results provide novel insight into the complex interplay among dietary plant chemicals, photoperiod, and animal reproduction. Importantly, they highlight the importance of local environmental cues, such as dietary chemicals, in the seasonal control of animal reproduction.

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558 Fig.1. Relative testis weight (A) and concentrations of luteinizing hormone (LH) (B),
559 follicle-stimulating hormone (FSH) (C), and testosterone (T) (D) in the serum of
560 male Brandt's voles (*Lasiopodomys brandtii*) exposed to long-day photoperiod
561 without 6-methoxybenzoxazolinone (LPW), low (5 µg), middle (50 µg), and high
562 (100 µg) doses of 6-MBOA and voles under short-day photoperiod without
563 6-MBOA (SPW). Error bars indicate standard error. Same letters connect bars
564 with no significant differences at $P < 0.05$ ($n = 8$). Note: Relative testis weight was
565 calculated as paired testes weight (g) divided by body weight (g).

566 Fig.2. Relative mRNA expression levels of *StAR* (A) and *CYP11a1* (B) in the testes of
567 male Brandt's voles (*Lasiopodomys brandtii*) exposed to long-day photoperiod
568 without 6-methoxybenzoxazolinone (LPW), low (5 µg), middle (50 µg), and high
569 (100 µg) doses of 6-MBOA and voles under short-day photoperiod without
570 6-MBOA (SPW). Error bars indicate standard error. Same letters connect bars
571 with no significant differences at $P < 0.05$ ($n = 8$).

572 Fig.3. Relative mRNA expression levels of *KiSS-1* in the anteroventral periventricular
573 nucleus (AVPV; A) and the arcuate nucleus (ARC; B), and relative mRNA
574 expression levels of *GPR54* (C) and *GnRH* (D) in the hypothalamus of male
575 Brandt's voles (*Lasiopodomys brandtii*) exposed to long-day photoperiod
576 without 6-methoxybenzoxazolinone (LPW), low (5 µg), middle (50 µg), and high
577 (100 µg) doses of 6-MBOA and voles under short-day photoperiod without
578 6-MBOA (SPW). Error bars indicate standard error. Same letters connect bars
579 with no significant differences at $P < 0.05$ ($n = 8$).

580 Fig.4. Differences in *KiSS-1* expression between the anteroventral periventricular
581 nucleus (AVPV) and the arcuate nucleus (ARC) of male Brandt's voles
582 (*Lasiopodomys brandtii*) exposed to long-day photoperiod without
583 6-methoxybenzoxazolinone (LPW), low (5 µg), middle (50 µg), and high (100
584 µg) doses of 6-MBOA and voles under short-day photoperiod without 6-MBOA
585 (SPW). Error bars indicate standard error. * means significant differences at $P <$
586 0.05 ($n = 8$).

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Table 1 Primers for quantitative real-time polymerase chain reaction used in the present study

	Forward (5'-3')	Reverse (5'-3')	Reference
<i>KISS-1</i>	TCTTGGCAGCTACTGCTTCTC	AACCCCAGATTTCCTTCTGC	
<i>GPR54</i>	AACTTGGCGGCGACAGACG	GAGACCTGCTGGATGTAGTTGA	
<i>GnRH</i>	TGCGCCCTGGAGGAAAGA	CAGACTTTCCAGAGCTCCTCGC	Dai et al.[14]
<i>StAR</i>	GGTCCTGCAAAAGATCGGGAA	GGCATCTCCCCAAAATGTGTG	
<i>CYP11a1</i>	AATCCCAGCGGTTTCATCG	TGTGCCGTAAGTCCCAGT	
<i>β-actin</i>	TTGTGCGTGACATCAAAGAG	ATGCCAGAAGATTCCATACC	Hegab et al.[34]
<i>GAPDH</i>	TGGCAAAGTGGAGATTGTTGCC	AAGATGGTGATGGGCTTCCCG	Lefever et al.[35]

Table 2 Body weight (mean $\pm$ standard error; n = 8) of male Brandt's voles (*Lasiopodomys brandtii*) under long-day photoperiod without 6-methoxybenzoxazolinone (LPW), low (5 μ g), middle (50 μ g), and high (100 μ g) doses of 6-MBOA and voles under short-day photoperiod without 6-MBOA (SPW).

Group	Body weight (g)			
	Day 1 st	Day 4 th	Day 8 th	Day 13 th
SPW	41.15 $\pm$ 0.79	42.21 $\pm$ 0.93	42.85 $\pm$ 1.13	44.40 $\pm$ 0.93
LPW	51.69 $\pm$ 2.14	50.59 $\pm$ 2.16	49.63 $\pm$ 1.91	49.76 $\pm$ 1.88
Low dose	51.21 $\pm$ 1.87	51.19 $\pm$ 1.49	51.70 $\pm$ 1.66	52.59 $\pm$ 1.68
Middle dose	54.29 $\pm$ 1.33	53.88 $\pm$ 1.44	54.10 $\pm$ 1.60	52.81 $\pm$ 2.10
High dose	53.99 $\pm$ 1.05	53.46 $\pm$ 0.76	53.15 $\pm$ 0.65	52.13 $\pm$ 1.52