THESES OF DOCTORAL (PhD) DISSERTATION

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POLYMORPHISM AND GENE EXPRESSION ANALYSIS OF SOME METABOLICALLY IMPORTANT GENES IN POULTRY SPECIES

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1 OBJECTIVES

The author's aim was to determine allele and genotype frequency of several genes that are potentially involved in growth and the development of body composition in a commercial hybrid broiler chicken population, such as *Spot14α* (a thyroid hormone responsive transcription factor), insulin-like growth factor binding protein 2 (*IGFBP-2*), somatostatin (*SST*), and prolactin (*PRL*). Furthermore, the author analysed the association between genotypes and various production traits (live weight at slaughter, carcass weight, thigh weight with skin and bone, breast muscle weight with and without skin).

In a further study, the author aimed to evaluate the expression of genes involved in growth and fat metabolism, such as peroxisome proliferator-activated receptor gamma (*PPARγ*), fatty acid desaturase 2 (*FADS2*), and insulin-like growth factor 1 (*IGF1*) in response to linseed oil supplementation in different tissues (breast, thigh, adipose tissue, liver) of male hybrid turkeys.

2 MATERIALS AND METHODS

2.1 Genotyping in the commercial broiler population

Feather samples were collected from 103 male ROSS-308 broiler chickens. Individual samples were stored in zip lock plastic bags at -20ºC until DNA isolation. Live weight, carcass weight, breast weight with and without skin, thigh weight (with skin and bone) was measured at the abattoir. Genotyping was carried out in the laboratories of Széchenyi István University, Faculty of Agricultural and Food Sciences, Department of Animal Science. DNA isolation from feather samples was done by means of the Wizard Genomic DNA Purification Kit (Promega, USA). Following DNA integrity test by agarose gel electrophoresis, NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) was used to determine DNA concentration. Oligonucleotides were designed with Primer3 application, and ordered from IDT. Analysed loci were amplified by polymerase chain reaction (PCR), and – where required – digestion by restriction enzymes (endonucleases) were applied to reveal genotypes. Genotypes of the following loci were determined:

- G645T single nucleotide polymorphism (SNP) in exon 2 region of insulin-like growth factor binding protein 2 gene (*IGFBP-2*)
- A213C SNP in exon 1 of thyroid hormone responsive transcription factor gene (*Spot14α*)

- 24 bp insertion/deletion (indel) in the promoter region of prolactin gene (*PRL*)
- A370G SNP in exon 2 of somatostatin gene (*SST*)

Data were recorded and organised in Microsoft Excel (2013, USA). IBM SPSS Statistics v.20.0 for Windows software was used for statistical analyses. Data normality was tested by Kolgomorov–Smirnov tests, whereas genotype-trait associations were analysed by Least Significant Difference (LSD) tests.

2.2 Gene expression analysis in turkeys

Changes in the expression of *FADS2*, *PPARγ*, *IGF-1* genes in response to linseed oil supplementation were analysed in several tissues of male hybrid Converter turkeys. Breast, thigh, abdominal fat, and liver samples were collected at a local abattoir in vials filled with RNAlater solution (Thermo Fisher Scientific), and then stored at room temperature until further processing. Sample processing and gene expression analyses were also carried out in the laboratories of the Department of Animal Science. Following total RNA extraction, concentration was evaluated by means of a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). RNA integrity was assessed via agarose gel electrophoresis. In order to eliminate potential DNA contamination, RNA samples were treated with DNase (RQ1 RNase-free DNase; Promega, Madison, WI, USA), then total RNA was reverse transcribed using iScript cDNA Synthesis kit (Bio-

Rad Laboratories, Hercules, CA, USA) supplied with random hexamer and oligo dt primers. Primers required for qPCR were designed with Primer3 based on available turkey gene sequences.

The expression of genes of interest was analysed by means of the $2^{-\Delta\Delta Ct}$ method normalised to two reference genes, namely *GAPDH* and *ACTB*. Statistical analysis was performed on the $2^{-\Delta\Delta Ct}$ values by means of the independent samples t-test using the IBM SPSS Statistics v.20.0 software.

3 RESULTS

3.1 Genotyping results in the broiler population

3.1.1 *Spot14α* genotyping

The A213C SNP in *Spot14α* was polymorphic in the broiler population, and three genotypes were detected (*AA*, *AC*, *CC*). Allele and genotype frequencies are presented in Table 1.

Table 1. *Spot14α allele and genotype frequencies, and the result of Chi-square test for Hardy–Weinberg equilibrium (degree of freedom (df)= 2)*

Allele frequency	Genotype frequency	x^2	p
A=0.11 *C*=0.89	*AA* (1) = 0.01 *AC* (20) = 0.20 *CC* (79) = 0.79	1.700	0.190

Based on the result of the Chi-square test the population was in HWE regarding the A213C polymorphism in *Spot14α*, as no significant (P>0,05) differences were detected between observed and expected genotype frequencies.

AA genotype was not included in genotype-trait association analysis due to small group size. There wer no significant (P>0,05) differences between *AC* and *CC* genotypes, with the exception of breast weight without skin % relative to live weight, where the birds with *CC* genotype demonstrated greater production compared to animals with *AC* genotype (Table 2).

Table 2. *Association of Spot14α genotype with the analysed traits in the broiler population*

Traits	Genotype	
	***AC* (n=20)**	***CC* (n=79)**
Live weight (g)	2534.61±279.19	2509.32±262.10
Cacass weight (g)	1929.72±212.47	1936.04±198.21
Breast weight with skin (g)	622.109±94.29	631.30±81.41
Relative to live weight (%)	27.38±3.54	28.53±4.23
Relative to carcass weight (%)	36.02±5.26	35.23±6.60
Breast weight without skin (g)	523.33±110.47	548.88±81.27
Relative to live weight (%)	**22.34±1.71[b]**	**23.87±2.97[a]**
Relative to carcass weight (%)	29.14±1.90	28.62±3.13
Thigh weight (g)	579.13±61.23	581.13±72.31
Relative to live weight (%)	25.61±3.18	26.23±3.61
Relative to carcass weight (%)	33.69±4.68	32.37±5.54

3.1.2 *IGFBP-2* genotyping

The G645T polymorphism in *IGFBP*-2 was present in the broiler population with two genotypes (*GG*, *GT*), whereas *TT* genotype was not detected (Table 3).

Table 3. *IGFBP-2 allele and genotype frequencies, and the result of Chi-square test for Hardy–Weinberg equilibrium (degree of freedom (df)= 2)*

Allele frequency	Genotype frequency	x^2	p
G=0.92 *T*=0.08	*GG* (86) = 0.84 *GT* (17) = 0.17 *TT* (0) = 0.00	0.657	0.417

Based on the result of the Chi-square test the population was in HWE regarding the G645T polymorphism in *IGFBP-2*, as no significant (P>0,05) differences were detected between observed and expected genotype frequencies.

The two detected genotypes (*GG*, *GT*) were significantly (P<0,05) different regarding several traits of the analysed population. Live weight, carcass weight, breast weight with and without skin of the heterozygous (*GT*) animals was significantly greater compared to homozygous chickens. Breast weight % relative to carcass weight was also significantly greater in the heterozygous birds (Table 4).

Table 4. *Association of IGFBP-2 genotype with the analysed traits in the broiler population*

Traits	Genotype	
	***GG* (n=86)**	***GT* (n=17)**
Live weight (g)	**2485.21±263.33[b]**	**2638.93±219.81[a]**
Cacass weight (g)	**1911.39±194.11[b]**	**2034.13±194.54[a]**
Breast weight with skin (g)	**618.64±80.83[b]**	**678.95±76.26[a]**
Relative to live weight (%)	28.31±4.17	28.53±3.90
Relative to carcass weight (%)	35.19±6.48	35.86±5.74
Breast weight without skin (g)	**528.39±81.20[b]**	**604.15±91.28[a]**
Relative to live weight (%)	23.46±2.94	24.33±2.74
Relative to carcass weight (%)	**28.37±2.93[b]**	**30.05±2.46[a]**
Thigh weight (g)	575.12±70.06	603.45±61.38
Relative to live weight (%)	26.31±3.58	25.35±3.14
Relative to carcass weight (%)	32.67±5.45	31.98±5.44

3.1.3 *PRL* genotyping

The 24 bp indel in the promoter region of *PRL* was present in the broiler population, three genotypes were separated (Table 5).

Table 5. *PRL allele and genotype frequencies, and the result of Chi-square test for Hardy–Weinberg equilibrium (degree of freedom (df)= 2)*

Allele frequency	Genotype frequency	χ^2	p
***D*=0.77** ***I*=0.23**	*DD* (66) = 0.56 *ID* (48) = 0.41 *II* (3) = 0.03	0.001	0.970

Based on the result of the Chi-square test the population was in HWE regarding the indel polymorphism in *PRL*, as no significant (P>0,05) differences were detected between observed and expected genotype frequencies. Remarkable differences were described between the *PRL* allele frequencies in different chicken breeds, and the deletion (*D*) allele was generally associated with lower/moderate egg production intensity.

Table 6. *Association of PRL genotype with the analysed traits in the broiler population*

Traits	Genotype	
	***DD* (n=59)**	***ID* (n=42)**
Live weight (g)	2533.64±283.97	2488.68±222.57
Cacass weight (g)	1946.65±208.28	1919.27±181.18
Breast weight with skin (g)	636.72±81.75	617.47±85.56
Relative to live weight (%)	28.74±4.17	27.48±3.88
Relative to carcass weight (%)	35.64±6.65	34.32±5.70
Breast weight without skin (g)	542.63±82.65	544.00±93.31
Relative to live weight (%)	23.78±2.89	23.39±3.00
Relative to carcass weight (%)	28.65±3.01	28.61±2.85
Thigh weight (g)	**593.34±71.47[a]**	**562.71±61.61[b]**
Relative to live weight (%)	**26.77±3.67[a]**	**25.07±3.06[b]**
Relative to carcass weight (%)	32.49±5.59	31.38±5.06

Homozygous *D* birds were characterised by greater (P<0.05) thigh weight and thigh weight relative to live weight (Table 6). Due to small group size, *II* individuals were not involved in the association study.

3.1.4 *SST* genotyping

In regard to the A370C SNP of *SST*, the A allele was fixed in the broiler population. There was no relevant literature available concerning allele and genotype frequencies or genotype-trait associations for the non-synonym A370G polymorphism in other breeds or hybrids.

3.2 Gene expression results in turkeys

3.2.1 *FADS2* expression results

A significant ($P<0.001$) increase was observed in *FADS2* hepatic expression in response to linseed oil (LO) supplementation compared to the control group. LO supplementation also affected muscle *FADS2* levels; however, a significant ($P<0.05$) increase was only described in thigh muscle samples, and not in the breast tissues. In contrast, adipose tissue *FADS2* expression was significantly lower in the LO-supplemented animals (Figure 1).

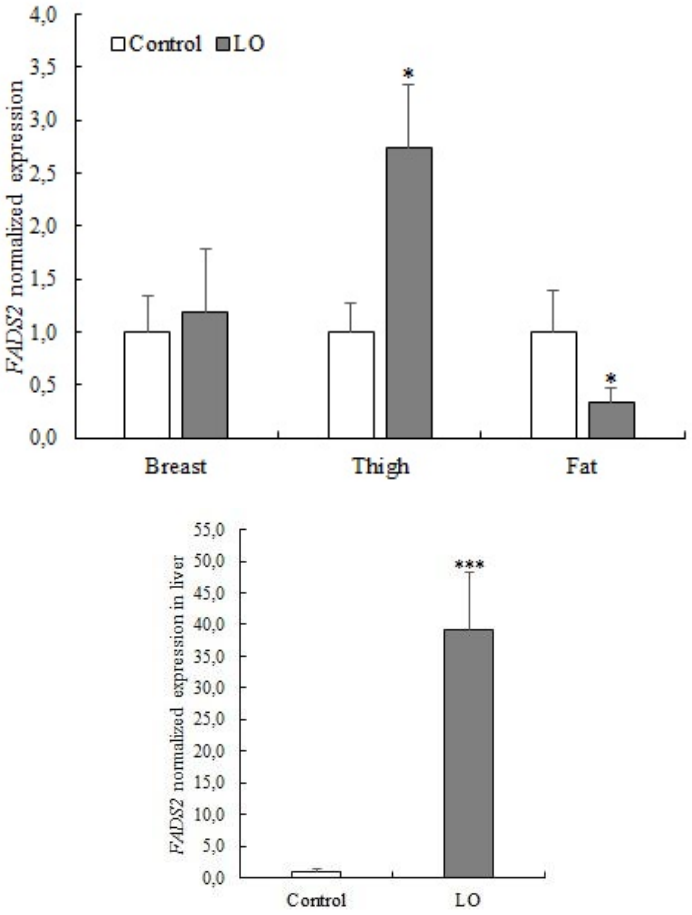

Figure 1. *Normalised FADS2 gene expression in breast, thigh, abdominal fat, and liver tissues of control and linseed oil (LO) supplemented male turkeys. Each bar represents mean±SEM of the analysed group. * and *** represent significant (P<0.05 and P<0.001, respectively) differences between control and LO groups*

3.2.2 *PPARγ* expression results

PPARγ mRNA levels significantly (P<0.05) decreased in thigh muscle samples of the LO supplemented turkeys. Abdominal fat *PPARγ* expression was significantly greater in the LO group, whereas breast

muscle and hepatic expression did not differ between the experimental groups (Figure 2).

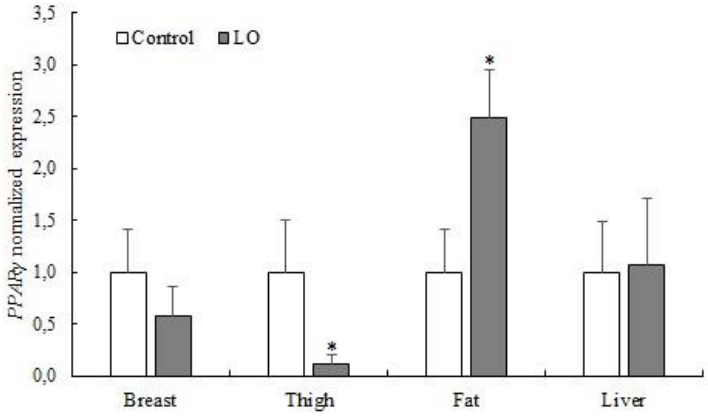

Figure 2. *Normalised PPARγ gene expression in breast, thigh, abdominal fat, and liver tissues of control and linseed oil (LO) supplemented male turkeys. Each bar represents mean±SEM of the analysed group. * represents significant (P<0.05) differences between control and LO groups*

3.2.3 *IGF-1* expression results

Elevated *IGF-1* expression was observed in muscle samples of the LO group; however, only thigh expression levels differed significantly (P<0.05). Conversely, *IGF-1* mRNA levels were lower in the adipose tissue of LO supplemented turkeys, whereas hepatic expressions did not differ (Figure 3).

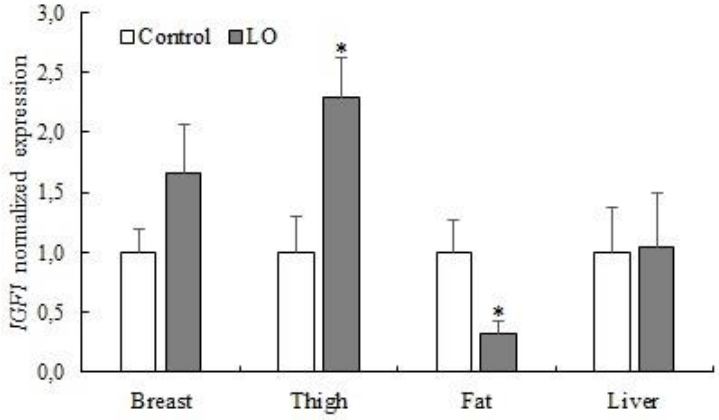

Figure 3. *Normalised IGF1 gene expression in breast, thigh, abdominal fat, and liver tissues of control and linseed oil (LO) supplemented male turkeys. Each bar represents mean±SEM of the analysed group. * represents significant (P<0.05) differences between control and LO groups*

4 NEW SCIENTIFIC RESULTS

1. The A213C SNP in *Spot14α*, G645T in *IGFBP-2*, A370C in *SST*, and the 24 bp indel in *PRL* genes were genotyped in ROSS-308 hybrid broiler chickens. Three genotypes (*AA*, *AC*, *CC*) of *Spot14α*, two genotypes (*GG*, *GT*) of *IGFBP-2*, and three genotypes (*DD*, *ID*, *II*) of *PRL* gene were discriminated. Observed and expected genotype frequencies did not differ significantly ($P>0.05$) indicating Hardy–Weinberg equilibrium for the three loci in the population. Allele *A* of the analysed *SST* polymorphism was fixed in the population.

2. Significant ($P<0.05$) associations were detected between the *IGFBP-2* genotype and production traits (live weight, carcass weight, breast weight with or without skin, breast (%) relative to carcass weight). Allele *T* was found beneficial for the analysed production and slaughter traits in the broiler population.

3. Effects of linseed oil (LO) supplementation on the expression of *FADS2* gene were first described in turkeys. Hepatic *FADS2* expression was higher ($P<0.001$) in LO supplemented animals compared to the control group. Thigh expression also increased ($P<0.05$) in response to LO feeding, whereas abdominal fat levels decreased in the experimental LO group.

4. Effects of LO supplementation on *PPARγ* mRNA levels were first described in different tissues of commercial turkeys. Thigh *PPARγ* expression significantly decreased ($P<0.05$), while abdominal fat level increased ($P<0.05$) in the LO supplemented animals.

5. Effects of LO supplementation on *IGF-1* expression in turkeys were first analysed. LO supplemental feeding increased ($P<0.05$) *IGF-1* expression in thigh muscle samples, while a decrease ($P<0.05$) in fat levels was described when compared to the control group.

5 LIST OF PUBLICATIONS

Scientific papers related to the dissertation

Scientific paper published in peer-reviewed journal (in Hungarian)

SZALAI KLAUDIA – TEMPFLI KÁROLY – BALI PAPP ÁGNES (2017): A tyúk géntérképezésének története és jelentősége. Magyar Állatorvosok Lapja, 139 (5). 295–305. (Literature review) (Q4; IF: 0.196)

Scientific papers published/under review in peer-reviewed journals (in English)

SZALAI KLAUDIA – TEMPFLI KÁROLY – LENCSÉS-VARGA ERIKA – BALI PAPP ÁGNES (2019): Genotyping of four loci in Hungarian yellow and broiler chickens. Acta Veterinaria Hungarica, *67(1)* pp. 1–10. (DOI: 10.1556/004.2019.001.) (Q2*; IF: 1.042*)

TEMPFLI KÁROLY – KISS BARBARA – SZALAI KLAUDIA – SIMON ZOLTÁN – PONGRÁCZ LÁSZLÓ – ÁGNES BALI PAPP (2016): Differential expression if six genes in fat-type Hungarian Mangalica and other pigs. Archiv für Tierzucht-Archives of Animal Breeding, pp. 259–265. (Q3; IF: 0.389)

Full text conference proceedings (in Hungarian)

TEMPFLI KÁROLY – HERCEG EMIL BALÁZS – SZALAI KLAUDIA – BALI PAPP ÁGNES (2019): Egyes baktérium nemzetségek relatív mennyisége különböző mangalica csoportokban. In: Kőszegi Irén Rita (szerk.) III. Gazdálkodás és Menedzsment Tudományos Konferencia, „Versenyképesség és Innováció. Kecskemét, Magyarország, Neumann János Egyetem, pp. 364–369.

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Full text conference proceedings (in English)

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TEMPFLI KÁROLY – KONRÁD SZILÁRD – KOVÁCSNÉ GAÁL KATALIN – SZALAI KLAUDIA – BALI PAPP ÁGNES (2014): Possible genetic markers for egg production traits in Hungarian Yellow hens. In: Bene Szabolcs (szerk.) 20th Youth Scientific Forum: University of Pannonia Georgikon Faculty, Keszthely, Magyarország, Pannon Egyetem, Georgikon Mezőgazdaságtudományi Kar, pp. 550–560.

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Conference abstracts (in English)

SZALAI KLAUDIA – TEMPFLI KÁROLY – LENCSÉS-VARGA ERIKA – BALI PAPP ÁGNES (2018): IGF1 and IGFBP2 polymorphisms in Hungarian Yellow and broiler chickens. In: EAAP, Scientific Committee (szerk.) Book of Abstracts of the 69th Annual Meeting of the European Federation of Animal Science, Wageningen, Hollandia. Wageningen Academic Publishers, p.602.

TEMPFLI KÁROLY – SZALAI KLAUDIA – LENCSÉS-VARGA ERIKA – KOVÁCSNÉ GAÁL KATALIN – BALI PAPP ÁGNES (2017): Genotyping of dopamine receptor D1 and somatostatin polymorphisms in Hungarian Yellow hens. In: EAAP, Scientific Committee (szerk.) Book of Abstracts of the 68th Annual Meeting of the European Federation of Animal Science, Wageningen, Hollandia. Wageningen Academic Publishers, p.373.

TEMPFLI KÁROLY – SZALAI KLAUDIA – LENCSÉS-VARGA ERIKA – SIMON ZOLTÁN – BALI PAPP ÁGNES (2017): Expression of perilipin 2 and leptin genes in muscle and backfat tissues of pigs. In: EAAP, Scientific Committee (szerk.) Book of Abstracts of the 68th Annual Meeting of the European Federation of Animal Science, Wageningen, Hollandia. Wageningen Academic Publishers, p.309.

SZALAI KLAUDIA – TEMPFLI KÁROLY – BALI PAPP ÁGNES (2016): Analysis of IGF1, IGFBP2, and SST DNA-polymorphisms in Hungarian Yellow hens. In: Gócza Elen – Kiss Erzsébet – Maráz Anna – Várallyay Éva (szerk.) Fiatal Biotechnológusok Országos Konferenciája „FIBOK 2016", Program és Összefoglalók p. 62.

Scientific paper under review (in English)

SZALAI KLAUDIA, TEMPFLI KÁROLY, ZSÉDELY ESZTER, LAKATOS ERIKA, GÁSPÁRDY ANDRÁS, BALI PAPP ÁGNES: Linseed oil supplementation affects *FADS2*, *PPARγ*, and *IGF1* expression in turkey (*Meleagris gallopavo*). Under review

Scientific paper not related to the dissertation (in Hungarian)

TEMPFLI KÁROLY – HERCEG EMIL BALÁZS – SZALAI KLAUDIA – BALI PAPP ÁGNES (2019): Egyes baktérium nemzetségek relatív mennyisége különböző mangalica csoportokban. In: Kőszegi Irén Rita (szerk.) III. Gazdálkodás és Menedzsment Tudományos Konferencia, „Versenyképesség és Innováció. Kecskemét, Magyarország, Neumann János Egyetem, pp. 364–369.