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Original Article

Utilization of Biotechnology, Neurotransmitter and Cytogenetic Indices in Selecting Pigeon Breeds

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ABSTRACT

Investigations into LDH-A and DR-D4 genes polymorphism, neurotransmitter values and cytogenetic indices of 3 sexed pigeon breeds; non-racing pigeons, (wild rock), racing long distances pigeons (Jan Aarden) and racing short distances pigeons (Janssen) have been performed. The long-distances pigeon showed the highest brain neurotransmitters concentration (p<0.001) among pigeon breeds. Both LDH-A and DR-D4 genes polymorphism indicate the presence of different biodiversity values among pigeon breeds. The variations appeared on the position length 389bp for LDH-A polymorphism, and on two positions length of 418bp and 524bp for DR-D4 polymorphism of long distances male pigeon indicate the presence of unique diversity and overall differences in the amino acids structure in this breed. The protein sequence of both genes showed that in the position of 60 for LDH-A gene the amino acid K (lys) was converted to E (glu), while, in the positions of 117 and 153 for DR-D4 gene the amino acid R (arg) and L (leu) were converted to S (ser) and F (phe) only in long distances male pigeon compared to the other breeds. Moreover, there were slight differences in cytogenetic indices detected among the three pigeon breeds. It can be concluded that both DR-D4 and LDH-A genes polymorphism and neurotransmitters estimations in the brain tissue of racing pigeon would be useful indices for the differentiation and genetic characterization of pigeon breeds and provide a foundation for developing sustainable genetic improvement and conservation programs of the breeding and selecting racing pigeon breeders.

INTRODUCTION

In the domestication of the wild rock pigeon (*Columba livia*), hundreds of years ago, a vast variety of pigeon breeds was produced involving a selection for particular phenotypes as appearance, acrobatic abilities, reproduction purposes, meat source (Squab pigeons) and flying /sporting capabilities (Zagel pigeon) (Ramadan *et al.,* 2013). Today, the biggest interest of pigeon producers is to have unique Zagel pigeons for racing purposes, a sport that is popular in virtually every country in the world, due to the unique ability of homing pigeons to fly and return back to their loft from huge distances and at such speeds. Therefore, these unique birds can compete on the basis of their characteristics and performance in the greatest competition race, ‘’a global sport of pigeon racing” in Olympic games, either for a short distance - up to 300 miles, or for long-distances - about 600 miles (Michael *et al.*, 2013; Proskura *et al.*, 2014; Hunnam *et al.*, 2019). It is a rich industry and the race winners accumulate large sums of money; up to $1 million when the pigeons return home fastest from a long race. Hence, the pigeon fanciers over the world give great attention to having good breeding stock with a top line pedigree in performance in order to produce the best long-distance racer pigeon that can find their way home quickly and win races. Accordingly, a better understanding of the genetic background of racing pigeons, especially the identification of dopamine receptors D4 (DR-D4) and the lactate dehydrogenase A (LDH-A) genes as genetic markers for this trait, might be useful for selecting breeding stock for further progress/ success in racing pigeon performance, and may determine the abilities of the bird to fly either short distance ranges from 100 to 600 km or fly extremely long distances over 600 km. In addition, the role of cytogenetic indices and neurotransmitters in racing pigeon's brain in emotion and wing muscle energy could provide an objective assessment of the body health status that's associated with flying performance and spatial orientation and could be an important source of information with valuable selection meaning. The lactate dehydrogenase A (LDH-A) is one of the genes named ''performance – associated gene" and found predominantly in muscle tissues. It considers the most important gene that are responsible for both the synthesis and recycling of lactic acid in the muscles (Coulter *et al.*, 2015; Allen & Laborde, 2014) The presence of LDH-A gene enable the body muscles to utilize the cumulated lactate, which is produced during the high physical effort of racing pigeon in the race and turn it into positive energy for mitochondrial respiration in skeletal muscle, allowing the bird to fly longer with speed and high-performance during the marathon racing (Dybus *et al.,* 2006; Kersting *et al.,* 2008; Hejjas *et al.,* 2009; Cuyas *et al.,* 2014). Dopamine receptors D4 (DR-D4), is another important gene named "neurotransmitter-associated gene", that is known to be associated with pigeon racing performance (Balestri *et al.*, 2014; Rangel-Barajas *et al.*, 2015). Hence, the presence of the dopamine gene in pigeon breeds provide stimulation/mood to the nervous system and causes the brain to produce, reabsorb or transmit higher dopamine resulting in a better muscle performance. The positive correlation between the presence degree of dopamine related gene in the pigeon breed and dopamine concentration confirms the vital role of neurotransmitter in brain functions. Hence, the brain needs neurotransmitters to regulate many necessary functions, including muscle movement, heart rate, mood, concentration, breathing, appetite, sleep cycles and digestion. Thus, it is important to study the neurotransmitter status in the racing pigeon's brain as a valuable index in the selection of pigeon breeds. The neurotransmitters are called powerful neurochemicals produced by activating neuron cells in the brain and influence synaptic activity, transmit signals from nerve cells to target cells, such as muscles, glands, or other nerves. In this respect, dopamine (DA), is a neurotransmitter, one of the brain's chemical messengers, used to send signals of pleasure and stimulates the growth hormone secretion. It is also involved in learning, memory formation, muscle movement and coordination abilities, and attention functions (Rizo, 2018). Gamma-aminobutyric acid (GABA), also, is a mood regulator. It has an inhibitory action in the central nervous system, essential for the memory-storage elements of the brain, vision, lowers stress and anxiety (Bowery & Smart 2006). While Serotonin (ST), is an inhibitory neurotransmitter present in the brain and other tissues, important factor in sleep cycle, helps regulate mood and emotions, appetite, gut motility, blood clotting, pain, body temperature, the body's circadian rhythm, cardiovascular function and plays a role in mood and anxiety (Berger *et al.*, 2009). Acetylcholine esterase (ACHE), is an excitatory neurotransmitter, critical for normal attention, memory, sleep cycle, stimulates muscle contraction. A balance of neurotransmitters is necessary to prevent certain health conditions, such as mood, anxiety and neurological disorders (Südhof, 2013). In addition, melatonine (MTN) is a neuromodulator, involved with the regulation of circadian rhythm, regulates various physiological processes, such as sleep, wake cycle, seasonal adaptation, neuroendocrine function, thermoregulation, feeding patterns and immune function (Huang *et al.*, 2013; Reiter *et al.,* 2016). On the other side of this research work, studying the cytogenetic diversity and the precise identification of chromosomes characterization (size, shape, position of centromere and structure) within different pigeon breeds may help delineate evolutionary genetic relationships and the genomic pattern for each breed. There are few or no data published about the LDH-A and DR-D4 gene polymorphism, neurotransmitter and cytogenetic indices for racing pigeon breeds. Therefore, the present study aimed to compare the values of DNA polymorphism LDH-A and DR-D4 gene, a number of neurotransmitters concentrations including: dopamine, serotonin, gamma-aminobutyric acid, acetylcholine esterase and melatonin and cytogenetic indices of clinically healthy individuals of the non- racing wild rock pigeon, racing long distances, and racing short distances pigeons.

MATERIAL AND METHODS

Pigeon breeds source

Three sexually mature pigeon breeds (racing long distances pigeons *Jan Aarden* breed, racing short distances pigeons *Janssen* breed and non-racing *wild* *rock* pigeons), were obtained from Egy-Nash pigeon housed loft of Nasr City, Cairo, Egypt. Thirty adult pigeons of known gender (15 ♂ and 15 ♀), weighing 400-520 g were used in this part of the study for biotechnology, neurotransmitter and cytogenetic estimations. The birds in Egy-Nash pigeon loft were maintained under the same management conditions of diet based on yellow corn, peas and soybean, grit, water, metal cages and 12:12 hour light-dark cycle. Each metal cage was 1.0 m wide X 1.2 m long X 0.5 m high, and equipped with a feeder and an automatic drinker and housed one couple of pigeons (1 ♂ and 1 ♀).

Statement of ethics

The study was approved by the scientific and ethics committee of Local Experimental Animals Care, Biological Application Department, Egyptian Nuclear Research Center, according to the guidelines of the National Institute of Animal Health for Animal Care and Use in the experiments.

Collection of data

Three estimations were performed on pigeon breeds as follows:

Genetic analysis

Ninety blood samples were obtained. For each pigeon breed (n = 30) individual for genomic DNA extraction, isolation and purification according to methods described by Sambrook *et al.,* (1989) as follows: a half milliliter of the blood sample was withdrawn from the wing vein on EDTA tube as anti-coagulant (0.2 mL of 0.5 M EDTA). DNA was freshly extracted from the whole collected EDTA-blood. Three milliliter of lysis buffer TSTM (20 mM Tris-HCl pH 7.6, 640 mM sucrose, 2% Triton X-100, 10 mM MgCl2) was added to the aliquot. The mixture was centrifuged, and the pellet was suspended in 150 µl proteinase K, 1.5 mL nuclei lysis buffer, and 110 µl SDS 20%. After overnight incubation at 37 °C, the proteins were removed by NaCl 6M, and the DNA was precipitated by ice-cold absolute ethanol. The genes were selected according to (Dybus *et al.,* 2006; Proskura *et al.,* 2015). Genes, primer sequences, and references are shown in Table (1).

PCR Reaction

LDH-A and DR-D4 gene

The PCR reaction mixture contained approximately 80 ng of genomic DNA, 10 pmol of each primer, 25 µL of master mix in a total volume of 50 µL. The following cycles were applied: denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 60 s, primer annealing at 57 and 60 °C for 30 s, and PCR products synthesis at 72 °C for 60 s, and then final synthesis at 72 °C for 10 min.

The LDH-A and DR-D4 genes were directly purified by Gene JET PCR purification kit and sequenced on ABI3730XL DNA Analyzer apparatus throughout Sigma Company. The sequenced fragments were aligned using (http://ncbi.nlm.nih.gov/BLAST/) with the published data in the NCBI databases (non-redundant nucleotide database) and submitted to the GeneBank using (http://www.ncbi.nlm.nih.gov/BankIt/) and the EMBL-EBI search and sequence analysis tools APIs by Madeira *et al.* (2019). Finch TV 1.4.0 (http://www.geospiza.com/finchtv/). CLC Sequence Viewer Version 6.8.2 (www.clcbio.com) and MEGA-6 using in alignment with three genotypes.

Karyotype Analysis

Preparation of bone marrow metaphase chromosome using technique described by Santos *et al.* (2020) minor modifications. Twenty cells of well-spread mitotic metaphase plates were used for chromosome measurements. The length of short arm (p) and the long arm (q) of each chromosome were measured, the chromosomes 1 to 8 and as well as sex chromosome Z and W, the total length (TL) was calculated as (p + q). The relative length (RL%) of each chromosome was estimated in percent of total length of complement according to the formula (TL/sum TL) x100. While, the formula used for the centromeric index (CI%) was (p/TL) x100.

GTG-banding technique

The GTG-banding technique was applied on the metaphase chromosomes of the pigeon breeds under investigation. The GTG-banding technique was adopted from Abd El-Gawad *et al.* (2019). The slides were dried on air and then soaked in working trypsin (0.025% trypsin EDTA) at 37°C until the termination of trypsin activity (5 to 10 seconds) by washing the slide

Table 1 – Primer sequence according to the following:

Genes	Primers sequences	References
DR-D4 Fragment (1)	F: TTTGGGATCGCTCGCTTACC R: ATGACAGGGGATGCTACAGC	Proskura *et al.,* (2015)
DR-D4 Fragment (2)	F: TTTGGGATCGCTCGCTTACC R: GCAGGACAACACAGCGTCTC	Proskura *et al.,* (2015)
DR-D4 Fragment (3)	F: GGGCCAACAGGAAGCTCTAT R: GCAGGACAACACAGCGTCTC	Proskura *et al.,* (2015)
LDH-A	F: ATCATGGGCTATTGGCCTCT R: AAAACCTGAGGAAGAGGA CAAA	Dybus *et al.,* (2006)

with phosphate buffer. The trypsinized slides were stained with 20% Giemsa solution for 30 minutes. The pattern of G bands was determined on the basis of an analysis of 1600 chromosomes obtained from 20 metaphase plates. The description of chromosome morphology and classification was done following (Fechheimer, 1990), and in accordance to the standard of G band identification on the chromosomes of Gallus domesticus (Ladjali-Mohammedi *et al.,* 1999).

Microscope examination, karyotyping and idiograming

Chromosomes examination was performed using a vertical fluorescence microscope (Leica DM4 B) equipped with a cooled digital color camera (Leica DFC450 C). Twenty cells with clearly observable and well spread chromosomes of each bird were examined and photographed at 100 × magnification under oil immersion. Chromosome counting and karyotyping were performed using the automated karyotyping & FISH software processing (Leica CW4000) system. Idiograms were constructed from complete chromosomes, which showed the maximum possible banding patterns in at least ten different metaphase plates.

Brain's neurotransmitters assay

Ten adult healthy birds, 12 months of age, of 450-550 g of body weight, from each pigeon breed were sacrificed, decapitated and whole brains tissue were dissected out from the skull. All brain samplings were done at 9:00 a.m. to remove circadian variations in concentrations and immediately frozen in liquid nitrogen gas for neurotransmitters; concentrations assay include: dopamine (DA), serotonin (ST), gamma-aminobutyric acid (GABA), acetyl choline esterase (ACHE) and melatonin (MTN) according to Manikkoth *et al.* (2016) method, described as following: wet tissue was weighed and homogenized in HCl–butanol for about 1 minute (in 1:10 ratio). The sample was then centrifuged for 10 minutes at 3000 rpm. An aliquot supernatant phase (1 mL) was removed and added to centrifuge tube containing 2.5 mL hexane and 0.3 mL of 0.1 M HCl. The aqueous phase (0.2 mL) was then taken for dopamine assay. All steps were carried out at 00C (on ice). To the 0.2 mL of aqueous phase, 0.05 mL 0.4 M HCl, and 0.1 mL of Sodium acetate buffer (pH 6. 9) were added, followed by 0.1 mL iodine solution (0.1 M in ethanol) for oxidation. The reaction was stopped after 2 min by the addition of 0.1 mL sodium sulphite solution. Acetic acid 0.1 mL was added after 15 minutes. The solution was then heated to 100°C for 6 minutes. When the sample again reached room temperature, excitation and emission spectra were read from the spectrofluorimeter at 330-375 nm. Tissue blanks for dopamine (DA), serotonin (ST), gamma-aminobutyric acid (GABA), acetyl choline esterase (ACHE) and melatonin (MTN) were prepared by adding the reagents of the oxidation step in reversed order (sodium sulphite before iodine).

Calculation

The neurotransmitter level is calculated using the following formula.

X neurotransmitter = Sample O.D – Blank O.D X Conc. Of Standard (500 pg/mL)

Standard O.D – Blank O.D

This gave the amount of *neurotransmitters* present in 1mL of the sample. The final reading of GABA, ACHE and MTN neurotransmitter levels are expressed as: pg/mL. While, dopamine DA, and serotonin (ST), concentrations were measured by radioimmunoassay with a kit which was produced by the Institute of Isotopes Co., Ltd. (Budapest, Hungary), and the samples were counted on Packard Gamma Counter (PerkinElmer Inc., Branford, CT, USA). The final reading of DA, and ST, neurotransmitter levels are expressed as: ng/mL.

Statistical analysis

All the neurotransmitters data were analyzed with the general linear model and variance procedure analysis between pigeon breeds using the statistical software SPSS (2010). Turkey's procedure for multiple comparison tests was used to identify significant differences of values at a significance level of 5%.

RESULTS

LDH-A gene polymorphism

Eleven polymorphic sites have been identified in LDH-A gene among three sexed pigeon breeds expressed as long distances racing, short distances racing and wild rock pigeons as showed in Table 2.

The polymorphism of long distances male pigeon was 5bp variation/deletion resulting in five positions length: 195bp, 385bp, 386bp, 387bp and 389bp (GenBank accession no: MW044915), while the polymorphism of long distances female pigeon (GenBank accession no: MW044598), and in both gender of short distances racing pigeon (GenBank accession no: MW072293 for female; GenBank accession no: MW072294 for male) were 3bp deletion and resulting in the same three positions length; 20bp, 362bp and 366bp. Furthermore, the polymorphism of

Table 2 – Deletion and variation in positions of lactate dehydrogenase A (LDH-A) and Dopamine Receptor D4 (DR-D4) genes in three sexed breeds (racing long distances, short distances and non-racing wild rock pigeons).

Genes /Breed	Female racing long distances pigeon			Male racing long distances pigeon		
	Position (bp)	Deletion	Variation	Position (bp)	Deletion	Variation
LDH-A	20	A		195		T→A
LDH-A	362	A		385	C	
LDH-A	366	C		386	A	
LDH-A				387	G	
LDH-A				389		A→G
DR-D4	17		T→C	418		A→C
DR-D4				524		C→T
	Female racing short distances pigeon			Male racing short distances pigeon		
	Position (bp)	Deletion	Variation	Position (bp)	Deletion	Variation
LDH-A	20	A		20	A	
LDH-A	362	A		362	A	
LDH-A	366	C		366	C	
DR-D4	457		C→T	17		T→C
DR-D4				496		T→C
	Female non-racing wild rock pigeon			Male non-racing wild rock pigeon pigeon		
	Position (bp)	Deletion	Variation	Position (bp)	Deletion	Variation
LDH-A	20	A		20	A	
LDH-A	362	A		261		C→T
LDH-A	366	C		362	A	
LDH-A	383		C→T	366	C	
LDH-A	386		A→C			
LDH-A	387		G→A			
LDH-A	388		A→G			

wild rock female pigeons was 7bp variation/deletion resulting in seven positions length: 20bp, 362bp, 366bp, 383bp, 386bp, 387bp and 388bp (GenBank accession no: MW039603), while, the polymorphism of male wild rock pigeons was 4bp variation/deletion resulting in four positions length of 20bp, 261bp, 362bp and 366bp (GenBank accession no: MW044597). The obtained results on LDH-A gene polymorphism indicate the presence of different biodiversity values among the mention pigeon breeds particularly, between long distances male pigeon and both gender of wild rock pigeons wherein they showed high variation in LDH-A gene polymorphism values, while the rest of breeds showed the same LDH-A gene polymorphism.

Furthermore, the genetic differentiation was evaluated using the nucleotide differentiation Table 3 and the frequency of LDH-A gene Table 4, the analysis showed that the count of A nucleotides ranged from 119 in long distances male pigeon to 117 in wild rock female pigeons. Also the count of T nucleotides ranged from 114 in both gender of wild rock pigeons to 112 in long distances male pigeon. The count of C nucleotides ranged from 68 in wild rock male pigeons to 69 in the rest of the other genotypes, while the count values of (G) were equal (86) in all six genotypes. The lowest value was 154 with wild rock male pigeons in (C+G) while the highest value was recorded in (A+T). The highest frequency value (0.601) was found in male

Table 3 – Counts of nucleotides of Dopamine Receptor D4 (DR-D4) and lactate dehydrogenase A (LDH-A) genes in three sexed breeds (racing long distances, short distances and non-racing wild rock pigeons).

Breed	Gender	Counts of nucleotides											
		DR-D4 gene						LDH-A gene					
		A	T	C	G	C+G	A+T	A	T	C	G	C+G	A+T
Racing long distances pigeon	Female	135	131	196	157	353	266	118	113	69	86	155	231
	Male	135	132	195	157	352	267	119	112	69	86	155	231
Racing short distances pigeon	Female	135	133	194	157	351	268	118	113	69	86	155	231
	Male	135	130	197	157	354	265	118	113	69	86	155	231
Non-racing wild rock pigeon	Female	134	133	195	157	352	267	117	114	69	86	155	231
	Male	134	133	196	157	352	267	118	114	68	86	154	232
Total		808	792	1173	942	1762	1333	708	679	413	516	929	1387

wild rock pigeons, while the same frequency values (0.598), were found in all genotypes.

Table 4 – Frequencies of Dopamine Receptor D4 (DR-D4) and lactate dehydrogenase A (LDH-A) genes in three sexed breeds (racing long distances, short distances and non-racing wild rock pigeons).

Pigeon Breeds	Gender	Genes	Frequencies of nucleotides		
			G/C	A/T	PIC
Racing long distances pigeon	Female	LDH-A	0.402	0.598	0.48
		DR-D4	0.570	0.430	0.49
	Male	LDH-A	0.402	0.598	0.48
		DR-D4	0.569	0.431	0.49
Racing short distances pigeon	Female	LDH-A	0.402	0.598	0.48
		DR-D4	0.567	0.433	0.49
	Male	LDH-A	0.402	0.598	0.48
		DR-D4	0.572	0.428	0.49
Non-racing wild rock pigeon	Female	LDH-A	0.402	0.598	0.48
		DR-D4	0.569	0.431	0.49
	Male	LDH-A	0.399	0.601	0.48
		DR-D4	0.402	0.598	0.48

In this part of the study, the variations appeared in LDH-A polymorphism of long distances male pigeon, particularly on the position length 389bp indicating the presence of unique diversity in this breed, hence A changed to G which means that the changes that occurred in the gene expression for LDH-A gene resulted in the differences in amino acids structure.

DR-D4 gene polymorphism

Six polymorphic locations have been discovered in the DR-D4 gene in three sexed pigeon breeds expressed as racing long distances, racing short distances and non-racing wild rock pigeons as showed in Table 2.

The polymorphism of long distances female pigeon was 1bp variation resulting in one position length: 17bp (GenBank accession no: MT982609). And the long distances male pigeon polymorphism was 2bp variation resulting in two positions length of 418bp and 524bp (GenBank accession no: MT982610). Also the short distances female pigeon polymorphism was 1bp variation resulting in one position length of 457bp (GenBank accession no: MT982612). And, the short distances male pigeon polymorphism was 2bp variation resulting in two positions length of 17bp and 496bp (GenBank accession no: MT982613). Finally, the wild rock female pigeon polymorphism was 7bp (GenBank accession no: MT982611).

The obtained results in DR-D4 gene polymorphism indicate the presence of different biodiversity values among the mention pigeon breeds in particular, among long distances male pigeon and both gender of wild rock pigeons. Regarding the count of nucleotides Table 3 and the frequency of DR-D4 gene Table 4, the analysis showed that the count of A nucleotides ranged from 134 in long distances male pigeon to 135 in the other breeds. Also, the count of T nucleotides ranged from 130 with short distances male pigeon to 133 with short distances female pigeon and long distances male pigeon. The count of C nucleotides ranged from 194 with short distances female pigeon to 197 in short distances male pigeon. While the count values of (G) were equal in all five genotypes. The lowest value was 351 with short distances female pigeon in (C+G) while the highest value was recorded in (A+T). The frequency values varied from 0.567 with short distances female pigeon to 0.572 with short distances male pigeon in (C+G) while in (A+T) the frequency values varied from 428 with male racing pigeon's short distances to 0.433 with short distances female pigeon. Moreover the frequency values varied from 0.569 with female wild rock pigeon to 0.402 with male wild rock pigeon in (C+G) while in (A+T) the frequency values varied from 0.431 with female wild rock pigeon to 0.598 with male wild rock pigeon. On the other side, the variations appeared in DR-D4 polymorphism of long distances male pigeon, particularly on two positions length of 418bp and 524bp indicate the presence of unique diversity in this breed, hence A changed to C and A changed to T, which means that the changes that occurred in gene expression for the DR-D4 gene resulted in overall differences in amino acids structure.

DR-D4 and LDH-A gene protein sequence

The data derived from the LDH-A gene protein sequencing (CDs) region in the pigeon breeds as shown in Figure (1A), showed that in the position of 60 the amino acid K (*lys*) was converted to E (glu) in the LDH-A gene only in long distances male pigeon compared to the other breeds. These amino acids consider the neurotransmitters in all the body, a precursor in synthesis. The identical match scored among three pigeon breeds was 98%, while, the conservation scored a total positive value of 2% in long distances male pigeon. Whereas, the amino acids E (glu) was scored 8.1% in long distances male pigeon, while in the other breeds scored 6.5% with LDH-A gene.

```
SM      VADLAETIMKNLRRVHPISTVVKGMHGIKEDVFLSVPCVLGSSGITDVVKMILKPEEEDK    60
SF      VADLAETIMKNLRRVHPISTVVKGMHGIKEDVFLSVPCVLGSSGITDVVKMILKPEEEDK    60
WF      VADLAETIMKNLRRVHPISTVVKGMHGIKEDVFLSVPCVLGSSGITDVVKMILKPEEEDK    60
LF      VADLAETIMKNLRRVHPISTVVKGMHGIKEDVFLSVPCVLGSSGITDVVKMILKPEEEDK    60
WM      VADLAETIMKNLRRVHPISTVVKGMHGIKEDVFLSVPCVLGSSGITDVVKMILKPEEEDK    60
LM      VADLAETIMKNLRRVHPISTVVKGMHGIKEDVFLSVPCVLGSSGITDVVKMILKPEEEDE    60
        *********************************************************** 

SM      LR      62
SF      LR      62
WF      LR      62
LF      LR      62
WM      LR      62
LM      LR      62
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Figure (1A) – LDH-A gene protein sequences of the three sexed pigeon breeds (racing long distances, short distances and non-racing wild rock pigeons).

In addition, the CDs region of the DR-D4 gene in the pigeon breeds also showed that in the positions of 117 and 153 the amino acid R (*arg*) and L (*leu*) were converted to S (*ser*) and F (*phe*) only in long distances male pigeon compared to the other breeds as shown in Figure (1B). These amino acids also consider the neurotransmitters in all the body, a precursor in synthesis. The identical match scored among three pigeon breeds was 99%. This means that the gap has been estimated of 1% for long distances male pigeon. Whereas the amino acids of S (*ser*) and F (*phe*) were scored as 7.14 and 5% in long distances male pigeon, while in the other breeds scored of 6.6 and 4.4% with DR-D4 gene.

```
LF    FIAVSIPLNYNRRQIDLRQLILISTTWIFAFAVASPVIFGLNNVPNRDPSLCQLEDDNYI    60
SM    FIAVSIPLNYNRRQIDLRQLILISTTWIFAFAVASPVIFGLNNVPNRDPSLCQLEDDNYI    60
WRF   FIAVSIPLNYNRRQIDLRQLILISTTWIFAFAVASPVIFGLNNVPNRDPSLCQLEDDNYI    60
SF    FIAVSIPLNYNRRQIDLRQLILISTTWIFAFAVASPVIFGLNNVPNRDPSLCQLEDDNYI    60
LM    FIAVSIPLNYNRRQIDLRQLILISTTWIFAFAVASPVIFGLNNVPNRDPSLCQLEDDNYI    60
      ************************************************************

LF    VYSSICSFFIPCPVMLVLYCAMFQGLKRWEEARKAKLRGSIYGANRKLYHPSTFIEREQT    120
SM    VYSSICSFFIPCPVMLVLYCAMFQGLKRWEEARKAKLRGSIYGANRKLYHPSTFIEREQT    120
WRF   VYSSICSFFIPCPVMLVLYCAMFQGLKRWEEARKAKLRGSIYGANRKLYHPSTFIEREQT    120
SF    VYSSICSFFIPCPVMLVLYCAMFQGLKRWEEARKAKLRGSIYGANRKLYHPSTFIEREQT    120
LM    VYSSICSFFIPCPVMLVLYCAMFQGLKRWEEARKAKLRGSIYGANRKLYHPSTFIESEQT    120
      ******************************************************** ***

LF    GLEPEECGPYAHSSHPGDYVMNNGIPTVSYPHLKYPPPGHGRKRAKINGRERKAMRVLPV    180
SM    GLEPEECGPYAHSSHPGDYVMNNGIPTVSYPHLKYPPPGHGRKRAKINGRERKAMRVLPV    180
WRF   GLEPEECGPYAHSSHPGDYVMNNGIPTVSYPHLKYPPPGHGRKRAKINGRERKAMRVLPV    180
SF    GLEPEECGPYAHSSHPGDYVMNNGIPTVSYPHLKYPPPGHGRKRAKINGRERKAMRVLPV    180
LM    GLEPEECGPYAHSSHPGDYVMNNGIPTVSYPHFKYPPPGHGRKRAKINGRERKAMRVLPV    180
      ******************************** ***************************

LF    VV      182
SM    VV      182
WRF   VV      182
SF    VV      182
LM    VV      182
      **
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Figure (1B) – DR-D4 gene protein sequences of the three sexed pigeon breeds (racing long distances, short distances and non-racing wild rock pigeons).

Karyotype analyses

The chromosome complement of the three sexed pigeon breeds (racing long distances, short distances and non-racing wild rock pigeons), is composed of 78 autosomes and a pair of sex Chromosomes (Z and W), thus making the diploid number of chromosomes, 2n=80 (Table 5, Figure 2). The relative length (RL%), centromeric index (CI%) and centromere position were estimated as displayed in Table (5).

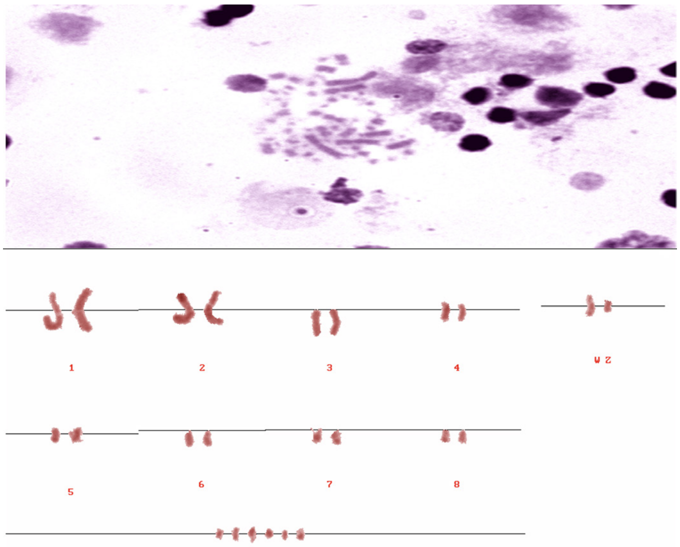

Figure 2 – Spread metaphase cells and male Karyotype of the three pigeon breeds.

In general, the morphological features of the chromosome complement of the three pigeons breeds were in accordance with the basic bird karyotype revealing the karyotype formula $2n = 80 = L^{sm}_{12} + L^{ac}_{8} + M^{sm}_{6+} + S^{ac}_{28}$+ micro chromosomes 26 included sex chromosomes, slight differences in the measured parameters were detected among the three pigeon breeds as shown in Table 5, and illustrated in the histograms (Figure 3 and 4).

The relative length of the 38 autosomal chromosomes across the three pigeon breeds ranged from 6.88% (chromosome 1) to 4.98% (chromosome 3). The relative length of the longest chromosome (chromosome 1) in the pigeon breed of short distances, wild rock and long distances breeds was 6.65, 6.88 and 6.64%, respectively. While, the shortest autosomal chromosome (chromosome 3) revealed a relative length of 4.98, 5.33 and 5.24% in the pigeon breed of short distances, wild rock and long distances breeds, respectively. Similarly, the three breeds revealed negligible differences in

Table 5 – The relative length (RL %), the centromere index (CI %) and centromere position (CP) for the three pigeon breeds.

	RL%			CI			CP
	1	2	3	1	2	3	
1	6.65	6.88	6.64	44.18	44.50	44.11	submetcentric
2	6.65	6.70	6.64	44.18	44.03	44.11	submetcentric
3	4.98	5.33	5.24	3.03	3.23	3.33	acrocentric
4	6.50	6.70	6.47	44.86	44.59	44.24	submetcentric
5	6.34	6.36	6.47	44.86	44.24	44.24	submetcentric
6	6.34	6.19	6.29	4.86	4.44	4.44	acrocentric
7	6.34	6.19	6.29	3.86	4.44	4.44	acrocentric
8	6.04	6.02	6.12	3.90	3.86	3.86	acrocentric
Z	3.29	3.27	3.15	44.86	44.37	44.44	submetcentric
W	2.50	2.56	2.60	43.90	43.72	43.80	submetcentric

the relative length of the Z (3.29, 3.27 and 3.15%) and W (2.50, 2.56 and 2.60%) chromosomes in the pigeon breed of short distances, wild rock and long distances breeds, respectively. These slight differences could be attributed to different levels of chromosome condensation among the three pigeon breeds.

The centromere index for the chromosomes of the three pigeon breeds are presented in Table 5 and illustrated as histogram in Figure 3. The results confirmed the metacentric nature of the three longest chromosomes. The centromeric index for the longest chromosome (chromosome1) was 44.18, 44.50 and 44.11% for the pigeon breed of short distances, wild rock and long distances breeds, respectively, the centromeric index for the shortest chromosome was 3.03, 3.23 and 3.33 for chromosome 3 for the pigeon breed of short distances, wild and long distances breeds, respectively. The rest of the autosomes were acrocentric with 0.0% centromeric index in the three breeds. In addition, the Z chromosome proved to be submetacentric with CI% of 44.86, 44.37 and 44.44% for racing short distances, non-racing wild rock and racing long distances pigeon breeds, respectively and the W chromosome proved to be acrocentric with CI% of 3.90, 3.72 and 3.80 for the pigeon breed of short distances, wild rock and long distances breeds, respectively.

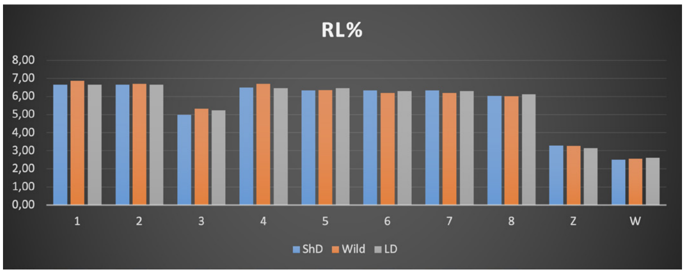

Figure 3 – Histogram showing the relative length (RL%) of the 8 autosomal pairs and the Z and W chromosomes in the three pigeon breeds.

GTG-banding analysis

The G-band idiogram of the chromosomes was developed based on twenty selected metaphases using automated karyotyping & FISH software processing (Leica CW4000).

Cytological examination of the G-banded chromosomes for the racing short distances, non-racing wild rock and racing long distances pigeon breeds revealed that the karyotype macrostructures were highly conserved and in general, in considerable accordance to the standard karyotype to the standard of G band identification on the chromosomes of Gallus domesticus (Ladjali-Mohammedi *et al.,* 1999).

The G-banding method using the photolytic enzyme trypsin affects the interaction that stabilizes the structure of different proteins and nucleic acid components of the chromatin. Therefore, the G-band mechanism is based mainly upon differences in protein composition and organization (Ali *et al.*, 2011; Abd El-Gawad *et al.,* 2019). It has also been suggested that trypsin treatment leads to the unfolding of protein loops and permits the protein structure associated with the alignment of AT-rich sequences, as reported by Abd El-Gawad *et al.* (2019).

Eight pairs of autosomes and sex chromosomes were analyzed. The short arm (p) of the chromosomes (1 and 2) and the long (q) arms of the chromosomes 2 and 4 as well as sex chromosome Z were divided into two regions. Meanwhile, the long (q) arms of chromosomes 1 and 3 were divided into three regions, but (p) arms of chromosomes (4, 5 and sex chromosomes Z and W) and (q) arms of chromosomes 6, 7 and 8 as well as sex chromosomes W observed into one region (Figures 3 and 4).

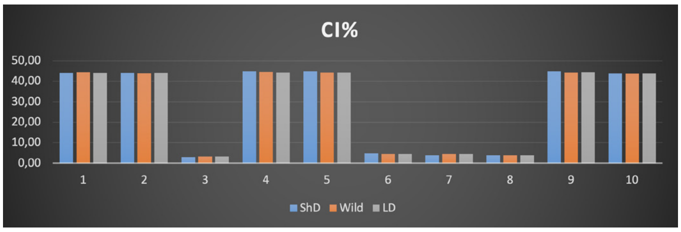

Figure 4 – Histogram showing the centromere index (CI%) of the 8 autosomal pairs and the Z and W chromosomes in the three pigeon breeds.

The G-banded ideogram of each of the three pigeon breeds comprised 114 bands in one set of haploid complements. The number of G-positive bands were 49 as stated in Table 6, while the total number of G-negative bands was 65. Twenty-two bands were detected in chromosome no. 1, nine bands were observed in the short arm, while thirteen bands were observed in the long arm, twenty bands were detected in chromosome no. 2, nine bands were observed in the short arm and eleven bands were observed in the long (q) arms of chromosomes. The same twelve bands were detected in chromosomes number (4, 5, and Z), five bands in the short arm and seven bands in the long arm. While in the acrocentric chromosome no. 3 eleven bands were detected, in the acrocentric chromosome no. 8 five bands were detected, but in the two acrocentric chromosomes no. 6 and 7 seven bands were detected. In the W chromosome 6 bands were detected, and three bands were observed in both ~~two~~ arms. By utilizing the relative length, the centromere index, and the G-banding for the 10 chromosome pairs of the three birds breeds (Tables 5 and 6), it was illustrated that the three pigeon breeds chromosomes are basically highly similar.

Table 6 – The total number of G- bands idiogram of 10 pairs of the three pigeon breeds, number of G-positive bands and G-negative bands.

		G-positive bands	G-negative bands		Total
1	p	4	5	9	22
	q	6	7	13	
2	p	4	5	9	20
	q	5	6	11	
3	p	5	6	11	11
4	p	2	3	5	12
	q	3	4	7	
5	p	2	3	5	12
	q	3	4	7	
6	p	3	4	7	7
7	P	3	4	7	7
8	p	2	3	5	5
Z	p	2	3	5	12
	q	3	4	7	
W	p	1	2	3	6
	q	1	2	3	
Total		49	65	114	

Neurotransmitters concentrations in brain tissues of pigeon breeds

Differences in neurotransmitters concentrations in brain tissues of three pigeon breeds are presented in Table (7). The pigeon breed of long distances racing showed the highest concentration (*p*<0.001) in all measured neurotransmitters; dopamine (DA), serotonin (ST), gamma-aminobutyric acid (GABA), acetyl choline esterase (ACHE) and melatonin (MTN) in brain tissues. In contrast, wild rock pigeon showed the lowest concentration among pigeon breeds in all measured brain's neurotransmitters. No significant gender-related differences were observed in the three pigeon breeds and therefore, the data in males and females were presented as averages.

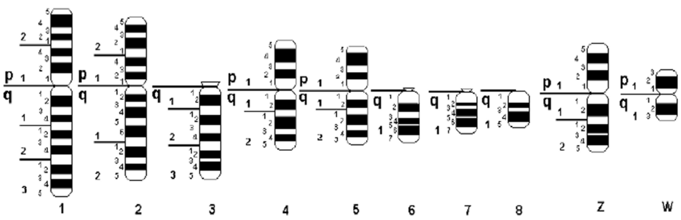

Figure 5 – Idiogram of the three pigeon breeds the 8 autosomal pairs and the Z and W chromosomes.

DISCUSSION

In this research work, the comparison of some neurotransmitters among three pigeon breeds; the non-racing wild rock pigeon and two racing pigeon breeds (long distance fly over 500 mils vs short distance fly up to 500 miles), showed that the pigeon breed of long distances racing showed the highest significant concentration (*p*<0.001) in all measured neurotransmitters including; DA, ST, GABA, ACHE and MTN in the brain tissues among other studied pigeon breeds (as seen in the result, Table 7). In contrast, the non-racing wild rock pigeon showed the lowest concentration in all measured brain's neurotransmitters. These results confirmed the important effects of brain neurotransmitters for high flying performance and back home in the racing competitions, particularly in this breed that flies (>500 mile). The reason for this difference is unknown. However, in the point view of the authors, the highest elevation in neurotransmitters concentration in the brain tissue of the pigeon breed of long distances racing may indicate the best health status, healthy brain functions rich with neurons, performance, high genetic structure and brain development of this breed explain a better race performance to fly long distance and back home faster than the pigeon breed of short distances racing and the wild rock pigeon, respectively. Despite, the information referring to neurotransmitters concentrations in brain tissues in different pigeon breeds to date are limited or missing, the analysis of neurotransmitters in the brain helps to understand brain function of pigeon breed and considering the fact that brain's neurotransmitters estimations have vital role in selecting racing pigeon breeders. Hence, the brain uses a variety of chemicals messengers called neurotransmitters to communicate between brain cells. Many studies showed the important part of neurotransmitters in the nervous system as they used to transmit many types of chemical messages "powerful neurochemicals or neurotransmitters" between

Table 7 – Differences in Neurotransmitters; dopamine (DA), serotonin (ST), Gamma-aminobutyric acid (GABA), acetyl choline esterase (ACHE) and melatonin (MTN) in brain tissues of Pigeon breeds.

Indices / Pigeon breeds	DA ng/ml	ST ng/ml	GABA pg/ml	ACHE pg/ml	MTN pg/ml
Racing long distances pigeon	0.666^{a}	159^{a}	258^{a}	68^{a}	197^{a}
Racing short distances pigeon	0.335^{b}	67^{b}	146^{b}	34^{b}	80^{b}
Non-racing wild rock pigeon	0.253^{b}	25^{c}	78^{c}	18^{b}	48^{c}
SEM	0.066	20.01	26.64	7.800	22.94
p -value	0.001	<0.001	<0.001	0.001	<0.001

Means in the same column within each classification bearing different letters are significantly different SEM standard error of means.

neurons of pigeon brain cells at the synapse, or from the nerve cells to target tissue throughout the body such as, muscles, glands, or other nerves. They enable the brain to provide a variety of functions, through the process of chemical synaptic transmission. The brain needs neurotransmitters, either excitatory or inhibitory to regulate many necessary bodily functions, including: heart rate, thinking and memory, breathing, sleep cycles, digestion, mood, concentration, appetite and muscle movement. The lack of these neurotransmitters are associated with some diseases and brain injuries. Many researchers reported that having low levels of neurotransmitters levels in certain regions of the brain tissue and central nervous system may lead to various brain diseases and major neurological disorders such as impaired motor coordination, learning and memory impairments, fatigue and muscle weakness, visual disturbances, cognitive impairment, hallucinations, mood and anxiety disorders (Pearl, 2013; Mastrangelo, 2021). One of the neurotransmitters is DA which is important for memory formation, influence several brain functions, including learning, blood flow, behavior, responsible for muscle movement co-ordination abilities, reward, emotion, alertness, decision-making, stimulates the secretion of growth hormone and executive functions (Rizo, 2018). The brain releases dopamine during pleasurable activities. GABA also, is a mood regulator and contributes to vision. It has an inhibitory action in the central nervous system, which stops neurons from becoming overexcited and is essential for the memory-storage elements of the brain, lowers stress and anxiety by slowing down heart rate, body temperature, and blood pressure (Bowery & Smart, 2006). While ST is an inhibitory neurotransmitter present in the brain and other tissues, particularly blood platelets "thrombocytes" and the lining of the digestive tract. In the brain, ST has been identified as an important factor in sleep cycle, helps regulate mood and emotions, metabolism and appetite, gut motility, blood clotting, perception of pain, body temperature, affect gastrointestinal processes like bowel motility, bladder control, the body's circadian rhythm, cardiovascular function and plays a role in mood and anxiety (Berger *et al.,* 2009). Furthermore, more than 90% of the body's total serotonin resides in the enterochromaffin cells in the gut, where it helps regulate the movement of the digestive system. An insufficient secretion of serotonin may result in decreased immune system function, emotional disorders like mood and disorder. MTN is a tryptophan-derived substituted indoleamine, acting as a neuromodulator, regulates various physiological processes, such as sleep-wake cycle, seasonal adaptation and immune function (Huang *et al.*, 2013; Reiter *et al.*, 2016). Acetylcholine is an excitatory neurotransmitter present in the brain secreted by motor neurons, critical for normal attention, memory, sleep cycle, stimulates muscle contraction, inhibit the heart muscle and controls the heartbeat. The low levels of ACHE in the brain can cause fatigue and muscle weakness. Thus, neurotransmitters play a role in nearly every function in the human body. A balance of neurotransmitters is necessary to prevent certain health conditions, such as mood, anxiety, neurological disorders and mental health (Südhof, 2013). Concerning the genetic measures, the current study investigated also the degree of genetic polymorphism (as seen in the result, Table 2), and protein sequence (as seen in the result, Figure 1A and B), among three pigeon breeds by focusing on the genes encoding dopamine receptor D4(DR-D4) named "neurotrans-mitter-associated gene" that are known to be associated with brain functions and the lactate dehydrogenase A (LDH-A) gene named ''performance –associated gene" which is found predominantly in muscle tissues and known to be associated with pigeon racing performance. The results clearly revealed major differences present between pigeon breeds in the DR-D4 and LDH-A genes polymorphism and protein sequence. Thereby, there were different biodiversity values for both genes among pigeon breeds particularly, between long distances male pigeon and both gender of wild rock pigeons, wherein, they showed high variation in genes polymorphism values, while the rest of breeds showed the same gene polymorphism. Furthermore, the variations appeared in LDH-A polymorphism of long distances male pigeon, particularly, on the position length 389bp or on two positions length of 418bp and 524bp for DR-D4 polymorphism indicate the presence of unique diversity in this breed, hence A changed to G for LDH-A and A changed to C and A changed to T for DR-D4 gene which means that high changes in gene expression occurred for both genes resulting in overall differences in the amino acids structure. The data derived from LDH-A gene protein sequence showed that in the position of 60 the amino acid K (*lys*) was converted to E (*glu*) in the LDH-A gene only in long distances male pigeon compared to the other breeds, while DR-D4 gene protein sequence in the pigeon breeds also showed that in the positions of 117 and 153 the amino acid R (*arg*) and L (*leu*) were converted to S (*ser*) and F (*phe*) only in long distances male pigeon compared to the other breeds. In spite of all pigeon breeds used in this study were subjected to the same managements as well as feeding, the results of DR-D4 and LDH-A gene protein sequence particularly, in long distances male pigeon compared to the other breeds confirmed the important role of amino acids: *glu* for LDH-A gene, *ser* and *phe* for DR-D4 gene polymorphism and functions (body muscle performance and neuro-transmitters status in brain tissue of pigeons), in addition to the important role of these amino acids as precursors for neurotransmitters building in all the body. In this respect, many studies have reported the importance of amino acids such as *phe, ser, glu, tryptophan, tyrosine, alanine, isoleucine, leucine, valine and histidine* which are present in brain tissues with highest concentration. Theses amino acids are taken up in the body either from protein-rich diet or synthesized by phenylalanine hydroxylation. They indirectly modulate synthesis of catecholamines, ST, GABA, DA and histamine (Fernstrom & Fernstrom, 2007; Featherstone, 2010; Coppola *et al.*, 2013; LaNoue *et al.*, 2013; Zhou & Danbolt, 2014; Dalangin *et al.*, 2020). To the authors' knowledge, little is known about the amino acid's roles for brain functions, health and genes polymorphism in the pigeon breeds. Therefore, further work is necessary in this topic. The variations in the DR-D4 gene polymorphism and protein sequence might have a role in increasing the levels of monoamines, particularly dopamine. Whenever, the variations in the LDH-A gene polymorphism and protein sequence might have role in increasing the levels of fuel source of energy for muscular activity and consequently the pigeon's flying performance. It is well known that, the DR-D4 gene of pigeon breeds affects the brain cells and enhances release of neurotransmitters such as ST and DA resulting in increased levels within the synaptic cleft leads to induce temporary improvements in physical and brain functions, mood, then elevate the body's ability to regulate muscles movement resulting in a better performance and powerful (Coyne *et al.,* 2015). Hence, DR-D4 gene is associated with the levels of monoamines in pigeon racing. Also DR-D4 gene protein sequence showed changes in the expression of genes that regulate tryptophan hydroxylase, an enzyme involved in ST synthesis particularly in long distance male pigeon breed. In contrast, no changes in the expression of genes for protein sequence in the other breeds and thereby may be led to low DA and ST. These can be the possible reasons for poor memory and depressed mood, neurodegenerative disorder, mood, anxiety, and attention processes. Further results agree with Proskura *et al.* (2015) who showed a number of different variations in DR-D4 gene between two racing pigeon either fly short distances races (<400 km) or middle distances races (>500 km). They attributed the variances in speed and distance performance between the two mentioned breeds at all distances (both middle and short distance), to the polymorphism in DR-D4 gene. The variations in this gene has been associated with mental capabilities and performance of racing pigeon (Hua *et al.*, 2014; Shao *et al.*, 2020). Regarding the results of LDH-A gene polymorphism and protein sequence, (Proskura *et al.*, 2015; Hunnam *et al.*, 2019) showed the efficiently of LDH-A genes in recycling and reusing the lactate in the muscles and consequently, leads to better speed performance Proia *et al.* (2016). Many studies showed that lactate is considered as an important fuel source of energy for muscular activity during strong exercises and racing competitions when oxygen is absent or in short supply and the rate of demand for energy is high; LDH-A enzyme converts pyruvate, the final product of glycolysis to lactate with nicotinamide adenine dinucleotide as a coenzyme (Ramadan *et al.,* 2013). Additionally, Dybus *et al.* (2006), Ramadan *et al.* (2018), and Gazda *et al.* (2018) attributed the racing pigeon survivability and homing abilities during racing the competitions to LDHA gene polymorphism and its role in physical and mental performance. From the previous results, testing the racing pigeon breeds for both LDHA the DRD4 genes is an important process in selecting new pigeon breeders and breeding perfect champion racing pigeons with high quality racing performance, capable of long flights and rapid return from distant released places through carrying possible variations of these genes and exclude the other pigeons that do not carry at least one of these racing performance genes from breeding program. Another indices studied in the current study and may be important in selecting racing pigeon breeds is cytogenetic indices including; chromosome structure and the precise identification of chromosomes using differential staining techniques such as G- banding and establishing a standard chromosome nomenclature for each breed. But, the study showed slight differences in the measured parameters of cytogenetic indices (the relative length, the centromere index and the G-banding for the 10 chromosome pairs), were detected among the three pigeon breeds. Therefore, form the above results discussed, the mentioned genes polymorphisms and their protein sequence ascertained the important role of neurotransmitters in selecting best racing pigeons breeders. Taking also into account there were slight differences in the measured parameters of cytogenetic indices detected among the three pigeon breeds.

CONCLUSION

In this article, it can be concluded that the values of both DR-D4 and LDH-A gene polymorphism and their protein sequence and neurotransmitters estimation (dopamine, serotonin, gamma-aminobutyric acid, acetyl choline esterase, and melatonin) in the brain tissue of pigeons would be useful indices for determining the differentiation, genetic characterization and performance fly of racing pigeon breeds and provide a foundation for developing sustainable genetic improvement and conservation programs of the breeding and selecting racing pigeons breeders.

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AUTHORS' CONTRIBUTIONS

Nashat Saeid Ibrahim (NSI), NMA and AEA participated in pigeon breeds sample collection and performed three breeds of Pigeons, maintained at the pigeons experimental farm, biochemical analysis of neurotransmitters and the statistical analysis of neurotransmitters data. NSI, nashaat1977@yahoo.com, Co-Author, participated in the conception, the design, data collection, interpretation of the results, wrote the manuscript and acted as corresponding author with MAE. Data discussion, data analyses. MAE, WSM and HAMA participated in genetic analyses of breeds and the data discussion and data analyses, and MEA, MAI measured parameters of cytogenetic among the three pigeon breeds. The authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ABBREVIATIONS

LM; males racing pigeon's long distances,
LF; females racing pigeon's long distances,
SM; males racing pigeon's short distances,
SF; females racing pigeon's short distances,
WM; males wild rock pigeons,
WF; females wild rock pigeons,
K (*lys*); Lysine,
E (*glu*); Glutamic acid,
R (*arg*); Arginine,
S (*ser*); Serine,
F (*phe*); Phenylalanine and
L (*leu*); Leucine.

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