# THESIS OF DOCTORATE (Ph.D.)

# DISSERTATION


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## PERFORMANCE AND EGG SHELL QUALITY OF HIGH CAPACITY PREDESTINATED LAYING HENS, DPENDING ON THE PHOSPHORUS SOURCE, IN THE ELONGATED LAYING TERM


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## 1. BACKGROUND OF RESEARCH, OBJECTIVES

The egg producer industries kept the layer hens in one-year laying period until the 2010's. In that one-year period, hens laid 300-320, averagely 63 gram heavy eggs. The modern hybrids were made for producing table eggs and in the elongated – 90-110 life weeks - laying period, they lay 410-420 eggs. The six-month-elongated persistency is caused by economic reasons, notedly the reduction of the total cost per egg. However, worldwide the most accepted international recommendation (NRC, 1994) considerably anachronistic, because neither for the modern layer hybrids, nor for the elongated laying period gives references. Updating of the recommendations is doubtlessly current. Additionally, concerning this topic, there is only a few publication in the national and Hungarian literature too.

Onward in the laying period, the egg size permanently grows and the bigger egg size the bigger shell surface, however, the Ca level for the egg formation is nearly constant during the whole laying period. For producing the egg shell, the laying hen capable of store the Ca in the medullary bones and mobilize it when it is necessary.

Data of the literatures show that in the common one-year laying period, the recommended P level by the NRC (1994) considerably can be reduced by around 55% without decreasing the egg production and egg shell quality. Little number of the literatures cover the whole period of the laying term and also in the point of view that the P demand of laying hens in the elongated laying period.

The results of the laying hens' examinations had been acknowledged that the slow degradable grained Ca sources are more preferable. The dynamic bone mineralization and the mineral circulate are not separable. There is nearly the same point of view in that during the classic one-year laying period, the P demand of laying hens can be 1.5-1.8 g/kg non-phytin phosphorus (NPP), in case the feed is supplemented with 300 FTU/kg phytase enzyme. Arise a question that in case of different velocity of P degradation but same level of NPP there is difference in the production of laying hens, especially in the last 6 months of elongated laying period, when the degradation of egg shell quality occurs.

Comparatively, a few examinations are made to evaluate the reaction of different laying hen genotypes on the different dietary P levels. According to our lights, there is no literature data what would discuss the comparison of the different genotypes in the last critical term of the elongated laying period.

The aims of the present work were the following:

- Examination of effects of the different genotypes of laying hybrids and the different level of total phosphorus, on the performance and egg shell quality, in the elongated (18 months persistency) laying term.

- The effect of dose pendent phytase enzyme studying on corn and soybean meals' NPP level *in vitro*, depending on the incubation time.

- Studying with the mixed trial feeds, based on the results of the *in vitro* studies, the different levels and degradation dynamics of NPP sources effect on the performance and egg shell quality and bone mineralization in the last 6 months of the elongated laying period.

## 2. MATERIALS AND METHODS

The dissertation covers 3 examinations, and in two studies from between were made on the performance of different genotype of laying hens with different levels of dietary phosphorus and one study was made *in vitro* in order to determine the NPP content of the diets.

### 2.1 PERFORMANCE AND EGG SHELL QUALITY OF DIFFERENT GENOTYPES OF LAYING HENS, IN THE ELONGATED LAYING TERM, WITH DIFFERENT PHOSPHORUS SOURCE

The examinations of the performance study in the elongated laying term were made at Kaposvár University, Faculty of Agricultural and Environmental Sciences, Nutrition Department, Poultry Test Room. Altogether, 120 two genotypes of long-life layer hybrids (19 life week) were conducted into the study: TETRA-SL LL (SL) and TETRA Blanca (BL). The total phosphorus content of the diets was adjusted: accordance to NRC (1994) recommendation (PC) and a 10.2% lower (NC) one. The performance and eggshell quality of the layers were examined in the elongated (18 months) laying term. The crude protein, amino acids, gross energy and the Ca content were the same level both in the two diets, according to the NRC (1994) recommendations. Study was conducted till the 72$^{nd}$ production week (90$^{th}$ life week) of the birds. Performance was examined in the whole laying term, from the 19$^{th}$ life week till the 90$^{th}$ life week (18 months) of the birds. Egg shell quality was examined from the 8$^{th}$ month, during 11 months. Live weight of the birds was measured monthly, feed intake weekly and the egg production and egg weight were measured daily, with gram precision. Performance of the birds was determined as following: production intensity

(%), egg weight (g), daily feed intake (g/day) and feed conversion ratio (kg diet per kg egg). Egg shell strength and thickness were determined based on 20 eggs per treatment, elected from the monthly collected eggs, such a way that the chosen eggs' average weight and scatter were the same as the four-week collected eggs' average weight and scatter. The egg shell strength was measured by Zwick Roell Z005 type instrument. The maximum power of egg shell breaking was described in Newton. Egg shell thickness was measured by electronic Mitutoyo micrometer. Data were analyzed by Two-Way-Anova in every single month of the laying term (SAS, 2004), using the following model: $Y_{ijk} = \mu + G_i + P_j + G_i*P_j + e_k$, where: $Y_{ijk}$ = dependent variable, $\mu$ = dependent mean, $G_i$ = effect of genotype, (i=2: SL, BL), $P_j$ = effect of dietary P level, (j=2: 4.4 g/kg, 4.9 g/kg), $G_i*P_j$ = interaction; $e_k$ = undefined error. In case of significant effect of treatments ($P<0.05$) the difference between the treatments was verified by Tukey-test (SAS, 2004).

## 2.2 *IN VITRO* KINETIKS OF DEGRADATION OF THE CORN AND SOYBEAN MEAL PHYTIN PHOSPHORUS, SUPPLEMENTED WITH DIFFERENT LEVELS OF PHYTASE ENZYME

A fermented *Schizosaccharomyces* yeast fungal enzyme was used which referenced to use in poultry and pig diets by the EU. The enzyme is characterized as a considerably thermostable (up to 95 °C) phytase. Guaranteed activity is 10 000 units/g. According to International Standard (2009) 1 phytase unit (FTU) is determined as the amount of enzyme required to release 1 μmol of inorganic phosphorus per minute from sodium phytate at 37 °C.

Two trials were accomplished, an enzyme activity test to determine the optimum pH of the product, and a dose response *in vitro* trial to study the effect of incubation time and additional enzyme activity on the Pi release from corn and soybean meal samples.

*Enzyme activity test*

The phosphate stock standard solution was diluted with 0.25 mol/l acetate buffer containing 0.01% mass fraction polisorbate. For the phosphate standard solution, 1080 µl 0.25 mol/l acetate buffer with 0.01% mass fraction polisorbate 20 were pipetted into a 2 ml tube. 120 µl diluted phosphate standard solution was added and sample mixed. Solutions were pre-incubated for 5 mins at 37° C. Then 2.4 ml acetate buffer solution was preheated to 37° C and incubated for 30 mins. After 30 mins, 2.4 ml stop reagent (1 volume ammonium vanadate with 1 volume ammonium heptamolybdate and 2 volumes dilute nitric acid – Nyannor *et al*, 2009) was added then mixed. Solutions were maintained for 10 mins at room temperature then centrifuged for 6 min. Released IP was measured by photo-spectrometer at 415 nm. Same method was used for determining phytase activity. All measurements were replicated three times from pH 1.0 to 8.5 by 0.5 steps and the results were averaged.

*Dose response test*

Based on the result of enzyme activity test, the hydrolysis trials in feed tests were performed on pH 5.5. Three determinations were performed for each extraction (maize and soybean meal, respectively) and results were averaged. For the determinations, 900 µl 0.25 mol/l acetate buffer was pipetted with 0.01% mass fraction plysorbate 20. 300 µl feed extract was added as the test portion. Contents were mixed and pre-incubated at 37° C. After 30, 60, 120 and 180 min, 2.4 ml stop reagent was added and then mixed. Contents were maintained for 10 mins at room temperature, and centrifuged for 6 mins. Inorganic phosphate in the sample contributed to colour formation, therefore, blanks were included for sample at zero moment. Released IP was measured by photo-spectrometer at 415 nm.

*Statistical Analysis*

According to Kim et al (2006), our data from the dose response study were analysed for Michaelis-Menten parameters ($V_{max}$ and $K_m$) using Gauss-Newton method, as following: $Y=V_{max}/(1+K_m/T)$, where Y is the released P from phytate bound, $V_{max}$ is maximum velocity of the reaction, $K_m$ is the time needed to release the half amount of max released P, determining the celerity of the curve, T=time of incubation. Statistical significance was set at 5% probability level.

## 2.3 PERFORMANCE, EGG SHELL QUALITY AND THE BONE MINERALIZATION OF THE LAYING HENS, DEPENDING ON THE DIETARY NPP CONTENT AND ITS' SOURCE

The examinations of the second performance study in the elongated laying term were made at Kaposvár University, Faculty of Agricultural and Environmental Sciences, Nutrition Department, Poultry Test Room. Altogether, 184 TETRA-SL LL layer hybrids were examined in the 45-68$^{th}$ laying weeks (mean 63-86$^{th}$ life weeks). Birds were classified into 2 groups by their genotype (A, B). Groups were allocated based on the previously examined egg shell parameters, relation of the producer company. Birds were placed into metabolic cages (1520 $cm^2$/cage) by three. Diets were conducted in two different NPP contents with or without phytase enzyme supplementation. Treatment PC was settled with 2.45 g/kg NPP (according to the NRC recommendation – 1994), treatment NC was settled with 2.15 g/kg NPP level. Treatments PCE and NCE NPP content was calculated based on the prior *in vitro* examination results, in which the released phosphorus was calculated with the added 300 FTU/kg phytase enzyme to the corn and soy meals (2.45 g/kg and 2.15 g/kg NPP content, respectively). Performance and eggshell quality were measured in the last 6 months of the elongated (17 months) laying term. Live weight of the birds was measured monthly, feed intake weekly and the egg production and egg weight were measured daily, with gram precision. Performance of the birds was determined as following: production intensity (%), egg weight (g), daily feed intake (g/day) and feed conversion ratio (kg diet per kg egg). Egg shell strength and thickness were determined based on 20 eggs per treatment, elected from the two-monthly collected eggs. The egg shell strength was measured by Zwick Roell Z005 type instrument. The maximum power of egg shell breaking was described in Newton. Egg shell thickness was measured by electronic Mitutoyo micrometer.

The Computed Tomography studies were made at Kaposvár University, Institute of Diagnostic Imaging and Radiation Oncology, Faculty of Agricultural and Environmental Sciences, used a Siemens Somatom Sensation 16 Cardiac scanner. The imagings were made on 144 healthy TETRA-SL LL genotype layer hybrids in 86$^{th}$ lifeweek, in the 68$^{th}$ layer week. The fed diets were the same as described in the part of written in the NPP diets trial. During the *in vivo* acquisition procedures, birds were fixed with belts in a special plastic container, without using any anaesthetics and three animals were scanned simultaneously. Acquisition parameters were set as 140 kV tube voltage, 200 mAs current, 16 x 1.5 mm collimation, 500 mm field of view, spiral data collection: pitch 0.7 (Donko et al., 2018).

Hens were slaughtered the day after the *in vivo* CT examination. The bones of the left leg were cleaned of surrounding muscles and soft tissues. The *tibiotarsal* bone was separated from the fibula. The bones were arranged

2 x 4 matrixes on the examination table. All the *tibiotarsal* bones were scanned using the above described protocol. Prior to the chemical analysis process, the breaking strength (N) of the *tibiotarsal* bones were determined on a Zwick Roell Z005 universal testing machine, equipped with a measuring cell (Zwick GmbH & Company KG, Ulm, Germany) of an operation range up to 5000 N, linked to a computer with TestXpert II 3.1 software (Zwick GmbH & Company KG, Ulm, Germany). The distance between the supports was set at the end part of the bones. The speed of the breaking blade was 20 mm / min. The dry matter, ash, Ca and P content of the *tibia* bones were measured accordance to AOAC 934.01 and AOAC 942.05 methods.

Data were analyzed by Two-Way-ANOVA in every single month of the laying term (SAS, 2004), using the following model: $Y_{ijk} = \mu + G_i + P_j + G_i*P_j + e_k$, where: $Y_{ijk}$ = dependent variable, $\mu$ = dependent mean, $G_i$ = effect of genotype, (i=2: A, B), $P_j$ = effect of dietary NPP level, (j=4: PC: 2.45 g/kg NPP, NC: 2.15 g/kg NPP, PCE: 2.45 g/kg NPP with 300 FTU/kg phytase enzyme, NCE: 2.15 g/kg NPP with 300 FTU/kg phytase enzyme), $G_i*P_j$ = interaction; $e_k$ = undefined error. In case of significant effect of treatments ($P<0.05$) the difference between the treatments was verified by Tukey-test (SAS, 2004).

## 3. RESULTS

### 3.1 PERFORMANCE AND EGG SHELL QUALITY OF DIFFERENT GENOTYPES OF LAYING HENS, IN THE ELONGATED LAYING TERM, WITH DIFFERENT PHOSPHORUS SOURCE

The brown egg layer TETRA-SL LL genotype hybrid had averagely 4.0 relative % higher laying intensity compared to the intensity of white egg layer TETRA Blanca genotype hybrid. The egg production of the 10% lowered total phosphorus content fed diet birds' was averagely 3.3% less from the $10^{th}$ month of elongated laying term. The feed intake of the TETRA-SL LL birds was 9.7% higher than the TETRA Blanca hybrids'. The lower content of total P diet fed birds ate 2.3% more than the NRC recommended total P content diet fed birds in the 8-11th and 15-17th months of the laying period. In the first 7 months of the laying term, the SL genotype hybrids laid averagely 4.5% heavier eggs compared to the BL hybrids' eggs, then in the rest months of the laying term, the egg weights were in the same range. The weight of the 4.4 g/kg total P contented diet fed birds' eggs were 2.5% smaller than the 4.9 g/kg tP diet fed birds' eggs, from the $10^{th}$ month of the laying period. The TETRA-SL LL genotype layers produced 9.5% more egg mass than TETRA Blanca genotype layers, till the 11th month of the laying period. The PC diet fed birds produced averagely 6.6% more egg mass than NC diet fed birds, but only in the last two months of the laying term. The feed conversion ratio was not affected neither by the genotype of the hybrids, nor the tP content of the diets. The egg shell thickness and egg shell strength were not affected neither by the genotype, nor the tP content of the diets.

### 3.2 *IN VITRO* KINETIKS OF DEGRADATION OF THE CORN AND SOYBEAN MEAL PHYTIN PHOSPHORUS, SUPPLEMENTED WITH DIFFERENT LEVELS OF PHYTASE ENZYME

During the laboratory examination, two well-detached pH optima were found in the phytase enzyme activity tests. At pH 3.5 the activity was 8828 FTU in one gram enzyme and at pH 5.5 the activity result was 12670 FTU, which was the 127% of the guaranteed activity. A smaller (4381 FTU/g) optimum was measurable at pH 7.5. In the corn and soymeal samples the hydrolysis of the phytate were measured on pH 5.5, supplemented with 100, 200 and 300 FTU/kg phytase enzyme, using Vmax and Km parameters. The most of the released phosphorus in corn were measured in the 30. and 60. minutes during the incubation time, and substrate was supplemented with 300 FTU. The same results were measured in the soymeal substrate, plus in the 120. incubation minute as well.

### 3.3 PERFORMANCE, EGG SHELL QUALITY AND THE BONE MINERALIZATION OF THE LAYING HENS, DEPENDING ON THE DIETARY NPP CONTENT AND ITS' SOURCE

In the study of the different NPP levels, it was determined that based on the eggshell quality measurement done by the producer company, the B line hybrids' laying intensity (%) was significantly higher in the whole examined period (last 24 weeks of the elongated – 17 months – laying term). The 12.2% less NPP contented diet supplemented with phytase enzyme fed birds and the same NPP level without enzyme supplementation diet fed birds had significant difference in the laying intensity (%). The feed intake was affected by the layers' line only in the 16-17$^{th}$ laying months. The A line birds consumed averagely 7.9% more feed in this period, than the B line birds did. The NPP content of the diets had no effect on the feed intake of the layers. Egg weight was no substantially affected by the hybrids' line but only in the 15-16th laying months. The average weight of the produced eggs in the elongated laying term was 62 gram. Overall, the test diets did not affect the individually weight of the eggs. Between the 13th and 16th months of the elongated laying term, the produced egg mass was affected by the NPP content of the diets. The lower NPP contented diet fed birds produced averagely lower egg mass, compared to the higher NPP contented diets fed birds. The eggshell strength values were not affected by the different lines of the hybrids. The egg shell of the genotype A birds was thicker than genotype B birds', however the NPP content of the diets had no effect on this parameter. The ratio of the broken eggshell was affected by the hybrids' line: the genotype B birds produced significantly less cracked egg shelled eggs, than genotype A birds did. The test diets had significant effect on this parameter such way: the most of the broken shelled eggs were found in the case of 2.45 g/kg NPP and the same NPP contented diet supplemented with phytase enzyme fed birds, in the 13th,14th and 16th months of the examined laying term.

During the *in vivo* Computer Tomography examination, in the first measuring date, the TETRA-SL LL genotype layer hybrids' A and B genotype birds had significantly different bone mineral density. The density of the A genotype birds was 3.2% higher than B genotype birds'. The volume of the total bone was 9% bigger in A genotype layers. The bone ratio in the whole body was the opposite the B genotype layers had 10.5% bigger result. In the first measuring date, the test diets did not affect the bone CT parameters. In the second measuring date, the bone density of the genotype A was averagely 2.6% higher, than genotype B birds'. The results of the *tibia* CT examinations, the genotypes of the birds and the NPP content of the diets did not affect the bone density. The A genotype birds bone volume was 9.5% higher than B genotype birds'. Interaction was observed in this parameter, which means, that the bone volume was affected by the genotype of the birds and the NPP content of the diet, in the same time. The genotype of the birds and the NPP content of the diets did not affect the breaking power of the bones, but the genotype had significant effect on the rest of parameters of this examination, such as the A genotype birds had better bone physical results than B genotype birds.

Some of the bone chemical parameters were affected by the hybrid genotype. The original and the extracted weight of the bones were heavier in A genotype birds. The bones' calcium content had statistically different amount, compared the two genotypes. The A genotype birds' had 20.6% higher Ca content in their *tibia* than B genotype birds'. The genotype did not affect the phosphorus content of the *tibia*. The different non-phytin phosphorus content of the test diets and the phytase enzyme supplementation had no effect on the bone chemical parameters.

## 4. CONCLUSIONS

During the 18 month-long laying period, the laying intensity of TETRA-SL LL genotype hybrid was better, than TETRA Blanca. The 10.2% less P content was adequate for keep the good intensity in the first 9 months of the elongated laying period, however, from the 10th laying month, the NRC (1994) recommended P content was needed. In the first 6 months of the examined period, TETRA-SL LL genotype hybrids' egg weight was higher then there was no significant difference in this parameter between the genotypes. According to the dietary P contents, the NRC (1994) recommended P content resulted bigger eggs in the second part of the laying term. TETRA-SL LL genotype hybrids produced 10% more egg mass, because this genotype had higher laying intensity and bigger eggs, in the first 6 months of the laying term. The P contents of the diets affect the egg mass only at the end of the laying term. Neither of the genotype, nor the dietary P content did significantly affect the feed conversion ratio of the hens. Produced eggs shell quality parameters were not affected by the genotype and the different P contents of the diets. It means, in the elongated laying period, the recommended (4.9 g/kg) dietary P content of the intensive laying hens, can be lowered by 10.2% without disimprovement of laying intensity and egg shell quality.

During the enzyme activity test, two well-detached pH optima were determined at pH 3.5 and 5.5, and a lower one at pH 7.5. Based on the results, hypothetically, the added phytase enzyme can take its effect in the gastro intestinal tract of the hens. The results of the *in vitro* phytate hydrolysis experiments show that added 300 FTU/kg phytase amount was adequate for the hydrolysis of the great rate of bounded P in the corn and soymeal. The hydrolysis did not substantially change between 2 and 3 incubation hours.

The performance examination studied in the last six months of the elongated laying period, the weaker egg shelled characterized B genotype TETRA-SL LL hybrid had the better laying intensity in the whole examined laying term. At the end of the 17 months laying term, both genotype hybrids produced above 70% laying intensity. The 2.15 g/kg NPP content diet fed birds' intensity was better than the same NPP content, but MCP based diet fed birds'. The dietary treatments with recommended (2.45 g/kg NPP) and 12.2% lowered NPP content source did not affect the feed consumption of the hens. The genotype of the hybrids did affect the egg weight only in the 15-16 months of the laying period, such way that A genotype hybrids' eggs were heavier. Overall, the genotype did not affect the egg size. The NPP content and the P source of the dietary treatments did not affect the egg weight in the last 6 months of the laying term, except in the 16$^{th}$ month. Likewise to the laying intensity, the egg mass was affected by the hybrid genotype. The B genotype hybrids produced more egg mass than the A genotype birds. At the end of the elongated laying term, the 2.15 g/kg NPP content diet fed birds – without reference to the velocity of the P degradation – produced less egg mass than the recommended (2.45 g/kg) NPP content diet fed birds. The breaking power of the egg shell was not affected neither by the genotypes of the hybrids, nor the dietary NPP content and the velocity of the P degradation. The genotype of the hybrids did affect the egg shell thickness such way, that the A genotype birds' egg shell was thicker during the whole laying term. The recommended NPP content of the diets and the enzyme supplementation did affect the eggshell thickness only in the last month of the laying term. It is statable, that in the second part of the elongated laying term, the lower NPP content of the diets can offer for the intensive laying hens, without degradation of egg shell quality. The A genotype hybrids produced more cracked shelled eggs in the whole examined laying term. In the elongated laying term – except the 15$^{th}$ month – the higher NPP content of the diets had significant effect on the ratio of cracked shelled eggs.

The *in vivo* CT examination shows, that the examined parameters were affected by only the genotypes of the hybrids. The body bone density and the body bone volume were bigger in the A genotype birds. The NPP content of the dietary treatments did not affect the CT parameters, none of the measuring times. The genetic background affected the *tibia* physical qualities. The diameter, length and cross-section of the A genotype hybrids' bones were higher than the B genotype birds. However, the breaking power of the bone strength was not affected by the genotype. Thus, it is statable that the breaking power of the bone strength is independent from the bone physical parameters.

With the 12.2% lowered dietary NPP can achieve same physical parameters in the *tibia*, than with the recommended NPP content, independently from the velocity of the P degradation. The A genotype hybrids *tibia* chemical composition had better results, except the P content of the bones, because that was on the same level both in A and B genotype's bones. The NPP contents of the dietary treatments and the velocity of the P degradation did not affect the chemical composition of the bones.

## 5. NEW SCIENTIFIC RESULTS

1. It is sufficient to maintaining to the high capacity predestinated TETRA-SL LL and TETRA Blanca genotype hybrids the lower 4.4 g/kg total phosphorus and 2.0 g/kg NPP (compared to the NRC /1994/ recommended 4.9 g/kg tP) level in the first 9 months of the 18 months elongated laying period. Beyond the common one year laying period, it is necessary to provide the recommended (NRC, 1994) 4.9 g/kg tP and 2.5 g/kg NPP, in case, the feed does not content any phytase supplementation and the egg weight is more than 64 grams.

2. TETRA hybrids (-SL LL and Blanca) egg shell quality does not declines over the common one year laying period, in case the layers' dietary tP is lower by 10%, compared to the NRC (1994) recommendation (4.4 g/kg tP and 2.0 g/kg NPP), either the egg weight is over 63 grams.

3. Regarding to TETRA hybrids (-SL LL and Blanca), it is sufficient to provide 1.0 g/kg NPP – supplemented with 300 FTU/kg phytase enzyme - in the corn-soy based dietary feed in the last 6 months of the elongated (till 68$^{th}$ laying week) laying period, in case the average egg weight is under 63 grams.

4. Based on the results that in the second part of the elongated laying term, it may be recommended to setting the dietary NPP content with decreasing the MCP rate and releasing the P with phytase enzyme.

5. The lower dietary P level and the source of the P do not affect the bones and the size, strength and chemical parameters of the *tibia* of the different egg quality TETRA-SL LL hybrids. The mineral circulation of the hybrids is balanced either at the end of the elongated laying period.

6. The better egg shell selected hybrids' tibia contains 20% more Ca, the egg shell is thicker, and in the same time, the cracked egg shell rate is higher in the last 6 months of the elongated laying period, compared to the non-selected hybrids.

# 6. SCIENTIFIC PAPERS AND LECTURES ON THE SUBJECT OF THE DISSERTATION

## 6.1 PEER-REVIEWED PAPERS PUBLISHED IN FOREIGN SCIENTIFIC JOURNALS

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## 6.3 PROCEEDINGS IN ENGLISH

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## 7.2 CONFERENCE PRESENTATION

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