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THE INFLUENCE OF *LDHA* GENE POLYMORPHISM ON RELATIVE LEVEL OF ITS EXPRESSION IN RACING PIGEONS

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ABSTRACT

The *LDHA/Hae*III polymorphism (g.2582481G>A) in pigeons is associated with physiological predispositions for rapid return and endurance, which are one of the most desired racing performance traits. Thus, the aim of the study was to analyse the association between the g.2582481G>A polymorphism with the relative expression level of *LDHA* in the group of young homing pigeons. The results demonstrated differences, but not significant in the relative *LDHA* expression level in the group of pigeons carrying different *LDHA* genotype. The highest expression of *LDHA* gene in pigeons carrying $LDHA^{AG}$ genotype was reported. Moreover, the differences in the average relative quantity of the *LDHA* gene were different in relation to gender, with a slightly higher expression level of the *LDHA* gene in females. In conclusion, the highest expression level of *LDHA* gene in homing pigeons with genotype *AG* may explain the better racing performance of $LDHA^{AG}$ pigeons reported in previous studies.

Key words: expression, *LDHA* gene, racing pigeons, real-time PCR

INTRODUCTION

Pigeon breeding has three main directions – utility, fancy and sporting/flying. The first one is using utility pigeons as a source of high quality meat (squab). The second one is focused on those pigeon breeds that are admired and bred for their various traits (e.g., patterns, shape, size, coloration). Hence, this group is qualified as a fancy pigeon. The third group – the sporting (homing) group is the most popular not only for recreational purposes, but can also be a prestigious and lucrative sport [Jerolmack 2007]. In recent years, it has become popular all over the word with annually organized The South African One Million Dollar Pigeon Race, Diamond Elite One-loft Race in China (first prize of more than 1 million EUR) or The Golden Island One-loft Race in Qinhuangdao (with a prize of 4 million EUR) [Jerolmack 2007, Proskura et al. 2015a]. To maximize pigeons' performance, appropriate type of feeding, training and veterinary support (including vaccinations, electrolyte repletion and medications) strategies are applied by many breeders. Another strategy in animal improvement is based on genetic marker identification enabling faster breeding progress for key traits of animals [Sandenbergh et al. 2016, Molotsi et al. 2017].

Potential genetic markers for racing performance traits has been investigated for many years, for example, in horses: Arabian [Ekiz and Kocak 2005], Australian and Hong Kong Thoroughbreds [Velie et al. 2016], Quarter horses [Corrêa and da Mota 2007] or in dogs, such as Whippets [Mosher et al. 2007] and Greyhounds [Iazbik at al. 2010]. Similarly, genetic markers of racing performance traits in homing pigeons have been studied for several years. The association between some genes and physiological and physical predispositions can be a useful tool for improving the selection of homing pigeons through marker assisted selection (MAS) in the near future [Dybus et al. 2013, Proskura et al. 2015a, Proskura et al. 2015b, Proskura et al. 2017, Gazda et al. 2018].

In vertebrates, at least three LDH isozymes have been characterized and all of them are encoded by different genes. In most vertebrates, LDHA is expressed specifically in skeletal muscles, LDHB in cardiac muscle and LDHC, in vertebrates and pigeons, mainly in the mature testis [Mannen et al. 1997, Li 1998]. The difference be-

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tween isoform A (LDHA) and isoform B (LDHB) in vertebrates is based on anaerobic or aerobic characteristics of tissues and the type of reaction – pyruvate reduction or L-lactate oxidation [Lupiáñez et al. 1996, Li 1998]. Lactate dehydrogenase isoform A is a member of a larger *LDH* gene family encoding L-lactate dehydrogenase (LDH, EC 1.1.1.27) that regulates aerobic and anaerobic metabolism. Those metabolic functions have an essential impact on the physiological condition of the organism, e.g. muscle endurance, recovery and aerobic capacity [Lupiáñez et al. 1996, Mannen et al. 1997].

The *LDHA-Hae*III (g.2582481G>A) polymorphism in pigeons has been identified and analysed as a marker of racing performance traits [Dybus and Kmieć 2002, Dybus et al. 2006]. Since then, g.2582481G>A has been tested in commercial genetics laboratories and genetic analyses become more popular every year.

Therefore, the aim of the study was to investigate the association between the g.2582481G>A polymorphism (located in intron 7 of the gene, very close to the splicing site) with the relative expression level of *LDHA* in the three genotype group of racing pigeons.

MATERIAL AND METHODS

The present study included a total of 24 young homing pigeons (12 hens and 12 cocks) from the university loft of the West Pomeranian University of Technology Szczecin (West Pomerania Province, Poland). Three- to five-month-old individuals were selected to the experiment prior to the racing season. All subjects selected for the investigation of the relative expression level of the *LDHA* gene were divided into three genotype groups. Each group included eight individuals (four hens and four cocks) with $LDHA^{AA}$, $LDHA^{AG}$ and $LDHA^{GG}$ (g.2582481G>A) (Dybus et al. 2006). DNA sex identification was determined as described by [Griffiths et al. 1998, Lee et al. 2007], with own modification (qPCR and melting curve analysis).

All blood samples (∼50–100 µl) were collected from the medial metatarsal vein into test tubes with anticoagulant (K_3EDTA). Crude DNA was isolated from 3 µl of whole peripheral blood using the MasterPure™ DNA Purification Kit for Blood version II (Epicentre Biotechnologies, Madison, WI, USA), according to the manufacturer's protocol. Young pigeons (n = 24) for qPCR analysis were selected from all 3-to-5 month old pigeons kept at the loft. Genotyping was performed using PCR-RFLP (Table 1) [Dybus et al. 2006]. PCR primers flanking a 423-base pair region of *LDHA* gene (GeneBank: NW_004973198.1). PCR reactions were carried out in 15 µl of reaction mixture containing ∼60 ng of DNA, 15 pmol of each primer, 1.5 µl of 10 × PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.3 unit of Taq polymerase (EUR_X, Gdańsk, Poland), and nuclease-free water (AppliChem, Darmstadt, Germany). The following thermal profile was applied: 5 min at 94°C followed by 33 cycles (denaturation at 94°C for 40 s, primer annealing at 57°C for 40 s and product synthesis at 72°C for 45 s) and then final elongation at 72°C for 5 min. PCR products were digested for 4 hours with 2 units of *Hae*III (EUR_X, Gdańsk, Poland) at 37°C and separated by horizontal electrophoresis (120 V, 40 min) in 2.5% agarose gel (Prona Agarose, EU).

The individuals' sex was identified by a modified PCR method using CHD1 primers (Table 1) [Griffiths et al. 1998, Lee et al. 2007]. PCR master mix contained ∼80 ng of DNA, 10 pmol of each primer, 3 µl of 5x AmpliQ HOT EvaGreen HRM Mix (Novazym, Poznań, Poland) and nuclease-free water (AppliChem, Darmstadt, Germany). PCRs were performed in a Rotor-Gene 6000 instrument (Corbett Research, Cambridge, UK) under the following thermal conditions: 15 min at 95°C for initial denaturation, subsequent 30 cycles (denaturation at 95°C for 15 s, primer annealing at 58°C for 20 s and product synthesis at 72°C for 40 s) and final extension at 72°C for 1 min. Pre-hold temperatures of 95°C for 15 s and subsequently 50°C for 15 s were used for product re-association and heteroduplex formation prior to continuous fluorescence data acquisition during melting phase (temperature changing from 70–95°C at a transition rate of 0.1°C each step). The melt curve analysis of PCR amplicons was performed using Rotor-Gene Q Series Software 2.3.1 (Qiagen GmbH, Hilden, Germany).

For gene expression analysis, 5 µl of blood was collected from the medial metatarsal vein directly to test tubes with RBCL buffer and total RNA was isolated immediately after sampling using Total RNA Kit (A&A Biotechnology, Gdańsk, Poland) following the vendor's instructions. Total RNA concentration was determined (mean of three measurements) using the Quant-iT™ RNA BR Assay Kit and a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). Genomic DNA was removed from RNA isolates by DNase I digestion (1U/1 µg of RNA; Thermo Fisher Scientific, Waltham, Massachusetts, USA). Two-step reverse transcription was performed using the RevertAid™ Premium First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) based on 1 ng of total RNA, oligo$(dT)_{18}$ primer and recombinant M-MuLV reverse transcriptase (RT), according to the vendor's protocol. Synthesized cDNA samples were preserved at –80°C until further use.

Before real-time PCRs, all cDNA samples were quantified using the Quant-iT™ ssDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). The qPCR mixture contained 7.5 µl of Kapa Sybr Fast qPCR Mix (Kapa Biosystems, Woburn, MA, USA), 200 nM of each pri-

Table 1. Primer sequences used for PCR-RFLP and quantitative PCR

Tabela 1. Sekwencje starterów wykorzystane w analizie PCR-RFLP oraz ilościowym PCR

Gene Gen	Primer sequences* Sekwencje starterów*	Size (bp)	RE	Accession No.
LDHA	F 5′-TGAAGGGGTACACATCATGG-3′ R 5′-CCTTCTGGATTCCCCAGAGT-3′	423	*Hae*III	NW_004973198.1
CHD1	F 5′-CTCCCAAGGATGAGRAAYTG-3′ R 5′-ATGGAGTCACTATCAGATCCAG-3′	289/270	–	NW_004973325
ACTB	F5′-ATCAGGGTGTGATGGTTGGT-3′ R5′-TCTCCATGTCATCCCAGTTG-3′	132	–	XM_005504502.1
LDHA	F5′-CATGGCAGCCTCTTCCTTAG-3′ F5′-AAGTTAAGGCGGCTCTCTCC-3′	128	–	NW_004973198

*All primer sequences were designed using Primer3 software (Untergrasser et al. 2012); for qPCR, primers sequences were designed to span an exon-exon junction.
*Wszystkie sekwencje starterów zostały zaprojektowane przy użyciu oprogramowania Primer3 (Untergrasser et al. 2012); dla qPCR sekwencje starterów zaprojektowano tak, aby obejmowały połączenie ekson-ekson.

mer (Table 1), cDNA template (40 ng) and nuclease-free water (AppliChem, Darmstadt, Germany) to a final volume of 15 µl. Real-time PCR thermal conditions were as follows: 4 min at 95°C followed by 40 cycles (95°C for 3 s, 60°C for 15 s, 72°C for 20 s) and 72°C for 3 min. qPCRs were performed in a Rotor-Gene 6000 (Corbett Research, Cambridge, UK). All samples were run in triplicate; no template control (NTC) was included in each run.

The expression of the *LDHA* gene and the *ACTB* (reference gene) was assayed using primers that flanked gene sequences between two exons to avoid genomic DNA amplification. The *ACTB* gene was chosen as a reference gene due to its constant expression in the study samples. The analysis of the relative expression level of the *LDHA* gene was preceded by relative transcript quantification with standard curves plotted using a three-fold serial cDNA dilution (from 40 ng to 0.04 ng) for *ACTB* (reference gene) and *LDHA* (gene of interest). The relative expression of the *LDHA* gene was analysed using the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen 2001, Pfaffl 2001]. Intra-run and inter-run variation was evaluated by the coefficient of variation (CV) using OriginPro 8.0 (OriginLab Corporation, Northampton, MA, USA) software. Differences in the relative level of *LDHA* expression between genotypes and genders were tested using non-parametric Kruskal Wallis ANOVA. Analyses were performed using the *STATISTICA* 12.5 (StatSoft Inc., Tulsa, OK, USA) software.

RESULTS

Firstly, PCR-RFLP method was performed to identify the SNP in *LDHA* gene (g.2582481G>A) of young pigeons kept at university's loft. PCR amplicons digested with *Hae*III resulted in the identification of three genotypes: $LDHA^{AA}$ – 395 and 28 bp, $LDHA^{AG}$ – 395, 311, 84 and 28 bp and $LDHA^{GG}$ – 311, 84 and 28 bp – Figure 1.

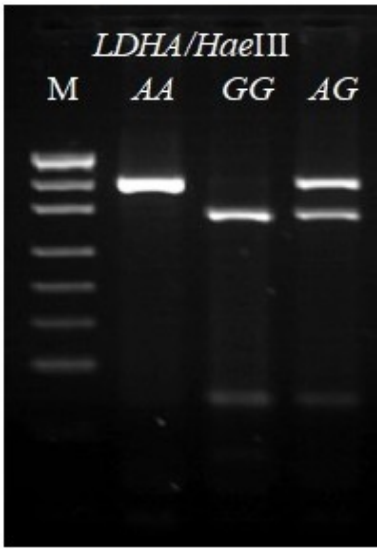

Fig. 1. Results of *LDHA/Hae*III genotyping*;* M – DNA marker (*pUC19*/*Msp*I)

Rys. 1. Wyniki genotypowania *LDHA/Hae*III; M – wzorzec DNA (*pUC19*/*Msp*I)

Secondly, gender identification was performed using melt curve analysis of PCR amplicons. Fluorescence data acquisition during the melting phase resulted in two peaks for females (corresponding to *CHD1-Z* and *CHD2-W* amplicons differing in size; Figure 2A) and a single peak for males (only *CHD1-Z* amplicon) (Figure 2B). From all pigeons genotyped, twenty four individuals were divided into three (genotype) groups.

Lastly, *LDHA* gene expression was measured in twenty-four young birds divided into three genotype groups (g.2582481G>A). Although the average Ct va-

lues in each group were slightly similar (in the range of 23.27 to 23.79), the average relative quantity (RQ) of *LDHA* gene expression, normalized using *ACTB* values, was the highest in the group of $LDHA^{AG}$ pigeons (RQ = 0.64) and the lowest in the $LDHA^{GG}$ (RQ = 0.52, see Figure 3). Ct values displayed the highest differences between $LDHA^{AA}$ homozygotes (with the highest SD) and $LDHA^{AG}$ ones in comparison to $LDHA^{GG}$ individuals. Statistical analysis did not reveal any significant differences between polymorphic groups.

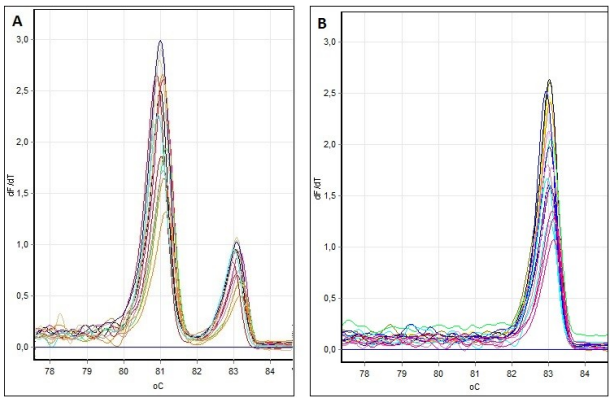

Fig. 2. Melt curve analysis for females (A) and males (B)

Rys. 2. Wyniki analizy krzywej topnienia dla samic (A) i samców (B)

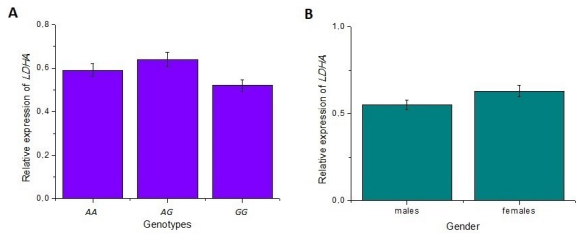

Fig. 3. The average relative expression level of *LDHA* in samples grouped into two categories – *LDHA/Hae*III genotypes (A) and gender (B)

Rys. 3. Średni względny poziom ekspresji genu *LDHA* w próbach pogrupowanych w zależności od genotypu *LDHA/Hae*III (A) oraz płci (B)

Reproducibility of the quantitation method was evaluated by comparing the results obtained from the replicate samples during the same reaction run – the intra-run variation – and the results obtained in different runs (data collected within two weeks) – the inter-run variation. The intra-run variation analysis was conducted using three Ct replicates of each sample in each run. The mean coefficient of variation (CV) of the *LDHA* gene was 0.52% for all analysed samples, whereas the average intra-run variation of the *ACTB* reference gene was 0.35%. The inter-run variation (reproducibility) was evaluated using real-time PCR data obtained from external control replicates (randomly selected sample) in each run within two weeks of the analysis. The average inter-assay precision was calculated; CV was 2.91%.

DISCUSSION

LDHA demonstrates high affinity for pyruvate and converts it to lactate, simultaneously oxidizing NADH to NAD^+ [Read et al. 2001, Valvona et al. 2016]. Regulation of *LDHA* synthesis is controlled by the *LDHA* promoter region (containing consensus sequences) and major transcription factors, such as hypoxia-inducible factor 1 (HIF1), c-Myc [Firth et al. 1995, Lewis et al. 1997] and Kruppel-like factor 4 (KFL4) [Shi et al. 2014]. It has also been reported that forkhead box protein M1 (FOXM1) binds to the *LDHA* promoter region and upregulates *LDHA* mRNA transcription, protein expression and lactate production [Cui et al. 2014]. *LDHA* transcription is also regulated by other factors, like miRNA [Hua et al. 2018].

In a previous association study on SNP g.2582481G>A in old racing pigeons, it was concluded that $LDHA^{AG}$ individuals demonstrated better racing performance. The effect of genotype on racing results for all race categories and short distance races was reported [Proskura et al. 2014]. The findings are consistent with the results presented in this study, where $LDHA^{AG}$ pigeons showed higher average relative quantity of the *LDHA* gene. A preliminary study [Dybus et al. 2006] demonstrated that the $LDHA^{AA}$ and $LDHA^{AG}$ genotypes for g.2582481G>A was more frequent in top-racing pigeons (from Poland and Taiwan/China). A similar observations were reported in other association studies in the groups of young homing pigeons, which showed that individuals with the rare $LDHA^{AA}$ genotype achieved better racing results in comparison to other genotypes [Proskura et al. 2015a]. It should be emphasized that this preliminary study was conducted on equal groups of individuals with three different genotypes. As mentioned in some publications [Dybus et al. 2006, Dybus 2009, Proskura et al. 2014, Proskura et al. 2015a] the $LDHA^{AA}$ genotype is extremely rare (0.007–0.092) in homing pigeons and none in non-homing ones [Dybus 2009]. In other study of *LDHA* gene in pigeons [Ramadan et al. 2013] six polymorphic sites were found in Japan and Egyptian homing and non-homing pigeon populations. The authors noticed that the frequency of *LDHA* loci might be related to selection history. Evolution diversity is a consequence of different physiological behaviours, resulting from different evolutionary targets and physio-

logical roles [Inoue-Murayama 2009], whereas human selection of homing pigeons has been strongly targeted for years towards the ability of rapid return and endurance [Ramadan et al. 2013]. Recently, Ramadan et al. [2018] reported that *LDHA* genotypes were significantly associated with estimated breeding value (EBV) values of longer total race distance; individuals carrying the S+ genotype had higher EBV (i.e., greater survivability).

The average relative quantity of the *LDHA* gene was also analysed in relation to pigeon sex. The average relative quantity of the *LDHA* gene in the group of males (RQ = 0.55) was lower in comparison with females (RQ = 0.63) (Figure 3). Statistical analysis of individuals grouped according to gender also did not reveal any significant statistical differences. The previous study demonstrated that gender did not affect racing ability of young pigeons [Proskura et al. 2015a], whereas the results obtained in the group of old pigeons showed that hens were characterised by a better racing performance than cocks throughout the racing season; they were characterised by significantly better racing results in all race categories – short and long distance races [Proskura et al. 2014].

The results of the racing pigeons are a combination of many different factors. The most important are gender, overall condition, individual motivation, training effect, weather conditions. *LDHA* genotype in racing pigeons is considered as a potential factor that may improve the racing performance traits (with commercial name in genotyping services – "*Speed Gene*"). It has been found that the g.2582481G>A polymorphic site is located very close the UT consensus splice donor site (–4 bp upstream). The proximity of the polymorphic site to the UT donor splice site may affect pre-mRNA splicing efficiency, RNA level variations and *LDHA* gene activity. The $LDHA^A$ allele seems to be more prevalent in racing pigeons than in non-homing pigeons, so the SNP in *LDHA* gene may affect and differentiate crucial traits of racing pigeons [Dybus et al. 2006, Dybus 2009]. It is also possible that the g.2582481G>A SNP is in linkage disequilibrium with other crucial (functional) SNP that affects physiological traits [Proskura et al. 2014].

Animals can be divided into two groups based on physical activity behaviour – distance runners or sprinters. Long distance runners/flyers display the capacity to maintain high energy consumption over a prolonged period of time, supported by type I muscle fibres, but are slow to accelerate, whereas sprinters have a short response time and the capacity for rapid acceleration executed by type IIb muscle fibres, but cannot maintain high velocity for a long time. Long distance animals exhibit high aerobic activity as opposed to anaerobic glycolysis that supports a quick response of sprinters [Lupiáñez et al. 1996, Meléndez-Morales et al. 2009]. Glycolysis in long-flight birds was found to have very high basal activity and its activation was low and slow. Short-flyers had a low basal activity but the activation was high and very fast [Lupiáñez et al. 1996]. In the pectoral muscle, activities of two enzymes are crucial – lactate dehydrogenase (LDH) and cytochrome c oxidase (CCO) – both involved in energy metabolism. LDH is an indicator of anaerobic glycolysis potential [Rioux and Blier 2006]. During increased physical activity, oxygen is absent or in short supply, while energy demand is high. In such physiological conditions, *LDHA* converts pyruvate to lactate – an important source of energy for muscle activity [Van Hall 2000, Van Hall et al. 2002]. Anaerobic glycolysis is a very rapid way to produced ATP, but requires glycogen reserves that cannot be maintained for a long period of time [Lupiáñez et al. 1996]. ATP source for aerobic metabolism are glucose and fatty acids necessary for endurance, while anaerobic glycolysis is required for sprint [Meléndez-Morales et al. 2009]; thus different metabolic characteristics can be observed in sprint-trained (with the highest LDH activity in the pectoral muscle) and endurance-trained birds (with the lowest LDH activity, but the highest lactate peak and fastest half-time response) pigeons [Chaplin et al. 1997].

The expression of *LDHA* gene and its regulation is much more complex than it was expected. As mentioned above, its regulation is dependent, among others, on different levels of lactate, cAMP or miRNA. Concentrations of some of these compounds are strictly dependent on physical activity in long- and short-distance flights. It cannot be excluded that LDH level might change with age, gender, developmental stages and genetic variability. Racing performance of homing pigeons is a result of the combination of different traits in the assessment of racing abilities [Negro Rama et al. 2016]. The desirable characteristics are controlled by certain genes as well as by their expression level, and the best animals for sprints do not have to achieve the best results in the group of long-distant runners/flyers [Gómez et al. 2010, Negro Rama et al. 2016]. *LDHA* expression level is only one factor that can determines velocity, endurance, muscle strength or stress resistance; myostatin (*MSTN*), cytochrome C oxidase subunit IV (*COX4*), alpha-globin (*AGLO*) and creatine kinase muscle gene (CKM) are some other genes that may be related to sporting performance [Velie et al. 2016], thus they can also exert a potential effect on LDHA activity.

CONCLUSIONS

The study demonstrated that *LDHA* genotype and gender might affect the relative expression level of the *LDHA* gene in homing pigeons and could be associated with racing performance. Due to the preliminary character of our study, further analysis, including top-racing pigeons du-

ring the racing season need to be conducted to verify the relative *LDHA* gene expression in relation to physical activity of sport pigeons.

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POLIMORFIZM W GENIE *LDHA* I JEGO WPŁYW NA WZGLĘDNY POZIOM EKSPRESJI U GOŁĘBI POCZTOWYCH

STRESZCZENIE

Polimorfizm *LDHA/Hae*III (g.2582481G>A) u gołębi związany jest z fizjologicznymi predyspozycjami do szybkiego powrotu do gniazda i wytrzymałością fizyczną, które stanowią jedne z najbardziej pożądanych cech użytkowych. Mając na uwadze powyższe, celem pracy była analiza zależności pomiędzy polimorfizmem g.2582481G>A w genie *LDHA* a względnym poziomem ekspresji tego genu w grupie młodych gołębi pocztowych. Uzyskane w toku analizy wyniki wskazały na różnice nieistotne statystycznie we względnym poziomie ekspresji *LDHA* u osobników o odmiennych genotypach. Najwyższy poziom ekspresji genu *LDHA* zaobserwowano u osobników z genotypem $LDHA^{AG}$. Dodatkowo, średni względny poziom ekspresji analizowanego genu był różny dla osobników podzielonych w zależności od płci, wskazując na nieznacznie wyższy jego poziom u samic. Podsumowując, wyższy poziom ekspresji genu *LDHA* u gołębi pocztowych o genotypie $LDHA^{AG}$ może wyjaśniać lepsze cechy powrotnolotowe tych osobników i potwierdzać wyniki wcześniejszych badań opartych na analizie genotypów.

Słowa kluczowe: ekspresja, gen *LDHA*, gołębie pocztowe, real-time PCR