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Genetic Analysis of Chromosomal Regions Affecting Sperm Mobility in the Chicken (Gallus gallus domesticus)

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ABSTRACT

This project is focused on determining the contributions of particular genetic loci to low sperm mobility in the chicken. Sperm mobility has been shown to be a major determinant of male fertility in broiler breeders. A whole genome SNP scan identified chromosomal regions (QTLs) that control sperm mobility. I used microsatellites from 4 chromosomal regions (15.151 on chromosome 6, 12.435 on chromosome Z, 12.341 on chromosome Z, and 17.214 on chromosome Z) to determine the association of these regions with sperm mobility in experimental lines divergently selected for mobility phenotype. Determination of the genetics underlying sperm mobility will identify the genetics of one of the major determinants of male fertility in commercial broiler males.

This thesis is approved for recommendation

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INTRODUCTION

SIGNIFICANCE OF THE PROJECT

The intent of this project is genetic analysis of chromosomal regions which contribute to low sperm mobility phenotype in broilers. Mobility is the ability of a chicken sperm to have directional motility. The aim of this project is using microsatellites to determine genetic linkage of mobility phenotype with previously identified regions.

Over the past several decades, there has been a significant decline in fertility of meat type chickens or broilers, and it is a major economic impediment to productivity in the broiler industry (Froman, 2006). There are different factors affecting fertility in chickens; in these several factors sperm mobility is a new and major determinant of fertility in poultry industry. For enhancing fertility in poultry it is essential to understand sperm mobility and motility.

Previous whole genome association studies identified more than seven chromosomal regions of interest which showed significant linkage disequilibrium in tested sample. These seven chromosomal regions are found within five autosomes and one sex chromosome. Further analysis of these regions showed one region on Gga 6 and two regions on Gga Z showed linkage disequilibrium with sperm mobility phenotype in non-related roosters. These regions show linkage disequilibrium for sperm mobility phenotype in replicate cohorts of commercial pedigree meat-type chickens. My research has been to assess linkage for these same regions in experimental lines divergently selected for sperm mobility phenotype. Linkage disequilibrium was assessed for two successive generations for these three regions.

Whole proteome analysis of high and low mobility sperm has shown that glycolysis and ATP metabolism are repressed in low mobility sperm (Froman, 2011). I measured relative RNA expression levels for several of these genes in testis RNA using quantitative PCR. The reduced levels of these proteins in sperm are not reflected in transcript levels in the total testis.

SPERM MOBILITY

According to Dr. David Froman, the expert on sperm mobility and mobility phenotype, ''sperm mobility, the net movement of a sperm cell population against resistance at body temperature, is a quantitative trait of the domestic fowl''. It is heritable, independent of time, and is the only trait that has been shown to be positively correlated with fertility in domestic fowl and turkeys. According to Froman and Feltmann, 1998, sperm mobility is a new quantitative trait in poultry. By doing non-competitive and competitive fertilization experiments, Froman and McLean, 1996, stated that sperm mobility is a primary determinant of male fitness in chicken. A greater proportion of high-mobility sperm can penetrate the inner perivitelline layer of eggs and is more likely to locate and fuse with the female pronucleus than low mobility sperm (Donoghue, 1998). High mobility sperm can also travel more rapidly between the sperm-storage tubules and the infundibulum than low-mobility sperm (T.R.Birkhead, 1999).

Previous research gave three important observations for sperm mobility phenotype. The first one is sperm mobility phenotype, which was independent of age in New Hampshire roosters (Froman *et al*. 1997). These results can be explained in terms of the concentration of motile sperm and the straight line velocity of individual sperm cells (Froman and Feltmann

2000). The third observation for sperm mobility phenotype is the relationship with mitochondrial function.

SPERM MOTILITY

Sperm cells are self propelled DNA delivery vehicles. Sperm motility is very critical for fertility because sperm have to travel from the hen's vagina to the region where the oviduct's sperm storage tubules are located (Bakst *et al*. 1994). Fertilization is primarily determined by the extent to which sperm are motile within the hen's vagina. Compared to other variables that affect fertility in chicken, sperm motility has not been studied extensively. According to Allen and Champion (1955), Cooper and Rowell (1958), Kamar (1960), and McDaniel and Craig (1962), the fecundity is more dependent upon sperm quality than sperm quantity. Reports of a correlation coefficient >0.70 between sperm motility and fertility proves the relationship (DP Froman, AJ Feltmann, 1997). Sperm motility can be measured objectively by spectrophotometry, videomicroscopy, and computer-assisted sperm motion analysis (Froman and McLean, 1996).

SPERM STRUCTURE

Usually most sperm possess four core elements: 1) a paternal haploid nucleus payload of DNA; 2) a source of energy in the mitochondrial and glycolytic metabolic systems; 3) a motor (the flagellum) to drive the sperm from the male to the egg; and 4) a mechanism for binding with and penetrating the egg (the acrosome). (Sperm biology, An Evolutionary Perspective)

The axoneme

The basic motor structure is the axoneme, the microtubule –based machinery of the eukaryotic cilium and flagellum (Satir & Christensen 2007). The microtubules polymerize from α- and β-tubulin heterodimers and are arranged as nine pairs of doublets, forming a cylinder around a central pair of single microtubules. Each doublet comprises two elements: the A subfiber is a complete microtubule with 13 protofilaments of tubulin subunits whereas the B is a partial one with only 10 or 11 protofilaments sitting anticlockwise to A. To the microtubules various protein structures are attached including the outer and inner dynein arms, radical spokes and central-pair projections. The protein structures comprise cellular enzyme motors, including protein kinases, A-kinase anchoring proteins and phosphatases (Satir & Christensen 2007).

SPERM STORAGE TUBULES

The biological basis of sustained fertility in broiler and turkey hens depends on the capacity to store sperm in the oviductal sperm storage tubules (SST) located in the uterovaginal junction. The sperm storage tubules are lined by a pseudo stratified layer of ciliated and non ciliated secretory cells. (M.R. Bakst, R.K. Bramwell, 2010). Fertilization success of high-mobility sperm depends upon, a greater proportion of high-mobility sperm entering the sperm-storage tubules (T.R.Birkhead, 1999). Donoghue (1998) showed that females inseminated with high-mobility sperm had a significantly greater number of sperm associated with the perivitelline layers of their eggs than females inseminated with the same number of lower-mobility sperm. Other studies on turkeys and domestic fowl have shown that the numbers of sperm on the

perivitelline layers of laid eggs reflect the numbers of sperm originally stored in the sperm-storage tubules (Brillard and Antoine, 1990). Because the space in the sperm-storage tubules is quite limited, only a few percent of the inseminated sperm are retained and stored. And the time available for sperm to enter the tubules is very limited, so high mobility sperm has more proportion to enter in to tubules (T.R. Birkhead, 1999). According to Wishart & Steele 1992, the avian vagina is an extremely hostile environment to sperm, and it can survive there only for a limited time. So before damage ensues, high mobility sperm may therefore be able to escape the vagina and enter the storage tubules.

SPERM COMPETITION

Sperm competition and cryptic female choice have been extremely active areas of research in the last two decades. Factors contributing to reduction of fertility and hatchability include male physical condition (Mc Daniel, 1978), sperm quality (Kirby *et al*. 1998), Muscular and skeletal conformation (Duncan *et al*. 1990) and selection for body weight (Siegel and Dunnington, 1985). Altered musculoskeletal conformation can also cause difficulty in successfully transferring sperm into the oviduct during mating (Mcgary *et al*. 2003). In addition to the above factors sperm competition plays a significant role in fertility reduction in poultry. Sperm competition is defined as the competition between ejaculates of different males for a given set of ova (Parker *et al*. 1970, 1998). Because female red jungle fowl and broiler breeders tend to mate with multiple males (Ligon and Zwartjes, 1995), sperm competition within the female oviduct may contribute to a differential male reproductive success (Birkhead, 1998). To outcompete another male's sperm, males have developed a number of adaptations in which

sperm mobility appears to have crucial effects on the outcome of sperm competition (Birkhead *et al*. 1999).

FERTILIZATION

Usually, a hen starts laying eggs from the age of three months at an average of five eggs a week. For fertilized eggs, the hen has to mate with a rooster. Usually the natural mating season of chickens starts in the spring. In avian males, sperm produced by the testes and carried in the semen. The male reproductive organs in the domestic fowl consist of two testes that are located internally, anterior to the kidneys and attached to the dorsal body cavity. Each testes have a deferent duct that leads from the testes to the cloaca. The deferent duct transports the sperm from the testes where they are formed to the cloaca from which they enter the oviduct of the female when mating. Roosters take only thirty seconds to deposit sperm in the oviduct. The sperm pass through the shell gland of the hen, then isthmus and magnum, finally the infundibulum. It takes almost one week for all this traveling to happen. After reaching infundibulum, the sperm can remain alive for one week, waiting for the eggs to undergo the process of formation.

In the ovaries of the hen, ovum is formed as egg yolk. The yolk released from ovaries travels to the infundibulum. If there is sperm in the infudibulum, the yolk will get fertilized. After fertilization the yolk passes through the same route, through which the sperm travelled to reach the infundibulum. But it travels in the opposite direction. When fertilized yolk is traveling back from infundibulum, it is surrounded by the egg white in the magnum, and formation of the

shell membrane starts in the isthmus. When yolk reaches the shell gland, the shell formation is complete, and the shell gets hardened. So now the egg is ready to lay.

POULTRY INDUSTRY

In general, chickens produce both eggs and meat. However, some breeds are good at producing more eggs, and others are good at producing more meat. To increase the production of both egg and meat, the poultry industry has bred chickens exclusively for production of either meat or egg products. In the US, these commercial breeds are the Broiler and the White Leghorn, respectively. Broilers are efficient in the production of meat type traits, and White Leghorns are efficient in egg production. This breed can easily lay over three hundred eggs a year. Broilers are selected for phenotypes such as increased growth rates, body weight, feed conversion, and breast yield. White Leghorns are selected for characteristics such as age at first lay, total number of eggs produced, and egg quality.

BREEDING PROCESS

All commercial poultry breeders use the same type of breeding methods to develop both broilers and Leghorns for production. These methods are designed to produce millions of high performance chickens from genetically selected elite-pedigree populations. The US poultry industries developed many intense selection methods and multi-generational amplification of breeding stocks. This approach enables hundreds to thousands of pedigree line birds to yield millions to billions of market birds. In the first step, geneticists and primary breeders focus on selection for trait improvements and the number of elite-pedigree populations. Some primary breeding companies work with 40 elite lines and others work with less than 10 elite lines

(Pollock, 1999). Male lines are selected for traits such as breast yield, growth rates and body weight. Female lines are selected on reproduction performance. Best performing progeny are selected from the elite pedigree, and used for next generation breeding. Genetic improvements are then delivered from the elite-pedigree population being selected to the commercial broilers through three multiplication generations (McKay, 2009). The multiplication process involves a series of crosses used to produce millions of chickens expressing a combination of desired traits that were selected for in the elite-pedigree.

PHOTO STIMULATION

In avian species reproductive success is determined by the following environmental variables: photoperiod, diet, ambient temperature, pathogens, toxins, as well as social interaction among birds within a flock. Photo stimulation induces egg and semen production. Specifically, photo stimulation induces lay and once a clutch of eggs is laid, a hen tends to cease ovulating and incubates her clutch. In general, 20 weeks of age is the conventional time at which chickens are photo stimulated, whereas turkeys are usually photo stimulated at 30 weeks of age. In addition to vision, light synchronizes various rhythmic activities in the body and induces hormone production. Both of these functions are essential for reproductive performance in chickens. According to Nestor, K.E and K.I. Brown, (1972), light intensity requirements are affected by the genetics of the hen, and specific requirements should be determined by strain. Chickens respond to all colors of light and reproductive performance is best with the longer (orange-red) wavelengths of visible light (Bissonette, T.H., 1932).

PREDICTING POULTRY FERTILITY

The sperm quality index (SQI) provides an estimate of overall semen quality of roosters (Mcdaniel *et al*. 1998) and toms (Neuman *et al*. 2002). The sperm quality index is determined by monitoring the disruption of a light path created only by the movement of motile sperm within an SQI capillary (P.R.Dumpala, 2006). The SQI is influenced by avian sperm concentration, viability, and motility and it is highly correlated with fertilizing ability of rooster sperm (McDaniel *et al* 1998). An alternative method for SQI that is used to predict poultry fertility is the sperm mobility index (SMI; Froman and Mc Lean, 1996). For the sperm mobility test, sperm concentration of a semen sample is determined by a spectrophotometer and semen is diluted. The suspension is over laid upon pre-warmed 6% Accudenz in a semi-micro polystyrene cuvet and incubated for five minutes at 41°C. After incubation, the cuvet is placed in a spectrophotometer and optical density is determined at 550nm. Absorbance is recorded as the sperm mobility score (Froman and Feltmann, 1998).

CHICKEN GENOME

The chicken (Gallus Gallus) is an important model organism for biomedical research, development and aging. The chicken genome along with large scale cDNA sequencing provided us 20,000+ candidate genes. The roles of most of these candidate genes are unknown. The Chicken was the first animal species in which mendelian inheritance was demonstrated: Additionally the chicken was the first among farm animals to have its genome sequenced. The sequencing of the chicken genome allows the chicken to be a major player in 21st century biology by providing an entry in to new technologies that can be used to explore any chicken

phenotype of interest (J.B. Dodgson, 2005). The chicken served as a model whose experimental populations and mutant stocks were used in basic and applied studies with broad application to other species, including humans (P.B. Siegel, 2006). The chicken karyotype (2n=78) is made up of 38 autosomes and one pair of sex chromosomes, with the female as the heterogametic sex (ZW female, ZZ male). All sequencing libraries were prepared from DNA of a single female of the inbred line of red jungle fowl (USD001) to lessen heterozygocity and provide sequence for both the Z and W sex chromosomes.

The evolutionary distance between chicken and human provides high specificity in detecting functional elements. When chicken genome is compared with mammalian genome there is nearly threefold difference in size between these two genomes, and this difference reflects a substantial reduction in interspersed repeat content, pseudogenes and segmental duplications within the chicken genome. The alignment of the chicken and human genome identifies 70 mega bases of sequence, and it is highly likely to be functional in both species (International Chicken Genome Sequencing Consortium).

MICROSATELLITES

Microsatellites, referred to as a variable number of tandem repeats or VNTRs, are short segments of DNA that have a repeated sequence such as CACACA, and they tend to occur in non-coding DNA. They are also known as simple sequence repeats (SSRs) or short tandem repeats (STRs). Because microsatellites are highly variable and widely dispersed in eukaryotic genomes and are PCR based, they have been used in different research areas. They are used as molecular markers in genetics and population studies. When the number of repeats are 10 or

more than 10, these markers present high levels of inter- and intra- specific polymorphism. Microsatellites have proved to be versatile molecular markers in most population studies. In Forensics microsatellites are generally known as Short Tandem Repeat (STR) loci and are used for forensic identification and relatedness testing and useful markers for early cancer detection.

RFLP and Fingerprint markers were used to enhance map coverage and merge smaller linkage groups (Levin *et al*. 1994). After that there was a growing move toward microsatellite markers in some of poultry genetics laboratories (Haberfeld *et al*. 1991). Although microsatellites are still in wide use, now-a-days virtually all segregating DNA polymorphisms can be used as markers, and single nucleotide polymorphisms (SNP) have become more popular (Siegel *et al. 2011*). Especially coding SNPs have been genotyped for over a decade, (Bumsted *et al*. 1994) and they are very useful in comparative mapping.

MATERIALS AND METHODS

CHICKEN POPULATION

High and low sperm mobility lines of New Hampshire chickens were selected, which reproduces annually. They were maintained by Dr. David Froman at the Oregon agricultural experiment station. The chicks hatched in March. Both high and low sperm mobility lines (n=600) were maintained as random bred flocks. Chicks were reared in floor pens, and then transferred to cages at 20 weeks of age and photo stimulated. Males were phenotyped at 27

weeks of age as outlined by Froman *et al*. (1999). To categorize roosters within lines, repeated measure analysis was used.

DNA

DNA was isolated from a 10µl semen sample from each male using a simple extraction procedure (Bailes *et al*. 2007).

MICROSATELLITE

Microsatellite loci were previously identified in our laboratory with the help of UCSC genome browser and SNPlotyping project (Dr. Douglas Rhoads). There were total seven regions of interest within five autosomes and one sex chromosome and showed significant linkage disequilibrium with respect to sperm mobility. The primers which I am using for amplifying these regions were designed by Dr. Douglas Rhoads, and synthesized by Eurofins-MWG-Operon. The loci, fluorescent labels, and primer sequences are presented in Table 1.

PCR REACTION

PCR was performed in 96 well plates using either an MJ research PTC – 100 thermo cycler (or) an Eppendorf master cycler gradient. PCR was done in 20µl reactions: 1x Buffer (50mM TrisCl pH8.3, 1mM $MgCl_2$ and 3mg/ml BSA), 0.2mM dNTP's, 1µM of mixed forward and reverse primer, and 3 units of Taq polymerase. PCR cycle conditions were as follows: 90°C for 30 secs for an initial denature followed by 42 cycles of 90°C denature for 15 seconds, 50°C annealing for 25 seconds, 72°C elongation for one minute and final elongation for 3 min at 72°C.

DENATURING POLY ACRYLAMIDE GEL ELECTROPHORESIS

PCR products were resolved in 6% denaturing polyacrylamide gels (38:2 acrylamide:MBA, 50% UREA) in 1X TEB (100mM Tris, 10mM boric acid, 2mM EDTA, pH 9.2). The DNA samples were prepared for electrophoresis by mixing 4µl of the PCR product with 4µl of loading buffer (95% formamide, 1X TEB, 0.1% bromo phenol blue). Samples were denatured at 90°C for 3 minutes and immediately placed on ice. Samples were loaded on a 30×40mm, 0.4mm thick 6% denaturing polyacrylamide gel and electrophoresed at 50 watts for 2-4 hours depending upon the predicted fragment sizes.

GEL SCANNING AND GENOTYPE ASSAY

The gels were scanned on a Typhoon fluorescence scanner 8600 (Molecular Dynamics, Amersham Bioscience, and Sunnyvale, California) to detect fluorescent PCR products. PCR fragments were sized using a DNA ladder (CxR, Promega corp).

TESTIS SAMPLE SELECTION AND RNA EXTRACTION

Three testis samples from each mobility line were chosen for mRNA isolation. Each frozen testis sample was mashed into pieces and weighed. GuSCN solution (4M Guanidine Isothiocyanate, 25mM Sodium citrate pH 7, 0.5% Sarkosyl, and 0.1M 2-Mercapto ethanol) was added (5ml per gram of tissue) and homogenized with a Biospec Products Tissue Tearor. The homogenate was mixed with 1/10^{th} volume of 2 M NaOAc pH4.2, then mixed with one volume of AE (50mM NaOAc, 10 mM EDTA, Ph 5.3) buffered phenol. After vigorous mixing, 0.2 volumes

of $CHCl_3$: IsoAmylAlcohol (24:1) was added. After another vigorous mixing, the sample was incubated in ice water for 15 minutes. The solution was centrifuged at 4°C and 10,000rpm for 20 minutes. The aqueous layer was transferred to a new centrifuge tube and mixed with one volume of Isopropanol and incubated at -20°C for 30 minutes. The sample was centrifuged at 10,000rpm for 10 minutes at 4°C, decanted, and the pellet was rinsed with 1 ml 70% ethanol then dried in a centrifugal evaporation system. The dried pellet (RNA) was dissolved in 1ml of TE (10mM TrisCl, 1mM EDTA pH 7.5). The RNA was quantified by spectrophotometry.

cDNA PRODUCTION FROM RNA FOR qPCR

Poly-A+ mRNAs were isolated using Poly Attract kit (Promega Life Sciences) according to the manufacturer's instructions. Forty micrograms of poly A+RNA were made 0.3 M with KOAc, precipitated with cold ethanol, and centrifuged. After drying, 20µl of Te (10mM TrisCl, 0.1mM EDTA pH7.5) was added to the pellet. Then 20µl 5x FS Buffer (Invitrogen), 1µl 20mM dNTPs, 2µl 30µM $CT_{23}V$ as primer, 5µl of 100mM DTT, and 47µl of water were added to the mRNAs. The mixture was first incubated at 50°C for ten minutes and then 37°C for 10 minutes. After incubation, 20 Ul of RNAsin and 1000 U RNAseH- MMLV Reverse Transcriptase SuperScriptII was added. After incubation at 37°C for one hour, the reaction was terminated with 100µl TE, 2.5µl of 10% SDS and 2.5µl of 250 mM EDTA. Finally the solution was extracted sequentially with 50:50 phenol: $CHCl_3$ AND $CHCl_3$-IAA. The cDNA was precipitated with ethanol and the DNA pellet dissolved in 200µl Te.

qPCR ANALYSIS

qPCR analysis was done on three glycolytic enzymes, which were phosphoglycerate kinase 1 and lactate dehydrogenase B and glucose transporter 3. UCSC genome browser and BLAT search were used to get the genomic sequence information for above three genes in chicken. Genomic sequence information was analyzed for all the three genes and the exonic information was retrieved using Edit seq software. Using this exonic sequence information and Primer 3 version 0.4 (http://frodo.wi.mit.edu/primer3/), 20 to22 nucleotide length primers were designed for qPCR analysis. PCR was performed in 20µl reactions: 1x SYBR Green buffer (50mM TrisCl pH 8.3, 1mM Mgcl2 and 3mg/ml BSA, 1:20000 SYBR Green- Lonza, Rockland, ME), 0.2mM dNTP's, 10µM of mixed forward and reverse primer and 3 units of Taq polymerase. PCR cycle conditions were as follows: 90°C for 30 seconds for an initial denature followed by 39 cycles of 90°C denature for 15 seconds, 55°C annealing for 15 seconds, 72°C elongation for 30 seconds and final elongation for 3 min at 72°C. qPCR reactions were ran in BioRad CFX96 using ribosomal protein S14 as a reference to compute delta-Ct values. Three individuals from each line were analyzed along with adult liver as a control.

STATISTICAL ANALYSIS

Genotype and allele counts were analyzed in MS Excel. For each locus we analyzed the population by dividing the population into subpopulations based on mobility score. Expected allele frequencies for a sub population were calculated based on the allele frequency observed in the entire population. Chi-square analysis was used to compare actual counts to expected counts with respect to phenotype.

RESULTS

GENOTYPIC ANALYSIS

For genotypic analysis I used the 2010 sperm DNA samples provided by Dr. David Froman (Oregon State University). These sperm DNA samples were from roosters whose mobility phenotypes had been determined by Dr. Froman and represented both the low and high lines. The goal was to determine genotypes for these samples for comparison to data we had obtained for the 2009 generation. In total we had 290 high line samples and 270 low line samples from 2010. From 2009, we had 191 high line samples and 192 low line samples. Based on mobility score, high line samples were divided into both high and low sub populations, and low line samples into low and high sub populations. For low line samples, we assigned mobility scores from 0-9 as the low mobility sub population, and mobility scores above 10 were classified in the high mobility sub population. In high line samples, mobility scores from 1-37 were classified in the low mobility sub population, and mobility scores above 37 were classified in the high mobility sub population. This roughly divides the low line into the lower $2/3^{rd}$ vs. upper $1/3^{rd}$ and the high line into lower $1/3^{rd}$ vs. higher $2/3^{rd}$. For the 2009 low line, we had 133 samples in the low mobility sub population, and 59 samples in the high mobility sub population. For the high line we had 60 samples in the low mobility sub population, and 131 samples in the high mobility sub population. In 2010, for the low line we had 195 samples in the low mobility sub population, and 70 samples in the high mobility sub population. In high line we had 47 samples in the low mobility sub population, and 141 samples in the high mobility sub population.

Analysis of Gga6 in the region of15.151Mbp used ADR57. In 2009, we had a total of eight alleles in both low and high line samples. The eight alleles were between 115 and 137 base pairs. Alleles 125, 121, and 115 were the major alleles in low line samples (Table2). There were a total of twelve genotypes found in low line (Table3). The genotypes 125/125, 133/125, and 125/121 were the major genotypes. Alleles 137, 121, and 119 were the major alleles in high line samples (Table4). In high line 137/137, 137.121, and 137/119 were the major genotypes (Table5). In 2009 high line samples, the chi square analysis of genotype 137/137 gave P value of 0.03. This significant P value suggests an association of genotype 137/137 with sperm mobility phenotype.

In 2010, analysis of the same region using ADR57 found eight alleles between 115 and 137 base pairs. In low line samples of 2010, we did not see any significant changes in allele number or allele frequency from 2009. The only change we found was allele 133 frequency decreased from 20% to 4% from 2009 to 2010 in high mobility sub population (Table2). There were no significant changes for number of genotypes and major genotypes in 2010 low line samples from 2009. The only change was genotype 125/125 was increased from 20% to 29% in low mobility sub population, and 1% to 26% in high mobility sub population (Table3). We observed significant changes in both allele and genotype frequencies of high line samples from 2009 to 2010. In high line low mobility sub population, allele 137 frequency decreased from 67% to 35%, and in high mobility sub population allele 137 frequency decreased from 56% to 43% (Table4). In high line low mobility sub population, the genotype 137/137 frequency decreased from 50% to 6%, and in high mobility sub population it was decreased from 29% to 20%

(Table5). Chi square analysis showed there was no significant association of allele and genotype with sperm mobility phenotype in both low and high line samples of 2010.

Analysis of Gga 'Z' in the region of 12.341Mbp used ADR58. In 2009, we had a total of three alleles in both low and high line samples. The three alleles were 92, 94 and 96 base pairs (Table6). In low line samples allele 92 was 39%, and allele 96 was 58% with 92/92 and 92/96 the major genotypes (Table7). In high line samples allele 92 was 25%, and allele 96 was 71% with 92/92 and 92/96 the major genotypes (Table8, 9). Chi square analysis showed there was no significant association of allele and genotype with sperm mobility phenotype in both low and high line samples of 2009.

In 2010, analysis of the same region using ADR58 found the same three alleles. In low line samples of 2010, we did not see any significant changes in allele frequency from 2009 (Table6). We observed changes in genotype frequencies of low line samples of 2010. Overall, the homozygous genotype 96/96 increased from 10% to 21% in low line samples. In low mobility sub population the same genotype increased from 11% to 22%, and in high mobility sub population it increased from 7% to 18%. Overall, the heterozygous genotype 92/96 decreased from 52% to 38% in low line samples. In low mobility sub population it decreased from 51% to 37%, and in high mobility sub population it similarly decreased from 54% to 39% (Table7). We did not see any major changes in both allele and genotype frequencies of high line samples from 2009 to 2010 (Table8). The only change was genotype 92/92 decreased from 59% to 40% and, the genotype 92/96 increased from 30% to 53% in low mobility sub population (Table9).

Chi square analysis showed there was no significant association of allele and genotype with sperm mobility phenotype in both low and high line samples of 2010.

Analysis of Gga 'Z' in the region of 12.435Mbp used ADR55. In 2009, we had one 121 base pair allele in low line samples (Table10), and two alleles in high line samples. The two alleles were 121 and 127 base pairs (Table12). Overall, the homozygous genotype 127/127 represented 28%, and the heterozygous genotype 121/127 represented 48% in high line samples (Table13). Genotype 121/121 was 30% in high mobility sub population, and 11% in low mobility sub population (P=0.02). This significant P value suggests an association of genotype 121/121 with sperm mobility phenotype in the high line.

In 2010, analysis of the same region using ADR55 found the same two alleles. Like 2009, the low line was homozygous for allele 121 (Table10). In high line, allele 121 frequency increased from 48% to 79% from 2009 to 2010. The other allele 127 frequency decreased from 52% to 21% from 2009 to 2010 (Table12). In 2010 high line samples, the genotype 127/127 decreased from 28% to 4%, and the other major genotype 121/127 decreased from 48% to 34%. The genotype 121/121 frequency increased from 23% to 62% in 2010 high line samples (Table13). Chi square analysis showed there was no significant association of allele and genotype with sperm mobility phenotype in high line samples of 2010.

Analysis of Gga 'Z' in the region of 17.214Mbp used ADR38. In 2009, we had two alleles of 108 and 130 base pairs in low line samples (Table14), and the high line was homozygous for allele 130. Overall, the homozygous genotype 108/108 represented 39%, and the heterozygote genotype 108/130 represented 43% in low line samples (Table15). Chi square analysis showed

there was no significant association of allele and genotype with sperm mobility phenotype in both low and high line samples of 2009.

In 2010, analysis of the same region using ADR38 detected alleles 108 and 130 in low line samples (Table14), and the high line was still homozygous for allele 130. We did not see any significant changes in both allele and genotype frequencies from 2009 to 2010 (Table15). Chi square analysis showed there was no significant association of allele and genotype with sperm mobility phenotype in both low and high line samples of 2010.

DIFFERENTIAL GENE EXPRESSION

Proteomic analysis of high vs. low mobility sperm identified significant differences in proteins for glycolysis and ATP metabolism (Froman *et al.* 2010). The proteins for these pathways identified as lower in low mobility sperm are listed in Table 18.

To test the gene expression levels of testis transcriptome in both high and low mobility lines, I analyzed three testis samples each from low and high lines. Low line samples were 112, 124 and 142, and high line samples were 203, 218 and 220. RNA was extracted from testes and cDNA was produced for qPCR analysis. From a list of glycolysis and ATP metabolism genes (Table 19), I selected three genes from the glycolysis pathway and four genes from ATP metabolism for qPCR reaction.

The glycolysis genes were phosphoglycerate kinase 1, lactate dehydrogenase B, and glucose transporter 3. The reasons for selecting these genes are phosphoglycerate kinase is the first glycolytic enzyme that generates ATP. Lactate dehydrogenase is near the end of glycolysis and

functions under low oxygen tension, and glucose transporter 3 helps in glucose uptake. The selected ATP genes were ATP5A1, ATP51, MYO9B and NDUFS1. The protein NDUFS1 has NADH dehydrogenase activity and oxidoreductase activity. It transfers electrons from NADH to the respiratory chain. Myosin 9B uses ATP for intracellular movements. The genes ATP5A1 and ATP51 are subunits of mitochondrial ATP synthase. Mitochondrial ATP synthase is an inner membrane complex that utilizes proton motive force to generate ATP from ADP and Pi.

Ribosomal protein S14 served as a reference to compute delta-Ct values. Three individuals from each line were analyzed along with adult liver as a control. In low line samples the delta-Ct values for glucose transporter 3 ranged from 9.7 to 13, and in high line samples the range is from 11.7 to 12.4. In low line samples the delta-Ct values for lactate dehydogenase B ranged from 11.6 to 15.4, and in high line samples the range is from 12.5 to 14.0. In low line samples the delta-Ct values for phosphoglycerate kinase ranged from 3.1 to 9.1, and in high line samples the range is from 2.6 to 7.6 (Graph 1).In low line samples the delta-Ct values for ATP51 ranged from 5.8 to 8.7, and in high line samples the range is from 6.8 to 8.1. In low line samples the delta-Ct values for ATP5A1 ranged from 4.3 to 7.0, and in high line samples the range is from 3.5 to 7.1. In low line samples the delta-Ct values for MYO9B ranged from -6.3 to -8.1, and in high line samples the range is from -5.4 to -8.3. In low line samples the delta-Ct values for NDUFS1 ranged from -6.8 to -7.9, and in high line samples the range is from -6.4 to -7.9 (Graph 2). Since the delta-Ct ranges were very similar for the low and high line testis samples, we can conclude that the differences in protein levels detected in sperm cells are not reflected in the RNA levels of the whole testis.

DISCUSSION

The analysis of 2009 sperm samples showed differences between phenotypes of both low and high line samples. There were significant differences for both allele and genotype frequencies between low mobility sub population and high mobility sub population. On chromosomal region Gga 6:15Mbp, in the high line the homozygous genotype 137/137 was 50% in low vs. 29% in high mobility sub population. For this same region, in the low line the homozygous genotype 125/125 was 20% in the low vs. 1% in the high mobility sub population. For chromosomal region Gga Z: 12.3Mbp, in the high line the heterozygous genotype 92/96 was 30% in the low and 44% in the high mobility sub population. For chromosomal region Gga Z:12.4Mbp, in the high line allele 127 was 62% in the low and 48% in high mobility sub population, while allele 121 was 38% in the low and 52% in the high mobility sub population. Additionally, genotype 121/121 was 11% in the low and 30% in the high mobility sub population, while genotype 121/127 was 54% in the low vs. 45% in the high mobility sub population.

When I compared allele frequencies and genotype frequencies of both 2009 and 2010 samples, overall there were not many substantial changes for the three loci. However, the genotypic differences between high and low sub-populations observed in 2009 were not repeated in 2010 sperm samples. The only significant differences I found in both allele and genotype frequencies were on chromosomal region Gga 6:15mbp. The reasons for the apparent shifts in allele and genotype frequencies from 2009 to 2010 are not known. They may be due to selection or genetic drift. Replication of the lines every generation has been through selection

of 40 to 50 sires representing the median mobility of each line and 60 to 70 hens. Semen is pooled from 5 males for artificial insemination onto approximately 10 dams. Which sires and dams are used is rotated on a daily basis. Each generation is approximately 500 to 600 progeny produced every year. Since semen is pooled and each dam is inseminated with different mixtures every 3 to 5 days, the contributions of each sire and dam to the next generation cannot be predicted. Therefore, shifts in allele and genotype frequencies are likely to represent a founder affect from the small number of sires and sperm competition in the oviduct.

When we analyzed three regions on two different chromosomes locus Gga6:15mbp showed association with sperm mobility phenotype. The remaining loci on GgaZ no longer showed any association with sperm mobility. Current efforts are directed at extending our analyses of Gga 6:15, because we have identified a candidate gene KCNMA1 in this region. KCNMA1 gene codes for a calcium activated potassium channel, as a possible locus for mutations that contribute to phenotypic variation in sperm mobility. In chickens, KCNMA1 has been shown to be differentially spliced in cochlear cells (Miranda Rottman et al., 2010). Previous research showed that alternative splicing is a frequent cause of mutations in several different genes controlling calcium-activated protein channels (Tian et al., 2001). If KCNMA1 has a significant role in sperm mobility, the testis-specific alternative splicing could be the cause of differentiation between the high and low mobility line. Further investigation of this chromosomal region by developing SNP assays for this region could further elucidate the genetics underlying low sperm mobility. Additional mapping is necessary possibly using a higher density SNP panel that is now available. The previous SNP mapping used on 1700 informative SNPs for a comparison of samples from the high and and low lines, along with a F2 cross of a single low mobility broiler male onto

leghorn females. There is a 60K SNP panel now available. If this was applied to reciprocal crosses between the high and low lines many more regions would likely be identified.

Research into gene expression and proteomics will enable us to decipher the contributions to sperm mobility phenotype. Knowledge of the DNA sequence is not enough on its own to explain how cells work and what goes wrong when disease strikes. To understand these complex processes, we need to study the expression of genes in the testis and developing sperm. This information gives clues to the biological role of the encoded protein. If sperm cell proteomes differ between low and high sperm mobility roosters, then the particular level of expression may be reflected only in particular cell types.

Differential gene expression studies using testis samples from both low and high mobility line roosters did not show any difference in gene expression levels. The absence of different testicular gene expression levels between low and high sperm mobility lines may be due to the fact that the sperm cell transcriptome is a minor component of the total testis transcriptome. Otherwise, there could be specific pathways like post-translational protein degradation (or) Mi RNA mediated pathways which are controlling the protein levels in low sperm mobility lines. I compared the expressed levels of the genes I analyzed in chickens with expression levels in humans. I used NCBI (National Center for Biotechnology Information) GENE viewer to obtain the relative expression levels based on EST data from human testis. I compared the obtained expression levels in humans and chickens relative to our reference gene rps14. In humans expression was estimated from sequences in EST data, while the chicken estimates are from my delta-Ct values from qPCR. While ATP5A1 is expressed nearly twenty five times higher that

rps14 in human testis, my data indicate it is 32 times higher in chicken testis. The other ATP gene ATP51 was expressed at 0.25x of rps14 in human, while 250x in chicken. The other two ATP genes MYO9B and NDUFS1 were expressed at levels approximately equal to rps14 in humans, but at 0.008x in chicken. The glycolysis gene PGK1 expressed around 8x in human, and 32x in chicken. The gene GLUT3 expressed around 45x in human, and nearly 4000x in chicken. LDHB expressed approximately 9x in human and 8000x in chicken. The above results indicating that the expression levels of ATP and Glycolysis genes are markedly different in human and chicken testes.

We are currently using NextGen sequencing to compare the total testis transcriptomes from the low and high lines. This will provide a clearer picture of how expression of particular genes correlates with sperm mobility. I am interested in determining whether there is differential expression of any genes in the two regions on chromosome Z on which we have focused. Specifically there are three candidate genes in this region: DMRT1, SOX9, and PTGDS. The gene DMRT1 is transcribed specifically in the testis and, it is believed to be the bird sex- determining gene. The SOX9 gene is specifically expressed in male differentiating Sertoli cells during chicken gonadal development. The gene PTGDS is a male specific gene during chicken testicular development and is temporally expressed in the same window as SOX9 in Sertoli cells. We hypothesize that these gene expression results will show some linkage for low mobility sperm in chickens. We need to design further experiments which address this hypothesis.

CONCLUSION

Fertility and hatchability are critical components of the broiler breeder industry, and they have decreased in recent years. Sperm mobility affords a means of identifying highly fertile males within a flock and can be used as a selection criterion, and this trait is heritable. To date, breeders have viewed the motile nature of sperm as an essential but uncontrollable variable. However genetic progress may be possible through selection.

Primer Name	Locus:Mb	Primer (5'→3') Forward	Reverse	Cycles	Annealing Temp.	Alleles	*Size Range (bp)*
ADR57	*Gga6:15.151*	*Fam-CACACCTTCCAGCTCCTATGAT*	*TGTTTTTAAATTTCTTTTGAAACTG*	*43*	*50*	*8*	*115-137*
ADR58	GgaZ:12.341	Cy3-CACCTATGTACTATTTCTATGTATC	CAGCATGCTACACTAGATGTC	43	50	3	*92-96*
ADR55	GgaZ:12.435	Cy5-ACACTTAAATGTTTCTCTGCTGT	GGTTAAAATGAACACTTGCTGCT	43	50	3	*121-133*
ADR38	GgaZ:17.214	Cy3-CAATCATTCACTGCTACTTTTGTCTC	GATTCTTCCTCAATAAATGTTGCACC	43	50	3	*108-130*

Table 1 Four chromosomal regions analyzed with four different primer pairs for allele and genotype counts. *The primer pairs with the chromosomal location, number of alleles and the sequence of both forward and reverse primers with annealing temperatures are listed. Forward primers were labeled on their 5' end with fluorescent tags as indicated.*

Low Line		2010	2009	2010	2009	2010	2009
ADR57		All	All	Low	Low	High	High
n		438	300	316	204	122	96
allele	size	Freq	Freq	Freq	Freq	Freq	Freq
1	137	0.05	0.00	0.03	0.00	0.11	0.00
2	135	0.01	0.05	0.01	0.04	0.00	0.05
3	133	0.06	0.15	0.07	0.12	0.04	0.20
4	125	0.50	0.44	0.52	0.46	0.44	0.40
5	121	0.19	0.19	0.17	0.21	0.22	0.16
6	119	0.02	0.00	0.01	0.00.	0.06	0.00
7	117	0.00	0.00	0.00	0.00	0.00	0.00
8	115	0.17	0.18	0.18	0.17	0.13	0.20

Table 2 Allele frequencies for chromosomal region Gga '6' on 15.151Mbp using marker ADR 57 in low sperm mobility lines. *Number of total alleles with different sizes and allele frequencies in 2009 and 2010 of all, low and high phenotype results are listed.*

Low Line	2010	2009	2010	2009	2010	2009
ADR 57	All	All	Low	Low	High	High
n	181	150	130	102	51	48
Genotype	Freq	Freq	Freq	Freq	Freq	Freq
11	0.03		0.01		0.06	
44	0.28	0.17	0.29	0.20	0.26	0.01
55	0.03	0.03	0.02	0.03	0.03	0.02
88	0.05	0.05	0.06	0.06	0.03	0.02
12	0.01		0.01		0.00	
15	0.03		0.01		0.06	
16	0.03		0.01		0.06	
24		0.06		0.07		0.04
25		0.01		0.02		0.00
28	0.01	0.02	0.01	0.00	0.00	0.06
34	0.08	0.17	0.12	0.14	0.00	0.25
35	0.03	0.09	0.01	0.1	0.06	0.08
36	0.01		0.00		0.03	
38	0.01	0.03	0.01	0.01	0.00	0.06
45	0.21	0.16	0.20	0.18	0.23	0.13
48	0.17	0.15	0.17	0.14	0.17	0.17
58	0.05	0.07	0.06	0.07	0.03	0.06

Table 3 Genotype frequencies for chromosomal region Gga '6' on 15.151Mbp using marker ADR 57 in low sperm mobility lines. *Different allele combinations and their frequency results of years 2009 and 2010 in all, low and high phenotype results are listed.*

High Line		2010	2009	2010	2009	2010	2009
ADR57		All	All	Low	Low	High	High
n		574	332	92	104	482	228
allele	size	Freq	Freq	Freq	Freq	Freq	Freq
1	137	0.42	0.59	0.35	0.67	0.43	0.56
2	135	0.08	0.08	0.15	0.02	0.07	0.11
3	133	0.02	0.02		0.02	0.03	0.02
4	125	0.06		0.09		0.05	
5	121	0.25	0.17	0.26	0.13	0.24	0.19
6	119	0.15	0.14	0.09	0.15	0.16	0.13
7	117	0.00	0.00	0.00	0.00	0.00	0.00
8	115	0.03	0.01	0.06	0.01	0.03	

Table 4 Allele frequencies for chromosomal region Gga '6' on 15.151Mbp using marker ADR 57 in high sperm mobility lines. *Allele frequencies in 2009 and 2010 of all, low and high phenotype results are listed along with total allels and allele number.*

High Line	*2010*	*2009*	*2010*	*2009*	*2010*	*2009*
ADR57	*All*	*All*	*Low*	*Low*	*High*	*High*
n	*286*	*165*	*46*	*52*	*240*	*113*
Genotype	*Freq*	*Freq*	*Freq*	*Freq*	*Freq*	*Freq*
11	*0.18*	*0.36*	*0.06*	*0.50*	*0.20*	*0.29*
22		*0.01*				*0.02*
33		*0.01*		*0.02*		
44	*0.02*				*0.02*	
55	*0.05*	*0.02*	*0.06*	*0.04*	*0.05*	*0.02*
66	*0.02*	*0.02*		*0.04*	*0.02*	*0.02*
88	*0.01*				*0.01*	
12	*0.09*	*0.07*	*0.12*	*0.02*	*0.09*	*0.09*
13	*0.02*	*0.02*			*0.02*	*0.04*
15	*0.22*	*0.22*	*0.35*	*0.13*	*0.20*	*0.25*
16	*0.15*	*0.17*	*0.12*	*0.19*	*0.15*	*0.16*
24	*0.01*		*0.06*			
25	*0.03*	*0.04*	*0.06*		*0.03*	*0.05*
26	*0.02*	*0.02*	*0.06*	*0.02*	*0.01*	*0.03*
28		*0.01*				*0.01*
34	*0.02*				*0.02*	
35	*0.01*				*0.01*	
45	*0.02*				*0.02*	
48	*0.03*		*0.12*		*0.02*	
56	*0.09*	*0.03*		*0.02*	*0.11*	*0.04*
58	*0.01*	*0.01*		*0.02*	*0.01*	

Table 5 Genotype frequencies for chromosomal region Gga '6' on 15.151Mbp using marker ADR 57 in high sperm mobility lines. *Different allele combinations and their frequency results of years 2009 and 2010 in all, low and high phenotype results are listed.*

Low Line		2010	2009	2010	2009	2010
ADR58		All	All	Low	Low	High
n		292	370	170	258	122
allele	size	Freq	Freq	Freq	Freq	Freq
1	96	0.40	0.39	0.40	0.40	0.41
2	94	0.00	0.00	0.00	0.00	0.00
3	92	0.60	0.58	0.60	0.57	0.59

Table 6 Allele frequencies for chromosomal region Gga 'Z' on 12.341Mbp using marker ADR58 in low sperm mobility lines. *Allele frequencies in 2009 and 2010 of all and low phenotype results are listed along with total alleles and their size*

Low Line	2010	2009	2010	2009	2010
ADR 58	All	All	Low	Low	High
n	221	185	161	129	60
Genotype	Freq	Freq	Freq	Freq	Freq
11	0.20	0.10	0.19	0.11	0.22
22	0	0	0	0	0
33	0.36	0.32	0.35	0.31	0.40
12	0	0	0	0	0
13	0.44	0.52	0.46	0.51	0.38

Table 7 Genotype frequencies for chromosomal region Gga 'Z' on 12.341Mbp using marker ADR58 in low sperm mobility lines. *Different allele combinations and their frequency results of years 2009 and 2010 in all and low phenotype results are listed*

High Line		2010	2009	2010	2009	2010
ADR 58		All	All	Low	Low	High
n		456	360	82	112	374
allele	size	Freq	Freq	Freq	Freq	Freq
1	96	0.32	0.25	0.28	0.23	0.33
2	94	0.00	0.00	0.00	0.00	0.00
3	92	0.68	0.71	0.72	0.74	0.67

Table 8 Allele frequencies for chromosomal region Gga 'Z' on 12.341Mbp using marker ADR58 in high sperm mobility lines. *Total alleles with their size and allele frequencies in 2009 and 2010 of all and low phenotype results are listed.*

High Line	2010	2009	2010	2009	2010
ADR58	All	All	Low	Low	High
n	232	180	41	56	191
Genotype	Freq	Freq	Freq	Freq	Freq
11	0.11	0.02	0.05	0.05	0.13
22	0	0	0	0	0
33	0.46	0.52	0.49	0.59	0.45
12	0	0	0	0	0
13	0.43	0.39	0.46	0.30	0.42

Table 9 Genotype frequencies for chromosomal region Gga 'Z' on 12.341Mbp using marker ADR58 in high sperm mobility lines. *Different allele combinations and their frequency results of years 2009 and 2010 in all and low phenotype results are listed.*

Low Line		2010	2009	2010	2009	2010	2009
ADR55		All	All	Low	Low	High	High
n		522	160	386	80	136	80
Allele	size	Freq	Freq	Freq	Freq	Freq	Freq
1	133	0	0	0	0	0	0
2	127	0	0	0	0	0	0
3	121	1.00	1.00	1.00	1.00	1.00	1.00

Table 10 Allele frequencies for chromosomal region Gga 'Z' on 12.435Mbp using marker ADR 55 in low sperm mobility lines. *Allele frequencies in 2009 and 2010 of all, low and high phenotype results are listed along with total allels and allele number.*

Low Line	2010	2009	2010	2009	2010	2009
ADR55	All	All	Low	Low	High	High
n	261	80	193	40	68	40
Genotype	Freq	Freq	Freq	Freq	Freq	Freq
11	0	0	0	0	0	0
22	0	0	0	0	0	0
33	1.00	1.00	1.00	1.00	1.00	1.00
23	0	0	0	0	0	0

Table 11 Genotype frequencies for chromosomal region Gga 'Z' on 12.435Mbp using marker ADR 55 in low sperm mobility lines. *Different allele combinations and their frequency results of years 2009 and 2010 in all, low and high phenotype results are listed.*

High Line		2010	2009	2010	2009	2010	2009
ADR55		All	All	Low	Low	High	High
n		536	324	90	108	446	216
allele	size	Freq	Freq	Freq	Freq	Freq	Freq
1	133	0	0	0	0	0	0
2	127	0.21	0.52	0.19	0.62	0.21	0.48
3	121	0.79	0.48	0.81	0.38	0.79	0.52

Table 12 Allele frequencies for chromosomal region Gga 'Z' on 12.435Mbp using marker ADR 55 in high sperm mobility lines. *Allele frequencies in 2009 and 2010 of all, low and high phenotype results are listed along with total allels and allele number.*

High Line	2010	2009	2010	2009	2010	2009
ADR55	All	All	Low	Low	High	High
n	268	162	45	54	223	108
Genotype	Freq	Freq	Freq	Freq	Freq	Freq
11	0	0	0	0	0	0
22	0.04	0.28	0.00	0.35	0.05	0.25
33	0.62	0.23	0.62	0.11	0.62	0.30
23	0.34	0.48	0.38	0.54	0.33	0.45

Table 13 Genotype frequencies for chromosomal region Gga'Z' on 12.435Mbp using marker ADR 55 in high sperm mobility lines. *Different allele combinations and their frequency results of years 2009 and 2010 in all, low and high phenotype results are listed.*

Low Line		2010	2009	2010	2009	2010	2009
ADR38		All	All	Low	Low	High	High
n		432	334	308	222	124	112
allele	size	Freq	Freq	Freq	Freq	Freq	Freq
1	130	0.48	0.40	0.47	0.40	0.50	0.38
2	122	0	0	0	0	0	0
3	108	0.52	0.60	0.53	0.60	0.50	0.62

Table 14 Allele frequencies for chromosomal region Gga 'Z' on 17.214Mbp using marker ADR 38 in low sperm mobility lines. *Allele frequencies in 2009 and 2010 of all, low and high phenotype results are listed along with total allels and allele number*

Low Line	2010	2009	2010	2009	2010	2009
ADR38	All	All	Low	Low	High	High
n	216	167	154	111	62	56
Genotype	Freq	Freq	Freq	Freq	Freq	Freq
11	0.25	0.18	0.23	0.18	0.29	0.18
22	0	0	0	0	0	0
33	0.28	0.39	0.28	0.38	0.29	0.41
13	0.47	0.43	0.49	0.44	0.42	0.41

Table 15 Genotype frequencies for chromosomal region Gga 'Z' on 17.214Mbp using marker ADR 38 in low sperm mobility lines. *Different allele combinations and their frequency results of years 2009 and 2010 in all, low and high phenotype results are listed.*

High Line		2010	2009	2010	2009	2010	2009
ADR38		All	All	Low	Low	High	High
n		474	230	76	76	398	154
allele	size	Freq	Freq	Freq	Freq	Freq	Freq
1	130	0.98	1.00	0.91	1.00	0.99	1.00
2	122	0	0	0	0	0	0
3	108	0.02	0	0.09	0	0.01	0

Table 16 Allele frequencies for chromosomal region Gga 'Z' on 17.214Mbp using marker ADR 38 in high sperm mobility lines. *Allele frequencies in 2009 and 2010 of all, low and high phenotype results are listed along with total allels and allele number.*

High Line	2010	2009	2010	2009	2010	2009
ADR38	All	All	Low	Low	High	High
n	237	115	38	38	199	77
Genotype	Freq	Freq	Freq	Freq	Freq	Freq
11	0.97	1.0	0.89	1.0	0.98	1.0
22	0	0	0	0	0	0
33	0.01	0	0.08	0	0	0
23	0.02	0	0.03	0	0.02	0

Table 17 Genotype frequencies for chromosomal region Gga 'Z' on 17.214Mbp using marker ADR 38 in high sperm mobility lines. *Different allele combinations and their frequency results of years 2009 and 2010 in all, low and high phenotype results are listed.*

Gene Index	Protein (*Gallus gallus*)	*P*-value[1]	Expression[2]
46048651	Facilitated glucose transporter member 3	< 0.0001	up
45383904	Hexokinase 1	--	--
45383696	Hexokinase 2	--	--
57524920	Phosphoglucose isomerase	< 0.0001	up
71895485	Phosphofructokinase	< 0.0001	up
157951672	Aldolase	< 0.0001	up
45382061	Triose phosphate isomerase	< 0.0001	up
46048961	Glyceraldehyde-3-phosphate dehydrogenase	< 0.0001	up
45384486	Phosphoglycerate kinase 1	< 0.0001	up
71895985	Phosphoglycerate mutase	< 0.0001	up
46048768	α-Enolase	< 0.0001	up
46048765	β-Enolase	< 0.0001	up
45382393	γ-Enolase	0.0263	up
45382651	Pyruvate kinase, muscle	< 0.0001	up
45384208	Lactate dehydrogenase, A chain	< 0.0001	up
4538766	Lactate dehydrogenase, B chain	< 0.0001	up

Table 18 Glycolytic enzymes and related proteins identified by mass spectrometry after differential detergent fractionation of sperm from roosters selected for low and high sperm mobility.

[1] Probability that protein expression differed between lines. In the case of missing values, the protein in question was detected but the amount was inadequate to insure a valid test between lines.

[2] Denotes high line relative to low line. In the case of missing values, the protein in question was detected but the amount was inadequate to confirm a difference in expression between lines.

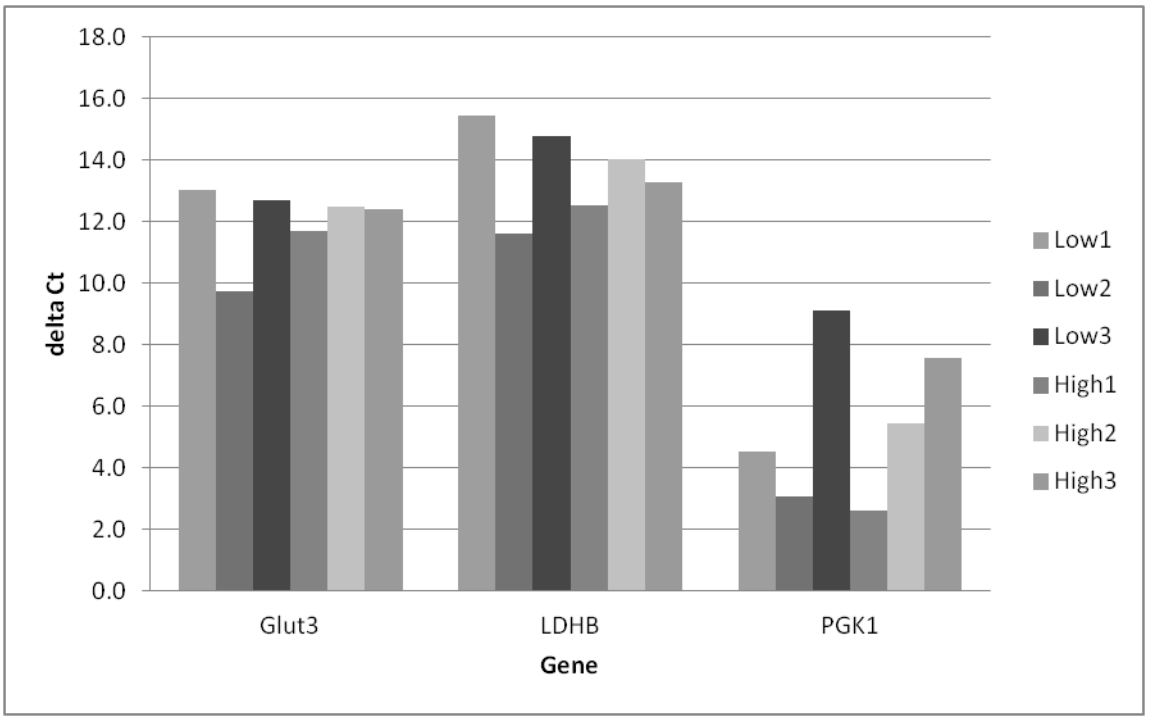
(reproduced from Froman et al., 2011).

GENE NAME	FUNCTION
PGK- Phosphoglycerate Kinase	Conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate
LDH-B- Lactate dehydrogenase B	reversible conversion of lactate and pyruvate
GLUT3- Glucose Transporter 3	main glucose transporter isoform
NDUFS1- NADH dehydrogenase Fe-S protein	Transfers electrons from NADH to the respiratory chain
ATP5A1- Mitochondrial ATP synthase	Required for ATP synthesis
ATP51- ATP Synthase subunit 5	Required for ATP synthesis
MYO9B- Myosin 1XB	ATPase activity, intracellular movements

Table 19 Glycolytic and ATP metabolism genes for qPCR reaction are listed with their main function in a cell

Primer Name	Primer Sequence, 5'→3'
PGK1F	GCTACATGCTGTGCGAAGTGG
PGK1R	CAGGAACAGGAACTGGGGTGA
LDHBF	GAACTGGGCCATTGGTCTTA
LDHBR	CAGAGGCACTCAGGACACAA
GLUT3F	GTTCCTGGTAGAGCGTGCAG
GLUT3R	TCTCAAAAGGGCCACAAAG
ATP5A1F	TCCCATGGCTATTGAGGAACA
ATP5A1R	AGCTTCCGTCTGGTCAGAGA
NDUFS1F	GCTGTTGTTGAAGGTGCTCAA
NDUFS1R	TTCTCTCCAGCCACAGGAGT
ATP51F	AGGCGGAGGAGAAGAAGAAG
ATP51R	ACATGCAAAGACCATCAACCA
MYO9BF	CGAGGCTCTGATGAGGAAAA
MYO9BR	GGAGGAAGCTTGATGTTTGCA

Table 20 Primers used for differential gene expression studies with their forward and reverse sequence



<u>Figure 1</u> Testicular gene expression for three glycolytic enzymes in high and low sperm mobility lines. *mRNA levels were determined by qPCR using ribosomal protein S14 as a reference to compute delta-Ct values.*

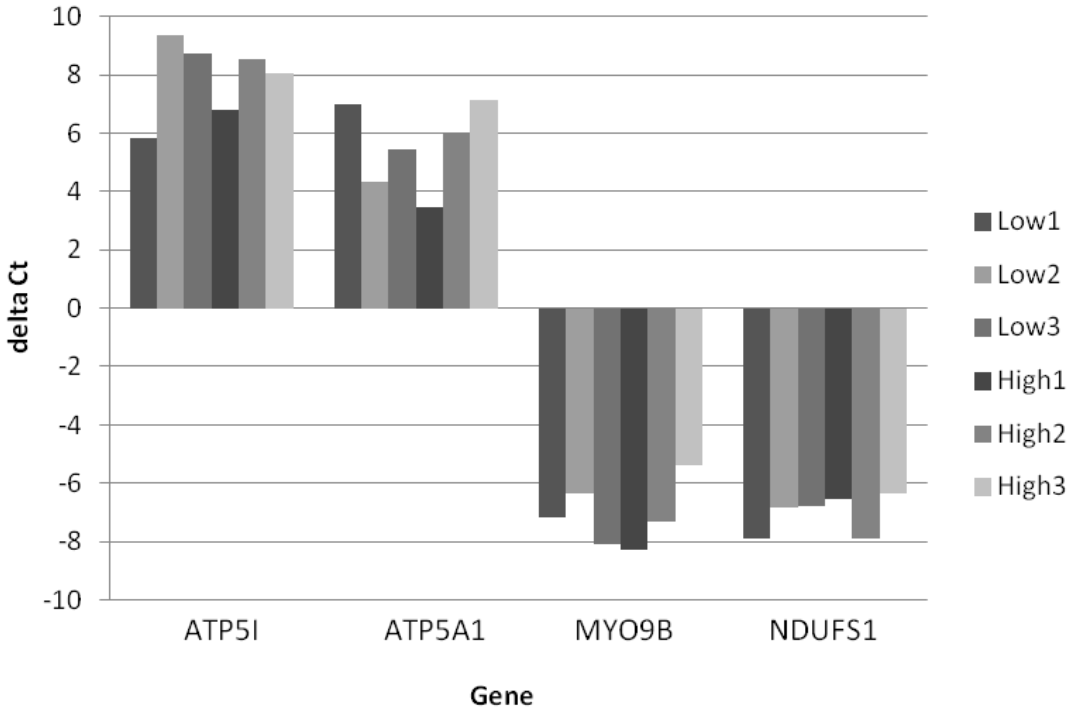


Figure 2 Testicular gene expression for three ATP genes in high and low sperm mobility lines. *mRNA levels were determined by qPCR using ribosomal protein S14 as a reference to compute delta-Ct values.*

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