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RESEARCH ARTICLE

# Apoptosis-mediated testicular alteration in Japanese quail (*Coturnix coturnix japonica*) in response to temporal phase relation of serotonergic and dopaminergic oscillations

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## ABSTRACT

Reproductive performance of many avian species, including Japanese quail, is reported to be modulated by specific temporal phase relation of serotonergic and dopaminergic oscillations. Accordingly, it has been shown that the serotonin precursor 5-HTP and the dopamine precursor L-DOPA given 8 h apart induce gonadal suppression and given 12 h apart lead to gonadal stimulation, while other temporal relationships were found to be ineffective. In the present study, we investigated the effects of 8- and 12-h phase relation of neural oscillations on testicular responses including expression of GnRH-I, GnIH, pro-apoptotic proteins (p53 and Bax), inactive and active executioner caspase-3, and the uncleaved DNA repair enzyme PARP-1. Testicular volume and mass decreased significantly in 8-h quail and increased in 12-h quail compared with controls. Expression of *ir*-GnIH, p53, Bax and active-caspase-3 increased and that of GnRH-I, pro-caspase-3 and uncleaved PARP-1 decreased in 8-h quail compared with controls. A TUNEL assay also confirmed testicular regression in these quail. Testes of 12-h quail exhibited significantly increased expression of GnRH-I, pro-caspase-3 and uncleaved PARP-1 compared with the control group. Our findings suggest that differential response of avian testes to 8- and 12-h phase relation of serotonergic and dopaminergic neural oscillations may be attributed to autocrine/paracrine action of GnIH expression, which is upregulated in regressed testes, leading to apoptotic changes, and downregulated in developed testes, causing apoptotic inhibition. It is concluded that specific phase relation of neural oscillations may modulate the local testicular GnRH-GnIH system and alter the apoptotic mechanism in quail testes. Moreover, these findings highlight the physiological effects of time-dependent drug delivery, including the specific time intervals between two drugs.

**KEY WORDS: Japanese quail, Testis, Neural oscillations, 5-HTP, L-DOPA, Apoptosis**

## INTRODUCTION

5-hyroxytryptophan (5-HTP, a serotonin precursor) and L-dihydroxyphenylalanine (L-DOPA, a dopamine precursor) administered daily at different intervals are used to induce a specific phase relationship between circadian serotonergic and dopaminergic oscillations as these precursors cross the blood–brain barrier, whereas serotonin and dopamine cannot cross (Bianchine, 1980). Injections of these precursor drugs, if given at 12-h intervals, stimulated and at 8-h intervals suppressed the gonadal systems in the autumn-breeding sedentary species lal munia, *Estrilda amandava* (Chaturvedi et al., 1994), and spotted munia, *Lonchura punctulata* (Chaturvedi and Prasad, 1991; Prasad and Chaturvedi, 1992, 1998), as well as summer-breeding migratory red-headed bunting, *Emberiza bruniceps* (Chaturvedi and Bhatt, 1990). In another summer-breeding but non-migratory species, the Indian weaver bird, *Ploceus phillipinus*, which lacks the characteristic post-reproductive photorefractory phase (Thapliyal and Saxena, 1964), daily administration of 5-HTP and L-DOPA at an interval of 12 h induced full breeding condition much in advance of the annual gonadal cycle, coupled with the nuptial plumage [luteinizing hormone (LH) dependent] and black bill (male hormone dependent) (Chaturvedi et al., 1997). Injections of 5-HTP and L-DOPA are thought to reset the phase of two circadian neuroendocrine oscillations with serotonergic and dopaminergic components and thereby determine seasonality (Meier et al., 1981), which has been also reported to be altered by the hormones corticosterone and prolactin given at specific intervals (Meier et al., 1971).

A large number of studies from our laboratory have reported the modulation of reproductive performance including photo-sexual responses by the specific temporal phase relation of serotonergic and dopaminergic oscillations. Accordingly, the serotonin precursor 5-HTP and the dopamine precursor L-DOPA given 8 h apart induce gonadal suppression, and if given 12 h apart, lead to gonadal stimulation, while other relationships (0, 4, 16 and 20 h apart) are found to be ineffective (Chaturvedi et al., 1991, 2006; Bhatt and Chaturvedi, 1992). Daily injections of 5-HTP and L-DOPA in specific temporal relation also determine seasonality (reproductive responsiveness to temperature) in the teleost fish *Fundulus grandis* (Emata et al., 1985), and have also established the reproductive and fattening effect in red-headed bunting (Chaturvedi and Bhatt, 1990). The hypothalamic reproduction regulatory peptides [gonadotropin-releasing hormone (GnRH)–gonadotropin-inhibitory hormone (GnIH) system] and hypophyseal hormones [LH, follicle-stimulating hormone (FSH) and prolactin] in combination control and/or regulate steroidogenesis as well as spermatogenesis. GnRH I and II serve as gonado-stimulatory peptides (GnRH I stimulates the synthesis and release of LH/FSH; GnRH II stimulates the reproductive behaviour; Ubuka et al., 2013a) and GnIH serves as a gonado-inhibitory hypothalamic peptide (see review Ubuka et al., 2013b). The presence of a gonadal GnRH–GnIH system provides further support for the local gonadal actions of these two neuropeptides. Bentley et al. (2008) investigated and confirmed the presence of GnIH and/or its receptor in the gonads of avian species. They also clearly demonstrated that GnIH and GnIH-R are expressed in the quail gonads and suggested that GnIH acts directly on the avian reproductive system (Bentley et al., 2008). It is also reported that *in vivo* administration of GnIH decreases seminiferous tubule diameter and the number of germ cells in quail via suppression of

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pituitary gonadotropin synthesis and release (Ubuka et al., 2006). The presence of GnIH-R has been detected in the testes and ovaries of several vertebrate species, including birds (McGuire and Bentley, 2010), and putative GnIH binding sites have been demonstrated in the granulosa cell layer by receptor fluorography in birds (Bentley et al., 2008). GnIH-R mRNA has been localized in the theca and granulosa cells, with significantly lower levels found in preovulatory follicles than in smaller growing follicles (Maddineni et al., 2008). Singh et al. (2011) has demonstrated the direct inhibitory effect of GnIH on ovarian steroidogenesis in mice. Anjum et al. (2014) showed the direct effect of RFRP3 *in vitro* and *in vivo* on testicular steroidogenesis in mice, and different doses of RFRP3 resulted in a significant decrease in testosterone synthesis in mice testes. GnIH modulates testosterone production and spermatogenesis directly as well as indirectly through its paracrine and endocrine function, mediated via its receptors present in the Sertoli, Leydig and germ cells (Bentley et al., 2008).

The temporal phase relation of serotonin and dopamine and other regulatory factors such as photoperiod may modulate the two neuropeptides GnRH and GnIH at the hypothalamic level, affecting the HPG axis, and subsequently induce testicular stimulation or regression. Alternatively, it is also possible that the testicular GnRH–GnIH system modulates testicular growth locally (Tsutsui et al., 2010; Anjum et al., 2014). However, a direct effect of photoperiod or other treatments (except intra-testicular injections) on the testicular activity bypassing the central/HPG axis seems unlikely. However, crosstalk between the hypothalamic and testicular GnRH–GnIH system may play a crucial role in the modulation of gonadal performance, especially because of the presence of GnIH receptors on the gonad (Bentley et al., 2008; Ubuka et al., 2013b).

Apoptosis is a programmed cell death that is broadly divided into an initiation phase, a signalling phase and an execution phase (Steller, 1995). Two major apoptotic pathways can induce the cell death – the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway – and molecules of one pathway can influence the other. The testicular cell number in the seminiferous tubules is maintained by a dynamic balance between cell proliferation and apoptotic cell death (Russell et al., 2002). Both spontaneous and increased cell death due to triggering stimuli such as increased GnIH, deprivation of testosterone and gonadotrophins, etc., occur via apoptosis. It is also well established that the seasonal testicular regression in photoperiodic birds is mediated by apoptosis (Young and Nelson, 2001; Young et al., 2001) and testicular apoptosis is mediated through the GnIH in quail (Ubuka et al., 2006). We hypothesized that the testicular regression in 8-h quail may be due to testicular apoptosis.

The nuclear transcription factor p53 (tumor suppressor protein-53) has also been found to play a critical role in apoptosis. It is also reported that p53, with hundreds of downstream targets, plays a potential role in DNA repair, cell-cycle regulation and cell death (Levine and Oren, 2009). Much literature is available focusing on the pathways that connect stresses such as DNA damage to p53 activation. p53 simply adjusts the sensitivity of a cell to respond to programmed cell death through transcriptional regulation of pro-apoptotic [Bcl-2-associated X protein (Bax)] and anti-apoptotic (Bcl-2) proteins. This protein (p53) may also directly impact Bcl-2 (B-cell lymphoma 2/Bcl-2 family of regulator proteins) activity as part of a transcription-independent program of cell death. In this process, cytoplasmic p53 binds to pro-apoptotic Bcl-2-family proteins, leading to permeabilization of mitochondria and apoptosis (Hemann and Lowe, 2006). It is well established that p53 alters the Bax/$Bcl_2$ ratio and promotes apoptosis through Bax activation (Bivik et al., 2006; Green, 2006). The activated Bax acts on inactive caspases (active-cysteine-aspartic acid proteases)/pro-caspases and converts them into active caspases. Inactive pro-caspase-3, upon activation, results in active-caspase-3, which cleaves its substrate poly (ADP) ribose polymerase-1 (PARP-1). PARP is a nuclear enzyme responsible for DNA repair. The active-caspase-3 cleaves PARP-1 and subsequently stalls the DNA repair and cell-cycle progression (Liu et al., 1996). We further hypothesized that the testicular regression in 8-h quail is mediated through the GnIH-induced, p53-dependent, Bax-mediated apoptotic pathway.

GnIH-mediated testicular regression is also thought to be mediated through apoptosis, but to explore the underlying molecular pathway/mechanism, we hypothesize that testicular apoptosis is GnIH induced, p53 dependent and Bax mediated. No report is available to date related to apoptosis in avian testes with special reference to simulated gonadal regression/stimulation. Hence, the present study was designed to investigate the alterations in the expression of GnIH, pro-apoptotic proteins (p53 and Bax), as well as pro-caspase-3, active-caspase-3 and uncleaved PARP-1 in stimulated (hyperactive) and suppressed/regressed gonads (testes) of 12-h and 8-h quail. To test this hypothesis, we investigated alterations in the expression of GnRH-I, GnIH, pro-apoptotic proteins (p53 and Bax), pro-caspase-3, active-caspase-3 and uncleaved PARP-1 in control, 8-h and 12-h testes. In combination, the pro-apoptotic proteins, pro-caspase-3, active-caspase-3 and uncleaved PARP-1 play a crucial role in the execution of apoptosis. In the present study, we elucidate the mechanistic pathways through which regressed testes in the 8-h quail exhibit GnIH-induced, p53-dependent, Bax-mediated testicular apoptosis, while reversal of this pathway promotes testicular hypertrophy in 12-h quail.

## MATERIALS AND METHODS

Sexually immature 3-week-old male Japanese quail raised under a long daylength photoperiod (16 h:8 h light:dark) were purchased from the Central Avian Research Institute, Izzatnagar, India. Quail were weighed and divided randomly into three groups ($n$=8 per group). The specific phase relation of circadian serotonergic and dopaminergic activity was induced as follows according to Chaturvedi and Bhatt (1990): (1) group I (control) received normal saline twice daily for 13 days (because two normal saline injections at either 8- or 12-h intervals had a similar effect, only one control was taken into account); (2) group II (8-h) received 5-HTP at 08:00 h and L-DOPA at 16:00 h, establishing an 8-h relationship between the two injections; and (3) group III (12-h) received 5-HTP at 08:00 h and L-DOPA at 20:00 h, establishing a 12-h relationship.

All injections (normal saline, 5-HTP and L-DOPA, 5 mg per 100 g body mass) were given intraperitoneally in 0.1 ml solution for 13 days under continuous light conditions to avoid possible photoperiodic interference. Thereafter, all the three groups were shifted to a long daylength (16 h:8 h light:dark) photoperiod (lights on at 06:00 h and off at 22:00 h) and maintained for 30 days (post-treatment), when the experiment was terminated (total period 13+30=43 days). The experiment was conducted in accordance with institutional practice and within the framework of the revised Animals (Scientific Procedures) Act of 2002 of the Government of India on animal welfare. This study is performed under the regulation of Institutional Animal Ethical Committee, Faculty of Science, Banaras Hindu University. The experiment was repeated thrice with the same result, and a single representative photomicrograph was provided for the immunohistochemistry of each antibody and for the TUNEL assay.

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**Table 1.** Mean (±s.e.m.) integrated optical density value for each antigen considered as arbitrary unit thresholds

| Antibody | Control | 8-h | 12-h |
|---|---|---|---|
| GnRH-I | 257.220±9.028 (moderate) | 38.679±5.002 (weak) | 523.865±23.379 (intense) |
| GnIH | 11.504±1.713 (moderate) | 91.561±4.859 (intense) | 2.018±0.365 (weak) |
| p53 | 41.739±2.847 (moderate) | 128.240±3.659 (intense) | 21.206±4.033 (weak) |
| Bax | 42.961±2.541 (moderate) | 105.551±3.017 (intense) | 28.352±2.555 (weak) |
| Pro-caspase-3 | 74.586±4.650 (moderate) | 29.832±1.189 (weak) | 113.704±2.450 (intense) |
| Active-caspase-3 | 11.691±0.339 (moderate) | 78.129±2.058 (intense) | 1.207±0.063 (weak) |
| PARP-1 | 97.041±2.557 (moderate) | 51.988±3.271 (weak) | 139.819±3.739 (intense) |

### Serum collection and testosterone assay

Birds were weighed individually, and blood samples (~1 ml) were obtained from the wing vein of anesthetized birds and kept at room temperature for 30 min and then centrifuged at 1254 ***g*** at 4°C for 15 min. The supernatant was collected as serum and frozen at −20°C until used for the testosterone assay.

For serum testosterone levels, an enzyme immunoassay was performed using a commercial RH-353-DS-EIA-Steroid-Testosterone kit (DSI, Saronno, Italy) following the manufacturer's protocol. The antiserum used in the assay was specific for testosterone. The cross-reactivity of the assay was 0.056% with progesterone, 0.004% with cortisol, 0.005% with estradiol, 4.8% with dihydrotestosterone, 3.6% with androstenedione, 0.048% with androsterone, 0.004% with cortisone, 0.002% with estriol and 0.007% with estrone. The analytical sensitivity of the assay was 0.0576 ng ml$^{-1}$. The intra-assay coefficient of variation (CV) was 5.6% whereas the inter-assay CV was 7.1%. The accuracy for this assay was 99%.

### Testes fixation and measurement

Quail were anesthetized with sodium pentobarbital (3–4 mg 100 g$^{-1}$ body mass), followed by whole body perfusion. Quail were perfused transcardially with phosphate buffered saline (PBS) followed by Zamboni's fixative (4% paraformaldehyde in 0.1 mol l$^{-1}$ sodium phosphate buffer; pH 7.4). The length and width of the left and right testes were measured *in situ* with dial calipers. Testicular volume of both the testis was calculated using Bissonett's formula $4/3\pi ab^2$ (where $a$=½ of the long axis and $b$=½ of the short axis) and averaged (Chaturvedi and Bhatt, 1990; Chaturvedi et al., 1993). Both the testes were excised and weighed. Testes were then post-fixed in fresh Zamboni's fixative. Twenty-four hours after fixation, testes were dehydrated in a graded series of alcohol, treated with xylene and embedded in paraffin wax. Sections 6 µm thick were cut using a Lieca RM2125 RT rotatory microtome (Leica Biosystems, Nussloch, Germany) and processed for immunohistochemistry of GnRH-I (generous gift from Dr R. Benoit, Montreal General Hospital, McGill University Health Centre), GnIH, full-length p53 (sc 6243, FL-393), Bax (sc 6236), pro-caspase-3 (sc 7148), active-caspase-3 (CST # 9661) and uncleaved/full-length PARP-1 (sc 8007) as follows using rabbit raised polyclonal antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Danvers, MA, USA).

### Immunohistochemistry

We carried out immunohistochemistry of GnIH using the antiserum raised against quail GnIH (Tsutsui et al., 2000), which cross-reacted not only quail GnIH but also RFRP-3, a mammalian GnIH (or a mammalian GnIH ortholog) (Ubuka et al., 2012). Specificity of immunolabeling was verified by pre-adsorbing a working dilution of the primary antiserum with a saturating concentration of the antigen (10 µg synthetic peptide ml$^{-1}$) as a control.

Immunohistochemistry for GnRH-I (dilution 1:2500), full-length p53 (sc 6243, F-393, dilution 1:50), Bax (sc 6236, dilution 1:50), pro-caspase-3 (sc 7148, dilution 1:50) and uncleaved/full-length PARP-1 (sc 8007, dilution 1:50) was performed in a two-step

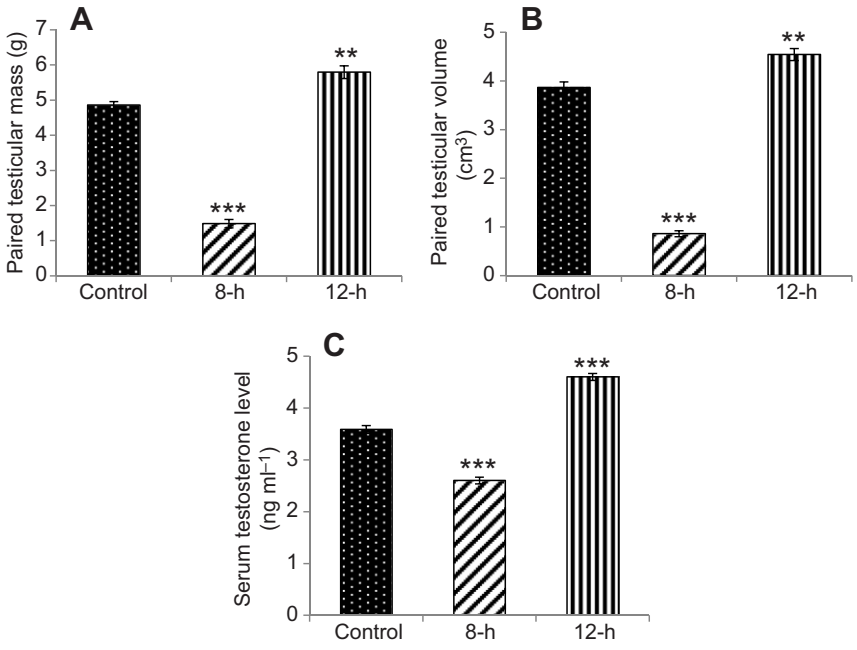


**Fig. 1. Effect of 8-h and 12-h phase relation of 5-HTP and L-DOPA on testicular mass, volume and serum testosterone.** (A) Paired testicular mass, (B) paired testicular volume and (C) serum testosterone level of control, 8-h and 12-h quail. Experimental quail were administered with 5-HTP and L-DOPA at an interval of 8 or 12 h. The control group received two daily injections of normal saline. Testicular mass, volume and serum testosterone level increased significantly in 12-h quail but decreased significantly in 8-h quail compared with controls. Values are expressed as means±s.e.m. ($n$=8). Asterisks indicate a significant difference from controls: **$P$<0.01, ***$P$<0.001.



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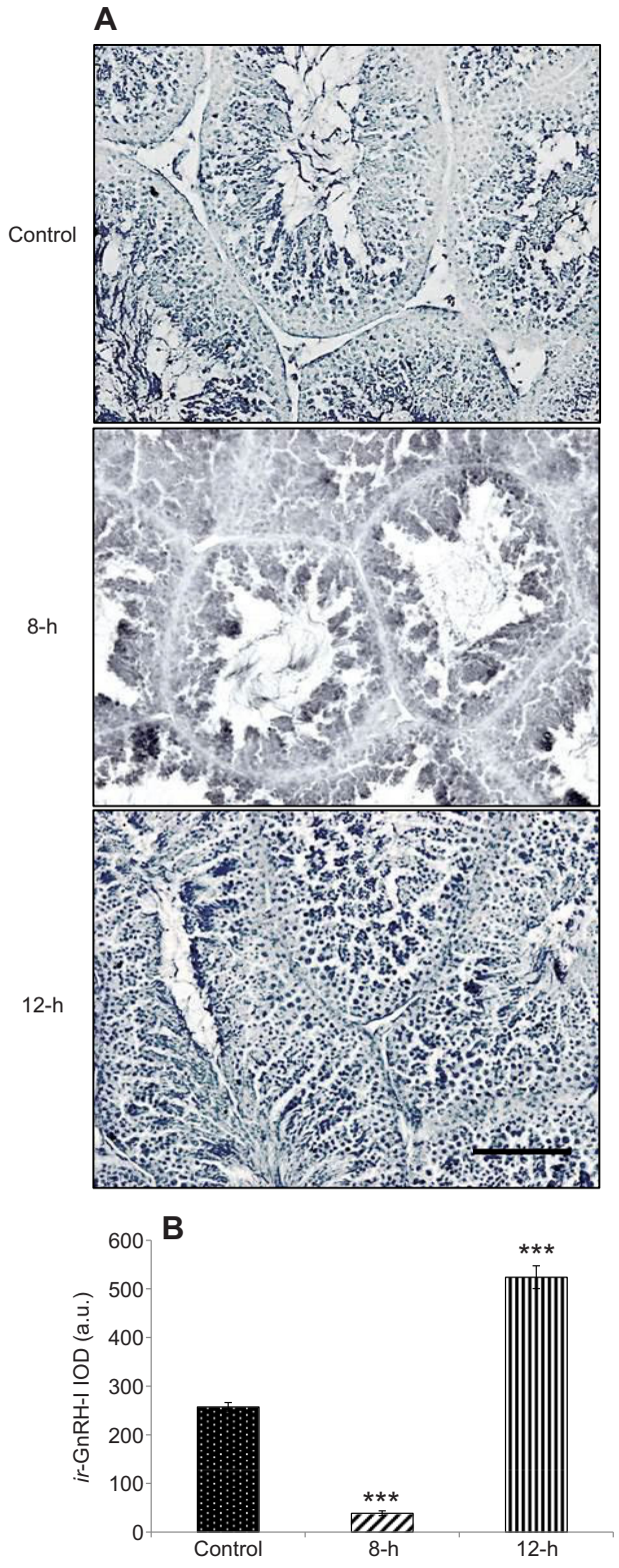


**Fig. 2. Expression of GnRH-I in control, 8-h and 12-h quail testes.** (A) Immunohistochemistry and (B) integrated optical density (IOD) of GnRH-I in testes of control, 8-h and 12-h quail. The 12-h quail testes showed very strong/intense immunoreactivity for GnRH-I in different stages of germ cells in the seminiferous tubules and Leydig cells. Control quail testes showed moderate immunoreactivity in a few cell stages only, while weak GnRH-I immunoreactivity was observed in 8-h quail testes. Scale bar, 100 μm. Values are expressed as means±s.e.m. ($n$=6). Asterisks indicate a significant difference from controls: ***$P$<0.001.

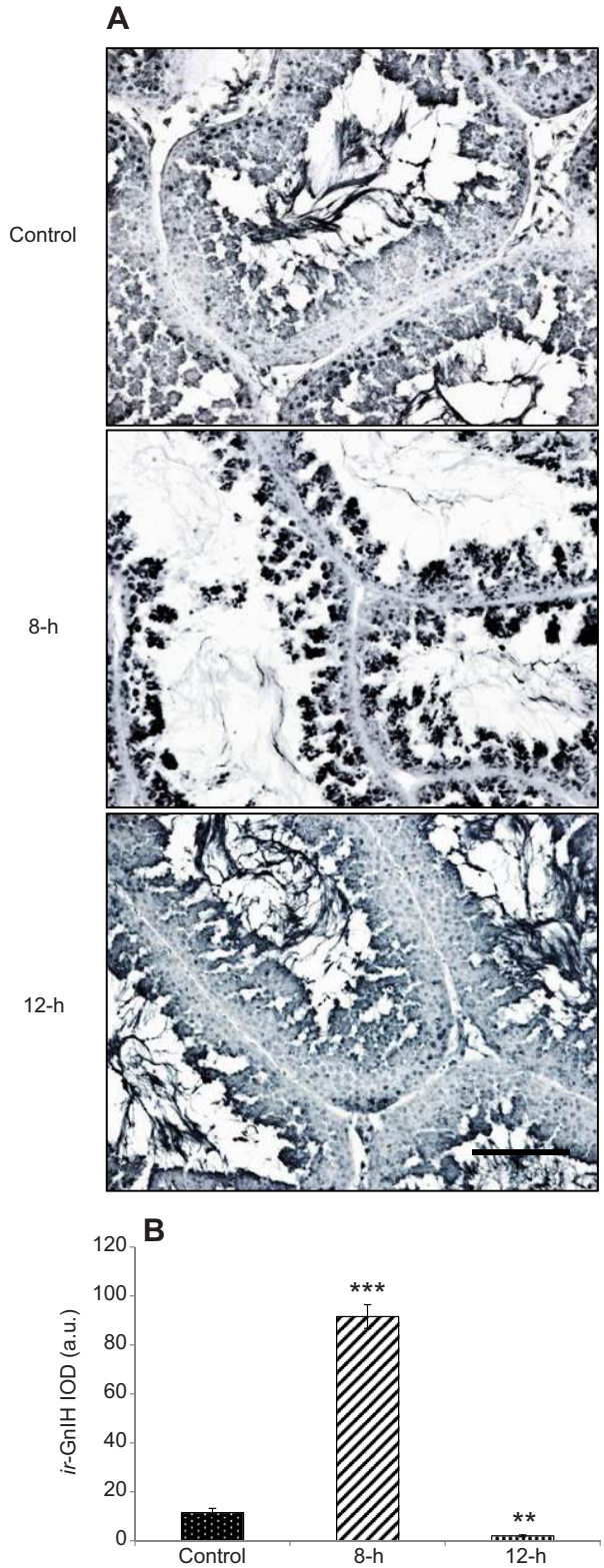


**Fig. 3. Expression of GnIH in control, 8-h and 12-h quail testes.** (A) Immunohistochemistry and (B) IOD of GnIH in testes of control, 8-h and 12-h quail. The control group showed moderate immunostaining in few spermatogonial cells of some tubules only. Very intense immunostaining of GnIH was observed in 8-h quail testes compared with controls. In contrast, 12-h quail showed weak immunoreactivity for GnIH in very few inner stages of germ cells of some seminiferous tubules. Scale bar, 100 μm. Values are expressed as means±s.e.m. ($n$=6). Asterisks indicate a significant difference from controls: **$P$<0.01, ***$P$<0.001.



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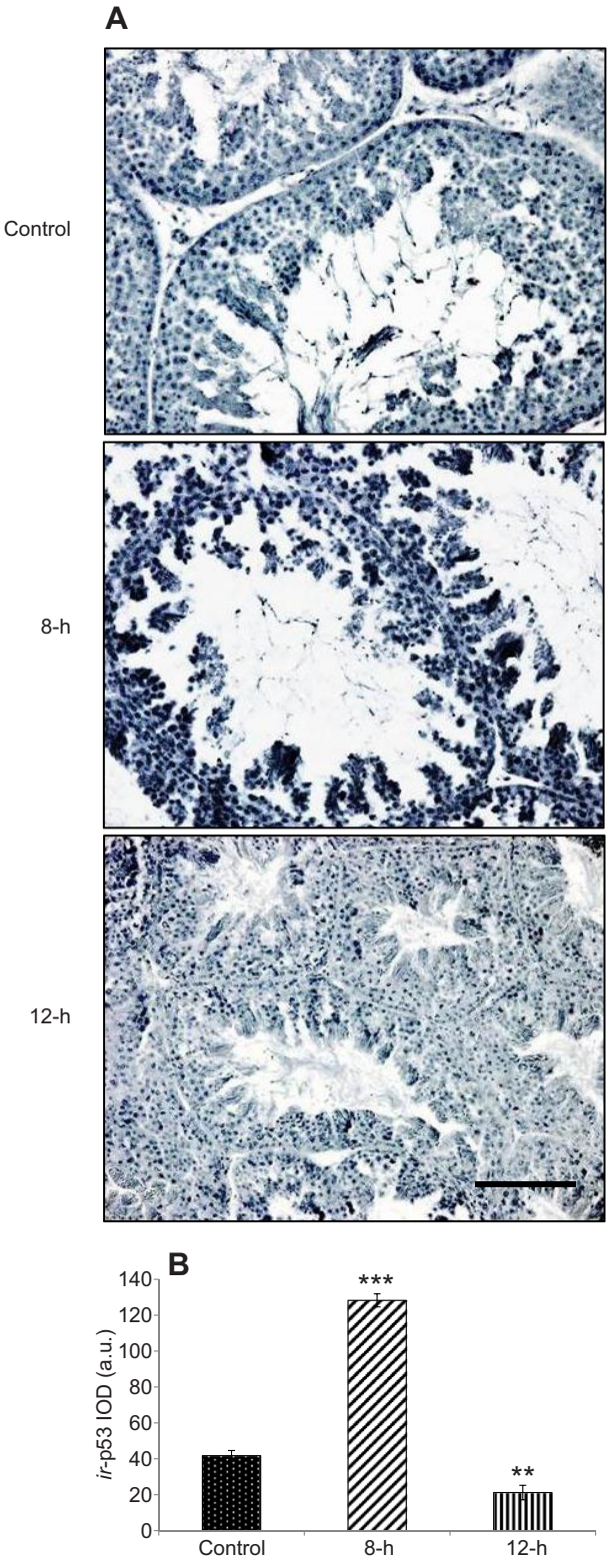


**Fig. 4. Expression of p53 in control, 8-h and 12-h quail testes.** (A) Immunohistochemistry and (B) IOD of full-length p53 in testes of control, 8-h and 12-h quail. Very intense immunostaining of p53 was observed in the testes of 8-h quail compared with the control group, which exhibited moderate immunoreactivity in the few spermatogonial and inner cell stages only. In contrast, 12-h quail testes showed weak p53 immunoreactivity mainly in the spermatogonial cells of the seminiferous tubule. Scale bar, 100 μm. Values are expressed as means±s.e.m. ($n$=6). Asterisks indicate a significant difference from controls: **$P$<0.01, ***$P$<0.001.

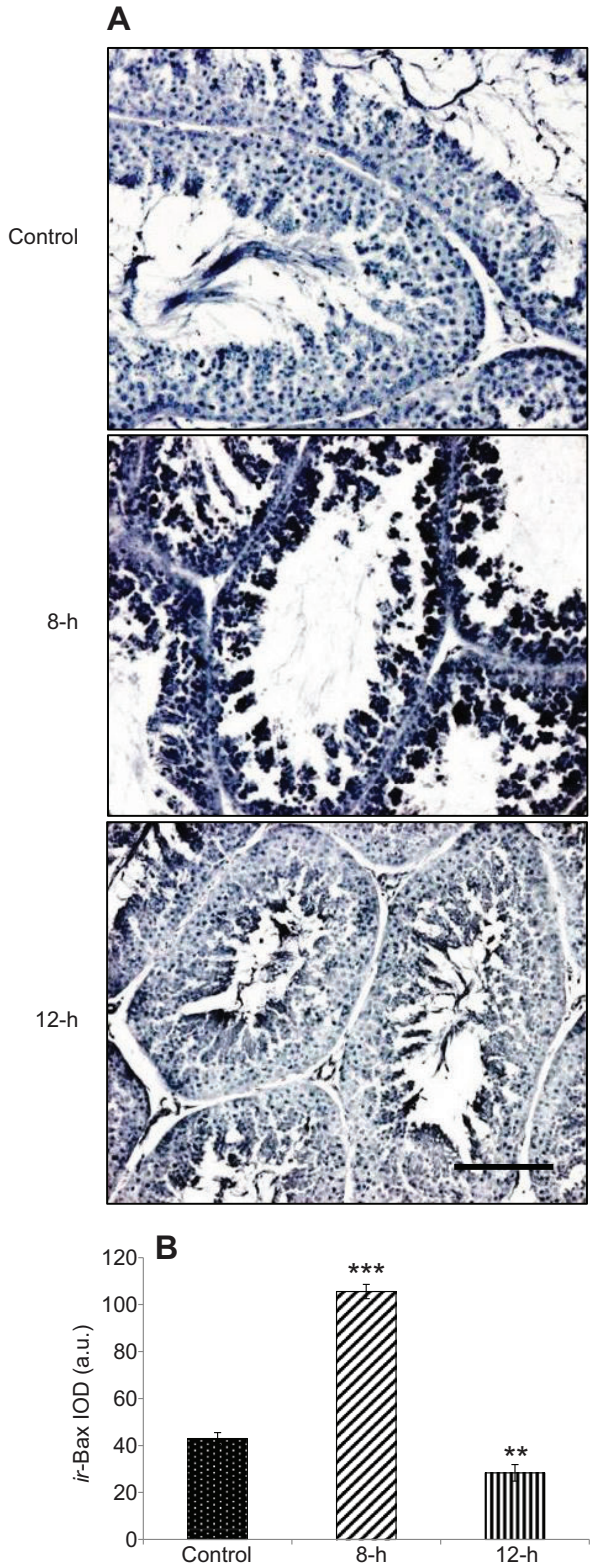


**Fig. 5. Expression of Bax in control, 8-h and 12-h quail testes.** (A) Immunohistochemistry and (B) IOD of Bax in testes of control, 8-h and 12-h quail. The 8-h quail testes showed highly intense immunostaining of Bax compared with control and 12-h quail testes. Control group testes showed weak Bax immunoreactivity in the inner germ cell stages of the seminiferous tubule while 12-h quail testes revealed still weaker immunoreactivity in the spermatogonial cells of the seminiferous tubule. Scale bar, 100 μm. Values are expressed as means±s.e.m. ($n$=6). Asterisks indicate a significant difference from controls: **$P$<0.01, ***$P$<0.001.



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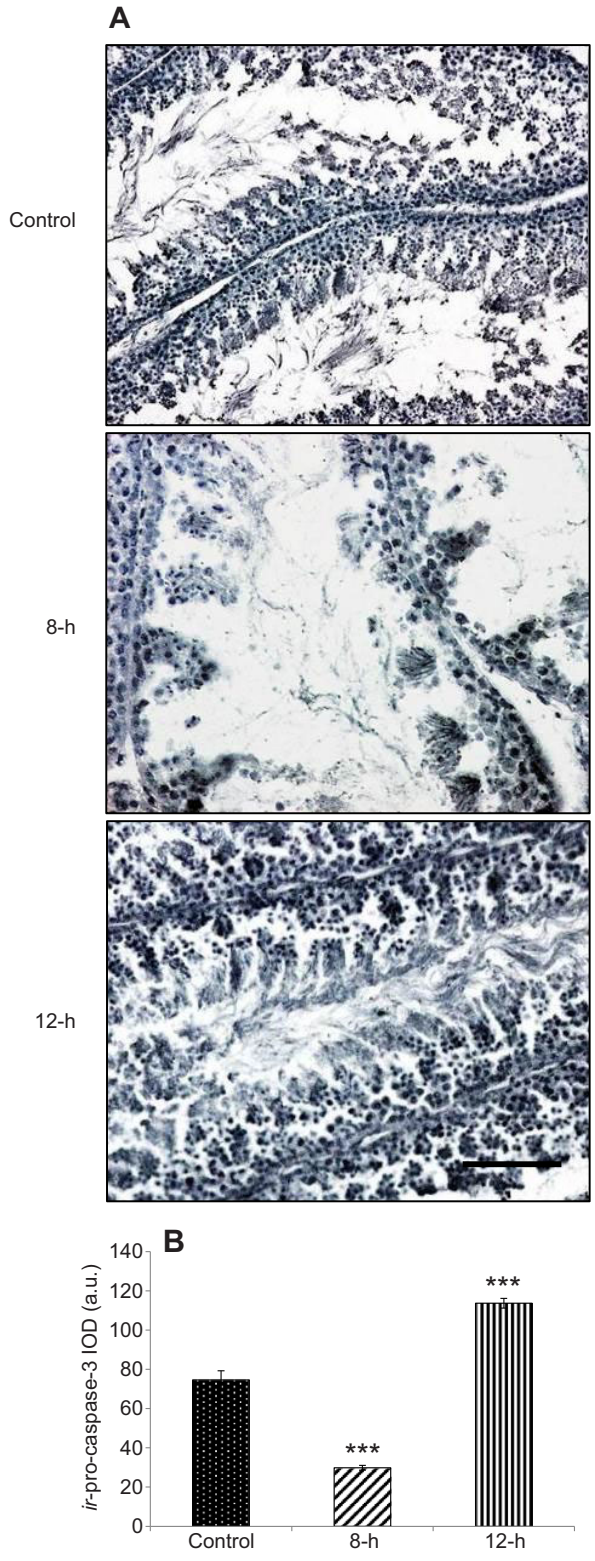


**Fig. 6. Expression of pro-caspase-3 in control, 8-h and 12-h quail testes.** (A) Immunohistochemistry and (B) IOD of pro-caspase-3 in testes of control, 8-h and 12-h quail. Control group testes revealed strong pro-caspase-3 immunoreactivity, and 12-h quail testes showed very strong/intense immunoreactivity in all the spermatogonial and inner germ cell stages of the seminiferous tubule. However, weak pro-caspase-3 immunoreactivity was observed in the spermatogonial cells of the seminiferous tubule of 8-h quail testes. Scale bar, 100 μm. Values are expressed as means±s.e.m. ($n$=6). Asterisks indicate a significant difference from controls: ***$P$<0.001.

procedure using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). In the first step, after initial deparafinization in xylene and rehydration in a graded series of alcohol, different sections of testes were incubated with antibodies with specificity to the GnIH, p53, Bax, pro-caspase-3 and uncleaved PARP-1 antibodies (purchased from Santa Cruz Biotechnology) for 24 h in a humid chamber (Shahin et al., 2014). The second step was incubation with horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin for 30 min. Finally, diaminobenzidine hydrochloride (SIGMA FAST DAB with metal enhancer, cat. no. D0426, Sigma-Aldrich, St Louis, MO, USA) was used as a chromogen molecule for the immunological detection. Sections were dehydrated through an ascending ethanol series, cleared in xylene and then mounted using DPX. The sections were viewed under a microscope (Axioskop 2 Plus; Carl Zeiss, Oberkochen, Germany), and images were captured with an Axioskop HRcam camera. A negative control, in which the tissue section was incubated with antibody diluent, without the primary antibody included, was processed for each antibody used.

### Image analysis

To measure the signal density of immunopositive testicular cells (GnIH, p53, Bax, pro-caspase-3 and uncleaved PARP-1), light microscopic examinations were made with a Carl Zeiss Axioskop 2 plus microscope with HRcam. Images of the testes of quail of all the groups were captured at the same magnification and stored as TIF files for immunohistochemistry signal analysis.

Each file was imported using ImageJ software (ImageJ 1.48, National Institutes of Health, Bethesda, MA, USA). Using the software's tool, integrated optical density (IOD) of all the testicular cells showing positive signals were measured. The IOD values (relative units) in the testes were averaged (four sections per testis and six testes per group) to determine signal density.

Average IOD values of testicular sections from each group were considered as arbitrary unit thresholds for each antigen (Table 1). Statistics were performed on the average arbitrary unit values (means±s.e.m.) and on the basis of the difference in the level of significance ($P$<0.05), the testicular section IOD was labeled or classified as intense, moderate or weak. One-way ANOVA followed by *post hoc* analysis (Dunnett's T3) was used to determine the significance among the three different groups. A $P$-value of <0.05 was considered significant. Data were analyzed using the statistical software package Statistical Analysis System (SPSS Statistics 17.0, IBM, Armonk, NY, USA).

### Active-caspase-3 and TUNEL analysis using confocal microscopy

Immunofluorescence for cleaved caspase-3 was performed in a two-step procedure. In the first step, testis section slides were processed for initial deparaffinization in xylene and rehydration in graded series of alcohol. Antigen retrieval was performed using 1 mol $l^{-1}$ citrate buffer (pH 6) in a microwave oven at 1000 W (3–4 min). Tissue sections were then treated with blocking solution for 2 h at room temperature [5% heat-inactivated goat serum (HIGS)] and sections were then incubated with rabbit anti-cleaved caspase-3 (Asp-175) polyclonal antibody (1:100) (CST#9661; Cell Signaling Technology) for 24 h in a humid chamber. In the second step, sections were washed with TBS (3×5 min) and then incubated with goat anti-rabbit IgG H&L (1:200) (conjugated with TRITC, ab6718, Abcam, Cambridge, MA, USA) for 3 h at room temperature in the dark. After

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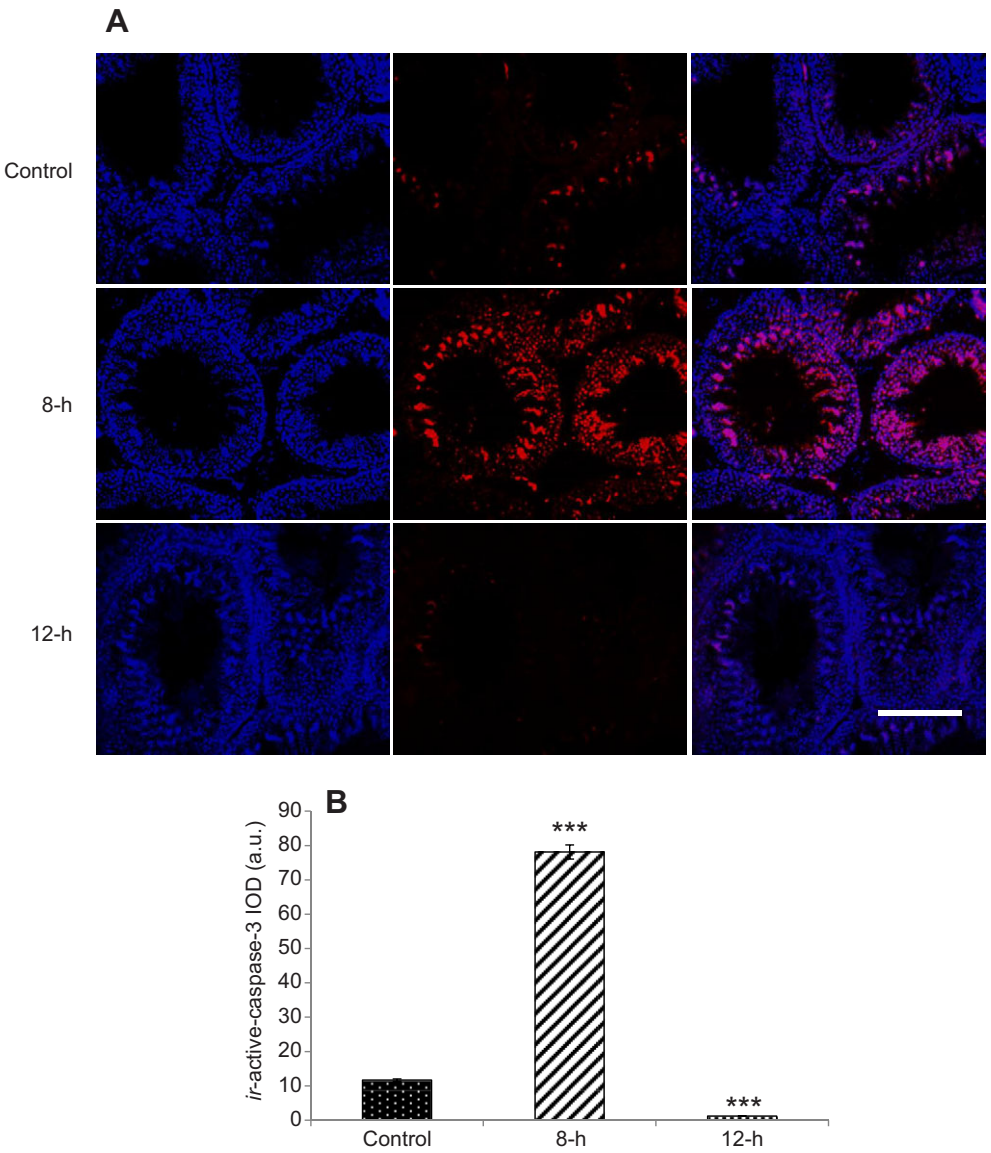


**Fig. 7. Expression of active-caspase-3 in control, 8-h and 12-h quail testes.** (A) Immunofluorescence and (B) IOD of cleaved/active caspase-3 in testes of control, 8-h and 12-h quail. Control group testes showed very weak cleaved/active caspase-3 immunoreactivity restricted to very few cells compared with the testes of 8-h and 12-h quail, which exhibited very weak or no immunoreactivity for cleaved/active caspase-3 in the spermatogonial cells of the seminiferous tubules. Scale bar, 100 μm. Column 1, DAPI; column 2, active-caspase-3; column 3, merged image (DAPI+active-caspase-3). Values are expressed as means±s.e.m. ($n$=6). Asterisks indicate a significant difference from controls: ***$P$<0.001

incubation, sections were again washed with TBST (3×5 min.) and two drops of the fluorescent medium (0.5% *N*-propyl gallate +20 mmol $l^{-1}$ Tris in 90% glycerol+DAPI) were applied to the sections. Coverslips were applied and then sealed with nail polish after ensuring the spread of the mounting medium over all the sections without any bubble formation. A Zeiss LSM510 Meta laser-scanning confocal microscope with a Plan-Apo 20.0×, 1.4-NA oil immersion objective was used to observe the sections, and the images were collected using LSM 510 Meta software. Nuclei were counterstained with DAPI (1 μg 10 $ml^{-1}$ PBS).

Apoptotic fragmentation of DNA in histological sections of quail testes was evaluated by TUNEL (terminal deoxynucleotide transferase dUTP nick end labeling) analysis according to the procedure of the kit (cat. no. A23210; APO-BrdUTM TUNEL Assay Kit, Invitrogen). Final detection of BrdU incorporation at DNA break sites was achieved through Alexa Fluor 488 dye-labeled anti-BrdU antibody. Standard protocols for paraffin sections were followed (Grataroli et al., 2002).

A Zeiss LSM510 Meta laser scanning confocal microscope with a Plan-Apo 20.0×, 1.4-NA oil immersion objective was used to observe the TUNEL-positive cells in the quail testicular sections, and the images were collected using LSM 510 Meta software. Final detection of BrdU incorporation at DNA break sites was achieved through Alexa Fluor 488 dye-labeled anti-BrdU antibody. Nuclei were counterstained with propidium iodide (PI) (10 μg $ml^{-1}$ PBS).

Immunofluorescence of cleaved/active caspase-3 and TUNEL-positive cells in quail testes of all the groups were captured at the same magnification and stored as TIF files using LSM 510 Meta software. Each file was imported using ImageJ 1.48. Using the software's tool, the IOD of all of the testicular cells showing positive signals was measured. The IOD values (relative units) in the testes were averaged (four sections per testis and six testes per group) to determine signal density. On the basis of intensity of signals, the terms intense, moderate and weak were applied.

### Statistical analysis

All data are presented as means±s.e.m. For statistical analysis, one-way ANOVA followed by *post hoc* analysis (Dunnett's T3) was used to determine the significance among the three different groups. A $P$-value of <0.05 was considered as significant. Data were analyzed using SPSS software.

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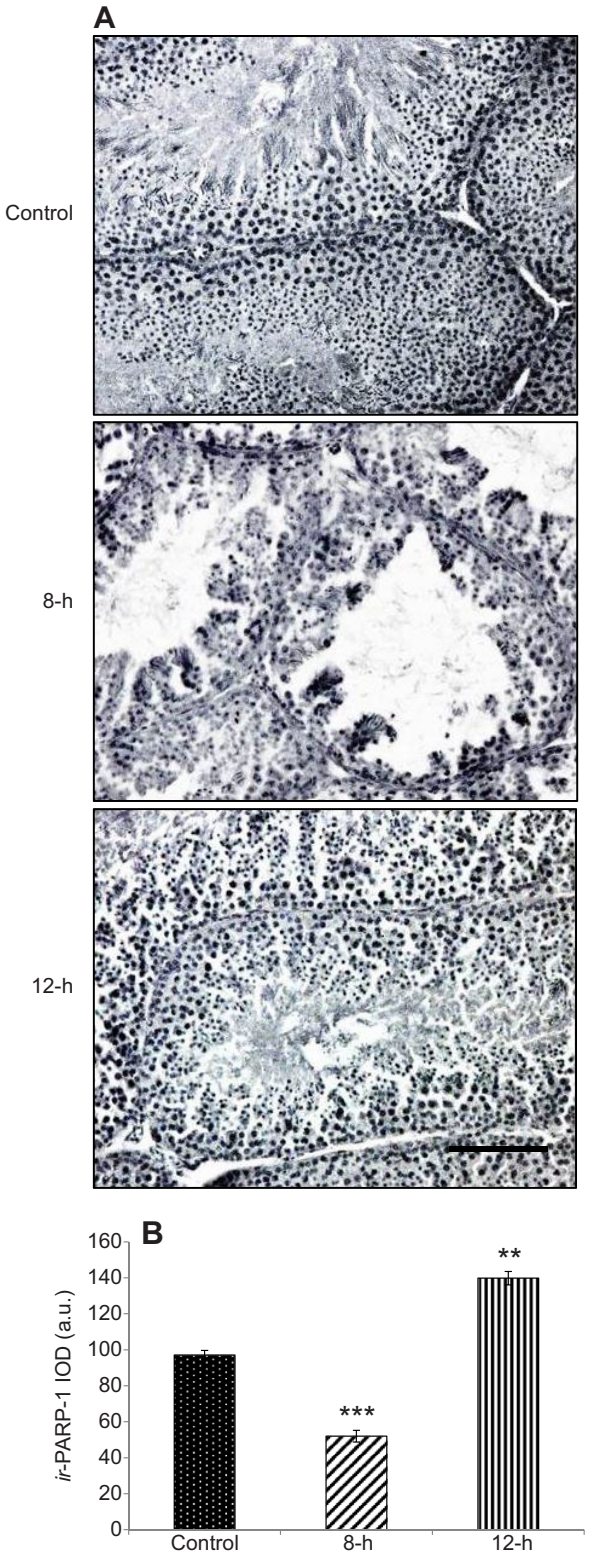


**Fig. 8. Expression of full-length PARP-1 in control, 8-h and 12-h quail testes.** (A) Immunohistochemistry and (B) IOD of uncleaved/full-length PARP-1 in the testes of control, 8-h and 12-h quail. Very weak uncleaved/full-length PARP-1 immunoreactivity was observed in the testes of 8-h quail restricted to very few cells compared with control and 12-h group quail testes, which exhibited very strong/intense immunoreactivity for uncleaved/full-length PARP-1 in the spermatogonial cells as well as inner germ cell stages of the seminiferous tubules. Scale bar, 100 μm. Values are expressed as means±s.e.m. ($n$=6). Asterisks indicate a significant difference from controls: **$P$<0.01, ***$P$<0.001.

## RESULTS

Paired testicular weight ($F_{2,21}$=269.634; Fig. 1A) and volume ($F_{2,21}$=334.461; Fig. 1B) decreased significantly in 8-h ($P$<0.001) and increased significantly in 12-h quail ($P$<0.01) compared with the control group. Serum testosterone increased significantly in 12-h quail ($P$<0.001) and decreased significantly in 8-h quail ($P$<0.001) compared with the control group ($F_{2,21}$=209.583; Fig. 1C).

To test the hypothesis of whether the induced gonadal stimulation in 12-h quail and the induced regression in 8-h quail occur via the prevention and induction of testicular p53-dependent, Bax-mediated apoptosis, respectively, we investigated the testicular expression of GnRH-I (Fig. 2), GnIH (Fig. 3), pro-apoptotic proteins (p53 and Bax) (Figs 4, 5), pro-caspase-3 (Fig. 6), active-caspase-3 (Fig. 7) and uncleaved PARP-1 (Fig. 8) in all three groups.

The testicular expression of GnRH-I ($P$<0.001, $F_{2,15}$=271.213; Fig. 2) increased significantly and that of GnIH ($P$<0.01, $F_{2,15}$=272.05; Fig. 3) decreased significantly in 12-h quail testis. In contrast, in 8-h quail, GnRH-I expression decreased significantly ($P$<0.001, $F_{2,15}$=271.213; Fig. 2) and GnIH expression increased significantly ($P$<0.001, $F_{2,15}$=272.05; Fig. 3). It may be the case that these two neuropeptides may, in combination, act on testicular development, maintenance and steroidogenesis (Ubuka et al., 2006, 2008, 2013a,b).

Compared with the control group, 12-h quail testes showed weak immunostaining for GnIH (Fig. 3), p53 ($P$<0.01, $F_{2,15}$=256.355; Fig. 4), Bax ($P$<0.01, $F_{2,15}$=228.405; Fig. 5) and active-caspase-3 ($P$<0.001, $F_{2,15}$=1314.00; Fig. 7), while the 8-h quail testes showed intense immunoreactivity ($P$<0.001) for these peptides/molecules, suggesting 8-h phase relation of serotonergic and dopaminergic oscillations induced p53-mediated apoptosis of 8-h quail testicular cells. Contrarily, intense immunostaining compare with the control was observed for pro-caspase-3 ($P$<0.001, $F_{2,15}$=181.936; Fig. 6) and uncleaved/full-length PARP-1 ($P$<0.01, $F_{2,15}$=110.980; Fig. 8) in 12-h quail testes, while 8-h quail testes revealed moderate and weak immunostaining for pro-caspase-3 ($P$<0.001, $F_{2,15}$=181.936; Fig. 6) and uncleaved PARP-1 ($P$<0.001, $F_{2,15}$=110.980; Fig. 8), respectively, in the testicular cells.

The number of TUNEL-positive cells (bright green spots) increased dramatically in the 8-h quail testes ($P$<0.001) compared with the control group, while 12-h quail testes showed lower numbers of TUNEL-positive cells compared with the control group ($P$<0.001). Nuclei were stained with PI ($F_{2,15}$=1855.00; Fig. 9).

## DISCUSSION

In the present study, intense immunoreactivity of GnIH, p53, Bax and active-caspase-3 was observed in the regressed testes of 8-h quail, unlike in the active testes of control and hyperactive/hypertrophied testes of 12-h quail. This indicates apoptotic changes in the regressed testes along with increased expression of GnIH and weak immunoreactivity of GnRH-I, pro-caspase-3 and uncleaved/full-length PARP-1. Previous studies have shown that the suppression of hypothalamic GnRH system as well as the potentiation of GnIH may lead to the suppression of pituitary LH/FSH release and also suppresses endogenous testosterone production by the Leydig cells, resulting in germ cell apoptosis (Ubuka et al., 2006, 2008, 2013a,b). As yet, however, the molecular mechanism by which germ cells undergo apoptosis is not understood. The present study, through the TUNEL assay as well as the pro-apoptotic proteins (p53 and Bax), pro-caspase-3, active-caspase-3 and uncleaved PARP-1, shows the underlying molecular mechanism of testicular suppression/regression following injections

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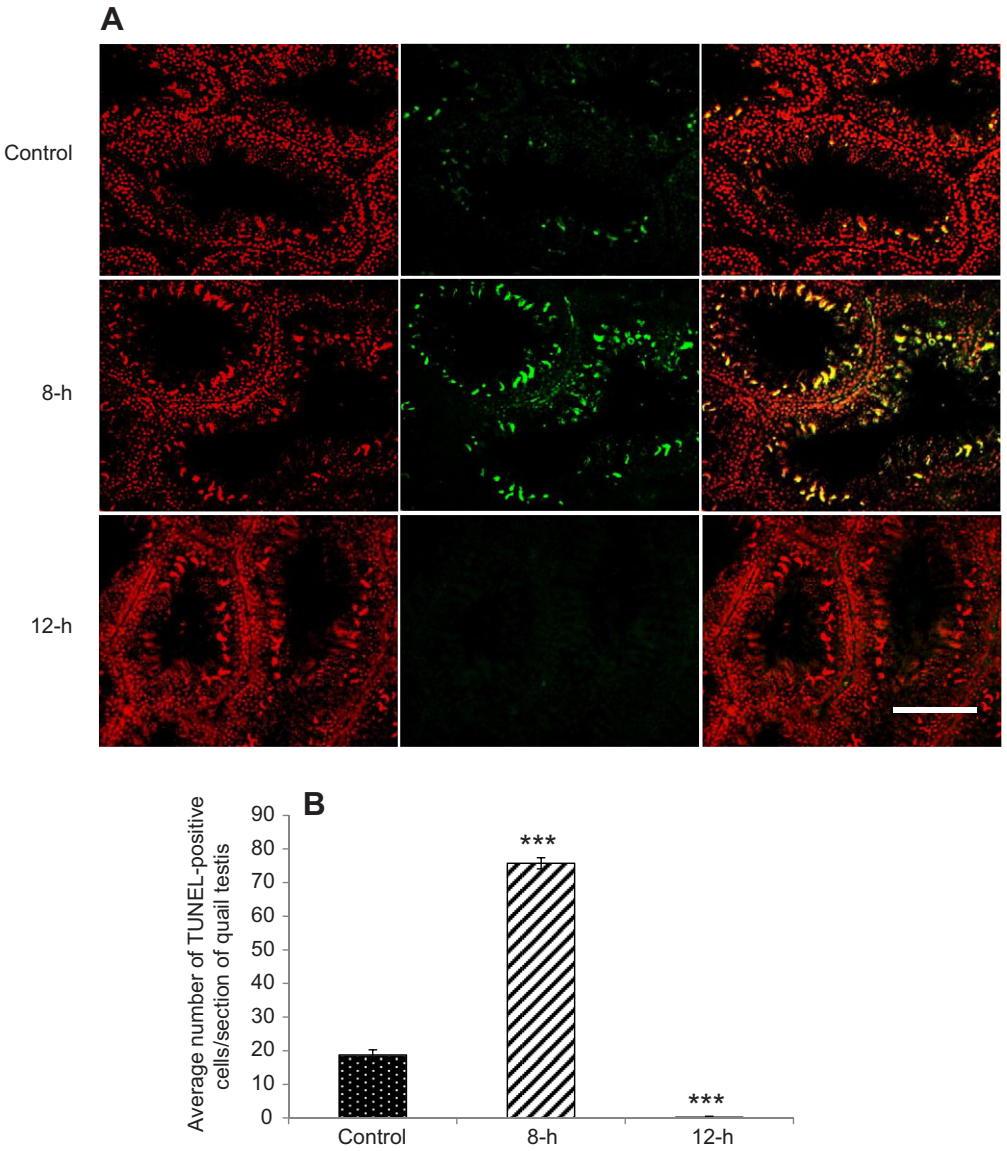


**Fig. 9. TUNEL assay in control, 8-h and 12-h quail testes.** (A) TUNEL assay and confocal microscopy. (B) Average number of TUNEL-positive cells in the testes of control, 8-h and 12-h quail. The number of TUNEL-positive cells (bright green or yellowish spots) increases dramatically in 8-h quail testes compared with controls. The TUNEL-positive cells are still much fewer and weakly stained in control testes and completely absent in 12-h quail testes. Red fluorescence corresponds to propidium iodide (PI) and green fluorescence corresponds to Alexa Fluor 488 (apoptotic or TUNEL-positive cells). Confocal images were recorded simultaneously in red and green fluorescence mode (i.e. green fluorescence overlaid on the red fluorescent image; co-localization of TUNEL-positive nuclei on the PI-stained nuclei). Scale bar, 100 µm. Values are expressed as means±s.e.m. ($n$=6). Asterisks indicate a significant difference from controls: ***$P$<0.001.

of 5-HTP and L-DOPA given at an 8-h interval. TUNEL staining also revealed that the predominant TUNEL-positive apoptotic cells seen in 8-h quail were dying in the lumen, apparently involving DNA fragmentation (Fig. 8). Thus, testicular stimulation/inhibition of sex steroid production and testicular cell survival/apoptosis appears to be the outcome of local (through testicular GnIH and its receptor) effects, which may be also altered by the temporal phase relation of neural oscillations. It is well documented from both *in vitro* and *in vivo* analyses that the mammalian GnIH ortholog RFRP3 has a direct effect on testicular steroidogenesis in mice (Anjum et al., 2014). The GnRH–GnIH system, through their receptors, may play a role in the autocrine/paracrine regulation of testosterone production and regulate optimal testicular activity in birds and mammals.

It has been reported that at the hypothalamic level, GnIH, coupled with melatonin, inhibits GnRH, pituitary gonadotropin, and gonadal steroid synthesis and release. In contrast, locally at the testicular level, GnIH inhibits spermatogenic activity, testicular growth and gonadal steroid production because GnIH receptors are located at spermatocytes, spermatids, myoid cells and Leydig cells in the testes (Bentley et al., 2008; Tsutsui et al., 2010, 2013; Ubuka et al., 2013b). Because testicular GnIH is reported to inhibit testicular activity directly through its receptors present in different testicular cell types (Ubuka et al., 2006, 2013b), it is possible that in 8-h quail, significantly increased testicular GnIH directly triggered the testicular apoptosis (Fig. 10). Moreover, significantly decreased testicular GnRH-I and serum testosterone further supports the local action of GnIH leading to apoptosis in 8-h quail, which subsequently results in the inhibition of spermatogenic activity. Accordingly, the testicular regression and atrophy in 8-h quail appears to be the direct effect of temporal phase relation in modulating the local testicular GnRH–GnIH system and testosterone, as these two drugs (5-HTP and L-DOPA) are likely to cross the blood–testis barrier as they can cross the blood–brain barrier. It may also be the case that temporal phase relation of serotonin and dopamine modulates the hypothalamic GnRH–GnIH system, which ultimately alters testicular activity. But, this central mechanism needs to be proved experimentally. However, the present study provides clear experimental evidence that inhibitory signals through testicular GnIH may activate and/or trigger the pro-apoptotic proteins, pro- and active-caspase-3, and uncleaved/full-length PARP-1 in the 8-h quail testes, leading to apoptosis. In

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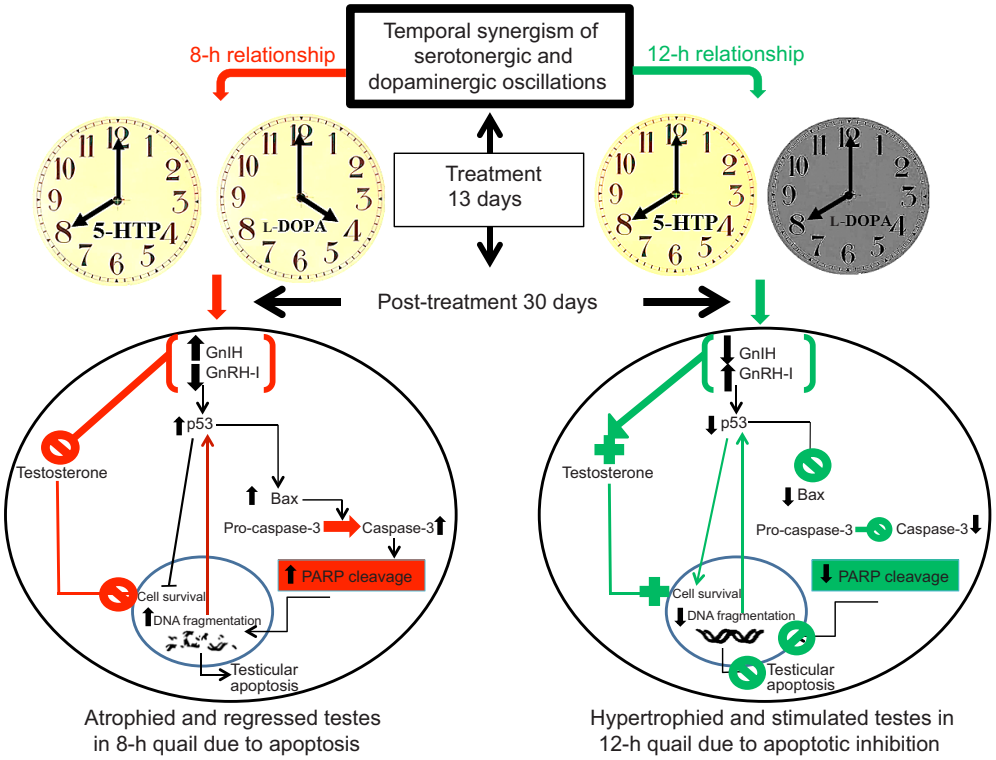


**Fig. 10. Hypothetical mechanism/ pathway that represent the temporal synergism of serotonergic and dopaminergic neural oscillation induced testicular apoptosis in quail.** Schematic depicting the temporal synergism of serotonergic and dopaminergic neural oscillation induced alteration in testicular neuropeptides (GnRH-I and GnIH) and subsequent underlying apoptotic pathway of testicular regression (atrophied testis) in 8-h quail. Hypertrophied and stimulated testis in 12-h quail results due to the apoptotic inhibition. This model includes the finding on the GnIH-induced, p-53-dependent, Bax-mediated pathway leading the testicular apoptosis in 8-h quail as observed in the present study.

contrast, in 12-h quail, similar to results reported by Ubuka et al. (2006), the testicular GnRH–GnIH system (lack of GnIH and significantly increased GnRH-I) potentiates the testicular growth, spermatogenic activity and testosterone production and thus results in hypertrophied testes.

p53, being the main tumor suppressor protein, mediates various cell-cycle checkpoints to prevent replication of damaged DNA via gene regulation to induce cell-cycle arrest or apoptosis (Lane et al., 1994). p53-mediated apoptosis can occur by way of trans-activation of Bax or trans-suppression of Bcl-2. p53 activation/overexpression stimulates pro-apoptotic Bax expression, which warps Bcl-2 and reduces the Bcl-2/Bax ratio (Laptenko and Prives, 2006). However, p53 possesses a dual mechanism that alternates between trans-activation and trans-suppression. Ho and Benchimol (2003) have suggested that p53 can also trigger apoptosis by repressing transcription of anti-apoptotic factors such as Bcl-2. Thus p53 functions as a transcription factor, thereby upregulating the transcription of pro-apoptotic genes such as *bax*, and possibly repressing the transcription of survival genes such as *bcl-2* (Miyashita et al., 1994). Bax is involved further downstream in mitochondrial disruption and contributes to the release of apoptogenic proteins, such as cytochrome *c* (Liu et al., 1996).

If the Bax level is constitutively high, p53 may mediate apoptosis through a transcriptionally independent pathway. By antagonizing Bcl-2 proteins, p53 can free Bax for apoptotic induction (Laptenko and Prives, 2006). By stimulating Bax expression, p53 skews the ratio of Bax and Bcl-2 and promotes apoptosis (Laptenko and Prives, 2006). Bcl-2 and its homologs, such as Bax, regulate caspase activation. Caspases are synthesized as zymogens, and undergo proteolytic activation at specific cleavage sites. Caspases may cleave other capsases. Caspases act in a cascade, in which upstream initiator caspases amplify and integrate pro-apoptotic signals that then activate downstream effector caspases (Chang and Yang, 2000). Jacobson et al. (1997) have also demonstrated that caspase inhibitors block apoptosis in animal cells.

Poly (ADP) ribose polymerase (PARP) is a nuclear enzyme responsible for the poly (ADP) ribosylation of chromosomal proteins and nuclear enzymes (Ohashi et al., 1983). The formation of DNA strand breaks during apoptosis is a potent stimulus for PARP-1 activation (Ferro and Olivera, 1982). The induction of PARP-1 may be an attempt by the dying cell to repair DNA damage caused by nuclease activation (Ohashi et al., 1983). PARP-1 is a substrate for the caspases (Lazebnik et al., 1994), in particular for one family member, active-caspase-3 (Tewari et al., 1995). This attempt to repair DNA damage during apoptosis would be futile as the proteases cleave PARP-1 into lower molecular weight fragments (Lazebnik et al., 1994).

Based on a large number of studies (for a review, see Chaturvedi and Yadav, 2013), it is well established that reproductive regulation involves temporal phase relation of circadian neural oscillations (serotonergic and dopaminergic). Our laboratory has not only substantiated the importance of the temporal phase relation of neurotransmitters in the maturation of the hypothalamo–hypophyseal–gonadal axis, but has also suggested a possible role of circadian organization/oscillation and their specific phase relation in the modulation of photosexual responses of Japanese quail (Phillips and Chaturvedi, 1995; Chaturvedi et al., 2006). Our findings revealed that the temporal phase relation of circadian neural oscillations induces specific reproductive/metabolic conditions in both seasonal (spotted munia, Prasad and Chaturvedi, 1992, 1998; Indian weaver bird, Chaturvedi et al., 1997; red-headed bunting, Chaturvedi and Bhatt, 1990) as well as continuous breeding bird species (Japanese quail, Chaturvedi et al., 1991, 2006; Bhatt and Chaturvedi, 1992; Kumar and Chaturvedi, 2008). The simulated induction of the specific phase relation of serotonergic and dopaminergic oscillation in this poultry species led to different gonadal responses. Out of six temporal relationships tested so far (0, 4, 8, 12, 16 and 20 h), the 8-h relation induced gonadal suppression, the 12-h relation induced gonadal stimulation, while the other relationships were found ineffective. Because daily doses of 5-HTP

and L-DOPA were the same in all experimental groups, obviously the specific testicular effects in 8-h and 12-h quail are executed as a function of the time interval relationship between the administration of these two precursor drugs (Chaturvedi et al., 1991, 2006).

Finally, in brief, in 8-h quail, deprivation of local testicular GnRH and gonadal (testosterone) hormones led to the overexpression of GnIH and induced GnIH-mediated testicular apoptosis (Ubuka et al., 2006, 2008). Instability in the chromatin and/or DNA can be potentially sensed by cellular p53 and, after sensing the DNA damage, p53 actively moves to the nucleus and trans-activates Bax. Bax activation and upregulation will permeabilize the mitochondrial outer membrane and consequently release the cytochrome *c* (apoptotic protein) from mitochondria, and subsequently serves in the activation of caspase-3, which ultimately leads to apoptosis via cleaving its substrate PARP-1. The active-caspase-3 cleaved PARP-1 in the nucleus, stalls the DNA repair and cell-cycle progression and thus mediates testicular apoptosis. This explanation of testicular suppression via changes in apoptotic proteins, pro-caspase-3, active-caspase-3 and uncleaved PARP-1 appears reasonable because changes observed in 8-h quail testis were also evident in the regressed testes of scotosensitive and photorefractory quail (S.B. and C.M.C., unpublished data). These explanations are further strengthened by the opposite findings noted in 12-h quail testis.

In 8-h quail testis, the significantly decreased GnRH-I and increased GnIH may potentially inhibit testicular development and maintenance, as well as testosterone production. The increased testicular GnIH may interact with the testicular Leydig, Sertoli and germ cells and play a key role in germ cell regression. The increased GnIH may stimulate the testicular germ cell DNA fragmentation (Ubuka et al., 2006), which may upregulate p53. Increased p53 alters the Bax/$Bcl_2$ ratio and promotes apoptosis through Bax activation. Activated Bax convert the procaspases into active caspases. Pro-caspase-3, after being converted into active caspase-3, cleaves its substrate PARP-1 and stalls the DNA repair and cell-cycle progression, and thus promotes the apoptosis.

In summary, we conclude that 12-h phase relation of the serotonergic and dopaminergic oscillations potentiates the testicular GnRH system and suppresses GnIH, thus driving gonadal growth, development and maturation in Japanese quail. In 12-h quail, testicular hyper-stimulation and over-maturation are promoted by the temporal phase relation of serotonergic and dopaminergic neural oscillation inducing the local GnRH–GnIH system and/or via the HPG axis. The testicular responsiveness in 12-h quail may preferably be mediated through stimulated GnRH production/GnIH suppression. But in 8-h quail, testicular regression is due to the GnIH-induced, p53-dependent, Bax-mediated testicular apoptosis. Based on the present experimental findings, it is suggested that specific phase relation of neural oscillations may also modulate the local action of GnIH in the testis via induction of the testicular apoptotic pathway.

The temporal phase relation of serotonin and dopamine may alter the testicular activity at the level of the hypothalamic GnRH–GnIH system or the testicular GnRH–GnIH system, or these two GnRH–GnIH systems may converge to execute the end effects. The experimental findings of the present study clearly indicate that temporal synergism of neural oscillations modulates the testicular GnRH–GnIH system, inducing testicular apoptosis in 8-h quail and the reversal of the apoptotic pathway in 12-h quail. Further research is required to elucidate the involvement of the hypothalamic GnRH–GnIH system and, if it is involved, how the hypothalamic and testicular GnRH–GnIH systems converge in executing the effects of temporal phase relation of neural oscillations on reproductive performance.

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#### Competing interests
The authors declare no competing or financial interests.

#### Author contributions
C.M.C. and S.B. designed the research. S.B. executed the experiments. S.B. and C.M.C. conceived the experiments, interpreted the results and prepared the manuscript prior to submission. K.T. gifted the GnIH antibody.

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