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# Visoka življenjska jubileja prof. dr. Jasne M.A. Stekar in prof. dr. dr. Franca Ločniškarja

V letu 2008 je prof. dr. Jasna M.A. Stekar praznovala 80., prof. dr. dr. Franc Ločniškar pa 85. rojstni dan. Oba jubilanta sta dogodka proslavila v krogu nekdanjih sodelavcev in priložnost izkoristila za živahen pomenek o dogodkih, povezanih s preteklostjo Oddelka za zootehniko in Biotehniške fakultete ter pokazala živahno zanimanje za tekoče raziskovalno in pedagoško delo na oddelku. Nekdanje sodelavce sta v pomenkih navdušila s smislom za humor in številnimi spomini, ki so tesno povezani z nastankom in delovanjem Oddelka za zootehniko.

Akademska kariera prof. Stekarjeve se je začela leta 1955 na Agronomski fakulteti v Ljubljani, kamor se je vrnila po diplomi, opravila jo je leta 1953 na isti fakulteti, in po opravljenem enoletnem stažu na državnem posestvu Ponoviče in krajši zaposlitvi v podjetju Vinosad Koper. Leta 1961 je bila izvoljena za asistentko pri predmetih živinoreja in prehrana domačih živali. Leta 1961 se je pet mesecev izpopolnjevala pri prof. Abgaroviču v Varšavi, januarja 1964 je zagovarjala dr. disertacijo z naslovom »Vpliv beljakovin in ogljikovih hidratov na potek vrenja v silažah«, kar je ostala pomembna tema njenega raziskovalnega dela vse do konca kariere, ko je delo in izkušnje zaokrožila z izdajo knjige Siliranje (1999). S svojim raziskovalnim delom je prišla do pomembnih temeljnih znanj o vplivu beljakovin na tvorbo mlečne kisline in razmerja med ogljikovimi hidrati in dušik vsebujočimi snovmi za potek fermentacije. Leta 1965 je sedem mesecev raziskovala v laboratoriju prof. McDonalda v Reedingu, enem vodilnih laboratorijev na področju konzerviranja krma v svetu, v tem času pa je sodelovala tudi s prof. Horvatom v Zagrebu.

Prof. Stekarjeva se je veliko ukvarjala s krmnimi dodatki, tako je npr. že v začetku 70-ih proučevala učinke probiotikov pri pujskih, za kar prejela tudi mednarodno priznanje. Ves čas je

aktivno skrbela za urejanje laboratorija in nabavo laboratorijske opreme ter uvajanje novih laboratorijskih in raziskovalnih metod. Prof. Stekarjeva se je udeležila številnih znanstvenih srečanj, pogosto kot vabljena predavateljica. Za svoje zasluge na področju prehrane in konzerviranja krme je letos v Opatiji na tradicionalnem kongresu Krmiva prejela posebno priznanje za dolgoletne zasluge za sodelovanje pri pripravi in izvedbi tega tradicionalnega srečanja.

Med njenimi sodelovanji v tujini je bilo najpomembnejše sodelovanje z INRA inštitutom v Clermont Ferrandu, kjer so se kasneje izpopolnjevali številni sodelavci Inštituta za prehrano. Prof. Stekarjeva ima bogato bibliografijo, ki obsega preko 600 enot, od tega okvirno 100 izvirnih znanstvenih člankov v domačih in mednarodnih revijah. Med priročniki so najpomembnejši Fliegov ključ, Siliranje in silaža, Kaj nam pove kemijska analiza ter Enote za oceno energijske vrednosti. Ves čas je sodelovala tudi z industrijo in državnimi inštitucijami. Prof. Stekarjeva je bila nosilka predmetov Splošna prehrana ter Krma in konzerviranja krme, bila je mentorica številnim diplomantom in devetim magistrskim in doktorskim študentom.

Izjemnega pomena za razvoj raziskovalnega dela na področju kmetijstva je njeno uredniško delo pri Zborniku Biotehniške fakultete, današnje *Acte agriculturae Slovenice*, ki ga je opravljala 25 let, od 1976 do 2001. Z urednikovanjem je gotovo naredila ogromno za razvoj stroke, oddelka, pa tudi jezika, na rabo katerega je bila ves čas zelo pozorna. Za svoje raziskovalno, pedagoško in strokovno delo je prof. dr. Jasna M.A. Stekar leta 1985 dobila tudi Jesenkovo nagrado.

## Prof. Dr. Jasna M.A. STEKAR CELEBRATED HER 80th AND Prof. Dr. Dr. Franc LOČNIŠKAR HIS 85th ANNIVERSARY

In 2008, Prof. Dr. Jasna M.A. Stekar celebrated her 80th and Prof. Dr. Dr. Franc Ločniškar his 85th anniversary. Both celebrated their jubilees in the frame of former co-workers and friends at Department of Animal Science and used this opportunity for vivid discussions about the past and present development at the Department of Animal Science and Biotechnical Faculty. It was really stimulating experience to talk with both professors about events, important for the history of the department and to share their sense of humor and many inspiring memories.

Prof. Dr. Jasna M.A. Stekar started her academic carrier after BSc at Agricultural Faculty as teaching assistant in 1961. She received her PhD from University of Ljubljana in 1964 and started a almost four decades of research work in the field of animal nutrition and conservation of feedstuff. During her career she had fruitful collaboration with Prof. McDonald in Reeding, Prof. Horvat in Zagreb and prof. Abgarovic in Varsaw. The most important for the development of the Institute of nutrition is her collaboration with the INRA institute for nutrition in Clermont Ferrand, where many colleagues from the institute performed their research and collaborated in joint projects. Bibliography of Prof. Dr. Stekar counts more than 600 units, among them more than 100 research articles in national and international peer reviewed journals, several books and numerous congress contributions. During her academic career Prof. Stekar was teaching nutrition and feedstuff conservation and was mentor to a number of BSc students and nine post graduate students. For her scientific achievements she received Jesenko award in 1985. For the development of research work in the field of animal science is crucial her editorial work for the Research Report of the Biotechnical Faculty, now *Acta agriculturae Slovenica*. She was Editor in Chief for 25 years and contributed significantly to the improvement of scientific merit of publications in the field as well as to proper use of Slovene language in agricultural science in general.

Peter Dovč, Editor

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# CARCASS QUALITY OF AUTOCHTHONOUS CIKA CATTLE

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## ABSTRACT

The Cika cattle is the only Slovenian autochthonous cattle breed, counting around 1,600 animals in 2007.. The breeding goal for Cika cattle is dual purpose with the emphasis on milk production. However, Cika cattle is mostly reared in the cow-calf system. To a smaller extent Cika cattle is still used for milk production in the traditional regions for Alpine dairy-farming. Our main interest was to get an insight into carcass quality of Cika cattle. The data used in this survey were collected in slaughterhouses in the years 2005 through 2007. Out of all slaughtered cattle in 2007 the share of slaughtered Cika cattle was 0.24%. According to the category, the slaughtered animlas were very heterogenic in carcass weight which is seen in rather large standard deviations. The average carcass weight of A category (bulls under 24 months of age) was 260.3 kg. The majority of bulls under 24 months of age and calves were classified in conformation class O (57.1 and 47.8%), whereas bulls over 24 months of age were mostly classified in the conformation class R. The majority of calves (88.1%), bulls under 24 months of age (55.8%) and bulls over 24 months of age (49.5%) were classified in fatness class 2. Most of Cika cattle are reared extensively on small farms, mostly on mountain pastures without any additional concentrates. This could present the basis and the opportunity for Cika breeders to promote beef from Cika cattle on the market and to increase their income. Autochthonous Cika is well adapted to the environment and helps to maintain biodiversity and sustainable agricultural production, especially in less favourable agricultural areas.

Key words: cattle / autochthonous breeds / Cika / carcass quality / Slovenia

# KLAVNA KAKOVOST AVTOHTONEGA CIKASTEGA GOVEDA

## IZVLEČEK

Cika je edina avtohtona pasma govedi v Sloveniji, katere populacija je v letu 2007 štela okoli 1600 živali. Rejski cilj za cikasto govedo je kombinirana usmeritev s poudarkom na prireji mleka. Kljub temu, cikasto govedo redijo v večini primerov v sistemu krava-tele. V manjši meri pa pasmo še vedno uporabljajo za prirejo mleka v tradicionalnih regijah za planšarstvo. Glavni namen raziskave je bil dobiti vpogled v klavno kakovost cikastega goveda, kakor je bila ovrednotena na klavni liniji. Podatki uporabljeni v raziskavi so bili zbrani v klavnicah v letih od 2005 do 2007. Cikasto govedo je v letu 2007 predstavljalo le 0,24 % vsega zaklanega goveda v Sloveniji. Zaklano govedo po kategorijah je bilo zelo heterogeno (raznoliko) v masi klavnih polovic, kar je vidno v precej velikih standardnih odklonih. Povprečna masa klavnega trupa kategorije A (biki do starosti 24 mesecev) je bila 260,3 kg. Večina bikov starih do 24 mesecev in telet je bila razvrščena v O razred za mesnatost (57,1 % in 47,8 %), medtem ko so bili biki starejši od 24 mesecev večinoma razvrščeni v R razred za mesnatost. Večina telet (88,1 %), bikov starih do 24 mesecev (55,8 %) in bikov starih nad 24 mesecev (49,5 %) je bilo razvrščeno

v razred 2 za zamaščenost. Cikasto govedo večinoma redijo ekstenzivno na majhnih kmetijah, pogosto na planinskih pašnikih brez dodajanja močnih krmil. To bi lahko bila priložnost za rejce cikastega goveda za promocijo govedine cikaste pasme na tržišču z namenom povečanja dohodka. Avtohtona cika je zelo prilagojena na okolje in pomaga ohraniti biodiverziteto in sonaravno kmetijsko proizvodnjo, še posebno na marginalnih območjih.

Ključne besede: govedo / avtohtone pasme / cika / klavna kakovost / Slovenija

## INTRODUCTION

The Cika cattle is the only Slovenian autochthonous cattle breed which has been preserved to the present days. In the second half of the 19th century, Cika cattle arose from the improving light-red, red or brown-red coloured Bohinj cattle with sires from Möelthal and Pinzgau (Austrian provinces). The population of Cika is very heterogeneous despite the increasing number of animals. The reason for phenotypic variability of population is the semen of Pinzgauer sires used for artificial insemination of Cika cows due to the agricultural - political decision after Second World War. In.2007, the population of Cika cattle is numbering 1625 animals (Sector for Identification and Registration at the Ministry for Agriculture, Forests and Food). Unfortunately in real autochthonous and original phenotype there are only about 20% of all Cika cattle, which totals around 300 animals.

Breeding goal for Cika cattle is dual purpose breed with the emphasis on milk production. However, Cika cattle is mostly reared in the cow-calf system. Suckler herds are kept for beef production only, which is not in accordance with the breeding goal. However, Cika cattle is to a smaller extent still used for milk production in traditional regions of Alpine dairy-farming, especially on Bohinj and Kamnik mountain pastures. Some breeders from those two regions have preserved traditional way of rearing this cattle breed. During the vegetation period, herds are moved from lowland farms to mountain pastures. The herds of Cika cows grazed on Alpine mountain pastures, where Alpine dairying and milk processing into products like cheese, cottage cheese, fresh butter and sour milk has been preserved. Alpine dairymen sell processed milk products to the mountaineers and to tourists visiting the mountains. The income from sold milk products nowadays represents an additional financial source, which was in the past the main income. However milk products, especially cheese from Cika milk made in the mountains represented the only source for the survival of the lowland farm in the winter time.

As mentioned previously, Cika herds are nowadays reared mostly as suckler herds for beef production. The aim of our work was to get an insight into carcass quality of slaughtered Cika cattle in Slovenia in the last three years.

## MATERIAL AND METHODS

The data used in this survey were collected in slaughterhouses between 2005 to 2007. Slaughtered Cika cattle were reared on Slovenian small farms using the traditional production system. In the cold months of the year, voluminous forage was used and during the vegetation period cattle were put on the pasture. Hay, silage and small amount of concentrates were used in the winter time. High percentage of slaughtered Cika cattle originated from ecological farms, where rearing technology is very extensive. After the slaughter, carcass weight was recorded and carcass conformation and fatness were scored according to the EUROP system (Pravilnik o izvajanju…, 2005). The net daily gain was calculated on the basis of carcass weight and age at slaughter.

## RESULTS AND DISCUSSION

In Table 1 the number of slaughtered Cika cattle is presented for years 2005 to 2007. Out of the total slaughtered cattle in the year 2007 in Slovenia the share of slaughtered Cika was 0.24% (Žgur *et al*., 2008). With the growing population of Cika cattle, the number of slaughtered Cika cattle has increased in the last three years from 145 to 287 animals. Especially the number of slaughtered Cika bulls under 24 months of age significantly increased in the last three years. The number of slaughtered Cika bulls over 24 months of age represented very extensive fattened bulls and culled sires for natural mating on farms. In the last few years a small number of Cika breeders have decided to rear steers, because steer's meat is believed to be more juicy and tasteful. Steers can graze in the herds together with other cows, calves and sire, which is very suitable for breeders. Usually, they reared steers just for self-supply on the farm. The number of culled cows above 5 years of age is higher than the number of culled cows under 5 years of age at slaughter. The number of slaughtered heifers is relatively low and we think that most of them were those born like twins together with a male calf or heifers with fertility disorders. In endangered population it is not advisable to slaughter calves, yet the number of slaughtered calves was relatively high. In the year 2005, 20.7%, in the year 2006, 12.2% and in the year 2007, 16.0% of slaughtered Cika cattle were calves. On the other hand, a calf is a by-product in the traditional milk production system with Cika cows. In the region of Bohinj, where the Alpine-dairy farming has still been preserved, the number of slaughtered calves proved to be higher. At the beginning of vegetation the cows are moved to the mountain pastures without calves, because the farmers need milk for cheese production. But nevertheless, female calves with well expressed autochthonous breed characteristics should not be slaughtered despite the traditional production system.

Table 1. The number of slaughtered Cika cattle from 2005 to 2007
Preglednica 1. Število zaklanega cikastega goveda od 2005 do 2007

| Category of cattle<br>Kategorija govedi | | Year of slaughter / Leto zakola | | |
|---|---|---|---|---|
| | | 2005 | 2006 | 2007 |
| A | Bulls under 24 months<br>Biki stari do 24 mesecev | 38 | 97 | 117 |
| B | Bulls over 24 months<br>Biki starejši od 24 mesecev | 37 | 38 | 48 |
| C | Steers<br>Voli | 5 | 6 | 6 |
| D1 | Cows under 5 years<br>Krave stare do 5 let | 9 | 14 | 14 |
| D2 | Cows over 5 years<br>Krave starejše od 5 let | 16 | 25 | 32 |
| E | Heifers<br>Telice | 10 | 7 | 24 |
| T | Calves<br>Teleta | 30 | 26 | 46 |
| | Total<br>Skupaj | 145 | 213 | 287 |

Carcass weights for all Cika cattle categories are shown in the Table 2. Great variability of Cika cattle is seen also in the large variability of carcass weight, which reflects in rather large standard deviations. The average carcass weight of bulls in A category (bulls under 24 months of age) was 260.3 kg at average 18.6 months of age. The lightest carcass weight was 104.0 kg and the heaviest carcass weight was 450.0 kg. The autochthonous phenotype of Cika cattle is lighter than Cika cattle improved with the Pinzgauer sires after Second World War. On the other hand, Cika cattle has the brachycerous origin, suitable for milk production, with low body weight and small frame. The differences in cows' linear body measurements (wither height, chest girth, width of chest, depth of chest) and their proportions of original and improved phenotype of Cika cattle have already identified (Kastelic *et al.* 2005).

Bulls reared to the age of more than 24 months (B category) were in average heavier (322.0 kg), and of course much older (30.3 months) but had lower net daily gain compared to bulls in A category. The lightest "B category" carcass weighed 166.8 kg and the heaviest 582.6 kg. Carcass weight of slaughtered steers was 247.2 kg, which was similar to the bulls in A category. But steers were much older at slaughtered than bulls in category A, and so they achieved lower net daily gain. Carcass weight of cows under 5 years had in average 235.6 kg, while carcass weight of cows over 5 years of age at slaughter weighed 257.5 kg. Average carcass weight of heifers was very low (193.9 kg). It would be better if breeders fattened heifers to higher weight at slaughter. The average carcass weight of calves was 91.3 kg at 4.6 months of age at slaughter.

Table 2. Carcass weight, net daily gain and age at slaughter for all categories of slaughtered Cika cattle in the years from 2005 to 2007

Preglednica 2. Masa klavnih polovic,dnevni neto prirast in starost ob zakolu za vse kategorije zaklanih govedi cikaste pasme v letih od 2005 do 2007

| Category of cattle<br>Kategorija govedi | | n | Average carcass weight<br>Povprečna masa klavnega trupa<br>± SD, kg | Average net daily gain, g/days<br>Povprečen dnevni neto prirast<br>± SD, g/dan | Average age, months<br>Povprečna starost<br>± SD, meseci |
|---|---|---|---|---|---|
| A | Bulls under 24 months<br>Biki stari do 24 mesecev | 249 | 260.3 ± 73.2 | 474.3 ± 96.8 | 18.6 ± 4.7 |
| B | Bulls over 24 months<br>Biki starejši od 24 mesecev | 123 | 322.0 ± 58.6 | 364.5 ± 83.6 | 30.3 ± 6.6 |
| C | Steers<br>Voli | 17 | 247.2 ± 71.2 | 328.4 ± 73.1 | 26.9 ± 14.3 |
| D1 | Cows under 5 years<br>Krave stare do 5 let | 34 | 235.6 ± 48.6 | - | 42.9 ± 8.5 |
| D2 | Cows over 5 years<br>Krave starejše od 5 let | 72 | 257.5 ± 55.5 | - | 106.1 ± 32.5 |
| E | Heifers<br>Telice | 41 | 193.9 ± 45.1 | 306.0 ± 78.0 | 22.2 ± 5.9 |
| T | Calves<br>Teleta | 102 | 91.3 ± 28.7 | 706.6 ± 176.0 | 4.5 ± 1.7 |

SD = standard deviation

Gil *et al.* (2001) reported carcass weight (Table 3) of low meat Spanish rustic cattle breeds, two breeds with small to medium size frame (Asturiana de la Montana, Morucha) and one medium sized frame breed (Avileña – Negra Ibérica). Cika bulls of A category had similar carcass weight as Morucha bulls, but the difference existed in age at slaughter. Cika bulls needed

almost four months longer fattening period to achieve the same carcass weight. On the other hand, Asturiana de la Montaña bulls needed 541 days to reach 249.9 kg carcass weight and had similar net daily gain (461.9 g/day) as Cika bulls (473.2 g/day). Avileña – Negra Ibérica had the highest net daily gain and the heaviest carcass weight compared to all breeds shown in the Table 3.

Table 3. Carcass weight and age at slaughter of three Spanish rustic breeds (Gil *et al.,* 2001) and of Cika cattle

Preglednica 3. Masa klavnega trupa in starost ob zakolu treh starih španskih pasem govedi (Gil *et al.,* 2001) in cikastega goveda

| Spanish rustic breed<br>Španske stare pasme | Asturiana de la Montaña | Morucha | Avileña–Negra Ibérica | Cika cattle (our study) |
|---|---|---|---|---|
| Carcass weight<br>Masa klavnih polovic, kg | 249.9 | 259.9 | 279.4 | 260.3 |
| Age at slaughter, days<br>Starost ob zakolu, dni | 541.0 | 438.9 | 363.3 | 556.8 |
| Net daily gain, g/days<br>Dnevni neto prirast, g/dan | 461.9 | 592.2 | 769.1 | 474.3 |

Petrič (2008) found higher carcass weight and net daily gain in similar study including other dual purpose and beef cattle breeds in Slovenia. Bulls under 24 months of age of Slovenian Brown cattle had carcass weight 323.1 kg and net daily gain 504 g/day, while Charolais and Limousine bulls had heavier carcass weights (372.0 kg, 352.3 kg) and higher net daily gain (609 g/day, 565 g/day), respectively.

Albertí *et al.* (2008) conducted a large study of carcass characteristics of bulls belonging to fifteen western European cattle breeds. In the study, 15 months old slaughtered bulls of beef, dairy as well as local breeds reared at very similar environmental conditions were included. The lightest breeds at slaughter were Highland, Jersey and Casina, which showed also great similarity in carcass weight and net daily gain to Cika bulls from our study. All others included bulls from different beef and dairy breeds, had better net daily gains and higher carcass weights. Carcass weight of Highland and Casina bulls were 245.1 kg and 244.7 kg compared to Cika bulls (260.3 kg). Average net daily gain of Cika bulls was similar to Jersey (457.4 g/day) and Highland bulls (480.0 g/day). The reason for similarity between Cika and Jersey bulls could be in brachycerous origin of both breeds.

As previously mentioned, Pinzgauer sires had a role in the past in development of Cika breed. So we compared also carcass characteristics of Pinzgauer and Cika bulls. Kogel *et al.* (1997) found that Pinzgauer bulls slaughtered at 500 days (16.7 months) had carcass weight of 360.3 kg and net daily gain of 721 g/day, which is higher compared to Cika bulls from A category slaughtered at an average age of 18.6 months. Pinzgauer bulls had 100 kg heavier carcass weight and better net daily gain, which points to the morphological differences between both breeds.

We decided to take a precise look on the conformation and fatness scores of bulls and calves (Table 4), because they represent the highest share of all slaughtered Cika cattle in Slovenia. The majority of bulls under 24 months of age and calves were classified in O conformation class (57.1 and 47.8%). However bulls from B category were slightly more often classified into class R (45.3%) than into class O (41.1%). However, 36.7% of bulls under 24 month of age and 41.8% of calves were classified in class R. The effect of brachycerous origin of Cika cattle and extensive fattening on pasture may be the reason for relatively low conformation scores.

Fatness scores (Table 4) were a little bit different by categories. The majority of calves (88.1%) were classified in fatness class 2; likewise 55.8% of carcasses of bulls under 24 months of age and 49.5% of bulls over 24 months of age. Problems of over fatness (fatness class 4 and 5) did not occur in slaughtered Cika cattle, whereas more than 10% of calves had too low fatness.

Table 4. The conformation and fatness scores for slaughtered bulls of A and B category and calves in the years from 2005 to 2007

Preglednica 4. Ocene za mesnatost in zamaščenost za zaklane bike A in B kategorije ter teleta v letih 2005 do 2007

| | Score<br>Ocena | Bulls under 24 months (A category)<br>Biki stari do 24 mesecev (A kategorija) (n = 197) | | Bulls over 24 months (B category)<br>Biki starejši od 24 mesecev (B kategorija) (n = 95) | | Calves (T category)<br>Teleta (T kategorija) (n = 67) | |
|---|---|---|---|---|---|---|---|
| Conformation<br>Mesnatost | E | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% |
| | U | 4 | 2.0% | 7 | 7.4% | 3 | 4.5% |
| | R | 72 | 36.7% | 43 | 45.3% | 28 | 41.8% |
| | O | 112 | 57.1% | 39 | 41.1% | 32 | 47.8% |
| | P | 8 | 4.1% | 6 | 6.3% | 4 | 6.0% |
| Fatness<br>Zamaščenost | 1 | 7 | 3.6% | 4 | 24.2% | 7 | 10.5% |
| | 2 | 110 | 55.8% | 47 | 49.5% | 59 | 88.1% |
| | 3 | 78 | 39.6% | 41 | 43.2% | 1 | 1.5% |
| | 4 | 2 | 1.0% | 3 | 3.2% | 0 | 0.0% |
| | 5 | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% |

Most of Cika cattle are reared extensively on small farms, mostly on mountain pastures without any additional concentrates. This could present the basis and the opportunity for Cika breeders to promote beef from Cika cattle and to increase their income.

## CONCLUSIONS

The obtained results showed the increased number of slaughtered Cika cattle in the last three years. Great variability in carcass traits of slaughtered Cika cattle, which indicate great heterogeneity also in phenotypic traits, was identified. The population is consisted of light phenotype of Cika cattle with the emphasis on milk production and a bit heavier phenotype of Cika cattle with larger frame and larger body weight very suitable for extensive beef production in cow-calf system on pastures. Nevertheless, autochthonous Cika of both phenotypes is well adapted to the environment and also helps to maintain biodiversity and sustainable agricultural production, especially in areas less suitable for agriculture.

## POVZETEK

Cika je edina avtohtona pasma govedi v Sloveniji, katere populacija je v letu 2007 štela okrog 1600 živali. Rejski cilj za cikasto govedo je kombinirana usmeritev s poudarkom na prireji mleka. Kljub temu cikasto govedo redijo v večini primerov v sistemu krava-tele. V manjši meri pa pasmo še vedno uporabljajo za prirejo mleka v tradicionalnih regijah za planšarstvo. Glavni namen raziskave je bil dobiti vpogled v klavno kakovost cikastega goveda, kakor je bila ovrednotena na klavni liniji. Podatki, uporabljeni v raziskavi, so bili zbrani v klavnicah v letih od

2005 do 2007. Cikasto govedo je v letu 2007 predstavljalo le 0,24 % vsega zaklanega goveda v Sloveniji. Zaklano govedo po kategorijah je bilo zelo raznoliko v masi klavnih polovic, kar je vidno v precej velikih standardnih odklonih. Povprečna masa klavnega trupa kategorije A (biki do starosti 24 mesecev) je bila 260,3 kg. Biki starejši od 24 mesecev (B kategorija) so bili v povprečju težji (322,0 kg), a so imeli manjši dnevni neto prirast v primerjavi z biki v A kategoriji. Masa klavnih polovic zaklanih volov je bila 247,2 kg in je zelo podobna masi klavnih polovic bikov iz A kategorije. Krave do starosti pet let so imele maso klavnih polovic v povprečju 235,6 kg, medtem ko so klavne polovice krav starejših od pet let tehtale v povprečju 257,5 kg. Povprečna masa klavnih polovic telic je bila zelo majhna (193,3 kg) in telet 91,3 kg. Teleta so imela tudi najboljše dnevne neto priraste (706,6 g/dan) izmed vseh kategorij zaklanih živali cikaste pasme. Večina bikov v starosti do 24 mesecev (57,1 %) in telet (47,8 %) je bila razvrščena v O razred za mesnatost. Večina bikov starejših od 24 mesecev (45,3 %) pa je imela ocenjeno mesnatost z R razredom. Večina telet (88,1 %) je bila razvrščena v 2 razred za zamaščenosti in prav tako 55,8 % bikov starih do 24 mesecev in 49,5 % bikov starih nad 24 mesecev. Cikasto govedo pogosto redijo na majhnih kmetijah, ekstenzivno na paši brez dodajanja močnih krmil. Tako je avtohtona cika zelo prilagojena na okolje in pomaga ohraniti biodiverziteto in sonaravno kmetijsko proizvodnjo, še posebno na marginalnih območjih. To bi lahko bila priložnost za rejce cikastega goveda, za promocijo pasme in na tak način prirejenega mesa.

## REFERENCES

Albertí, P./ Panea, B./ Sañudo, C./ Olleta, J.L./ Ripoll, G./ Ertbjerg, P./ Christensen, M./ Gigli, S./ Failla, S./ Concetti, S./ Hocquette, J.F./ Jailler, R./ Rudel, S./ Renand, G./ Nute, G.R./ Richardson, R.I./ Williams, J.L. Live weight, body size and carcass characteristic of young bulls of fifteen European breeds. Livestock Science, 114(2008), 19–30.

Gil, M./ Serra, X./ Gispert, M./ Oliver, M.A./ Sañudo, C./ Panea, B./ Olleta, J.L./ Campo, M./ Oliván, M./ Osoro, K./ Garcia-Cachán, M.D./ Cruz-Sagredo, R./ Izquierdo, M./ Espejo, M./ Martín, M./ Piedrafita, J. The effect of breed-production systems on the myosin heavy chain 1, the biochemical characteristics and the colour variables of Longissimus thoracis from seven Spanish beef cattle breeds. Meat Science, 58(2001), 181–188.

Kastelic, M./ Žan Lotrič, M./ Kompan, D. Linear body measurements of Cika cattle in comparison to Pinzgauer cattle. *Acta agriculturae Slovenica*, 86(2005), 85–91.

Kogel, J./ Reinsch, N./ Kustermann, W./ Eichinger, H./ Thaller, G./ Pirchner, F. Beef performance of the numerically endangered Bavarian cattle breeds. 1st communication: fattening performance and carcass quality. Zuchtungskunde, 69(1997)4, 244–253.

Petrič, P. Učinki gospodarskega križanja rjave pasme z različnimi mesnimi pasmami na klavne lastnosti. Diplomsko delo. Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za zootehniko, 2008. 47 p.

Pravilnik o izvajanju uredb Sveta (EGS) in uredb Komisije (EGS) o razvrščanju trupov goveda. Uradni list RS, št. 120/2005. (29.12.2005)

Žgur, S./ Čepon, M./ Kovač, M./ Malovrh, Š./ Petrič, N./ Kovačič, K./ Pavlin, S./ Ule, I. Rezultati ocenjevanja govejih trupov in polovic na klavni liniji v letu 2007. Domžale, Biotehniška fakulteta, Oddelek za zootehniko, 2008, 35 p.

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# GENETIC PARAMETERS FOR GROWTH IN CHAROLAIS CALVES

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## ABSTRACT

Genetic parameters for birth weight (BW), weight at the beginning (WB), in the middle (WM), and at the end of grazing season (WE), as well as weight at the age of one year (WY) were estimated. Data were collected on 319 Charolais calves. The total number of records, including pedigree data (parents and grandparents) was 377 animals. Variance and covariance components were estimated by REML method using the VCE-5 package. The effects of sex, parity and year of birth were included in the models for all traits. Age of calves at the beginning of grazing season was included as linear regression in models for all traits except for birth weight. The age of calves in the middle, at the end of grazing season, and age at approximately one year were included as linear regression in the models for corresponding weights. Direct additive genetic effect was included in models for all traits as random effect. Estimated heritabilities for BW, WB, WM, WE and WY were 0.62 ± 0.06, 0.23 ± 0.09, 0.35 ± 0.08, 0.29 ± 0.07 and 0.23 ± 0.07, respectively.

Key words: cattle / calves / breeds / Charolais / genetic parameters / body weight / variance / heritability / genetic correlation

# GENETSKI PARAMETRI ZA RAST PRI TELETIH ŠAROLE PASME

## IZVLEČEK

Ocenjeni so bili genetski parametri za rojstno maso (BW), telesno maso na začetku (WB), sredini (WM) in na koncu pašne sezone (WE) ter ob starosti enega leta (WY). Podatki so bili zbrani na 319 teletih šarole pasme. Skupno število zapisov vključno s podatki o poreklu (starši in stari starši) je bilo 377 živali. Komponente varianc in kovarianc so bile ocenjene z metodo REML in uporabo VCE-5 paketa. Vplivi spola, zaporedne telitve in leta rojstva so bili vključeni v modele za vse proučevane lastnosti. Starost telet na začetku paše je bila kot linearna regresija vključena v modele za vse lastnosti razen v model za rojstno maso. Starost telet na sredini in na koncu paše ter starost ob približno enem letu pa je bila kot linearna regresija vključena v modele za pripadajoče telesne mase. Direktni aditivni genetski vpliv je bil vključen v modele za vse lastnosti kot naključni vpliv. Ocenjene heritabilitete za BW, WB, WM, WE in WY so bile 0,62 ± 0,06; 0,23 ± 0,09; 0,35 ± 0,08; 0,29 ± 0,07 in 0,23 ± 0,07.

Ključne besede: govedo / teleta / pasme / šarole / genetski parametri / telesna masa / varianca / heritabiliteta / genetska korelacija

## INTRODUCTION

Ratio between dairy and suckler cows has been changed since the introduction of milk quotas in EU and consequently in Slovenia, too. In Slovenia, suckler cows represent about 40% of the total cow population and the percentage is still increasing. The most widespread breed in suckler herds is locally adapted Simmental cattle. Slovenian Brown cattle are used as suckler cows in a few herds only. Recently, an increased number of purebred beef cattle, like Charolais and Limousine, are reared in cow-calf system on pastures. The first purebred Charolais animals were imported to Slovenia from France in 1965. Lately, small groups of Charolais cattle and semen of sires were imported, too. In the last five years beef recording and selection of purebred beef breeds is getting more intensive. The semen of the best sires is still imported from France to prevent inbreeding, and to achieve faster genetic progress in purebred Charolais herds.

A number of studies have been done in different parts of the world on larger populations of Charolais cattle included. Heritabilities for weaning weight were reported to range between 0.13 and 0.33 (Phocas and Laloë, 2004; Donoghue and Bertrand, 2004; Crews *et al*., 2004; Bennett and Gregory, 1996; Duangjinda *et al*., 2001) while heritability for weight at one year was 0.34 (Bennett and Gregory, 1996). On the other hand in Slovenia, only one study has reported genetic parameters for Charolais and Limousine calves has been performed so far (Simčič *et al.*, 2006). According to the well known fact that genetic evaluation is useful if genetic and non-genetic parameters for each population are estimated, we have decided to estimate genetic parameters for Charolais calves in one of Slovene herds for the start.

The aim of this study was to estimate the genetic parameters for weaning weight (weight at the end of grazing season) and weight at one year of age in Charolais calves. Birth weight, weight at the beginning and weight in the middle of grazing season were also analysed.

## MATERIAL AND METHODS

### Material

Data was collected on 319 Charolais calves (171 males and 148 females) reared at the Educational and Research Animal Husbandry Centre Logatec (Slovenia). Calves were born in years 1995 to 2005 in late winter or spring calving season from January to June. During grazing season, cows and calves had no additional concentrate on pasture, except mineral-vitamin mixture fed *ad libitum*. The average grazing season lasted from the beginning of May to the end of October. The herd was on all-day grazing at 470 m above the sea level on a karst plateau, with short vegetation period. Moreover the plateau is located on the passage of mild Mediterranean and cold Alpine climate. The amount of rainfall differs among years. The end of grazing season coincided with the weaning period/time. Weaned bulls were housed in performance test unit until the age of one year. Weaned heifers were housed during winter period and next spring, at the average age of one year, they were put on pasture.

Besides animal measurements, pedigree file included sires, dams and grandparents, altogether 377 animals. In the analysed period, 24 sires had progeny in the herd. Sires in natural mating had more progeny than AI sires.

In the study, birth weight (BW), body weight at the beginning of grazing season (WB), body weight in the middle of grazing season (WM), body weight at the end of grazing season (WE), as well as body weight at the average age of one year (WY) were analysed. On average calves were weighed five times: at birth, three times during grazing season (beginning, middle, the end) and at the approximate age of one year (Table 1).

Table 1. Descriptive statistics for birth weight (kg) and body weights (kg) up to one year
Preglednica 1. Opisna statistika za rojstno maso (kg) in telesne mase (kg) do enega leta starosti

| | Sex / Spol | Male / Biki | Female / Telice |
|---|---|---|---|
| Birth<br>Rojstvo | n | 171 | 148 |
| | BW ± SD, kg | 47.7 ± 6.6 | 46.0 ± 6.1 |
| Beginning of grazing season<br>Začetek paše | n | 146 | 123 |
| | WB ± SD, kg | 95.9 ± 25.4 | 91.7 ± 26.3 |
| | Age / Starost ± SD, days / dni | 59.5 ± 24.0 | 57.5 ± 24.37 |
| Middle of grazing season<br>Sredina paše | n | 135 | 110 |
| | WM ± SD, kg | 201.7 ± 48.6 | 194.6 ± 47.5 |
| | Age / Starost ± SD, days / dni | 144.4 ± 30.4 | 147.8 ± 31.8 |
| End of grazing season<br>Konec paše | n | 144 | 124 |
| | WE ± SD, kg | 270.6 ± 51.9 | 251.4 ± 52.7 |
| | Age / Starost ± SD, days / dni | 204.5 ± 30.9 | 202.1 ± 34.8 |
| Average age of one year<br>Povprečna starost eno leto | n | 114 | 88 |
| | WY ± SD, kg | 453.3 ± 54.9 | 371.0 ± 52.2 |
| | Age / Starost ± SD, days / dni | 358.33 ± 10.6 | 403.7 ± 52.0 |

BW – birth weight / rojstna masa; WB – body weight at the beginning of grazing season / telesna masa ob začetku paše; WM – body weight in the middle of grazing season / telesna masa na sredini paše; WE – body weight at the end of grazing season / telesna masa na koncu paše; WY – body weight at the age of one year / telesna masa ob starosti enega leta; n – number of calves / število telet; SD – standard deviation / standardni odklon

## Methods

Variance and covariance components were estimated by REML procedures using the VCE-5 package (Kovač *et al.*, 2002). The fixed effects in the model [1] differed among traits. The effects of sex, parity, and year of birth were included in models for all traits. Age of calves at the beginning of grazing season was included as linear regression in models for all traits except for the birth weight. Age of calves in the middle, at the end of grazing season, and age at one year were included as linear regression in the models for corresponding weights. Direct additive genetic effect was treated as random effect in the models for all traits.

$$\mathbf{y} = \mathbf{X\beta} + \mathbf{Zu} + \mathbf{e} \quad [1]$$

$$E(\mathbf{y}) = \mathbf{X\beta} \quad [2]$$

$$\text{var}(\mathbf{y}) = \mathbf{V} = \mathbf{ZGZ'} + \mathbf{R} \quad [3]$$

$$\text{var}(\mathbf{u}) = \mathbf{G} = \mathbf{A} \otimes \mathbf{G_0} \quad [4]$$

$$\text{var}(\mathbf{e}) = \mathbf{R} = \sum^{\otimes} \mathbf{R_{0i}} \quad [5]$$

Where:

**y** = vector of observations
**X** = incidence matrix for fixed effects
**β** = vector of parameters for fixed effects
**Z** = incidence matrix for random effects
**u** = vector of random direct additive genetic effects
**e** = residual vector
**V** = phenotypic matrix of variances and covariances
**G** = matrix for variances and covariances of additive genetic effect
$\mathbf{G_0}$ = matrix for variances and covariances of genetic effect among five traits
**A** = relationship matrix
**R** = matrix for residual variances and covariances
$\mathbf{R_0}$ = matrix for residual variances and covariances among traits

Expected value for observations [2] was equal to the fixed part of the model. Phenotypic matrix of variances and covariances had two components [3] – additive genetic and residual variance. Matrix with genetic variances and covariances [4] was a Kronecker product between the relationship matrix and matrix of genetic effect among five traits. Covariance matrix of residuals [5] was block-diagonal matrix, where matrices $R_{0i}$ were different due to missing measurements for same weights. We assumed the residuals to be identical, independent and normally distributed. Covariances between genetic and environmental effects were assumed to be zero and no variances due to dominance or epistatic effect were assumed to exist.

## RESULTS AND DISCUSSION

For the first time genetic variances, covariances and heritabilities were estimated for Charolais population in Slovenia. Although the herd was small in comparison with literature data, genetic parameters belonged to the animals in Slovenian environmental conditions. Genetic variances of BW, WB, WM, WE and WY were 21.78 $kg^2$, 56.42 $kg^2$, 303.23 $kg^2$, 365.47 $kg^2$ and 457.13 $kg^2$, respectively (Table 2). Bennett and Gregory (1996) found lower genetic variance for BW (13.12 $kg^2$) and for weight on 200 days (106.86 $kg^2$) on Charolais calves. Covariance between BW and weight on 200 days was also estimated by Bennett and Gregory (1996) and was lower (14.42 $kg^2$) compared to covariance between BW and WE (57.68 $kg^2$) in our study. Crews *et al.* (2004) also found lower genetic variance of BW and at 205 days weight for Canadian Charolais cattle, which was 11.02 $kg^2$ and 188.80 $kg^2$, respectively. Genetic and phenotypic standard deviations (SD) of our research were computed for easier interpretation and comparison with literature. Genetic standard deviations were 4.67 kg, 7.51 kg, 17.41 kg, 19.12 kg and 21.38 kg for BW, WB, WM, WE and WY, respectively. Lower genetic standard deviation for BW (2.45 kg) had Charolais calves reared in Czech Republic (Jakubec *et al.*, 2003), who also had lower genetic SD (16.53 kg) for weight at 210 days, compared to SD for WE. Říha *et al.* (2001) reported of lower SD (16.00 kg) for weight at 120 days in comparison with SD for WM.

Table 2. Genetic variances, phenotypic variances, genetic covariances (above diagonal) and phenotypic covariances (below diagonal) among BW, WB, WM, WE and WY

Preglednica 2. Genetske variance, fenotipske variance, genetske kovariance (nad diagonalo) in fenotipske kovariance (pod diagonalo) med BW, WB, WM, WE in WY

| | $\sigma_g^2$, $kg^2$ | $\sigma_{ph}^2$, $kg^2$ | Covariance / Kovariance, $kg^2$ | | | | |
|---|---|---|---|---|---|---|---|
| | | | BW | WB | WM | WE | WY |
| BW | 21.78 | 35.29 | | 14.98 | 35.21 | 57.68 | 63.47 |
| WB | 56.42 | 240.41 | 37.26 | | 83.94 | 94.68 | 55.70 |
| WM | 303.23 | 866.34 | 52.44 | 295.19 | | 322.13 | 331.40 |
| WE | 365.47 | 1280.21 | 66.37 | 328.04 | 880.60 | | 381.38 |
| WY | 457.13 | 1952.64 | 78.02 | 332.00 | 828.17 | 1131.08 | |

$\sigma_g^2$ – genetic variance / genetska varianca, $\sigma_{ph}^2$ – phenotypic variance / fenotipska varianca, BW – birth weight / rojstna masa; WB – body weight at the beginning of grazing season / telesna masa ob začetku paše; WM – body weight in the middle of grazing season / telesna masa na sredini paše; WE – body weight at the end of grazing season / telesna masa na koncu paše; WY – body weight at the age of one year / telesna masa ob starosti enega leta

Phenotypic variance of BW, WB, WM, WE and WY were 35.29 $kg^2$, 240.41 $kg^2$, 866.34 $kg^2$, 1280.21 $kg^2$ and 1952.64 $kg^2$, respectively (Table 2). Phocas and Laloë (2004) estimated lower

phenotypic variances for BW (20.0 $kg^2$) and for weaning weight (1141 $kg^2$) in Charolais calves reared in France. In a large study, Donoghue and Bertrand (2004) compared phenotypic variance for BW and for weaning weight of Charolais calves reared in four countries. Phenotypic variance for BW and weaning weight were 18.24 $kg^2$ and 686.58 $kg^2$ for calves in Australia, 23.08 $kg^2$ and 838.94 $kg^2$ for calves in Canada, 18.05 $kg^2$ and 721.25 $kg^2$ for calves in the USA, and 25.90 $kg^2$ and 930.74 $kg^2$ for calves reared in New Zealand. However, phenotypic variances for BW and weaning weight in herds from different countries shown, that we estimated higher values for phenotypic variances. Phenotypic variance for BW (20.95 $kg^2$) and weaning weight (837.50 $kg^2$) in Charolais calves in Canada were also estimated by Crews *et al.* (2004). The only suitable explanation could be environmental conditions.

Phenotypic standard deviations in Charolais calves of our study were computed from phenotypic variance and were 5.94 kg, 15.50 kg, 29.43 kg, 35.78 kg and 44.19 kg for BW, WB, WM, WE, and WY, respectively. Říha *et al.* (2001) reported of lower SD for BW (4.92 kg), but higher SD for weight at 120 days (32.00 kg) and weight at 210 days (43.00 kg) in Charolais calves reared in Czech Republic, compared to SD for WM and WE. On the other hand, Jakubec *et al.* (2003) reported of SD for BW (4.90 kg), weight at 210 days (33.05 kg) and weight at 365 days (52.73 kg) in Charolais calves also reared in Czech Republic.

Heritabilities estimated in this study were between 0.23 and 0.62 (Table 3). The highest heritability was estimated for BW. Phocas and Laloë (2004) estimated lower heritability for BW (0.33) and for weaning weight (0.13) in Charolais calves. Their research included much larger number of calves from 236 herds compared to our 319 calves from one herd. This could be one of the reasons for the difference in estimated heritability. On the other hand, Bennett and Gregory (1996) reported heritability 0.43 for BW, 0.16 for 200-day weight and, 0.34 for 368-day weight in Charolais calves. Only the heritability for weight at 368 days (0.34) was higher than heritability for WY (0.23) in our study. Donoghue and Bertrand (2004) compared heritabilities for BW and weaning weight of Charolais calves reared in four countries. Heritability for BW and weaning weight were 0.34 and 0.22 for calves in Australia, 0.55 and 0.27 for calves in Canada, 0.47 and 0.25 for calves in the USA, and 0.31 and 0.21 for calves reared in New Zealand. Similar to our results, heritability for BW (0.62) and weaning weight (0.29) had calves in Canada (0.55 for BW) and (0.27 for weaning weight). Crews *et al.* (2004) also estimated similar heritability for BW (0.53) and for weaning weight (0.23) for Canadian Charolais calves compared to our estimation.

Table 3. Heritabilities (on the diagonal) for BW, WB, WM, WE, WY, genetic correlations (above diagonal) and phenotypic correlations (below diagonal) among them with standard errors

Preglednica 3. Heritabilitete (na diagonali) za BW, WB, WM, WE in WY in genetske (nad diagonalo) ter fenotipske korelacije (pod diagonalo) med njimi s standardnimi napakami

| | BW | WB | WM | WE | WY |
|---|---|---|---|---|---|
| BW | 0.62 ± 0.06 | 0.43 ± 0.20 | 0.43 ± 0.15 | 0.65 ± 0.14 | 0.64 ± 0.14 |
| WB | 0.40 | 0.23 ± 0.09 | 0.64 ± 0.15 | 0.66 ± 0.15 | 0.35 ± 0.25 |
| WM | 0.30 | 0.65 | 0.35 ± 0.08 | 0.97 ± 0.03 | 0.89 ± 0.08 |
| WE | 0.31 | 0.59 | 0.84 | 0.29 ± 0.07 | 0.93 ± 0.07 |
| WY | 0.30 | 0.48 | 0.64 | 0.72 | 0.23 ± 0.07 |

BW – birth weight / rojstna masa; WB – body weight at the beginning of grazing season / telesna masa ob začetku paše; WM – body weight in the middle of grazing season / telesna masa na sredini paše; WE – body weight at the end of grazing season / telesna masa na koncu paše; WY – body weight at the age of one year / telesna masa ob starosti enega leta

However, Duangjinda *et al.* (2001) estimated heritability for weaning weight (0.33) for Charolais calves included in American International Charolais Association. Although different method for heritability estimation was used, the results were very similar to heritability for WE (0.29) estimated in our study.

Genetic correlations among weights were positive and ranged from 0.35 to 0.97 (Table 3). On the other hand, phenotypic correlations ranged between 0.30 and 0.84. The lowest genetic correlation (0.35) was between WB and WY, but the highest one (0.97) was between WM and WE. That genetic correlation showed, that we would quite good estimate WE with WM. BW was more highly correlated with WE and WY (0.65, 0.64) than with WB and WM (0.43 for both). Estimated correlation between BW and WE was 0.65, and was higher compared to the correlation between BW and weaning weight (0.39) estimated by Phocas and Laloë (2004). Crews *et al.* (2004) also found lower genetic correlation (0.33) between BW and weaning weight.

## CONCLUSIONS

Genetic parameters for body weights of Charolais calves were estimated for the first time in Slovenia. Data used in our study was collected for a ten year period in a herd reared on the Educational and Research Animal Husbandry Centre Logatec. Genetic and phenotypic variances for body weights estimated in our study were higher compared to literature, because the herd was genetically very heterogeneous and very suitable for intensive selection. The herd was included in a suckler herd recording scheme. With the intention to promote genetic progress, each year, semen of the best sires is imported from France for artificial insemination of best cows in the herd. Higher phenotypic variances could be explained with very changeable environmental conditions in Logatec. It was difficult to successfully adapt the management of the herd to changes in environmental conditions. However, heritabilities for birth weight and for weaning weight were similar or slightly higher to those reported in literature. They might be higher, because of high genetic variances for body weights of genetically very variable calves.

## POVZETEK

Krave dojilje predstavljajo v Sloveniji 40 % staleža vseh krav. Najbolj razširjena pasma krav v čredah dojilj je lisasta, v manjši meri pa slovenska rjava. V zadnjem času se povečuje tudi število čistopasemskih čred krav dojilj mesnih pasem šarole in limuzin. Prve živali pasme šarole so bile v Slovenijo uvožene iz Francije v letu 1965. Kontrola in selekcija v čredah pasme šarole postaja vedno bolj intenzivna. Z namenom preprečevanja inbridinga in za doseganje hitrejšega genetskega napredka v čistopasemskih čredah se za osemenjevanje uporablja seme najboljših bikov šarole pasme iz Francije.

V raziskavi smo proučili rast pri teletih šarole pasme. Ocenili smo genetske parametre za rojstno maso, telesno maso na začetku, sredini in koncu paše ter ob starosti enega leta. Podatke smo zbrali od 319 šarole telet (171 ♂, 148 ♀) vzrejenih na Pedagoško – raziskovalnem centru za živinorejo Logatec. Teleta so bila rojena v letih 1995 do 2005 v zimsko-pomladanski telitveni sezoni. Paša je v povprečju trajala od maja do oktobra. Konec paše je sovpadal z odstavitvijo. Zapisi o poreklu so vključevali starše in stare starše telet, skupaj 377 živali.

Komponente varianc in kovarianc so bile ocenjene z REML proceduro s paketom VCE-5. Sistematski vplivi v modelu 1 so se razlikovali glede na lastnost. Vplivi spola, zaporedne telitve in leta rojstva so bili vključeni v model za vse lastnosti. Starost telet na začetku paše je bila vključena kot linearna regresija v modele za vse lastnosti z izjemo modela za rojstno maso. Starost telet na sredini in na koncu paše ter ob enem letu je bila kot linearna regresija vključena v

modele za pripadajoče telesne mase. Direktni aditivni genetski vpliv je bil vključen kot naključni vpliv v modele za vse lastnosti.

Variance, kovariance in heritabilitete za telesne mase so bile s to raziskavo prvič ocenjene za teleta šarole pasme v Sloveniji. Genetske in fenotipske variance za telesne mase ocenjene v naši raziskavi (pregl. 2) so bile večje v primerjavi s podatki iz literature. Vzrok je v genetsko zelo raznoliki čredi, ki je zelo primerna za intenzivno selekcijo. Velike fenotipske variance bi lahko razložili z zelo spremenljivimi vplivi okolja v Logatcu. Ocenjene heritabilitete za telesne mase so bile med 0,23 in 0,62 (pregl. 3). Največja je bila za rojstno maso. Tudi heritabilitete so bile večje v primerjavi s podatki iz literature. Eden izmed vzrokov bi lahko bilo večje število čred z večjim številom telet v čredah vključenih v podobne raziskave v literaturi v primerjavi z eno čredo v naši raziskavi. Genetske korelacije med telesnimi masami so bile pozitivne, v rangu od 0,35 do 0,97.

## REFERENCE

Bennett, G.L./ Gregory, K.E. Genetic (co)variances among birth weight, 200-day weight, and postweaning gain in composites and parental breeds of beef cattle. J. Anim. Sci., 74(1996), 2598–2611.

Crews, D.H.Jr./ Lowerison, M./ Caron, N./ Kemp, R.A. Genetic parameters among growth and carcass traits of Canadian Charolais cattle. Can. J. Anim. Sci., 84(2004), 589–597.

Donoghue, K.A./ Bertrand, J.K. Investigation of genotype by country interactions for growth traits for Charolais populations in Australia, Canada, New Zealand and USA. Livest. Prod. Sci., 85(2004), 129–137.

Duangjinda, M./ Bertrand, J.K./ Misztal, I./ Druet, T. Estimation of additive and nonaditive genetic variances in Hereford, Gelbvieh, and Charolais by Method R. J. Anim. Sci., 79(2001), 2997–3001.

Jakubec, V./ Schlote, W./ Říha, J./ Majzlík, I. Comparison of growth traits of eight beef cattle breeds in the Czech Republic. Arch. Tierzucht., 46(2003)2, 143 – 153.

Kovač, M./ Groeneveld, E./ Garciá-Cortés, L.A. VCE-5, a package for the estimation of dispersion parameters. In: Proceedings of the 7th World Congress on Genetics Applied to Livestock Production, Montpellier, 2002-8-19/23. Castanet-Tolosan, INRA, cop, 33(2002), 741–742.

Phocas, F./ Laloë, D. Genetic parameters for birth and weaning traits in French specialized beef cattle breeds. Livest. Prod. Sci., 89(2004), 121–128.

Říha, J./ Jakubec, V./ Golda, J./ Majzlík, I. Comparison of preweaning growth traits of six beef cattle breeds in the Czech Republic. Czech J. Anim. Sci., 46(2001)4, 152–158.

Simčič, M./ Malovrh, Š./ Čepon, M. Different parameters affecting body weights of Charolais and Limousine calves from birth to weaning. Acta Agraria Kaposváriensis, 10(2006)2, 127–133.

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# CALCULATION OF PRP GENOTYPE AND NSP TYPE PROBABILITIES IN SLOVENIAN SHEEP

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## ABSTRACT

The PrP genotype probabilities in non genotyped Slovenian sheep were calculated. Altogether 36 083 ewes and rams of various breeds were included into analysis. The PrP genotype was known for 10 504 animals. Five different PrP alleles were present in the data set. Pedigree and genotype data structure differed between breeds. Iterative allelic peeling with incomplete penetrance model was used for the calculation of genotype probabilities for each animal given the genotype data of relatives. Analyses were performed for each breed separately. Additionally, NSP (National Scrapie Plan) type probabilities and the average NSP value were calculated from the genotype probabilities. Results were presented for live animals only. There were no animals with additionally identified PrP genotype or NSP type with certainty. The PrP genotype was additionally identified with 95% probability for 0.0 to 5.7% animals of different breeds. NSP type was additionally identified with the same probability for 0.0 to 34.9% animals of different breeds. We assume that the low number of additional identifications was due to: a large number of alleles, intermediate allele frequencies, data structure, a uniform prior, and the use of incomplete penetrance model. Additional identifications provided some cost savings, but did not prove useful in the selection for scrapie resistance of the entire population. The average NSP value should be used instead, since it can be calculated for all animals and encompasses all information from genotype probabilities.

Key words: sheep / genotype probabilities / PrP genotype / NSP type / scrapie / Slovenia

# IZRAČUN VERJETNOSTI GENOTIPOV PrP IN SKUPIN NSP PRI OVCAH V SLOVENIJI

## IZVLEČEK

Izračunali smo verjetnosti genotipov PrP za negenotipizirane ovce v Sloveniji. V analizo smo zajeli 36 083 ovc in ovnov različnih pasem. Genotip PrP je bil znan za 10 504 živalih. V podatkih je bilo prisotnih pet različnih alelov PrP. Struktura porekla in podatkov o genotipu PrP se je med pasmami razlikovala. Z metodo alelnega lupljenja in modelom nepopolne penetrance, smo na podlagi genotipa sorodnikov iterativno izračunali verjetnosti genotipov PrP za vse živali. Analize smo opravili za vsako pasmo posebej. Iz verjetnosti genotipov smo preračunali verjetnosti za skupine NSP (National Scrapie Plan) ter iz teh povprečno vrednost NSP. Rezultate smo predstavili le na živih živalih. Prav nobeni živali nismo uspeli z gotovostjo dodatno določiti genotipa PrP ali skupine NSP. Za 0,0 do 5,7 % živali različnih pasem smo lahko dodatno določili genotip PrP s 95 % verjetnostjo, medtem ko smo lahko z enako verjetnostjo dodatno določili skupino NSP za 0,0 do 34,9 % živali različnih pasem. Menimo, da so vzroki za majhno število dodatnih določitev genotipa PrP sledeči: veliko število alelov, intermediarne frekvence alelov,

struktura podatkov, uniformna apriorna verjetnost in uporaba modela nepopolne penetrance. Dodatne določitve genotipa PrP in skupine NSP predstavljajo prihranek za rejski program. Zaradi majhnega števila dodatnih določitev metoda ni uporabna za selekcijo celotnih populacij na odpornost proti praskavcu. V ta namen lahko uporabimo povprečno vrednost NSP, saj ta parameter združuje vse verjetnosti genotipov PrP in ga lahko izračunamo za vse živali.

Ključne besede: ovce / verjetnosti genotipov / genotip PrP / skupina NSP / praskavec / Slovenija

## INTRODCTION

Scrapie is a transmissible spongiform encephalopathy (TSE) disease of sheep, like BSE in cattle. In the past, scrapie was not known in sheep in Slovenia, but in the last few years some cases have been identified. Transmissible spongiform encephalopathies are a group of diseases, where a changed form of prion protein accumulates in the central nervous system (Prusiner, 1998). The primary cause of these diseases has not been discovered yet. Research results have shown that in the same flock only some animals die due to scrapie, while others do not. This led to the detection of PrP genotype (also marked as PrnP), which plays a major role in genetic predisposition for susceptibility to scrapie (Hunter, 1997). Differences at codons 136, 154 and 171 codon of the PrP gene are strongly associated with the resistance or susceptibility to scrapie and with the age of the disease onset (Hunter, 1997). Because the mentioned codons are very close, the polymorphisms in these three codons (haplotypes) are often reported as an allele named by the initial letters of amino acids, such as A136R154R171, or just ARR. The most frequent alleles are: ARR, AHQ, ARH, ARQ and VRQ. New alleles are still being discovered (Lühken *et al.*, 2008; Ulvund, 2008). Allele ARR is associated with the highest resistance to scrapie, while allele VRQ is associated with the highest susceptibility to scrapie infection (Hunter, 1997). PrP genotypes are grouped into 5 groups based on scrapie susceptibility. These groups are usually called NSP types after National Scrapie Plan in UK (Dawson *et al.*, 2008). NSP types (Table 1) follow from 1 (the highest resistance) to 5 (the highest susceptibility). Ulvund (2008) provides a recent review about scrapie in sheep.

Table 1. Classification of PrP genotypes by NSP type (Dawson *et al.*, 2008)
Preglednica 1. Razvrstitev genotipov PrP v skupine NSP (Dawson in sod., 2008)

| NSP type<br>Skupina NSP | PrP genotype<br>Genotip PrP |
|---|---|
| 1 | ARR/ARR |
| 2 | ARR/ARQ, ARR/AHQ, ARR/ARH |
| 3 | AHQ/AHQ, AHQ/ARH, AHQ/ARQ, ARH/ARH, ARH/ARQ, ARQ/ARQ |
| 4 | ARR/VRQ |
| 5 | AHQ/VRQ, ARH/VRQ, ARQ/VRQ, VRQ/VRQ |

Scrapie in sheep has been known for more than 250 years, with no evidence of harmful effects on humans so far. The European Commission has accepted the regulation 2003/100/EC (European Commission, 2003), due to the incidence of BSE in cattle, possible spontaneous transmission of scrapie from sheep to cattle. At that time there was also a lack of tools to distinguish between BSE and scrapie in sheep. The regulation requires the acceptance of selection programme for increasing scrapie resistance in sheep in all member states. The aim of the programme is the elimination of the VRQ allele and the increase of the ARR allele

frequency. In Slovenia, such programme was initiated in the year 2005. The programme is based on the selection of performance recorded rams and ewes genotyped for the PrP gene.

Both, genotyping as well as tissue collection represent a considerable cost for the breeding programme. However, the genotype of an individual can be (partially) determined from the genotype of his relatives, given that the pedigree and genotype data are accurate. If the parents are homozygous, the genotype of an offspring can be determined with certainty, i.e., parents with ARQ/ARQ and ARR/ARR genotypes will definitely have offspring with ARR/ARQ genotype, given that there are no mutations or data errors. Definite genotype identification from the genotypes of relatives is possible only in special cases. However, genotype probabilities for the individual can be calculated from the genotype data collected on relatives (e.g. Jacquard, 1974). Elston and Stewart (1971) developed a general method for the calculation of genotype probabilities. They used their method in the context of human genetics. Livestock pedigrees are usually much larger and more complex due to inbreeding, multiple matings, and a larger number of offspring per parent (see Cannings *et al.*, 1978). Several methods were developed to handle these complexities (e.g. Van Arendonk *et al.*, 1989; Janss *et al.* 1995; Kerr and Kinghorn, 1996; Fernández *et al.*, 2001; Thallman *et al.*, 2001a; Henshall and Tier, 2003; Gengler *et al.*, 2007). The aim of this paper is to present the results of the calculation of PrP genotype probabilities and NSP type probabilities in Slovenian sheep using allelic peeling method (Thallman *et al.*, 2001a) as proposed by Gorjanc and Kompan (2008).

## MATERIAL AND METHODS

### Material

Seven breeds from the Slovenian sheep breeding programme were included in the analysis: Jezersko-Solcava (JS), Improved Jezersko-Solcava (JSR), Bela Krajina Pramenka (BP), Texel (T), Bovec (B), Improved Bovec (VFB) and Istrian Pramenka (IP). Data on PrP genotype were collected in the frame of the selection programme for TSE (scrapie) resistance in Slovenia. More than 10 000 ewes and rams (Table 2) were genotyped (at codons 136, 154 and 171 of the PrP gene) by the end of the year 2007.

Pedigree data was also included into the analysis to provide relationship information. Initially, the complete pedigree for each breed was obtained, considering all living and dead animals registered in the herdbook. Some animals did not contribute information for the calculation of genotype probabilities due to missing genotype data or non informative links within the pedigree. Non-informative animals were excluded (pruned) iteratively generation by generation in the direction from ancestors to descendants. The following criteria had to be met for the exclusion of an animal: known date of death or culling, only one offspring, and no PrP genotype data. All the data (PrP genotype data and pedigree) were obtained from the sheep and goat database of the Centre for extension service at the Animal Science Department of Biotechnical Faculty.

The largest pedigree was obtained for JSR breed (15 054; Table 2), followed by JS breed with 10 429 animals and B breed with 5101 animals. Pedigrees of other breeds were smaller, according to the size of breed populations in Slovenia. The highest percentage of genotyped animals was in BP breed (61.2%; Table 2). The percentage of genotyped animals was also high in IP (43.9%), B (36.5%), and JS breed (35.2%). In other breeds the percentage of genotyped animals was lower than 30%. Animals with many relatives are very informative for the calculation of genotype probabilities. The highest percentage of genotyped sires was in IP breed (41.0%). Almost every third sire was also genotyped in JS (28.7%) and BP (27.0%) breed. The percentage of genotyped dams was generally much higher than for sires (Table 2). However,

dams of T breed mostly did not have PrP genotype data, because tissue collection programme has not been introduced yet in the flocks of T breed. The highest number of live non-genotyped animals was in the most numerous breed, the JSR breed (3795 animals; Table 2). JS breed followed with 2673 live non-genotyped animals. However, JS breed had much narrower (better) ratio (0.7 for JS and 1.4 for JSR breed) between the number of live non-genotyped animals and the number of all genotyped animals. In other breeds the number of non-genotyped animals was lower and the ratio did not exceed the value of 0.3, with the exception of T breed (Table 2).

Table 2. Data structure
Preglednica 2. Struktura podatkov

| Breed<br>Pasma | Animals<br>Živali | Known genotype, %<br>Znan genotip, % | | | Number of live non-genotyped animals<br>Št. živih negenotipiziranih živali | Ratio[1]<br>Razmerje[1] |
|---|---|---|---|---|---|---|
| | | Animals<br>Živali | Sires<br>Očetje | Dams<br>Matere | | |
| JS | 10 429 | 35.2 | 28.7 | 40.9 | 2673 | 0.7 |
| JSR | 15 054 | 18.4 | 11.8 | 20.0 | 3795 | 1.4 |
| BP | 1297 | 61.2 | 27.0 | 70.0 | 159 | 0.2 |
| T | 555 | 16.8 | 15.4 | 1.0 | 189 | 2.0 |
| B | 5101 | 36.5 | 12.8 | 42.4 | 522 | 0.3 |
| VFB | 1711 | 26.7 | 8.9 | 23.8 | 128 | 0.2 |
| IP | 1936 | 43.9 | 41.0 | 48.7 | 263 | 0.3 |

JS – Jezersko-Solcava; JSR – Improved Jezersko-Solcava; BP – Bela Krajina pramenka; T – Texel; B – Bovec; VFB – Improved Bovec; IP – Istrian pramenka; [1] Ratio between the number of live non-genotyped animals and the number of all genotyped animals / [1] Razmerje med številom živih negenotipiziranih živali in vseh genotipiziranih živali

## Methods

The presented PrP genotype data and pedigree data were used in the calculation of PrP genotype probabilities for the non-genotyped animals. These calculations are based on probability laws of gene segregation given the known genotype data and pedigree structure. We used the allelic peeling method (Thallman *et al.*, 2001a) as implemented in the GenoProb programme (Thallman, 2002), where genotype probabilities of an individual animal are calculated as a function of genotype probabilities in ancestors and offspring. A uniform prior distribution was used for allele probabilities in founders (animals with unknown ancestors). Exact calculations were not possible, due to complex pedigrees. We used the iterative procedure as presented by Thallman *et al.* (2001b). Exploratory runs revealed that 100 iterations were enough to achieve convergence in all breeds, except in BP breed where around 250 iterations were needed.

Pedigree and genotype data might contain errors due to various reasons. We used the incomplete penetrance model to enable the use of all data (Thallman *et al.*, 2001b). With incomplete penetrance model the observed genotype data was conceptually treated as "phenotype" data (Lincoln and Lander, 1992). Inconsistencies between "phenotype" data and calculated genotype probabilities were governed by the penetrance function (Thallman *et al.*, 2001b). We used a uniform penetrance function with the error rate set to 0.1.

We obtained a vector $\mathbf{g}$ with 15 PrP genotype probabilities [1] for each animal. These probabilities were summed into a vector $\mathbf{n}$ of five NSP type probabilities [2] with regard to the resistance/susceptibility to scrapie infection (Table 1).

$$\mathbf{g}' = \left[\Pr(ARR/ARR), \Pr(ARR/AHQ), \ldots, \Pr(VRQ/VRQ)\right] \quad [1]$$

$$\mathbf{n}' = \left[\Pr(NSP_1), \Pr(NSP_2), \ldots, \Pr(NSP_5)\right] \quad [2]$$

Bearing in mind the resistance to scrapie it is desirable that the animals are of as low NSP type as possible. As shown by Gorjanc and Kompan (2008) the average NSP value was calculated as an average of NSP type values weighted with NSP type probabilities [3]. We also calculated the average NSP value for the entire population, where the NSP type probabilities were derived from the frequencies of PrP genotype in the entire population.

$$\overline{NSP} = \mathbf{a}' * \mathbf{n} \qquad [3]$$

## RESULTS AND DISSCUSSION

### Allele frequency

Besides the number of animals with genotype data and pedigree structure, allele frequency is also important in the calculation of genotype probabilities (Tier and Henshall, 2005). Six alleles (haplotypes) of a PrP gene have been identified so far in Slovenia: ARR, AHQ, ARH; ARQ; VRQ and VRR. The VRR allele has been identified in one case only and it has been excluded from the analysis. Allele frequencies (Table 3) were calculated as simple percentages, i.e., relationship dependencies among the collected genotypes of relatives were not taken into the consideration (e.g. Bohenke, 1991). For all breeds, except for T, a high frequency of allele ARQ was observed (from 50.0 to 69.7%). The frequency of VRQ allele (associated with the high susceptibility to scrapie) was less than 5% in all breeds. The ARR allele (associated with the high resistance to scrapie) was more frequent than the VRQ allele. The frequency of this favourable allele was 52.7% in T breed, around 35.0% in BP and IP breed, and around 20% in JS, JSR, B and VFB breed. The frequency of AHQ and ARH alleles was quite diverse and ranged between 0.1 and 25.5%. Lühken *et al.* (2008) estimated allele frequencies for 56 breeds from 15 European and Near East countries. They showed that there is a considerable variation between breeds. Allele frequencies in breeds in Slovenia are within the range of values reported by Lühken *et al.* (2008).

Table 3. PrP allele frequencies, %
Preglednica 3. Frekvence alelov PrP, %

| Breed<br>Pasma | Allele / Alel | | | | |
|---|---|---|---|---|---|
| | ARR | AHQ | ARH | ARQ | VRQ |
| JS | 17.4 | 7.4 | 8.3 | 63.2 | 3.7 |
| JSR | 17.8 | 7.9 | 0.9 | 69.7 | 3.8 |
| BP | 35.0 | 4.3 | 0.6 | 58.6 | 1.4 |
| T | 52.7 | 4.8 | 12.4 | 26.3 | 3.8 |
| B | 16.9 | 17.4 | 7.6 | 57.1 | 1.0 |
| VFB | 22.0 | 24.6 | 2.5 | 50.0 | 0.9 |
| IP | 32.5 | 7.5 | 0.1 | 57.2 | 2.6 |

JS – Jezersko-Solcava; JSR – Improved Jezersko-Solcava; BP – Bela Krajina pramenka; T – Texel; B – Bovec; VFB – Improved Bovec; IP – Istrian pramenka

**PrP genotype and NSP type identification**

From here onwards, the results are presented for live animals only, because only the living animals are of interest for the selection process. However, all known animals (dead and alive) were used in the process of the calculation of genotype probabilities. We calculated genotype probabilities for all 15 possible PrP genotypes for each animal. The success of analysis was evaluated with the number of additionally identified or excluded PrP genotypes and NSP types. If the probability of a particular genotype was 100%, then that genotype was identified with certainty. The success was assessed also for the probability of identification of 99 and 95%. In case of five alleles, fifteen genotypes are possible. Therefore, the ratio between the number of confirmed and excluded genotypes was 1:14. This means that for a particular animal we could confirm one genotype and exclude 14 genotypes.

Table 4. Number of additional PrP genotype identifications or exclusions
Preglednica 4. Število dodatno potrjenih ali ovrženih genotipov PrP

| Breed<br>Pasma | Additional PrP genotype identifications<br>Dodatno potrjenih genotipov PrP | | | Additional PrP genotype exclusions<br>Dodatno ovrženih genotipov PrP | | |
|---|---|---|---|---|---|---|
| Probability<br>Verjetnost | 1.00 | 0.99 | 0.95 | 1.00 | 0.99 | 0.95 |
| JS | | | | | | |
| No. / Št. | 0 | 66 | 101 | 0 | 2045 | 2673 |
| % | 0 | 2.5 | 3.8 | 0 | 5.1 | 6.7 |
| JSR | | | | | | |
| No. / Št. | 0 | 105 | 145 | 0 | 2243 | 3795 |
| % | 0 | 2.8 | 3.8 | 0 | 3.9 | 6.7 |
| BP | | | | | | |
| No. / Št. | 0 | 0 | 9 | 0 | 137 | 159 |
| % | 0 | 0.0 | 5.7 | 0 | 5.7 | 6.7 |
| T | | | | | | |
| No. / Št. | 0 | 0 | 0 | 0 | 161 | 189 |
| % | 0 | 0.0 | 0.0 | 0 | 5.7 | 6.7 |
| B | | | | | | |
| No. / Št. | 0 | 11 | 22 | 0 | 478 | 522 |
| % | 0 | 2.1 | 4.2 | 0 | 6.1 | 6.7 |
| VFB | | | | | | |
| No. / Št. | 0 | 7 | 7 | 0 | 122 | 128 |
| % | 0 | 5.5 | 5.5 | 0 | 6.4 | 6.7 |
| IP | | | | | | |
| No. / Št. | 0 | 10 | 14 | 0 | 219 | 263 |
| % | 0 | 3.8 | 5.3 | 0 | 5.6 | 6.7 |

JS–Jezersko-Solcava; JSR–Improved Jezersko-Solcava; BP–Bela Krajina pramenka; T–Texel; B–Bovec; VFB–Improved Bovec; IP–Istrian pramenka

We were not able to identify any additional PrP genotype or NSP type with certainty not even for one animal in any breed (Table 4 and 5). This is inherently due to the use of incomplete penetrance model as discussed by Gorjanc and Kompan (2008). Results were only a bit better (data not shown) when the success was evaluated on all (alive and dead) animals in the pedigree. However, those results are of no practical significance, since dead animals are not interesting for selection. The number of animals with additionally identified or excluded PrP genotype was just slightly higher at 99 or 95% probability of identification (Table 4). The percentage of animals with additionally identified PrP genotype with 95% probability ranged from 0.0 to 5.7% of animals (Table 4). For all breeds together, we additionally identified PrP genotype (with 95% probability) in 298 animals. There were about 4 to 6% of excluded PrP genotypes with 99%

probability and surprisingly 6.7% of excluded PrP genotypes with 95% probability for all breeds. Success of the analysis practically did not differ between breeds, even though the data structure was diverse (Table2).

The success was better with the additional identification of NSP types (Table 5). The highest percentage of animals with additionally identified NSP type with 95% probability were achieved in B breed (34.9%) and VFB breed (25.8%). In other breeds the percentages were mostly lower than 10% (Table 5). Considering all breeds together, we additionally identified NSP type in 561 animals with 99% probability or 818 animals with 95% probability. The percentage of excluded NSP type with 95% probability varied between 15 and 20%, i.e., around three to four times higher in comparison to excluded PrP genotypes (Table 4 and 5).

The absolute number of additionally identified PrP genotypes and NSP types is not negligible, as there is no need to genotype these animals. Since tissue collection and genotyping are still of considerable cost, additional identifications provide economical savings for the breeding programme. Nevertheless, we can conclude that the percentage of additional genotype identifications was very low. Different data structure between breeds (Table 2) had practically no effect. In what follows, we discuss reasons for such a low number of additional identifications. We also discuss a novel parameter (average NSP type), which encompasses all information from genotype probabilities and can be used effectively in the selection for scrapie resistance.

Table 5. Number of additional NSP type identifications or exclusions
Preglednica 5. Število dodatno potrjenih ali ovrženih skupin NSP

| Breed<br>Pasma | Additional NSP type identifications<br>Dodatno potrjenih skupin NSP | | | Additional NSP type exclusions<br>Dodatno ovrženih skupin NSP | | |
|---|---|---|---|---|---|---|
| Probability<br>Verjetnost | 1.00 | 0.99 | 0.95 | 1.00 | 0.99 | 0.95 |
| JS | | | | | | |
| No. / Št. | 0 | 209 | 283 | 0 | 1583 | 2488 |
| % | 0 | 7.8 | 10.6 | 0 | 11.8 | 18.6 |
| JSR | | | | | | |
| No. / Št. | 0 | 213 | 293 | 0 | 1469 | 3402 |
| % | 0 | 5.6 | 7.7 | 0 | 7.7 | 17.9 |
| BP | | | | | | |
| No. / Št. | 0 | 2 | 11 | 0 | 123 | 148 |
| % | 0 | 1.2 | 6.9 | 0 | 15.5 | 18.6 |
| T | | | | | | |
| No. / Št. | 0 | 0 | 1 | 0 | 75 | 158 |
| % | 0 | 0.0 | 0.5 | 0 | 7.9 | 16.7 |
| B | | | | | | |
| No. / Št. | 0 | 101 | 182 | 0 | 456 | 512 |
| % | 0 | 19.3 | 34.9 | 0 | 17.5 | 19.6 |
| VFB | | | | | | |
| No. / Št. | 0 | 26 | 33 | 0 | 111 | 123 |
| % | 0 | 20.3 | 25.8 | 0 | 17.3 | 19.2 |
| IP | | | | | | |
| No. / Št. | 0 | 10 | 15 | 0 | 164 | 223 |
| % | 0 | 3.8 | 5.7 | 0 | 12.5 | 17.0 |

JS – Jezersko-Solcava; JSR – Improved Jezersko-Solcava; BP – Bela Krajina pramenka; T – Texel; B – Bovec; VFB – Improved Bovec; IP – Istrian pramenka

First of all, five alleles of PrP gene were included into the analysis: ARR, AHQ, ARH, ARQ and VRQ. These alleles are the most frequent in our as well as in other populations of sheep (Lühken *et al.*, 2008; Ulvund, 2008). Five alleles give rise to 15 different genotypes, which is not

negligible. According to Tier and Henshall (2005), Elston and Stewart (1971) developed a method for the calculation of genotype probabilities in human genetics, where pedigrees under inspection were limited to a smaller number of families and loci with rare lethal alleles. In such cases, the aim was to determine the carriers of lethal alleles with the use of genotype probabilities. Tier and Henshall (2005) assessed the limits of additional genotype identifications for beef cattle, sheep and pigs. They concluded that better results were obtained in cases with a small number of alleles having diverse frequencies. This is in accordance with the data in human genetics that inspired Elson and Stewart (1971). In our study, the situation was opposite. We had higher number of alleles (five) with intermediate frequencies (Table 3). Kinghorn (1999) assessed the percentage of additional genotype identifications. He reported that in the case of two alleles, complete penetrance model, and 10, 20, and 80% of genotyped animals, 50, 60, and even 100% of additional identifications could be achieved, respectively. We also could merge several alleles into two groups, i.e., ARR and other alleles. However, this is not optimal as some flocks did not have any animal carrying ARR allele, but they had a substantial variation for other alleles.

The next limitation in the view of larger number of identifications was used prior for allele probabilities in founders. Prior probabilities are the initial allele probabilities for the animals at the top of the pedigree. Thallman (2002) proposed a uniform distribution for allele probabilities in founders, resulting in our data. This proposal is the handiest, as it is the least informative. Prior probabilities in founders are used in the calculation of genotype probabilities via the Bayes theorem of conditional probability and are as such subsequently used in the calculation of genotype probabilities for all relatives in the pedigree. Until the collected genotype data (the likelihood) do not dominate the genotype probabilities (the posterior) the effect of a prior does not vanish (e.g. Gelman *et al.*, 2004, Sorensen and Gianola, 2007). The uniform prior therefore adds to the uncertainty in genotype probabilities and lowers the number of additional PrP genotype identifications with certainty or high probability. Results by Lühken *et al.* (2008) and many others show that some PrP alleles are more frequent than others. For example ARQ allele is the most frequent in the majority of breeds and it seems logical to assign higher prior probability to ARQ allele than say VRQ allele. Instead of uniform allele probabilities, the sample allele frequencies could be used as a prior information. However, Tier and Henshall (2002) warned against such use, because the allele frequencies in founders can be entirely different to the ones in the collected data. Furthermore, the allele frequencies can also be markedly different between families (Tier and Henshall, 2002). The estimated allele frequencies in founders (e.g. Bohenke, 1991) could be used instead. Kerr and Kinghorn (1996) have established, using simulation, that the uniform prior probability decreases the number of erroneous genotype exclusions. Gorjanc and Kompan (2008) argued that it is of paramount importance that inference about VRQ/* genotypes is correct and recommended the uniform prior as a conservative choice.

In Slovenia, the selection programme for scrapie has started in the year 2005. Regarding this fact, we can divide animals in our study into three groups. The first group represents genotyped animals. The majority of these animals are still alive. The second group represents non-genotyped ancestors that are already dead. Finally, the third group represents live non-genotyped animals. Our aim was to identify the PrP genotype for animals in the third group. Animals of the first group were used as a primary source of genotype information, while animals of the second group provided additional link between the first and the third group through the pedigree. Kerr and Kinghorn (1996) have shown that the success is much lower in such scenario compared to the one, where genotype data spans over generations.

The use of incomplete penetrance model (Thallman *et al.*, 2001b) also added to the uncertainty and therefore lowered the number of additional PrP genotype identifications. Errors in genotype and/or pedigree data can cause deviations between laboratory reported genotype and genotype inferred from genotype data of relatives. Erroneous data can be corrected, but it is often

hard to determine which record is erroneous in the case of large and complex pedigrees with incomplete genotype data at one locus with multiple alleles. Thallman *et al.* (2001b) used a model of incomplete penetrance in order to conceptually treat genotype data as "phenotype" data via penetrance function (Lincoln and Lander, 1992). This enables the use of data with potential errors. Unfortunately, the incomplete penetrance model adds to the uncertainty of the results as manifested in small probabilities for all genotypes that are not consistent with the "phenotype". These small probabilities are also transferred throughout the pedigree as it is the case with prior allele probabilities in the founders. Consequently, the number of additional genotype identifications is lowered (Table 4 and 5). Gorjanc and Kompan (2008) showed the effect of use of incomplete penetrance model on calculated PrP genotype probabilities and the number of additional PrP genotype identifications. Nevertheless, Thallman *et al.* (2001b), Gengler at al. (2007), and Gorjanc and Kompan (2008) argued that incomplete penetrance is very useful or even essential for the applied work on field data in animal breeding.

**Average NSP value**

The selection for scrapie resistance in sheep is based on the elimination of the VRQ allele and on the increase of the ARR allele frequency. In order to apply selection to the whole population of performance recorded sheep, we tried to identify the PrP genotype and NSP type for non-genotyped animals. Unfortunately, we were not able to identify (with certainty or high probability) PrP genotype and NSP type for the majority of non-genotyped animals. Instead of singly identified PrP genotype and NSP type, all PrP genotype probabilities can be used for the selection process. Van Arendonk *et al.* (1989) have given similar suggestion, but for a gene with only two alleles. Work with probabilities for all fifteen PrP genotypes for each animal is, of course, cumbersome. Therefore, we used the average NSP value as suggested by Gorjanc and Kompan (2008). The average NSP value is a weighted average of NSP type values weighted with NSP type probabilities derived from PrP genotype probabilities. Therefore, this parameter combines all information in PrP genotype probabilities into a single value.

Distribution of the average NSP value for the non-genotyped animals agreed to a great extent with the distribution of NSP type for the genotyped animals in all sheep breeds (Fig. 1). Similar distribution between non-genotyped and genotyped animals is expected because the PrP genotype probabilities and subsequently NSP types were derived from the collected genotype data. In some breeds peaks were observed at values around 1.5, 2, 2.5, 3, 3.5, 4, and 5. This can be attributed to the small number of NSP types (five) and discrete NSP type values.

To increase resistance to scrapie, breeders should select animals having low NSP type value. Apart from selecting only among genotyped animals, non-genotyped animals could also be included in the selection process with the means of average NSP value. Breeders should select animals with average NSP value below the population average NSP value (dashed line in Fig. 1). As seen in Table 6, the use of average NSP value increased the pool of animals for selection by 2034 animals in all breeds together. The increase varied between breeds; from 19.0% in T breed to 45.9% in BP breed (Table 6). The increased pool of animals inherently provides more space for selection for other economically important traits in sheep and lowers the rate of inbreeding. The later is very important for the autochthonous rare breeds: Bela Krajina pramenka, Istrian pramenka, and Bovec breed. Jezersko-Solcava breed is also autochthonous but the size of the population (performance recorded flocks and others flocks) is sufficiently large to avoid close inbreeding.

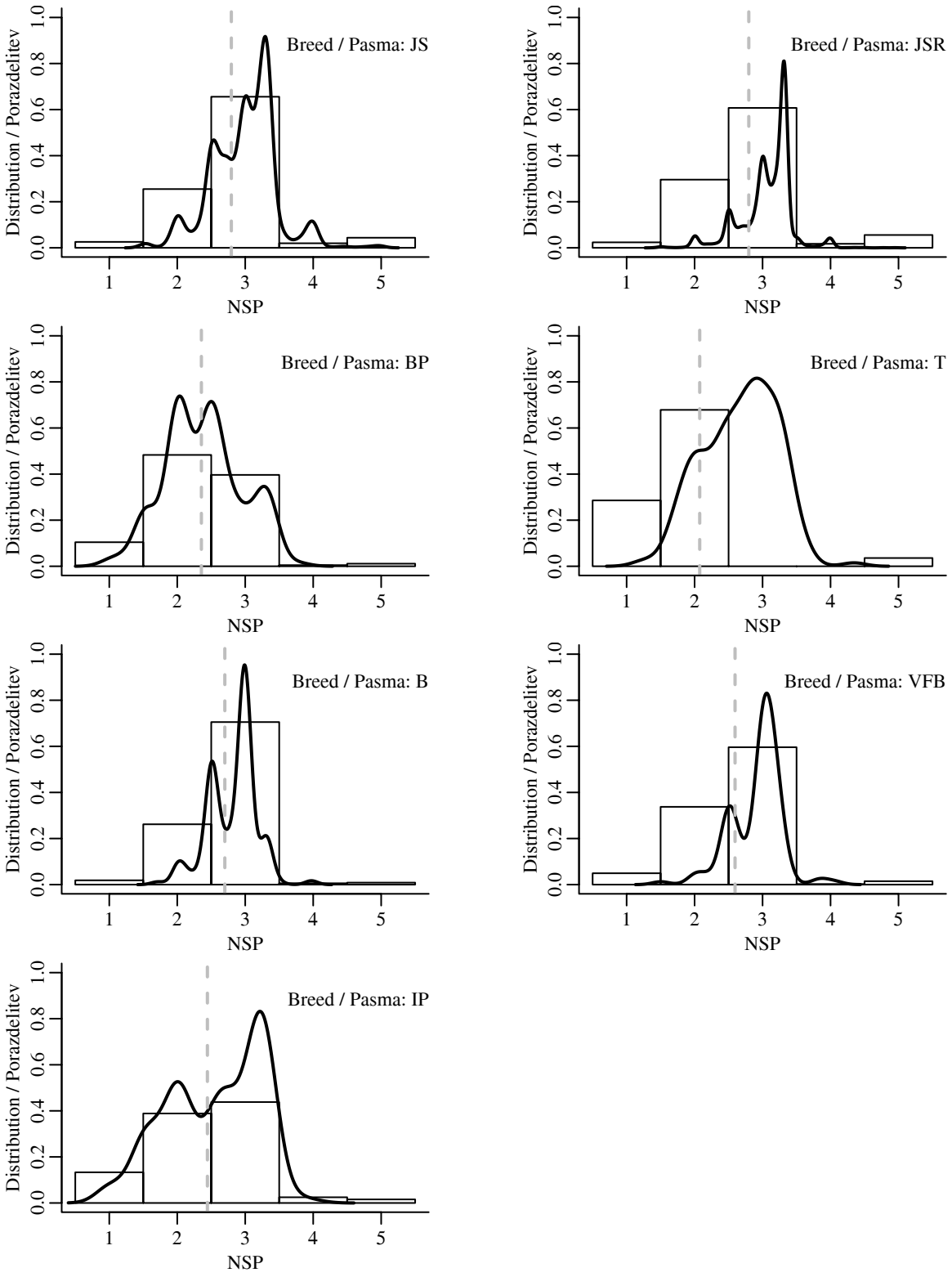


Figure 1. Distribution of animals by average NSP value bars – genotyped animals, superimposed curve – non-genotyped animals, vertical dashed line –average NSP value in population

Slika 1. Porazdelitev živali glede na povprečno vrednost NSP: stolpci – genotipizirane živali, naložena krivulja – negenotipizirane živali, navpična črtkana črta – populacijska povprečna vrednost NSP

Table 6. Live non-genotyped animals bellow population average NSP value
Preglednica 6. Žive negenotipizirane živali pod populacijskim povprečjem vrednosti NSP

| Breed<br>Pasma | Number of live non-genotyped animals<br>Število živih negenotipiziranih živali | $\overline{NSP}_i < \overline{NSP}_p$ | |
|---|---|---|---|
| | | No. / Št. | % |
| JS | 2673 | 879 | 32.9 |
| JSR | 3795 | 726 | 19.1 |
| BP | 159 | 73 | 45.9 |
| T | 189 | 36 | 19.0 |
| B | 522 | 185 | 35.4 |
| VFB | 128 | 32 | 25.0 |
| IP | 263 | 103 | 39.2 |

JS – Jezersko-Solcava; JSR – Improved Jezersko-Solcava; BP – Bela Krajina pramenka; T – Texel; B – Bovec; VFB – Improved Bovec; IP – Istrian pramenka

Besides the additional genotype identifications and selection, genotype probabilities can be used for the calculation of genotype probability index (Kinghorn, 1997). This index is used to create a list of animals for the next round of genotyping in such a way that the newly identified genotypes will provide maximal gain of information in the pedigree. Use of this index can therefore help breeders to use resources in a more efficient way. Additionally, genotype probabilities can also be used in association analyses to increase the statistical power as has been, for example, shown by Vitezica *et al.* (2005).

## CONCLUSIONS

PrP genotype probabilities and NSP type probabilities were calculated for non-genotyped ewes and rams. The success of PrP genotype or NSP type identification was low. We maintain that the main reasons for these results are: a high number of alleles, intermediate allele frequencies, data structure (genotype data known for animals of recent generations only), a uniform prior, and the use of incomplete penetrance model. Nevertheless, PrP genotype probabilities can be used for the calculation of the average NSP value, which is a useful and practical parameter in the selection for scrapie resistance of the entire population.

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## REFERENCES

Bohenke, M. Allele frequency estimation from data on relatives. Am. J. Hum. Gen., 48(1991), 22–25. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1682747 (31. mar. 2008)

Cannings, C./ Thompson, E.A./ Skolnick, M.H. Probability functions on complex pedigrees. Adv. Appl. Prob., 10(1978), 26–61.

Dawson, M./ Moore, R.C./ Bishop, S.C. Progress and limits of PrP gene selection policy. Vet. Res., 39(2008), 25–37. http://dx.doi.org/10.1051/vetres:2007064 (10.mar. 2008)

European Commission, 2003/100/EC: Commission Decision of 13 February 2003 laying down minimum requirements for the establishment of breeding programmes for resistance to transmissible spongiform encephalopathies in sheep. Official Journal, L04(2003), 0041–0045. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32003D0100:EN:HTML (31. mar. 2008)

Elston, R.C./ Stewart, J. A general model for the genetic analysis of pedigree data. Hum. Hered., 21(1971), 523–542.

Fernández, S.A./ Fernando, R.L./ Guldbrandtsen, B./ Totir, L.R./ Carriquiry, A.L. Sampling genotypes in large pedigrees with loops. Genet. Sel. Evol., 33(2001), 337–367. http://dx.doi.org/10.1051/gse:2001122 (31. mar. 2008)

Gelman, A./ Carlin, J.B./ Stern, H.S./ Rubin, D.B. Bayesian data analysis. Boca Raton, Chapman & Hall/CRC, 2004, 668 p.

Gengler, N./ Mayeres, P./ Szydlowski, P. A simple method to approximate gene content in large pedigree populations: application to the myostatin gene in dual-purpose Belgian Blue cattle. Animal, 1(2007), 21–28. http://dx.doi.org/10.1017/S1751731107392628 (31. mar. 2008)

Gorjanc, G./ Kompan, D. Use of genotype probabilities in selection on PrP genotype in sheep. Liv. Sci., 2008, submitted.

Henshall, J.M./ Tier, B. An algorithm for sampling descent graphs in large complex pedigrees efficiently. Genet. Res., 81(2003), 205–212. http://dx.doi.org/10.1017/S0016672303006232 (31. mar. 2008)

Hunter, N. PrP genetics in sheep and the implications for scrapie and BSE. Trends in Microbiol., 5(1997), 331–334. http://dx.doi.org/10.1016/S0966-842X(97)01081-0 (31. mar. 2008)

Jacquard, A. The genetic structure of populations. Berlin, Springer-Verlag, 1974, 569 p.

Janss, L.L.G./ Van Arendonk, J.A.M./ Van der Werf, J.H.J. Computing approximate monogenic model likelihoods in large pedigrees with loops. Genet. Sel. Evol., 27(1995), 567–579. http://www.gse-journal.org/articles/gse/pdf/1995/06/GSE_0999-193X_1995_27_6_ART0007.pdf

Kerr, R.J./ Kinghorn, B.P. An efficient algorithm for segregation analysis in large populations. J. Anim. Breed. Genet., 113(1996), 457–469.

Kinghorn, B.P. An Index of Information Content for Genotype Probabilities Derived from Segregation Analysis. Genetics, 145(1997), 479–483. http://www.genetics.org/cgi/content/abstract/145/2/479 (31. mar. 2008)

Kinghorn, B.P. Use of segregation analysis to reduce genotyping costs. J. Anim. Breed. Genet., 116(1999), 175–180. http://dx.doi.org/10.1046/j.1439-0388.1999.00192.x (31. mar. 2008)

Lincoln, S.E./ Lander, E.S. Systematic detection of errors in genetic linkage data. Genomics, 14(1992), 604–610. http://dx.doi.org/10.1016/So888-7543(05)80158-2 (31. mar. 2008)

Lühken, G./ Lipsky, S./ Peter, C./ Erhardt, G. Prion protein polymorphisms in autochtonous European sheep breeds in respect to scrapie eradication in affected flocks. Small. Rum. Res., 75(2008), 43–47. http://dx.doi.org/10.1016/j.smallrumres.2007.07.010 (31. mar. 2008)

Prusiner, S.B. Prions. Proc. Natl. Acad. USA, 95(1998), 13363–13383. http://www.pnas.org/cgi/content/abstract/95/23/13363 (31. mar. 2008)

Sorensen, D./ Gianola, D. Likelihood, Bayesian and MCMC Methods in Quantitative Genetics. New York, Springer, 2007, 760 p.

Thallman, R.M./ Bennet, G.L./ Keele, J.W./ Kappes, S.M. Efficient computation of genotype probabilities for loci with many alleles: I. Allelic peeling. J. Anim. Sci., 79(2001a), 26–33. http://jas.fass.org/cgi/content/abstract/79/1/26 (31. mar. 2008)

Thallman, R.M./ Bennet, G.L./ Keele, J.W./ Kappes, S.M. Efficient computation of genotype probabilities for loci with many alleles: II. Iterative method for large complex pedigrees. J. Anim. Sci., 79(2001b), 34–44. http://jas.fass.org/cgi/content/abstract/79/1/34 (31. mar. 2008)

Thallman, R.M. User's Manual for GenoProb Version 2.0. Computation of genotype and phase probabilities in complex pedigrees by iterative allelic peeling. 2002, 1–18.

Tier, B./ Henshall, J.M. Founder allele frequencies and segregation analysis. In: Proceedings of the 7th World Congress on Genetics Applied to Livestock Production, Montpellier, 2002-8-19/23. Castanet-Tolosan, INRA, cop, 2002, Communication, 16–17.

Tier, B./ Henshall, J.M. Limits to genotypic probabilities for single loci. In: Application of new genetic technologies to animal breeding. Proceedings of the 16th Conference of the Association for the Advancement of Animal Breeding and Genetics, Noosa, 2005-09-25/28, Australia, 2005, 366–369.

Ulvund, M.J. Ovine scrapie disease: Do we have to live with it? Small Rum. Res., 76(2008), 131–140. http://dx.doi.org/10.1016/j.smallrumres.2007.12.004 (31. mar. 2008)

Van Arendonk, J.A.M./ Smith, C./ Kennedy, B.W. Method to estimate genotype probabilities at individual loci in farm livestock. Theor. Appl. Genet., 78(1989), 735–740. http://dx.doi.org/10.1007/BF00262571 (31. mar. 2008)

Vitezica, Z.G./ Elsen, J.M./ Rupp, R./ Diaz, C. Using genotype probabilities in survival analysis: a scrapie case. Genet. Sel. Evol., 37(2005), 403−415. http://dx.doi.org/10.1051/gse:2005008 (31. mar. 2008)

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# LONG-TERM DIVERGENT SELECTION FOR 8-WEEK BODY WEIGHT IN CHICKENS – A REVIEW OF EXPERIMENTS

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## ABSTRACT

In order to study the effects of long-term selection on genetic parameters, the effect of selection on selected and correlated traits and to develop lines for various physiological, biochemical and molecular genetic studies a comprehensive selection study for body weight in chickens has been conducted at the Biotechnical Faculty of University in Ljubljana. Long-term divergent selection in chickens for 8-week body weight for 31 generations produced a high weight (D+) and low weight (D–) line. Body weight at 8 weeks of age was the exclusive selection criterion. Selection lines were compared for body weight and for unselected traits including carcass traits, feed conversion, egg and meat quality traits, reproduction traits, muscle characteristics, nutritional and physiological traits. This paper describes the most important results of different experiments that were performed on animals from both lines in various generations of selection.

Key words**:** poultry / chickens / divergent selection / growth / correlated traits

# REZULTATI POSKUSOV IZ DVOSMERNE SELEKCIJE NA TELESNO MASO PIŠČANCEV PRI 8. TEDNIH STAROSTI

## IZVLEČEK

Z namenom ocenjevanja učinkov dolgotrajne selekcije na genetske parametre, selekcionirane in korelirane lastnosti ter za potrebe izvajanja raziskav s področij fiziologije, biokemije in molekularne genetike je bil na Biotehniški fakulteti Univerze v Ljubljani izpeljan obsežen dvosmerni selekcijski poskus na telesno maso piščancev. Enaintrideset generacij smo odbirali piščance na večjo (D+ linija) in manjšo (D– linija) telesno maso pri osmih tednih starosti, ki je bila vseskozi edini selekcijski kriterij. V posameznih generacijah smo proučevali direktne (telesna masa) in korelirane učinke selekcije (klavne lastnosti, izkoriščanje krme, kakovost jajc in mesa, reprodukcijske lastnosti, lastnosti mišičnih vlaken, prehranske in fiziološke lastnosti). V članku so zbrani najpomembnejši rezultati omenjenih raziskav.

Ključne besede: perutnina / piščanci / dvosmerna selekcija / rast / korelirane lastnosti

## INTRODUCTION

Farmers have been implementing artificial selection in chickens for thousands of years. However, in the last century artificial selection has become a complex scientific business. A limited number of artificial selection effects can be deduced from the selection theory but many can only be demonstrated by conducting experiments. Long-term (exceeding ten generations) selection experiments can provide a substantial amount of information and have a practical value

for chicken improvement. By affecting many regions of the genome simultaneously, they can reveal relationships among interacting genetic pathways. Because correlated responses may have economic consequences, the impact of selection for actual target(s) of selection on total economic merit of chickens should be monitored. Long-term divergently selected lines are also a unique resource for dissecting the genetic basis underlying line divergence. Understanding the genetic architecture of traits such as growth and body composition has become a primary focus for biomedical and agricultural research.

At the University of Ljubljana we conducted a selection experiment over a period of 28-years (31 generations of selection) to measure long-term response to selection. Divergent selection on 8-week body weight produced a high weight (D+) and a low weight (D–) line. The work on the experiment is still in pregress. Many other long-term selection experiments have also been conducted in chickens and other domestic poultry species for a range of traits (e.g. Dunnington and Siegel, 1996; Marks, 1996; Nestor *et al*., 1996). Our investigation differs from earlier studies on responses to body weight selection by the duration of selection and by monitoring correlated effects in traits which differ from the traits used in other studies. The aim of this review is to describe the most general findings that have emerged from the long-term bidirectional mass selection for 8-week body weight.

## MATERIAL AND METHODS

The base population for this experiment was stock from a commercial heavy sire line of the Slovenian provenance Prelux-bro. Two lines were established by selecting from the base population 10 males and 50 females that were the heaviest at 8 weeks of age and those that were the lightest. Within each line, the high-weight males and females were mated at random to establish a high-weight (D+) line, and the low-weight males and females used similarly to establish a low-weight (D–) line. After formation in the first generation of selection, the lines were closed, with parents for subsequent generations chosen as the extreme weighing males and females from each of the lines. A control line was not propagated. Progeny of each generation was obtained from two or even three hatches. The total number of animals per generation varied between 324 animals in the 30$^{th}$ generation and 1283 animals in the 18$^{th}$ generation. The proportion kept for breeding ranged from 22.7% to 56.5% per generation (Fig. 1).

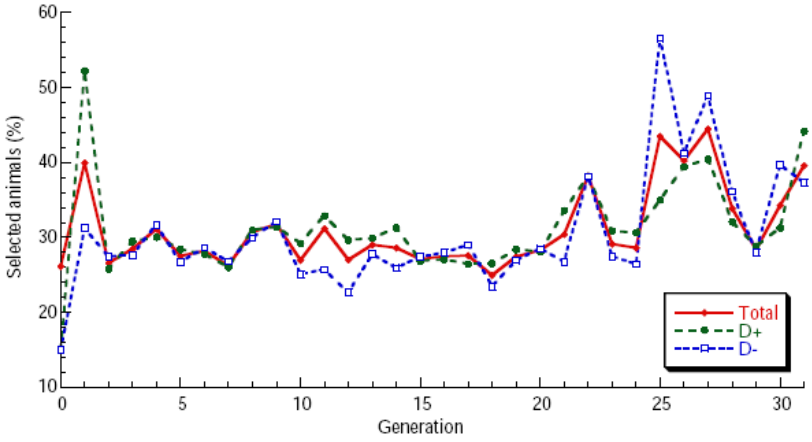


Figure 1. The proportion of animals selected to be parents of the next generation (D+ = high weight line; D– = low weight line; Total = both lines together).

Slika 1. Delež odbranih živali za starše naslednje generacije (D+ = linija selekcionirana na večjo telesno maso; D– = linija selekcionirana na manjšo telesno maso; Total = obe liniji skupaj).

Up to the age of 8 weeks growing chickens were reared together in a windowless house with a deep litter system. Afterwards selected parents of each line were reared in separate pens within the same poultry house. Pens were supplied with hanging bell waterers and feeders to provide *ad libitum* access to water and feed. Environmental factors were controlled so as to provide conditions that were as similar as possible across generations and hatches. Animals were exposed to a set-up lighting program. This program started from a constant short (8 hour) day length. At the age of 18 weeks the day length was increased by increments of 1 hour per week until day length reached 15 hours.

## RESULTS AND DISCUSSION

After conducting divergent individual mass selection for 8-week body weight for seven generations, Holcman (1986) evaluated the direct (body weight) and correlated (age at maturity, egg number, egg weight, weight of one day old chicks, hatchability) selection responses. At each generation, the cumulated response to selection was computed as the difference between the phenotypic mean in the downward line and the phenotypic mean in the upward line. The mean realized heritability for body weight at 8 weeks was estimated as the regression of response to selection on accumulated selection differential. The rate of inbreeding per generation was calculated as $\Delta F = (1/8N_m) + (1/8N_f)$ where $N_m$ is the number of males and $N_f$ is the number of females. For calculating the cumulative inbreeding in generation t the following formula was used: $F_t = \Delta F + (1–\Delta F)F_{t–1}$. The total effect of divergent selection in seven generations was 752 g (783 g for males and 720 g for females). The realized heritability for 8-week body weight after 7 generations of selection was 0.26 (0.27 for males and 0.25 for females). The average rates of inbreeding per generation were 3.2% and 2.0% for lines D(+) and D(–), respectively. There was an increase in the cumulative inbreeding on successive years up to the mean of 20.9% in D(+) and 13.4% in D(–) line. Selection for high 8-week body weight resulted in a delay in age at sexual maturity and in an increase in egg weight. Thus, indirect selection for higher body weight had a positive effect on the weight of one day old chicks. According to the Witt and Schwalbach (2004) there is a strong positive correlation between egg weight and hatching weight. A negative relationship has been observed between high body weight and hatchability. In the seventh generation of divergent selection the average ages at sexual maturity were 27 and 22 weeks, the average egg weights were 65.0 and 55.0 g and the average percentages of hatchability were 52.3 and 68.1 for lines D(+) and D(–), respectively.

In a subsequent report Terčič *et al*. (2006) presented data for 24 additional generations of selection for high and low 8-week body weight. In the 30$^{th}$ generation of selection, the D(+) males and females weighed 2304 and 1679 grams more, respectively, compared with D(–) males and females. These differences represent a 7.5-fold increase in body weight at 8 weeks of age for the D+ compared to the D– line. Selection responses for generations 0 to 31 in the D(+) and D(–) line are shown in Fig. 2. It can be seen that patterns of responses are unpredictable. Substantial responses that were observed in both lines were followed with periods of little or no response to selection (Fig. 2). Dunnington and Siegel (1996) suggested two possible explanations for the phenomenon of irregular response: a) after many generations of selection, genotypes are more sensitive to microenvironmental factors that facilitate irregular responses or b) spontaneous mutations may have occured periodically. Regressions of mean body weights on generation across the 30 generation period yielded respective regression coefficients of 25.46 g and -36.07 g per generation in the D(+) and D(–) line. The differences in selection intensity values within lines represent plausible reason for asymmetry between upward and downward selection responses. The realized heritability of body weight (mean ± s.e.) was 0.12 ± 0.014 in the D(+) and 0.22 ± 0.015 in the D(–) line. The realized heritability was computed as the slope of the

linear regression of the generation means, measured as deviations from founder population, on the cumulated selection differential. Realized heritability estimates for generations 1-7 were relatively high, however, after 30 generations of selection, heritability estimates were reduced. Because of the variation in the genetic constitution of the founder populations and/or effects of population size it is difficult to compare the current estimates for realized heritability with published estimates obtained in different experiments.

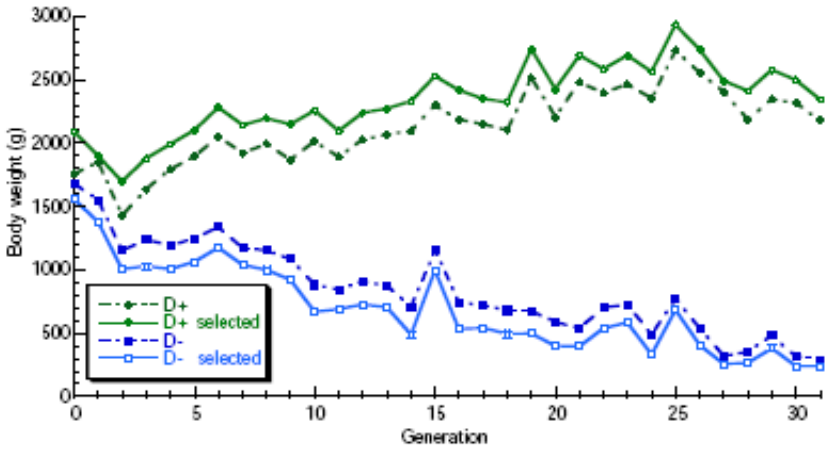


Figure 2. Mean 8-week body weights of D(–) and D(+) line from generations 0 to 31.
Slika 2. Srednje vrednosti za telesne mase piščancev D(–) in D(+) linij iz generacij 0 do 31.

Growth performance, feed conversion, nutrient digestibility, carcass traits and blood serum parameters were explored on the male chickens from the tenth generation of divergent selection for 8-week body weight (Mužic, 1990). Thirty-two birds from each line were placed in individual cages. Nutritionally complete diets were formulated for starter and finisher periods. Time of feeding the diets was adjusted to 0 to 3 weeks for starter, and 4 to 7 weeks for finisher. Feed and water were provided for *ad-libitum* consumption. Body weights and feed consumption were recorded at weekly intervals. The traditional method of total excreta collection was applied for all animals from 36 to 40 day of age to determine the digestibility coefficients for the dry matter, organic matter, crude protein, crude fibre, crude fat and nitrogen-free extract. Blood samples were collected from the wing (brachial vein) at 6 weeks of age, to obtain the total cholesterol, triglycerides, total protein and total fat concentration in the serum. All birds were slaughtered to determine dressing percentage and parts yield at 49 days of age. Differences were reflected in the performance of the lines. High weight males had higher ($P < 0.05$) body weights (1.94 vs 1.12 kg), lower (better) ($P < 0.05$) feed conversion rates (1.95 vs 2.16 kg), and higher ($P < 0.05$) carcass yields (66.99 vs 64.72%). The absolute weight of the abdominal fat pads as well as the weight of the abdominal fat pads expressed as a percentage of body weight were higher ($P < 0.05$) in D(+) males (48.11 g; 2.46%) compared to D(–) males (20.23 g; 1.78%). The nutrient digestibility studies showed a higher ($P < 0.05$) digestibility of crude fibre and crude fats in D(+) males. There was no difference ($P > 0.05$) between the digestibility coefficients for crude protein, nitrogen-free extract, organic matter and dry matter obtained in D(+) males and the values obtained in D(–) males. Blood serum total protein and total cholesterol concentrations were higher ($P < 0.05$) in D(+) males than in D(–) males, whereas total fat and trygliceride concentrations did not differ ($P > 0.05$) between the birds from the two lines.

To study the effects of divergent selection for 8-week body weight on growth and feed conversion Holcman (1992) designed an experiment with animals from fifteenth generation of selection. Two hundred and forty-one chickens [112 from line D(+) and 129 from line D(–)]

were reared in two separate pens in deep litter within the same poultry house. All birds were fed a common starter diet in mash form. Body weights were recorded individually, whereas feed conversion was recorded by line. All measurements were done at weekly intervals up to nine weeks of age. The increases in body weight per 1-wk periods were plotted as a percentage of values for body weight at the start of the week (relative increase in body weight). The relative increase in body weight of the D(+) line was superior to the relative increase in body weight of the D(–) line during the first two weeks after hatching and in the fourth and in the fifth week of age. At the age of three weeks and from the sixth week onwards the relative growth rate was higher in D(–) line. Differences among lines in weekly feed conversion ratios were most striking in the second week of the experiment. Birds from D(–) line had higher (poorer) weekly feed conversion rations during the whole experiment. Cumulative feed conversion ratios of birds from the D(–) and D(+) line were 2.34 and 2.04 kg, respectively. Thus selection for high body weight in chickens, is positively associated with efficiency of feed utilization as already demonstrated the experiment of Mužic (1990). These findings are not in accordance with findings of Siegel and Wisman (1966) who demonstrated that under *ad libitum* feeding there was no difference between high weight and low weight lines in feed efficiency to a fixed age. But when the feed intake of high weight chickens was limited to that of the low weight line counterparts, they utilized feed more effectively, indicating that correlated response for feed consumption masked those for feed conversion. In subsequent generations this difference between lines has increased (O'Sullivan *et al*., 1992).

The purpose of the investigation carried out by Holcman and Bevc (1992) in the fourteenth generation of selection was to determine differences in physical characteristics of eggs produced by two divergently selected lines of chickens at two different ages. External (shape index, egg weight, shell colour, shell weight, shell thickness) and internal (albumen height, Haugh units, yolk colour, blood & meat spots) quality traits were examined in fresh eggs obtained at the age of 39 weeks (group I) and at the age of 43 weeks (group II). At each age 300 eggs per line were collected and analyzed. A set of electronic instruments (Technical Services and Supplies, York, UK) was used to measure egg quality traits. Egg quality values showed differences ($P < 0.05$) in all analysed characteristics. Egg weight, albumen height, Haugh units, yolk colour, shell weight and shape index were better ($P < 0.05$) in D(+) line compared with D(–) line. Additionally, D(+) hens had higher ($P < 0.05$) percent of eggs with blood & meat spots. The D(–) hens have layed eggs with paler ($P < 0.05$) and thicker ($P < 0.05$) shells than did the hens from D(+) line. Internal and shell quality increased ($P < 0.05$) with age with increments in albumen height, Haugh units, yolk colour, shell weight and egg weight.

In the thirty-first generation of divergent selection the actual egg cholesterol content was determined. Hens from both divergently selected lines were fed with a commercial diet for laying hens. Cholesterol level in the yolk was measured using the enzymatic-spectrophotometric method (Boehringer Mannheim, Germany). Ten eggs from each line were analysed when the hens were 49 weeks of age. The egg yolk and egg white were separated, and weighed. Each yolk sample was analysed in duplicate. The mean yolk cholesterol contents (mean ± s.d.) expressed in mg per 100 g of egg yolk mass were 1605.00 ± 127.17 and 1373.39 ± 91.00 in the D(+) and D(–) line, respectively. The average contents of cholesterol per egg were 341.47 ± 32.72 mg in D(+) line and 212.37 ± 17.85 mg in D(–) line. The lines differed ($P < 0.001$) in cholesterol content per 100 gram of yolk and per egg. However most of the variation in cholesterol content per egg was attributable to line differences in egg weight. The D(+) line showed higher ($P < 0.001$) average egg weight (77.24 ± 4.91 g) than the D(–) line (48.42 ± 1.37 g). The average percentages of yolk per egg were 27.6% and 31.9% in the D(+) and D(–) lines, respectively. In comparison to D(–) line, hens from D(+) line produced eggs with higher cholesterol concentrations per 100 g of yolk, which was less pronounced when expressed per egg, due to the low yolk content of the eggs.

In the seventeenth generation of selection, a study was conducted to study the effects of divergent selection for 8-week body weight on body weight gain, dressing percentage, abdominal fatness and chemical composition of the meat (Holcman *et al.*, 1995). Chickens reared on *ad libitum* intake of conventional starter diet (first 14 days) and finisher diet (last 33 days) were placed into cages. Average body weight was determined at day 47 on 45 and 42 animals from lines D(+) and D(–), respectively. Samples from eight-animals per line (four males and four females) were taken to determine chemical composition. Dressing percentage was determined using either traditional carcass weight (carcass together with lungs, kidneys, head, neck, lower parts of legs, giblets and abdominal fat) or grill carcass weight (carcass with lungs and kidneys) as a proportion of body weight. Abdominal fat was used as an indicator of the fat content of the carcass and was calculated as a percentage of body weight. Chickens from the D(+) line had higher ($P < 0.01$) dressing percentages and also higher ($P < 0.001$) share of abdominal fat from live weight than the chickens from the D(–) line. The line difference in traditional dressing percentage was 2.99%, 4.25% in grill dressing percentage and 2.2% in abdominal fatness. These results are consistent with those from Dunnington and Siegel (1996) where high weight chickens had higher percentage of body fat than those from the low weight line. Calabotta *et al*. (1985) pointed out that regardless of feeding state (fasted/nonfasted chickens) lipogenic and lipolytic capacity was higher in low weight chickens than in high weight chickens indicating that fat deposition is more dependent upon lipid degradation than lipid synthesis. Referring to chemical composition of meat from breasts, thighs and drumsticks (with skin), meat from chickens that were selected for a higher body weight contained less moisture ($P < 0.01$) and more fat ($P < 0.01$) in comparison to meat from chickens that were selected for lower body weight. No differences ($P > 0.05$) were found in the content of protein and ash. Thigh and drumstick meat with skin was found to contain 69.9 vs 70.1% moisture, 17.2 vs 17.6% protein, 12.7 vs 10.9% fat and 0.7 vs 0.8% ash in D(+) and D(–) lines, respectively. The breast meat with skin in lines D(+) and D(–) contained 71.5 and 72.8% moisture, 22.0 and 21.5% protein, 4.5 and 4.1% fat, and 1.09 and 1.0% ash, respectively.

In the fifteenth generation of selection an experiment was carried out to evaluate the cock's semen from both selection lines (Holcman *et al.*, 1993). Sperm quality (expressed as progressive motility, percentage of fertility, percentage of hatchability and percentage of morphologically normal/abnormal/dead sperm cells) and sperm quantity (expressed as sperm concentration and semen volume) were the parameters evaluated in each cock. The results showed that ejaculate volume, sperm concentration, mobility, percentage of fertility and percentage of hatchability were higher in the D(–) line than in the D(+) line, values being (mean ± s.d.) $0.27 \pm 0.02$ vs $0.23 \pm 0.08$ ml, $1.81 \times 10^9 \pm 0.41 \times 10^9$ vs $1.69 \times 10^9 \pm 0.69 \times 10^9$ spermatozoa/ml, $4.75 \pm 0.50$ vs $4.0 \pm 1.15$, $86.19 \pm 4.86$ vs $80.15 \pm 8.57$% and $76.56 \pm 10.02$ vs $62.10 \pm 9.20$%. High weight cocks were superior to the low weight cocks in percentage of abnormal and dead spermatozoa, values being $11.57 \pm 2.15$ vs $12.25 \pm 3.30$% and $8.14 \pm 3.08$ vs $8.25 \pm 1.50$%. The results revealed that percentage of hatchability was the only variable ($P < 0.05$) affected by the line. All other variables did not change ($P > 0.05$) in the lines.

Dahmane *et al*. (1995a,b) analysed structural and histochemical characteristics of the muscles *biceps femoris* (BF) and *pectoralis profundus* (PP) in cockerels from the sixteenth generation of divergent selection for 8-week body weight. Seven samples of each line D(+) and D(–) were collected at 3, 6, 9 and 12 weeks of age, respectively. Transverse sections of 10 μm were cut from both muscles and placed onto coverslips for immediate histochemical assay. Quantitative histochemical determination of succinic dehydrogenase activity (SDH) and menadion-linked α-glycerol phosphate dehydrogenase activity (GPDH) were used as estimates of oxidative and glycolytic energy supply, respectively. SDH and GPDH activities were determined by using the histophotometer linked to a personal computer with special software for measuring absorbtion

within muscle fibres. Within each of four age groups fifty muscle fibres per slice were investigated and for each muscle and line under investigation informations about mean SDH and GPDH activities as well mean fibre diameter were obtained. Selection for body weight had no effect on energy metabolism, but it induced an increase in cross sectional areas of muscle fibres. Cockerels from the D(+) line had ($P < 0.05$) larger mean diameter than those from the D(–) line. From comparisons of two chicken lines, one selected for high juvenile (8 week) and high adult (36 week) body weight and the other selected for low juvenile (8 week) and adult (36 week) body weight Remignon *et al*. (1995) concluded that the change in muscle size appears to be in number and size of fibers rather than in myosin isoform profiles. In the D(+) line giant cells were observed which are the sign of morphologic alteration. The SDH and GPDH showed high activity in the third and sixth week of age, whereupon a slight ($P = 0.065$) decrease occured. These changes in enzyme activities were in accordance with animal growth rates which initially (third, sixth week of age) steadily increased and then (nineth, twelveth week of age) declined as the birds moved to their mature live weight. At all four ages the mean fibre diameters were larger ($P < 0.05$) in the *m. pectoralis profundus* compared to the *m. biceps femoris*.

In the eighteenth generation of selection two divergently selected lines of chickens were analysed by DNA profiling using random amplification of genomic DNA (RAPD) pooled from 10 individuals of each line with 200 different 10-base primers (Terčič, 1997). Only one primer was used per reaction. Amplification products obtained with primers UBC-563 (5′-CGCCGCTCCT-3′) and UBC-788 (5′-CCTTCCCTCT-3′), were clearly different between the pools. These two primers were further investigated on single DNA samples to determine the proportion of animals carrying the pool-specific DNA fragments. Primer UBC-788 revealed one (≈ 300 bp) D– line-characteristic RAPD fragment, while UBC-563 amplified two (≈ 1000 bp and ≈ 600 bp) D– line specific RAPD fragments which can be useful markers in the linkage analysis with quantitative trait loci (QTLs) for body weight.

In the twenty-fifth generation of divergent selection for 8-week body weight an experiment was conducted to determine what effect genotype [D(+) versus D(–) line)] has on nitrogen balance. The birds were kept in individual metabolism cages and fed on a practical feed mixture in pellet form which was calculated to contain (expressed on dry matter basis) 12.86 MJ ME/kg, 235.53 g/kg crude protein, 23.34 g/kg crude fat, 26.81 g/kg crude fibre and 64.40 g/kg crude ash. At the beginning of the trial animals were 42 days old, and the trial period lasted for 16 days. The entire trial period was divided on four four-day periods. Nitrogen balance was analysed for 6 males and 6 females from each line. Body-weights were recorded at the end of four-day periods. At the end of each daily collection, excreta were weighed and stored at − 20 C until chemical analyses. The birds were fed once daily on 220 g (D+) and 120 g (D–) of the test diet, a quantity that was not completely consumed. Unconsumed feed was collected for each bird on daily basis and fresh feed was weighed and offered to the birds. The nitrogen content in feed and excreta was determined using the Kjeldahl method and crude protein was calculated as Kjeldahl $N \times 6.25$. Nitrogen balance was calculated as the difference between the nitrogen intake in an individual bird and the total nitrogen excretion. There were differences ($P < 0.05$) between lines in daily nitrogen retention. Animals from the D(+) line retained more nitrogen than animals from the D(–) line. Males retained more nitrogen than females irrespective of line. In the last four-day period D(+) males and females retained less ($P < 0.05$) nitrogen in comparison with the first four-day period, whereas in D(–) males and females this difference was not significant. In chickens 42 to 58 days of age, protein retention expressed in grams per kilogram of body weight decreased from 10.1 to 5.2 g/kg in D(+) males, from 11.1 to 5.1 g/kg in D(+) females, from 16.1 to 6.6 g/kg in D(–) males and from 16.9 to 7.6 g/kg in D(–) females. In all trial periods except in the first period for D(–) females, D(–) chickens showed greater feed nitrogen utilization efficiency than D(+) birds.

## CONCLUSIONS

A divergent selection experiment with chickens, using body weight at eight weeks of age as the selection criterion, was undertaken for 31 generations. A high (D+) line and a low (D–) line were made up in 1979 with 50 females and 10 males each. Despite the fact that the body weight at 8 weeks of age was the only selection criterion variation within and between lines in other production/physiological traits was recorded in some generations and the genetic control of different traits involved was assessed, as well as direct and correlated responses to selection. This paper is a review of experiments conducted over a period of twenty-eight years on abovementioned lines of chickens.

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## REFERENCES

Calabotta, D.F./ Cherry, J.A./ Siegel, P.B./ Jones, D.E. Lipogenesis and lipolysis in fed and fasted chickens from high and low body weight lines. Poult. Sci., 64(1985), 700–704.

Dahmane, R./ Holcman, A./ Eržen, I. The energy metabolism and diameter of muscle fibres in chicken from two-way selection for body weight. Zb. Bioteh. Fak. Univ. Ljubl., Kmet. Zooteh., 66(1995a), 53–62.

Dahmane, R./ Holcman, A./ Eržen, I. Influence of growth on the energy metabolism and diameter of chicken skeletal muscle fibres. Zb. Bioteh. Fak. Univ. Ljubl., Kmet. Zooteh., 66(1995b), 63–77.

De Witt, F./ Schwalbach, L.M.J. The effect of egg weight on the hatchability and growth performance of New Hampshire and Rhode Island Red chicks. S. Afr. J. Anim. Sci., 34(2004)Suppl. 2, 62–64.

Dunnington, E.A./ Siegel, P.B. Long-term divergent selection for eight-week body weight in White Plymouth Rock chickens. Poult. Sci., 75(1996), 1168–1179.

Holcman, A. Two way selection for body weight in chickens. Zb. Bioteh. Fak. Univ. Edvarda Kardelja Ljubl., Kmet. Živin., 48(1986), 121–167.

Holcman, A. Differences in growth of chickens of two strains from two-way selection for body weight. Zb. Bioteh. Fak. Univ. Ljubl., Kmet. Živin., 60(1992), 61–72.

Holcman, A./ Bevc, B.1992. The influence of two-way chicken mass selection on some physical egg-characteristics. Zb. Bioteh. Fak. Univ. Ljubl., Kmet. Živin., 60(1992), 1–19.

Holcman, A./ Fabijan, R./ Kovač, M. The effect of the two-way selection on the mass of 56 days old chickens to some reproduction characteristics of cooks. Zb. Bioteh. Fak. Univ. Ljubl., Kmet. Zoot., 62(1993), 143–151.

Holcman, A./ Žlender, B./ Kmecl, A. Dressing percentage and the chemical components of meat of chickens from two-way selection for body weight. Zb. Bioteh. Fak. Univ. Ljubl., Kmet. Zooteh., Supplement 22(1995), 219–223.

Marks, H. Long-term selection for body weight in Japanese quail under different environments. Poult. Sci., 75(1996), 1198–1203.

Mužic, S. Hranidbeno, fiziološko-anatomske karakteristike dviju u suprotnom smjeru selekcioniranih linija tovnih pilića. Doctoral dissertation. Agronomski fakultet Sveučilišta u Zagrebu, 1990, 153 str.

Nestor, K.E./ Bacon, W.L./ Anthony, N.B./ Noble, D.O. Divergent selection for body weight and yolk precursor in Coturnix coturnix japonica. 10. Response to selection over thirty generations. Poult. Sci., 75(1996), 303–310.

O'Sullivan, N.P./ Dunnington, E.A./ Larsen, A.S./ Siegel, P.B. Correlated responses in lines of chickens divergently selected for 56-day body weight. 2. Organ growth, DNA, RNA and protein content. Poult. Sci., 71(1992), 598–602.

Remignon, H./ Gordahaut, M.F./ Marche, G./ Ricard, F.H. Selection for rapid growth increase number and the size of muscle fibres without changing their typing in chickens. J. Muscle Res. Cell Motility, 16(1995), 95–102.

Siegel, P.B./ Wisman, E.L. Selection for body weight at 8 weeks of age. 6. Changes in appetite and feed utilization. Poult. Sci., 45(1996), 1391– 1397.

Terčič, D. Identification of genetic markers for body weight in chicken (*Gallus domesticus*). Master of science thesis. Domžale, Biotehniška fak., Odd. za zootehniko, 1997, 131 p.

Terčič, D./ Flisar T./ Malovrh, Š./ Kovač, M./ Holcman, A. Response to divergent selection for eight week body weight in chickens. In: Book of abstracts: supplement of the World's Poult. Sci. J. (World's Poultry Science Journal, Supplement). Verona, World's Poult. Sci. Assoc., 2006, 215.

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# UGOTAVLJANJE PRISOTNOSTI EKOTIPOV KRANJSKE ČEBELE (*Apis mellifera carnica* Pollman) V SLOVENIJI NA PODLAGI RAZLIK V OŽILJENOSTI PREDNJIH KRIL

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## IZVLEČEK

Kranjska čebela (*Apis mellifera carnica* Pollman 1879) v Sloveniji, je bila v preteklosti na podlagi morfoloških kriterijev razdeljena na več skupin oz. ekotipov, česar pa genetska analiza ni potrdila. Zaradi neenotnih rezultatov smo populacijo kranjske čebele v Sloveniji še enkrat preučili, tokrat na podlagi ožiljenosti kril. Pregledali smo 273 vzorcev čebel iz Slovenije, za primerjavo pa smo uporabili še 68 vzorcev čebel iz Hrvaške, Češke, Grčije in nekaterih selekcioniranih linij. Na vsakem krilu smo označili 19 točk, na osnovi katerih smo izmerili in izračunali 37 značilnosti. Analizirali smo slovensko populacijoin jo primerjali z vzorci čebel iz tujine. Razlike med slovenskimi in ostalimi skupinami vzorcev so bile statistično značilne, razlike znotraj populacije v Sloveniji pa ne. Med skupinami vzorcev smo ugotovili razlike na podlagi diskriminantne analize, analize glavnih komponent in analize variance. Tudi meritve kubitalnega indeksa so nakazovale razlike med skupinami. Enak pristopi pri analizi populacije čebel v Sloveniji ni podprl teze o obstoju različnih ekotipov. Na podlagi rezultatov sklepamo, da je populacija čebel v Sloveniji homogena in kot taka ne nudi dodatne variabilnosti, ki bi jo bilo mogoče uporabiti za selekcijske ali ohranitvene namene.

Ključne besede: čebele / kranjska čebela / *Apis mellifera carnica* / ekotipi / ožiljenost / krila / Slovenija

# CARNIOLAN BEE (*Apis mellifera carnica* Pollman) POPULATION DEFINITION AS BASED ON DIFFERENCE IN WING VENATION STRUCTURE

## ABSTRACT

Based on morphology of Carniolan bee (*Apis mellifera carnica* Pollman 1879) the population in Slovenia has been divided in more subgroups or ecotypes, but these differences could not be vonfirmed using genetic methods. Therefore one more time Slovenian population of honeybees were studied, this time based on wing venation analysis. Two hundred seventythree samples from Slovenia and 68 samples from Croatia, Czech Republic, Greece including some selection lines from Austria, Germany, Poland and France were used. On each wing 19 points were marked from which 37 wing characteristics were measured and calculated. Slovenian population of honeybees were analyzed and compared with other groups. Differences, based on discriminant and PCA analysis as well as analysis of variance were found between groups. Differences between groups were also confirmed using measurements of cubital index. On the other hand, the same approach did not reveal any differences within Slovenian populations. According to our results we can conclude that Slovenian population of honeybees is homogenous and as such does not offer additional variability which coulnd be exploited for selection or preservation purposes.

Key words: bees / Carniolan bee / *Apis mellifera carnica* / ecotypes / wing venation / Slovenia

## UVOD

Medonosna čebela (*Apis mellifera*) je prvotno naseljevala Afriko, Evropo in Bližnji ter Srednji vzhod. Med populacijami so se izoblikovale velike morfološke razlike predvsem zaradi raznolikosti okolja, ki so ga naseljevale. Na osnovi morfoloških raziskav je Ruttner s sod. (1978) in Ruttner (1988) opisal 24 podvrst, ki jih je glede na njihov izvor uvrstil v tri različne filogenetske linije: afriško (A), severno-zahodnoevropsko (M) in jugo-vzhodnoevropsko (C). Tej razvrstitvi so Arias in Sheppard (1996), Franck in sod. (2000) ter Palmer in sod. (2000) dodali še linijo O, ki naj bi bila prisotna na Bližnjem in Srednjem vzhodu (pregl. 1). Poleg linij A, C, M in O se po navedbah Francka in sod. (2001) pojavlja tudi linija Y, ki se razprostira na območju Etiopije.

Preglednica 1. Podvrste vrste *Apis mellifera*
Table 1. *Apis mellifera* subspecies

| Centralnosredozemska in jugovzhodna evropska skupina (C) | Bližnjevzhodna skupina (O) | Zahodnosredozemska in severozahodnoevropska skupina (M) | Afriška skupina (A) |
|---|---|---|---|
| *sicula* | *adami* | *iberica* | *adansonii* |
| *ligustica* | *anatoliaca* | *intermissa* | *capensis* |
| *cecropia* | *armeniaca* | *mellifera* | *lamarckii* |
| *macedonica* | *caucasica* | *sahariensis* | *litorea* |
| *carnica* | *cypria* | | *monticola* |
| | *meda* | | *scuteliata* |
| | *syriaca* | | *unicolur* |
| | | | *yemeniticia* |

### Kranjska čebela

Kranjsko čebelo oz. kranjsko sivko (*Apis mellifera carnica* Pollman 1879) uvrščamo v jugo-vzhodnoevropsko skupino čebel. Je čebela z izrazito dolgim rilčkom in v primerjavi z drugimi podvrstami precej temna. Na oprsju ima rjavkaste dlačice, zadkovi obročki pa so usnjeno rjave barve z včasih nakazanimi svetlejšimi pegami na prvem in drugem obročku. Med čebelarji je zelo priljubljena, saj je druga najbolj razširjena podvrsta čebel na svetu, takoj za italijansko čebelo *A. m. ligustica*. Glavna odlika kranjske čebele je njena mirnost. Poleg tega je znana po delavnosti, dolgoživosti, izkoriščanju paše, dobrem prezimovanju in tudi po skromni porabi zimske zaloge hrane. V primerjavi z drugimi podvrstami zelo dobro izkoristi pelodno pašo (Ruttner, 1988). Zaradi svojih lastnosti so jo velikokrat uporabili pri selekciji novih linij čebel.

Njeno izvorno območje je opredeljeno severno in južno od Karavank, na obeh straneh meje med Avstrijo in Slovenijo (Ruttner, 1988). Podvrsta se je oblikovala po koncu zadnje ledene dobe pred približno 10.000 leti. Celotna populacija kranjske čebele je na podlagi morfoloških znakov razdeljena na tri večje skupine (Ruttner in Hänel, 1992):

- alpska (Slovenija, Avstrija, Slovaška),
- panonska (Madžarska, Romunija),
- mediteranska (Hrvaška, Bosna in Hercegovina, Srbija, Črna Gora).

Ker se Slovenija nahaja na stičišču vseh treh skupin, je Poklukar (1998) na podlagi morfoloških znakov (kubitalni indeks, dolžina rilčka, dolžina goleni) preučil populacijo čebel v

Sloveniji in celotno populacijo razdelil na tri večje skupine oz. ekotipe: panonski, alpski in dinarski. Ozemlje ob italijanski meji je označil kot nedefinirano območje zaradi mešanja kranjske čebele z italijansko čebelo. Da bi meje med ekotipi natančneje določili so Sušnik in sod. (2004) celotno populacijo preučili še na podlagi genetskih markerjev, vendar razlik niso našli.

Ostaja torej temeljno vprašanje, ali med populacijo kranjske čebele v Sloveniji obstajajo opisane razlike in s tem ekotipi ali ne? Če so ekotipi prisotni, jih je potrebno določiti in zavarovati, da se bodo ohranili tudi v bodoče. Poleg tega lahko nudijo tudi dodatno variabilnost, ki bi jo bilo mogoče uporabiti v selekcijske namene. Obstaja domneva, da smo tovrstno pestrost v zadnjih letih s prekomernim mešanjem genetskega materiala, zlasti z nenadzorovanim razpošiljanjem mladih matic od vzrejevalcev do čebelarjev, že izgubili. V naši raziskavi smo celotno polulacijo kranjske čebele še enkrat analizirali, tokrat na podlagi ožiljenosti kril.

**Ožiljenost kril**

Za ločevanje podvrst čebel so najprej uporabljali barvo čebel, ki je najbolj očitna lastnost. Kasneje se je uveljavilo merjenje določenih morfoloških lastnosti, s tem pa natančnejše razvrščanje podvrst čebel. Analizo ožiljenosti kril je vpeljal Goetze (1940), pristop pa je pogosto uporabljen v večini vzrejnih in ohranitvenih programov vsake že določene podvrste (Leclercq, 1999), saj z njo enostavno dokažemo pripadnost določeni podvrsti. Na krilih lahko merimo in izračunavamo številne lastnosti, med katerimi so najbolj informativne in največkrat uporabljene naslednje lastnosti: 10 kotov na žilnem sistemu (A4, B4, D7, E9, G18, J10, J16, K19, L13, O26), razdalje a, b, c, in d ter kubitalni indeks (Kauhausen-Keller, 1994; Nazzi, 1992).

V naši raziskavi smo za preučevanje populacije kranjske čebele v Sloveniji uporabili analizo ožiljenosti kril, ker menimo, da, če znotraj populacije razlike in ekotipi obstajajo, jih bi s to metodo lahko identificirali. Ta pristop je malenkostno drugačen od tistega, ki ga je uporabil Poklukar (1998), vendar na nek način predstavlja nadgradnjo njegove raziskave. Z našimi rezultati želimo populacijo kranjske čebele v Sloveniji prikazati v dodatni luči in skupaj z ostalimi sorodnimi raziskavami postaviti osnovo za nadaljne raziskave populacije kranjske čebele pri nas. Rezultati so pomembni tudi za nadaljno organizacijo selekcije kranjske čebele, kot tudi za vse čebelarje, ki bi morali morebiten obstoj haplotipov ohranjati v bodoče.

Preglednica 2. Lokacije vzorčenja in število analiziranih vzorcev
Table 2. Sampling locations and numbers of analysed honeybees

| Podvrsta<br>Subspecies | Lokacija vzorčenja<br>Sampling locations | Št. vzorcev<br>No. of samples |
|---|---|---|
| *A. m. carnica* | Slovenija | 273 |
| *A. m. carnica* | Hrvaška | 13 |
| *A. m. macedonica* | Grčija | 20 |
| *A. m. carnica* | Češka | 10 |
| Selekcionirane linije kranjske čebele | 1 Hohen Neuendorf (Nemčija) | 5 |
| | 2 Buckfast (Nemčija) | 5 |
| | 3 Polen (Poljska) | 5 |
| | 4 K111 (Avstrija) | 5 |
| | 5 Toulouse (Francija) | 5 |

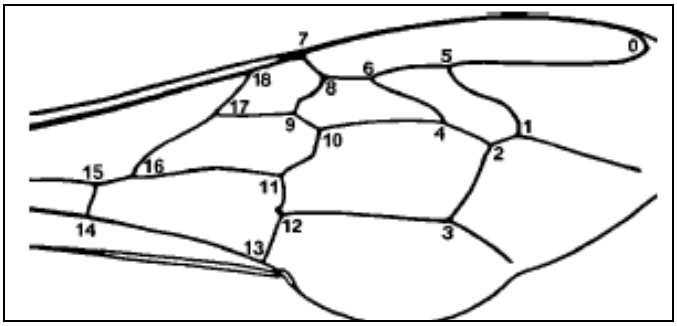
## MATERIAL IN METODE

### Vzorčenje

V raziskavo smo vključili 273 vzorcev čebel iz Slovenije. Vsak vzorec je predstavljal eno družino, razdalja med posameznimi vzorci je bila najmanj 2 km. Pri zbiranju vzorcev smo upoštevali, da čebelar čebelari z najmanj 10 čebeljimi družinami in da v zadnjih treh letih ni kupil čebel ali matic. Za primerjavo smo uporabili še 68 vzorcev s Hrvaške, Grčije, Češke, ter selekcioniranih linij kranjske čebele iz Poljske, Avstrije, Nemčije in Francije (pregl. 2). Vzorci so bili zbrani v letu 2001 in so bili do pričetka analize shranjeni na temperaturi –80 ºC, oz. v 96 % etanolu.

### Priprava kril in merjenje

Desno prednje krilo smo pri bazi odrezali od toraksa in ga suho preparirali na prozorno folijo ter prenesli v digitalno obliko. Slike smo v programu Slikar za Windows povečali in jih pripravili za merjenje z računalniškim programom Beewings 1.20. S programom smo na vsakemu krilu označili 19 stičišč žilc, v predpisanem vrstnem redu (slika 1). Program je na podlagi razdalj izračunal 37 meritev za vsako krilo (pregl. 3). Od vseh opisanih metod za merjenje lastnosti kril smo izbrali metodo, ki jo uporabljajo v selekcijskem centru Brno na Češkem. (Morphometrical analysis … of honey bees by wing characters, 2000).




Slika 1. Merjene točke na žilnem sistemu (Kauhausen in Keller, 1994).
Figure 1. Measured points on the wing venation (Kauhausen in Keller, 1994).

### Statistična obdelava

Pridobljene podatke meritev posameznih morfoloških lastnosti smo iz programa BeeWings prenesli v program Excel v okolju Windows, ter jih pripravili za statistično analizo. Statistično obdelavo podatkov smo opravili s statističnim programom STATGRAPH ver. 15.2. Najprej smo primerjali celotno populacijo kranjske čebele v Sloveniji s skupinami čebel od drugod. V ta namen smo uporabili diskriminantno analizo, s katero smo želeli v naprej določene skupine vzorcev čim bolje ločiti med sabo. Uporabili smo še analizo glavnih komponent (PCA analiza), in analizo variance. Za vsako skupino smo izračunali še kubitalni indeks.

Nato smo analizirali populacijo čebel v Sloveniji. Za izhodišče smo uporabili razmejitveno karto treh ekotipov pri nas (Poklukar, 1998). Najprej smo vse nabrane vzorce v Sloveniji razdelili na štiri skupine, glede na lokacijo, kjer so bili vzorci nabrani. V skupino 1 smo uvrstili vzorce nabrane na območju opisanega panonskega ekotipa, v skupino 2 nabrane vzorce na območju opisanega alpskega ekotipa in v skupino 3 nabrane vzorce na območju opisanega

dinarskega ekotipa. V četrto skupino smo uvrstili vzorce, ki so bili na nedefiniranem območju in na mejnih območjih med ekotipi. Prve tri skupine smo nato primerjali med sabo z diskriminantno analizo, analizo glavnih komponent (PCA) ter analizo variance. Prav tako smo za vse tri skupine izračunali kubitalni indeks. Da bi preverili, ali se skupine vzorcev grupirajo še kako drugače, smo naredili še analizo uvrščanja v skupine (cluster analysis).

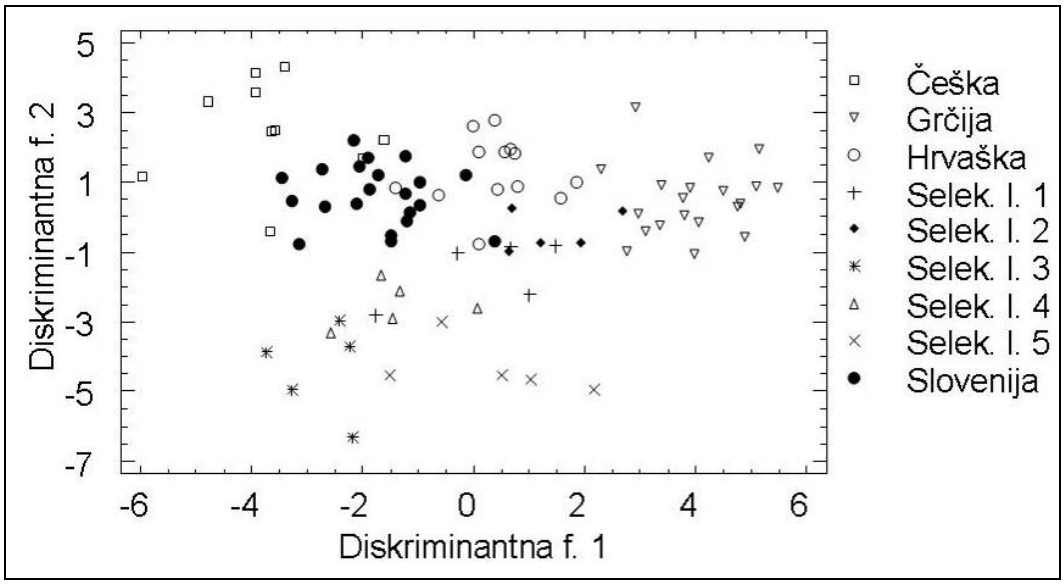
Preglednica 3. Značilnosti, merjene na krilih in točkovni sistem, ki smo ga uporabili pri izračunih
Table 3. Wing characteristics and point system used for calculations

| KOTI angles | Točke points | KOTI angles | Točke points | Indeksi index | Točke points |
|---|---|---|---|---|---|
| A1 | 2,1,4 | M17 | 7,8,18 | Kubitalni | 2,4/1,2 |
| A4 | 4,1,5 | N23 | 9,18,17 | prekubitalni | 4,9/8,10 |
| B3 | 1,4,3 | O26 | 15,14,16 | dumb-dell | 1,4/5,6 |
| B4 | 1,4,5 | Q21 | 11,16,17 | radialni | 0, 7, 3 |
| D7 | 4,3,13 | DOLŽINE length | Točke points | Celice Cells | Točke points |
| E9 | 6,5,10 | Radialna | 0,7 | Cub. c. 1 | |
| G7 | 3,13,4 | A | 2,4 | Cub. c. 2 | |
| G18 | 12,13,14 | B | 1,2 | Cub. c. 3 | |
| H12 | 11,10,12 | C | 3,4 | Disk. c.1 | |
| J10 | 6,9,10 | D | 11,15 | Disk. c.2 | |
| J16 | 8,9,18 | Notranja d. | 1,14 | Bra. c. | |
| K19 | 12,11,14 | Notranja š. | 7,13 | Bra. c. 1 | |
| L13 | 5,7,6 | diskoidalni odklon | 0, 7, 3 | površina c. Area6 | 1,2,3,12,13,14, 15,16,17,18,7,6,5, |

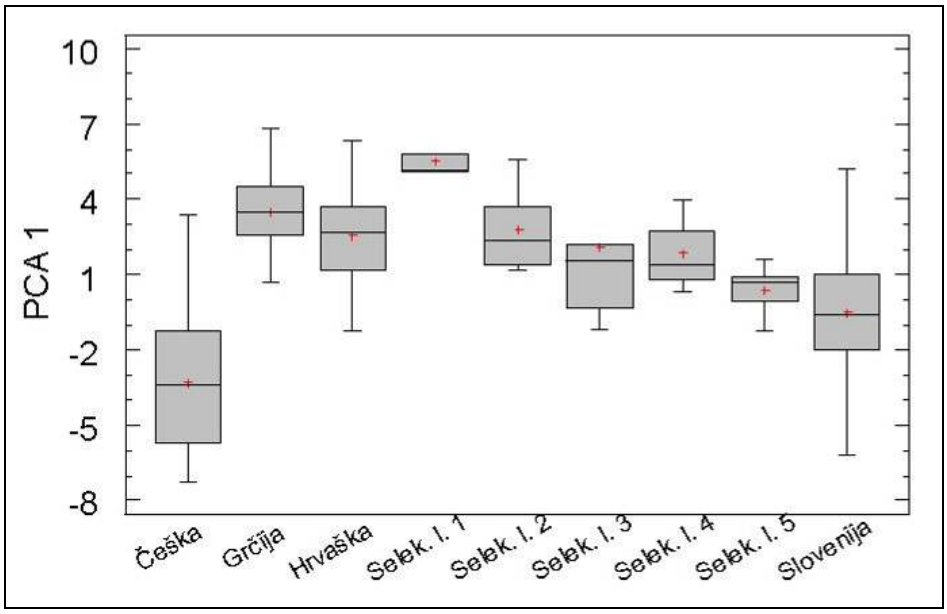
## REZULTATI

Z diskriminantno analizo smo variabilnost vseh 37 izračunanih lastnosti preračunali v osem diskriminantnih funkcij, od katerih prve štiri statistično značilno ($p < 0,05$) delijo analizirane skupine vzorcev, ostale pa ne. Vrednosti za prvi dve spremenljivki smo uporabili tudi za prikaz vzorcev čebel v dvodimenzionalnem grafu (slika 2). Iz njega je razvidno, da vzorci posameznih skupin tvorijo skupine, ki se med sabo delno prekrivajo. Še najbolj se od drugih skupin vzorcev razlikujejo vzorci iz Grčije. Slovenskim vzorcem so najbližji vzorci iz Hrvaške, pa tudi vzorci iz Češke. Vzorci iz selekcioniranih linij so razporejeni malo stran od ostalih in kažejo na določeno medsebojno sorodnost.

V nadaljevanju smo maredili analizo glavnih komponent s katero smo variabilnost vseh 37 lastnosti skrčili na štiri, ki skupaj pojasnujejo več kot polovico variabilnosti (51,04 %). Prvo komponento, ki pojasnuje 20,31 % variabilnosti smo izrisali tudi na grafu (slika 3). Iz njega je razvidno, da razlike med skupinami obstajajo in da se od ostalih vzorcev najbolj razlikujejo vzorci iz Češke. Izstopajo tudi vzorci iz selekcionirane linije 1. Ta skupina vzorcev je na grafu izrisana najvišje, zanjo pa je značilno tudi, da ima najmanjši razpon variabilnosti. Variabilnost, ki jo je moč oceniti iz grafa je manjša pri selekcioniranih linijah kot pri ostalih skupinah vzorcev.




Slika 2. Korespondenčna analiza vzorcev čebel iz različnih virov.
Figure 2. Correspondence analysis of honey bee samples from different origin.




Slika 3. Razlike med skupinami vzorcev na podlagi prve komponente PCA analize.
Figure 3. Differences between groups based on the first component of PCA analysis.

Iz štirih novih izračunanih komponent smo naredili še analizo variance, s katero smo izračunali razlike med skupinami in preverili ali se le te statistično razlikujejo med sabo (pregl. 4). Iz dobljenih rezultatov smo ugotovili, da se vzorci čebel iz Slovenije statistično razlikujejo od vseh ostalih skupin vzorcev, razen od skupine vzorcev selekcionirane linije 5. Tudi češki vzorci se statistično razlikujejo od vseh ostalih skupin vzorcev, razen od skupine vzorcev iz Hrvaške. Selekcionirane linije se med sabo statistično značilno ne razlikujejo, le vzorci iz selekcionirane linije 1 se razlikujejo od linij 3, 4 in 5.

Za vsako skupino vzorcev smo izračunali še kubitalni indeks in ga primerjali med skupinami (pregl. 5). Ugotovili smo, da imajo najmanjši kubitalni indeks vzorci iz Grčije. Sledijo jim vzorci iz Hrvaške in komercialne linije 2 ter vzorci iz Slovenije. Ostali vzorci imajo večji kubitalni

indeks (od 2,82 do 3,07). Zanimiv je predvsem rezultat vzorcev čebel iz Češke, pri katerih smo izračunali presenetljivo visok kubitalni indeks in pri čebelah selekcionirane linije 2, pri katerih smo izračunali občutno manjšo vrednost kubitalnega indeksa kot pri ostalih linijah. Najnižjo standardno deviacijo smo izračunali za vzorce iz selekcionirane linije 2, najvišjo pa za vzorce iz selekcionirane linije 3, ki je bila selekcionirana na Poljskem.

Preglednica 4. Razlike med skupinami vzorcev na podlagi analize variance (* – statistično značilno; ns – ni statistično značilno)
Table 4. Differences between groups based on variance analysis (* – statistical significant; ns – not statistical significant)

| | Slovenija | Hrvaška | Češka | Grčija | Sel. l. 1 | Sel. l. 2 | Sel. l. 3 | Sel. l. 4 | Sel. l. 5 |
|---|---|---|---|---|---|---|---|---|---|
| Slovenija | | * | * | * | * | * | * | * | ns |
| Hrvaška | 3,03 | | * | ns | * | ns | ns | ns | ns |
| Češka | –2,77 | –5,80 | | * | * | * | * | * | * |
| Grčija | 4,01 | 0,98 | –6,78 | | ns | ns | ns | ns | * |
| Sel. l. 1 | 6,01 | –2,98 | –8,78 | –2,00 | | ns | * | * | * |
| Sel. l. 2 | 3,33 | –0,30 | –6,10 | 0,68 | 2,68 | | ns | ns | ns |
| Sel. l. 3 | 2,59 | 0,44 | –5,36 | 1,42 | 3,42 | 0,74 | | ns | ns |
| Sel. l. 4 | 2,34 | 0,69 | –5,11 | 1,67 | 3,67 | 0,99 | 0,25 | | ns |
| Sel. l. 5 | 0,88 | 2,15 | –3,65 | 3,14 | 5,13 | 2,45 | 1,71 | 1,46 | |

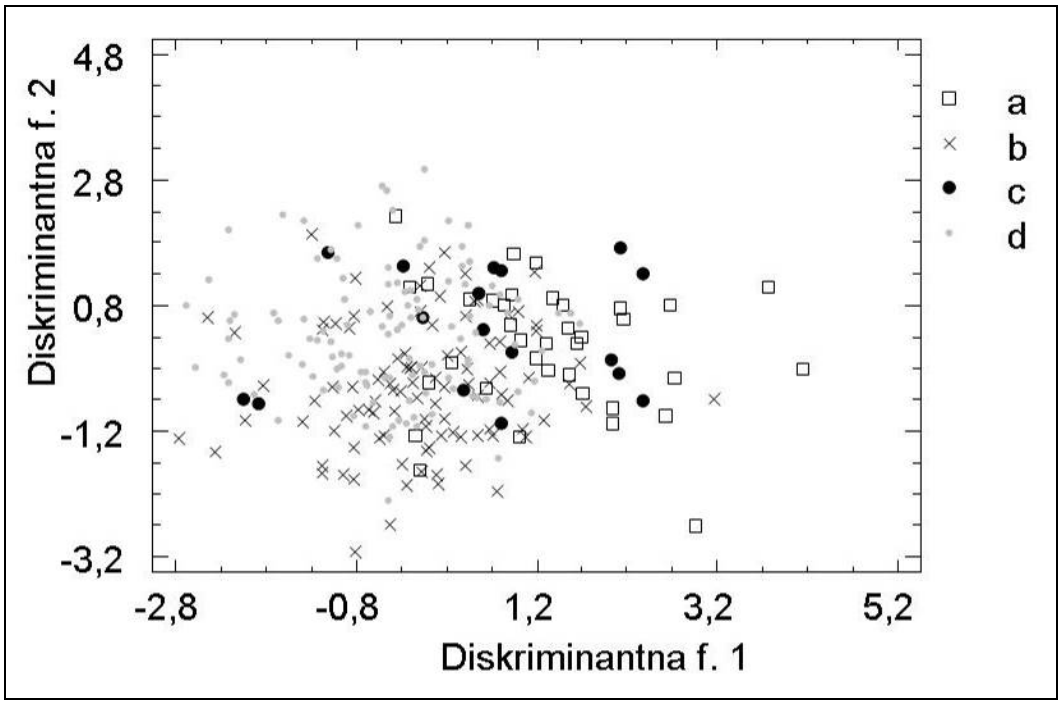
Preglednica 5. Vrednosti kubitalnega indeksa za analizirane vzorce skupin
Table 5. Values of cubital index for analysed samples groups

| Podvrsta<br>Subspecies | Lokacija vzorčevja<br>Sample orign | Kub. indeks<br>Cub. index | Stand. dev.<br>Stand. dev. |
|---|---|---|---|
| *A. mellifera carnica* | Slovenija | 2,53 | 0,40 |
| *A. mellifera carnica* | Hrvaška | 2,46 | 0,51 |
| *A. mellifera carnica* | Češka | 3,07 | 0,67 |
| *A. mellifera macedonica* | Grčija | 2,34 | 0,50 |
| Selekcionirane linije kranjske čebele | 1 Hohen Neuendorf (Nemčija) | 2,98 | 0,53 |
| | 2 Buckfast (Nemčija) | 2,50 | 0,20 |
| | 3 Polen (Poljska) | 2,82 | 0,83 |
| | 4 K111 (Avstrija) | 2,85 | 0,56 |
| | 5 Toulouse (Francija) | 3,07 | 0,54 |

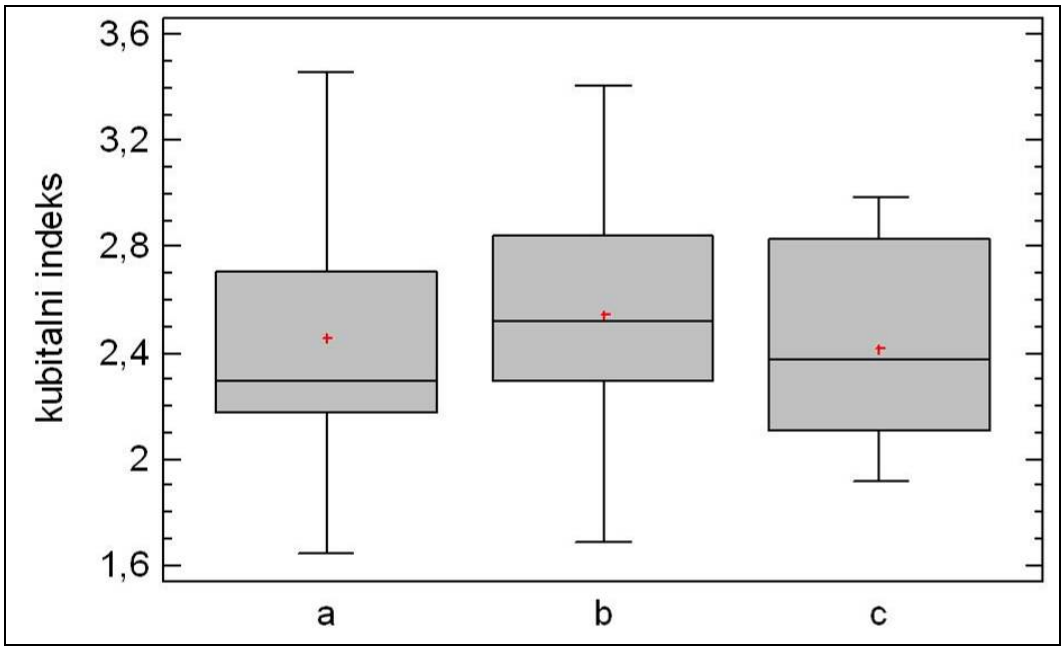
**Rezultati analize slovenske populacije čebel**

Z diskriminantno analizo smo variabilnost vseh 37 izračunanih lastnosti preračunali v tri diskriminantne funkcije, od katerih le prva statistično značilno ($p < 0,05$) deli analizirane, v naprej določene skupine vzorcev. Vrednosti za prvi dve spremenljivki smo uporabili tudi za prikaz vzorcev v dvodimenzionalnem grafu (slika 4). Iz njega je razvidno, da vzorci posameznih skupin sestavljajo skupine, ki med sabo niso ločene ampak se prekrivajo.

Tudi analiza variance med skupinami ni pokazala statističnih razlik med skupinami. V nadaljevanju smo med skupinami primerjali še vrednosti kubitalnih indeksov, za katere smo ugotovili, da razlike med njimi niso statistično značilne. Srednje vrednosti ter standarna deviacija so podane v pregl. 6 in na sliki 5.




Slika 4. Korespondenčna analiza slovenskih vzorcev čebel, razdeljenih v 4 skupine.
Figure 4. Correspondence analysis of Slovenian honey bee samples divided in 4 groups.




Slika 5. Grafični prikaz vrednosti kubitalnega indeksa za tri skupine vzorcev (a, b, c).
Figue 5. Box plot graph for values of cubital index for three groups (a, b, c).

S klastrsko analizo smo vzorce razdelili v tri in enkrat v dve novi skupini. Uporabili smo Wardovo metodo in kvadrirano evklidsko razdaljo. Po natančnem pregledu smo posamezne vzorce razdelili v skupine. Po izrisu posamičnih vzorcev na karti Slovenije smo ugotovili, da so le-ti pomešani in da med njimi ni mej, ki bi jih razločevale.

Preglednica 6. Število vzorcev, srednja vrednost ter standardna deviacija za kubitalni indeks treh skupin

Table 6. Number of samples, mean value and standard deviations for cubital index for three groups

| Skupina<br>Group | Št.vzorcev<br>No. of samples | Srednja v.<br>Medium value | St. deviacija<br>St. deviation |
|---|---|---|---|
| a | 37 | 2,46 | 0,43 |
| b | 102 | 2,55 | 0,39 |
| c | 17 | 2,42 | 0,38 |

## RAZPRAVA IN SKLEPI

Razlike, ki smo jih ugotovili med slovensko populacijo čebel in ostalimi skupinami, so bile v večji meri pričakovane. Z diskriminantno analizo smo skupine vzorcev ločili med sabo, vendar med njimi nismo našli jasnih razlik. Ugotovili smo, da se od ostalih skupin jasno razlikujejo le grški in češki vzorci, ostale skupine pa se medsebojno ne ločijo jasno. Vzorci čebel selekcioniranih linij so na korespondenčnem grafu malenkostno zamaknjene. Na temelju tega domnevamo, da gre za linije, ki so nastale z mešanjem ostalih populacij in da so si na določen način te linije sorodne. Na grafu predstavljena prva komponenta analize glavnih komponent kaže določeno odstopanje vzorcev iz Češke in vzorcev selekcionirane linije 1, pri kateri je tudi variabilnost znotraj skupine najmanj izražena. Skupine selekcioniranih linij so pričakovano manj variabilne kot ostale skupine, ki predstavljajo večje populacije.

Na podlagi statističnih razlik med skupinami smo ugotovili, da se na podlagi ožiljenosti kril od ostalih skupin najbolj razlikujejo vzorci iz Češke. Češki čebelarji čebelarijo večinoma s kranjsko čebelo avstrijskega in slovenskega izvora ter z deželno čebelo, ki je vmesna oblika med kranjsko in avtohtono temno čebelo (*A. m. mellifera*) (Poklukar, 1999). Zaradi prisotnosti njihove temne čebele lahko razlagamo tolikšno razliko do ostalih skupin vzorcev. Slovenski vzorci se statistično razlikujejo od skupine vzorcev iz Hrvaške, kakor tudi od vseh ostalih, razen od selekcionirane linije 5. Razlika med skupino vzorcev iz Slovenije in Hrvaške je presenetljiva, saj gre za isto populacijo čebel. Tudi na podlagi genetskih raziskav niso bile ugotovljene razlike (Sušnik in sod., 2004). Selekcionirane linije se statistično ne razlikujejo med sabo, le selekcionirana linija 1 se loči od linij 3, 4 in 5.

Rezultati analize kubitalnega indeksa so pokazali, da med vsemi skupinam vzorcev obstajajo določene razlike. Najmanjši kubitalni indeks smo izračunali za vzorce čebel iz Grčije. Tudi Cermak (1999) navaja, da ima ta podvrsta čebel od kranjske čebele nižjo vrednost kubitalnega indeksa (2,59). Za kranjsko čebelo podaja vrednost 2,83, kar je višja vrednost kot smo jo izračunali v naši raziskavi (2,53). Podobno vrednost navaja tudi Poklukar (1998) v svojem poročilu (2,44). Zanimiv je rezultat za vzorce iz selekcionirane linije 2, ki izvira iz Nemčije. Vrednost kubitalnega indeksa je blizu vrednosti, ki smo jo izračunali za populacijo čebel iz Slovenije in Hrvaške zaradi česar sklepamo, da je v tej liniji prisotnih nekaj genov kranjske čebele iz tega območja. Rezultat podpira tudi dejstvo, da so nekateri slovenski trgovci v obdobju velike trgovine s čebelami v Nemčijo prodali več družin kranjske čebele (Zaletel, 1998), kjer je sicer značilna podvrsta *A. m.* mellifera.

Za vzorce čebel iz Češke smo izračunali največjo vrednost kubitalnega indeksa (3,07) prav tako kot tudi za vzorce čebel iz selekcionirane linije 5, ki izvira iz Francije. Standardna deviacija je bila v enakih mejah (0,67 in 0,54) iz česar sklepamo, da je v tej liniji prisotnih veliko genov češkib čebel. Za ostale tri selekcionirane linije čebel smo izračunali vrednosti med 2,85 in 2,89,

kar nam nakazuje, da so si te tri linije med sabo bolj sorodne, pa tudi, da bi lahko predstavljale križance med kranjsko čebelo, ki je prisotna pri nas in tisto, ki je prisotna na Češkem.

Rezultati diskriminantne analize za vzorce nabrane v Sloveniji niso nakazali delitev v več podskupin ali ekotipov. Že razdelitev vzorcev na podlagi diskriminantne analize je nakazovala na homogeno populacijo brez večjih razlik, saj so bili vzorci zgoščeni v sredini (slika 3). Izračunane razlike med vnaprej določenimi skupinami se niso izkazale za dovolj velike, da bi med njimi našli pomembnejše razlike. Tudi s ponovno razdelitvijo vzorcev v nove skupine, s pomočjo analize uvrščanja v skupine nismo našli razlik med dobljenimi skupinami, ki bi nakazovale delitev vzorcev v skupine, ki bi odražale geografsko porazdelitev v Sloveniji. Malenkostne razlike smo dobili le pri izračunavanju vrednosti za kubitalni indeks v naprej določenih skupinah, vendar le te niso bile statistično značilno različne. Tudi nobena od drugih analiziranih lastnosti kril na podlagi analize variance ni izkazala statističnih razlik med skupinami. Iz rezultatov meritev kubitalnega indeksa je razvidno le, da so vzorci čebel iz tretje skupine imeli nižjo vrednost, vzorci iz druge skupine pa najvišjo vrednost. Tudi iz Poklukarjevih rezultatov (Poklukar 1998) je razviden enak trend med alpskim (2,47), dinarskim (2,39) in panonskim (2,43) ekotipom. Vrednosti standardne deviacije so pri obeh analizah podobne. Naši rezultati se skladajo z genetsko analizo, ki jo je opravila Sušnik s sod. (2004) in ne podpira delitve populacije v več ekotipov.

Na podlagi dobljenih rezultatov sklepamo, da je populacija kranjske čebele v Sloveniji enotna. Menimo, da znotraj nje ni podskupin, ki bi nakazovale prisotnost krajevnih ekotipov. Rezultati potrjujejo hipotezo, da je populacija kranjske čebele v Sloveniji homogena in kot taka ne nudi dodatne regijske variabilnosti, ki bi lahko bila uporabljena v selekcijske namene. Rezultati ne izključujejo dejstva, da so pred časom obstajali krajevni tipi čebel, ki pa so se lahko v zadnjih desetletjih zaradi prekomernega mešanja genetskega materiala znotraj Slovenije izgubili.

## SUMMARY

*Apis mellifera* is highly polytypic species. Based on morphometrics, 24 recognized subspecies in the Old World can be grouped in four evolutionary lineages. Carniolan honey bee, *Apis mellifera carnica* Pollman 1879, is one of the subspecies of the C phylogenetic lineage (sub group of the South Eastern honey-bee group). It is native to Slovenia, former Yugoslavia, Austria (south of the Alps), and parts of Hungary, Romania and Bulgaria.

Population of Carniolan bee from Slovenia was analyzed in this study. Based on wing venation characteristics, 273 samples from Slovenia and 55 samples from other countries (Croatia, Greece, Czech Republic) or selected lines (from Germany, Austria, Poland, France), all collected in 2001, were surveyed. Right sided forewing was removed, scanned and analyzed with computer program BeeWings 1.20. 37 characteristic for each wing were calculated. With discriminant and PCA analysis differences between groups were found and estimated. Differences between Slovenian populations were not confirmed. Therefore we suppose that Carniolan bee in Slovenia is homogenous and that differences between different regions do not exist.

## VIRI

Arias, M.C./ Sheppard, W.S. Molecular phylogenetics of honey bee subspecies (*Apis mellifera* L.) inferred from mitochondrial DNA sequence. Molecular phylogenetics and Evolution, 3(1996), 557–566.

Cermak, K. Results of morphometrical analysis and taxonomical classification of honey bees by wing characters. (1999). Bee research institute Dol. Ltd. http://www.beedol.cz/dawino/protokol_EN_data.html (15. oktober. 2007)

Goetze, G. Die beste Biene. Leipzig, Liedloff, Loth & Michaelis Verlag, 1940, 200 str.
Franck, P./ Garnery, L./ Loiseau, A./ Oldroyd, P./ Helburn, H.R./ Solignac, M./ Cornuet, J.M. Genetic diversity of the honeybee in Africa: Microsatellite and mitochondrial data. Heredity, 86(2001), 420–430.
Franck, P./ Garnery, L./ Solignac, M./ Cornuet, J.M. Molecular confirmation of a fourth lineage in honeybees from the Near East: Taxonomy and evolutionary biology of the honeybees. Apidologie, 31(2000), 167–180.
Kauhausen-Keller, D./ Keller, R. Morphometrical control of pure race breeding in the honeybee (*Apis mellifera* L). Apidologie, 25(1994), 133–143.
Leclercq, B. Biometry. Text and illustrations-extracts from CDROM "The Honeybee and man" (1999) http://www.beekeeping.com/leclercq/biometry.htm (8.oktober 2007)
Morphometrical analysis and taxonometrical classification of honey bees by wing characters. Bee Research Institute Dol. 2000. (9. 5. 2007) http://www.beedol.cz/beedolan.html (29. 5. 2007).
Nazzi, F. Morphometric analysis of honey bees from an area of racial hybridization in northeastern Italy. Apidologie, 23(1992), 89–96.
Palmer, M.R./ Smith, D.R./ Kaftanoglu, O. Turkish honeybees: genetic variation and evidence for a forth lineage of *Apis mellifera* mtDNA. The Journal of Heredity, 91(2000), 42–46.
Poklukar, J. Kranjska čebela je osvojila Češko. Slovenski čebelar, 10(1999), 277–278.
Poklukar, J. Zaključno poročilo o rezultatih raziskovalnega projekta v letu 1998. Kmetijski inštitut Slovenije, 1998, 12 str.
Ruttner, F./ Tassencourt, L./ Louveaux J. Biometrical-statistical analysis of the geographic variability of *Apis mellifera* L., Apidologie 9(1978), 363–381.
Ruttner, F. Biogeography and taxonomy of honeybees. Berlin, Springler-Verlag, 1988, 284 str.
Ruttner, F. Naturgeschichte der Honigbienen. München, Ehrenwirth Verlag, 1992, 357 str.
Ruttner, F./ Hanel, H. Active defense against Varroa mites in a Carniolan strain of honeybee (*Apis mellifera carnica* Pollman). Apidologie 23(1992), 173–187.
Susnik, S./ Kozmus, P./ Poklukar, J./ Meglic, V. Molecular characterization of indigenous *Apis mellifera carnica* in Slovenia. Apidologie 35(2004), 623–636.
Zaletel, P. Zgodovina slovenskega čebelarstva in čebelarske organizacije. Slovenski čebelar, 12(1998), 317–337.

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# UČINKOVITO KODIRANJE ZAPOREDIJ DNA

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## IZVLEČEK

V zadnjem obdobju smo priča znatnemu naraščanju uporabe mikroračunalnikov pri raziskavah in analizah zaporedij DNA. Molekule DNA so računalnikom najpogosteje predstavljene v obliki zapisov v formatu FASTA , ki kodirajo sekvence DNA v obliki ASCII niza štirih nukleotidnih oznak A, G, C in T, katerim se po potrebi pridružijo še degenerativne kode in znak za presledek, ko gre za množice med seboj poravnanih zaporedij DNA. Zapis FASTA je dojemljiv za biologa in enostaven za programerja, ki razvija računalniški program, saj si pri razvoju lahko pomaga z bogatim naborom obstoječih knjižnic za delo z znakovnimi polji. Kljub omenjenim prednostim ima zapis FASTA določene slabosti, kot je manj učinkovito iskanje zaporedij nukleotidov, še posebej ob prisotnosti degenerativnih kod. Druga slabost izvira iz dejstva, da vsak posamezni znak FASTA za presledek zasede po en zlog računalniškega pomnilnika, kar je ob prisotnosti velikega števila presledkov neučinkovito in tudi dodatno manjša hitrost iskanja nukleotidnih zaporedij. Zaradi omenjenih slabosti predstavljamo alternativni zapis zaporedij DNA, ki omogoča hitrejše iskanje nukleotidnih zaporedij in učinkovitejše shranjevanje informacij o poravnavi, kar vodi v hitrejše delovanje programov in odpira možnost shranjevanja večjega števila zapisov DNA v delovni pomnilnik računalnika.

Ključne besede: molekularna genetika / bioinformatika / DNK / kodiranje zaporedij

# EFFICIENT CODING OF DNA

## ABSTRACT

Microcomputers have become ubiquitous tools for DNA research and analysis. Before DNA sequences can be fed into computer programs they need to be suitably coded, which is usually done in a widely accepted FASTA format. According to this scheme, DNA sequence is represented as an ASCII string of four nucleotide characters A, G, C and T, possibly extended with additional codes for representation of degenerated sites, and a character code for FASTA blanks when dealing with aligned DNA sequences. FASTA representation is intuitive for biologists and it eases development of programs since developers can utilize a myriad of available libraries for working with ASCII strings. Despite the mentioned advantages, FASTA format possesses certain drawbacks like inefficient searching for substrings, especially in the presence of degenerative codes. The second disadvantage is inefficient storage of FASTA blank characters, since each such character occupies one byte of memory. Substring searching speed is also negatively affected in the case of excessive number of blanks. Due to the stated drawbacks, we propose an alternative coding of DNA sequences, which enables faster searching of substrings and efficient storage of FASTA blanks, with the result that a greater set of DNA sequences can be held in working memory of a computer and processed faster.

Key words: molecular genetics / bioinformatics / DNA sequences / coding

## UVOD

Za mikrobne združbe sta v splošnem značilni visoka gostota celic ($10^9$/g) in visoka pestrost ($10^7$ vrst/g) (Gans in sod., 2005). Z razvojem novih tehnologij sekvenciranja (angl. 454 pyrosequencing technology) (Neufeld in sod., 2004; Greena in Kellera, 2006; Roesch in sod., 2007) se je število sekvenc v posamezni klonski knjižnici, narejeni iz vzorcev iz okolja, povzpelo do pred kratkim nedoumljive številke 300.000. S trenutno najbolj uporabljanimi metodami za analizo sekvenc in ugotavljanje filogenetskih odnosov so do nedavnega raziskovalci obdelovali le nekaj sto, redko več tisoč sekvenc naenkrat (Felsenstein, 2006; Ronquist and Huelsenbeck, 2003). Tako sedanje najboljše metode za analizo sekvenc čedalje bolj zaostajajo za tehnologijo pridobivanja podatkov. Zaradi vse večjih količin podatkov lahko pričakujemo, da današnji in bodoči mikroračunalniki ne bodo mogli izvesti želenih analiz v doglednem času. Ker je veliko takih projektov v teku in je posledično možno pričakovati bistveno povečanje števila sekvenc, je poleg novih načinov analiz potrebno tudi izboljšati algoritme iskanja, na osnovi katerih je moč bistveno pospešiti računske operacije.

V podatkovnih bazah so sekvence zapisane v obliki različnih formatov. Med najpogostejšimi so FASTA, EMBL, GCG, GenBank, IG in drugi (Felsenstein, 2006; Felsenstein, 2005; Ronquist, 2004; http://www.genomatix.de/). Format FASTA je splošno razširjen format, v katerem je kodirana večina sekvenc v podatkovnih bazah in ki ga bere večina filogenetskih programov, četudi so izhodne datoteke v drugih formatih (Tamura in sod., 2007; Felsenstein, 1989; Thompson in sod., 1999; Swofford, 2002). Tako upravičeno domnevamo, da je zaradi obsega raziskav in analiz sekvenc format FASTA med najbolj uporabljanimi formati v raziskavah.

Klub razširjenosti formata FASTA, le-ta ni računalniško najbolj učinkovit, zaradi česar tudi zmogljivosti mikroračunalnikov pri analizi zaporedij DNA niso optimalno izrabljene. To narekuje raziskavo možnosti drugačnega kodiranja zapisov, s katerim bi se hitrost analize sekvenc na mikroračunalnikih (tako osebnih računalnikih kot na strežnikih) bistveno povečala.

## MATERIALI IN METODE

### Opis zapisa FASTA.

Zaporedje DNA je v zapisu FASTA predstavljen kot niz znakov ASCII A, G, C in T, s katerimi zakodiramo zaporedje nukleotidov. V naboru so lahko vsebovani tudi znaki za degenerirana mesta, s katerimi popišemo negotovost pri določanju zaporedja DNA na sekvenatorjih, polimorfizem v primeru degeneriranih začetnih oligonukleotidov ter (ne)selektivnost prepoznavnih mest za rezanje nekaterih restrikcijskih endonukleaz. Za vsako kombinacijo nedoločenosti dveh, treh ali vseh štirih nukleotidov obstaja predpisan znak, s čimer dobimo množico petnajstih ($2^4$–1) možnih znakov, kot prikazuje pregl. 1.

Nukleotidi so v nekaterih formatih označeni tudi z malimi črkami namesto z velikimi, vendar to za samo branje ni pomembno (Felsenstein, 2004). Velikost črk pri iskanju začetnih oligonukleotidov ali prepoznavnih mest za endonukleaze navadno ignoriramo.

Pri poravnavi dveh ali več zaporedij DNA med seboj potrebujemo tudi znak za presledek '–' (v novejših programih tudi '~') , s katerim označimo po eno vrinjeno mesto. Pri kodiranju prepoznavnih mest za endonukleaze, potrebujemo še znak '^', s katerim določimo mesto prekinitve verige (zaporedja) DNA.

Kot primer si oglejmo naslednji izsek iz bistveno daljše sekvence DNA:

```
5'—...----G---T---A----A---C----G----G---...—3'.
```

Pri nezanesljivem sekvenciranju se pojavijo degenerirana mesta:

```
5'—...----G---T---W----A---C----K----G---...—3'.
```

Preglednica 1. Kode nukleotidov v zapisu FASTA
Table 1. Nucleotide codes according to FASTA format

| znak<br>symbol | ime<br>name | pomen<br>meaning |
|---|---|---|
| *popolnoma določen nukleotid*<br>*totally determined nucleotide* | | |
| A | Adenin<br>Adenosine | A |
| G | Gvanin<br>Guanine | G |
| C | Citozin<br>Cytosine | C |
| T | Timin<br>Thymidine | T |

| znak<br>symbol | ime<br>name | pomen<br>meaning |
|---|---|---|
| *nedoločenost dveh nukleotidov*<br>*indeterminism among two nucleotides* | | |
| Y | Pirimidin<br>Pyrimidine | C ali T<br>C or T |
| R | Purin<br>Purine | A ali G<br>A or G |
| W | šibek<br>weak | A ali T<br>A or T |
| S | močan<br>strong | G ali C<br>G or C |
| K | keto<br>keto | G ali T<br>G or T |
| M | amino<br>amino | A ali C<br>A or C |

| znak<br>symbol | ime<br>name | pomen<br>meaning |
|---|---|---|
| *nedoločenost treh nukleotidov*<br>*indeterminisem among three nucleotides* | | |
| D | ni C<br>not C | A, G ali T<br>A, G or T |
| V | ni T<br>not T | A, G ali C<br>A, G or C |
| H | ni G<br>not G | A, C ali T<br>A, C or T |
| B | ni A<br>not A | G, C ali T<br>G, C or T |
| *nedoločenost štirih nukleotidov*<br>*indeterminisem among four nucleotides* | | |
| N (X) | neznan<br>unknown | katerikoli<br>any |

Glede na pregl. 1 pomeni znak W nedoločenost nukleotidov adenin in timin, medtem ko znak K pomeni negotovost med nukleotidoma timin in gvanin. Primer prepoznavnih mest za endonukleaze v formatu FASTA predstavlja vzorec `CG^CG`, ki poleg zaporedja nukleotidov določa tudi mesto rezanja zaporedja DNA med drugim in tretjim nukleotidom znotraj prepoznavnega mesta za endonukleaze.

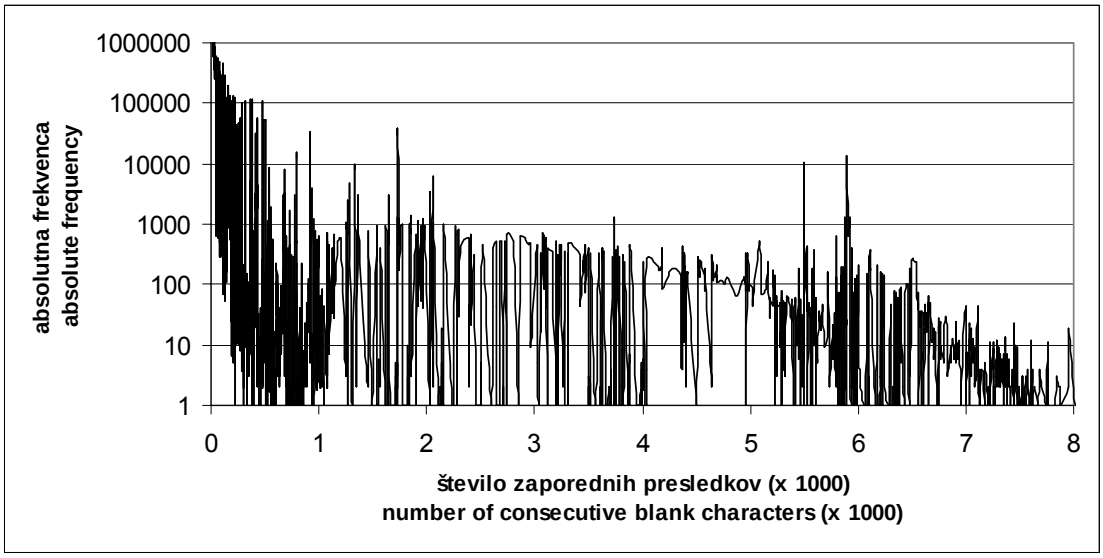
## Slabosti zapisa FASTA.

Zapis FASTA kljub prikladnosti na prvi pogled ni najprimernejši s stališča implementacije učinkovitih računalniških postopkov analize vzorcev DNA, kot je iskanje nizov (prepoznavnih mest za endonukleaze, mesta prileganja začetnih oligonukleotidov) v vzorcu. Slabost izvira iz dejstva, da so simboli v pregl. 1 izbrani glede na biološki pomen (informativnost in spremenljivost mest znotraj kodona; nekodirajoča zaporedja) in ne glede na vsebino bitnega vzorca, ki pripada določeni črki, kar bi bolje ustrezalo delovanju računalnikov. Npr. črki G in K (slednja pomeni G ali T; pregl. 1) sta v računalniškem pomnilniku predstavljeni s številkama 71 ($0100\ 0111_2$) in 75 ($0100\ 1101_2$), pri čemer na ravni dvojiškega zapisa simbol G ne označuje podmnožice nukleotidov simbola K, saj enice (ali ničle) kode črke G niso npr. podmnožica istoležnih enic (ali ničel) kode črke K.

Posledica opisanega je, da pri iskanju nizov ne moremo primerjati ujemanja vzorca prepoznavnega mesta za endonukleazo z zaporedjem DNA s preprostimi bitnimi operacijami, ki jih računalniki izvajajo hitro in učinkovito. Namesto tega moramo pri testiranju ujemanja uporabiti kompleksne (sestavljene) pogojne stavke, ki se izvajajo počasneje od preprostih bitnih operacij. Alternativna možnost je uporaba tabele, v kateri so v smislu kartezijevega produkta zajeti vsi možni pari simbolov v pregl. 1, katerim je prirejen indikator ujemanja oziroma neujemanja. Uporaba tabel najprej zahteva izračun lokacije polja na podlagi primerjanih simbolov (črk) in nato dostop do tako določene pomnilniške lokacije v tabeli; noben od teh korakov se niti sam zase ne more izvesti hitreje od preprostih bitnih operacij. S prisotnostjo tako velikih kot malih črk se postopki še neznatno upočasnijo zaradi pretvorbe vseh simbolov v eno velikost črk (alternativno je možno povečati tabelo ujemanja, kar tudi lahko upočasni dostop zaradi slabše izrabe predpomnilnika).

Druga slabost zapisa FASTA pride do izraza ob prisotnosti velikega števila presledkov v zaporedjih DNA, kar je pogost rezultat poravnave sekvenc. Slika 1 prikazuje histogram dolžin zaporednih presledkov v bazi RDP II (http://rdp.cme.msu.edu/), ki je v izdaji 9.50 vsebovala 125 208 sekvenc gena za 16S rRNA, daljših od 1200 baznih parov. Slika je zaradi preglednosti

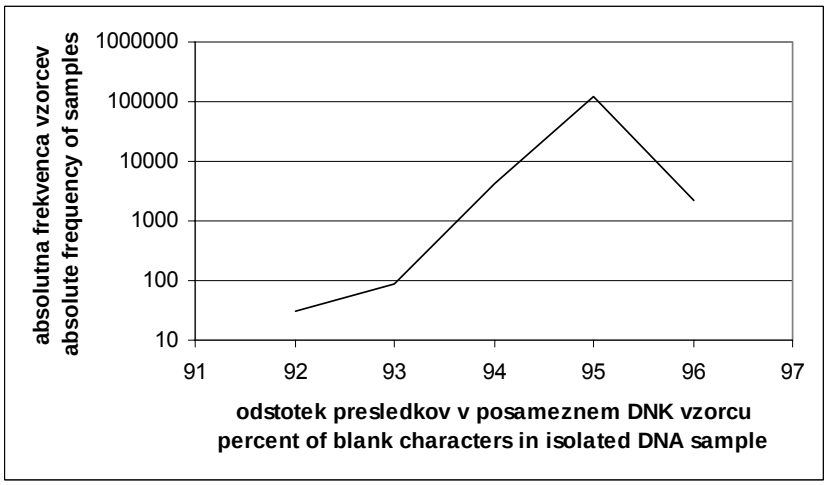
prirezana in vsebuje samo najzanimivejši del dejanskega histograma, ki se po abscisni osi razteza do deset tisoč in po ordinatni osi do petindvajset milijonov (logaritemsko merilo do sto milijonov), vendar je v odstranjenem območju tako malo podatkov, da se na sliki skoraj ne vidijo.




Slika1. Histogram dolžin zaporednih presledkov baze RDP s 125 208 zaporedji (prirezan).
Figure 1. Histogram of consecutive blanks for RDP database with 125 208 sequences. (trimmed).

S slike razberemo, da se najpogosteje pojavljajo zaporedja presledkov do dolžine okvirno 500 znakov, vidimo pa, da velikostni red 6000 zaporednih presledkov in več ni redkost. Najdaljše zaporedje presledkov v obravnavani bazi je dolgo 9922 znakov.

Pri zapisu FASTA se vsak presledek obravnava in shrani v pomnilniku kot ločen znak, zaradi česar lahko presledki zasedejo levji delež delovnega pomnilnika, kar ima za posledico manjšanje števila zaporedij DNA, ki jih lahko hkrati obdelujemo na učinkovit način. Razmere pri obravnavani RDP bazi prikazuje slika 2.




Slika 2. Histogram zaporedij DNA z določenim odstotkom vsebovanih presledkov.
Figure 2. Histogram of DNA sequences with certain percentage of blank characters.

Pri popolnoma vseh 125 208 zaporedjih se vsebovanost presledkov nahaja v intervalu med 92 % in 96 %, pri čemer je daleč najpogostejši delež enak 95 %. Ugotovitev je vredna razmisleka, saj nakazuje, da je izkoriščenost delovnega pomnilnika računalnika zgolj 5 % v primeru, da presledkov ne moremo zavreči. Poleg pomnilniške potratnosti so zaradi presledkov upočasnjeni postopki analize zaporedij. Pri iskanju nizov (prepoznavnih mest za endonukleaze ali mest prileganja začetnih oligonukleotidov) je potrebno vsaj s trivialnim pogojnim stavkom pregledati vse znake zaporedja, čeprav je 95 % le-teh nekoristnih za določitev mesta prileganja.

Opisane slabosti nakazujejo, da bi računalniški postopki analize zaporedij DNA postali učinkovitejši (časovno in pomnilniško) z izbiro drugačnega kodnega zapisa namesto ustaljenega formata FASTA. Tak zapis predstavljamo v nadaljevanju.

**Izhodišča za razvoj novega kodnega zapisa**

Pri snovanju novega kodnega zapisa zaporedij DNA smo upoštevali naslednja izhodišča. Zapis mora omogočati učinkovito iskanje nizov (mesta prileganja začetnih oligonukleotidov, prepoznavna mesta endonukleaz) s pomočjo preprostih bitnih operacij. Iskanje mora biti učinkovito v obeh smereh preiskovanja DNA verige, kar je pomembno pri lociranju prednjih in zadnjih začetnih oligonukleotidov in prav tako pri iskanju mest rezanja restrikcijskih endonukleaz v obeh smereh. Druga zahteva je kompakten in pomnilniško varčen zapis verige presledkov FASTA, ki bi iskanje nizov čim manj upočasnil. Pri tem je potrebno paziti, da je kljub prisotnosti presledkov še vedno možno izvajati iskanje podnizov v obeh smereh, kar je pri zapisu FASTA v osnovi zagotovljeno, saj je vsak znak za presledek avtonomen in ga lahko enostavno prepoznamo in preskočimo ne glede na smer preiskovanja, ni pa nujno da to velja za vsak kodni zapis.

**Učinkovitejše kodiranje nukleotidov.**

Omenili smo, da so v računalniškem pomnilniku podatki predstavljeni kot binarna števila in da je izvajanje bitnih operacij nad njimi učinkovito. Danes praktično vsi računalniki operirajo z operandi (registri in pomnilniškimi lokacijami) velikosti osmih bitov (en zlog) ali določenim mnogokratnikom tega števila (16, 32 in 64), zato je smiselno ohraniti lastnost zapisa FASTA, da se posamezna koda nukleotida shrani v ločen zlog pomnilnika, vendar moramo posameznim bitom znotraj zloga prirediti drugačen pomen.

Predlagamo shemo, po kateri od osmih bitov porabimo prve štiri (od 0 do 3) za shranjevanje informacije o nukleotidu, pri čemer je vsakemu nukleotidu prirejen ločen bit, kar prikazuje slika 3.

| **bit** | 7 | 6 | 5 | 4 | 3 | 2 | 1 | 0 |
|---|---|---|---|---|---|---|---|---|
| **pomen**<br>**meaning** | presledek<br>blank | rezerviran<br>reserved | degeneriran<br>degenerated | mala črka<br>lower case | Timin<br>Thymidine | Gvanin<br>Guanine | Citozin<br>Cytosine | Adenin<br>Adenosine |

Slika 3. Predlagana shema kodiranja nukleotidov.
Figure 3. Recommended nucleotide coding scheme.

**Primer.** Če zlog predstavlja nukleotid adenin, ima bit 0 vrednost 1, medtem ko imajo ostali biti vrednost 0; binarna koda adenina je torej $0000\ 0001_2$. Podobno ugotovimo, da je koda citozina enaka $0000\ 0010_2$. Gvaninu in timinu pripadata kodi $0000\ 0100_2$ in $0000\ 1000_2$.

Za popis nukleotida porabimo štiri od osmih bitov, zato lahko v preostale štiri bite shranimo dodatne podatke. Bit 4 nosi podatek o velikosti črke, pri čemer njegova vrednost 0 pomeni veliko črko in 1 malo črko. Na ta način smo podatek o velikosti črke povsem ločili od kode nukleotida, zato lahko velikost shranimo, ne da bi ta manjšala učinkovitost iskanja nizov v zaporedju DNA.

V primeru, da zlog hrani podatek o degeneriranem mestu v zaporedju, se priredi vrednost 1 vsem bitom, ki pripadajo možnim nukleotidom, poleg tega se bit 5 nastavi na 1. Po tej shemi FASTA simbolu K, ki pomeni nedoločenost med gvaninom in timinom, pripada binarna koda $0010\ 1100_2$. Na podoben način ugotovimo, da FASTA simbolom Y (C ali T), W (A ali T) in H (A, C ali T) pripadajo kode $0010\ 1010_2$, $0010\ 1001_2$ in $0010\ 1011_2$, medtem ko simbolu N (katerikoli nukleotid) pripada koda $0010\ 1111_2$.

Pomen bita 5 je v tem, da lahko s preverjanjem enega samega bita ugotovimo, ali je nukleotidno mesto degenerirano ali ne. To ponavadi ne pomaga pri iskanju nizov v zaporedju DNA, ampak je koristno v drugih primerih. S pomočjo bita 5 lahko učinkovito preštejemo število degeneriranih mest v zaporedju DNA ali v njegovem določenem odseku (na primer med prednjim in zadnjim začetnim oligonukleotidom), kar je pomembno pri kontroli kakovosti zaporedja DNA. Zapis FASTA tega ne omogoča na preprost način, saj simboli A, C, G, T in degenerativni simboli (pregl. 1) niso ločeni niti glede na binarni zapis niti glede na zaporedje njihovih ASCII kod (npr. tako da bi črke A, B, C in D pomenile štiri osnovne nukleotide in nadaljnje črke degenerirana mesta).

Bit 6 je neizkoriščen in prestavlja možnost za bodoče razširitve kodnega zapisa. Ta bit mora biti nastavljen na nič, sicer kasneje predstavljene operacije ne delujejo pravilno.

Tudi iskane podnize zaporedja DNA (mesta prileganja začetnih oligonukleotidov, prepoznavna mesta endonukleaz) moramo ustrezno kodirati. Pri tem uporabimo enako shemo kot pri kodiranju zaporedja DNA (slika 3), pri čemer bit 5 ne nastavimo na 1 v primeru degeneriranih oziroma neselektivnih mest prileganja. Na primer, če se na določenem mestu začetni oligonukleotid ujema z nukleotidoma A in T, bo to mesto iskanega podniza zakodirano s kodo $0000\ 1001_2$ in ne s kodo $0010\ 1001_2$.. Isto pravilo velja za neselektivnost prepoznavanja mest endonukleaz.

Prav tako morata biti bita 4 in 6 vedno na nič. Zakaj je temu tako, bomo videli pri opisu operacij v nadaljevanju.

Zaradi razlogov, ki jih bomo predstavili kasneje, je včasih (vendar ne vedno) bolje za kodiranje iskanih podnizov uporabiti nekoliko spremenjeno kodno shemo, po kateri bite od 0 do 3 na sliki 3 negiramo (zamenjamo ničle z enicami in obratno). Na ta način bi simbola A in W (nedoločenost med A in T) označili s kodama $0000\ 1110_2$ in $0000\ 0110_2$, simbol N za povsem nedoločen nukleotid, pa je predstavljen s kodo $0000\ 0000_2$. Poudarjamo, da tako kodno shemo uporabljamo samo za kodiranje iskanih nizov in nikoli za kodiranje zaporedja DNA.

**Kodiranje presledkov.**

V poravnani bazi zaporedij DNA lahko večinski delež kod v formatu FASTA predstavljajo presledki (sliki 2 in 1), zato moramo njihovemu učinkovitemu kodiranju posvetiti posebno pozornost. V ta namen predlagamo kodno shemo, po kateri se v pomnilnik računalnika ne shranjuje vsak posamezni presledek posebej ampak število le-teh v neprekinjenem zaporedju.

Ker moramo zapis števila presledkov ločiti od zapisa nukleotida, porabimo za namen razločevanja en bit zloga, ki je v našem primeru bit 7 (skrajno desni bit na sliki 3). Ko je ta bit nastavljen na vrednost 0, nosi zlog informacijo o nukleotidu, tako kot prikazuje slika 3, v nasprotnem primeru spodnjih sedem bitov (od 0 do 6) nosi število zaporednih presledkov v zapisu DNA. S sedmimi biti lahko zapišemo število v območju med 0 in 127, kar pomeni, da 127 zaporednih presledkov lahko shranimo v en sam zlog, medtem ko bi v zapisu FASTA zanje potrebovali 127 zlogov.

Za primer si oglejmo naslednji izsek zaporedja DNA: A- - -*...80 zaporednih presledkov...*- - - C. V zapisu FASTA bi tak izsek zasedel 82 zlogov pomnilnika, pri čemer bi po en zlog porabili za shranjevanje začetne črke A in končne črke C, med njiju pa bi vrinili 80 znakov za presledek '-'. Po predlagani kodni shemi porabimo za isti odsek samo 3 zloge. Prvi zlog vsebuje binarno

število $0000\,0001_2$, ki predstavlja kodo A (slika 3). Naslednji zlog vsebuje binarno število $1101\,0000_2$, pri čemer bit 7 (skrajno desni) označuje, da gre za niz presledkov in ne za nukleotid, medtem ko preostanek bitov 101 0000 predstavlja v binarnem številskem sistemu zapisano desetiško število 80. Sledi tretji zlog zapisa, ki vsebuje binarno število $0000\,0100_2$ za predstavitev kode C (slika 3). Vidimo, da je pri velikem številu zaporednih presledkov prihranek pomnilnika znaten, saj smo v prikazanem primeru porabili sedemindvajsetkrat (82/3) manj zlogov, kot bi jih pri uporabi kodnega zapisa FASTA.

S slike 1 razberemo, da se v poravnanem zapisu DNA pogosto pojavljajo zaporedja presledkov, ki so bistveno daljša od 127 (do okvirno 10 000), zato se postavi vprašanje, kako jih kodirati. Ena možnost je, da za vsakih 127 zaporednih presledkov porabimo en zlog. Na ta način bi najdaljše zgoraj omenjeno zaporedje 9922 presledkov zakodirali s 79 zlogi, pri čemer bi prvih 78 zlogov vsebovalo binarno število $1111\,1111_2$ (127 presledkov), zadnji zlog pa bi vseboval število $1001\,0000_2$ (16 presledkov), saj je število 9922 enako $78 \times 127 + 16$.

Bistveno boljši izkoristek pomnilnika dosežemo, če binarno število, ki predstavlja dolžino zaporedja presledkov, razbijemo na sedem bitov dolge odseke in te odseke shranimo v zaporedne zloge, ki imajo nastavljen bit 7 na 1. Na primer, število 9922 se zapiše kot $10\,0110\,1100\,0010_2$, torej zanj potrebujemo 14 bitov. Glede na predlagano kodno shemo bi prvih (skrajno desnih) sedem bitov shranili v prvi zlog, ki označuje zaporedje presledkov, medtem ko bi zadnjih sedem (skrajno levih) bitov shranili v naslednji zlog. Zaporedje 9922 presledkov bi bilo tako predstavljeno z zlogoma $1100\,0010_2$ in $1100\,1101_2$, pri čemer pri obeh zlogih skrajno levi bit, nastavljen na 1 indicira zaporedje presledkov in ne nukleotid.

Glede na predlagano shemo zapišemo odsek: `A---`*...9922 zaporednih presledkov...*`---C` s samo štirimi zlogi, od katerih prvi in zadnji zlog kodirata nukleotida A in C, medtem ko srednja dva kodirata 9922 znakov dolgo zaporedje presledkov. Pri zapisu FASTA potrebujemo za isti odsek 9924 zlogov pomnilnika, kar je 2481-krat več.

Po opisani shemi lahko dva zloga shranita največ 16 383 ($2^{14}$–1) zaporednih presledkov. Če to ni dovolj, lahko trije zlogi shranijo 2 097 151 ($2^{21}$–1) zaporednih presledkov, kar bi moralo zadostovati za vse potrebe. Nadalje lahko štirje zlogi shranijo absurdnih 268 milijonov ($2^{28}$–1) zaporednih presledkov.

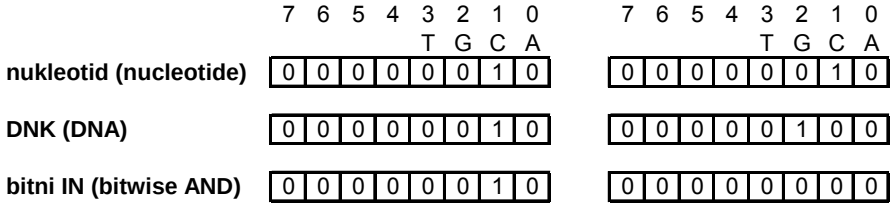
## Uporaba predlagane kodne sheme

Smisel vpeljave nove kodne sheme je učinkovito izvajanje postopkov analize zaporedij DNA. Omenili smo že štetje degeneriranih mest v zaporedjih ali določenem odseku le-tega, za kar predlagana kodna shema nudi direktno podporo. Pri štetju mora postopek samo preleteti zaporedje DNA oziroma njegov usterzni odsek in prešteti nukleotidne zloge, pri katerih ima bit 5 vrednost 1 (slika 3). Pri tem moramo paziti, da ne štejemo zlogov, ki kodirajo zaporedje presledkov, zato štejemo zloge, pri katerih je bit 5 enak 1 in hkrati je bit 7 enak 0, kar je še vedno dovolj enostavno za realizacijo. Zapis FASTA ne omogoča tako enostavne izvedbe tega opravila.

Bolj zanimivo od štetja degeneriranih mest je iskanje nizov v zaporedju DNA, sestavni del česar je preverjanje, ali se na določenem mestu nahaja ustrezen nukleotid. Predlagana kodna shema je tako zasnovana, da omogoča hitro preverjanje ustreznosti nukleotida tako v ekzaktnem kot degeneriranem primeru ne glede na to, ali je degenerirana sama DNA veriga (negotovost sekvenciranja), iskani niz (degenerirani začetni oligonukleotid ali neselektivno prepoznavno mesto endonukleaze) ali oboje, kar v zapisu FASTA zahteva relativno zapletene pogojne stavke ali že omenjeno uporabo tabele ujemanja.

Denimo, da želimo preveriti, ali se na določenem mestu verige DNA nahaja nukleotid C. Da dobimo odgovor na vprašanje, izvedemo operacijo bitni IN med kodo nukleotida C in vsebino DNA verige ter pogledamo, ali je rezultat (celoten zlog) različen od nič. Dogajanje prikazuje slika 4, pri čemer je uporabljena kodna shema, pri kateri nukleotidni biti niso degenerirani. Pri

izvajanju operacije bitni IN je posamezni bit rezultata enak 1, če sta istoležna bita obeh operandov enaka 1.



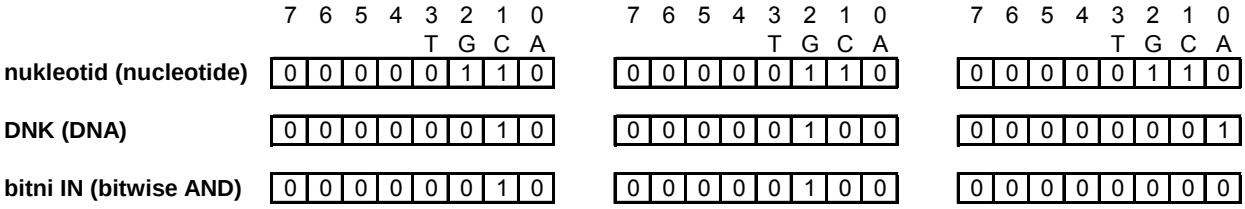

Slika 4. Preverjanje prisotnost nukleotida C (levo: prisoten C, desno: prisoten G).
Figure 4. Checking presence of nucleotide C (left: C is present, right: G is present).

V primeru, da se na mestu preverjanja DNA verige resnično nahaja nukleotid C (slika 4 levo), sta ustrezna bita v kodi nukleotida in v DNA verigi nastavljena na vrednost 1, zato je istoležni bit rezultata operacije bitni IN enak 1 in s tem je celotni zlog rezultata različen od nič, kar nakazuje ujemanje.

Slika 4 desno prikazuje situacijo, ko se na mestu preverjanja DNA verige nahaja nukleotid G. V tem primeru nima noben bit nukleotidove kode vrednosti 1 na istem mestu kot bit DNA verige in rezultat operacije bitni IN je enak nič, kar nakazuje neujemanje.

Primera na sliki 4 jasno ponazorita, zakaj je koristno uporabiti kodno shemo, pri kateri je vsakemu nukleotidu prirejen ločen bit. Bitne operacije spadajo med najhitrejše operacije, ki jih poznajo digitalni računalniki in preverjanje ujemanja verige DNA z vzorcem na ta način je teoretično najhitrejše možno in tudi pomnilniško učinkovito (ne potrebujemo tabele ujemanja).

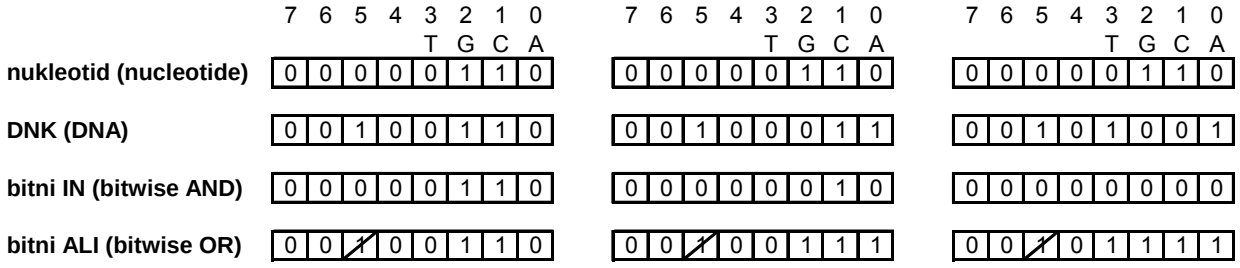
Zgornji primer razširimo tako, da dovolimo ujemanje DNA verige z večimi nukleotidi (npr. v primeru degeneriranega začetnega oligonukleotida), kar prikazuje slika 5; v prikazanem primeru želimo preveriti, ali se na izbranem mestu v DNA verigi nahaja eden od nukleotidov C ali G, čemur ustreza nukleotidna koda, ki ima oba bita C in G nastavljena na 1.




Slika 5. Preverjanje prisotnost nukleotida C ali G (levo: prisoten C, sredina: prisoten G, desno: prisoten A).
Figure 5. Checking presence of nucleotide C or G (left: C is present, middle: G is present, right: A is present).

Levi primer na sliki prikazuje dogajanje ob prisotnosti nukleotida C v zaporedju DNA , kjer je rezultat operacije bitni IN različen od nič, saj sta bita C nastavljena na 1 tako v nukleotidni kodi kot v zaporedju DNA. Analogno dogajanje lahko spremljamo na sredini slike 5, le da tokrat opis velja za bit nukleotida G. Desni primer kaže situacijo ob prisotnosti nukleotida A v zaporedju DNA. Sedaj noben bit nukleotidne kode ni hkrati na vrednosti 1 z istoležnim bitom DNA verige in rezultat operacije bitni IN je enak nič, zato ujemanja ni.

Najsplošnejši primer nastopi, ko imamo degenerirana mesta tako v specifikaciji nukleotida kot v zaporedju DNA. V tem primeru samo ena bitna operacija ne zadostuje oziroma ne daje nujno želenega rezultata. Situacijo prikazuje slika 6, pri čemer bomo zadnjo vrstico uporabili kasneje.




Slika 6. Preverjanje prisotnost nukleotida C ali G (levo: možen C ali G, sredina: možen C ali A, desno: možen T ali A).

Figure 6. Checking presence of nucleotide C or G (left: possible C or G, middle: possible C or A, right: possible T or A).

Levi primer na sliki ponazarja situacijo, kjer se v zaporedju DNA lahko nahaja nukleotid C ali G, zato se mesto ujema s specificirano množico nukleotidov. Rezultat operacije bitni IN je različen od nič (tokrat sta dva bita rezultata različna od nič), kar nakazuje prileganje.

Srednji primer na sliki ponazarja možnost prisotnost nukleotida C ali A v zaporedju DNA. Ker se vsaj en nastavljen bit zaporedja DNA ujema z istoležnim bitom nukleotidne kode, je rezultat različen od nič, kar nakazuje ujemanje. Tak rezultat ni nujno zaželen, saj se v dejanskem zaporedju DNA lahko na tem mestu nahaja nukleotid A namesto nukleotida C.

Desni primer na sliki prikazuje situacijo, kjer je v zaporedju DNA možen nukleotid A ali T. Ker je množica dovoljenih nukleotidov C in D tuja množici možnih nukleotidov A in T, je rezultat operacije bitni IN enak nič in ujemanja zanesljivo ni. Sedaj tudi vidimo, zakaj bita 5 (tudi 4 in 6) ne smemo uporabljati pri kodiranju podnizov; v desnem primeru na sliki 6 bi postopek razglasil ujemanje, saj bi bil bit 5 nastavljen na 1 v obeh operandih, zaradi česar bi imel tako vrednost tudi pripadajoči bit rezultata in celotni rezultat bi bil različen od 0 (ujemanje).

Zaključimo, da je rezultat opisanega testa ujemanja pozitiven, čim je vsaj en možen nukleotid v zaporedju DNA enak vsaj enemu dovoljenemu nukleotidu v iskanem nizu. Tako iskanje je koristno v primeru, da želimo identificirati vsa mesta v DNA verigi, kjer bi se iskani niz lahko nahajal, čeprav obstaja možnost, da se ne.

Navadno si želimo, da bi postopek iskanja niza ugotovil ujemanje samo, če ni nikakršne možnosti napačnega ujemanja, torej če množica možnih nukleotidov v zaporedju DNA predstavlja podmnožico dovoljenih nukleotidov. Da to dosežemo, potrebujemo pri obravnavani kodni shemi vsaj dve bitni operaciji. Postopek ponovno prikazuje slika 6, pri čemer sedaj uporabimo njeno zadnjo vrstico.

Želeni test prileganja dobimo tako, da že opisani postopek nekoliko spremenimo. Namesto operacije bitni IN izvedemo operacijo bitni ALI. Pri tej operaciji je posamezni bit rezultata nastavljen na 1, če je vsaj v enem operandu (ali obeh) ustrezni bit nastavljen na 1. Zaporedje DNA se prilega kodi nukleotida, če je tako dobljeni rezultat enak izhodiščni kodi nukleotida, sicer je možno, da se v DNA verigi nahaja nukleotid, ki ga koda ne dovoli.

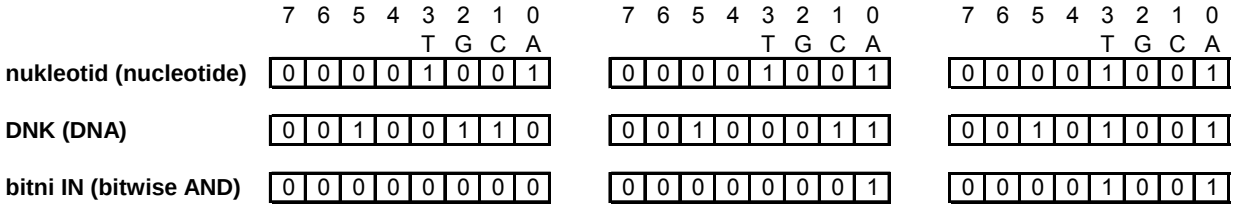
Na sliki 7 levo si ponovno oglejmo primer, ko množica dovoljenih nukleotidov vsebuje C in G. Prav ta nukleotida vsebuje tudi množica možnih nukleotidov v zaporedju DNA. Rezultat operacije bitni ALI je enak kodi nukleotida, zato razglasimo ujemanje. Na sliki vidimo, da bit 5, ki označuje degenerirano mesto, moti, zato ga pred preverjanjem enakosti brezpogojno izbrišemo. Isto bi veljalo za bit 4, ki nakazuje malo črko namesto velike. V splošnem izbrišemo vse štiri zgornje bite, kar je možno izvesti z eno samo dodatno operacijo.

Na srednji sliki je rezultat operacije bitni ALI različen od kode nukleotida, zato je možno, da se v zaporedju DNA nahaja nedovoljen nukleotid in razglasimo neujemanje. Na desni sliki sta

oba možna nukleotida v zaporedju DNA različna od dovoljenih in v tem primeru spet pravilno razglasimo neujemanje.

Na videz smo tudi restriktivnejše iskanje rešili na še vedno eleganten način samo z uporabo bitnih operacij. Kljub temu je predlagani postopek vreden razmisleka, saj poleg osnovne bitne operacije potrebujemo še pomožno (brisanje zgornjih štirih bitov). Poleg tega je za določitev ujemanja potrebno rezultat primerjati z vrednostjo nukleotida, torej ne z vrednostjo nič, za kar so računalniki posebej optimirani. Zaključimo, da se na ta način test ujemanja izvrši okvirno trikrat počasneje od izvedbe ene same binarne operacije.

Situacijo lahko izboljšamo z uporabo že omenjene kodne sheme, kjer nukleotide iskanega podniza zakodiramo negirano. To interpretiramo, kot da biti kode iskanega podniza, ki so enaki 1, pomenijo nukleotide, ki jih v DNA verigi ne sme biti na pripadajočem mestu. Ujemanje DNA z iskanim nizom testiramo tako, da izvedemo operacijo bitni IN med obema kodama in pogledamo, ali je rezultat enak nič. Situacijo prikazuje slika 7.




Slika 7. Ekzaktno preverjanje prisotnost nukleotida C ali G z negiranim zapisom iskanega niza (levo: možen C ali G, sredina: možen C ali A, desno: možen T ali A).

Figure 7. Exact checking of presence of nucleotide C or G with negated nucleotide code of searched substring (left: possible C or G, middle: possible C or A, right: possible T or A).

Levi primer na sliki ponovno prikazuje situacijo, kjer se v zaporedju DNA lahko nahaja nukleotid C ali G; ta pogoj je ekvivalenten pogoju, da se na tem mestu ne sme nahajati niti nukleotid A niti nukleotid T. Pogoj je prikazan tako, da sta bita, ki pripadata nukleotidoma A in T, nastavljena na 1. Rezultat operacije bitni IN je enak nič, kar pomeni, da noben od možnih nukleotidov v zaporedju DNA ni vsebovan v množici prepovedanih nukleotidov iskanega (ujemanje).

Srednji primer na sliki prikazuje situacijo, ko se v zaporedju DNA lahko nahaja nukleotid C ali A. Rezultat operacije bitni IN, ki je različen od nič, nakazuje, da je možno prepovedano stanje, zato razglasimo neujemanje.

V desnem primeru na sliki sta dva možna nukleotida v zaporedju DNA vsebovana v množici prepovedanih nukleotidov, zato sta dva bita rezultata različna od nič, s čimer je tudi celotni rezultat različen od nič in s tem je neujemanje pravilno detektirano.

Z opisano kodno shemo smo tudi restriktivno testiranje ujemanja uspeli realizirati z eno samo bitno operacijo, kar pomeni, da je tak test teoretično najhitrejši možen.

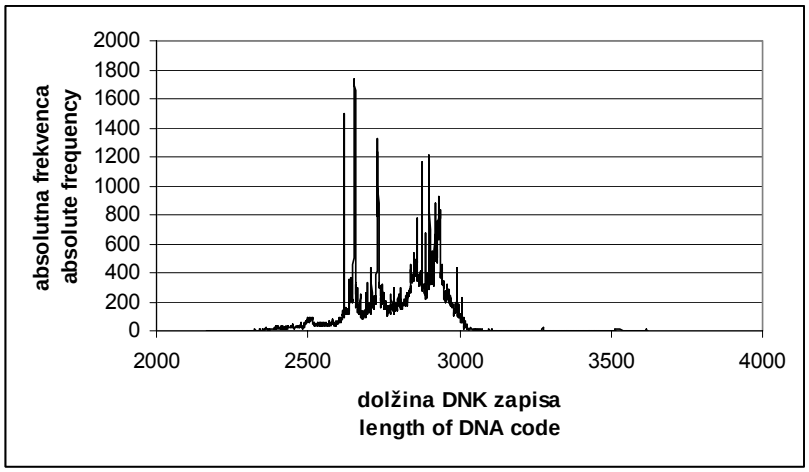
## REZULTATI IN RAZPRAVA

Predhodno podani opis nove kodne sheme sekvenc DNA nakazuje, da le-ta odpravlja določene slabosti zapisa FASTA. Na tem mestu nas zanima, kaj in koliko pridobimo z vpeljavo predlagane sheme v računalniške programe. Primerjavo naredimo s pomočjo predhodno omenjene baze RDP II (9.50).

Pomnilniško učinkovitost ocenimo tako, da primerjamo velikost pomnilnika, ki ga v povprečju potrebujemo za shranjevanje DNA v obeh zapisih. Ker je baza RDP II (9.50)

poravnana, imajo vsi zapisi DNA enako dolžino, in sicer 27 934 znakov (skupaj nukleotidnih kod in presledkov); to je tudi število zlogov pomnilnika, ki jih potrebujemo za pomnenje enega zaporedja DNA v primeru uporabe kodne sheme FASTA.

Ker naša kodna shema shranjuje presledke učinkoviteje, pričakujemo znatno zmanjšanje potrebnega pomnilniškega prostora, kar potrjuje histogram dolžin zapisa DNA na sliki 8.




Slika 8. Histogram dolžin zapisa zaporedij DNA v bazi RDP II (9.50).
Figure 8. Histogram of length of DNA sequence code in database RDP II (9.50).

Natančno izračunana povprečna dolžina zapisa je enaka 2798 zlogov (standardni odklon je 138 zlogov). To pomeni, da lahko s predlagano kodno shemo v isti pomnilniški prostor shranimo skoraj desetkrat več zaporedij DNA, kot pri zapisu FASTA.

Za primerjavo hitrosti procesiranja v primeru uporabe obeh kodnih shem smo vsakemu zaporedju DNA v bazi RDP II (9.50) poskusili določiti lokacijo šestih parov začetnih oligonukleotidov (vse možne kombinacije med prednjima začetnima oligonukleotidoma 968f in 341f in zadnjimi začetnimi oligonukleotidi 1492r, 1062r in 926r) (http://www.microbial-ecology.net/probebase/). Za vsak par začetnih oligonukleotidov, ki se je prilegal zaporedju DNA, smo poskusili locirati prvo in zadnje mesto rezanja endonukleaz AbaI, AbsI in AccII (http://rebase.neb.com). Tako smo za vsako zaporedje DNA potencialno poskusili locirati osemnajst različnih kombinacij oligonukleotidov in encimov, pri čemer smo encim iskali dvakrat (enkrat za prednjim in drugič pred zadnjim začetnim oligonukleotidom). Nadalje smo pri vsaki od lociranih kombinacij prešteli število vsake nukleotidne kode v segmentu DNA od začetka prednjega začetnega nukleotida do najbližjega mesta rezanja endonukleaze ter podobno od zadnjega mesta rezanja endonukleaze do konca zadnjega začetnega oligonukleotida; skupno smo imeli šestnajst števcev: enega za vsoto vseh nukleotidov (dolžino segmenta) in ostalih petnajst za posamezno nukleotidno kodo v pregl. 1. Test je potekal na prenosnem računalniku s procesorjem Intel (R) Celeron (R) M 1,5 GHz in delovnim pomnilnikom (RAM) 512 MB.

Izvršni čas analize zaporedij DNA pri uporabi kodne sheme FASTA znašal 387,1 s, medtem ko je pri uporabi naše kodne sheme znašal 42,5 s, kar pomeni 9,1-krat večjo hitrost delovanja pri uporabi naše kodne sheme. Poudarjamo, da je to zgolj čas obdelave podatkov, v katerega ni vključen čas branja zaporedij DNA z diska, ki nikakor ni zanemarljiv, saj baza RDP II (9.50) zavzame 3,4 GB prostora na disku. Zato že samo branje datoteke in preverjanje pravilnosti zapisa zaporedij DNA traja več minut in pri manjših obdelavah podatkov kodna shema ne igra nobene vloge pri celotnem času izvajanja, saj ozko grlo predstavlja strojna oprema. V tej luči predhodno prikazani rezultati hitrostnega testa nakazujejo, asimptotično pohitritev, ki jo lahko pričakujemo z vpeljavo naše kodne sheme pri obsežnih obdelavah zaporedij DNA.

Navedimo še test celotnega izvršnega časa, ki je bil potreben za izvedbo testa: 699,2 s v primeru kodiranja FASTA in 310,3 s pri uporabi naše kodne sheme, kar pomeni 2,25-krat hitrejše celotno izvajanje z uporabo naše kodne sheme. Pri obsežnejših analizah (npr. pri lociranju mest prileganja večjega števila parov začetnih oligonukleotidov in prepoznavnih mest rezanja endonukleaz) pričakujemo, da bi se to razmerje asimptotično približevalo predhodno podanemu razmerju 9,1, saj bi bilo potrebno zaporedja DNA prebrati z diska samo enkrat (fiksni del časa), nadaljnja analiza pa bi se vršila s hitrostjo, ki jo določa izbrana kodna shema.

## SKLEPI

Članek opisuje novo kodno shemo za kodiranje zaporedij DNA, ki je bila razvita z namenom odpraviti glavne slabosti uveljavljene kodne sheme FASTA. Empirični testi kažejo, da predlagani pristop omogoča okvirno desetkrat boljšo izrabo delovnega pomnilnika računalnika in asimptotično omogoča več kot devetkrat hitrejše delovanje v članku preizkušenih postopkov analize zaporedij DNA (iskanje podnizov in štetje nukleotidov v določenih podsegmentih zaporedij DNA).

## SUMMARY

The article presents new DNA coding scheme, which was developed as a worthy replacement for the well-established FASTA coding scheme. Empirical tests show that our proposition utilizes working memory of computer roughly ten times better than the original FASTA scheme. In addition, our scheme asymptotically increases the speed of DNA analysis for more than nine times on tested algorithms (searching for substring and counting of nucleotides in DNA segments).

## VIRI

Felsenstein, J. PHYLIP – Phylogeny Inference Package (Version 3.2). Cladistics 5(1989), 164–166.

Felsenstein, J. PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, 2005.

Felsenstein, J. Inferring Phylogenies. 2004, Sinauer Associates.

Greena, B.D. / Kellera, M. Capturing the uncultivated majority. Current Opinion in Biotechnology, 17(2006), 236–240.

Gans, J./ Wolinsky, M./ Dunbar, J. Computational Improvements Reveal Great Bacterial diversity and High Metal Toxicity in Soil. Science, 309(2005), 1387–1390.

Neufeld, J.D./ Yu, Z./ Lam, W./ Mohn, W.W. Serial analysis of ribosomal sequence tags (SARST): a high-throughput method for profiling complex microbial communities. Environ. Microbiol., 6(2004), 131–144.

Roesch, L.F.W./ Fulthorpe, R.R./ Riva, A./ Casella, G./ Hadwin, A.K.M./ Kent, A.D./ Daroub, S.H./ Camargo, F.A.O./ Farmerie, W.G./ Triplett, E.W. Pyrosequencing enumerates and contrasts soil microbial diversity. The ISME Journal, 1(2007), 283–290.

Ronquist, F./ Huelsenbeck, J.P. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics, 19(2003), 1572–1574.

Ronquist, F. Bayesian inference of character evolution. Trends in Ecology and Evolution, 19(2004), 475–481.

Swofford, D. L. PAUP*: Phylogenetic Analysis Using Parsimony (and Other Methods) 4.0 Beta. Florida State University, 2002.

Tamura, K./ Dudley, J./ Nei, M./ Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution, 24(2007), 1596–1599.

Thompson, J.D./ Plewniak, F./ Poch, O. A comprehensive comparison of multiple sequence alignment programs. Nucleic Acids Res., 27(1999), 2682–2690.

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# SMERNICE NA PODROČJU POSKUSOV NA ŽIVALIH

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## IZVLEČEK

V juniju 2007 je v Italiji potekalo skupno srečanje FELASA (Federation of European Laboratory Animal Science Associations) in ICLAS (International Council for Laboratory Animal Science), katerega se je udeležilo preko 1100 strokovnjakov iz vseh kontinentov. Na simpoziju je bil predstavljen obsežen pregled najnovejših znanstvenih dognanj in tehnologije s področja znanosti o laboratorijskih živalih, ki je potekal v znamenju predstavitev, konzultacij ter pripravljanja smernic za prihodnost. Na srečanju predstavljene teme se nanašajo predvsem na izboljšave in inovativno tehnologijo eksperimentalnih postopkov, oskrbo in uporabo živali v poskusih, zakonodajo, usposabljanje osebja ter etike in so v prispevku podrobneje opisane. V prispevku namenjamo nekaj poudarka tudi evropski zakonodaji, ki je v procesu dopolnjevanja in spreminjanja. Vse v EU sprejete spremembe bodo namreč vplivale tudi na predpise v Sloveniji.

Ključne besede: zakonodaja / etika / poskusi na živalih / izobraževanje / usposabljanje / izboljšave

# GUIDELINES IN THE FIELD OF ANIMAL EXPERIMENTATION

## ABSTRACT

The FELASA (Federation of European Laboratory Animal Science Associations) and ICLAS (International Council for Laboratory Animal Science) Joint Meeting took place in Italy in June 2007. The meeting has attended over 1100 experts from the whole world. The international symposium has provided a comprehensive overview of the most recent developments in the field of laboratory animal sciences and technologies. It was organized to present, consult and create guidelines for the future. Main topics of the meeting were refinment and innovative techologies of experimental procedures, housing and use of laboratory animals, legislation, education and training of personel and ethics, which are described in article. In article special attention is also devoted to legislation in European Union, which is in the process of supplementing and changing, because all accepted amendments will affect legislation in Slovenia.

Key words: legislation / ethics / animal experimentation / education / training / refinement

## UVOD

V zadnjih štiridesetih letih je prišlo do nastanka nove multidisciplinarne veje znanosti, znanosti o laboratorijskih živalih, ki se zelo hitro razvija (Vergara in Demers, 2003). Sočasno so nastajala različna znanstvena združenja o laboratorijskih živalih, med katerimi je v Evropi najpomembnejša FELASA (Federation of European Laboratory Animal Science Associations), ki je sestavljena iz neodvisnih evropskih nacionalnih in regionalnih združenj o laboratorijskih živalih in deluje že od leta 1978 (Vergara in Demers, 2003). Danes jo sestavlja 15 združenj, v katerih so zastopani znanstveniki in tehnologi s področja laboratorijskih živali iz preko 20

evropskih držav. FELASA deluje kot ugledno znanstveno združenje, ki daje nasvete pri pripravi predpisov na osnovi najnovejših znanstvenih dognanj ter pripravlja priporočila na področju usposabljanja osebja in drugih področij, ki se tičejo dobrobiti poskusnih živali (Vergara in Demers, 2003). Združenja in priporočila so objavljena na njihovi spletni strani (http://www.felasa.eu/).

Najpomembnejša organizacija na področju znanosti o laboratorijskih živalih na svetovni ravni je nevladna organizacija ICLAS (International Council for Laboratory Animal Science), ki je bila ustanovljena leta 1956 pod okriljem različnih organizacij, med katerimi je bil tudi UNESCO. Njegovo poslanstvo je podpirati in pospeševati mednarodno sodelovanje na področju znanosti o laboratorijskih živalih, vzpostaviti standarde kvalitete in kontrole laboratorijskih živali, zbirati in širiti podatke, ki se nanašajo na znanost o laboratorijskih živali ter pospeševati humano uporabo živali v poskusih na osnovi etičnih načel in znanstvene odgovornosti (Vergara in Demers, 2003).

Čeprav znanost o laboratorijskih živalih hitro napreduje, pa združenja ugotavljajo, da je na področju ugotavljanja dobrobiti (angl. welfare) poskusnih živali še vedno premalo raziskav (Zurlo, 2007). Kajti glavni cilj znanosti o laboratorijskih živalih kot tudi združenj je pospeševati kvaliteto poskusov na živalih in skrbeti za dobrobit poskusnih živali (Vergara in Demers, 2003). Temu cilju se pridružuje tudi EU, ki namerava v naslednjih letih dvigniti raven zaščite živali in poskrbeti za njihovo dobrobit na vseh področjih vključujoč poskuse na živalih (Council of the European Communities, 2006).

## SPREMEMBA EVROPSKE ZAKONODAJE

Ključna internacionalna predpisa v evropskem prostoru, ki urejata področje uporabe živali v znanstveno-raziskovalne in druge namene sta Konvencija ETS 123 in Direktiva 86/609/EEC, ki sta bila sprejeta v letu 1986 in sta močno vplivala na nacionalno zakonodajo držav članic EU. Po 20-ih letih od njunega sprejetja, je prišlo do spremembe in dopolnitve. Za boljši vpogled v proces priprave sprememb in dopolnitev ključnih predpisov na področju poskusov na živalih v EU, navajamo nekaj razlag in obrazložitev.

## REVIZIJA KONVENCIJE ETS 123

Konvencija ETS 123 je bila sprejeta leta 1986 in je stopila v veljavo 1. januarja 1991. V letu 1987 je bil s strani Sveta ministrov sprejet predlog po spremljanju izvajanja Konvencije ETS 123, ki je določal večstranska posvetovanja predstavnikov držav podpisnic Konvencije ETS 123 na vsakih pet let. Večstranska posvetovanja namenjena spremljanju Konvencije ETS 123 so potekala v letih 1992, 1993 in 1997. Ker so ugotovili, da so navodila za nastanitev in oskrbo poskusnih živali zelo uporabna in dobro sprejeta, da pa so znanstvena dognanja in izkušnje na področju znanosti o laboratorijskih živalih od sprejetja Konvencije ETS 123 napredovala, so se že na tretjem posvetu (leta 1997) dogovorili o reviziji Konvencije ETS 123 v delu, ki vsebuje navodila za nastanitev in oskrbo živali uporabljenih v poskusih, t.j. v dodatku A (Forbes in sod., 2007). Za pripravo novega predloga dodatka A h Konvenciji ETS 123 so ustanovili 7 strokovnih skupin, ki so preučile volumen kletk v katerih so nastanjene živali (talna površina in višina) glede na specifične potrebe posamezne živalske vrste, socialno sestavo skupine živali posamezne živalske vrste, njihovo starost in uporabo (živali v vzreji ali v poskusih, pri slednjem upoštevajoč še naravo in trajanje eksperimentalnih postopkov) ter potrebo po obogatitvi okolja (Forbes in sod., 2007).

Po desetih letih temeljitega dela, pri katerem so sodelovali številni strokovnjaki in organizacije s področja znanosti o laboratorijskih živali, med njimi tudi FELASA, je nastal končni osnutek revidiranega dodatka A (Forbes in sod., 2007). Po zaslugi poenostavljenega

postopka (Directive 2003/65/EC) je bil končni osnutek sprejet 15. junija 2006 v Strasbourgu na četrtem večstranskem posvetu in je stopil v veljavo 15. junija 2007 (Forbes in sod., 2007). V letu 2003 je namreč Svet evropske unije sprejel poenostavljen postopek za spremembo dodatkov konvencije, ki omogoča spreminjanje in dopolnjevanje dodatkov Konvencije ETS 123 brez njihove potrditve, saj »so določbe dodatkov h konvenciji tehnične narave in morajo odražati najnovejši znanstveni in tehnični razvoj ter izsledke raziskav na področjih, ki jih pokriva navedena konvencija« (Directive 2003/65/EC).

Revidiran dodatek A temelji na najnovejših znanstvenih dognanjih in izkušnjah, v nekaterih delih (kjer znanost še ne razpolaga z zadostnimi podatki) pa na mnenju stokovnjakov in dobri laboratorijski praksi (Forbes in sod., 2007). Spremenjen in dopolnjen dodatek prinaša veliko novosti, saj vsebuje preko 100 strani in priporoča za živali prijaznejše pogoje oskrbe in nastanitve, podrobneje se posveča posameznim živalskim vrstam, med katerimi so tokrat še dihurji, dvoživke, plazilci in ribe, ki v prejšnjem dodatku niso bili posebej navedeni (Council of Europe, 2006).

Konvencijo ETS 123 je od leta 1986 pa do danes podpisalo in ratificiralo 20 držav. Lista podpisnic je objavljena na spletu (http://www.conventions.coe.int). Slovenija je podpisala Konvencijo ETS 123 v letu 2002, s čimer je izrazila strinjanje z njeno vsebino. Z ratifikacijo, 15. decembra 2006, pa se je Slovenija zavezala, da bo začela slediti njenim določilom s 1. julijem leta 2008 (Council of Europe, 2007).

## REVIZIJA DIREKTIVE 86/609/EEC

Danes se kažejo cilji EU po zaščiti živali na različne načine. V obdobju od 2006 do 2010 načrtuje EU na internacionalni ravni izvesti ukrepe zagotavljanja zaščite in dobrobiti živali in sicer z zvišanjem minimalnih standardov, pospeševanjem razvoja alternativ živalskim poskusom, določitvijo indikatorjev dobrobiti živali, zagotavljanjem boljše informiranosti strokovnjakov in javnosti ter podpiranjem internacionalnih pobud po zaščiti živali (Commission of the European Communities, 2006).

Cilj v letu 1986 sprejete Direktive 86/609/EEC je bil izboljšati kontrolo uporabe poskusnih živali, postaviti minimalne standarde nastanitve in oskrbe poskusnih živali ter usposabljanja osebja, ki dela s poskusnimi živalmi, zmanjšati število v poskusih uporabljenih živali z zahtevo po prepovedi uporabe živali v primeru obstoja alternativne metode ter pospeševati razvoj in uzakonitev alternativ živalskim poskusom.

Od sprejetja Direktive 86/609/EEC pa do danes je prišlo na področju znanosti o laboratorijskih živalih do velikega napredka. Prav tako je prišlo do razvoja novih tehnik kot so kloniranje in transgeneza, ki niso zajete v Direktivi 86/609/EEC. V današnjem času vse pomembnejši poudarek na etično upravičljivi uporabi živali v poskusih vključuje tudi težnje po izboljšanju dobrobiti laboratorijskih živali, doslednjemu upoštevanju 3R načel (angl. replacement - zamenjava, reduction - zmanjšanje, refinement - izboljšanje), ki je splošno priznani pristop za zmanjšanje uporabe poskusnih živali, močni podpori razvoju alternativnih metod ter upeljavi procesa etičnega presojanja poskusov na živalih. Zaradi navedenega je Evropska komisija v letu 2003 ustanovila Tehnično delovno skupino strokovnjakov, ki je obsegala 4 podskupine. Njihova naloga je bila pripraviti mnenje o ključnih točkah revizije Direktive 86/609/EEC in sicer področje obsega direktive, proces etične presoje, ocenjevanje etične upravičljivosti ter avtorizacijo.

Mnenje so pridobili tudi s strani strokovnjakov s pomočjo vprašalnika za strokovnjake. Rezultati so pokazali, da se strokovnjaki strinjajo s predlogi EU v točkah projektov etičnega ocenjevanja, procesov etične presoje na ustanovah, inšpekcij s strani EU, zahtevah po usposabljanju in kompetenci osebja ter statističnem poročanju, nestrinjanje pa so izrazili v

točkah prepovedi uporabe $CO_2$ ter povezavi med visoko ravnijo dobrobiti živali in zmanjšanjem tveganja pred nasilnimi ekstremisti.

O vprašanjih uporabe živali v poskusih je Evropska komisija pridobila tudi mnenje javnosti. Dobili so 42 655 mnenj, kar je bil tretji največji odziv javnosti na spletne vprašalnike Evropske komisije. Rezultati pa so pokazali, da javnost podpira ukrepe EU, s katerimi bi se dobrobit poskusnih živali povečala. Močno izraženo je bilo tudi mnenje, da bi morala EU nameniti več sredstev razvoju in uzakonitvi alternativam živalskih poskusov. Vsi navedeni dokumenti se nahajajo na straneh Evropske komisije (http://ec.europa.eu/environment/chemicals/lab_animals/works_en.htm).

18. junija 2007, leto dni po sprejetju revidiranega dodatka A h Konvenciji ETS 123, je Evropska komisija sprejela Priporočila o navodilih za nastanitev in oskrbo živali uporabljenih v poskusne in druge znanstveno-raziskovalne namene (Recommendation on guidelines for the accommodation and care of animals used for experimental and other scientific purposes), ki v celoti povzemajo v letu 2006 sprejeti dodatek A Konvencije ETS 123.

## PRIPOROČILA ZA PROGRAME USPOSABLJANJA OSEBJA, KI DELA S POSKUSNIMI ŽIVALMI

Države v Evropi se razlikujejo po kulturnih in moralnih vrednotah, razvoju in zakonodaji (Direktiva 86/609/EEC predpisuje le minimum). V skladu s prizadevanji EU po odstranitvi nepotrebnih meja in pregrad, ustvarjanju enotnega trga, internacionalni izmenjavi znanstvenikov ter medsebojnem sodelovanju znotraj EU, je FELASA v letih med 1995 in 2000 pripravila in objavila priporočila za izobraževanje in usposabljanje osebja udeleženega pri delu s poskusnimi živalmi znotraj evropskega prostora (FELASA, 1995; Nevalainen in sod., 2000; Berge in sod., 1999). V letu 2003 je FELASA v skladu s svojimi priporočili vzpostavila tudi sistem akreditacije tečajev usposabljanja (Nevalainen in sod., 2002), ki zagotavlja razvoj visoko kvalitetnega usposabljanja za oskrbovalce poskusnih živali (kategorija A1-A4) (FELASA, 1995), izvajalce (kategorija B) (Nevalainen in sod., 2000) in vodje poskusov na živalih (kategorija C) (FELASA, 1995) ter specialiste (kategorija D) (Berge in sod., 1999) po celotni Evropi. Od leta 2003 pa do danes je FELASA odobrila tečaje usposabljanja v petih evropskih državah. Cilj FELASA je omogočiti vsem evropskim državam vzpostavitev visoko kvalitetnih in mednarodno priznanih tečajev usposabljanja o znanosti o laboratorijskih živalih ter posledično olajšati izmenjavo osebja znotraj Evropskih držav (Vergara in sod., 2007).

Na svetovni ravni potekajo prizadevanja po harmonizaciji programov usposabljanja osebja s strani ICLAS. Zavedajoč se, da potekajo usposabljanja v različnih državah sveta v odvisnosti od njihove lokalne oziroma nacionalne zakonodaje, običajev in vrednot, je njihov cilj najprej določiti skupno prakso, področja in teme pri ustvarjanju programov usposabljanja in zagotavljanju kompetentnosti osebja (Dennis, 2007).

Potreba po ustrezno usposobljenem osebju, ki dela s poskusnimi živalmi, namreč izhaja predvsem iz etičnih načel humanega ravnanja s poskusnimi živalmi, odgovorni uporabi živali v poskusih ter zahvaljujoč miselnosti, da dobra znanost nastaja po zaslugi dobro usposobljenih ljudi. S tem namenom je bila s strani FELASA v letu 2006 ustanovljena delovna skupina za pripravo priporočil o kontinuiranem izobraževanju osebja, ki dela s poskusnimi živalmi (kategorije A,B,C in D). Naloga delovne skupine je določiti minimalne kriterije za navedene kategorije in programe tečajev ter razmisliti o sistemu za akreditacijo tovrstnih tečajev. Priporočila bodo objavili v letu 2008 (Smith D in sod., 2007).

## ETIČNA PRESOJA POSKUSOV NA ŽIVALIH

V današnjem razvitem svetu je veliko govora o etiki na različnih področjih znanosti. O etični presoji je bilo veliko povedanega tudi na srečanju, kjer so bili znanstveniki in strokovnjaki mnenja, da je etična presoja uporabe živali v poskusu eden izmed pomembnih delov raziskovalnih projektov s stališča zaščite živali kot tudi ustvarjanja mnenja javnosti. Je namreč način preko katerega lahko javnost verjame in zaupa znanstveni skupnosti, da so poskusi izvedeni v skladu s predpisi in etičnimi normami. Prav tako priporočajo, da so znanstveniki dostopni za javnost in z njo kumunicirajo o vzrokih in načinih izvajanja raziskav, ki vključujejo poskuse na živalih. Pred tem pa morajo vsekakor temeljito prediskutirati vsa vprašanja o možnostih uporabe alternativ in za izvedbo raziskave izbrati ustrezna orodja, ki so na voljo (od in vitro raziskav do živalskih poskusov). Če namreč nespornost (validnost) znanstvenih raziskav ne more biti izkazana, potem se živali ne sme uporabljati v poskusih, ki povzročijo potencialno bolečino, trpljenje, stres ali trajne poškodbe, saj so etično neupravičeni (Eklof, 2007).

Večina držav članic EU je v preteklem desetletju ustanovila etične komisije na podlagi lastne iniciative, z namenom etičnega presojanja poskusov na živalih (Smith JA in sod., 2007). Ker so etične norme odvisne od zgodovine, razvoja in običajev posamezne družbe, je eden izmed ciljev EU tudi harmonizacija procesa etične presoje in etičnih norm na področju poskusov na živalih znotraj držav članic. S tem namenom sta bili ustanovljeni 2 podskupini znotraj Tehnične delovne skupine strokovnjakov, ki sta preučili proces etične presoje uporabe živali v poskusih in pripravili navodila o sestavi etičnih komisij, njihovem delu in pristojnosti ter vrednotenju posameznih poskusov glede na zgornjo mejo trpljenja, ki ga utrpijo živali v poskusu. Mnenje Tehnične delovne skupine strokovnjakov je, da so etične komisije za presojanje etične upravičenosti vseh poskusov v znanstveno-raziskovalne namene, ki sodijo v okvir Direktive 86/609/EEC, nujne. Etično komisijo naj bi poleg znanstvenikov sestavljali še strokovnjaki s področja etike kot so filozofi ali moralni teologi lahko tudi strokovnjaki s področja o dobrobiti živali. Povdarili so, da je treba vzpostaviti tak sistem, da se bodo sklepi Etičnih komisij udejanili tudi v praksi (http://ec.europa.eu/environment/chemicals/lab_animals/works_en.htm). O procesu etične presoje je smernice pripravila tudi FELASA (Smith JA in sod., 2007). Tako Tehnična delovna skupina strokovnjakov kot FELASA so mnenja, da mora proces etične presoje zagotoviti, da je na vseh korakih znanstveno-raziskovalnega dela, ki zajema uporabo živali (torej od začetka načrtovanja do zaključka poskusa in pregleda rezultatov) jasno izražena etična upravičljivost uporabe živali, ki je v vsakem koraku podvržena kritični presoji.

V zadnjih letih je vse več razprav tudi o retrospektivnih raziskavah poskusov na živalih s stališča ovrednotenja etičnosti uporabe živali po opravljenem poskusu in pridobljenih rezultatih. Na ta način naj bi se v prihodnosti izoblikovali boljši kriteriji etičnega ocenjevanja, ki bi temeljili na izkušnjah (Smith JA in sod., 2007; Jenings in sod., 2007).

## ALTERNATIVE ŽIVALSKIM POSKUSOM

Težnje EU po zmanjšanju uporabe živali v poskusih, ki so bile nakazane že v Direktivi 86/609/EEC, se danes že kažejo na področju preskušanja varnosti kozmetičnih produktov in kemikalij.

Na področju kozmetičnih produktov je EU izdala Direktivo 93/35/EEC o prepovedi trgovanja s kozmetičnimi produkti testiranimi na živalih že leta 1993. Prepoved naj bi stopila v veljavo s 1. januarjem 1998, vendar pa so jo zaradi nezadostnega napredka pri razvoju in uzakonitvi alternativnih metod, prestavili kar dvakrat (Hartung in sod., 2003). Končno so v letu 2003 sprejeli Direktivo 2003/15/EC, s katero so uvedli takojšnjo prepoved testiranja na živalih za končne kozmetične izdelke in takojšnjo prepoved trgovanja z novimi kozmetičnimi produkti (končni produkti in sestavine), ki so bili testirani na živalih namesto na že sprejetih alternativnih

metodah. Nenazadnje, Direktiva 2003/15/EC predpisuje dokončno prepoved uporabe živali za testiranje kozmetičnih sestavin po 6-ih oziroma 10-ih letih od sprejetja (slednje v primeru proučevanja reproduktivne toksičnosti in toksikokinetike).

Tudi na področju testiranja oziroma določanja toksičnosti kemikalij se kažejo vse večje težnje po zmanjševanju uporabe živali, predvsem v tistih delih testiranja, ki temeljijo na lokalni toksičnosti in določanju praga toksičnosti (Hartung in sod., 2003; Zvinavashe in sod., 2007; Van de Sandt, 2007). Evropski parlament je namreč pred kratkim sprejel nov sistem kontrole kemikalij, t.i. REACH (Registration, Evaluation and Authorization of Chemicals), s katerim omejuje uporabo živali v testiranjih in pospešuje razvoj in uzakonitev alternativ. Izračuni so namreč pokazali, da bi bilo treba za ocenjevanje varnosti kemikalij (angl. safety assessment of chemicals) uporabiti preko 3,9 milijona živali, nekateri govorijo celo o 12,8 milijonih vretenčarjev (Hartung in sod., 2003; Van de Sandt in sod., 2007). Nekatera testiranja na živalih lahko danes v celoti nadomestimo z uporabo ene alternativne metode (npr. test na kožno korozivnost in test fototoksičnosti), medtem ko je določanje sistemske (med katere sodijo tudi karcinogenost in reproduktivna toksičnost) in kronične toksičnosti veliko zahtevnejše in je zato testiranje na živalih zaenkrat edina ustrezna metoda. Ključna slabost razvoja alternativnih metod na področju sistemske toksičnosti je odsotnost procesov kot so adsorpcija, distribucija, metabolizem in izločanje, ki v organizmu določajo razporejanje učinkovine do tarčnih tkiv. Vendar pa ugotavljajo, da bi bilo mogoče doseči dobre rezultate s kombinacijo različnih alternativnih metod (Hartung in sod., 2003; Van de Sandt in sod., 2007). Zato potekajo na področju razvoja in uzakonjenja alternativnih metod v EU obsežne raziskave na mednarodni ravni v okviru ECVAM (the European Centre for the Validation of Alternative Methods), ki je bil v ta namen ustanovljen v letu 1991 na podlagi Direktive 86/609/EEC. ECVAM je danes vodilni mednarodni center v EU za koordinacijo uzakonitve alternativnih metod na področju biomedicine, pretežno toksikologije (Gribaldo, 2007). Na spletnih straneh ECVAM je zaslediti, da je danes razvitih in znanstveno sprejetih že 21 metod, ki bodo zamenjale uporabo živali v toksikoloških testiranjih, med njimi je 7 tudi že uzakonjenih (http://ecvam.jrc.it/index.htm).

## IZBOLJŠAVE POGOJEV NASTANITVE IN POSTOPKOV

Kljub močnim težnjam po čimhitrejšem razvoju alternativnih metod v zadnjih dveh desetletjih, se znanstveniki in strokovnjaki danes vse bolj zavedajo, da je razvoj alternativ živalskim poskusom počasen proces. Na srečanju je bilo večkrat povdarjeno, da je uporaba živali v znanstveno-raziskovalne namene v biomedicini še vedno neizbežna, še posebno na področju bazičnih raziskav. Zato je danes potreba po izboljšanju pogojev nastanitve živali in postopkov, ki se izvajajo na živalih v poskusih, vse bolj izražena in dobiva vse večji pomen in podporo tudi s strani EU. Z namenom poskrbeti za zaščito in dobrobit živali na način, da bodo živali v poskusih utrpele čim manj nelagodja, bolečin ali trpljenja, potekajo številne raziskave. Znanstveno neizpodbitno je dejstvo, da nesporne, zanesljive in ponovljive rezultate lahko dobimo le na zdravih živalih, ki imajo zadovoljene fiziološke in etološke potrebe. Z uporabo takih živali je najpogosteje tudi variabilnost rezultatov manjša, ki posledično vpliva tudi na zmanjšano število uporabljenih živali v poskusu.

Čeprav imajo danes poskusne živali boljše nastanitvene pogoje in je zanje bolje poskrbljeno tudi s strani oskrbovalcev in izvajalcev, ki morajo biti izkušeni in ustrezno usposobljeni, je v znanosti o laboratorijskih živalih vse več raziskav usmerjenih v iskanje ustreznih bioloških označevalcev stresa, bolečine (Roughan, 2007) oziroma dobrobiti živali (Serviere, 2007), izboljšav tehnik in postopkov v poskusih (Hoyt, 2007), optimalnih nastanitvenih oziroma obogatitvenih pogojih (Spangenberg in sod., 2007). Ker se vse več poskusov (tudi kirurških) dela na glodalcih, ki so v primerjavi s psi ali mačkami bistveno manjši, je treba poskrbeti tudi za

ustrezno (mikro)kirurško opremo (Hoyt, 2007) in kadar je le mogoče med poskusom uporabljati analgezijo in anastezijo. Strokovnjaki za laboratorijske živali namreč ugotavljajo, da se analgezija in anestezija pri glodalcih premalo uporabljata (Flecknell, 2007).

Vse večji je poudarek na humanih končnih ciljih (angl. humane endpoints), kjer se pri bolečih poskusih na živalih išče ustrezen čas zaključka poskusa za pridobitev ustreznih rezultatov (vsekakor predno bi ta utrpela bolečine in trpljenje zaradi katerih bi poginila). Gre za določanje končnih ciljev poskusa pri katerih bolečino ali trpljenje živali v poskusu končamo s humano evtanazijo ali zmanjšamo s prekinitvijo bolečih postopkov oziroma s protibolečinskim zdravljenjem (Morton, 1998; Castle, 1998).

## HARMONIZACIJA ŽIVALSKIH POSKUSOV NA SVETOVNI RAVNI

Že nekaj časa so na področju poskusov na živalih prisotne težnje o vpeljavi mednarodno uveljavljenih standardih na svetovni ravni. Strokovnjaki ugotavljajo, da čeprav večina nacionalnih sistemov temelji na osnovnih principih humane znanosti, vključujoč 3R, so standardi oskrbe in uporabe živali v državah na različnih koncih sveta različni. Zato je otežkočeno primerjanje rezultatov živalskih poskusov, saj je njihova ponovljivost vprašljiva, kar oteži globalno sprejetje znanstvenih podatkov in upočasni mednarodno znanstveno sodelovanje (Demers in sod., 2007).

## TRENUTNO STANJE V SLOVENIJI

Področje poskusov na živalih je v zadnjem času zelo živahno tudi v Sloveniji, kjer potekajo na terenu komisijski pregledi in odobritve organizacij za delo s poskusnimi živalmi. Živahno je tudi na zakonodajni ravni, saj so bili v Sloveniji od 2006 dalje sprejeti kar trije predpisi s področja poskusnih živali in sicer Pravilnik o načinih usmrtitve poskusnih živali, Pravilnik o pogojih za izvajanje poskusov na živalih, ki je v celoti zamenjal Pravilnik o strokovnih, kadrovskih in tehničnih pogojih za opravljanje poskusov na živalih ter novela Zakona o zaščiti živali. Kljub pred kratkim sprejetim predpisom, pa lahko v bližnji prihodnosti pričakujemo nove spremembe, predvsem na področju nastanitve in oskrbe poskusnih živali, ki jih prinaša sprememba Konvencije ETS 123.

## ZAKLJUČEK

Ker so živali živa bitja, ki občutijo nelagodje, bolečino, trpljenje in stisko, je skrb za dobrobit živali in njihovo etično upravičeno uporabo v poskusih v današnji družbi prisotna na vseh nivojih družbenega življenja. Poskusi na živalih so v biomedicini še vedno nenadomestljivi. In ker je proces razvoja in uzakonjenja alternativnih metod počasnejši kot je bilo pričakovati, je danes vse več pozornosti namenjeno raziskavam na področju dobrobiti živali, v smislu zagotavljanja optimalnih pogojev nastanitve za posamezno živalsko vrsto in izboljševanja tehnik, ki so sestavni del poskusov na živalih. Tovrstne raziskave in spoznanja podpira tudi EU, ki si je zadala cilj izboljšati minimalne standarde in pogoje oskrbe in nastanitve poskusnih živali na mednarodni ravni. Kar se bo posledično odražalo tudi v naši zakonodaji.

## VIRI

Berge, E./ Gallix, P./ Jilge, B./ Melloni, E./ Thomann, P./ Waynforth, B./ van Zutphen, L.F. FELASA guidelines for education of specialists in laboratory animal science (Category D). Report of the Federation of Laboratory Animal Science Associations Working Group on Education of Specialists (Category D) accepted by the FELASA Board of Management. Lab Anim 33(1999), 1-15.

Castle, P. The European Pharmacopoeia and humane endpoints. V: Humane endpoints in animal experiments for biomedical research. Proceeding of the International Conference, 23-25 November 1998. The Netherlands. (ur.: Hendriksen, C.F.M./ Morton, D.B.). London, RMS Press LTD, 1998, 15-19.

Commission of the European Communities. Communication from the Commission to the European Parliament and the Council of 23 January 2006 on a Community Action Plan on the Protection and Welfare of animals 2006-2010. (Text with EEA relevance) Brussels, 2006. Official Journal of the European Communities L 49 of 28.02.2006.

Council of Europe. Appendix A of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS No. 123). Guidelines for accommodation and care of animals (Article 5 of the Convention) approved by the multilateral consultation. Strasbourg; 15. June 2006.

Council of Europe. European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123). Strasbourg, 1986.

Council of Europe. European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123). Chart of signatures and ratifications. Strasbourg, Junij 2007.

Council of the European Communities. Council Directive on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (86/609/EEC). Brussel, 1986. Official Journal of the European Comunities L 358; 1986.

Council of the European Communities. Directive 93/35/EEC of 14 June 1993 amending for the sixth time Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmentic products. Official Journal of the European Communities L151, 32-37.

Demers, G. Impact of harmonization at the international level. FELASA-ICLAS joint meeting abstract book; 2007 June 11-14; Cernobbio, Italy, 2007, 62.

European Parliament, Council of the European Communities. Directive 2003/15/EC of the European Parliament and of the Council of 27 February 2003 amending Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products (Text with EEA relevance) Official Journal of the European Union L66, 26-35.

European Parliament, Council of the European Communities. Directive 2003/65/EC of the European Parliament and of the Council of 22 July 2003 amending Council Directive 86/609/EEC on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. Official Journal of the European Communities L230, 32-33.

Dennis, M.B. Training of research animal users around the world.. FELASA-ICLAS joint meeting abstract book; 2007 June 11-14; Cernobbio, Italy, 2007, 111.

Eklof, A.C. Ethical evaluation for animal experimentation. A question of cost/benefit, research development and communication. FELASA-ICLAS joint meeting abstract book; 2007 June 11-14; Cernobbio, Italy, 2007, 86-87.

FELASA. FELASA recommendations on the education and training of persons working with laboratory animals: categories A and C. Reports of the Federation of European Laboratory Animal Science Associations Working Group on Education accepted by the FELASA Board of Management. Lab Anim 29(1995), 121-131.

Flecknell, P. Refining laboratory animal anasthesia and analgesia – scientific, ethical and practical issues. FELASA-ICLAS joint meeting abstract book; 2007 June 11-14; Cernobbio, Italy, 2007, 122.

Forbes, D./ Blom, H./ Kostomitsopoulos, N./ Moore, G./ Perretta, G. Euroguide on the accommodation and care of animals used for experimental and other scientific purposes (Based on the revised Appendix A of the European Convention ETS 123). London, RMS Press LTD, 2007.

Gribaldo, L. Alternative methods for refinement and reduction of animal use in biomedical sciences. FELASA-ICLAS joint meeting abstract book; 2007 June 11-14; Cernobbio, Italy, 2007, 102.

Hartung, T./ Bremer, S./ Casati, S./ Coecke, S./ Corvi, R./ Fortaner, S./ Gribaldo, L./ Halder, M./ Roi, A.J./ Prieto, P./ Sabbioni, E./ Worth, A./ Zuang, V. ECVAM's Response to the Changing Political Environment for Alternatives: Consequences of the European Union Chemicals and Cosmetics Policies. ATLA 31(2003), 473-481.

Hoyt, R.F. Challenges in developing a state-of-the-art rodent surgical facility. FELASA-ICLAS joint meeting abstract book; 2007 June 11-14; Cernobbio, Italy, 2007, 18.

Jenings, M./ Howard, B.R./ Berdoy, M. Retrospective review as an integral part of ethical review processes. FELASA-ICLAS joint meeting abstract book; 2007 June 11-14; Cernobbio, Italy, 2007, 88.

Morton, D.B. Humane endpoints in animal experimentation for biomedical research: ethical, legal and practical aspects. V: Humane endpoints in animal experiments for biomedical research. Proceeding of the International Conference, 23-25 November 1998. The Netherlands. (ur.: Hendriksen, C.F.M./ Morton, D.B.). London, RMS Press LTD, 1998, 5-11.

Nevalainen, T./ Dontas, I./ Forslid, A./ Hoeard, B.R./ Klusa, V./ Kaesermann, H.P./ Melloni, E./ Nebendahl, K./ Stafleu, F.R./ Vergara, P./ Verstegen, J./ FELASA. FELASA recommendations on the education and training of persons working with laboratory animals (category B). Report of the Federation of European Laboratory Animal

Science Associations Working Group on Education of Persons Carrying out Animal Experiments (Category B) accepted by the FELASA Board of Management. Lab Anim 34(2000), 229-235.

Nevalainen, T./ Blom, H.J.M./ Guaitani, A./ Hardy, P./ Howard, B.R./ Vergara, P. FELASA recommendations for the accreditation of laboratory animal science education and training. Report of the Federation of Laboratory Animal Science Associations Working Group on Accreditaion of laboratory animal science education and training. Lab Anim 36(2002), 373-377.

Pravilnik o načinih usmrtitve poskusnih živali. Ur. l. RS, št. 140/2006.

Pravilnik o pogojih za izvajanje poskusov na živalih. Ur. l. RS, št. 88/2006.

Pravilnik o strokovnih, kadrovskih in tehničnih pogojih za opravljanje poskusov na živalih. Ur. l. RS, št. 36/2004 in 40/2004.

Roughan, J. Assessing and alleviating pain in laboratory animals. FELASA-ICLAS joint meeting abstract book; 2007 June 11-14; Cernobbio, Italy, 2007, 119.

Serviere, J. Towards new interpretations of biological markers used for the assessment of animal welfare? FELASA-ICLAS joint meeting abstract book; 2007 June 11-14; Cernobbio, Italy, 2007, 54.

Smith, D./ Dorier, A./ Hack, R./ Scand, M.S./ Visa, J./ Zeller, W./ Ruane, B./ Weiss, J. Felasa working group: Guidelines/recommendations for continuing education. FELASA-ICLAS joint meeting abstract book; 2007 June 11-14; Cernobbio, Italy, 2007, 112.

Smith, J.A./ van den Broek, F.A.R./ Canto Matorell, J./ Hackbarth, H./ Ruksenas, O./ Zeller, W. Principles and practice in ethical review of animal experiments across Europe: summary of the report of a FELASA working group on ethical evaluation of animal experiments. Lab Anim 41(2007), 143-160.

Spangenberg, E./ Dahlborn, K./ Essen-Gustavsson, B./ Cvek, K. Assessing the effect of different housing conditions on the welfare of laboratory rats, with focus on physical fitness. FELASA-ICLAS joint meeting abstract book; 2007 June 11-14; Cernobbio, Italy, 2007, 56.

Spletne strani Evropske komisije (poročila in vprašalniki):
http://ec.europa.eu/environment/chemicals/lab_animals/pdf/finalreportscope.pdf
http://ec.europa.eu/environment/chemicals/lab_animals/pdf/finalreportethicalreviewprocess.pdf
http://ec.europa.eu/environment/chemicals/lab_animals/pdf/finalreportcostbenefit.pdf
http://ec.europa.eu/environment/chemicals/lab_animals/pdf/finalreportauthorisation.pdf
http://ec.europa.eu/environment/chemicals/lab_animals/questionnaire2.htm
http://ec.europa.eu/environment/chemicals/lab_animals/questionnaire1.htm

Zvinavashe, E./ Murk, A.J./ Vervoort, J./ Soffers, E.M.F./ Freidig, A./ Rietjens, I.M.C.M. ZonMW Project 3170.0066. Development of a computational chemistry-based QSAR approach that sets priorities in toxicity testing of selected groups of EINECS list chemicals. NCA. Newsletter; 22(2007), 3-5.

Van de Sandt, J.J.M. Intelligent testing under the new European chemicals regulation REACH. NCA. Newsletter; 22(2007), 14-15.

Vergara, P./ Demers, G. Laboratory animal science and service organization. V:. Handbook of laboratory animal science: essential principles and practices. 2nd ed. (ur.: Hau, J./ Van Hoosier, G.L.) Boca Raton, CRC Press LLC, 2003, 77-105.

Vergara, P./ Costa, P./ Hau, J./ Howard, B./ Kaesermann, H.P./ Nebendhal, K. FELASA accreditation for teaching and training: harmonization from diversity. FELASA-ICLAS joint meeting abstract book; 2007 June 11-14; Cernobbio, Italy, 2007, 110.

Zurlo, J. International efforts to identify research needs in support of science-based guidelines for laboratory animal care. FELASA-ICLAS joint meeting abstract book; 2007 June 11-14; Cernobbio, Italy, 2007, 64.

Zakon o spremembah in dopolnitvah zakona o zaščiti živali (ZZZiv-B). Ur. l. RS, št. 14/2007.

*Acta agriculturae Slovenica*, 92(december 2008)2, 173–183.

http://aas.bf.uni-lj.si

Agris category codes: L51 COBISS Code 1.01

# UGOTAVLJANJE ŠTEVILA PROBIOTIČNIH MIKROORGANIZMOV V KRMNIH MEŠANICAH

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## IZVLEČEK

V prehrani živali se uporablja širok spekter probiotičnih mikroorganizmov, ki pripadajo mlečnokislinskim bakterijam, kvasovkam ali rodu *Bacillus*. V raziskavi smo ugotavljali število mikroorganizmov oziroma njihovih spor v različnih krmnih mešanicah in premiksih, ki so vsebovali komercialne probiotične pripravke Bioplus 2b, Vebac ali Biosaf. Pri bakterijskih sevih *Bacillus subtilis* in *Bacillus licheniformis*, ki smo ju osamili iz pripravka Bioplus 2b, smo ugotavljali občutljivost za antibiotike, protimikrobno aktivnost ter sposobnost preživetja v simuliranem želodčnem in črevesnem soku. Ugotovili smo, da je bilo v vseh testiranih proizvodih število probiotičnih mikroorganizmov oziroma spor manjše od deklariranega. Razlika med deklariranim in ugotovljenim številom je bila največja pri proizvodih z dodatkom pripravkov Vebac in Biosaf. Izolata *B. subtilis* in *B. licheniformis* sta občutljiva za vse izbrane antibiotike razen metronidazola. Test preživetja je pokazal, da simulirana želodčni oz. črevesni sok nimata večjega vpliva na preživetje spor, vegetativne celice obeh sevov *Bacillus* pa so zelo občutljive za nizke vrednosti pH in žolčne soli. *B. licheniformis* je inhibiral rast predvsem po Gramu pozitivnih bakterij, medtem ko je bil *B. subtilis* učinkovitejši proti po Gramu negativnim bakterijam.

Ključne besede: živinoreja / prehrana živali / krmne mešanice / mikrobiologija / probiotiki / *Bacillus* / antibiotiki / protimikrobna aktivnost

# ENUMERATION OF PROBIOTIC MICROORGANISMS IN FEED MIXTURES

## ABSTRACT

In animal nutrition a wide range of probiotic microorganisms belonging to lactic acid bacteria, yeasts and *Bacillus* genus are used. In this study we determined the number of microorganisms in different complete feed mixtures and premixtures, containing commercial probiotic supplements Bioplus 2b, Vebac or Biosaf. *Bacillus subtilis* and *Bacillus licheniformis* bacterial strains, which were isolated from product Bioplus 2b, were tested for antibiotic susceptibility, antimicrobial activity and ability to survive in simulated gastric and intestinal juice. We found that all tested products contained lower number of microorganisms than declared. The difference between declared and established value was the highest in products with Vebac and Biosaf. *B. subtilis* and *B. licheniformis* isolates were sensitive to all selected antibiotics except metronidazol. Survival test showed that simulated gastric or intestinal juices did not have considerable effect on spore survival, while vegetative cells of *Bacillus* strains were very sensitive to low pH and bile salts. *B. licheniformis* inhibited mostly Gram positive bacteria, while *B. subtilis* was more efficient against Gram negative bacteria.

Key words: animal husbandry / animal nutrition / feed mixtures / microbiology / probiotics / *Bacillus* / antibiotics / antimicrobial activity

## UVOD

Probiotiki se v prehrani živali uporabljajo že vrsto let. Zanimanje za njihovo uporabo pa se je s prepovedjo uporabe antibiotikov kot pospeševalcev rasti v Evropski uniji z letom 2006 še povečalo. Probiotični mikroorganizmi predstavljajo v prehrani živali alternativo antibiotikom, saj lahko s svojo prisotnostjo oz. metabolično aktivnostjo uravnavajo mikrobno ravnotežje v prebavilih ter bolj ali manj učinkovito vplivajo na imunski status živali. Praksa je pokazala, da živali z 'zdravo' mikrofloro bolje priraščajo, porabijo manj krme za enoto prirasta in imajo bistveno manj zdravstvenih težav (Ortwin, 2005).

Komercialni probiotični dodatki in krmila morajo v deklaraciji vsebovati informacijo o vrsti mikroorganizma (identifikacijska številka seva) in minimalnem številu kolonijskih enot na gram oz. mililiter proizvoda (Uredba 1831/2003/ES). Analize pogosto pokažejo razkorak med deklarirano in ugotovljeno mikrobno sliko. Največkrat gre za manjše število celic od deklariranega, nemalokrat celo za nepravilno ali pomanjkljivo identifikacijo mikroorganizmov (Green in sod., 1999; Duc in sod., 2004; Hoa in sod., 2000), kar je z vidika varovanja zdravja zelo sporno.

Učinek določenega probiotika, ki ga dodajamo živalim v krmo ali vodo za napajanje, je odvisen predvsem od starosti, zdravstvenega stanja in vrste živali. Znano je, da so učinki probiotikov opaznejši pri mladih in stresu izpostavljenih živalih. Nerazvita prebavna mikroflora ali pa porušeno ravnovesje le-te (disbioza) največkrat vodita v slabšo telesno kondicijo, zahiranost in razne prebavne težave, npr. driske, ki jih povzročajo bakterijski toksini. Probiotiki s svojo prisotnostjo in/ali aktivnostjo vzdržujejo mikrobno ravnovesje prebavil (evbiozo), tako da zavrejo oz. preprečijo razrast neželenih mikroorganizmov (De Vrese in Marteau, 2007).

Eden pomembnejših aspektov uporabe probiotikov v živalski prehrani je njihova uporabnost oziroma primerne tehnološke lastnosti. Mikroorganizmi, ki jih dodajamo krmilom, morajo preživeti bolj ali manj agresivne tehnološke procese, kot so peletiranje, mešanje, briketiranje ali ekspandiranje. Zelo pomembno je, da ohranijo viabilnost tudi med daljšim skladiščenjem. Mikroorganizme lahko v ta namen zaščitijo s posebnimi postopki kot sta enkapsulacija ali 'coating', kjer žive celice obdajo z zaščitnimi plastmi, sestavljenimi iz mikrogranul, odmrlih celic ali drugih snovi. Tako lažje preživijo visoko temperaturo in pritisk, ki nastajata pri omenjenih postopkih (Ortwin, 2005).

V prehrani živali se uporabljajo različni probiotični mikroorganizmi, med katerimi prevladujejo bakterijske vrste iz rodov *Enterococcus (E.)* ali *Bacillus (B.)* ter kvasovke *Saccharomyces (S.)*. Posebej zanimivi so mikroorganizmi iz rodu *Bacillus*, ki sicer niso nativni prebivalci prebavil, ampak bolj prehodni. Kot probiotiki so zanimivi predvsem zato, ker tvorijo endospore, ki so odporne proti različnim ekstremnim okoljskim razmeram (temperatura, pH, encimi, kemikalije, radiacija). Spore rodu *Bacillus* brez večjih izgub preživijo proizvodne procese in prehod preko prebavil do debelega črevesa, kjer lahko učinkujejo (Spinosa in sod., 2000). Zaradi svoje prehodne narave imajo velik potencial lokalnega stimuliranja imunskega sistema prebavil, o čemer poroča vse več študij (Sanders in sod., 2003; Duc in sod., 2004a; Midilli in sod., 2008).

Namen naše raziskave je bil ovrednotiti število probiotičnih mikroorganizmov v krmilih, ki so bila izdelana z različnimi postopki. Pri dveh probiotičnih mikroorganizmih iz rodu *Bacillus*, ki smo ju osamili iz produkta Bioplus 2b, smo preučili še sposobnost preživetja skozi želodec in tanko črevo, občutljivost za antibiotike in protimikrobne aktivnosti. Omenjene lastnosti so namreč za probiotike posebej pomembne.

## MATERIAL IN METODE

### Material

Obravnavali smo 16 vzorcev krmil za živali slovenskega proizvajalca, od tega 14 dopolnilnih oziroma vitaminsko-mineralnih mešanic in dve popolni krmni mešanici. Vzorci so bili stari največ tri dni. Razen po obliki, t. j. peletirani, drobljeni oziroma prašnati, so se razlikovali po probiotičnem dodatku. Osem vzorcev je vsebovalo pripravek Bioplus 2b (Christian Hansen, Danska), pet vzorcev Vebac (Medipharm AB, Švedska), trije vzorci pa Biosaf (S.I. Lesafre, Francija). Vsebovani mikroorganizmi in deklarirano število so podani v pregl. 1.

Preglednica 1. Vrste in oznake sevov probiotičnih mikroorganizmov v komercialnih probiotičnih pripravkih ter deklarirano število
Table 1. Species and strain designation of probiotic microorganisms in commercial probiotic supplements and declared number

| Ime probiotičnega pripravka<br>Name of probiotic supplement | Vrsta in oznaka seva<br>Species and strain designation | Število v g, KE/g<br>Number per g, CFU/g |
|---|---|---|
| Bioplus 2b | *Bacillus subtilis* (DSM 5750)[a] | $1{,}6 \times 10^9$ |
| | *Bacillus licheniformis* (DSM 5749)[a] | $1{,}6 \times 10^9$ |
| Vebac | *Enterococcus faecium* M74 (NCIMB 11181) | $5{,}0 \times 10^{10}$ |
| Biosaf | *Saccharomyces cerevisiae* (NCYC sc 47) | $5{,}0 \times 10^9$ |

[a] spore/ spores; KE, kolonijske enote; CFU, colony forming units

### Metode

Ugotavljanje števila mikroorganizmov

Za ugotavljanje števila mikroorganizmov v krmnih mešanicah smo uporabili validirane metode, kot jih navajajo Leuschner in sod. (2002, 2003, 2003a). Vzorce smo homogenizirali v 0,2 % raztopini NaOH oziroma fosfatnem pufru, decimalne redčitve pa smo izvedli v peptonski vodi (pepton 1g/l, NaCl 8,5 g/l, Tween 80 0,01 %, pH 7,0 ± 0,2). Raztopine vzorcev, ki so vsebovali spore bacilov, smo temperirali v vodni kopeli 10 minut pri 80 °C, s čemer smo uničili vegetativne celice in spodbudili germinacijo spor. Za spore *Bacillus* smo uporabili gojišče TSA (triptic soy agar) (Biolife, Italija), za enterokoke BEA (bile esculin azid) in za kvasovke CGYE (chloramphenicol glucose yeast extract). Vzorce z dodatkom pripravka Bioplus 2b smo inkubirali 24 ur pri 30 °C. To modifikacijo smo izvedli zato, ker so bile kolonije *B. subtilis* in *B. licheniformis* po 16 urni inkubaciji pri temperaturi 37 °C tako velike, da je to oteževalo štetje. Enterokoke smo inkubirali 24 ur pri 37 °C, kvasovke pa 48 ur pri 35 °C.

Ugotavljanje občutljivosti za antibiotike

Pri mikroorganizmih *B. subtilis* in *B. licheniformis,* ki smo ju osamili iz preparata Bioplus 2b, smo testirali občutljivost za deset izbranih antibiotikov s pomočjo sistema E-test (AB Biodisk, Švedska). Upoštevali smo navodila proizvajalca. Celice iz 16-urne kulture inkubirane v gojišču BHI (Merck, Nemčija) pri 30 °C, smo centrifugirali (3500 g, 10 min), sprali z ¼ Ringerjevo raztopino ter resuspendirali v primerni količini iste raztopine, tako da je bila optična gostota pri valovni dolžini 625 nm med 0,04 in 0,05, kar je ustrezalo koncentraciji $10^7$ celic/ml. Po 100 µl suspenzije smo razmazali na petrijeve plošče z agarjem Mueller-Hinton (Merck, Nemčija),

pustili, da se inokulum vpije in površina nekoliko osuši, nato pa nanesli trakove z antibiotiki. Plošče smo inkubirali pri 30 °C, 20 do 24 ur, ter odčitali minimalne inhibitorne koncentracije (MIC) (μg/ml) posameznih antibiotikov.

Ugotavljanje občutljivosti za prebavne sokove

Celice *B. subtilis* in *B. licheniformis* iz 18-urne kulture smo izpostavili simuliranemu okolju, kakršno vlada v prebavilih. V ta namen smo pripravili simuliran želodčni sok in simuliran sok zgornjega tankega črevesa (Barbosa in sod., 2005). Za prvega smo izotoničnemu pufru z Bott-Wilson-ovimi solmi (1,24 % $K_2HPO_4$, 0,76 % $KH_2PO_4$, 0,1 % trinatrijev citrat, 0,6 % $[NH_4]_2SO_4$, pH = 6,7) dodali 0,85 % NaCl in 1 mg/ml pepsina (Sigma, Nemčija) in s HCl umerili vrednost pH na 2. Za simuliran sok tankega črevesa pa smo v izotoničnem pufru raztopili 0,2 % žolčnih soli št.3 (Biolife, Italija) in dodali pankreatin (mešanica pankreasnih encimov) (Sigma, Nemčija). Testirali smo občutljivost spor kot tudi vegetativnih celic obeh organizmov.

Ugotavljanje protimikrobne aktivnosti

Protimikrobno aktivnost *B. subtilis* in *B. licheniformis* smo ocenili z metodo, ki so jo opisali Barbosa in sod. (2005). Točkasto inokulirani kulturi testiranih sevov bacilov, ki so rasli 16 ur na ploščah z Luria-Bertani agarjem, smo izpostavili kloroformnim hlapom za 30 do 60 minut. Plošče smo prepihali z zrakom (20 min) v laminariju in prelili s 5 ml poltrdih gojišč (7,5 g/l agar-agarja) MRS (Merck, Nemčija), BHI (Merck, Nemčija) oziroma RCM (Merck, Nemčija). Ta so bila inokulirana s 50 μl različnih indikatorskh kultur, ki smo jih gojili aerobno 24 ur pri 37 °C. Pri isti temperaturi smo gojili tudi *C. prefringens* vendar v anaerobnih pogojih, 48 ur. Indikatorske kulture so izvirale iz zbirk American type culture collection (ATCC), National collection of dairy organisms (NCDO), Deutsche sammlung von microorganismen (DSM) in zbirke mikroorganizmov Inštituta za mlekarstvo, Biotehniške fakultete (IM). Velikost con inhibicije smo izmerili po 20 do 24 urah inkubacije pri 37 °C oziroma 30 °C (za *Lb. sakei*).

## REZULTATI IN RAZPRAVA

Definicija o probiotikih pravi, da moramo za dosego pozitivnih oz. želenih učinkov le-te zaužiti v zadostni količini (Guarner in Schaafsma, 1998). Vrsta študij na živalih je pokazala, da premajhne količine dodanih probiotikov nimajo učinka ne na prirast, ne na imunski odziv živali, na drugi strani pa tudi presežne količine nimajo nič večjega učinka kot priporočljive (Jin in sod., 1998; Pelicano in sod., 2003; Abdollahi in sod., 2002; Chumpawadee in sod., 2008). Proizvajalci morajo upoštevati izgube živosti mikroorganizmov, ki nastajajo v proizvodnji krmil, sicer v končnem proizvodu ni dovolj živih probiotikov.

V pregl. 2 je prikazano število mikroorganizmov v krmnih mešanicah, ki smo ga ugotovili s štetjem na ploščah in deklarirano število probiotikov, ki smo ga izračunali iz deleža dodanega probiotičnega pripravka.

Vsi vzorci krmil so vsebovali manj probiotičnih mikroorganizmov, kot je deklariral proizvajalec. Razlike med ugotovljenim in deklariranim številom variirajo od 20 do 100 %. Največje izgube smo ugotovili pri proizvodih, ki so vsebovali preparata Vebac in Biosaf, kar ni presenetljivo, saj vsebujeta žive celice *E. faecium* oziroma *S. cerevisiae*. V štirih vzorcih od petih z dodanim *E. faecium* so bile razlike med ugotovljenimi in deklariranimi vrednosti za več kot 96 %. Proizvod Vebac sicer vsebuje enkapsulirane enterokoke, ki po zagotovilih proizvajalca preživijo temperature do 65 °C, zato tako velikih izgub med tehnološkim postopkom ni bilo pričakovati, sploh ker omenjeni izdelki niso bili peletirani.

Preglednica 2. Število (log KE/g) probiotičnih mikroorganizmov v krmnih mešanicah, ugotovljeno s štetjem na ploščah, ter primerjava z deklariranim številom (log KE/g)

Table 2. The number (log CFU/g) of probiotic microorganisms in feed mixtures determined by plate counting and comparison with the number labeled (log CFU/g)

| Vzorec | % dodanega probiotika | Deklarirano št., log KE/g | Ugotovljeno št., log KE/g | Izguba, % [a] |
|---|---|---|---|---|
| Mešanice s pripravkom Bioplus 2b | | | | |
| 1 [b] | 0,07 | 6,35 | 6,12 | 41,7 |
| 2 [p] | 0,04 | 6,11 | 5,75 | 55,6 |
| 3 [d] | 0,03 | 5,98 | 5,86 | 24,9 |
| 4 [p] | 0,06 | 6,28 | 6,19 | 19,8 |
| 5 [p] | 0,06 | 6,28 | 6,18 | 20,8 |
| 6 [b] | 10,00 | 8,51 | 7,92 | 74,0 |
| 7 [b] | 8,00 | 8,41 | 7,83 | 73,7 |
| 8 [b] | 8,00 | 8,41 | 5,65 | 99,8 |
| Mešanice s pripravkom Vebac | | | | |
| 9 [b] | 0,05 | 7,40 | 5,79 | 97,5 |
| 10 [b] | 0,05 | 7,40 | 5,40 | 99,0 |
| 11 [b] | 0,002 | 6,00 | 4,64 | 95,6 |
| 12 [b] | 0,002 | 6,00 | 5,40 | 75,0 |
| 13 [b] | 0,004 | 6,30 | 4,83 | 96,6 |
| Mešanice s pripravkom Biosaf | | | | |
| 14 [d] | 0,8 | 7,90 | 5,73 | 99,3 |
| 15 [b] | 0,8 | 7,90 | 7,04 | 86,4 |
| 16 [p] | 0,07 | 6,85 | 6,11 | 81,7 |

[a] Izguba = (deklarirano št. (KE/g) – ugotovljeno št.(KE/g))/deklarirano (KE/g)*100; [b] prah / powder; [p] peleti / pellets; [d] drobljenec / crum

Vzorci 2 do 5, 14 in 16 so šli skozi proces peletiranja, ki pa ni presegel temperature 80 °C. Izgube živih celic pri vzorcih 14 in 16, ki so vsebovali *S. cerevisiae,* so bile od 82 do 99 %, čeprav naj bi kvasovke po zagotovilih proizvajalca v celoti preživele peletiranje do 83 °C. Za spore mikroorganizmov *B. subtilis* in *B. licheniformis*, iz produkta Bioplus 2b proizvajalec prav tako zagotavlja 100 % preživetje peletiranja. Rezultati naše študije so pokazali za 20 do 56 % premalo živih probiotičnih mikroorganizmov. Domnevamo, da je visoka temperatura (60 do 80 °C) aktivirala del spor, da so germinirale. Germinacija je relativno hiter proces (Levinson in Mildred, 1970), tako je mogoče, da je visoka temperatura poškodovala tudi že del populacije germiniranih spor. Raziskav, ki bi obravnavale vpliv peletiranja na preživetje probiotikov, nismo zasledili. V objavah, ki obravnavajo učinke probiotikov na živalih, pa pogosto navajajo zgolj število mikroorganizmov, ki je dodano krmni mešanici pred procesom peletiranja. Naši rezultati kažejo, da izgub pri peletiranju vendarle ne velja spregledati, saj so precejšnje.

Pri mešanicah z Bioplus 2b, je zanimiva ugotovitev, da smo v izdelkih, ki so bili podvrženi zgolj mešanju sestavin (vzorci 6-8), zabeležili celo večje izgube kot v peletiranih (vzorci 2-5). Vzorci 6-8 so bili odvzeti iz treh premiksov, katerih priprava je nekoliko drugačna od priprave dopolnilnih krmnih mešanic. Mešanje v proizvodnji premiksov je namreč izredno počasno in dolgotrajno. Pri tem nastajajo precej velike tlačne obremenitve na sestavine, od katerih je mnogo

soli v obliki kristalov. Kombinacija kristalov in velikega tlaka v mešalnem stroju bi lahko povzročila poškodbe vegetativnih celic in tudi spor. Literaturnih podatkov, ki bi potrdili te domneve, sicer nismo zasledili. Druga možna razlaga za izgubo viabilnosti probiotičnih mikroorganizmov v določenih krmilih pa je lahko tudi prisotnost posameznih sestavin (soli, minerali, kisline), ki so živim mikroorganizmom škodljive ali celo letalne. Tudi nekateri terapevtiki, ki se dodajajo v krmo (npr. kokcidiostatiki), imajo lahko vpliv na mikroorganizme, zato pri probiotičnih mikroorganizmih, ki se uporabljajo v prehrani pitovne perutnine, vedno ugotavljajo tudi občutljivost za izbrani kokcidiostatik (EFSA opinion, 2007).

Natančne analize prisotnosti genov za odpornost proti antibiotikom in zunajkromosomskih elementov (plazmidi in transpozoni) bi morale biti opravljene pri vsakem probiotičnem mikroorganizmu preden pride na tržišče. Evropska komisija za zdravje se pri tej problematiki opira na mnenji strokovega odbora za prehrano živali (SCAN) iz leta 2003 in odbora FEEDAP iz leta 2005, da mikroorganizmov (sevov), ki bi utegnili prenašati pridobljeno odpornost proti določenemu antibiotiku, ne smemo uporabljati v prehrani živali. Zato je bilo s tržišča v zadnjem času umaknjenih kar nekaj probiotičnih preparatov tako za animalno kot humano prehrano (Hong in sod., 2004; Cartman in sod., 2007). Poročilo odbora SCAN (2000) zagotavlja, da *B. subtilis* in *B. licheniformis* ne vsebujeta plazmidov, sta pa odporna proti flavomicinu in cink-bacitracinu, slednji pa tudi proti klindamicinu. Odpornost proti bacitracinu se dokazano ne prenaša niti *in vitro* niti *in vivo*.

*B. subtilis* DSM 5749 in *B. licheniformis* DSM 5750 sta občutljiva za vse testirane antibiotike (pregl. 1), razen za metronidazol, katerega MIC je večja od 256 µg/ml. V literaturi nismo zasledili podatka o mejni MIC za metronidazol, zato ne moremo zaključiti, ali sta testirana seva odporna proti temu antibiotiku. So pa za *B. licheniformis* Handal in sod. (2003) prav tako zabeležili MIC večjo od 256 µg/ml. *B. licheniformis* je v naši študiji pokazal občutljivost tudi za klindamicin, saj je bila MIC (1,5 oz. 2 µg/ml) nižja od mejne vrednosti MIC za odpornost, kot jih navajajo v mnenju FEEDAP (2005). Kar zadeva odpornost proti kloramfenikolu, pa smo pri *B. licheniformis* ugotovili mejno vrednost, to je 8 µg/ml.

Preglednica 3. Minimalne inhibitorne koncentracije (µg/ml) izbranih antibiotikov za *B. subtilis* in *B. licheniformis,* osamljenih iz izdelka Bioplus 2b

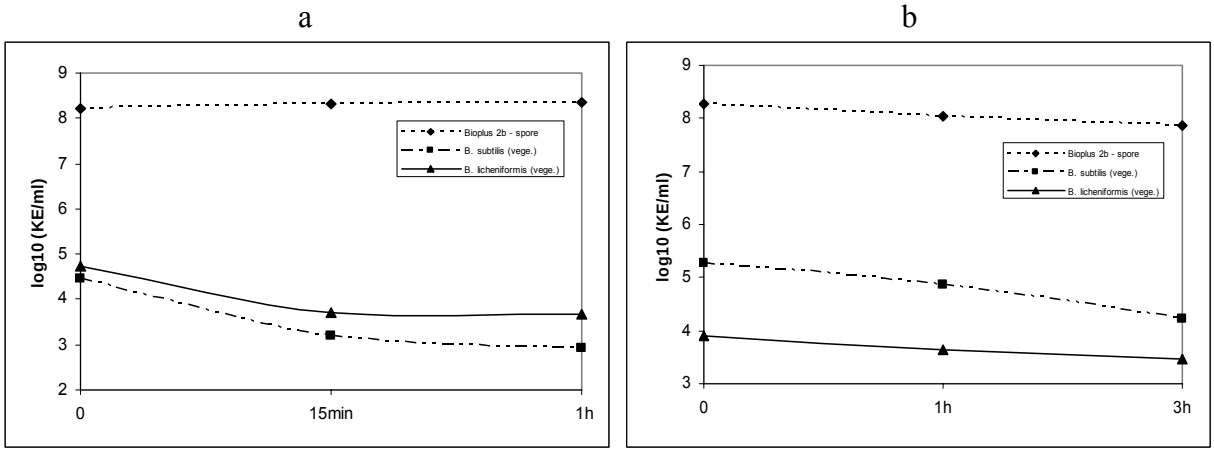
Table 3. Minimal inhibitory concentrations (µg/ml) of selected antibiotics for *B. subtilis* and *B. licheniformis* isolated from Bioplus 2b product

| Antibiotik<br>Antibiotic | MIC, µg/ml | | | | | |
|---|---|---|---|---|---|---|
| | *B. subtilis* DSM 5749 | | *B. licheniformis* DSM 5750 | | Meje za rezistenco (breakpoint MIC) | |
| | Vzorec 1<br>Sample 1 | Vzorec 2<br>Sample 2 | Vzorec 1<br>Sample 1 | Vzorec 2<br>Sample 2 | FEEDAP | SCAN |
| Streptomycin | 6 | 4 | 4 | 4 | 8 | 64 |
| Gentamycin | 0,38 | 0,38 | 0,25 | 0,25 | 4 | 8 |
| Tetracycline | 0,19 | 0,19 | 0,75 | 1 | 8 | 16 |
| Ampicillin | 0,023 | 0,023 | 0,5 | 0,5 | nd | 2 |
| Metronidazol | > 256 | > 256 | > 256 | > 256 | nd | nd |
| Clindamycin | 0,38 | 0,5 | 1,5 | 2 | 4 | nd |
| Chloramphenicol | 1,5 | 1,5 | 8 | 8 | 8 | 16 |
| Erithromycin | 0,32 | 0,32 | 2 | 3 | 4 | 4 |
| Rifampicin | 0,23 | nd | 0,64 | 0,94 | nd | 4 |
| Vancomycin | 0,38 | 0,38 | 0,25 | 0,25 | 4 | 4 |

FEEDAP opinion (2005); SCAN opinion (2003); nd = ni podatka / no data

Prehod skozi želodec in tanko črevo je za probiotične mikroorganizme ključnega pomena. Glavne ovire na tej poti so nizka vrednost pH v želodcu in proteolitični encim pepsin, v duodenumu tankega črevesa pa produkti žolča (žolčne soli) in encimi trebušne slinavke (lipaze, proteaze in amilaze). Preživetje *B. subtilis* in *B. licheniformis* v simuliranem želodčnem in črevesnem soku prikazuje slika 1. Testirali smo občutljivost spor in vegetativnih celic za nizke vrednosti pH in pepsin v želodcu ter za žolčne soli in pankreatin v začetnem delu tankega črevesa. Spore obeh mikroorganizmov so bile odporne proti nizkemu pH in encimu pepsinu, kakor tudi proti žolčnim solem in encimom trebušne slinavke. Upad števila spor v primeru simuliranega soka tankega črevesa je verjetno posledica germinacije dela spor, ki so jo izzvale žolčne soli. Podobno opažanje so navedli Duc in sod. (2004) v primeru dveh sevov *B. cereus*. Tako imenovan aktivacijski šok lahko povzročijo tudi drugi dejavniki, npr. temperatura in vrednost pH. Spore takrat germinirajo, ker pa je v okolju še vedno prisoten agresiven dejavnik (kislina, žolčne soli, visoka temperatura), vegetativne celice odmrejo, kar se kasneje pokaže v manjšem številu spor (Duc in sod., 2004).

Občutljivost vegetativnih celic se lepo odraža na slikah 1 a in 1 b, kjer je mogoče opaziti drastičen upad števila celic takoj, ko so le te prišle v stik s kislino oz. žolčnimi solmi. Začetno število celic je bilo pri *B. subtilis* $1{,}31 \times 10^7$ KE/ml, pri *B. licheniformis* pa $9{,}79 \times 10^6$ KE/ml, kar pomeni za več kot 99 % manjše število KE v simuliranem želodčnem soku in za 99 % oziroma za 100 % manjše število v simuliranem soku tankega črevesa takoj po izpostavitvi kislini oziroma žolčnim solem. O podobnih (> 98 %) izgubah so poročali tudi Barbosa in sod. (2005). Spinosa in sod. (2000) so ugotovili zelo nizke inhibitorne koncentracije konjugiranih in nekonjugiranih žolčnih soli za *B. subtilis,* in sicer 195 in 78 µg/ml. Nadaljnje padanje števila vegetativnih celic (po 15min, 1h in 3h) pa je verjetno posledica različnih razvojnih faz obeh mikroorganizmov.




Slika 1. Test preživetja spor in vegetativnih celic mikroorganizmov *B. subtilis* in *B. licheniformis* v simuliranem želodčnem soku (a) in soku zgornjega tankega črevesa (b).
Figure 1. Survival test of spores and vegetative cells of *B. subtilis* and *B. licheniformis* in simulated gastric's (a) and upper small intestine's juices (b).

Produkcija protimikrobnih substanc je eden od pomembnejših zaželenih mehanizmov delovanja probiotičnih mikroorganizmov. Predstavniki rodu *Bacillus* so znani po produkciji različnih protimikrobnih snovi, med katerimi najdemo tako bakteriocine in baktericinom podobne inhibitorne snovi (subtilin, koagulin), kot tudi antibiotike (bacitracin, polimiksin, dificidin, idr.) (Hong in sod., 2005). Produkcija teh snovi in encimov poteka le v celicah v vegetativni obliki, ne pa v sporah. V pregl. 4 je prikazana protimikrobna aktivnost dveh izolatov *Bacillus* proti različnim po Gramu negativnim in po Gramu pozitivnim mikroorganizmom. *B.*

*subtilis* je zaviral rast predvsem po Gramu negativnih bakterij, najbolj zaviralno pa je deloval proti trem sevom *E. coli*. O močni inhibiciji različnih sevov *E. coli* s strani *B. subtilis* MA139 in *B. subtilis* PY79 so poročali tudi Guo s sod. (2006) in La Ragione s sod. (2001). Nekoliko manjši inhibitorni vpliv je imel *B. subtilis* tudi proti *S. typhimurium*, ki je prav tako kot testirane *E. coli*, pogosta povzročiteljica drisk pri živalih. Proti nekaterim drugim po Gramu pozitivnim mikroorganizmom je *B. subtilis* pokazal manj izrazito inhibicijo, saj cone inhibicije niso bile večje od 2 mm, pri nekaterih ponovitvah testov pa se sploh niso pokazale. Omenjeno variabilnost lahko pripišemo manjšim razlikam v fiziološkem stanju testiranih kultur oziroma v pogojih izvedbe testov (starost gojišč, pogoji med inkubacijo...). *B. licheniformis* na drugi strani pa je zaviral rast zgolj po Gramu pozitivnih bakterij, med njimi večino laktobacilov, ki so bili osamljeni iz prebavil prašiča, kar ne govori v prid uporabi tega probiotičnega pripravka. Nasprotno pa je zelo ugodno, da *B. licheniformis* inhibira rast prašičjega izolata *C. perfringens*, ki priprada vrsti, ki lahko povzroča hude driske in s tem velike izgube pri brojlerjih in novorojenih pujskih (Van Immerseel in sod., 2004; Czanderlova in sod., 2006).

Preglednica 4. Protimikrobna aktivnost *B. subtilis* in *B. licheniformis*
Table 4. Antimicrobial activity of *B. subtilis* and *B. licheniformis*

| | Cone inhibicije / inhibitory zone, mm ± std | |
|---|---|---|
| | *B. subtilis* DSM 5749 | *B. licheniformis* DSM 5750 |
| *Salmonella typhimurium* ATCC 14028 | 3,0 ± 0,0 | / |
| *Escherichia coli* O8 K88+ Ent+ (IM 263) | 5,6 ± 0,6 | / |
| *Escherichia coli* ATCC 25922 | 9,3 ± 0,6 | / |
| *Escherichia coli* O8 K88+ Ent- (IM 262) | 11,0 ± 1,0 | / |
| *Clostridium perfringens* 2P 119 (IM 71) | / | 9,5 ± 0,7 |
| *Staphylococcus aureus* ATCC 29213 | / | +/– |
| *Enterococcus faecalis* ATCC 19433 | +/– | 2,5 ± 0,7 |
| *Lactobacillus sakei* NCDO 2714 | +/– | 10,0 ± 0,0 |
| *Lactobacillus reuteri* 12/26 (IM 300) | +/– | 5,0 ± 0,0 |
| *Lactobacillus vaginalis/reuteri* 13/26 (IM 302) | +/– | 2,5 ± 0,7 |
| *Lactobacillus reuteri* 10/26 (IM 301) | / | 3,0 ± 0,0 |
| *Lactobacillus reuteri* 9/26 (IM 278) | / | 1,5 ± 0,7 |
| *Bacillus subtilis* DSM 5749 | 1,0 ± 0,0 * | 2,0 ± 0,0 |

+/– = variabilni rezultati/variable results; / = ni inhibicije / no inhibition; * = avtokompeticija / autocompetition

## SKLEPI

Vsi vzorci krmil, ki smo jih preiskali, so vsebovali manjše število probiotičnih mikroorganizmov, kot je deklariral proizvajalec. Večja odstopanja smo odkrili pri proizvodih, ki so vsebovali preparata Vebac in Biosaf, saj le ta vsebujeta žive mikroorganizme vrst *E. faecium* in *S. cerevisiae*. Presenetile so nas izgube viabilnosti spor v proizvodih s preparatom Bioplus 2b, saj bi v splošnem spore morale dobro preživeti neugodne razmere, tudi takšne, ki vladajo med proizvodnim procesom. Seva *B. subtilis* in *B. licheniformis,* osamljena iz probiotičnega pripravka Bioplus 2b, sta občutljiva za vse testirane antibiotike, razen za metronidazol. Oba brez težav preživita prehod skozi želodec in tanko črevo v obliki spor, vegetativne celice pa so popolnoma neodporne proti nizkim vrednostim pH in žolčnim solem. Njuna protimikrobna aktivnost je

komplementarna, saj *B. subtilis* zavira predvsem po Gramu negativne, *B. licheniformis* pa po Gramu pozitivne bakterije .

## SUMMARY

Probiotics are live microbial feed supplements that can benefit the host by improving its intestinal balance (Fuller, 1989) if ingested in adequate concentration (Guarner and Schaffsma, 1998). They are considered as alternatives to antibiotics in animal feed used as growth promoters for farm animals. Their prime mode of action is modification of the microbial population by different mechanisms, such as stimulation of immune response, inhibition of pathogens by aggregation with pathogenic bacteria, by competitive adhesion to epithelial receptors, by production of specific substances (organic acids, bacteriocins, antibiotics) or by competition for nutrients. In order to achieve these effects, the probiotic strains must reach the intestine in a viable form and in sufficient number. This requires the survival of probiotics during feed processing, including pelleting by heat, during feed storage over weeks and finally during the passage through the stomach with adverse low pH condition and through the small intestine with high bile salts concentrations (Ortwin, 2005). The objective of the present work was to determine the number of viable probiotics in 16 different complete feed mixtures and premixtures, containing one of three commercial probiotic supplements, and to estimate the effect of feed processing on microbial survival rate. In addition, some probiotic characteristics of two *Bacillus* strains isolated from a commercial probiotic supplement were determined.

Eight samples of 16 tested contained product Bioplus 2b (Chr. Hansen, Denmark) with spores of two *Bacillus* strains, five samples contained product Vebac (Medipharm AB, Sweden) with *Enterococcus faecium* strain, and three samples contained probiotic supplement Biosaf (S.I. Lesaffre, France) with *Saccharomyces cerevisiae* strain. To our surprise a significant deviation from declared concentrations of viable probiotics was observed in all samples. The biggest losses occured in samples that contained products Vebac and Biosaf. Although *Enterococcus faecium* and *Saccharomyces cerevisiae* are present in two above mentioned products in a form protected by encapsulation and coating, they experienced 80 to 100% losses. Naturally resistant endospores of *Bacillus subtilis* and *Bacillus licheniformis* present in Bioplus 2b, also showed sensitivity to processes such as pelleting and mixing. The losses that occured in the samples of pelleted feeds were surprisingly lower (20 to 55%) than the losses in premixed samples which underwent mixing only.

*Bacillus subtilis* DSM 5749 and *Bacillus licheniformis* DSM 5750 isolates from product Bioplus 2b were subjected to some common tests for probiotic microorganisms. We prepared simulated gastric and small intestine juices and observed the survival rate of spores and vegetative cells of both strains. The tests showed that spores survived the exposure to low pH conditions (pH=2) as well as the presence of bile salts in concentrations similar to those in small intestine, without any loss. Nevertheless, a small part of spores which probably germinated, were lethally damaged by the bile salts during the incubation. On the contrary, vegetative cells were extremely sensitive to both low pH and bile salts and were killed almost immediately after exposure. Test for sensitivity to antibiotics showed that both *B. subtilis* and *B. licheniformis* were sensitive to almost all tested antibiotics, except to metronidazol. Most of *B. licheniformis* strains are, according to SCAN (2000) opinion, resistant to clindamycin, however the results of the presented tests showed sensitivity to this antibiotic. *B. subtilis* inhibited various *E. coli* and *S. typhimurium*. Some representatives of the latter two species can cause severe diarrhoea in weanling pigs. The inhibition of most of the other Gram positive microorganisms was weaker, sometimes even non-detectable. *Bacillus licheniformis* inhibited mostly the representatives of Gram positive bacteria, among which the inhibition of *C. perfringens* is of particular importance,

since this species is one of the major diarrhoea causative species in animals. The inhibitory spectra of both Bacillus isolates are interesting especially because they are complementary.

"… even when probiotics seem to work … we know too little abaut the normal gut ecosystem to understand why", (Abbott, 2004).

## VIRI


Abbott, A. Gut reaction. Nature, 427(2004), 284–286.

Abdollahi, M.R./ Kamyab, A./ Bazzazzadekan, A./ Shahneh, A.Z. Effect of different levels of probiotic (*Bacillus subtilis* and *Bacillus licheniformis*) on broilers performance. Poultry Science, 81(2002)1, 134.

Barbosa, T.M./ Serra, C.R./ La Ragione, R.M./ Woodward, M.J./ Henriques, A.O. Screening for *Bacillus* isolates in the broiler gastrointestinal tract. Applied and environmental microbiology, 2(2005)71, 968–978.

Chumpawadee, S./ Chinrasri, O./ Somchan, T./ Ngamulan, S./ Soychuta, S. Effect of dietary inclusion of cassava yeast as probiotic source on growth performance, small intestine (ileum) morphology and carcass characteristic in broilers. International journal of poultry science, 7(2008)3, 246–250.

Czanderlova, L./ Hlozek, P./ Chmelar, D./ Lany, P. Clostridium perfringens in suckling piglets with diarrhoea and its PCR typing and prevalence in the Czech republic in 2001–2003. Veterinarni medicina, 51(2006), 461–467.

De Vrese, M./ Marteau, P.R. Probiotics and prebiotics: effects on diarrhea. The journal of nutrition, 137(2007), 803–811.

Duc, L.H./ Hong, H.A./ Barbosa, T.M./ Henriques, A.O./ Cutting, S.M. Characterization of *Bacillus* probiotics available for human use. Applied and environmental microbiology, 4(2004)70, 2161–2171.

Duc, L.H./ Hong, H.A./ Uyen, N.Q./ Cutting, S.M. Intracellular fate and immunogenicity of *B. subtilis* spores. Vaccine, 22(2004a), 1873–1885.

Fuller, R. Probiotics in man and animals. Journal of applied bacteriology, 66(1989), 365–378.

Guarner, F./ Schaffsma, G.J. Probiotics. International journal of food microbiology, 39(1998), 237–238.

Guo, X./ Li, D./ Lu, W./ Piao, X./ Chen, X. Screening of *Bacillus* strains as potential probiotics and subsequent confirmation of the *in vivo* effectiveness of *Bacillus subtilis* MA139 in pigs. Antonie van Leeuwenhoek, 90(2006), 139–146.

Handal, T./ Caugant, D.A./ Olsen, I. Antibiotic resistance in bacteria isolated from subgingval plaque in a Norwegian population with refractory marginal periodontitis. Antimicrobial agents and chemotherapy, 47(2003)4, 1443–1446.

Hoa, N.T./ Baccigalupi, L./ Huxham, A./ Smertenko, A./ Van, P.H./ Ammendola, S./ Ricca, E./ Cutting, S.M. Characterisation of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. Applied and environmental microbiology, 66(2000)12, 5241–5247.

Hong, H.A./ Duc, L.H./ Cutting, S.M. The use of bacterial spore formers as probiotics. FEMS Microbiology Reviews, 29(2005), 813–853.

Jin, L.Z./ Ho, Y.W./ Abdullah, N./ Jalaludin, S. Growth performance, intestinal microbial populations and serum cholesterol of broilers fed diets containing *Lactobacillus* cultures. Poultry science, 77(1998), 1259–1265.

La Ragione, R.M./ Casula, G./ Cutting, S.M./ Woodward, M.J. *Bacillus subtilis* spores competitively exclude *Escherichia coli* O78:K80 in poultry. Veterinary Microbiology, 79(2001), 133–142.

Leuschner, R.G.K./ Bew, J. Enumeration of probiotic bacilli spores in animal feed: Interlaboratory study. Journal of AOAC int., 3(2003)86, 568–575.

Leuschner, R.G.K./ Bew, J./ Bertin, G. Validation of an official control method for enumeration of authorised probiotic yeast in animal feed. Systematic and applied microbiology, 26(2003a), 147–153.

Leuschner, R.K.G./ Bew, J./ Domig, K.J./ Kneifel, W. A collaborative study of a method for enumeration of probiotic enterococci in animal feed. Journal of applied microbiology, 93(2002), 781–786.

Levinson, H.S./ Mildred, H.T. Activation energy for glucose-induced germination of *Bacillus megaterium* spores. Journal of bacteriology, 1970, 270–271.

Midilli, M./ Alp, M./ Kocabagh, N./ Muglah, H./ Turan, N./ Yilmaz, H./ Danir, S. Effects of dietary probiotic and prebiotic supplementation on growth performance and serum IgG concentration of broilers. South african journal of animal science, 37(2008)1, 21–27.

Opinion of the Scientific Committee on Animal Nutrition on the criteria for assessing the safety of microorganisms resistant to antibiotics of human and veterinary importance. European Commission, health and consumer protection directorate-general. Available at: http://europa.eu.int/comm/food/fs/sc/scan/out108_en.pdf.

Opinion of the scientific panel on additives and products or substances used in animal feed on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance. The European food safety authority journal, 223(2005), 1–12.

Opinion of the scientific panel on additives and products or substances used in animal feed on compatibility of the microbial product 035, a preparation of Bacillus subtilis with lasalocid sodium, maduramicin ammonium, monensin sodium, narasin, salinomycin sodium and semduramicin sodium. The European food safety authority journal, 575(2007), 1–7.

Ortwin, S. Microorganisms as feed additives – probiotics. Advances in Pork Production, 16(2005), 161–167.

Pelicano, E.R.L./ De Souta, P.A./ De Souza, H.B.A./ Oba, A./ Norkus, E.A./ Kodawara, L.M./ De lima, T.M.A. Effect of different probiotics on broiler carcass and meat quality. Brazilian journal of poultry science, 5(2003)3, 207–214.

Sanders, M.E./ Morelli, L./ Tompkins, T.A. Sporeformers as human probiotics: *Bacillus*, *Sporolactobacillus* and *Brevibacillus*. Comprehensive reviews in food science and food safety, 2(2003), 101–110.

Spinosa, M.R./ Braccini, T./ Ricca, E./ De Felice, M./ Morelli, L./ Pozzi, G./ Oggioni, M.R. On the fate of ingested *Bacillus* spores. Research in microbiology, 151(2000), 361–368.

Uredba Evropskega parlamenta in Sveta (ES) št. 1831/2003 o dodatkih za uporabo v prehrani živali, 22. 9. 2003.

Van Immerseel, F./ De Buck, J./ Pasmans, F./ Huyghebaert, G./ Haesebrouck, F./ Ducatelle, R. *Clostridium perfringens* in poultry: an emerging threat for animal and public health. Avian pathology, 33(2004), 537–549.

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# POLYMORPHISM ANALYSIS OF THE PROMOTER OF COW LACTOFERRIN GENE WITH PCR-RFLP AND ITS CORRELATION WITH SUBCLINICAL MASTITIS

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## ABSTRACT

CMT was used to detect the incidence of mastitis. One hundred twenty cows were selected and assigned into 2 groups, 60 animals in each group: control group (healthy cows), experimental group (cows with subclinical mastitis) and the relationship between cow′s subclinical mastitis and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the promoter of cow lactoferrin gene was explored. The results showed that polymorphism existed in the promoter of bovine lactoferrin gene, which suggested that this polymorphism could be associated with mastitis susceptibility.

Key words: cattle / dairy cows / diseases / subclinical mastitis / molecular genetics / lactoferrin gene / PCR-RFLP

# PCR-RFLP ANALIZA POLIMORFIZMOV V PROMOTORJU LAKTOFERINSKEGA GENA PRI GOVEDU IN POVEZAVA S SUBKLINIČNIM MASTITISOM

## IZVLEČEK

Mastitis smo ugotavljali na osnovi kliničnih znakov. Sto dvajset krav smo razdelili v dve skupini, 60 živali je bilo v kontrolni skupini (zdrave živali), drugih 60 pa v poskusni skupini (živali s subkliničnim mastitisom) in ugotavljali povezavo med subkliničnim mastitisom in polimorfizmom restrikcijskih fragmentov (PCR-RFLP) promotorske regije gena za laktoferin. Rezultati kažejo, da bi bil polimorfizem v promotorski regiji govejega laktoferinskega gena lahko povezan z dovzetnostjo za mastitis.

Ključne besede: govedo / krave / molznice / bolezni / subklinični mastitis / molekularna genetika / geni / laktoferin / PCR-RFLP

## INTRODUCTION

The milk lactoferrin (Lf) is synthesized mainly by breast epithelial cells and neutrophils and secreted as the non-heme iron-binding protein, belonging to the transferrin family (Plaffl *et al*., 2003). In some studies authors reported that in infected cows, the milk and serum concentration of Lf will change (Hirvonen *et al*.,1999; Barkema, 1998). In cows with mastitis Lf may compete with bacteria for iron ions, so the RFLP analysis of genetic polymorphism in Lf gene, and relationship with udder infections may have theoretical and practical significance.

Through this study Lf gene promoter region was analyzed using polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP), and possible association with mastitis susceptibility was tested.

## MATERIALS AND METHODS

### Sample Collection

Animals from the Xinjiang Shihezi dairy cattle farm were divided into a healthy group (control group) and group with subclinical mastitis (test), 60 in each group. From each animal was collected 10 mL blood, mixed with anticoagulant and frozen at –20 °C.

### Test Method

The PCR was performed in a final volume of 25μL containing 40 ng of template DNA, 20 pmole of each primer (5'-CACATTACAAGCAGGATCTTTTGCTG-3' and 5'-CTGGCCAATGAGCCCTATATGTGT-3'), PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 1 mM $MgCl_2$, 0.25 mM of dNTPs, and 0.5 U of *Taq* DNA polymerase. This solution was initially denatured at 94 °C for 4 min. followed by 35 cycles of denaturation (94 °C for 30 s), annealing (60 °C for 45 s), and elongation (72 °C for 45 s) and a final extension at 72 °C for 10 min. The PCR products were electrophoresed on 1.5% agarose gels in order to check the quality and specificity of DNA fragment amplification. To examine the nucleotide sequence variation at the *Lf* locus, the *Hin*f I restriction enzyme was chosen. The resulting DNA fragments were separated on 6% PAGE gels, using *pBR322* as molecular marker. Gels were photographed under UV light with a Gel Doc 1000 system (BioRad) after ethidium bromide staining and the relative migration of the DNA bands was estimated.

## RESULTS

Amplification of the Lf gene fragment revealed a 1143 bp long product after electrophoresis of PCR product (Fig. 1). After restriction enzyme digestion with *Hin*fI the two alleles were characterized by two and one restriction fragment, respectively. The heterozygotes showed three bands (Fig. 2, Table 1)

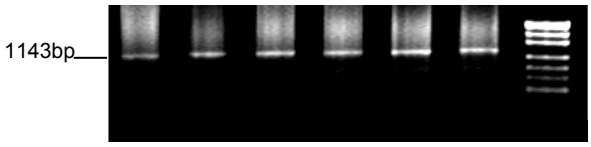


Figure 1. Gel electrophoresis of PCR products of the lactoferrin gene promoter fragment.
Slika 1. Gelska elektroforeza PCR produkta promotorja laktoferinskega gena.

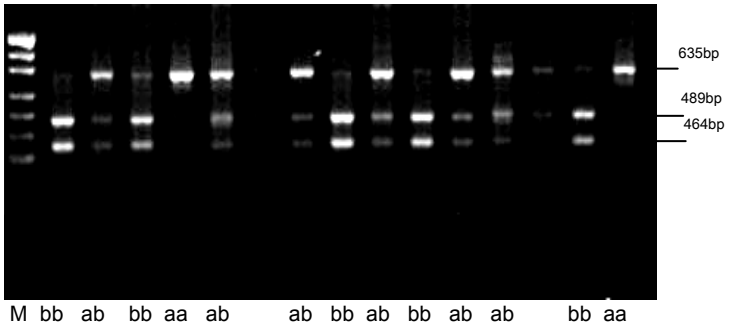


Figure 2. PCR-RLFP patterns of lactoferrin gene promoter PCR products digested with *Hin*fI.
Slika 2. PCR-RLFP vzorci fragmenta laktoferinskega promotorja po restrikciji s *Hin*fI.

Table 1. Genotype frequency and gene frequency in the promoter of lactoferrin gene
Preglednica 1. Frekvence genotipov in alelov v promotorju laktoferinskega gena

| Groups | Sample Sizes | Genotype frequency | | | Gene frequency | |
|---|---|---|---|---|---|---|
| | | A / A | A / B | B / B | A | B |
| Control group | 60 | 0.5 | 0.4 | 0.1 | 0.78 | **0.22** |
| Experimental group | 60 | 0.15** | 0.23 | 0.62** | 0.17** | 0.83** |

** $P < 0.01$

**Hardy-Weinberg Equilibrium Test**

The results of the exact Fisher test for the significance of Hardy-Weinberg probabilities are shown in Table 1. The frequencies for homozygotes AA and BB, as well as allele frequencies in the experimental group show significant departure from H-W equilibrium.

## DISCUSSION

This study revealed variation in RFLP banding pattern of the Lf gene promoter PCR fragment, which might be associated with the level of the Lf gene expression. Our results support the hypothesis that this mutation might be associated with mastitis susceptibility. Further research is needed to investigate the possible effect of the described mutation on Lf mRNA and protein level in milk.

## REFERENCES

Barkema, H.W. Incidence of clinical mastitis in dairy herds grouped in three categories by bulk milk somatic cell counts[J]. J Dairy Sci, 81(1998)2, 411–419.

Hirvonen, J./ Eklund, K./ Teppo, A.M. *et al.* Acute phase response in dairy cows with experimentally induced mastitis[J]. Acta Vet Scand, 40(1999), 35–46.

Plaffl, M.W./ Wittmann, S.L./ Meyer, H.H.D./ Bruckmaier, R.M. Gene expression of immunologically important factors in blood cells,milk cells and mammary tissue of cows [J]. J Dairy Sci, 86(2003)3, 538–545.

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## SUBJECT INDEX BY AGROVOC DESCRIPTORS

## PREDMETNO KAZALO PO DESKRIPTORJIH AGROVOC

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## VSEBINSKO KAZALO PO PREDMETNIH KATEGORIJAH AGRIS

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## ABECEDNO KAZALO AVTORJEV

## AUTHOR'S INDEX

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# NAVODILA AVTORJEM

## Prispevki

Sprejemamo izvirne znanstvene članke, predhodne objave in raziskovalne notice s področja zootehnike (genetika, mikrobiologija, imunologija, prehrana, fiziologija, ekologija, etologija, mlekarstvo, ekonomika, živalska proizvodnja in predelava živalskih proizvodov, tehnologija in dokumentalistika) v slovenskem in angleškem jeziku, znanstveno pregledne članke samo po poprejšnjem dogovoru. Objavljamo tudi prispevke, podane na simpozijih, ki niso bili v celoti objavljeni v zborniku simpozija. Če je prispevek del diplomskega, magistrskega ali doktorskega dela, navedemo to in tudi mentorja na dnu prve strani. Navedbe morajo biti v slovenskem in angleškem jeziku.

Pri prispevkih v slovenskem jeziku morajo biti preglednice, grafikoni, slike in priloge dvojezični, povsod je slovenščina na prvem mestu. Naslovi grafikonov in slik so pod njimi. Slike in grafikoni so v besedilu. Priloženi morajo biti tudi jasno označeni izvirniki slik (fotografije ali ločene grafične datoteke). Na avtorjevo željo jih vračamo. Grafikoni morajo biti črno-beli, brez rastrov. Dovoljeni so vzorci v črno-beli kombinaciji. Latinske izraze pišemo ležeče. V slovenščini uporabljamo decimalno vejico, v angleščini decimalno piko. Prispevki v angleščini morajo imeti povzetek v slovenščini in obratno.

Prispevki naj bodo strnjeni, kratki, največ 12 strani. Uporabljamo Microsoft Word 97 ali novejšo verzijo (Windows); pisava v besedilu in preglednicah je Times New Roman, velikost črk 12, v obsežnih preglednicah je lahko 10, pisava v grafikonih in slikah je Ariel, velikost črk najmanj 9, pisava za primerjave nukleotidnih in aminokislinskih zaporedij je Courier; zunanji rob 2,0 cm, notranji 2,5 cm, zgoraj živa *pagina* v eni vrstici, velikost črk 10 z avtorjem oz. avtorji in naslovom prispevka, zaključenim s piko. Če je naslov daljši, ga smiselno okrajšamo. Primera: Štuhec, I. in Siard, N. Obnašanje prašičev. Stibilj, V. in sod. Določitev maščobno-kislinske sestave … vzorcev mleka v Sloveniji.

## Prva stran

Na prvi strani prispevka na desni strani označimo vrsto prispevka v slovenščini in angleščini, sledi naslov prispevka, pod njim avtorji. Ime avtorjev navedemo v polni obliki (ime in priimek). Vsak avtor naj bo označen z indeksom, ki ga navedemo takoj pod avtorji, in vsebuje polni naslov ustanove ter znanstveni in akademski naslov; vse v jeziku prispevka. Navedemo sedež ustanove, kjer avtor dela. Če je raziskava opravljena drugje, avtor navede tudi sedež te inštitucije. Na željo avtorjev bomo navedli naslov elektronske pošte.

Pod naslovi avtorjev je datum prispetja in datum sprejetja prispevka, ki ostaneta odprta. Sledi razumljiv in poveden izvleček z do 250 besedami. Vsebuje namen in metode dela, rezultate, razpravo in sklepe. Sledijo ključne besede.

Izvlečku v jeziku objave sledi naslov in izvleček s ključnimi besedami v drugem jeziku.

Predlogo za pomoč pri oblikovanju prve strani prispevka najdejo avtorji na domači strani: http://aas.bfro.uni-lj.si/predloga-aas.dot.

## Viri

V besedilu navajamo v oklepaju avtorja in leto objave: (priimek, leto). Če sta avtorja dva, pišemo: (priimek in priimek, leto), če je avtorjev več, pišemo: (priimek in sod., leto). Sekundarni vir označimo z »navedeno v« ali »cv.«. Seznam virov je na koncu prispevka, neoštevilčen in v abecednem redu. Vire istega avtorja, objavljene v istem letu, razvrstimo kronološko z a, b, c. Primer: 1997a. Navajanje literature naj bo popolno: pri revijah letnik, leto, številka, strani; pri

knjigah kraj, založba, leto, strani. Za naslove revij je dovoljena uradna okrajšava, za okrajšanimi besedami naj bodo vedno pike. Navedbo zaključimo s piko. Nekaj primerov:

Fraser, A.F./ Broom, D.M. Farm animal behaviour and welfare. London, Bailliere Tindall, 1990, 437 str.

Hvelplund, T. Protein evaluation of treated straws. V: Evaluation of straws in ruminant feeding (ur.: Chenost, M./ Reiniger, A.). London, Elsevier Applied Science, 1989, 66–74.

Stekar, J.M.A. Vsebnost makro elementov v slovenski mrvi. V: Posvetovanje o prehrani domačih živali »Zadravčevi-Erjavčevi dnevi«, Radenci, 1997-10-27/28. Murska Sobota, Živinorejsko-veterinarski zavod za Pomurje, 1997, 105–117.

Stekar, J.M.A./ Golob, A./ Stibilj, V./ Koman Rajšp, M. Sestava in hranilna vrednost voluminozne krme v letu 1990. Zb. Bioteh. Fak. Univ. Ljubl., Kmet. Živin., 58(1991), 149–155.

Stekar, J.M.A./ Pen, A. Sadržaj natriuma, cinka i mangana u stočnoj hrani sa travnatih površina. Agrohemija, 21(1980)1–2, 7–15.

## Oddaja

Avtorji prispevke oddajo v natisnjenem in elektronskem izvodu. Priložijo tudi izjavo s podpisi vseh avtorjev, da avtorske pravice v celoti odstopajo reviji.

Prispevke recenziramo in lektoriramo. Praviloma pošljemo mnenje prvemu avtorju, po želji lahko tudi drugače. Če urednik ali recenzenti predlagajo spremembe oz. izboljšave, vrne avtor popravljeno besedilo v 10 dneh v natisnjenem in elektronskem izvodu. Ko prvi avtor vnese še lektorjeve pripombe, odda popravljeno besedilo v natisnjenem in elektronskem izvodu.

Prispevke sprejemamo vse leto.

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Papers in Slovenian language should have tables, graphs, figures and appendices in both languages, Slovenian language being the first. Titles of graphs and figures are below them. Figures and graphs are part of the text. Clearly marked original figures should be added (photographs or separate graphic files); they can be returned upon request. Latin expressions are written in italics. Decimal coma is used in Slovenian and decimal point in English. Papers in English should contain abstract in Slovenian and *vice versa*.

The papers should be condensed, short and should not exceed 12 pages. Microsoft Word 97 or later version (Windows) should be used, fonts Times New Roman, size 12 in text and tables (in large tables size 10 is allowed), Ariel for graphs and figures (letter size at least 9) and Courier for nucleic- and amino acid sequence alignments should be used; right margin 2.0 cm, left margin 2.5 cm; *pagina viva* in one line, size 10, author(s) and abbreviated title of the paper ending with a full stop. Examples: Štuhec, I. and Siard, N. Pig Behaviour. Stibilj, V. *et al.* Determination of fatty acids composition ... milk samples in Slovenia.

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The type of the paper should be indicated on the first page on the right side in Slovenian and English language following by title of the paper and authors. Full names of authors are used (first name and surname). Each name of the author should have been added an index, which is put immediately after the author's name, and contains address of the institution and academic degree of the author, in the language of the paper. The address of the institution in which the author works is indicated. If the research was realised elsewhere, the author should name the headquarters of the institution. E-mail is optional.

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Fliegerová, K./ Pažoutová, S./ Hodrová, B. Molecular genotyping of rumen fungi based on RFLP analysis. Zb. Bioteh. Fak. Univ. Ljubl., Kmet. Zooteh., 72(1998), 95–98.

Fraser, A.F./ Broom, D.M. Farm animal behaviour and welfare. London, Bailliere Tindall, 1990, 437 p.

Hvelplund, T. Protein evaluation of treated straws. In: Evaluation of straws in ruminant feeding (Eds.: Chenost, M./ Reiniger, A.). London, Elsevier Applied Science, 1989, 66–74.

Ristič, M./ Klein, F.W. Schlachtkoerperwert von Broilern verschiedener Herkunfte. Mitteilungsblatt der Bundesanstalt fuer Fleischforschung, Kulmbach, 101(1988), 8045–8051.

Stekar, J.M.A. Silage effluent and water pollution. In: 6th International Symposium "Animal Sciences Days", Portorož, 1998-09-16/18, Slovenia. Zb. Bioteh. Fak. Univ. Ljubl., Kmet. Supl., 30(1998), 321–325.

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