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RFRP-3 synchronized with photoperiods regulates the seasonal reproduction of striped hamsters

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Research Article

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Summary

The purpose of this study was to investigate the effect of RFRP-3 synchronized with photoperiods on regulating the seasonal reproduction of striped hamsters. The striped hamsters were raised separately under long-day (LD; 16 h light/8 h dark), medium-day (MD; 12 h light/12 h dark) or short-day (SD; 8 h light/16 h dark) conditions for 8 weeks. *RFRP-3* and gonadotropin-releasing hormone (*GnRH*) mRNA levels in the hypothalamus, testis or ovaries in three groups were detected using reverse transcription polymerase chain reaction (RT-PCR). Melatonin (MLT), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) concentrations in serum were detected using enzyme-linked immunosorbent assay (ELISA). The correlation between *RFRP-3* and *GnRH* mRNA and FSH and LH concentrations was also analyzed. MLT negatively regulated the expression of *RFRP-3*. Significant differences for *RFRP-3* mRNA existed in the three groups, which positively correlated with the *GnRH* and the FSH and LH concentrations. *RFRP-3* mRNA levels in the hypothalamus were significantly higher than those in ovaries or testis. *RFRP-3* levels in the hypothalamus were significantly lower in female than in male under SD conditions, while those in ovaries were significantly higher than those in testes under LD conditions. MLT decreased RFRP neuron activity, and *RFRP-3* regulated the reproduction of striped hamsters.

Introduction

Seasonal reproduction is a main cause in fluctuation of population abundance, therefore studying seasonal reproduction mechanisms is beneficial to rationally control population abundance. Seasonally reproductive mammals can regulate fertility by controlling photoperiodic signals (Goldman, 2001). Photoperiod information is translated by the pineal hormone melatonin in the brain and variation in melatonin levels is synchronized with photoperiod. Therefore, the melatonin rhythm is important for synchronizing reproduction with seasons (Pitrosky and Pévet, 1997; Helfer *et al.*, 2019).

Gonadotropin-releasing hormone (GnRH) is released mainly around the capillaries of the pituitary portal system (Hahn and Coen, 2006). It mainly acts in the hypothalamic–pituitary–gonadal (HPG) axis to control gonadotropin, luteinizing (LH), and follicle-stimulating (FSH) hormone secretion in mammals. LH and FSH stimulate testicular spermatogenesis, follicular growth, and estradiol and testosterone release, therefore feeding back to and regulating the HPG axis. Therefore, GnRH is the most important factor for regulating reproductive activity in animals.

The ways in which the melatonin signal transfers to GnRH neurons are less understood, and melatonin acts indirectly on GnRH neurons (Urbanski *et al.*, 1991). Revel *et al.* (2008) found that the RFamide-related peptide (*RFRP*) gene was expressed in Siberian and Syrian hamsters (Revel *et al.*, 2008). In addition, RFRP-immunoreactive fibres were found to directly contact GnRH neurons (Smith and Clarke, 2010; Rizwan *et al.*, 2012). Therefore, RFRP neurons may be a factor in melatonin signalling (Simonneaux *et al.*, 2013); this indicated that RFRP may centrally control the reproductive axis. Alternatively, RFRP levels in the hypothalamus were strongly regulated by melatonin, corresponding to the length of the photoperiod (Revel *et al.*, 2008), which further indicated that RFRP may be a bridge between melatonin and GnRH neurons to translate its effects on reproduction (Simonneaux *et al.*, 2013). The striped hamster is a main rodent pest in northern China farmland and has typical seasonal reproductive characteristics (Mu *et al.*, 1999). Its reproductive activity intensity is higher in spring and autumn than in summer and winter (Luo *et al.*, 2000). Whether photoperiod regulates *RFRP-3* levels in hypothalamus and gonads and the role of *RFRP-3* in the reproduction of striped hamsters are all unknown. To explore whether *RFRP-3* levels are controlled by MLT, *RFRP-3* levels in hypothalamus, ovarian or testis of striped hamsters from LD, MD and SD photoperiods were detected. To further assess whether *RFRP-3* regulates the seasonal reproduction of the

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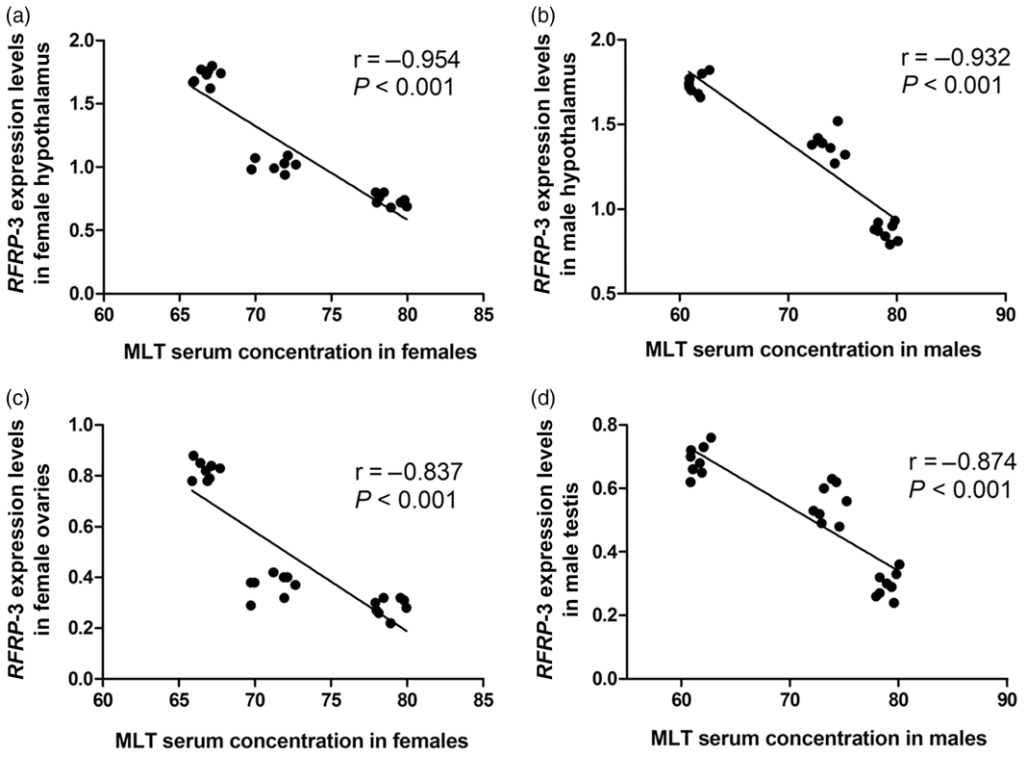

Figure 1. Relationship between serum MLT concentration and *RFRP-3* mRNA in the hypothalamus and gonads of striped hamsters. (a) Correlation between serum MLT concentration with *RFRP-3* mRNA in the female hypothalamus. (b) Correlation between serum MLT concentration and *RFRP-3* mRNA in the male hypothalamus. (c) Correlation between serum MLT concentration and *RFRP-3* mRNA in the female ovaries. (d) Correlation between serum MLT concentration and *RFRP-3* mRNA in the male testis.

striped hamster, the relationships between *RFRP-3* levels and *GnRH*, FSH or LH were also analyzed.

Materials and methods

Animals and protocols

Striped hamsters were captured in May 2011 in Wu Village, Qufu City of Shandong Province. The trapped striped hamsters were sexed, weighed, and appraised to provide information on size, age and reproductive condition. Twenty-four adult male and female striped hamsters weighing 22–24 g were selected at random and housed in the animal experiment centre of Qufu Normal University under natural photoperiod and temperature conditions for 1 week. Then, the hamsters were randomly raised under long (LD; 16 h light/8 h dark), medium (MD; 12 h light/12 h dark) or short day lengths (SD; 8 h light/16 h dark) for 2 months. The estrous cycle of the striped hamsters was examined at 5 p.m. to 6 p.m. every day. For estrous, hamsters were immediately killed by CO_2 asphyxiation. The flow rate of CO_2 displaced was no more than 20% of the chamber volume per minute. Their blood samples, hypothalamus, ovaries or testes were collected. All experiments performed were approval by the Animal Ethics Committee of Qufu Normal University.

qRT-PCR

Total RNA from hypothalamus, ovaries or testis of striped hamsters in the LD group, MD group and SD group were extracted using a tissue total RNA isolation kit (TaKaRa, Japan) under conditions recommended by the manufacturer. Total RNA was reverse transcribed into cDNA using reverse transcriptase XL AMV with an oligodeoxythymidylate primer (TaKaRa, Japan). The primers (forward primer: 5′-AACCTGCCCCTGAGATTTG-3′ and reverse primer: 5′-ACTCTGGATTTCTTGATGCTGG-3′) for *RFRP-3*, (forward primer: 5′-GAGACCTTCAACACCCCAGC-3′ and reverse primer: 5′-ATGTCACGCACGATTTCCC-3′) for *β-actin*, and forward primer: 5′-CTGGTCCTATGGGTTGCG-3′ and reverse primer: 5′-GAAGTGCTGGGGTTCTGCT-3′ for *GnRH*) were used to carry out qRT-PCR. qRT-PCR was carried out using the Brilliant II SYBR Green QPCR Master Mix (TaKaRa) to detect the quantity of the products. Amplification efficiency was verified using a standard curve (Rutledge and Stewart 2008). *β-Actin* was used as the control (Livak and Schmittgen, 2001).

Measurement of serum melatonin, FSH and LH concentration

Blood samples were centrifuged (2400 *g*) for 10 min at 4°C. The concentrations of MLT, FSH and LH in serum were detected using ELISA and a Labsystems Multiskan MS 352 spectrophotometer.

Statistical analysis

Data were expressed as means ± standard deviation. Data were compared by independent sample *t*-test and analysis of variance (ANOVA) using SPSS Statistics v.22.0 software (International Business Machines, Corp., Armonk, NY, USA). Values of $P < 0.05$ or $P < 0.01$ were considered as significantly different. Pearson's correlation between *RFRP-3* mRNA and *GnRH* mRNA, serum melatonin, and FSH and LH concentrations were analyzed using SPSS v.22.0.

Results

Correlation between serum MLT concentration with RFRP-3 *mRNA in striped hamsters*

The relationship between serum MLT concentration and *RFRP-3* mRNA in the hypothalamus and gonads is shown in Figure 1. In female striped hamsters, serum MLT concentration was

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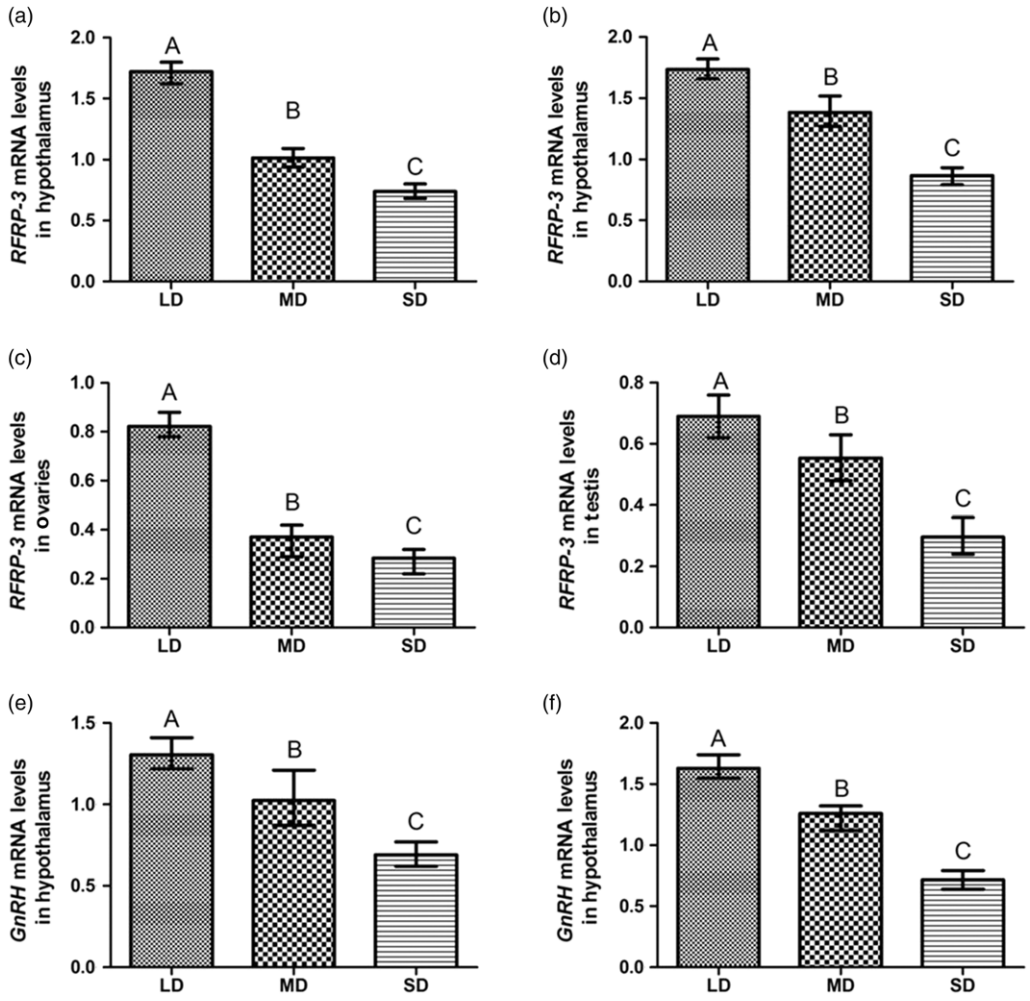

Figure 2. *RFRP-3* in striped hamsters. (a) *RFRP-3* expression in the hypothalamus of females under LD, MD and SD conditions. (b) *RFRP-3* expression in the hypothalamus of males under LD, MD and SD conditions. (c) *RFRP-3* expression in the ovaries of females under LD, MD and SD conditions. (d) *RFRP-3* expression in the testis of males under LD, MD and SD conditions. (e) *GnRH* expression in the hypothalamus of females under LD, MD and SD conditions. (f) *GnRH* expression in the hypothalamus of males under LD, MD and SD conditions. Relative quantities with different capital letters differ significantly ($P > 0.01$).

significantly negatively correlated with *RFRP-3* mRNA in the hypothalamus ($r = -0.954$, $P < 0.001$) (Figure 1a) and the ovaries ($r = -0.837$, $P < 0.001$) (Figure 1c). In male striped hamsters, serum MLT concentration was also significantly negatively correlated with *RFRP-3* mRNA in the hypothalamus ($r = -0.932$, $P < 0.001$) (Figure 1b) and the testis ($r = -0.874$, $P < 0.001$) (Figure 1d). Our results suggested that MLT is an inhibitory factor to control *RFRP-3* levels in hypothalamus and gonads, and the role of MLT in *RFRP-3* expression was sex independent.

RFRP-3 *and* GnRH *in hamsters from different photoperiodic conditions*

mRNA expression of *RFRP-3* in the hypothalamus and gonads and *GnRH* in the hypothalamus were quantitatively determined in striped hamsters from different photoperiods (Figure 2). The results suggested that *RFRP-3* mRNA levels in hypothalamus and ovaries or testis were significantly different among striped hamsters from different photoperiods ($P < 0.01$). The highest *RFRP-3* levels in hypothalamus and in ovaries or testis were detected in individuals under LD conditions, and the lowest levels were detected in individuals from SD conditions (Figure 2A–D). In addition, *GnRH* mRNA levels in striped hamsters from LD conditions were notably higher than those from MD and SD conditions. Lowest *GnRH* mRNA levels were determined in the hypothalamus of hamsters under SD conditions (Figure 2E, F). These results further indicated that photoperiod was a main environmental factor for regulating the expression of *RFRR-3* in striped hamsters.

Correlation between RFRP-3 *and* GnRH

In female hamsters, *RFRP-3* mRNA levels in hypothalamus were significantly positively correlated with *GnRH* mRNA levels in hypothalamus ($r = 0.941$, $P < 0.001$) (Figure 3a) and those in ovaries were also significantly positively correlated with the *GnRH* in hypothalamus ($r = 0.864$, $P < 0.001$) (Figure 3c). In male hamsters, *RFRP-3* mRNA levels in hypothalamus were significantly positively correlated with *GnRH* in hypothalamus ($r = 0.973$, $P < 0.001$) (Figure 3b) and those in testis were also significantly positively correlated with *GnRH* in hypothalamus ($r = 0.948$, $P < 0.001$) (Figure 3d).

Correlation of RFRP-3 *mRNA levels in hypothalamus with serum FSH and LH concentrations*

In female hamsters, *RFRP-3* mRNA levels in hypothalamus were significantly positively correlated with serum FSH and LH concentrations ($r = 0.961$, $P < 0.001$; $r = 0.745$, $P < 0.00$; Figure 4a, c). In male hamsters, *RFRP-3* mRNA levels in hypothalamus were also significantly positively correlated with serum FSH and LH concentrations ($r = 0.902$, $P < 0.001$; $r = 0.928$, $P < 0.001$; Figure 4b, d). These results showed that *RFRP-3* immediately regulated the synthesis and secretion of FSH and LH in hamsters.

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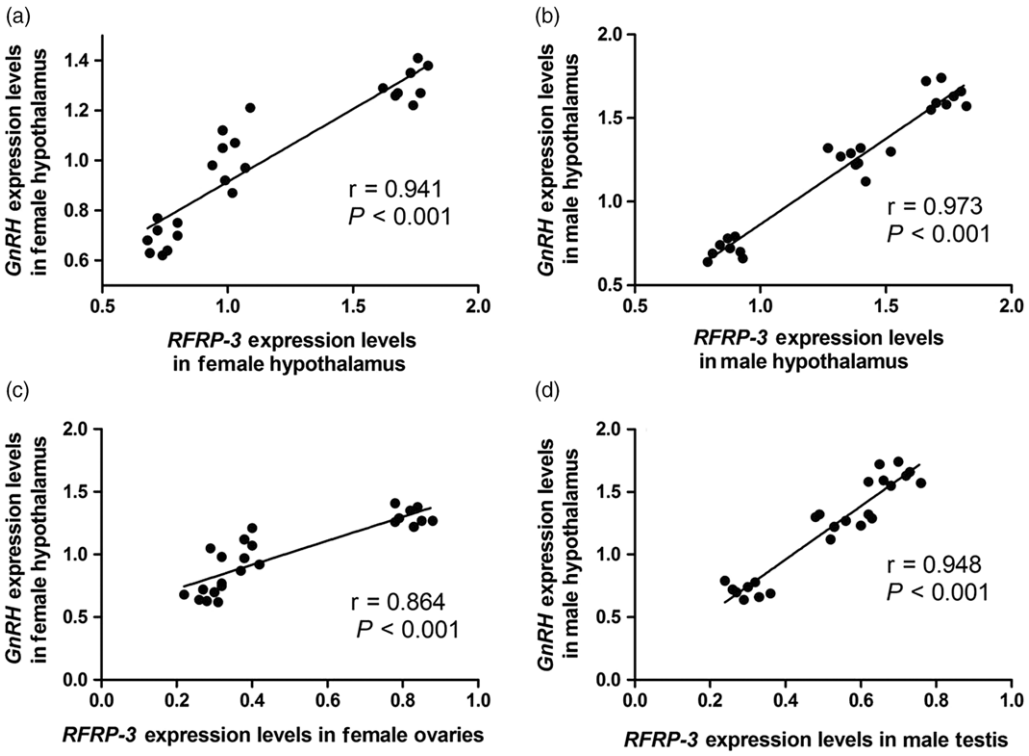

Figure 3. Relationships of *RFRP-3* mRNA in the hypothalamus and gonads with that of *GnRH* mRNA in the hypothalamus of striped hamsters. (a) Correlation between *RFRP-3* and *GnRH* in the female hypothalamus. (b) Correlation between *RFRP-3* and *GnRH* in the male hypothalamus. (c) Correlation between *RFRP-3* in ovaries and *GnRH* in the hypothalamus in females. (d) Correlation between *RFRP-3* in the testis with that of *GnRH* in the hypothalamus in males.

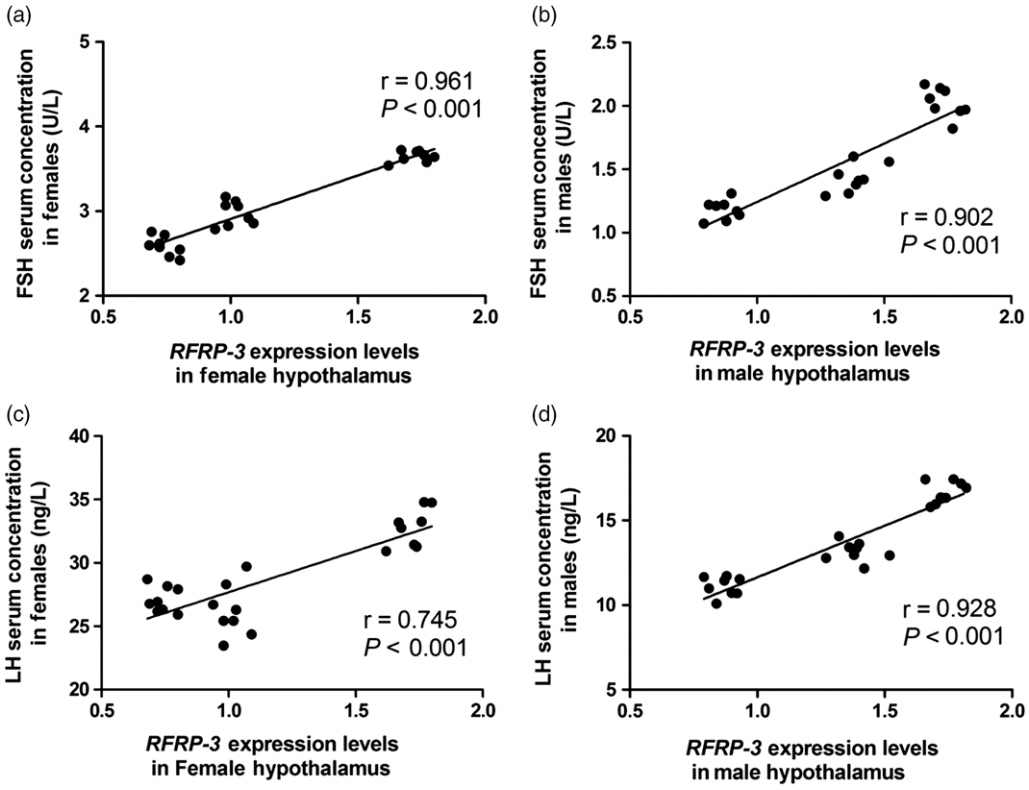

Figure 4. Relationships between *RFRP-3* mRNA in the hypothalamus with serum FSH and LH concentrations in the striped hamsters. (a) Correlation between the *RFRP-3* mRNA in the hypothalamus with the serum FSH concentration in female striped hamsters. (b) Correlation between the *RFRP-3* mRNA in the hypothalamus with serum FSH concentration of male striped hamsters. (c) Correlation between the *RFRP-3* mRNA in the hypothalamus with serum LH concentration of female striped hamsters. (d) Correlation between the *RFRP-3* mRNA in the hypothalamus with serum LH concentration of male striped hamsters.

RFRP-3 *in hamsters between sexes and tissues*

RFRP-3 mRNA levels in male hypothalamus were markedly higher than those in female hypothalamus from MD and SD conditions. However, there were no significant differences in the hypothalamus between the males and females from LD conditions. *RFRP-3* mRNA levels in testis were significantly higher than those in ovaries of hamsters from LD and MD conditions, while there were no significant differences between testis and ovaries from SD conditions (Figure 5). These results indicated that the difference between *RFRP-3* mRNA levels between sexes was photoperiod dependent.

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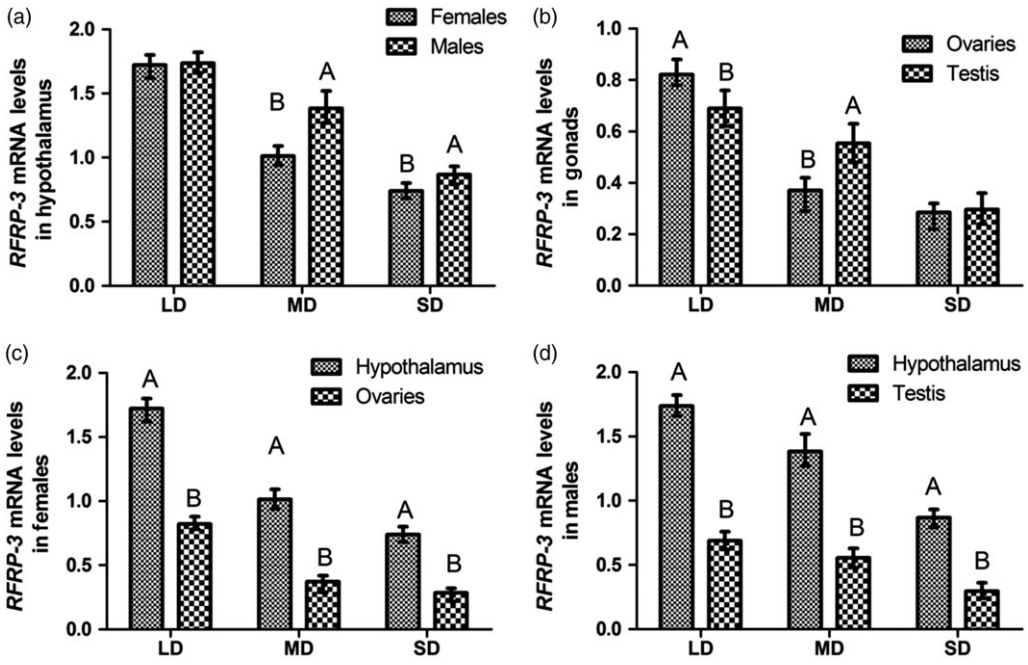

Figure 5. Differences of *RFRP-3* mRNA between sexes and tissues of striped hamsters from different photoperiods. (a) Differences of *RFRP-3* mRNA in the hypothalamus between males and females under LD, MD and SD conditions. (b) Differences of *RFRP-3* mRNA between ovaries and testis of striped hamsters under LD, MD and SD conditions. (c) Differences of *RFRP-3* mRNA between the hypothalamus and ovaries of females under LD, MD and SD conditions. (d) Differences of *RFRP-3* mRNA between the hypothalamus and testis of males under LD, MD and SD conditions. Relative quantity with different capital letters are significantly different ($P > 0.01$).

RFRP-3 mRNA levels in hypothalamus were significantly higher than those in ovaries and testes of hamsters from LD, MD or SD conditions (Figure 5). These results indicated that the difference between *RFRP-3* mRNA levels in hypothalamus and gonads was photoperiod independent.

Discussion

Photoperiod is an important environmental seasonal cue affecting a broad spectrum of physiological parameters including reproductive status (Pitrosky and Pévet, 1997). Photoperiodic mammals rely on the annual cycle of melatonin changes in nocturnal secretion to regulate their reproductive activity (Bronson, 1989). Striped hamsters are photoperiod-sensitive mammals, and the intensity of their reproductive activity significantly varies with seasons (Lu *et al.*, 2000). If *RFRP-3* is involved in the control of reproductive activity in striped hamsters, *RFRP-3* levels should be regulated by photoperiod and melatonin. In our study, we found that the mRNA levels of *RFRP-3* were significantly correlated with serum melatonin concentration, and there were significant differences in mRNA levels for *RFRP-3* among individuals under different photoperiods. These results further proved that striped hamsters were photoperiodically sensitive mammals. Our results were also consistent with findings that *RFRP* neurons were strongly downregulated by melatonin in Syrian hamsters (Simonneaux and Ancel, 2012) and Siberian hamsters (Ubuka *et al.*, 2012). However, the expression levels of gonadotropin inhibitory hormone (*GnIH*), orthologous to *RFRP-3*, were also regulated by melatonin in an opposite manner when compared in mammals (Ubuka *et al.*, 2005). Therefore, the effect of melatonin on expression of *RFRP-3* is species dependent. For most seasonal mammalian species, *RFRP* expression is decreased under SD conditions and increased under LD conditions, regardless of reproduction carried out under SD or LD conditions. In striped hamster, *RFRP-3* mRNA levels were significantly lower under SD conditions than those under LD, indicating an inhibitory effect of pineal melatonin on *RFRP* neurons. This result was consistent with the discoveries in Syrian hamsters (Revel *et al.*, 2008; Mason *et al.*, 2010) and sheep (Dardente *et al.*, 2008), while a discrepancy was founding with quail (Ubuka *et al.*, 2005).

In the Siberian hamster, the mRNA expression levels of *GnIH* were higher under LD conditions than those under SD conditions, and the mRNA expression levels of *GnIH* were suppressed by melatonin, which is a nocturnal pineal hormone (Ubuka *et al.*, 2012). *RFRP* mRNA levels in hypothalamus from hamster significantly varied among different photoperiodic conditions, and there were reduced expression levels under SD conditions, which were consistent with the observation that the number of *RFRP* neurons was higher and they were more intensive in hamsters under LD conditions compared with SD conditions (Revel *et al.*, 2008).

mRNA expression levels of *RFRP-3* in the hypothalamus or in the gonads were significantly positively correlated with the quantity of *GnRH* mRNA in the hypothalamus and serum FSH and LH concentrations in striped hamsters. These results were in line with a study that showed that *RFRP-3* could increase *GnRH* neuron activity and gonadotropin secretion in the Syrian hamster (Simonneaux and Ancel, 2012), and were not correlated with findings in sheep, in which expression of *RFRP-3* was decreased under SD conditions and increased under LD conditions, indicating that *RFRP-3* was an inhibitory factor (Dardente *et al.*, 2008; Smith *et al.*, 2008). In the Syrian (Simonneaux *et al.*, 2013), Siberian (Revel *et al.*, 2008; Ubuka *et al.*, 2012) and European (Simonneaux and Ancel, 2012) hamsters and jerboa (Janati *et al.*, 2013) considered as LD breeders, expression of *RFRP-3* was decreased under SD conditions and increased under LD conditions (Revel *et al.*, 2008). While in sheep considered as an SD breeder, expression of *RFRP-3* was increased under SD conditions and decreased under LD conditions. These results suggested that the action of *RFRP-3* on seasonal reproduction was species dependent. However, in non-photoperiodic sensitive rats, the mRNA levels of *RFRP* were not regulated by photoperiodic conditions (Revel *et al.*, 2008). Those findings indicated that whether photoperiod changes the

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expression of *RFRP-3* in the hypothalamus is based on the photoperiodic sensitivity of the mammal. In seasonally reproductive mammals, photoperiod regulates the expression of *RFRP-3*. We hypothesized that the expression levels of *RFRP-3* varied between the SD and LD mammals, and whether the expression of *RFRP-3* varied with photoperiods could be thought as a clue to photoperiod sensitivity or not.

In the present study, expression levels of *RFRP-3* in the striped hamster were positively correlated with serum FSH and LH concentrations, indicating that *RFRP-3* acts as an activator to regulate the synthesis or secretion of FSH and LH. These results did not correlate with the results showing that *RFRP-3* inhibited LH secretion in Syrian hamsters (Kriegsfeld *et al.*, 2006) and rats (Murakami *et al.*, 2008) *in vivo*, and also inhibited gonadotropin release from cultured pituitary cells in sheep (Clarke *et al.*, 2008; Sari *et al.*, 2009) and cattle (Kadokawa *et al.*, 2009). However, a central injection of *RFRP-3* to male Syrian hamsters under SD conditions could increase gonadotropin release, and 5 weeks of continuous central administration of *RFRP-3* to male Syrian hamsters under SD conditions also could reactive the reproductive axis (Simonneaux and Ancel, 2012). This indicated that *RFRP-3* was an activator under certain conditions, and was in line with our results that *RFRP-3* could increase gonadotropin release. The effect of *RFRP-3* on secretion of FSH and LH varied with species, photoperiod, environmental conditions, and even stage of the estrous cycle, therefore this complicated mechanism needs further study.

Central administration of hamster *RFRP-3* inhibited LH release in the Siberian hamster after administration under LD conditions, while *RFRP-3* stimulated LH release in the Siberian hamster after administration under SD conditions (Ubuka *et al.*, 2012). These results indicated that the effect of *RFRP-3* on the reproduction of the mammals may be photoperiod dependent. In our study, if the samples were analyzed separately in LD, MD or SD groups, the correlation between mRNA expression of *RFRP-3* in the hypothalamus with serum LH concentration was not significant. If the samples were analyzed together without considering photoperiods, mRNA expression of *RFRP-3* in the hypothalamus was significantly correlated with serum LH concentration. This difference may be due to the number of fewer samples, the small effect, the various administrated periods under different photoperiods, and so on.

For most seasonal mammalian species, the effect of photoperiod on expression of *RFRP-3* in the hypothalamus was consistent with expression levels of *RFRP-3* in the hypothalamus of the Syrian hamster, Siberian hamster and sheep, which were increased under LD and decreased under SD conditions. However, the effect of *RFRP-3* on seasonal reproduction varied between the LD and SD breeders (Simonneaux and Ancel 2012). That meant that the mechanism of action of *RFRP-3* on seasonal reproduction was complicated.

In conclusion, taken together, the present results indicated that pineal melatonin decreased mRNA expression of *RFRP-3*, and that *RFRP-3* increased *GnRH* neuron activity and the release of FSH and LH in the striped hamster. The differences in *RFRP-3* mRNA levels between male and female striped hamster was photoperiod dependent.

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Statement of ethics. The study was approved by the Animal Ethics Committee of Qufu Normal University.

Disclosure of statement. The authors declare that they have no competing interest.

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Author contributions. Huiliang Xue, Jinhui Xu and Laixiang Xu are dedicated to guarantee of the integrity of the entire study. Huiliang Xue and Jinhui Xu carried out the study concepts, definition of intellectual content, manuscript preparation and manuscript editing. Huiliang Xue, Lei Chen and Lei Zhao were involved in the study design. Huiliang Xue and Lei Chen handled the literature research. Huiliang Xue and Lei Zhao were dedicated to the studies. Huiliang Xue, Jinhui Xu, Lei Chen and Lei Zhao carried out the experimental studies. Huiliang Xue, Lei Chen and Ming Wu were involved in data acquisition and statistical analysis. Lei Chen and Lei Zhao carried out data analysis. Jinhui Xu and Lei Chen were involved in the manuscript review. All authors have read and approved this article.

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