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Molecular Characterization and Expression of *LDHA* and *LDHB* mRNA in Testes of Japanese Quail (*Coturnix japonica*)

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ABSTRACT : The LDH isozymes are key catalysts in the glycolytic pathway of energy metabolism. It is well known that the distribution of the LDH isozymes vary in accordance with the metabolic requirements of different tissues. The substrates required for energy production change noticeably at successive stages of testes development suggesting a significant flexibility in the expression of glycolytic enzymes. Therefore, expression of *LHDA* and *LDHB* mRNAs was examined in adult and prepubertal quail testis. The mRNA of both *LDHA* and *LDHB* were expressed and no significant difference was observed in prepubertal testes. The mRNA levels of *LDHB* significantly increased during testicular development. In the adult testis, *LDHA* mRNA was not expressed. Expression studies revealed the presence of different LDH isozymes during testicular development. In contrast, electrophoresis of both testicular samples revealed only single band at a position indicative of an extreme type of LDH isozyme in quail testes. Furthermore, nucleotide and amino acid sequence analysis revealed significant similarity to chicken, duck and rock pigeon. These sequence results confirmed the similarity of LDHA and LDHB subunit protein in different avian species. (**Key Words :** Lactate Dehydrogenase, Testes, Lactate, Isozymes, Messenger RNA)

INTRODUCTION

Spermatogenesis is a very complex process that highly dependent upon the hormonally (testosterone and follicle-stimulating hormone) regulated sertoli cells (Sharpe, 1994; Griswold, 1995). Sertoli cells provide regulatory factors, such as growth factors (Benahmed, 1996; Gnessi et al., 1997) and nutrients (Grootegoed et al., 1986) to the germ cells. Among all the nutrients, lactate used as energy substrates by developing germ cells. Several observations have indicated that postmeiotic germ cells use sertoli cell lactate rather than glucose as energy substrate (Grootegoed et al., 1986). A similar metabolic cooperation involving lactate as an energy metabolite also occurs in other tissues such as the quail brain (Singh et al., 2010). In addition to this we have recently reported that lactate is also used as an energy substrate by quail spermatozoa (Singh et al., 2011a) that is provided by cloacal gland foam (Singh et al., 2011b). During spermatogenesis, spermatogonia and mature sperm utilize glucose as their major energy substrate (Nakamura et al., 1984). However, spermatocytes and spermatids suffer a rapid decline in their ATP content in the presence of glucose and require lactate/pyruvate for the maintenance of their ATP concentration (Jutte et al., 1981; Mita and Hall, 1982).

Several biochemical steps are involved in lactate production, including glucose uptake, glycolysis and the interconversion of lactate and pyruvate. Lactate dehydrogenase (LDH; EC.1.1.1.27) catalyzes this interconversion with nicotinamide adenine dinucleotide (NAD+) as coenzyme. Mammals have three different subunits of LDH that are encoded by three genes, *LDHA*, *LDHB* and *LDHC* (Markert et al., 1975). However, birds have only two subunits of LDH that are encoded by two genes *LDHA* and *LDHB* except pigeon (Zinkham et al., 1964). The A and B subunits form together five tetrameric isoenzymes: A4, A3B1, A2B2, A1B3, and B4. Although these hybrid forms occur in most tissues, the B-type subunit predominates in aerobic tissues such as heart and is superior for lactate oxidation, whereas the A-type subunit predominates in tissues that are subject to anaerobic conditions, such as skeletal muscle and liver, and is best

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suited for pyruvate reduction. The third subunit C codes a unique isozyme LDH-X (C4) among the LDH isozymes with respect to its restricted distribution within the germinal epithelium of the mammalian and pigeon testes (Markert et al., 1975) which involved in the energy metabolism of spermatozoa and helps in capacitation (Flaherty et al., 2002). Previously it was thought that LDH isozyme activity is tissue specific rather than species specific and is related to the physiological activity and microenvironment of the tissue (Cahn et al., 1962) but recent studies have demonstrated the expression of LDH enzyme is both tissue and species specific (Echigoya et al., 2009).

Expression of *LDHA* and *LDHB* mRNA has been confirmed in premiotic, meiotic cells and early spermatids of chicken (Arias et al., 2000) but no literature is available on quail. The reproductive strategy of male Japanese quail is different from chicken. The testes of Japanese quail are large (2.26% of body weight) with a high rate of sperm (3.08×10^9) production (Clulow and Jones, 1982). Furthermore, male quail germ cells (spermatozoa) are different among all the avian species in having higher number (>1,400) of mitochondria (Korn et al., 2000) collectively indicated a need of unique energy substrate to meet the higher energy requirement. The substrates required for energy production change noticeably at successive stages of testes development suggesting a significant flexibility in the expression of glycolytic enzymes. Since LDH is a key enzyme of glycolytic pathway which mediates both aerobic and anaerobic energy metabolism through its different isozymes. Therefore, we studied the expression of *LDHA* and *LDHB* mRNA in adult and prepubertal quail testes. The sequences of these genes were not available in GenBank for quail, so it is imperative to characterize mRNA sequences for these genes in quail.

MATERIALS AND METHODS

Experimental birds

Male Japanese quail of four and ten weeks old from same hatch were used in this study. The birds were housed in individual cages and were maintained under uniform standard management conditions with 14 h light:10 h dark and provided feed and water *ad libitum*. This experiment was performed in accordance with the rules of "Animal Ethics Monitoring Committee" of the Institute.

Preparation of tissue extracts

Testes of prepubertal and adult Japanese quail were collected after sacrificing birds and washed in ice-cold normal saline. Tissue was homogenized (10% w/v) in 0.02 M Tris-HCl buffer (pH 7.4) by using a Polytron homogenizer (PT 1600E). Tissue homogenates were centrifuged at 40,000×g for 20 min at 4°C in P40ST rotor using Himack CP80B-Hitachi ultracentrifuge. The supernatant were collected and used for non-denaturing PAGE. Tissue extracts prepared in the similar manner from quail brain was used as reference for electrophoresis.

Collection of samples

Four male Japanese quail from each age group (4 and 10 week) were sacrificed and testes were removed. A small piece of testes was collected aseptically. Special care was taken to avoid blood contamination during sampling. Each collected sample was divided into two parts, one part used immediately for RNA isolation and other part was stored separately in RNA stabilization solution (RNAlater, Ambion Inc. USA) as per manufacturer's instructions for further use if required.

RNA isolation and reverse transcription

Total RNA from individual testes samples was extracted by 'RNAgents-Total RNA isolation system' (Promega, Madison, WI, USA) according to the manufacturer's instructions. Approximately, 25 mg of tissue samples were used for RNA isolation. The concentration and puritiy of RNA preparations were determined spectrophotometrically at A260 and A280. The possible traces of genomic DNA were removed by treating 5 μg of each RNA samples with 5 U of RNase-free DNase at 37°C for 1 h. The DNase was subsequently inactivated by incubation at 65°C for 10 min. Each DNase treated total RNA sample (1 μg) 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 had all components except reverse transcriptase.

Quantification of LDHA and LDHB expression

The amplification of *LDHA* and *LDHB* mRNA were carried out by gene specific primers designed by Beacon designer software (Premier Biosoft International, USA). Primers were designed from coding region of chicken *LDHA* and *LDHB* mRNA sequences (Accession No. NM_205284.1 and NM_204177.1, Imagawa et al., 2006) available in GenBank considering the close relationship between chicken and quail in phylogeny and sequences. Forward and reverse primer for *LDHA*, *LDHB* and *beta-actin* used for the PCR are shown in Table 1. PCR reactions were performed in a thermalcycler (iCycler Bio-Rad, Hercules, CA, USA) with equal amount of cDNA samples from testes in separate tubes in duplicate. The amplification was carried out in 25 μl volume containing 10 pmoles each primer, 0.1 mM dNTPs mix, 1 unit of Taq DNA polymerase (Platinum® Pfx DNA Polymerase, Invitrogen) and 2 μl

Table 1. Primer used for semi-quantitative RT-PCR

Gene name	Primer sequence	Amplicon size	Accession number
β-actin	F 5'GGA AGT TAC TCG CCT CTG3' R 5'AAA GAC ACT TGT TGG GTT AC3'	114	L08165
LDH-A	F 5'GACTTGGCAGATGAACTTACC3' R 5'CACAAGGAACACTTAGGAAGAC3'	720	NM_205284.1
LDH-B	F 5'ACGGTTCCCAGCAACAAG3' R 5'GGCACTCAGGACACAAGG 3'	808	NM_204177.1

cDNA in 1× Taq polymerase buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 2.5 mM $MgCl_2$). PCR cycling conditions included an initial period of denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C; 45 s, annealing 56°C (for *LDHA, LDHB* and β-actin) for 1 min and extension 72°C for 1 min. and final extension at 72°C for 10 min. Amplicons were analyzed on ethidium bromide stained 1.0% agarose gel (Gel documentation system, Syngene, USA). Amplification with gene specific LDH primers, samples yielded products of the expected sizes in the samples from testes.

Semi-quantitative RT-PCR was chosen to estimate the transcripts of *LDHA* and *LDHB* genes. The estimation of mRNA of these genes in testes of prepubertal and adult birds was carried out according to Sundaresan et al. (2005). To control the variation in the efficiencies of the RT step among different experimental samples, mRNA concentrations of β-actin, a housekeeping gene, presumed to be expressed at constant amounts in quail testes were also calculated, along with mRNA concentrations of targeted genes, by densitometry analysis using Image J software (NIH). Relative expression was determined as arbitrary units, defined as the ratio of mRNA level to the corresponding β-actin mRNA level after subtraction of background intensity (value = (intensity; gene of interest-intensity; background)/(intensity; β-actin-intensity; background). Mean values of four measurements for adult and prepubertal testes bands were taken for analysis.

Cloning and sequencing of LDHA and LDHB cDNA

The PCR product was purified by 'QIAquick Gel Extraction Kit' (QIAGEN). The purified PCR product was ligated to pTZ57R/T cloning vector (InsT/A clone kit, MBI Fermantas) by overnight incubation at 4°C and transformed into freshly prepared *Escherichia coli* DH5α competent cells. Plasmids were isolated from the overnight grown culture and insert was confirmed by colony PCR. Both strands were sequenced by M13 forward and reverse primers using an automated ABI PRISM 310 Genetic Analyzer (Applied Biosystem, Foster City, CA). Nucleotide sequence was analyzed using Lasergene Software (DNA Star, USA), both at nucleotide and predicted amino acid level using National Centre for Biotechnology Information (NCBI) BLAST network sources (http://www.ncbi.nlm.nih.gov/BLAST).

Non-denaturing polyacrylamide gel electrophoresis (PAGE)

LDH isozymes in quail testes homogenates were analyzed on non-denaturing 8% PAGE according to the method described by Trigun et al. (2006). After electrophoresis, the gels were subjected to LDH enzyme specific stain. Staining solution consisted of 0.125 M Tris-HCl (pH 7.4), 0.5 mM $MgCl_2$, 0.1 mM Na-Lactate, 1 mg/ml NAD, 0.01 M NaCl, 0.25 mg/ml nitro blue tetrazolium (NBT), and 0.025 mg/ml phenazine methosulúte (PMS). The isozyme bands in the gel were characterized by comparing their migration pattern.

Statistical analysis

Electrophoretic band intensities of PCR products were quantified. The mRNA expression level of each gene was normalized against β-actin mRNA level. The mRNA expression level was analyzed using one way ANOVA and means compared using Duncan's multiple range test (Duncan, 1955).

RESULTS

Expression of LDHA and LDHB in quail testes

The expression of *LDHA* and *LDHB* in testes of prepubertal (4 weeks old) and adult (10 weeks old) birds was studied by using specific primers on the basis of chicken. Expression of *LDHA* (720 bp) and *LDHB* (808 bp) mRNA was detected in testes of prepubertal birds whereas only *LDHB* expression was detected in adult birds (Figure 1A). In prepubertal birds expression of *LDHA* and *LDHB* mRNA was not significant (Figure 1D). However, the expression of *LDHB* in adult testes was significant ($p<0.05$) higher as compare to testes of prepubertal birds (Figure 1C).

Isozyme separation by electrophoresis

Zymogram of adult and prepubertal quail testes homogenate revealed only single band at identical position when subjected to LDH enzyme specific stain (Figure 2). In order to determine the LDH isozyme in both testicular

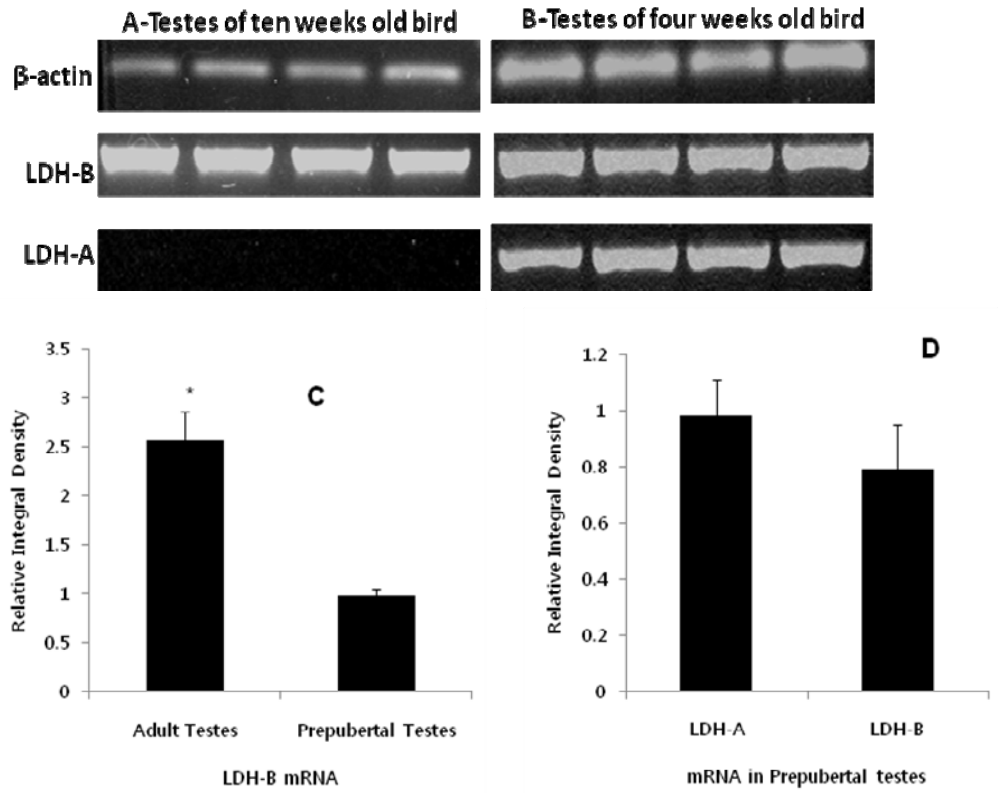


Figure 1. RT-PCR analysis of *LDHA* and *LDHB* mRNA in the testes of ten weeks (panel A) and four weeks old Japanese quail (panel B). C) Difference of *LDHB* mRNA expression in adult (ten week old birds) and prepubertal testes (four week old birds). D) Difference of *LDHA* and *LDHB* mRNA expression in prepubertal testes. Graph represent the mean of the normalized integral density for each mRNA band (mean±SE; n = 4). Normalization was performed by dividing each value by the value of β-actin band in the same sample. Asterisk (*) indicates a significant difference ($p<0.05$) between mRNA expression.

samples on the basis of their electrophoretic mobility, quail brain tissue homogenate was run along with seminal plasma for further verification. Quail brain tissue homogenate was used as reference because LDH isozymes present in quail brain were well identified. Electrophoresis of quail brain revealed four bands. The electrophoretic mobility of LDH isozyme present in both testicular samples was identical to the LDH-4 of quail brain.

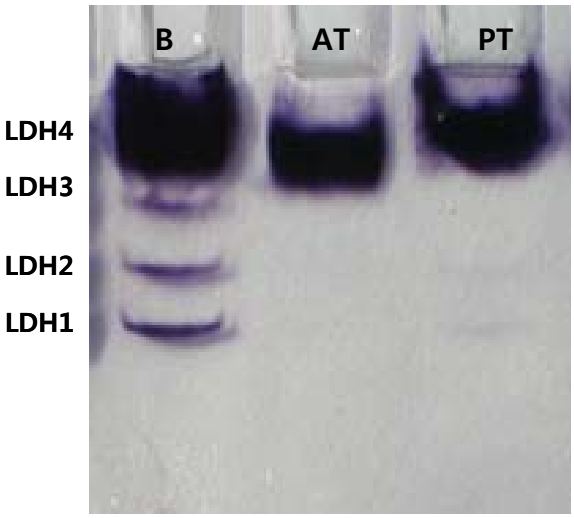
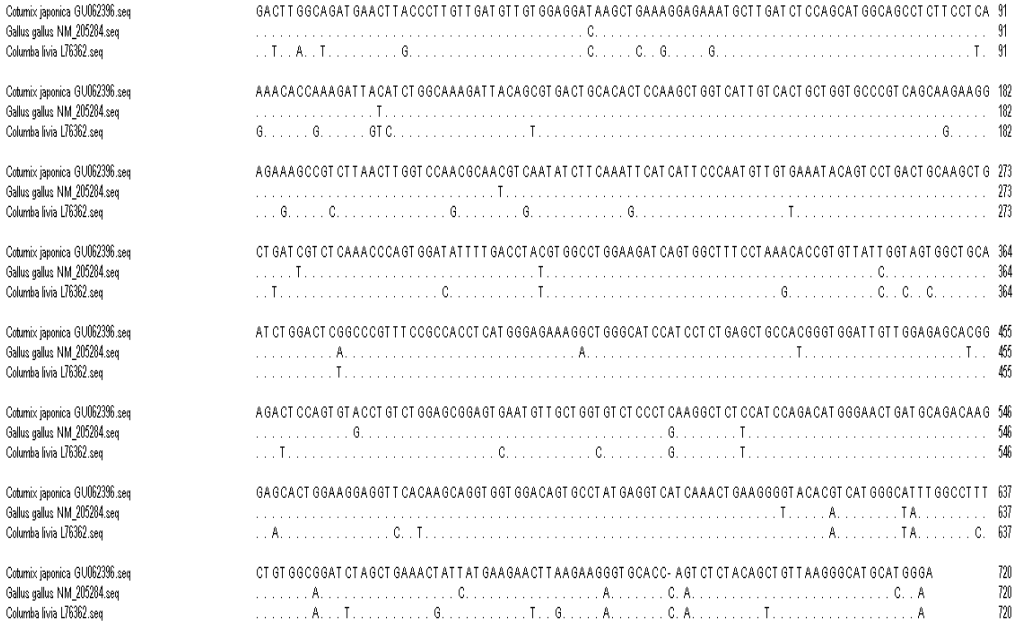


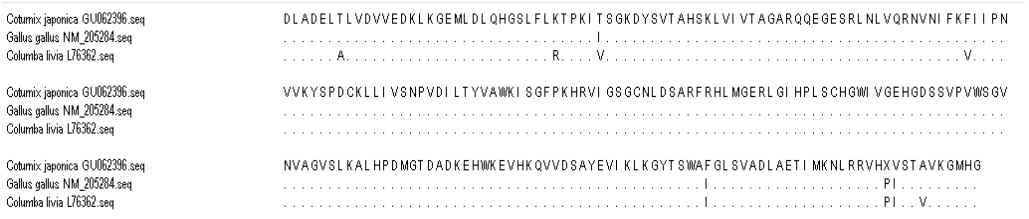
Figure 2. Electrophoretic pattern of LDH isozymes in adult (10 wks) and prepubertal quail testes homogenate (B = quail brain, AT = adult testes and PT = prepubertal testes). The isozymes migrated from top (Cathode) to bottom (Anode).

Sequence analysis of LDHA and LDHB mRNA

The mRNA and deduced protein sequences of *LDHA* and *LDHB* of Japanese quail were analyzed with CLUSTAL W (MegAline) software using IUB matrix. The proteins encoded by these sequences consisted of 240 (Figure 3B) and 269 (Figure 4B) amino acids. The enzyme active site in LDHA was present between 148 to154 amino acid with a motif of VEGHGDS however; in LDHB active site was present between 174 to 180 amino acid with a motif of LGEHGDS. The deduced amino acid sequence of LDHA (Figure 3C) showed 95.0 and 93.8% identity and 5.2 and 6.5% diversity with chicken (NM205284) and rock pigeon (L76362). Similarly, sequences of LDHB (Figure 4C) revealed 82.9, 81.0 and 80.3% identity and 19.1, 21.5 and 22.5% divergence with chicken (NM204177), duck (J03869) and rock pegion (L79957). Further the nucleotide sequence of Japanese quail *LDHA* and *LDHB* revealed that



(A)



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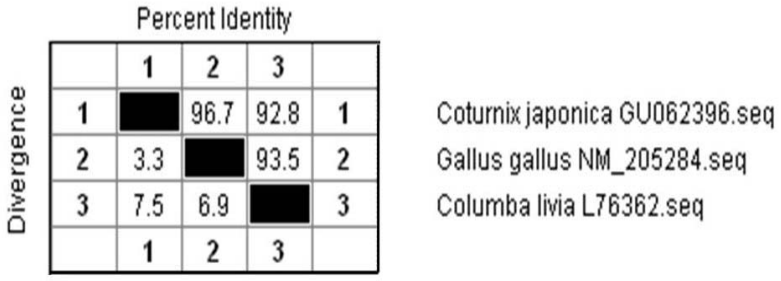
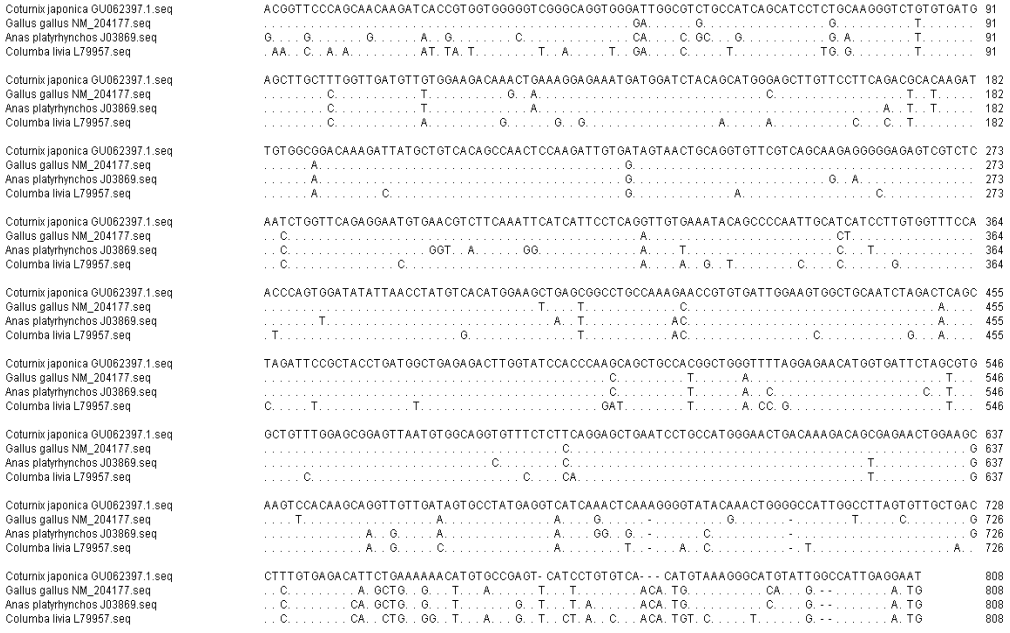
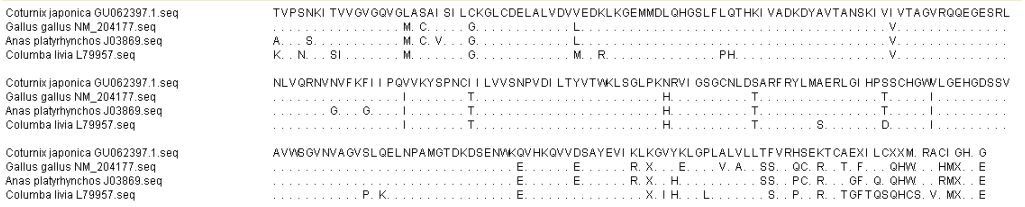


(C)

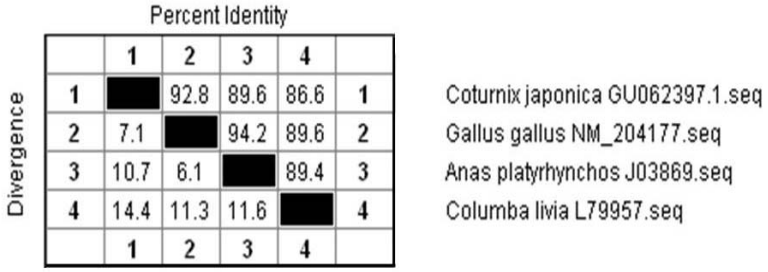
Figure 3. Nucleotide and amino acid sequence of the *LDHA*. (A) Alignment of nucleotide sequences of *LDHA* of quail, chicken and rock pigeon. (B) Alignment of deduced amino acid sequences of LDHA in quail, chicken and rock pigeon. (C) Percent identity of nucleotide sequences among quail, chicken and rock pigeon. The sequence has been deposited in the GenBank database under the accession number GU062396.



(A)



(B)



(C)

Figure 4. Nucleotide and amino acid sequence of the *LDHB*. (A) Alignment of nucleotide sequences of *LDHB* of quail, duck, chicken and rock pigeon. (B) Alignment of deduced amino acid sequences of LDHB in quail, duck, chicken and rock pigeon. (C) Percent identity of nucleotide sequences among quail, duck, chicken and rock pigeon. The sequence has been deposited in the GenBank database under the accession number GU062397.

LDHA sequence was 96.8 and 92.9 percent identity and 3.3 and 7.5 percent divergence to the chicken and rock pegion (Figure 3A), respectively whereas, *LDHB* indicates 92.8, 89.6 and 86.6 percent identity and 7.1, 10.7 and 14.4 percent divergence with chicken, duck and rock pegion (Figure 4A). Phylogenetic analysis of *LDHA* and *LDHB* also confirmed the closeness of Japanese quail with chicken followed by duck and rock pegion (Figure 5A and 5B). The cloned cDNA sequences have been submitted in NCBI under accession numbers GU062396 (*LDHA*) and GU062397 (*LDHB*).

DISCUSSION

The present study reveals difference in *LDH* transcript expressed in testes of prepubertal and adult Japanese quail. *LDHA* and *LDHB* mRNA expression was observed in testes of prepubertal birds whereas only *LDHB* mRNA expression was noticed in adult quail testes. These results reflected a difference in energy metabolism of adult testes as compare to prepubertal testes due to the difference in LDH isozyme. Expression of *LDHA* and *LDHB* mRNA has been confirmed in mammalian (Thomas et al., 1990) and chicken (Arias et al., 2000) testes however, *LDHB* expression was higher in chicken adult testes than in prepubertal testes. A similar situation has been described for the testes specific *LDHC* in mammals (Thomas et al., 1990). This is in support of our results that significant higher expression of *LDHB* was noticed in adult testes as compare to prepubertal testes. These results indicated an energy metabolic shift during development of male germ cells. During spermatogenesis, spermatogonia and mature sperm utilize glucose as their major energy substrate (Nakamura et al., 1984). However, spermatocytes and spermatids suffer a rapid decline in their ATP content in the presence of glucose and require lactate/pyruvate for the maintenance of their ATP concentration (Jutte et al., 1981; Mita and Hall, 1982). Grootegoed et al. (1989) reported that glucose cannot maintain the cellular ATP content, and exposure of isolated spermatids to glucose without other energy substrates soon results in ATP depletion. This is a reason of utilization of lactate as an alternative substrate for ATP production. Since lactate is oxidized by LDH-B isozyme which is encoded by *LDHB* gene. So this could be a reason of higher *LDHB* mRNA expression in adult testes.

The LDH-B isozyme converts lactate into pyruvate which served as fuel for Krebs cycle and oxidative phosphorylation for ATP generation. This type of tissue energy metabolism is present in highly aerobic tissues like heart where lactate is used as preferred energy substrate. Same type of metabolism predominantly found in quail brain (Singh et al., 2010) and probably in germ cells also (Singh et al., 2011a). In the physiological context, lactate may play a key role in at least two conditions. Firstly, lactate is used as an energy substrate, particularly in different tissues, including gonads and brain. In the testes, the concept that sertoli cells metabolize glucose to lactate for the use of germ cells arose because of the capability of cultured sertoli cells to produce high amounts of lactate and the efficient use of lactate, but not glucose, by germ cells. These observations have led to the concept that one of the nurse cell functions of the Sertoli cells is to provide lactate for energy production in spermatocytes and spermatids.

Previous reports are confliction on the expression of *LDHA* in developing germ cells and inversely correlated with its polypeptide. Arias et al. (2000) reported abundant mRNA in mature testes as comparison to immature testes and most somatic tissues with the exception of skeletal muscle (Markert et al., 1975; Markert, 1984; Li et al., 1983; Thomas et al., 1990; Jungmann et al., 1998). The relative LDHA subunit concentration declines in the developing testis, whereas *LDHA* mRNA rises parallel with that of *LDHC* (Alcivar et al., 1991) in mouse. Skidmore and Beebee (1991) reported that relative LDHA subunit activity and concentration decline with age in the developing rat testis whereas Salehi-Ashtiani and Goldberg (1995) reported an increase in *LDHA* mRNA. We could not detect expression of *LDHA* mRNA in adult quail testes. This indicated presence of only LDH-B isozyme in adult quail testes. This type of LDH isozyme is primarily found in

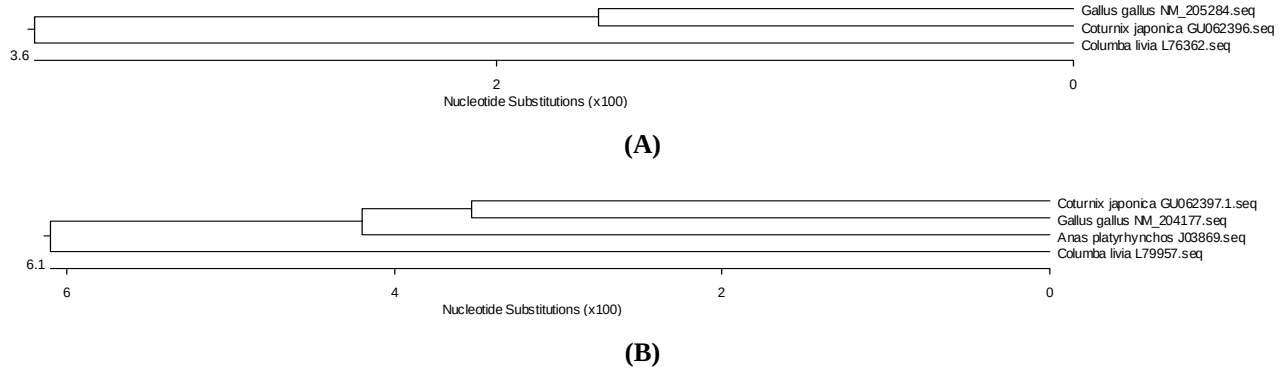


Figure 5. Phylogenic analysis of *LDHA* (A) and *LDHB* (B) sequences of Japanese quail with chicken, duck and rock pigeon.

aerobic tissues like heart where lactate is oxidized. It has been recognized for nearly 20 year that sertoli cells convert glucose to lactate to influence the survival of germ cells (Grootegoed et al., 1989). These cells convert glucose into lactate which is mediated through transcription of *LDHA* mRNA and many other endocrine factors (Boussouar and Mohamed, 2004). Absence of *LDHA* mRNA in adult quail testes seems either lactate producing mechanism in sertoli cells is not mediated by *LDHA* or presence of an extreme type LDH isozyme. However, further studies are needed on quail testes to confirm these assumptions.

Zymogram of both testicular samples revealed only single band at identical position. This indicated presence of same type of LDH isozyme in adult as well as prepubertal testes but expression studies are showing that LDH present in prepubertal testes is made up of association of both A and B subunit whereas in adult testes it is only made up of B subunit. Nucleotide and amino acid sequence analysis of *LDHA* and *LDHB* mRNA revealed significant similarity with chicken, duck and rock pigeon. This indicated presence of two similar type of subunit in these avian species but LDH isozyme may differ because isozyme formed due to the association of two different subunits. The finding of only single band at identical position is anomalous. These results support the idea that isozyme present in quail testes constitute extreme type LDH and that although they have nearly identical electrophoretic mobility. Synthesis of LDH isozyme in this species appears to be different from the usual case of random association of subunits into tetramer.

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