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INVESTIGATION OF THE EFFECT OF DOMESTICATION ON MATERNAL REPRODUCTIVE BEHAVIOR IN RABBITS

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1. AIM AND BACKGROUND OF THE RESEARCH

In mammals, nest-building often happens in exact order of the behaviour forms. This is also true in the case of the wild rabbit (*Oryctolagus cuniculus*), where maternal care involves burrow digging and nest building. For wild rabbit does, this complicated nest-building process is the maternal care itself, which is a significant investment before parturition. The delay may influence the reproductive success of the does in this process.

In rabbits, the mortality rate for the entire litter during the nesting period is high (40%), although 75% of the causes of death remain unexplained. So far, we have little information on how nest-building behaviour affects birth mortality. The delayed timing of the nest building under natural conditions is a possible explanation for birth mortality in the species. In terms of the nest-building behaviour, which is a well-repeatable trait, there is a difference between the individuals. Although, the information about the factors (timing, nest quality) influencing the nest-building is limited concerning wild rabbits. Such factors are experience, genetic background, size of the social group, status of the individual in the social ranking, number of competing kits, hormonal effects, and stress. It has already been described that social stress can result in delayed nest-building, poor nest quality and higher nesting mortality.

Aims:

Since strictly defined hormonal changes precede the steps of nest building, the objective of this work was to know more about the genetic background of the hormonal influence. Whether polymorphisms in the progesterone and prolactin receptor genes (PGR, PRLR) do affect the timing of nest-building behaviour and the nest quality, and if so, what is the proportion of its genetic variation.

The second aim of the study was to see more clearly, whether the effect of stress (cortisol) can change the hormone (progesterone) levels of a pregnant

rabbit doe and yet, to what extent. Whether it affects the collecting of nest material, and thus the number and mortality of kits.

The domesticated rabbit has enough calm nature in contrast to the wild rabbit since avoiding predators needs to jump at every little sign. The recent domestication changed primarily their behaviour, mainly due to many small changes in their genetic pool. As a result, rare variants present in rabbit populations have become frequent in the domesticated form. Having the full spectrum of the behaviour, the wild rabbit is an excellent subject for behavioural testing.

As a first step, we tested if the specific behavioural responses of rabbits could be identified in certain situations or if they are more context-dependent.

Furthermore, we wanted to clarify how personality (measured by a novel food - in novel arena test) affects the reproductive traits of the rabbit and whether the mother's personality influences the behaviour of the offspring.

2. MATERIALS AND METHODS

The examinations performed on wild rabbits (*Oryctolagus cuniculus*) were approved by the Institutional Animal Welfare and Ethics Committee of the Hungarian University of Agriculture and Life Sciences (permit number: MÁB / 2-2 / 2019).

Experimental animals: The experiments were performed on 30 (in the PRLR gene study on 40, and in the sex allocation study on 15), 10–12-month-old mature wild rabbits, of which the first parturitions were compared. In the effect of stress on the sex allocation study, the sex of the first offspring of 15 does (n = 76) was determined at the age of 60 days.

Housing: The lighting period in the barn was 16 hours (15.4 ± 1.6 hours) illumination, and in addition to the light entering the windows, artificial illumination was provided by timer lamps. The animals were placed individually into cages (60 * 60 * 45 cm) equipped with farrowing boxes (34 * 25 * 31 cm). The cages were made of welded wire mesh with hand-fill self-feeders and hayracks attached to the front, while a galvanised steel sheet manure store running on rails was installed under each cage. The cages were arranged in two rows in the barn.

Feeding: commercially available rabbit feed for rabbits ad libitum (DE: 10.6 MJ / kg, crude protein: 16.3%, crude fat: 3.8%, crude fibre: 17.7%), hay (100 g / day) and water was provided using nipple drinkers.

2.1. Examination of the timing of nest-building behaviour

For the examination of grass collection behaviour during nest-building, dry grass was provided, additionally to the standard amount of hay, as nesting material 6 days before the expected time of parturition (from day 25). The nest-building elements were recorded (except the digging process, which is not detectable adequately in a welded cage). The starting time of the

collecting grass by the pregnant rabbit or extracting fur from its body was recorded in 12 hours periods. The building activities were recorded daily. Approaching the day of parturition, the animals were checked every 2-3 hours. When the new-born kittens were discovered, a sliding door was used to close down the farrowing box from the direction of the cage. Next, the kittens were taken out through the opening top door and weighed separately on a Sartorius scale to one decimal accuracy, and then placed back into the nests. The total number and weight of kittens were recorded as well as the number of incidental stillbirths.

2.2. Nest analysis

When the kittens achieved the age of 21 days, the nests were removed from the farrowing boxes. The kittens' urine makes the bottom of the nest wet, so it was dried out first. Once dry, the total weight of the nests was measured; the fur and the grass were arranged evenly in order to get a homogeneous mix. Ten samples were taken out (a pinch weighed appr.1-1.5 g). Every sample was split into hay and hair and weighed separately on Sartorius scales to two decimal accuracy, in grams. This produced the hay vs. fur ratio in the ten samples, which enabled us to estimate the hay and fur weights of the whole nests.

2.3. Determination of hormone from faeces

The levels of progesterone and cortisol levels were measured from excrement on the basis of breakdown products. Stools were collected from day 28 of gestation until the day following parturition every 24 hours (p.m. 8.00.). In the study on the effect of the stress on sex ratio in the offspring, faeces samples were collected 24 hours after natural mating. The mesh placed in the trays under the cages was used to prevent faecal contamination of the faeces.

After collecting the samples, completely clean trays were placed under the rabbits. The GCM was extracted from the stool, and samples were stored at -20 °C until extraction. The amount of hormone was determined in the Endocrinology Laboratory of the University of Veterinary Medicine.

2.4. Sequencing of the progesterone receptor gene (PGR)

Extraction of the DNA was carried out from fur samples, by cutting the hair roots and with the help of 5% Chelex resin. A 558 bp long segment from the promoter region of the PGR gene was amplified from the genomic DNA (primer sequences: PGR-F 5'GAAGCAGGTCATGTCGATTGGAG3 'and PGR-R 5'-UTR 5'CGCCTCTGGTGCCAAGTCTC3'). The conditions were as follows: 10 min at 95˚C, followed by 35 cycles, 30 min at 95˚C, 60 s at 66˚C, 90 s at 72˚C. The final extension step was 15 min at 72˚C. The final volume of the reaction mixture was 20 µL, which contained the following components: 2.5 µL genomic DNA solution (55 ng/µL), 10 µL 2×Platinum Superfi Master Mix, 5 µL 5×Enhancer, 1.25 µL PGR-F and PGR-R primers (10 µM in stock solution). The resulting 558 bp long product went through silica membrane purification and was used for sequencing reaction with the help of a BigDye terminator 3.1 sequencing kit. The temperature profile of the sequencing reaction was as follows: 96˚C for 3 minutes, 96˚C for 10 seconds, 55˚C for 20 seconds, 60˚C for 1 minute 15 seconds, and then 4˚C. The final volume of the reaction mixture was 10 µL, its components were: 0.8–2 µL sample, 1.4 µL BigDye reaction mix, M-13 sequencing primer, distilled water as described in the manual. Sequencing was carried out with the help of an ABI 3100 genetic analyser. The sequences of the progesterone receptor gene of the 30 mother rabbits were aligned to a GenBank sequence (identification number X06623.1) with the program Clustal Omega.

2.5. Sequencing of the prolactin receptor gene (PRLR)

Extraction of the DNA was carried out from fur samples, by cutting the hair roots and with the help of 5% Chelex resin, similar to the PGR gene region. Primers for amplification were designed using the Primer3 + program (Primer sequences: 5 'ATAGCTCCCTGAGGCTTGGT 3' and 5 'TGGGACGTG GAGATCCATTG 3'). These conditions were as follows: 95˚C for 10 minutes, followed by 94˚C for 30 seconds, 55˚C for 60 seconds, 72˚C for 90 seconds. The final extension step was 15 min at 72˚C. The final volume of the reaction mixture was 20 µL, which contained the following components: 2.5 µL genomic DNA solution (55 ng/µL), 10 µL 2×Platinum Superfi Master Mix, 5 µL 5×Enhancer, 1.25 µL PRLR-F- and PRLR-R primers (10 µM in stock solution). The resulting 1210 bp product was sequenced similarly to the PGR gene region after purification on silica membrane. The sequence of the 40 maternal prolactin receptor genes was aligned with the appropriate region of the GenBank sequence (NC_013679.1) using the Clustal Omega program to identify point mutations. In the promoter region (1210 bp) we found a microsatellite with the sequence: CTCCTCCTCCTCCTC (from the forward direction), for which the primers were designed using Primer3 + program. The sequence of the primers is as follows: forward primer 5'TGTTTGGACCACTGACCCTT3', reverse primer 5'GAGAGCCTCGGTGTCAAATT3'. The final volume of the reaction mixture was 10 µl, which contained the following components: 1 µl of genomic DNA solution (55 ng / µl), 5 µl of 2x Platinum Superfi MasterMix, 2 µl of 5x Enhancer, 0.5 - 0.5 µl of 10 forward - and reverse primers, 1 µl distilled water. The temperature conditions are as follows: 95˚C for 15 minutes, then 35 cycles (95˚C for 30 seconds, 58˚C for 30 seconds, 72˚C for 45 seconds), and finally 15 minutes at 72˚C. A forward primer with NED-fluorescent end-labelling was used for DNA amplification. For fragment length polymorphism

analysis, the LIZ-500 size standard was performed on an ABI 3500 genetic analyser, and the results were evaluated according to GeneMapper 4.1. program.

2.6. Measurement of production characteristics

After parturition, the kits were removed from the nest, the number of nestlings was recorded, and their weight was measured individually. In addition to the litter weight at birth, the 21-day-old litter weight was also recorded. Measurements were performed on a Sartorius balance with 0.1-gram accuracy. Maternal milk production was recorded daily during the first 21 days of lactation. Shortly, the kits were individually weighed before and after lactation, and the amount of milk consumed was determined as the difference between post-feed and pre-feed weight. In the study, we calculated the total amount of milk under 21 days of age.

2.7. Behavioural testing

Behavioural testing was performed at the age of 60 days (N = 86). The repetition of the test in the broodstock was realized ten months later, at the age of 12 months. First, we tested 24 individuals, which later became broodstock (N = 24), nine males and fifteen females, and then their offspring (N = 62).

A novel object test in a novel arena was performed in order to determine exploratory behaviour. Because of its biological relevance, novel food represented the novel object. The rabbit was transported to a same-sized cage in the barn, separated from its companions. After three minutes of habituation, subsequently, as a novel object, we provided previously unknown food to the individual, which was placed in the first third of the cage, in the middle about 10 cm from the grid. In the first test (for all 86 individuals), the object was an apple slice (a quarter of the apple), while in the replication with the parent

stock, it was an approximately 7 cm long carrot. During the test, each individual received a new apple/carrot. The latency of the first touch of the novel object was measured, and the number of attempts was recorded. The study lasted five minutes. However, if the animal started consuming food, the fact of this was also recorded, and the test was terminated.

2.8. Investigation of factors influencing the behaviour of offspring generation

Offspring in the behavioural study (N = 62) were individually labelled with a microchip, and their sex was determined. Behavioural testing was performed at 60 days of age, and body weight was measured at 5 months of age.

2.9. Statistical processing (examination of the genetic background of nest building)

Genetic diversity was determined by calculating the observed heterozygosity (H_0) and unbiased expected heterozygosity (H_e), the effective number of alleles (Ne) and by testing the Hardy Weinberg equilibrium in each SNPs using GENALEX version 6.5. Polymorphism information content (PIC) was calculated by CERVUS 3.0.7 software. Linkage disequilibrium data were analysed with the DNAsp 5.10 program. To test whether there was an association between the hay carrying behaviour, hay weight and the PGR polymorphisms, we used a general linear model (GLM) procedure implemented in SPSS 17.0, according to the following model: dependent variables were time of hay carrying and hay weight, fixed factors were SNP2464, SNP2682 and SNP 2866 genotypes, covariates were the progesterone and cortisol hormone levels at the day of parturition. Partial Eta-squared was calculated to determine the effect sizes of the factors. Two-step cluster analysis was used to assign the rabbit does into groups according to the

start of stay carrying, and Chi-squared test (linear by linear association test) was used to determine the significance of the difference in the genotype distributions of groups. Narrow sense heritability of the SNP2464G>A was calculated by the standard procedure with the following equations $\mathbf{h^2=V_A/V_P}$ where $\mathbf{V_A}$, the additive variance and $\mathbf{V_P}$ is the phenotypic variance. $\mathbf{V_A}$ was calculated by the equation $\mathbf{V_A = 2\ p\ q\ \alpha^2}$ where $\mathbf{\alpha = a + d\ (q-p)}$ and **p** and **q** are the frequencies of the two alleles, **a** is the genotypic value of the homozygote and **d** is the genotypic value of the heterozygote.

2.10. Statistical processing (influence of the physiological stress at parturition on nest-building and the offspring)

For the examination of differences in cortisol levels at parturition and during gestation as well as correlations with levels of progesterone at parturition, linear regression was applied. The arrangement of groups based on differences in cortisol levels at parturition and during gestation was carried out by cluster analysis (k-means cluster), where the number of clusters was two and the number of iterations was limited to 10. The differences in levels of progesterone between the two groups were examined with two-sample T-test at parturition and during gestation, at the time of hay stacking, for the quality of the collected hay and hair and the number of the kittens born. The time of fur stacking did not indicate a normal distribution, so there the non-parametric Kruskal-Wallis- test was applied. The kitten mortalities in the groups were compared with the Chi-squared test. The normality of the data was checked with the Shapiro-Wilk-test, while their homogeneity with the Levene-test. Differences between groups were considered significant at the $P < 0.05$ level. The statistical analysis was carried out with SPSS 17.0 software.

2.11. Statistical processing (effect of stress on sex allocation of offspring)

The homogeneity and normal distribution of the samples were tested and confirmed with Levene test and Shapiro Wilk test. The does were classified into two groups using k-means cluster analysis (maximum iterations: 10). The significance of the differences between the two groups in the litter size and the sex ratios was estimated by the independent samples t-test. Linear regression was calculated between the faecal cortisol metabolite (FCM) values and the female sex ratio and the progesterone values and the female sex ratio. Pearson correlation was calculated between the progesterone and the FCM values. All statistical analyses were calculated using SPSS 17.0 software.

2.12. Statistical processing (association between the differences in exploratory behaviour and the reproductive success)

Spearman correlation was used to the values obtained in the novel object test to estimate the relationship between the reproductive properties of the parent stock (litter size, milk production, neonatal and 21-day-old weight) and the results of the novel food test (latency, frequency) and the offspring test results (also latency, frequency). The repeatability of the tests was estimated based on the intragroup correlation coefficient (ICC). The effect of does, sex and offspring weight on the behaviour was analysed using a generalized linear mixed model (GLMM). This included the doe as a random factor, sex, and weight as fixed factors. In the case of latency, the effect of the former factors was performed by survival analysis using a Cox proportional hazard regression model. The sex (categorical variable) as a covariate and the weight and the mean of maternal latency were included in the model. Mean of the latency was given as a categorical variable, on the basis of which the mothers were divided into two clusters by cluster analysis (k-means cluster analysis) (neophile: 18 individuals, latency: 77.06 s, and neophobic: 44 individuals, latency: 215.59 s). SPSS 17.0 software and SAS programs were used for statistical analyses.

3. RESULTS

3.1. Examination of the polymorphism of the PGR gene.

Based on the sequences obtained as a result of the Sanger sequencing, we identified the point mutation at position 2464G> A in the promoter region, as well as a point mutation not yet described in the promoter region at position 2682T> C. The point mutation already described at 2866G> T in the exon-1 region of the gene was also detected. *Table 1* shows the distribution of the observed genotypes, observed heterozygosity (H_o), expected heterozygosity (H_e), effective allele size (Ne), and PIC value. Examination of the distribution of genotypes shows that they are in Hardy – Weinberg equilibrium for all three SNPs (P> 0.05). The PIC values indicate a moderate polymorphism in the rabbit population.

Table 1. The genotype distribution and gene diversity data of the three SNPs located in the PGR gene.

SNP	Observed genotype						H_o	$_uH_e$	HWE		Ne	PIC
									$\chi 2$	P		
2464G>A	GG	19	GA	8	AA	1	0.286	0.299	0.019	0.890	1.415	0.250
2682T>C	TT	17	TC	11	CC	0	0.393	0.321	1.673	0.196	1.461	0.266
2866G>T	GG	13	GT	14	TT	1	0.500	0.416	1.418	0.234	1.690	0.325

H_0: observed heterozygosity, $_uH_e$: unbiased expected heterozygosity, Ne: effective allele size, PIC: polymorphism information content, $\chi 2$: Chi-squared value, HWE: a Hardy-Weinberg equilibrium, P: significance level

The linkage disequilibrium data between SNPs in the promoter region and exon 1 are shown in *Table 2.* Based on our results, there is no significant association between the two SNPs in the promoter region (2464G> A and 2682T> C) and either of the pairs in the SNP in exon 1 (the SNPs are independently inherited).

Several factors, such as the two hormones (progesterone and cortisol) and the 2464G> A SNP found in the PGR gene, have a significant effect on the start time of hay collection (*Table 3*). The effects neither of the other two SNPs nor the interactions were significant. Regarding the amount of hay collected, a significant effect was found for the interaction of SNP 2-3; however, the effect of SNPs did not prove to be significant.

Table 2. The allele and haplotype distribution and linkage disequilibrium on the examined SNPs

	Allele frequency		Haplotype frequency		D’	r	χ^2	P
SNP1-2	G	0.82	GT	0.68	0.018	0.016	0.029	NS
	A	0.18	GC	0.16				
	T	0.80	AT	0.13				
	C	0.20	AC	0.03				
SNP1-3	T	0.80	TT	0.57	-0.632	-0.181	3.651	NS
	C	0.20	TG	0.24				
	T	0.71	CT	0.15				
	G	0.29	CG	0.04				
SNP2-3	G	0.82	GT	0.55	-0.239	-0.077	0.662	NS
	A	0.18	GG	0.27				
	T	0.71	AT	0.16				
	G	0.29	AG	0.02				

SNP1-2464G>A, SNP2-2682T>C, SNP3-2866G>T, D’: distance from the linkage equilibrium, r: correlation coefficient, $\chi 2$: Chi-squared value, P: significance level

Table 3. The association between the start point of the hay collection behaviour and the polymorphisms observed in the PGR gene. General linear model (GLM), the cortisol and progesterone levels were included as covariants. The values highlighted in bold are significant at the level p<0.05

	df	Start point of the hay collection			
		MS	F	P	Partial Eta squared
Intercept	1			**0.018**	**0.288**
Progesterone	1	**9.873**	**6.203**	**0.023**	**0.267**
cortisol	1	**8.298**	**5.214**	**0.036**	**0.235**
SNP1 (2464G>A)	2	**7.085**	**4.452**	**0.028**	**0.344**
SNP2 (2682T>C)	1	0.299	0.188	0.670	0.011
SNP3 (2866G>T)	2	1.595	1.002	0.388	0.105
SNP1 * SNP2	1	0.129	0.081	0.780	0.005
SNP1 * SNP3	1	0.035	0.022	0.883	0.001
SNP2 * SNP3	1	0.333	0.209	0.653	0.012

df: degree of freedom, MS: mean square, F: F value, P: significance level

The mothers were classified into two clusters according to the starting time of hay collection. Fifteen individuals were classified in the early group (time of collection 3.6 ± 0.78 days before parturition) and thirteen individuals in the late group (time of collection 0.88 ± 0.54 days before parturition). The genotype distribution of 2464G> A SNP in the two groups is shown in *Figure 1*. The early group contained 87% of the GG genotype and 13% of the heterozygous alleles (GA), and no AA genotype at all. In the late group, the GG ratio decreases significantly, while the GA ratio increases significantly and the AA genotype is also found in this group. (Linear by linear association χ^2 = 5.184, df = 1, p = 0.023). The time to start hay collection was 2.78 ± 0.35 days for the GG genotype, 1.5 ± 0.36 days for the GA genotype, and 0.5 days for the AA genotype.

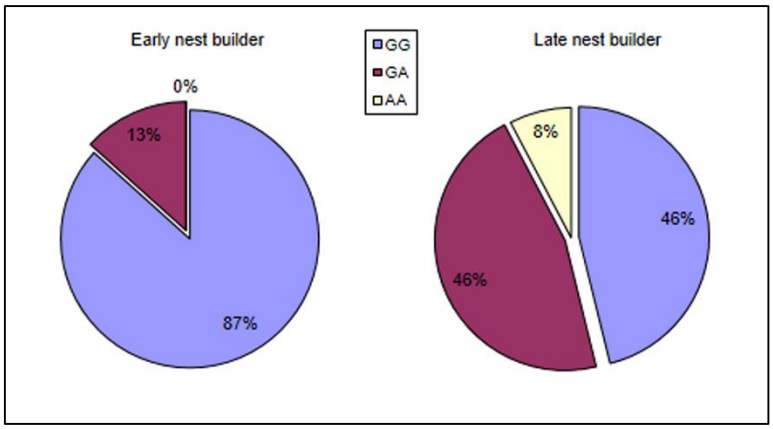

Figure1. The genotype distribution of the 2464G >A SNP in the groups the mothers showing early and late nest building behaviour.

The calculated additive genetic variance (Va = $2pq*\alpha^2$, α = 0,909) for the 2464G>A SNP was Va = 0.2444 The heritability of the trait associated for PGR 2464G>A mutation, based on the equation $h^2 = Va/Vp$ (Vp = 2,353), was $h^2_{PRG2464} = 0.10$.

3.2. The examination of the polymorphisms in the PRLR gene

Sequencing of the promoter region of the PRLR gene revealed four point mutations located at SNP1-407G> A, SNP2-496G> C, SNP3-926T>C and SNP4-937A> C. In addition to SNPs, a microsatellite was detected at position 574. *Table 8* shows the distribution of observed genotypes, observed heterozygosity (H_o), expected heterozygosity (H_e), effective allele size (Ne), and PIC value. Examination of the distribution of genotypes shows that they are identical to Hardy – Weinberg equilibrium for SNPs 293G> T and 339G> A (P> 0.05), while they are not in equilibrium for the other two SNPs; (complete absence of heterozygotes is observed). The PIC values indicate that the rabbit population shows a moderate polymorphism for each point mutation. (*Table 4*)

Table 4. The genotype distribution and gene diversity data of the four SNPs located in the promoter region of the PRLR gene.

SNP	Observed genotype						H_0	H_e	HWE		Ne	PIC
									χ^2	P		
293G>T	GG	21	GT	15	TT	4	0.375	0.415	0.287	0.592	1.694	0.326
339G>A	GG	21	GA	15	AA	4	0.375	0.415	0.287	0.592	1.694	0.326
770G>C	GG	21	GC	0	CC	19	0.000	0.505	40.000	<0.001	1.995	0.374
869T>C	TT	28	TC	0	CC	12	0.000	0.425	40.000	<0.001	1.724	0.332

H_0: observed heterozygosity, χ^2: Chi-squared value, H_e: expected heterozygosity, Ne: effective allele size, PIC: polymorphism information content, HWE: a Hardy-Weinberg equilibrium, P: significance level

Table 5 shows the linkage disequilibrium between SNPs. Based on our results, all five SNP pairs showed significant linkage disequilibrium (linked inheritance). In the stock, the four SNPs were segregated into the following four genotypes: GGGGGGTT, TTAACCCC, GTAACCTT, GTGACCCC.

Table 5. The allele and haplotype distribution and linkage disequilibrium for the examined SNPs.

	Allele frequency		Haplotype frequency		D’	r	χ^2	P
SNP1-2	G	0.713	GG	0.60625	0.228	1.000	40.000	<0.001
	T	0.288	GA	0.08125				
	G	0.713	TG	0.08125				
	A	0.288	TA	0.23125				
SNP1-3	G	0.713	GG	0.525	0.310	0.907	32.894	<0.001
	T	0.288	GC	0.1625				
	G	0.525	TG	0				
	C	0.475	TC	0.3125				
SNP1-4	G	0.713	GT	0.6125	0.233	0.745	22.185	<0.001
	T	0.288	GC	0.075				
	T	0.700	TT	0.0875				
	C	0.300	TC	0.225				
SNP2-3	G	0.713	GG	0.525	0.310	0.907	32.894	<0.001
	A	0.288	GC	0.1625				
	G	0.525	AG	0				
	C	0.475	AC	0.3125				
SNP2-4	G	0.713	GT	0.6125	0.233	0.745	22.185	<0.001
	A	0.288	GC	0.075				
	T	0.700	AT	0.0875				
	C	0.300	AC	0.225				
SNP3-4	G	0.525	GT	0.525	0.323	0.688	18.947	<0.001
	C	0.475	GC	0				
	T	0.700	CT	0.175				
	C	0.300	CC	0.3				

D’: distance from the linkage equilibrium, r: correlation coefficient, χ2: Chi-squared value, P: significance level

Several factors have a significant effect on milk production, including the number of kits and the microsatellite found in the PRLR gene, as well as the genotypes formed by SNPs (*Table 6*).

Table 6. The association between the milk production (total amount of the milk during the first 21 days of lactation) and polymorphisms located in the PRLR gene promoter region. (General linear model (GLM), the number of kits was included as covariant)

	df	Milk productions			
		MS	F	P	Partial Eta-squared
Corrected model	7	603986.881	5.419	**0.000**	0.542
Intercept	1	1659510.225	14.888	**0.001**	0.318
Number of kits	1	1239088.433	11.116	**0.002**	0.258
Genotype	3	487348.278	4.372	**0.011**	0.291
MS574	2	758532.337	6.805	**0.003**	0.298
MS574*genotype	1	2304.989	0.021	0.887	0.001
Error	32	111466.214			

df: degree of freedom, MS: mean square, F: F value, P: significance level

For genotypes, the homozygous TTAACCCC genotype showed higher milk production (1564.7 ± 444.7 g) compared to the other three genotypes (GGGGGGTT 1399.1 ± 326.8g; GTGACCTT 1403.8 ± 517.1 g; GTGACCCC 1220.0 ± 666.2g). The interaction between microsatellite and SNP genotypes was not significant. When the dead nestling was excluded from the analysis, the effect of SNP genotypes was not statistically significant (P = 0.071); however, the effect of the number of kits (P = 0.002) and microsatellite remained significant (P = 0.025). The milk production in the microsatellite genotypes is shown in *Figure 2*. The short repeat, a 167-base fragment, resulted in higher milk production (1623.8 ± 525.1 g) than the long repeat (170 bases,

1300.4 ± 458.6 g), while the heterozygous form (167/170) represented an intermediate value (1460.4 ± 411.5 g). The differences persist even after excluding the dead nestling, as only the value of the long-repeat group changes (1359.5 ± 368.9 g).

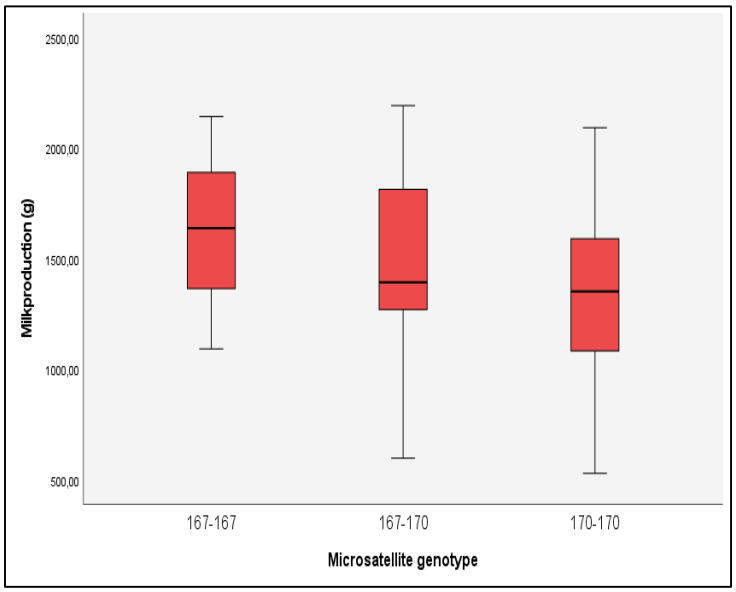

Figure 2. The milk production of the groups of does with different microsatellite genotypes. *The 167/167 and 170/170 represent the homozygote genotypes, and the 167/170 represents the heterozygote genotype.*

The weight of hair collected into the nest by the rabbit does did not show a significant correlation with the polymorphisms in the PRLR gene (SNPs and microsatellites) (*Table 7*). The amount of hair in the different genotypes varied as follows: TTAACCCC 21.3 ± 8.8 g; GGGGGGTT 20.8 ± 12.1 g; GTGACCTT 16.21 ± 7.7 g; GTGACCCC 15.9 ± 8.0 g. In the case of the microsatellite, the short repetition gave an average of 21.8 ± 10.7 g in terms of hair volume, the long repetition 19.8 ± 12.1 g, and the heterozygote form 17.3 ± 7.2 g.

Table 7. The association between the amount of hair in the nest and the polymorphisms located in the PRLR gene promoter region. (General Linear Model (GLM)).

	df	Hair weight			
		MS	F	P	Partial Eta-squared
Corrected model	6	60.758	0.520	0.789	0.086
Intercept	1	5289.150	45.564	0.001	0.578
Genotype	3	92.605	0.793	0.507	0.067
MS574	2	63.261	0.541	0.587	0.032
MS574*genotype	1	11.594	0.099	0.755	0.003
Error	33	116.850			

df: degree of freedom, MS: mean square, F: F value, P: significance level

3.3. The effect of stress on the nest-building behaviour

There was a significant correlation between the increase in cortisol levels in the last three days of pregnancy and the level of cortisol measured on the day of parturition ($R = 0.843$, $F = 61.617$, $df = 26$, $p = 0.001$). The mean cortisol level at parturition in the study stock was 826.01 ± 479.29 ng / mg. Cluster analysis based on the difference between cortisol levels during pregnancy (3 days before parturition) and cortisol levels on the day of parturition divides the animals into two groups. Seven individuals (sensitive group) showed a large increase (mean increase: 925.48 ± 424.75 ng / mg; mean cortisol level at parturition 1462.09±511.37 ng/mg); while in the other (normal) group, cortisol levels decreased (mean increase: -152.09±236.55 ng/mg), representing twenty individuals (mean cortisol level at parturition 603.38±174.74 ng/mg). The results of the cluster analysis are shown in *Figure 3*.

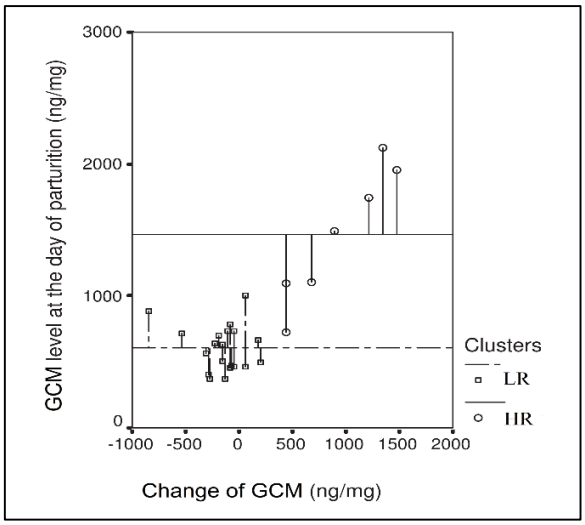

Figure 3. Groups formed by the cluster analysis based on the cortisol level increase until the day of parturition (HR- high response, stress-sensitive /LR low response, normal).

Cortisol and progesterone values measured on the day of parturition showed a moderate correlation for all does. The linear regression model was significant (R = 0.567, F = 11.871 p = 0.002) (*Figure. 4*).

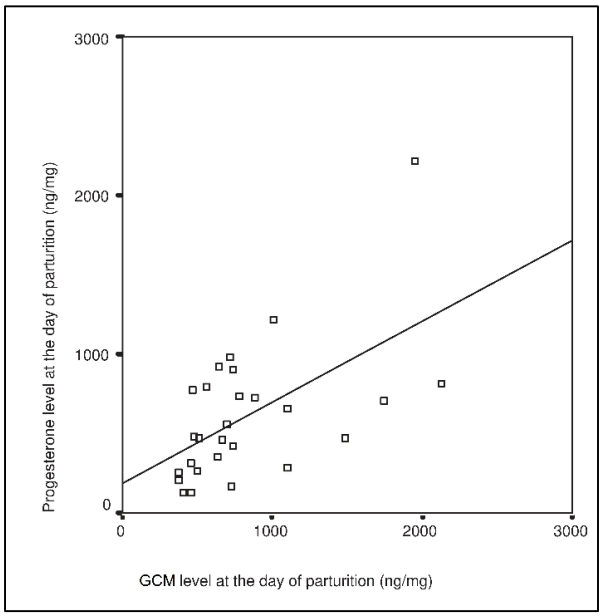

Figure 4. Correlation of the cortisol and progesterone values measured on the day of parturition.

We hypothesised that higher maternal cortisol levels would modify the time of hay collection, and the amount of hay and hair collected. In the case of the time of the start of hay collection, a significant (t = -2.238 df = 25 p = 0.034) difference was found, in which case the group means were 26.14 ± 31.39 hours (sensitive) and 76.10 ± 55.57 hours (normal), presented in *Figure 5*.

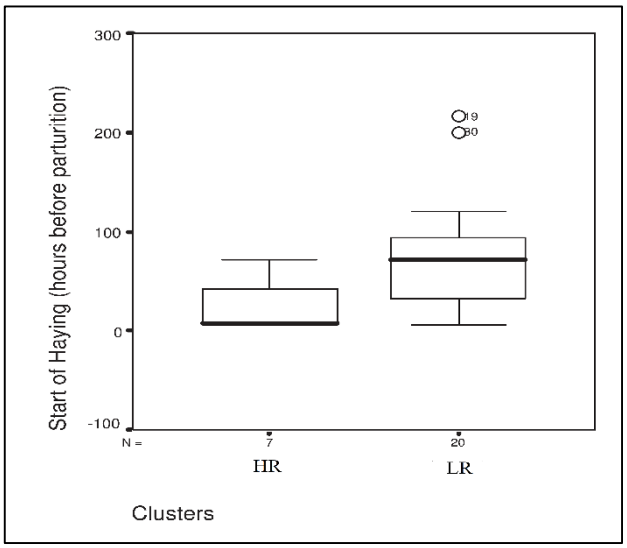

Figure 5. The time of hay collection differed significantly in the groups formed based on the stress response. (HR- high response, stress-sensitive /LR low response, normal)

The amount of hay did not differ significantly (t = -0.558, df = 25, p = 0.582) in the two groups (sensitive: 146.21 ± 37.89 g and normal: 160.10 ± 61.51 g). The amount of hair (sensitive: 10.91 ± 6.15 g and normal: 14.20 ± 13.18 g) was also very similar between groups (t = -0.631 df = 25 p = 0.534).

Based on previous studies, we expected that cortisol levels and their change might be related to the number of kits. There was a significant difference in the average number of kits (t = -2.185, df = 25, p = 0.038), resulting lower value in the sensitive group. Mortality at birth was significantly (Chi^2 = 5.092, df =

1, p = 0.024) higher in the sensitive group compared to the normal group (Figure 6).

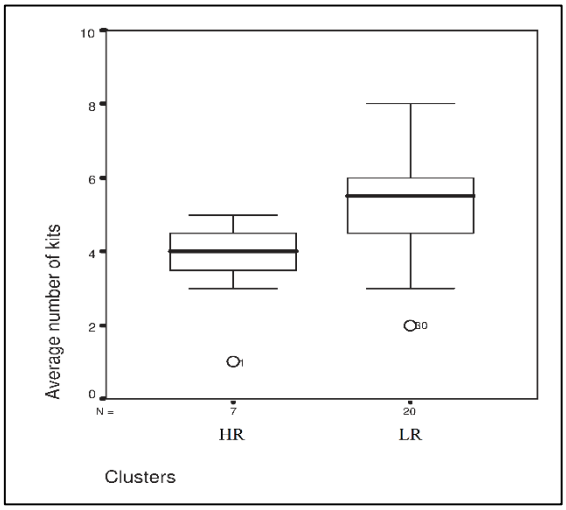

Figure 6. The association between the groups formed based on the stress response and the kit number/mortality was significant. (HR- high response, stress-sensitive /LR low response, normal)

Based on our results, the increase in cortisol levels in the last three days of pregnancy in the rabbit can be used to determine how strong the stress response of the individual to parturition is and how sensitive the animal is to stress. This had an effect on the production of progesterone in the pregnant animal, which was supported by a significant correlation between cortisol and progesterone values measured on the day of parturition.

3.4. The effect of stress on the sex ratio of the offspring

Fifteen does in the study were divided into two groups based on faecal cortisol metabolite (FCM) levels measured on the day of mating. The group with a low FCM value (471.94 ± 69.3 ng/mg) consisted of seven individuals, and the group with a high FCM value (797.25 ± 102.7 ng/mg) was represented by eight individuals. The mean litter size did not differ significantly between the two groups. The ratio of the two sexes was equal in the 76 offspring (38 males and

38 females). The linear regression between FCM values measured on the day of conception and the proportion of female offspring in litters (female ratio = 0.001 * FCM - 0.024, *Figure 7*) was significant (F = 6.997; P = 0.020; r^2 = 0.350). The proportion of females was higher in offspring of does with a higher cortisol response.

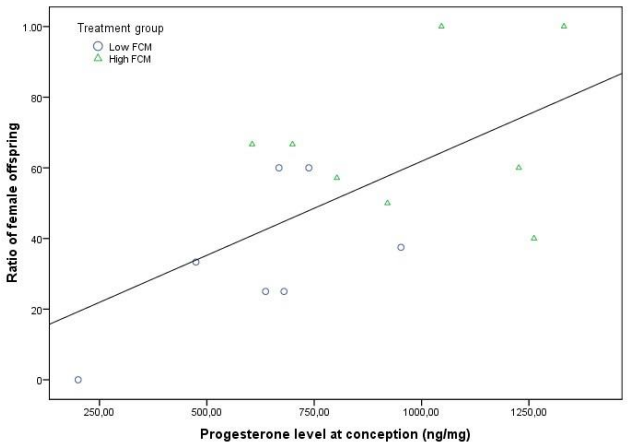

Figure 7. Linear regression between the FCM measured on the day of mating and the female sex ratio of the offspring (F = 6.997; P = 0.020; r^2 = 0.350).

Progesterone levels showed a similar regression to the proportion of female offspring than FCM levels. The linear regression between the two variables (female ratio = 0.001 * P + 0.086) was significant (F = 7.884, P = 0.015, r^2 = 0.378). Faecal progesterone and cortisol metabolite levels showed a moderate, positive correlation (Pearson correlation: r = 0.626; P = 0.013; N = 15).

3.5. The repeatability of the exploration behaviour

The mean repeatability of the novel food test was 2.3 ± 2.3, with values ranging from 0 to 8. The mean latency of the exploratory behaviour was 188.1 ± 107.6 sec. The novel food test showed good repeatability in a novel environment.

However, of the correlations considered for the two parameters (Spearman and Intraclass correlation coefficients), only the latency of the first touch gave a significant correlation with a medium correlation value (*Table 8*).

Table 8. The repeatability of the two parameters in the novel food test

Test	N	Spearman-correlation (r; p-value)	Intraclass correlation coefficient		
			R	95% CI (lower / upper bound)	p-value
Frequency of the exploration	24	0.239; 0.260	0.377	-0.440; 0.730	0.132
Latency of the first touch	24	**0.599; 0.002**	**0.615**	**0.110; 0.834**	**0.013**

N: Sample number, R: correlation coefficient, CI: confidence interval, the values highlighted in bold are significant at the level $P < 0.05$.

3.6. Examination of the association between the exploratory behaviour and the production traits

The mean litter size was 5.0 ± 1.5 (values ranged from 2 to 8), and the mean milk production was 1356.8 ± 335.7 g (values ranged from 741.0 g to 2098.9 g). The mean litter weight at birth and litter weight at 21 days were 203.9 ± 59.7 g (values ranged from 67.9 g to 294.9 g) and 960.8 ± 227.9 g (values ranged from 496.3 to 1353.8). The does with lower values of behavioural latency, i.e., more exploratory individuals, had higher milk production and higher litter weight at birth and on the 21 days. The data is shown in *Table 9*.

Table *9. The association between the parameters of the novel food test and the reproductive traits of the wild rabbit does.*

Test	N	Spearman-correlation (r), p-value			
		litter size	milk production	weight at birth	weight at the 21st day
Frequency of the exploration	15	0.121; 0.667	0.051; 0.857	0.045; 0.872	-0.065; 0.817
Latency of the first touch	15	-0.472; 0.076	**-0.673; 0.006**	**-0.611; 0.015**	**-0.516; 0.049**

The values highlighted in bold are significant at the level $P < 0.05$.

3.7. Examination of the factors influencing the behaviour of the offspring

Based on the GLMM model, only the effect of the offspring's weight was statistically significant in terms of the frequency of exploratory behaviour (Chi^2 = 13.12; p = 0.0003, df = 1). The frequency of the behaviour and the weight were significantly correlated r = 0.267 (p = 0.036). The sex of the offspring did not affect the frequency of their exploratory behaviour (Chi^2 = 1.41; p = 0.235, df = 1). The effect of the does on the latency of the first touch of the novel food in the case of offspring was negligible (estimate = 0.1857; SE = 0.1332).

Sex also had no significant effect on the latency of the first touch in the offspring, although the hazard value (Exp (B)) was above one (*Table 11*). Bucks showed lower values of 127.1 ± 122.9 s than females 139.0 ± 126.8 s. There was a significant correlation between the weight of the offspring and the latency of the first touch with the novel food (Spearman correlation r = −0.269; p = 0.034; n = 62); this factor had the strongest effect on the behaviour (*Table 10*).

Table 10. The effects of the factors influencing the latency of the novel food test in the offspring

	B	SE	Wald-chi^2	df	p-value	Exp(B)	95% CI	
							Lower	Upper
Sex	0.341	0.326	1.098	1	0.295	1.407	0.743	2.665
Weight	0.972	0.378	6.607	1	0.010	2.644	1.260	5.550
Latency of the does	0.736	0.345	4.547	1	0.033	2.087	1.061	4.104

B: regression coefficient, SE: standard error, Wald chi^2: Wald-chi^2 value, df: degree of freedom, Exp(B): hazard ratio, CI: confidence interval

The latency of maternal exploration for novel food also showed a significant and robust hazard value (*Table 10*). The offspring of neophile does show demonstrably lower latency in the novel food test (*Figure 8*).

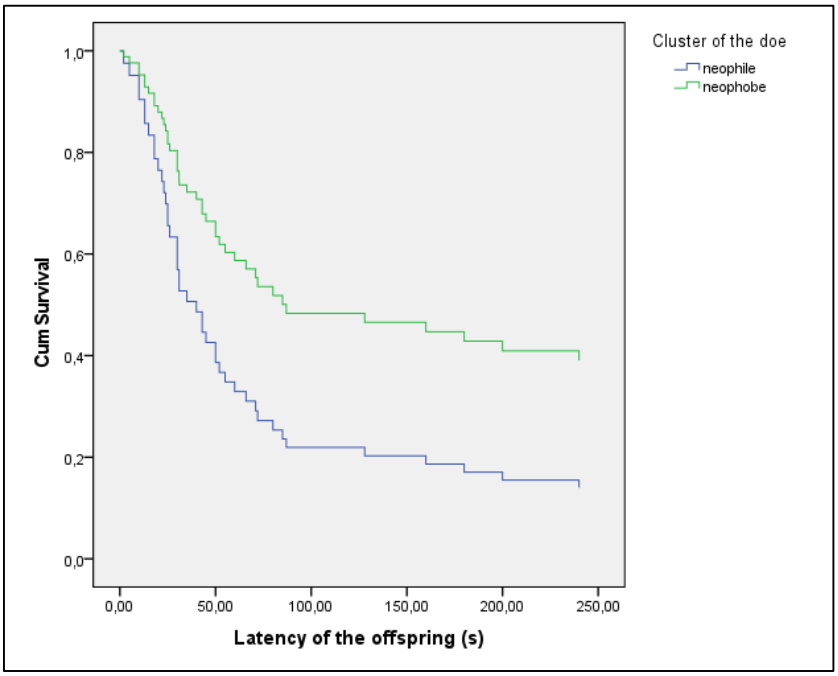

Figure 8. The effect of the maternal groups with different exploration behaviour on the latency of the offspring

4. CONCLUSIONS AND RECOMMENDATIONS

Examination of the progesterone receptor gene

In addition to the polymorphisms already described, a new point mutation was detected by sequencing the promoter region of the gene. However, the linkage between SNPs could not be demonstrated. The point mutation at 2464G> A showed an association with the timing of nesting but not with nest quality. The candidate role of the PGR gene in the genetic background of the behaviour was supported, the h^2 value of the property was 0.1. In addition to the higher uterine capacity already described, the GG genotype also results in early nesting, which is adaptive not only in domesticated lines but also in nature. The selection for uterine capacity (number of kits) may change the nest-building behaviour, which can greatly reduce behavioural variance after colonisation in breeding programs. Further studies are needed to explore the natural performance of the genotypes (fitness).

Examination of the prolactin receptor gene

Four SNPs (SNP1-407G> A, SNP2-496G> C, SNP3-926T> C and SNP4-937A> C) were successfully described by sequencing the promoter region of the prolactin receptor gene, and in addition, a microsatellite was detected. SNPs showed linkage disequilibrium and were segregated into four haplotypes. In the case of rabbits, these polymorphisms have not been previously described. Since polymorphisms in the PRLR gene in other species (cattle, buffalo, goat) are associated with milk production, we examined the relationship between the detected markers and milk production. SNPs did not, but microsatellite proved to be associated with this trait, and the short fragment length resulted in higher milk production. The most useful result for current

rabbit breeding is related to the increase in milk production, since the development of suckling rabbits is influenced by several factors (birth weight, fetal nutrient supply, litter size, environmental conditions). However, the milk production of the does is dominant in the first three weeks. Further studies are needed to explore the regulatory effect of the microsatellite on milk production in differently selected domesticated breeds (and lines) to develop a marker-based selection. As the nutrient requirement of suckling rabbits, especially in larger litters, is high during the first three weeks, the does can satisfy this demand with difficulties. The marker-based selection could offer a solution for it. Because the PRLR gene, together with the PRL gene, affects the process of fur pulling during nest-building behaviour in the wild rabbit, we hypothesized that polymorphisms will also affect this trait. Contrary to our expectations, none of the polymorphisms showed an association with the amount of fur in the nest. It is likely that the PRL gene affects this trait and the receptor gene may only be involved in the timing of the behaviour. Its confirmation requires further targeted tests.

Investigation of the effect of stress on nest-building behaviour

Our study demonstrated that the increase in cortisol levels during the three days before parturition showed large individual differences and correlated with the change in progesterone levels before parturition. Pre-parturition social stress in free-range rabbit colonies and its negative effect on nest-building behaviour have been partially described, but our study confirmed that late (immediately before parturition) nesting of stress-sensitive mothers is not the result of intra-group fighting but also a consequence of progesterone release due to individual stress sensitivity (high cortisol response). We also pointed out that although this has / can have a significant effect on the nest quality and, thus the survival of the kits under natural conditions, this is only true with

limited resources. If there is available amount of sufficient hay, the stress-sensitive mother can also build a good quality nest in a short time. Neither the timing nor the amount of fur pulling is affected by high cortisol levels, so this factor does not cause a difference in nest-building. Although reproductive success is not affected by high cortisol levels due to nest quality, its effect on progesterone levels resulted in a significantly lower number of kits and higher birth mortality in stress-sensitive mothers. Our study points out that personality type (one of the indicators of stress sensitivity) has a significant effect on reproductive traits, which can often lead to misinterpreted results in studies performed in the natural environment. In rabbit breeding, group keeping is emerging as a new direction, affecting even mother rabbits. Social ranking can be a serious factor in stress sensitivity. My results highlight that stress-sensitive mothers start to build the nest late, although based on nest quality, the reproduction success is not affected by high cortisol levels, the number of kits is significantly lower, and the mortality is higher in stress-sensitive mothers. Consequently, it would be worthwhile in practice to carry out further studies on large-scale breeds, and in the case of a similar result, mother stocks selected for low stress sensitivity could be established.

Investigation of the effect of stress on the sex ratio of offspring

Our results show that the number of females is significantly higher among the offspring of stress-sensitive mothers compared to normal does. Cortisol can inhibit progesterone recognition, uterine blood circulation, capillary permeability, thereby preventing implantation, disrupting and impairing synchronization between the uterus and the embryo. That is why we need to pay attention to its consequences. In the group with high FCM values, the proportion of female offspring was higher, which is mainly due to the increase in the number of females and not to the decrease of males, although the latter

was also tendentious. The proportion of females was also correlated with cortisol and progesterone values measured on the day of mating. Our results will help us get one step closer to understanding the complex processes discussed above.

Investigation of exploratory behaviour

The results of our exploratory studies trigger several thoughts. One of the directions concerns the rabbit re-introduction programs, during which caged animals, not appropriate individuals, are released into their natural environment. This is because in the absence of predators in caged housing, exploratory individuals predominate, which eat better, have larger kits and according to our results, reproduce more progeny. However, when returned to the natural environment, they are easily disappearing from the system under high predatory pressures because, although they learn faster in new situations, their behaviour is not adaptive. Another line of thought is that we can select stocks to improve milk production with the help of exploration studies against novel food, as the milk production of the more exploring individuals is higher. However, it has to be mentioned that our studies were performed on wild rabbits. In domesticated lines, where there is a complete lack of predation, it is also conceivable that more confidential, more eating, more fertile individuals remained in the system and this property is no longer characteristic of domesticated lines.

5. NEW SCIENTIFIC RESULTS

1. I identified an undescribed point mutation in the wild rabbit by sequencing the promoter region of the PGR gene at position 2682T> C.
2. The point mutation at 2464G> A showed an association to the timing of nest-building but not to nest quality. The GG genotype results in early nest building behaviour with a heritability of $h^2 = 0.1$.
3. By sequencing the promoter region of the PRLR gene, in addition to four new SNPs (SNP1-407G> A, SNP2-496G> C, SNP3-926T> C and SNP4-937A> C), a microsatellite has been identified at position 577. Point mutations showed linkage disequilibrium, segregating into four haplotypes in the study population. Contrary to my assumptions, the point mutations did not, but the microsatellite showed an association with milk production. The short fragment length caused higher milk production compared to the longer section (1623.8 ± 525.1 g and 1300.4 ± 458.6 g, respectively).
4. I found a correlation between the change in cortisol levels measured in the three days before parturition and the progesterone levels of rabbit mothers. High cortisol levels kept progesterone levels elevated until parturition. The high cortisol response resulted in significantly delayed nest-building (grass-carrying) behaviour but did not affect the time of fur pulling.
5. Under ideal conditions, the nest quality of stress-sensitive mothers did not differ from that of normal mothers, but the number of kits was lower.
6. The latency of the exploratory behaviour towards novel foods is a well-repeatable parameter ($r = 0.599$, $p = 0.002$), while its frequency is poorly repeatable ($r = 0.239$, $p = 0.260$). The latency of the exploration shows a negative correlation with milk production ($r = -0.673$), birth weight ($r = -0.611$), and 21-day weight ($r = -0.516$).
7. The exploratory latency of the offspring is significantly influenced by the weight of the individual and the exploratory behaviour of the mother; the

offspring of neophile mothers showed demonstrably lower latency in the new food test.

6. PUBLICATIONS ON THE TOPIC OF THE DISSERTATION

1. Benedek, Ildikó ; Altbäcker, Vilmos ; Molnár, Tamás ✉ Stress reactivity near birth affects nest building timing and offspring number and survival in the European rabbit (*Oryctolagus cuniculus*) PLOS ONE 16 : 1 Paper: e0246258 , 15 p. (2021)

2. Benedek, Ildikó ✉ ; Altbäcker, Vilmos ; Zsolnai, Attila ; Molnár, Tamás ✉ Exploring the Genetic Background of the Differences in Nest-Building Behavior in European Rabbit ANIMALS 10 : 9 Paper: 1579 , 12 p. (2020)

3. Benedek, Ildikó ✉ ; Altbäcker, Vilmos ; Zsolnai, Attila ; Molnár, Tamás Maternal cortisol level around conception is associated with offspring sex ratio in captive European wild rabbit ACTA AGRARIA KAPOSVÁRIENSIS – accepted.