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Research Article

EFFECT OF STRESS ON THE PERCENT BODY WEIGHT CHANGE AND MRNA EXPRESSION OF IGF-1, SURVIVINE AND HSP-70 GENE IN THE HIERARCHIAL FOLLICLES OF JAPANESE QUAIL

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ABSTRACT: The present study was carried out to explore the effect of stress on body weight and the mRNA expression of IGF-1, Survivine and HSP-70 gene in the hierarchial follicles of Japanese quail. A total 24 birds (10 weeks) were taken and stress was induced by immobilization daily for 2hrs (between 9.00 - 11.00 AM) throughout the study (10 days). Four birds were sacrificed on 1, 2, 4, 6, 8 and 10 days of the treatment. Hierarchial follicles (F1, F2 & F3) were aseptically collected to quantify the expression of IGF-1, Survivine and HSP-70 gene using real-time PCR technique. The percent body weight reduction increased and reached highest (21.30%) on 10th day. The fold expression of IGF-1 gene was significantly ((P=0.05) down regulated in advance to the time of experiment. However, the fold expression of survivine gene was significantly (P=0.05) up regulated and the intensity was highest (17 fold) in F-3 follicle on 4th day of experiment. No significant change in the mRNA expression of HSP-70 gene was evident in this study. From this study it may be concluded that stress brings physio-molecular change through HPA activation, which not only causes tissue regression also modifies the cellular mechanism.

Key words: Stress, Body weight, Expression, Hierarchial follicle, Japanese quail.

INTRODUCTION

Japanese quail (*Coturnix coturnix japonica*) is the only smallest domesticated avian species, which brings suitable diversification in the chicken dominated poultry industry (Panda and Singh 1990, Thiagasundaram 1989). Considering the anatomy and physiological homology to chicken, the species has been introduced as a model animal in the heavy expensive bio-medical (embryology, endocrinology, genetics, toxicology etc.) research particularly for their fast growing genetic makeup, early sexual maturity and short generation interval (Parkhurst and Mountney 1988). Similar to other poultry species, they are sensitive and highly susceptible to stress which adversely affect the reproductive functions through Hypothalamo Pituitary Adrenal Axis (HPA) activation (Moberg 1993). Besides, stress accelerates metabolic changes such as

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hepatic glycogenolysis, protein catabolism and gluconeogensis to mobilize energy reserves to assist an individual to evade the stressors (Vellucci 1997). Such response redirects animals to a life-saving state or emergency life-history stage allowing them to overcome stress and re-establish homeostasis in the best possible physical condition (Wingfield and Ramenofsky 1999). The small ovarian follicles are more susceptible to atresia followed by F1 and F2-F5 follicle during stress (Moudgal *et al.*,1991). Stress associated molecular consequences in birds represents the follicular expression of IGFs which have been shown to play a crucial role in avian reproduction as they hasten dose dependent gonadal steroid hormone synthesis, cell proliferation, selection and inhibition of follicular apoptosis by inhibiting oligonucleosome formation (Lovell *et al.*, 2002, Tosca *et al.*,2008). Survivin has been proposed to control cell proliferation and death (Li *et al.*, 1998, Wheatley and McNeish 2005) and over expression is only reported in embryonic and fetal tissues though it remains undetected in many normal adult tissues (Ambrosini *et al.*,1997). The cells with increased Heat Shock Proteins exhibit tolerance against the additional stress hence they are often called as stress markers (Mahmoud *et al.*, 2003, Figueiredo *et al.*, 2007).

However, little information is available so far on the molecular mechanism associated with the initiation and the cyclic activity of the ovary and stress regulated tissue regression (atresia) in Japanese quail. Hence, the present study was an attempt to elucidate the effect of stress on the percent body weight change and mRNA expression of IGF-1, Survivine and HSP-70 gene in the hierarchial follicles of Japanese quail.

MATERIALS AND METHODS

Experimental birds

A total of twenty four healthy Japanese quail hens at 10 weeks of age were randomly selected from the institute quail farm. The protocols involving the care and use of animals for the experiments was in accordance with the rules of the 'Animal Ethics Monitoring Committee of the Central Avian Research Institute' and the revised framework of animals (Scientific Procedures) Act of 2002 of Government of India on Animal welfare. Selected birds were individually caged (20x20x20 cm^3) under uniform husbandry conditions at 14 h photo-schedule with *ad-libitum* clean drinking water and quail layer ration (Table 1).

Induction of stress

Birds selected for this experiment were undergone into immobilization stress which was practiced by movement restriction daily for 2 hrs (between 9.00 to 11.00 AM) throughout the experimental period (10 days).

Sample collection

Four birds were sacrificed each time by decapitation on 1, 2,4,6,8 and 10^{th} day of the experiment. Immediately before slaughter, body weight of individual birds was accounted (0.01g specificity) to specify the percent of body weight change. The gene expression study was carried out in the F1, F2 and F3 ovarian follicles. Follicles were excised from the ovary and almost aseptically cut open transversely along the stigma to drain the yolk material. The follicular membranes were washed three times with ice-cold sterile normal saline (0.9%) to devoid of adhering yolk material. Each sample was divided into two parts, one part used immediately for RNA isolation and other part

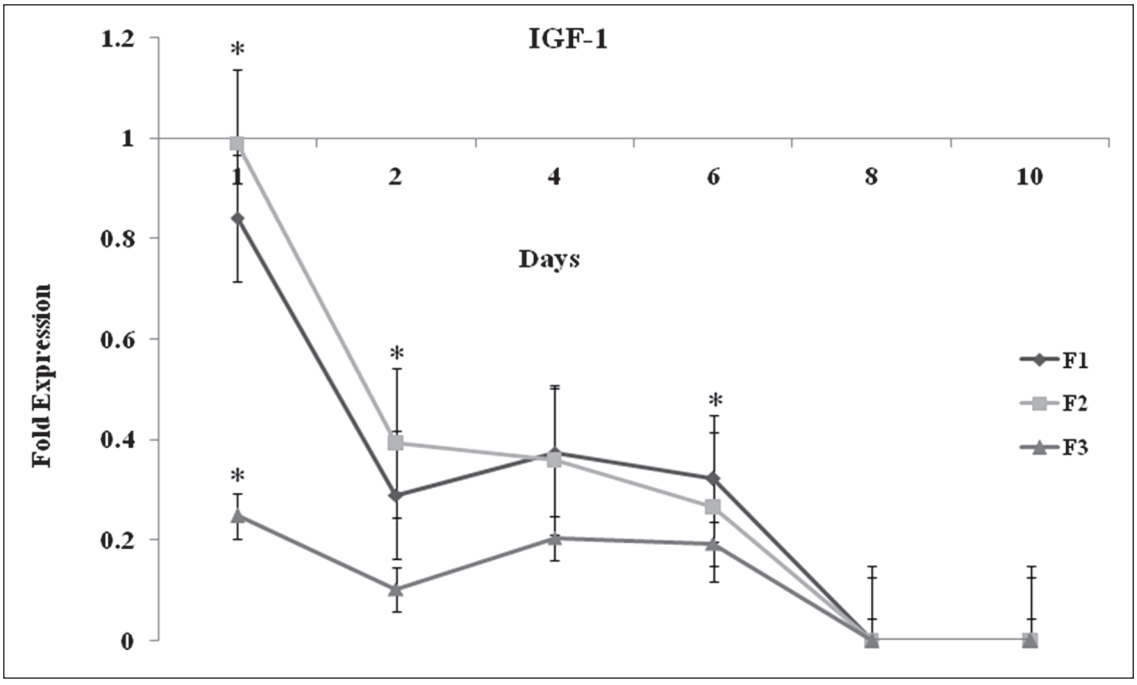

Fig. 1: Fold expression of IGF-1 gene in hierarchical follicles (F1, F2 & F3) of Japanese quail during stress (mean ± SE; N=4). Star above the bars indicates significant (p= 0.05) difference in the means.

was stored separately in RNA stabilization solution (RNAlater, Ambion Inc. USA) as per manufacturer's instructions for further use if needed.

RNA isolation and reverse transcription

Total RNA from each follicle was extracted by 'RNAgents-Total RNA isolation system' (Promega, Madison, WI, USA) according to the manufacturer's instructions. The RNA integrity was checked by 1% denaturing agarose gel electrophoresis. The concentration and puritiy of RNA preparations were measured through Nano Drop (Thermo2000) and the ratio of 260/280 was >2.0 for all the samples. The possible traces of genomic DNA were removed by treating 5ìg of each RNA samples with 5U of RNase-free DNase (Biogene,CA,USA) at 37°C for 1h. The DNase was subsequently inactivated by incubation at 65°C for 10 min. Each DNase treated total RNA sample (1ìg) was reverse transcribed using the 'RevertAid First strand cDNA synthesis kit' (MBI Fermentas, Hanover, MD, USA) according to the manufacturer's instructions. The resultant cDNA was stored frozen at -20°C till used. Negative controls were performed using all components except reverse transcriptase.

Quantification of IGF-1, Survivine and HSP-70 gene expression by real-time PCR

After reverse transcription the target mRNA into cDNA, it was quantified by real-time PCR technology using Syber Green master mix in IQ5 cycler (Bio-Rad, USA). The amplification of mRNAs was carried out by gene specific primers designed by Beacon designer software (Premier Biosoft International, USA). Primers

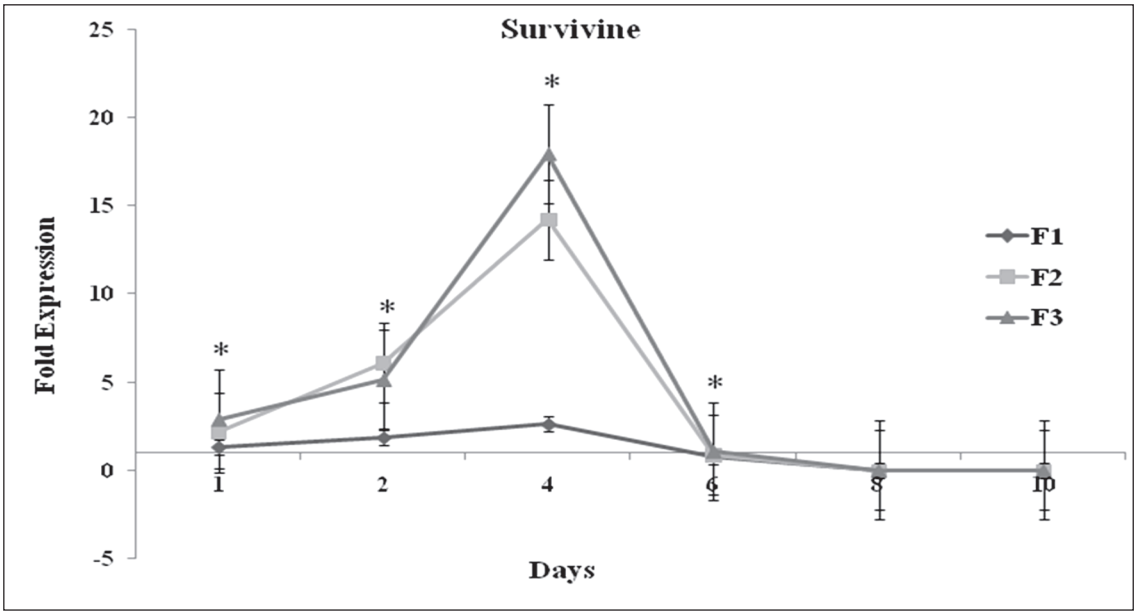

Fig. 2: Fold expression of survivine gene in hierarchical follicles (F1, F2 & F3) of Japanese quail during stress (mean ± SE; N=4). Star above the bars indicates significant (p= 0.05) difference in the means.

were designed from coding region of chicken mRNA sequences available in Gene Bank considering the close relationship between chicken and quail in phylogeny and sequences. The sequence of forward and reverse primer for the IGF-1, Survivine, HSP-70 and beta- actin used as reference gene are shown in Table 2.

For RT-qPCR assay the reaction was set in triplicate with each cDNA sample. The amplification was carried out in 25 ml volume containing 1x QuantiTect SYBR Green PCR master mix (SyberGreen1dye, Hot Start TaqDNA polymerase and dNTPs in optimized buffer components; QIAGEN GmBH, Germany), a 0.2µM Concentration of each gene-specific primer and 1µl of cDNA template. RT-qPCR cycling conditions includes initial denaturation of 95^0C for 10 min followed by 40cycles of denaturation 95^0C for 30s, annealing (Table 2) for 30s and extension 72^0C for 45s. For each gene of interest negative and positive Controls were included. Negative controls were samples in which cDNA was not added. A melting curve was performed for each sample after completion of amplification and analyzed in comparison to negative and positive controls, to determine the specificity of PCR reaction. To generate gene-specific standard curves, plasmids containing each of the different genes were serially diluted from 10-1 to 10-5. Each RT-qPCR experiment contained triplicates of test samples, one no-template-control and a log10 dilution series. Regression analysis of the standard curve was used to calculate the slopes of the gene specific log10 dilution series. The relative expression was determined following the delta-delta Ct method (Pfaffl 2001).

Statistical analysis

The data collected on body weight was analyzed by one-way analysis of variance

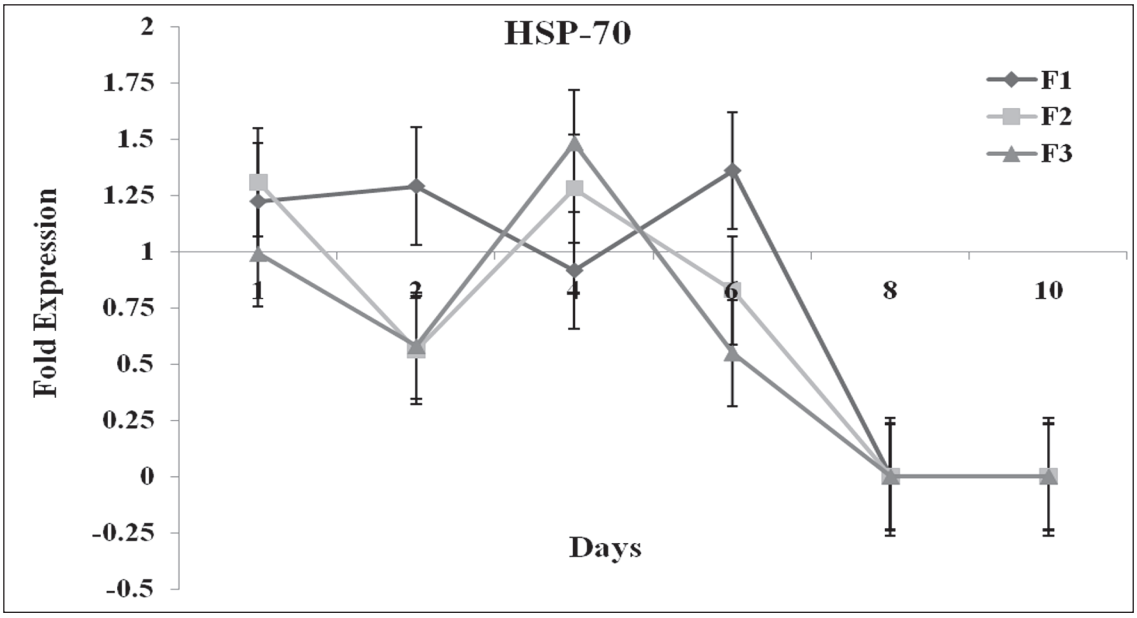

Fig. 3: Fold expression of Heat Shock Protein (HSP)-70 gene in hierarchical follicles (F1, F2 & F3) of Japanese quail during stress (mean ± SE; N=4).

(ANOVA) and means compared using Duncan's multiple range test (Duncan 1955). The real time expression data of corresponding genes was analyzed by SPSS ver. 16 (SPSS 16.0, SPSS. Inc.NY) using following model

$Yij = \mu + Geni + eij$

Where, Yij is the jth observation under ith genotype, m is the overall mean, Geni is the effect of ith genotype and eij is the random error associated with mean '0' and variance ó2.

RESULTS AND DISCUSSION

Stress is defined as biological response elicited when an individual perceives a threat to its homeostasis (Moberg 2000). Among vertebrates, exposure to a variety of potentially threatening stimuli (environmental, nutritional, physical, social, physiological, psychological and pathological) results in activation of the hypothalamo–pituitary–adrenal (HPA) axis thereby reducing productive and reproductive performance (Feltenstein *et al.,* 2003, Cheng and Muir 2004, Corzo *et al.*, 2005). Similar to other poultry species, Japanese quail are sensitive and highly susceptible to stress which adversely affect their productive and reproductive functions (Moberg 1993).

Changes in percent body weight

The present study revealed a gradual body weight reduction in advance to the time of experiment. Though it was recorded 8.86 percent on day-1 but on 10th day, birds lost their body weight by 21.30 percent (Table 3). Initially the percent reduction followed a symmetrical change when it was drastic in the last two days of the experiment. Similar to chicken (Edens and Siegel 1975, Ben Nathan *et al.,* 1976), turkeys (El Halawani *et al.,* 1973) and pigeons (Pilo *et al.*, 1985), stress elevate plasma adreno-cortical hormones in Japanese quail (Bhattacharyya and Ghosh 1972). However, this

Table 1: Formula and chemical composition of quail layer ration.

Ingredients	(%)
Maize	56.50
Soyabean	29.44
Sunflower	02.50
RSM	02.50
M. Mix (ISI)	00.50
Marble chips	04.00
Limestone	03.00
Di-calcium phosphate	00.80
Salt	00.30
DL-methionine	00.09
Lysine	00.02
TM premix*	00.10
Vitamin premix**	00.15
B-complx	0.015
Choline chloride	00.03
Toxine binder	00.05
Total	100.00
Nutrient composition	
M Energy (Kcal/kg)	2716.21
Crude protein (%)	20.03
Calcium (%)	03.06
Available phosphorus (%)	00.33
Lysine (%)	01.09
Methionine (%)	00.45

***Trace mineral premix: Mg- 300, Mn- 55, I- 0.4, Fe-56, Zn-30 and Cu- 4 mg kg-1**

**** Vit. premix: vit. A- 8250IU, vit. D3- 1200 ICU, vit. K- 1mg,**
vit. E- 40IU, vit. B1- 2mg, vit. B2- 4mg, vit. B12-10mcg, niacin- 60mg,
pantothenic acid- 10mg, choline- 500mg kg-1 diet.
Percent of values specified by NRC, 1994.

outcome is in accordance to McCormick and Cunningham (1984) who reported that the body weight losses of hens corresponded to the rate of the feed intake during study. With the interaction to neuro-endocrine axis, the hypothalamopituitary-adrenal (HPA) axis in birds plays an essential role in adjustment of their morphology, physiology and behavior in response to overarching environmental cues which may correspond to the present findings.

Gene expression study

Evidently, the locally produced factors such as growth factors, steroids, cytokines, neuropeptides and adipokines have essential modulatory roles in the regulation of ovarian functions. Follicle recruitment into the pre-ovulatory hierarchy is associated with FSH-induced cAMP accumulation which leads to the acquisition of p450 side chain cleavage and increased basal levels of LH receptor (LH-R) mRNA within the rapidly differentiating granulosa cell layer. Expression of p450scc enzyme which converts cholesterol to ultimately progesterone in granulosa cells of pre-ovulatory follicle may be considered as markers for follicle selection.

In this experiment, hierarchial follicles (F1, F2 & F3) were considered for gene expression study. Because of tissue regression effect of stress, no hierarchial follicles were available in the ovary after 6th day of the experiment so gene expression could not be possible in the last phase of the experiment.

a. Insulin like growth factor (IGF)-1

Similar to mammals, avian ovary also contains the full complement of the members of the insulin-like growth factors (IGFs) system including the peptides, receptors and binding

Table 2: Oligonucleotide primer pairs used for gene expression study in hierarchial follicles of Japanese quail hens.

Gene Name	Primer sequence (5'-3')	Annealing Temperature	Amplicon size (bp)	Reference/ Accession number
IGF-1	F- 5'TGTATTGTGCTCCAATAAAGC3'	58^0C	127	Guernec, *et al.* (2003)
	R- 5'CTGTTTCCTGTGTTCCCTCTACTTG3'			
Survivine	F- 5'TCGAAGATGTAGCCAAGG3'	56^0C	98	HM588003
	R- 5'CAGCGTGGCAGTGTC3'			
HSP-70	F- 5'GGCACCATCACTGGGCTT3'	56^0C	74	HM587997
	R- 5'TCCAAGCCATAGGCAATAGCA3'			
Beta-actin	F-5'GGAAGTTACTCGCCTCTG3'	58^0C	114	L08165
	R-5'AAAGACACTTGTTGGGTTAC3'			

Table 3: Overallchanges in the percent body weight reduction during stress in the Japanese quailhens (mean +_ SE; N=4).

	DAY					
Body Weight reduction (%)	D-1	D-2	D-4	D-6	D-8	D-10
	8.86±2.45	9.81±1.78	11.18±3.02	12.37±1.37	12.66±1.51	21.31±2.39

proteins. IGFs play a crucial role in avian reproduction as they hasten dose dependent gonadal steroid hormone synthesis, cell proliferation, selection and inhibition of follicular apoptosis by inhibiting oligonucleosome formation (Roberts *et al.*, 1994, Onagbesan and Peddie 1995, Onagbesan *et al.*, 1999a, b, 2000, Johnson *et al.*, 2001, Lovell *et al.*, 2002, Tosca *et al.*, 2008). The relative mRNA expression of the IGF-1 gene in hierarchial follicles (F1, F2 & F3) revealed a significant (P=0.05) down regulation in hierarchial follicles towards the progress of the experiment (Fig 1). However, the intensity of the fold expression was shown comparatively lower in the smallest follicles. Any sort of stress disrupts the normal ovarian functions upon dose dependent gonadal hormone synthesis and cell proliferation which may correspond to the present findings. Interestingly, in avian species both IGF-1 and IGF-2 exerts their action after binding to the same receptor IGF-R (type-1). The mRNA and protein encoding for the IGF's receptor are expressed in immature ovary (Heck *et al.*, 2003), granulosa and theca cells of the developing follicles (Armstrong and Hogg 1996, Tosca *et al.*, 2008).

b. Survivine

Survivin is an inhibitor of apoptosis (IAP) protein with a single baculovirus IAP repeats (BIR) domain and has been proposed to like cell proliferation and death (Li *et al.*, 1998, Wheatley and McNeish 2005). The Survivin because of its conserved single BIR domain associates with HSP-90 and this association accommodates its multiple molecular interactions for mitotic regulation, stress response and cell death (Fortugno *et al.*, 2002, Marusawa *et al.*, 2003). A significant (P=0.05) up regulation was evident in the mRNA expression of survivine gene in hierarchial follicles till 4th day of the experiment (Fig. 2). However, the level of expression was comparatively higher in the small follicles and the intensity was recorded highest (17 fold) in F-3 follicle when it was 2 and 14 fold in F1 and F2 follicle respectively on the same day. This result suggests that tissues in atresia undergo into severe regression and lost almost all their physiological functions which could be the reason of less mRNA expression. In addition, F2 and F3 follicles may be considered mitotically more active and they are under regular cell cycle (G2/M phase) where survivin is a factor (Johnson and Howerth 2004). Evidently, survivin has been found to inhibit apoptosis either by inhibiting the cytochrome c induced proteolytic processing events in the cytoplasm or by directly interfering the activities of terminal effector cell death protease such as caspases-3, -7 and -9 (Tamm *et al.*, 1998, Shin *et al.*, 2001).

c. Heat shock protein (HSP)-70

Heat shock proteins (HSPs) are a group of proteins that are present virtually in all animals but expressed at high levels when they are exposed to stressors. It regulates cellular functions by playing institutive roles in protein folding, unfolding, assembling, disassembling and translocation (Pelham 1986, Randall and Hardy 1986, Murukami *et al.*, 1988, Wang and Edens 1998). The cells with increased HSPs exhibit tolerance against the additional stress hence they are often called as stress markers (Mahmoud *et al.*, 2003, Figueiredo *et al.*, 2007). An inconsistent variation was recorded in the mRNA expression of HSP-70 gene in hierarchical follicles (Fig.3). However, no significant change was found in the relative expression though in relation to the standard gene it invariably represented up-regulation in its expression level. This consequence is in agreement to the findings of Mahmoud *et al.* (2003) who demonstrated that heat shock proteins (HSPs) expressed at high level only when cells are exposed to high or low temperature. It would be promising that HSP-70 gene may not be involved in modulation of stress related events in the hierarchal follicles in Japanese quail. However, further study is required to confirm the involvement of HSPs family protein in hierarchical follicles of Japanese quail under stress.

CONCLUSION

In conclusion, birds subjected to immobilization stress under this study reduced their body weight by 21.30 percent in a period of 10 days. The relative mRNA expression of IGF-1 gene was significantly down regulated in the small follicles when the reverse picture was true for the inhibition of apoptosis protein (IAP), the survivine gene. However, the fold expression of heat shock protein (HSP)-70 did not show any significant change in the hierarchial follicles of Japanese quail.

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