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UTILIZATION OF CYTOCHROME B – MITOCHONDRIAL DNA IN BROILER RABBIT SELECTION

Vladimír PARKÁNYI[1], Ľubomír ONDRUŠKA[1], Ján RAFAY[1,2]*, Dušan VAŠÍČEK[1], Jaromír VAŠÍČEK[1,3], Andrej BALÁŽI[1], Alica NAVRÁTILOVÁ[3]

[1]NPPC – Research Institute for Animal Production Nitra, Lužianky, Slovak Republic
[2]University of Ss. Cyril and Methodius in Trnava, Faculty of Natural Sciences, Trnava, Slovak Republic
[3]Slovak University of Agriculture in Nitra, Nitra, Slovak Republic

ABSTRACT

Cytochrome b haplotypes (GenBank OCU07566, NCBI, USA) of mitochondrial DNA (mtDNA) were described in meat lines of rabbits – cyt b 430 and cyt b 306. The haplotype cyt b 430 represents 571A (190Threonine-T)+877G(292Alanine -A). Haplotype cyt b 306 with polymorphism A571G mtDNA-cell line LEU-RAB is registered as BioSample: SAMN03701526; Sample name: cyt b O.C. (Model organism or animal sample from *Oryctolagus cuniculus*), in NCBI, USA. The vitality of offspring of the cyt b 306 haplotype, represented by the average number of weaned youngs at 42 days of age, was significantly higher compared to the haplotype cyt b 430 (ẍ = 7.00 vs. ẍ = 5.69, t (0.01) = 2.91^{++}). These results are confirmed also by the immunogenetic results determined using flow cytometry. Haplotype cyt b 306 is characterized by a significant increase (P = 0.041^{+} to P = 0.049^{+}) of the flow cytometric parameters at the frequency of T lymphocytes, which results in activation of lymphocytes of the type pT2 and CDS4 (pT2 = 22.02 ± 4.45 % and CD4 = 17.08 ± 3.43 %), compared to the cyt b 430 haplotype, which is represented by significantly lower values of the flow cytometric parameters in activation of lymphocytes of the pT2 and CD4 type (pT2 = 15.32 ± 6.11 % and CD4 = 11.8 ± 4.83 %).
Meanwhile, in the reproduction parameters such as the average number of live-born kits, no differences (8.45 vs. 8.00) were determined between the studied haplotypes (cyt b 306 vs. cyt b 430). Similarly, production parameters represented by carcass value (carcass without the head and skin) of both haplotypes (cyt b 306 vs. cyt b 430) were not influenced by the targeted selection and remained on the same level of 54 % (54.41 vs. 54.26).

Key words: polymorphism; cyt b; rabbit; reproduction

INTRODUCTION

Mitochondrial DNA is traditionally used in population genetics as a selection marker for characteristics of phylogenesis (Avise *et al*., 1987; Zink & Barrowclough, 2008). Many researchers highlighted the functional role of mtDNA and the opportunities for direct utilization of mitochondrial data in ecology and evolution (Ballard & Whitlock, 2004; Gemmell *et al*., 2004; Dowling *et al*., 2008). Multicellular organisms are characterised by a closed circular mtDNA molecule. Animals in general have a small mtDNA (15 – 20 kb) genome containing 37 genes. Genome mtDNA codes 13 enzymes of oxidative phosphorylation (OXPHOS complexes). Rapid changes in amino acid composition influence OXPHOS complexes and have a significant impact on the function of an organism.

Repair mechanisms on the mtDNA level usually eliminate most of the damaging mutations fast. However, unrepaired mtDNA sequences influence the quality of mitochondrial enzyme production

***Correspondence:** E-mail: rafay@vuzv.sk
Ján Rafay, NPPC – Research Institute for Animal Production Nitra, Hlohovecká 2, 951 41 Lužianky, Slovak Republic

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and therefore also the effective share on the total energy metabolism of the cell. Based on the matroclinal inheritance of mtDNA, the mutation haplotype will be present in the population along the female line. This effect was named "mother's curse effect" (Gemmell *et al*., 2004).

The central role in the 3 OXPHOS complexes of mitochondria is played by cytochrome b. Cytochrome b is a protein present in the mitochondria of eukaryotic cells. It forms a part of the electron transport chain and is the primary subunit of the transmembrane cytochrome bc1, known also as ubiquinol-cytochrome c reduction. These complexes participate on the transport of electrons, extraction of protons and generation of the proton-motive force (PMF). Then the proton gradient is used for the production of ATP, therefore these processes play a significant role in cells (Brand, 1997). It is most often used as a mtDNA sequence to determine phylogenetic relationships between organisms. However, cytochrome b finds application in clinical practice as well. Mutations on the cytochrome b cause significant changes in the respiratory tract, which leads to its damages and decreased health of the affected organism (Wei, 1998).

This paper presents the results observed in study of the relationship between cytochrome haplotype frequencies and vitality traits of rabbits.

MATERIAL AND METHODS

Animals

The experimental animals (broiler rabbits) were kept at an accredited facility of the Research Institute for Animal Production Nitra (RIAP) within the National Agricultural and Food Centre in Nitra, Slovakia. Cytochrome b mtDNA was analysed from peripheral blood samples (sampled from *a. auricularis centralis* into heparinised vials) from males (18 animals) and females (24 animals) of a parent generation and their F1 generation of the original meat lines (M91 and P91) of rabbits kept at RIAP Nitra. Females of the parent generation were divided into two groups: 1. – experimental group (12 animals) underwent divergent selection with strict selection criteria (the selected females had to have at least three consecutive litters of 7 – 10 live-born kits). 2. – control group (12 animals) with three consecutive litters with significant variability in the number of live-born kits (1 – 15). The animals were housed in single-level cages under constant light regiment of 14 hours of light and 10 hours of darkness per day. The temperature and humidity in the housing area was continuously monitored using a hydrothermograph, which was situated on the same level as the cages (the average humidity and temperature during the year were maintained at values of 60 ± 5 % and 17 ± 3 °C). The experimental rabbits were fed a commercial diet for growing animals (KV, Tekro Nitra, s.r.o) *ad libitum* and water was supplied through drinkers *ad libitum*. The experiment was approved by the State Veterinary and Food Administration of the Slovak Republic, no. SK CH 17016, SK U 18016.

In the following generations of the progeny of the experimental females selected into the program of divergent selection, in the 24rd to 83rd litter we evaluated also the reproduction and production parameters of the two studied mitochondrial haplotypes (cyt b 430 and cyt b 306), such as the average number of live-born kits per litter, average number of weaned kits, live weight at the age of 75 days, carcass weight and carcass value.

From the gathered results of the particular genotype groups, basic variation-statistical parameters were calculated. Significance of the differences in the arithmetic averages was estimated using the t-test. In order to test a set, Scheffe's test was used. Statistic differences were evaluated on the level of significance $P \leq 0.05$; $P \leq 0.01$ a $P \leq 0.001$.

Cytochrome b-mtDNA (mitochondrial DNA)

To the heparinized rabbit blood samples (Heparin, 25 000 I.U., 6 µl.ml^{-1}) was added a lysis solution (200 µl 1x PCR buffer (50 mM KCl, 20 mM Tris-HCl pH 8.4), enriched with 50 mM DTT, 1 % Triton X-100 and 400 ng.µl^{-1} proteinase K. The samples were incubated overnight at 56 °C. After the incubation, they were deactivated and denaturised for 5 minutes at 96 °C, after which they were cooled down to laboratory temperature. We used technology of amplification of specific small sequences of isolated mitochondrial (mtDNA) male and female rabbit DNA via polymerase chain reaction (PCR). To isolate mtDNA from the heparinised peripheral blood, Maxwell DNA Purification Kit was used. Concentration of DNA

in samples (2.495-2.994 ng.µl^{-1}) was measured using NaNoPhotometer (Implen) spectrophotometer. As a reference solution, we used an elution solution used in DNA elution during purification.

PCR conditions (PTC-200 DNA Engine; BioRad) were 95 °C for 2 minutes, 94 °C for 30 s, 54 °C for 30 s, 35 cycles with the last step of extension at 72 °C for 10 minutes. PCR reaction volume (25 µl) contained 10 mM Tris-HCl (pH 8.6 at 25 °C, 50 mM KCl, 1.5 mM $MgCl_2$, 25 units.ml^{-1} Taq DNA polymerase, 0.2 mM dNTPs each, 5 % glycerol, 0.08 % IGEPAL® CA-630, 0.05 % Tween-20) (New England Biolabs), 10 pmol.µl^{-1} each average (ORYCTO-cyt b-FOR-21nt a ORYCTO-cyt b-REV-20nt) and from 2.495 to 2.994 ng.µ^{-1} from every mtDNA sample. Amplified mtDNA was electrophoreticaly separated to 2 % agarose gel containing ethidium bromide at 80 mA, 120 V in 10 mM lithium borate buffer, pH 8.0 for 90 minutes. PCR products were visualized under UV light and photographed using documentation system MiniBis Pro (Bio-Imaging Systems) (Figure 1).

The detected fragment was 692 bp long, located in the area of *Oryctolagus cuniculus* – cytochrome b (GenBank OCU07566, NCBI, USA) of the rabbit mitochondrial DNA. To locate and restrict the analysed mtDNA sequence, oligonucleotides designed and synthetized under laboratory conditions were applied:

Oligonucleotides for detection of the partial cytochrome b *Oryctolagus cuniculus*:

	Tm
ORYCTO-cyt b-FOR 5'- CTA TCA GCA ATC CCA TAT ATC -3'	54.0 °C
ORYCTO-cyt b-REV 5'- CTT CAT TTG AGG ATT TTG TT -3'	54.0 °C

After evaluation of the amplified PCR product via Agarose electrophorese, the amplicons were then broken by AluI (5 U/ 20 µl) restriction enzyme (New England Biolabs). AluI recognizes a specific sequence 5'- ...AG'CT ...-3' , resp. 3' - ...TC'GA ...- 5', and breaks down the given PCR product, depending on the presence of a given sequence in the amplified sequence under optimal reaction conditions (37 °C), Figure 1.

Flow Cytometry

Samples of peripheral rabbit blood (PB) were taken from *a. auricularis centralis* into heparinized vials. Mononuclear cells from the peripheral blood (PBMC) were isolated using centrifugation with Ficoll according to the original protocol: Isolation of mononuclear cells from human peripheral blood via centrifugation in dense gradient (Miltenyi Biotec, 2008). In each sample, 10 000 to 50 000 cells were measured using flow cytometer FACS Calibur (Becton Dickinson, Mountain View, CA). 7-AAD dye solution (BD Biosciences, USA) was used to eliminate dead cells from the analysis. Frozen and then thawed cells were divided into prepared vials and dyed with various clones of anti-rabbit monoclonal antigens: anti-IgM (NRBM, Bio-Rad AbD Serotec GmbH, Nemecko), anti-CD4 (RTH1A, WSU, Pullman, WA) anti-CD8 (ISC27A, WSU, Pullman, WA), anti-pan T2 (pT2, RTH21A, WSU, Pullman, WA).

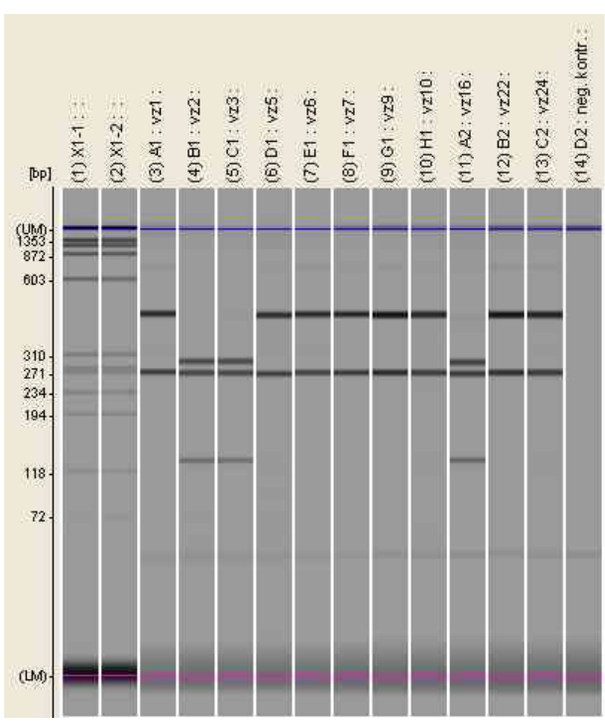

Figure 1. Cytochrom b of rabbit mitochondrial DNA: results of PCR-RFLP (AluI). Microchip electrophoresis MCE® -202 MultiNA.

RESULTS AND DISCUSSION

AluI restriction analysis of the PCR product results in the creation of fragments, which were analysed by microchip electrophoresis MCE® -202 MultiNA, Figures 1 and 2. Based on the restriction fragments, 2 types of AluI RFLP -cyt b mt DNA (GenBank OCU07566, NCBI, USA) were detected: 430-262bp and 306-262-124bp. Based on them, the experimental rabbits were divided into 2 haplotype groups cyt b 430 and cyt b 306.

Haplotype cyt b 430 represents 571A (Threonine-T)+877G (Alanine-A). Haplotype cyt b 306 is represented by substitution of nucleotides A571G, that is 571G (Alanine-A) +877G (Alanine-A).

This substitution of nucleotides is the causal result of conversion and translation alteration in the peptide chain, where the essential amino acid Threonin-T is replaced with amino acid Alanin-A, T190A. Haplotype cyt b 430 is characterized by one restriction position (877G; Figure 3). While cyt b 306 is a new haplotype defined by two restriction positions 571G and 877G.

Sudden changes in the sequence of amino acids in cytochrome b influence the expression of OXPHOS mitochondrial complexes of somatic and generative cells and have a large influence also on the function of tissue and organs and therefore on the bodily functions as well.

Due to the aforementioned reason, the occurrence of new haplotypes in cytochrome b mtDNA of rabbits (cyt b 306 and cyt b 430) is studied also from the aspect of prospective benefit as a candidate biomarkers associated with select physiological, functional, selection, reproductive, and production parameters, such as the number of live-born kits in a litter, the average number of weaned kits, live weight at the age of 75 days, carcass weight and carcass value (Table 1).

In the reproduction parameters such as the average number of live-born kits, no significant differences (8.45 vs 8.00) were determined between the studied haplotypes (cyt b 306 vs cyt b 430).

Similarly, the production parameters represented by carcass value of both haplotypes (cyt b 306 vs cyt b 430) were not negatively influenced by the targeted selection and remained at the same level of 54 % (54.41 vs. 54.26).

Despite this, it can be stated that the cyt b 430 haplotype reached higher live weight (g) at the slaughter age of 75 days compared to cyt b 306 haplotype (3076 vs 2884.44). These parameters

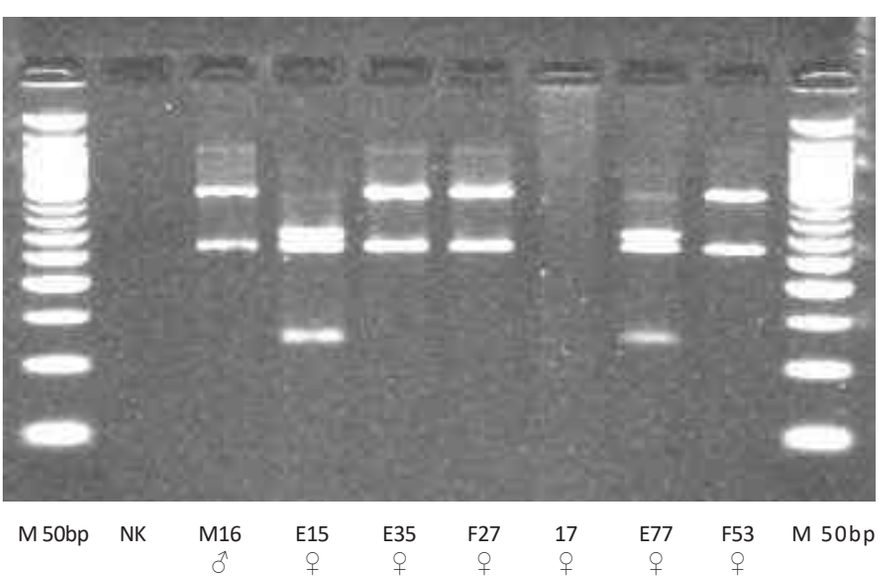

Figure 2. Alu I cleavage of cytochrome b 692 bp PCR product visualised on 2 % Agarose gel by MiniBis Pro (Bio-Imaging Systems). Haplotype cyt b 430 = samples M16, E35, F27, F53; haplotype cyt b 306 = samples E15 and E77; NK = negative control, M = 50 bp DNA Ladder (Jena Bioscience).

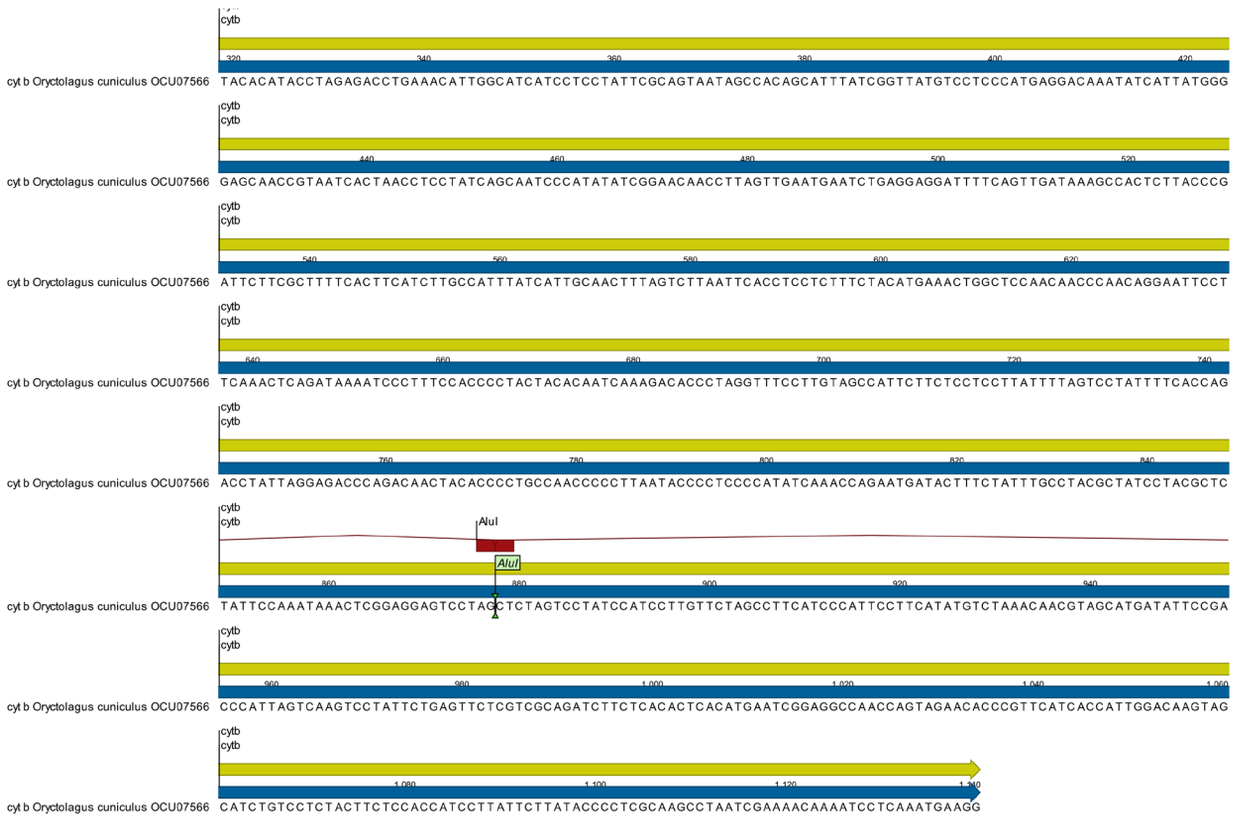

Figure 3. Haplotype cyt b 430: AluI Cytochrome B (GenBank OCU07566 = 430-262b, 571A(190Threonine-T)+ 877G(292Alanine-A)

of cyt b 430 however represent the influence of the higher weight of the head and skin (g) compared to cyt b 306 haplotype (672.00 vs 613.89). The results from evaluation of slaughter weight (g) are significantly favourable to cyt b 430 haplotype (1669.00 vs 1569.44).

However, for an important production and economic parameter – vitality of kits represented by the number of weaned kits at the age of 42 days, haplotype cyt b 306 showed significantly higher values than haplotype b 430 ($\ddot{x}$ = 7.00 vs. $\ddot{x}$ = 5.69, t (0.01) = 2.91^{++}). These results are confirmed also by the immunological data gathered from flow cytometry. The changes in amino acid composition of cytochrome b influence also the OXPHOS complexes of mitochondria and significantly affect the cells of the immune system as well – such as T and B lymphocytes, Table 2. T-lymphocytes are created in thymus in the form of CD4^{+} or CD8^{+} cells. CD4^{+} cells help B lymphocytes during cell-propagated immune response via lyphozine secretion. CD8^{+} cells specialize on cytotoxic killing of other cells, especially virally infected cells or tumour cells under experimental conditions. Their functions can partly overlap, when CD8^{+} cells produce lymphokines and CD4^{+} cells can kill other cells. The most important difference is, however, that CD4^{+} cells recognise antigen peptide in coordination with MHC molecules of Class II while CD8^{+} cells cooperate with Class I MHC molecules (Nossal, 1997). When the CD4^{+} T cells are activated, they start to secrete various types of cytokines (Kelso *et al*., 1991). When the immunity response reaches its peak, however, cases may occur when either (Th)-1 response or (Th)-2 response prevails (Mosmann a Coffman, 1989). Th-1 response causes production of antigens, including production of TgG1 and IgE antigens (Finkelman *et al*., 1990). B lymphocytes are responsible for the production of antigens (Nossal *et al*., 1968). At the beginning, the IgM and B cells can cause an immune response without activation of T cells, but most long-term immunity responses involving IgG, IgA

Table 1. Reproduction and production traits of mtDNA-cytochrome b haplotype rabbits

Traits	mtDNA haplotypes		
	Cytochrome b 306		Cytochrome b 430
1. Number of live born animals per litter: ẍ	8.45		8.00
$s_{\ddot{x}}$	3.50		3.18
df		85.00	
P		0.27	
$t_{(0,05)}$		0.61^{-}	
2. Number of weaning animals at age 42 days: ẍ	7.00		5.68
$s_{\ddot{x}}$	1.46		2.54
df		78.00	
P		0.002	
$t_{(0,01)}$		2.91^{++}	
3. Live weight at 75 days (g): ẍ	2884.44		3076.00
$s_{\ddot{x}}$	159.01		142.14
df		26.00	
P		0.002	
$t_{(0,01)}$		3.16^{++}	
4. Carcass weight (g): ẍ	1569.44		1669.00
$s_{\ddot{x}}$	95.51		83.19
df		26.00	
P		0.005	
$t_{(0,01)}$		2.76^{++}	
5. Carcass utility (%) without head and skin: ẍ	54.41		54.26
$s_{\ddot{x}}$	1.49		0.96
df		26.00	
P		0.39	
$t_{(0,05)}$		0.29^{-}	
6. Weight of head and skin (g): ẍ	613.89		672.00
$s_{\ddot{x}}$	38.22		56.73
df		26.00	
P		0.002	
$t_{(0,01)}$		3.24^{++}	

or IgE antigens requires the help of activated T cells (Miller, 1972). Knowledge of the physiological values is required in order to recognise changes in the distribution of particular types of lymphocytes (Faldyna *et al*., 2001). Haplotype cyt b 306 is characterized by a significant increase ($P = 0.041^{+}$ to $P = 0.049^{+}$) of the flow cytometric parameters in the frequency of the T lymphocytes, which is expressed as activation of pT2 and CD4 (pT2 = 22.02 ± 4.45 % and CD4 = 17.08 ± 3.43 %) type lymphocytes. Compared to the cyt b 430 haplotype, which is represented by significantly lower values of flow cytometric parameters in activation of pT2 and CD4 (pT2 = 15.32 ± 6.11 % and CD4 = 11.48 ± 4.83 %) (Table 2).

From these results it is apparent that genetic polymorphism in cyt b gene is a useful genetic marker in selection and breeding of broiler rabbits.

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Table 2. Influence of haplotype mtDNA=cyt b 306 and cyt b 430 on Flow cytometric parameters for F1 generation at age 62 days (youngs from 56 to 63 days are characterized by maturation of gastrointestinal tract – microbiotical and chemical digestibility

Sample	Flow cytometric parameters/ age of F1 generation	pT2 (%)	CD4 (%)	CD8 (%)	$CD4^+CD8^+$ (%)	IgM (%)	pT2/IgM (ratio)	CD4/CD8 (ratio)	CRP (%)
E15-1 cyt b 306	62 days	17.55	13.27	4.74	0.26	15.13	1.16	2.80	0.04
E15-2 cyt b 306	62 days	28.02	18.32	7.77	0.22	8.83	3.17	2.36	0.02
E77-16 cyt b 306	62 days	20.22	21.19	5.23	0.18	18.06	1.12	4.05	0.07
E77-18 cyt b 306	62 days	22.30	15.52	5.38	0.90	8.46	2.64	2.89	0.17
E17-11 cyt b 430	62 days	6.09	4.63	1.45	0.27	9.53	0.64	3.19	0.02
E17-12 cyt b 430	62 days	10.40	8.16	2.38	0.11	10.21	1.02	3.43	0.03
E17-13 cyt b 430	62 days	17.34	13.92	3.81	0.20	7.44	2.33	3.65	0.03
E5-8 cyt b 430	62 days	16.10	11.11	4.73	0.22	15.87	1.01	2.35	0.07
E35-5 cyt b 430	62 days	23.02	18.67	11.30	0.29	25.97	0.89	1.65	0.08
Statistics	cyt b 306	22.02 ± 4.4	17.08 ± 3.4	5.78 ± 1.3	0.39 ± 0.3	12.62 ± 4.7	2.02 ± 1.04	3.03 ± 0.72	0.08 ± 0.0
	cyt b 430	15.32 ± 6.1	11.48 ± 4.8	4.84 ± 3.48	0.26 ± 0.12	14.25 ± 6.7	1.17 ± 0.59	2.76 ± 0.78	0.04 ± 0.0
		$P = 0.049$	$P = 0.041$	$P = 0.314^-$	$P = 0.203^-$	$P = 0.345^-$	$P = 0.067^-$	$P = 0.302^-$	$P = 0.155^-$
		$t = 1.8^+$	$t = 1.99^+$	$t = 0.50$	$t = 0.88$	$t = 0.41$	$t = 1.66$	$t = 0.54$	$t = 1.08$

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