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Analysis of the weaned piglets reaction on the handling material with coloured balls and possibility of exploration and chewing

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Abstract: *Analysis of the weaned piglets reaction on the handling material with coloured balls and possibility of exploration and chewing*. Maintaining proper pig welfare levels in intensive production systems requires the use of appropriate handling materials. At present, there are many elements commonly referred to as toys in breeding and production, but not all of them meet safety and functional requirements. The purpose of the study was to analyze the reaction of weaned piglets on a toy pen – an "abacus" with coloured balls (test version). Direct observations were made on the behaviour of piglets of the Pulawska and Polish Large White (PLW) breeds using the ethogram, taking into account motor activity with elements of interest in an "abacus", including: climbing on an "abacus", shifting balls (interest in yellow, blue, red), rest, excretory behaviour, nutritional behaviour. It was found that piglets of the Pulawska breed climbed on an "abacus" for more time (15.2 min, i.e. 3.6 min longer than the PLW breed), and the PLW breed moved balls longer (26.2 mi., i.e. 9.6 min longer than the Pulawska breed). Piglets of the two breeds had the least interest for red balls. Piglets of the Pulawska breed preferred the most blue and then yellow balls. Polish Large White piglets were more likely to choose yellow and blue balls. Eating time and water intake were higher in Pulawska breed, respectively 10.8 and 5.4 min compared to 6.6 and 3.0 min in PLW breed.

Key words: piglets, weaning, behaviour, toys

INTRODUCTION

Behaviour of pigs manifested in a specific ontology and production environment is an important element used in the shaping of modern breeding and production principles of this species of livestock. Age-appropriate behaviours are in addition to the production and health indicators, the most determinant criterion for assessing well-being (Marć-Pieńkowska et al. 2014, Adamczyk et al. 2015). The intensive rearing system exposes the individual pig production groups to a number of stressors. These factors affect their vital functions, they change the pattern of behaviour (Frindt et al. 2006), which often leads to behavioural disorders such as stereotypes or aggression (Kondracki et al. 2014). Based on the results of observing the behaviour of particular groups of pigs, also post-weaning piglets, it is possible to modify the breeding environment, e.g. by enriching them with manipulative materials (Nowicki et al. 2015). Straw is best material, but also in this case it is possible to apply materials of the marginal interest. These elements are used to distract pigs, which is especially useful in the post-weaning period. Materials of this type should arouse interest in exploration e.g. easy access, colour

variability and enable oral manipulation, e.g. biting, chewing (Council Directive 2008/120/EC, Commission Recommendation (EU) 2016/336).

The aim of the study was to analyze the reaction of piglets to a handling material – an "abacus" with coloured balls, towards the possibility of its use as an element of improving well-being by limiting the influence of stress factors in post-weaning period.

MATERIAL AND METHODS

Animals

The study was performed in the Lublin region (East-Central Poland) with two groups of piglets: Pulawska breed and Polish Large White (PLW) breed. Piglets were kept in two farms. Piglets were weaned at 35th day of age. Each research group was two litters. The size of the group was in the range: for the Pulawska breed 18–22 pcs, for the PLW breed 20–24 pcs. The sex share in the groups was close to even. Observations were conducted for five groups of the Pulawska breed (100 piglets) and five groups of the PLW breed (110 piglets). Piglets were maintained on traditional straw and they were kept in pens in which the average area for 1 pc was 0.25 m^2. The construction of pens and welfare conditions (temperature, humidity, air movement, lighting) in farms were comparable and consistent with the requirements (2010 Minister of Agriculture and Rural Development Regulation). Piglets were fed *ad libitum*. Permanent access to water was provided by nipple drinkers. Litters were subjected to preventive measures, standardized for this age group.

Behavioural observations

The handling material was placed in the pen on the first day after weaning – the experimental period. Direct observations were performed in three phases during the experimental phases: 7:00–8:00, 12:00–13:00, 16:00–17:00, which was associated with the activity of piglets resulting from the length of daylight. The record was made by the service staff, which eliminated the impact of additional novelty in the piglets environment. The animals showed no interest in the service staff. The ethogram included: motor activity with elements of interest an "abacus", including: climbing on an "abacus", moving balls (interest in yellow, blue, red), rest, excretory behaviour, nutritional behaviour. Behavioural observations included: frequency of each behaviour in three periods, duration of each behaviour per hour of observation in the experimental period. Observations were also made on behavioural anomalies, i.e. stereotypies acute aggression beyond normal behaviour in terms of group hierarchies, tails and/or ears biting.

Construction of handling material

The proposed manipulation element to maintain optimum piglet behaviour, as well as to reduce stress, was an "abacus" with coloured balls (Fig.), mounted in a pen. The counter frame has been made of rectangular tubes that are fit with steel bars (stainless steel) with movable coloured balls. The rods were secured with locking elements. The balls were used in three colours: yellow, blue, red, all made of material harmless to the health

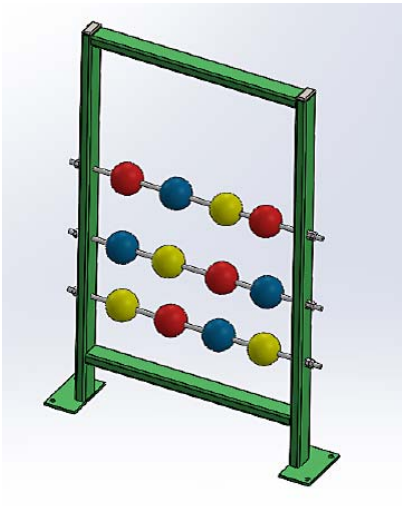

FIGURE. Design of an "abacus" with coloured balls

and life of piglets while easy to clean and disinfect. The base of the counters allows them to be attached to the floor in the pen by screws. Dimensions of the toy were adjusted to the size of piglets aged 5–6 weeks. The height of the counter was 65 cm and the width was 40 cm. With the increase in piglets, it is possible to adjust the height of the counter by moving the bars to the higher levels. The construction and assembly of the toy made it possible to exchange the balls and to disinfect the construction at any time. The toy was mounted in a pen on the border of the feeding and storage areas.

All data were statistically analysed by calculating the arithmetic means and standard deviations (*SD*) for each type of behaviour. Normal distribution of data was confirmed by the Shapiro–Wilk test. The results were statistically analysed by significance of differences between means was determined by Student's t- test, using Statistica software ver. 6.0.

RESULTS AND DISCUSSION

Exploration is one of the most important components of the behaviour of pigs. Basic exploratory behaviour of pigs includes, among others, touching, chewing and sniffing (Olsen et al. 2000, Pisula 2003, Studnitz et al. 2007). Intrinsic instinct is particularly manifested by young individuals who are acquainted with the environment (Empel 2005). In no group of piglets it was found any behaviour different from the standard behaviour for age and hierarchy in the newly formed group (Grudniewska 1998). The highest motor activity of litters occurred in the morning and the lowest in the afternoon. In both breeds, it was similar in the individual observation intervals (Table 1). Although the motor activity of the piglets with interest in an "abacus" remained at a similar level, statistically significant differences ($P < 0.05$) were observed for the individual behaviour of an "abacus".

TABLE 1. Mean (±*SD*) number of events in individual behaviours of piglets

Type of behaviour	Observation 7:00–8:00		Observation 12:00–13:00		Observation 16:00–17:00	
	Pulawska	PLW	Pulawska	PLW	Pulawska	PLW
	SD		*SD*		*SD*	
Motor activity with elements of interest in an "abacus"	14.8 ±6.1	14.4 ±6.9	9.7 ±4.5	10.1 ±4.4	8.9 ±3.6	7.9 ±3.1
climbing on an "abacus"	6.5[a] ±2.9	4.6[a] ±1.8	4.7 ±1.9	3.8 ±1.1	3.9 ±1.4	2.8 ±1.0
moving and biting balls	7.2[a] ±3.1	9.5[a] ±3.2	4.8[a] ±1.2	6.7[a] ±2.2	4.7 ±1.8	4.8 ±1.8
– interest in yellow	1.8[a] ±0.7	4.7[a] ±1.9	1.0[a] ±0.3	3.6[a] ±1.1	1.0[a] ±0.3	3.7[a] ±1.2
– interest in blue	4.6 ±1.7	2.9 ±0.9	3.6 ±1.2	1.9 ±0.4	2.9[a] ±1.1	1.0[b] ±0.7
– interest in red	1.1 ±0.3	1.8 ±0.5	0.0	1.1 ±0.4	1.0 ±0.5	0.0
Eating	3.4 ±1.4	1.8 ±0.5	1.8 ±0.4	1.9 ±0.6	2.7 ±1.0	1.9 ±0.7
Drinking	1.9 ±0.5	1.1 ±0.3	1.7 ±0.5	1.1 ±0.5	1.1 ±0.3	1.0 ±0.2
Excretion behaviour	1.0 ±0.2	0.9 ±0.4	1.1 ±0.3	1.0 ±0.3	1.0 ±0.4	0.9 ±0.1
Rest	0.9 ±0.3	1.0 ±0.3	0.9 ±0.2	1.1 ±0.3	0.9 ±0.3	1.1 ±0.5
Behavioural anomalies	lack					

Means with the same letters differ significantly between groups in a observation, $P \leq 0.05$.

Climbing on an "abacus" took the Pulawska breed 15.2 min, e.g. 3.6 min longer than the PLW breed. On the other hand, in the case of piglets of the PLW breed, the time spent on ball handling was increased by 5.6 min (Table 2). Such a distribution of values may mean that individuals of the Pulawska breed prefer active exploration of new objects, while the PLW breed selects stable explora-

TABLE 2. Mean (±*SD*) time spent of individual behaviours per 1 hour of observation

Type of behaviour	Duration (min)	
	Pulawska	PLW
	SD	
Motor activity with elements of interest in an "abacus"	31.8 ±18.8	37.8 ±22.3
climbing on an "abacus"	15.2[a] ±7.9	11.6[a] ±5.6
moving and biting balls	16.6[a] ±9.6	26.2[a] ±12.1
Eating	10.8 ±6.6	6.6 ±3.9
Drinking	5.4 ±2.9	3.0 ±1.6
Excretion behaviour	3.9 ±2.1	2.9 ±1.3
Rest	8.1 ±4.7	9.7 ±4.3

Means with the same letters differ significantly between groups, $P \leq 0.05$.

tion, which may be due to the genetic characteristics of the breed (Pastwa et al. 2016).

Using knowledge of pig behaviour, the characteristics of the exploratory material should be defined, e.g. the possibility of chewing, shape and colour, contact, edibility and safety (Santen and Donselaar 2014). An important asset of the exploratory material for pigs is the maintenance of the so-called novelty effect (Tynes 2015). The more introduced to the pen the element stands out against the background of the environment, e.g. colour, the greater the interest of the animal.

Pulawska breed were more likely to climb on an "abacus", and the PLW breed was more likely to move the balls (Table 1).

As a result of observations of pig behaviour, it has been shown that these animals have the ability to distinguish basic colours (Herbut et al. 2006, Stelios et al. 2006, Kittawornrat and Zimmerman 2010, Klocek et al. 2010, Klocek et al. 2016) and piglets prefer blue and red.

In our own studies it was shown that piglets of the two breeds had the least interest for red balls. Piglets of the Pulawska breed preferred the most blue and then yellow. Polish Large White piglets, on the other hand, were more likely to choose yellow and blue (Table 1). The information obtained is an important element in the application of the appropriate colour to piglets. Thanks to the use of preferential colours in an "abacus" it stands out against the background of the living environment of piglets, which increases their interest in handling material.

During observation it was also shown that piglets from Pulawska breed were more likely to eat than PLW breed. An analogous trend was observed with respect to water intake (Table 1). Feeding time and water intake were higher in Pulawska breed, respectively 10.8 and 5.4 min compared to 6.6 and 3.0 min in PLW breed (Table 2). On the other hand, the multiplicity of excretory and resting behaviours remained similar in both breeds (Table 1). Piglets of the Pulawska breed spent 3.9 and 8.1 min respectively, while 2.9 and 9.7 min were used in PLW breed (Table 2). However, the differences noted were statistically insignificant.

CONCLUSION

The perineal period is considered to be one of the critical stages in the rearing of piglets. Therefore, measures should be taken to limit the effects of stressors on animals, e.g. by using properly selected handling material. The test version of an "abacus" with coloured ball has been shown to meet the requirements as an environmental enrichment factor for piglets. This is indicated by the number of approaches and time spent with an "abacus". The ability to regularly clean and periodically change the colour of the balls in an "abacus" is a solution that allows the pigs to be more interested in the subject when their natural curiosity about the dirty or damaged element is noticeable.

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Streszczenie: *Analiza reakcji prosiąt odsadzonych na materiał manipulacyjny z kolorowymi piłkami i możliwością eksploracji oraz żucia.* Utrzymanie właściwego poziomu dobrostanu świń w intensywnym systemie produkcji wymaga zastosowania odpowiednich materiałów manipulacyjnych. Obecnie w praktyce hodowlanej i produkcyjnej funkcjonuje wiele elementów określanych potocznie mianem zabawek, lecz nie każdy z nich spełnia wymagania odnośnie bezpieczeństwa i funkcjonalności. Celem pracy była analiza reakcji prosiąt odsadzonych na

umieszczoną w kojcu zabawkę – „liczydło" z kolorowymi piłkami (wersja testowa). Wykonano obserwacje bezpośrednie behawioru prosiąt rasy puławskiej i wielkiej białej polskiej (wbp) z wykorzystaniem etogramu, uwzględniając aktywność motoryczną z elementami zainteresowania liczydłem, w tym: wspinanie się na „liczydło", przesuwanie piłek (zainteresowanie kolorem żółtym, kolorem niebieskim, kolorem czerwonym), odpoczynek, zachowania wydalnicze, zachowania żywieniowe. Stwierdzono, że prosięta rasy puławskiej wspinały się na „liczydło" przez dłuższy czas (15,2 min, czyli o 3,6 min dłużej od rasy wbp), a rasa wbp dłużej zajmowała się przesuwaniem piłek (26,2 min, tj. o 9,6 min dłużej od rasy puławskiej). Prosięta obu ras najmniejsze zainteresowanie wykazywały piłkami w kolorze czerwonym. Osobniki rasy puławskiej najbardziej preferowały niebieskie, a następnie żółte piłki. Prosięta rasy wbp częściej wybierały żółte i niebieskie piłki. Czas pobierania paszy i wody był dłuższy dla rasy puławskiej, odpowiednio 10,8 i 5,4 min w porównaniu z czasem rasy wbp (odpowiednio 6,6 i 3,0 min).

Słowa kluczowe: prosięta, odsadzanie, behawior, zabawki

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Effect of *Bacillus subtilis* and *Bacillus licheniformis* inclusion in turkey diets on growth performance

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Abstract: *Effect of* Bacillus subtilis *and* Bacillus licheniformis *inclusion in turkey diets on growth performance.* The aim of this study was to evaluate the influence of a probiotic preparation containing spores of *Bacillus subtilis* and *Bacillus licheniformis*, added to feed, on growth performance of female turkeys reared until 84th day of age. A total of 300-day-old Hybrid Converter female turkeys were randomly assigned to 20 pens. The pens were randomly divided into two treatment groups: T1 received basal diets, and T2 received the same diets as group T1 supplemented with the probiotic ($1.28 \cdot 10^6$ CFU/g feed). It can be concluded that the probiotic feed additive had a beneficial influence on growth performance and significantly increased the final body weights and average daily gains of female turkeys.

Key words: female turkeys, probiotic, *Bacillus subtilis*, *Bacillus licheniformis*, growth performance

INTRODUCTION

Poultry production has been growing rapidly in recent years due to the fast growth rate and short fattening period of birds. This prompted the search for new solutions in poultry nutrition, to improve productivity while maintaining the desirable taste of meat as well as adequate standards of poultry health and welfare. Feed additives, including probiotics, are increasingly used in poultry nutrition on account of their positive effects on gut microbiota (Fallah et al. 2013). An improvement in the intestinal environment may contribute to increasing the efficiency of nutrient digestion and absorption (Pelicano et al. 2004). Probiotic strains produce bacteriocins with bacteriostatic activity (Oelschlaeger 2010). Dietary supplementation with probiotics exerts positive effects on local (GALT) and humoral immunity in poultry (Alloui et al. 2013). Probiotic bacteria produce their own enzymes and activate the host's enzymes, thus improving the growth rate of birds and feed conversion ratio (Yirga 2015). *Bacillus subtilis* and *Bacillus licheniformis* strains are widely used in probiotic products (Hong et al. 2005). The aim of this study was to evaluate the influence of a probiotic preparation containing spores of *B. subtilis* and *B. licheniformis*, added to feed, on the growth performance of female turkeys reared until 84th day of age.

MATERIAL AND METHODS

The experiment was conducted at the experimental poultry farm of the Department of Poultry Science, University of Warmia and Mazury in Olsztyn (Poland). There were two dietary treatments in the experiment: T1 – control treatment without dietary supplementation, and T2 – experimental treatment with dietary supplementation with a probiotic. The test material (BioPlus 2B, Chr.Hansen A/S, Denmark) contained strains of *B. subtilis* and *B. licheniformis* at a ratio of 1 : 1 ($1.6 \cdot 10^9$ *B. subtilis* spores and $1.6 \cdot 10^9$ *B. licheniformis* spores per 1 g of the preparation). *Bacillus subtilis* and *B. licheniformis* were added to T2 diets at $1.28 \cdot 10^6$ CFU/g feed (400 g/t feed). A total of 300-day-old healthy female turkey poults (Hybrid Converter) were obtained from a commercial hatchery. The turkeys were allocated at random to 20 floor pens. Each treatment comprised 10 pens (replicates) of 15 birds each. Pen surface area was 4 m^2 (stocking density of 3.75 birds per 1 m^2). Wood shavings were used as bedding material, and the litter was replenished as necessary. The house was provided with artificial programmable lights and climate, heating by gas heating system, and forced ventilation. The environmental conditions were consistent with the Hybrid recommendations. The trial was conducted for 84 days, and involved three feeding phases. The diets were formulated and the calculations were performed based on the dietary recommendations for female turkeys proposed by Smulikowska and Rutkowski (2005). Each pen was equipped with a feeder, and feed was offered *ad libitum*. Feeders were re-filled with pre-weight feed amounts when required. All diets were offered in crumbled/pelleted form. Drinking water was supplied *ad libitum* by bell-type drinkers. All experimental procedures involving animals were approved by the local Animal Experimentation Ethics Committee at the University of Warmia and Mazury in Olsztyn.

The birds were weighed on the day of arrival, and then the body weight (BW) of birds in each pen was recorded on a pen basis at 28th, 56th and 84th day of age. Feed intake was calculated as the difference between the offered feed and refusals. The results were used to determine feed conversion ration (FCR) for all experimental periods, and the European productivity index – EPI [(livability × × final BW × 100)/(duration of the study × × FCR)] for the entire experiment. All diets were analyzed for the content of crude nutrients by the VDLUFA method (Naumann and Bassler 1993), and for the content of *Bacillus* spores.

The results were analyzed by a one-way analysis of variance (ANOVA), and significant differences between treatments were determined by Duncan's multiple range test. The Statistica software package ver. 10.0 was used for statistical calculations. The data in tables are given as means and standard deviations. Treatment differences were considered significant at $P \leq 0.05$. Replicate-pen was the experimental unit for all variables measured.

RESULTS AND DISCUSSION

A proximate feed analysis showed that the actual nutrient concentrations were consistent with the calculated values (Table 1). The mean concentrations of

TABLE 1. Composition and nutrient density of the diets

Composition (%)	Feeding phase		
	Starter (1–28 days)	Grower (29–56 days)	Finisher (57–84 days)
Wheat	42.711	46.417	53.805
Sunflower meal	3.000	4.000	5.000
Soybean meal	40.172	37.411	27.804
Rapeseeds	3.000	4.000	5.000
Potato protein	3.000	–	–
Soybean oil	2.928	2.760	2.477
Animal fat	–	1.000	2.000
Na-bicarbonate	0.100	0.100	0.100
Salt	0.229	0.207	0.214
Limestone	1.824	1.518	1.524
MCP	1.898	1.419	0.978
Choline chloride	0.070	0.070	0.070
DL-methionine	0.313	0.303	0.268
L-lysine	0.372	0.438	0.384
L-threonine	0.134	0.107	0.126
Vitamins + trace minerals[1, 2]	0.250	0.250	0.250
Nutrient density[3]			
Age (weeks)	0–4	5–8	9–12
ME (kcal/kg)	2 800	2 880	3 000
Crude protein (g/kg)	280.0/277.2[4]	255.0/248.7[4]	225.0/221.1[4]
Methionine (g/kg)	7.2	6.7	6.00
Methionine + Cysteine (g/kg)	11.8	11.0	10.0
Lysine (g/kg)	17.5	16.0	13.5
Threonine (g/kg)	11.6	10.0	9.0
Arginine (g/kg)	17.7	16.3	14.2
Calcium (g/kg)	12.0	10.0	9.0
Phosphorus (g/kg)	6.0	5.0	4.0

[1] Content per kg premix for weeks 1–8: 5,000,000 IU Vitamin A; 1,330,000 IU Vitamin D_3; 670,000 IU Vitamin D_3 HyD; 40,000 mg Vitamin E; 1,600 mg Vitamin K_3; 1,800 mg Vitamin B_1; 6,000 mg Vitamin B_2; 2,000 mg Vitamin B_6; 16 mg Vitamin B_{12}; 1,400 mg Folic acid; 11,200 mg Pantotenic acid; 44,000 mg Nicotinic acid; 150 mg Biotin; 64,000 mg Manganese; 64,000 mg Zinc; 32,000 mg Iron; 10,000 mg Coper; 1,000 mg Iodine; 120 mg Selenium.

[2] Content per kg premix for weeks 9–12: 3,840,000 IU Vitamin A; 1,920,000 IU Vitamin D_3; 24,000 mg Vitamin E; 1,200 mg Vitamin K_3; 800 mg Vitamin B_1; 4,800 mg Vitamin B_2; 2,000 mg Vitamin B_6; 10 mg Vitamin B_{12}; 1,000 mg Folic acid; 9,200 mg Pantotenic acid; 34,000 mg Nicotinic acid; 150 mg Biotin; 48,000 mg Manganese; 48,000 mg Zinc; 16,000 mg Iron; 10,000 mg Coper; 800 mg Iodine; 120 mg Selenium.

[3] Calculated (Smulikowska and Rutkowski 2005).

[4] Analyzed (Naumann and Bassler 2004).

B. subtilis and *B. licheniformis* in turkey diets were $9.35 \cdot 10^5$, $9.23 \cdot 10^5$ and $1.26 \cdot 10^6$ viable cells per 1 g of feed in the starter, grower and finisher phases, respectively. The probiotic strains were not detectable in control diets (< $1.0 \cdot 10^4$ CFU/g feed). The results were satisfactory and corresponded to the target values, 0 and $1.28 \cdot 10^6$ CFU/kg feed in groups T1 and T2, respectively. The livability was very good in both treatments – 100%. During the experiment, 22 birds (6.67 and 8% in T1 and T2, respectively) were culled. The main reason for culling was enlarged crop (20 birds), and it was no relationship between the reason of culling and the use of the probiotic preparation.

During the first feeding phase, the tested probiotic had no significant effect of feed intake in female turkeys fed crumbled/pelleted diets (Table 2). Differences in feed intake were noted in the period of 1–56 days when birds fed probiotic-supplemented diets consumed more feed than control group birds ($P = 0.048$). Over the entire experimental period of 84 days the female turkeys from the probiotic group (T2) consumed 2.7% more feed than the control group (T1) birds. The difference in feed intake (1–84 days) was not significant ($P = 0.230$), but it could suggest that probiotic bacteria (*B. subtilis* and *B. licheniformis*) exerted a stimulatory effect. An increase in feed intake due to dietary probiotic supplementation has been found to improve the growth performance of birds. However, in the study of Midilli et al. (2008), where the BioPlus 2B feed additive was used, no differences were observed in feed intake and consequently in the body weights or average daily gains of broiler chickens between treatments. Similar results (an improvement of growth performance) were reported by Gohain and Sapcota (1998). In contrast, Safalaoh (2006) reported improved body weight gain (BWG) and FCR in broilers supplemented with a microbial preparation (Effective Micro-organisms – EM), despite a decrease in feed intake in the experimental group.

The average body weights and weight gains of turkeys are presented in Tables 3 and 4. No significant differences in body weight gains were found between treatments in the first stage of the study (days 1–28). During the second experimental period (days 29–56), turkeys fed the BioPlus 2B diet (T2) were significantly heavier and gained significantly more than birds fed the control diet (+4.9%,

TABLE 2. The results of feed intake of female turkeys (g/day/bird)

Period (days)	Group		*SEM*	*P*
	T1	T2		
1–28	43.8 ±1.4	43.7 ±1.7	0.344	0.894
29–56	171.9 ±6.8	176.9 ±6.9	1.598	0.124
1–56	109.7^b ±3.9	113.8^a ±4.7	1.046	0.048
57–84	288.8 ±25.3	300.8 ±16.2	4.821	0.223
1–84	172.6 ±7.9	177.3 ±8.8	1.894	0.230

Values in same rows with no common superscript are significantly different ($P \leq 0.05$).

TABLE 3. The results of body weight of female turkeys (kg)

Age of birds (day)	Group		*SEM*	*P*
	T1	T2		
1st	0.057 ±0.001	0.057 ±0.001	0.000	0.280
28th	1.043 ±0.033	1.044 ±0.036	0.008	0.966
56th	3.831^{b} ±0.096	3.970^{a} ±0.124	0.029	0.012
84th	7.363^{b} ±0.243	7.659^{a} ±0.268	0.065	0.019

Values in same rows with no common superscript are significantly different ($P \leq 0.05$).

TABLE 4. The results of weight gain of female turkeys (g/day)

Period (days)	Group		*SEM*	*P*
	T1	T2		
1–28	35.2 ±1.2	35.2 ±1.3	0.268	0.981
29–56	99.6^{b} ±2.7	104.5^{a} ±3.6	0.892	0.003
1–56	67.4^{b} ±1.7	69.9^{a} ±2.2	0.516	0.012
57–84	126.1 ±8.1	131.8 ±6.4	1.711	0.102
1–84	87.0^{b} ±2.9	90.5^{a} ±3.2	0.776	0.019

Values in same rows with no common superscript are significantly different ($P \leq 0.05$).

104.5 vs. 99.6 g, $P = 0.003$). From 56th day of the trial, turkeys fed the probiotic diet (T2) were characterized by significantly higher average body weight (+3.5%, 3.831 vs. 3.970 g, $P = 0.012$) and gained significantly more in comparison with birds fed control diet (+3.7%, 69.9 vs. 67.4 g, $P = 0.012$). During the third feeding phase (days 57–84), a numerical improvement in body weight gains was noted in turkeys fed the BioPlus 2B diet (T2) relative to birds fed control diet. At 84th day, birds fed the T2 BioPlus 2B diet were heavier than birds fed the control diet (+4.02%, 7.659 vs. 7.363 kg), and the observed difference was statistically significant ($P = 0.019$). Over the entire experimental period (days 1–84), turkeys fed the BioPlus 2B diet (T2) were characterized by significantly higher body weight gains in comparison with birds fed the control diet (+4.02%). Previous studies have showed the efficacy of *B. licheniformis* and *B. subtilis* in turkey production. Fallah et al. (2013) demonstrated that a probiotic preparation containing *B. subtilis* and *B. licheniformis* had a positive influence on body weight gains in broiler chickens. In a study by Blair et al. (2004), the body weights of 18-week-old turkeys fed diets supplemented with *B. subtilis* were higher than the body weights of control group birds (14.32 vs. 13.41 kg). Shivaramaiah et al. (2011) reported significantly higher body weights in male turkeys receiving *B. subtilis*, compared with control group birds.

In the present study, dietary probiotic supplementation had no impact on FCR in any of the feeding phases and throughout the experiment (Table 5). Midilli et al.

TABLE 5. The results of feed conversion ratio (FCR) of female turkeys (kg feed/1 kg body weight gain) and European productivity index (EPI)

Period (days)	Group		*SEM*	*P*
	T1	T2		
FCR				
1–28	1.244 ±0.036	1.241 ±0.038	0.008	0.848
29–56	1.712 ±0.047	1.694 ±0.037	0.009	0.369
1–56	1.590 ±0.032	1.579 ±0.032	0.006	0.429
57–84	2.329 ±0.099	2.261 ±0.141	0.028	0.229
1–84	1.936 ±0.050	1.900 ±0.058	0.012	0.147
EPI				
1–84	453^{b} ±23	481^{a} ±28	6.451	0.028

Values in same rows with no common superscript are significantly different ($P \leq 0.05$).

(2008) noted a positive effect of dietary probiotic and prebiotic supplementation on feed intake. In an experiment with broiler chickens, Jayaraman et al. (2013) demonstrated that *B. subtilis* bacteria provided health benefits and decreased FCR.

Female turkeys from group T2, fed probiotic-supplemented diets, achieved higher productive performance throughout the experiment, and were characterized by a significantly higher EPI (+6.1%, 481 vs. 453, $P = 0.028$). Loeffler (2014) pointed out that there is evidence to support that multi-species probiotic supplementation is more efficient than single strain probiotics in turkey nutrition. In this sense, Blanch and Rouault (2016) recently concluded that the high efficiency of BioPlus 2B diet could be elucidated by the complementarity between the different modes of action of the two strains enclosed in the product, being *B. licheniformis* extremely efficient in pathogen growth inhibition and *B. subtilis* in digestive enzyme activation.

CONCLUSIONS

The results of this study indicate that a probiotic preparation containing *B. subtilis* and *B. licheniformis* positively affected the growth performance of female turkeys. Birds fed probiotic-supplemented diets were characterized by significantly higher final body weight and body weight gain, and significantly higher values of the EPI.

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Streszczenie: *Wpływ zastosowania do paszy* Bacillus subtilis *i* Bacillus licheniformis *na wyniki odchowu indyków.* Doświadczenie przeprowadzono w celu określenia wpływu zastosowania preparatu probiotycznego zawierającego spory *Bacillus subtilis* i *Bacillus licheniformis* dodanego do paszy na wyniki odchowu indyczek odchowywanych do 84. dnia życia. Trzysta jednodniowych indyczek Hybrid Converter zostało przydzielonych do 20 kojców, po 15 indyczek w każdym. Zostały one podzielone na dwie grupy (10 powtórzeń/10 kojców w każdej): T1 żywione paszą bazową oraz T2 żywione paszą bazową z dodatkiem preparatu probiotycznego w ilości $1{,}28 \cdot 10^6$ CFU/g paszy. Reasumując, dodatek probiotyku do paszy miał korzystny wpływ na wyniki odchowu indyczek i istotnie poprawiał ich końcową masę ciała oraz średnie przyrosty masy ciała.

Słowa kluczowe: indyczki, probiotyk, *Bacillus subtilis*, *Bacillus licheniformis*, wyniki odchowu

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Relationship between parity and oxidative stress in high-performance Polish Holstein-Friesian cows after the peak of lactation

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Abstract: *Relationship between parity and oxidative stress in high-performance Polish Holstein-Friesian cows after the peak of lactation.* There are many factors, which may expose cows attacks of free radicals. The highest level of oxidative stress appears in the parturition period and at the peak of lactation. Therefore, the purpose of this study was to demonstrate the relationship between the parity and oxidative stress in high-performance Polish Holstein-Friesian (PHF) cows after the peak of lactation. Seventy PHF cows were selected for the experiment according to: age (35 primiparous and 35 multiparous in the second lactation) and stage of lactation (after the peak of lactation; at days 61–90 in milk). Samples of milk and blood were collected in monthly intervals, from 61st–90th till about 271st day of lactation. Study results demonstrated a significant impact of the parity and days in milk of cows on the formation of oxidative stress markers. The primiparous cows were characterized by lower levels of GluRed and Gpx. The lowest level of oxidative stress was observed in the months after the peak to about 250 days of lactation. Based on study results, it can be concluded that that younger animals were more exposed to free radicals and oxidative stress.

Key words: oxidative enzymes, dairy cows, Holstein-Friesian breed, milk, blood, parity

INTRODUCTION

The total antioxidant capacity is the body's ability to remove excess of oxygen free radicals, understood as the sum of all the antioxidant elements in the organism. The organism has many antioxidant systems that are important in preventing oxidative stress. Free radicals may be neutralized by some active substances, such as: vitamin C, vitamin E, uric acid, bilirubin, thiols and glutathione (Crujeiras et al. 2008, Puppel et al. 2015).

There are two main groups of antioxidants: endogenous and exogenous ones. The endogenous antioxidants are produced in natural reactions in the organism. Most of them are antioxidant enzymes, which are the first line of body defense against oxidative stress, like e.g.: superoxide dismutase (SOD), glutathione peroxidase (GSH-Px-E), and glutathione reductase (GluRed). Selected enzymes reduce or break down free radicals (Chiumiento et al. 2006, Puppel et al. 2015). Unfortunately, it is difficult to correctly determine levels of

antioxidants for cows, because of their different needs depending on the stage of lactation, and also because of the lack of external symptoms. Therefore, it is good to know levels of oxidative stress induced at each stage of the whole lactation period (Gaál et al. 2006, Pedernera et al. 2010, Celi 2011). The antioxidant potential decreased during physiological changes, and this may be an indirect cause of inflammation or mastitis (Stefanon et al. 2005, Sordillo and Aitken 2009). Because of the strain on the body's metabolism, high-performance cows are more exposed to oxidative stress. In this situation, the probability of metabolic diseases development increases and problems may appear with reproduction. Even with proper feeding of cows, the "metabolic depletion" of the animal may induce oxidative stress (Lykkesfeldt and Svendsen 2007, Celi 2011, Puppel et al. 2015).

It has already been proved that the highest level of oxidative stress appears in the parturition period or the peak of lactation (Bernabucci et al. 2005, Pintea et al. 2006, Kapusta et al. 2018). It can be considered as an organism effort to adapt to higher reactive oxygen species (ROS) production. Considering also cows welfare, it is however important to know the antioxidants capacity in the latest stage of lactation. Researchers have shown that the level of anti-oxidative enzymes decreases at the beginning of lactation. After the peak of lactation, it slowly returns to normal in natural conditions (Castillo et al. 2006, Pintea et al. 2006, Celi et al. 2010). The age of cows seems to be an interesting aspect in this respect. Some researchers reported that younger cows, especially the primiparous ones, were more exposed to oxidative stress. Lactation is something new to those animals, and connected with a lot of negative factors, e.g. stress caused by the milking process, effort caused by milk production or calving (Piccione et al. 2007).

Therefore, the purpose of this study was to demonstrate the relationship between the parity and oxidative stress in high-performance Polish Holstein-Friesian cows after the peak of lactation.

MATERIAL AND METHODS

The study was carried out at the experimental dairy farm of the Warsaw University of Life Sciences – SGGW (WULS-SGGW). Cows were kept in a free-stall dairy shed and fed a total mixed ration (TMR) *ad libitum*. The ingredient composition of TMR was (kg/day): maize silage – 21; alfalfa silage – 9.50; corn silage – 5.0; soybean meal – 2.10; pasture ground chalk – 0.1; vitamin mix – 0.16; rapeseed meal – 2.20; and magnesium oxide – 0.05. While, its chemical composition (g/kg DM) was as follows: ash – 50; crude protein – 85; acid detergent fiber – 223; and neutral detergent fibre – 354.

Seventy Polish Holstein-Friesian cows were selected for the experiment according to: parity (35 primiparous and 35 multiparous in the second lactation), and stage of lactation (after the peak of lactation; at days 61–90 in milk).

The cows were milked daily at 07:00 and 18:30 using a milking parlor and milk meters. The combined milk from the morning and afternoon milking was a representative sample for analysis.

The milk was placed in sterile bottles (250 mL) and immediately transported to the Cattle Breeding Division for analysis.

Blood samples (10 mL) were taken from each cow by jugular venipuncture into a heparinized tube, separated by centrifugation at room temperature and transported to the WULS-SGGW laboratory.

Samples of milk and blood were collected in monthly intervals (VIII samplings): sampling I – cows were between day 61 and 90 of lactation; sampling II – cows were between day 91 and 120 of lactation; sampling III – cows were between day 121 and 150 of lactation; sampling IV – cows were between day 151 and 180 of lactation; sampling V – cows were between day 181 and 210 of lactation; sampling VI – cows were between day 211 and 240 of lactation; sampling VII – cows were between day 241 and 270 of lactation; and sampling VIII – cows were beyond day 271 of lactation.

Milk gross composition including contents of: fat, total protein and lactose, was determined using a Milko-Scan FT--120 analyzer (Foss Electric, Hillerod, Denmark).

Malondialdehyde (MDA) concentration in milk was determined using a NanoQuant Infinite M200 Pro analyzer (Tecan Austria GmbH, Grödig, Austria) at a wavelength of 532 nm according to the methodology described by Kapusta et al. (2018).

Concentrations of GluRed, GPx, SOD, TAS in blood plasma (TASp) and TAS in milk (TASm) were established using a NanoQuant Infinietie M200Pro analyzer (Tecan Austria GmbH, Grödig, Austria) with a dedicated ELISA Kit, according to the methodology described by RANDOX (Randox Laboratories, Crumlin, United Kingdom).

The obtained data were analyzed statistically by two-way ANOVA, and Tukey post-hoc test using SPSS23 software. Data were presented as least squares means with standard error of the mean. Only the interactions between factors whose influence was statistically significant ($P \leq 0.05$) were considered. The correlations were determined by the Pearson coefficient.

The statistical model was:

$$Y_{ijkl} = \mu + A_i + B_j + (A_i \cdot B_j) + e_{ijk}$$

where:

Y_{ijkl} – dependent variable;
μ – overall mean;
A_i – fixed effect of the parity (i = 1, 2);
B_j – fixed effect of stage of lactation (j = 1–7);
$(A_i \cdot B_j)$ – interaction between parity and stage of lactation;
e_{ijkl} – residual error.

RESULTS AND DISCUSSION

The study showed that milk from primiparous cows contained more fat, and less protein and lactose during the whole lactation period, compared to milk of the multiparous cows (Table 1). In addition, it demonstrated a direct relationship between malondialdehyde (MDA) level and fat content; i.e. the higher level of fat was associated with a higher level of MDA. A similar correlation was demonstrated by Kapusta et al. (2018). In turn, Pedernera et al. (2010) reported that factors associated with a high level of

TABLE 1. Milk content regarding lactation stage of primiparous (PC) and multiparous (MC) cows

Days in lactation		Fat (%)		Protein (%)		Lactose (%)	
		PC	MC	PC	MC	PC	MC
61–90	*LSM*	4.36^{ABCDEF}	2.69^{ABCDEF}	$3.06^{ABCDEFG}$	$3.12^{ABCDEFG}$	4.95^{ABCDEF}	5.15^{ABCDEF}
	SEM	0.797	0.335	0.216	0.258	0.120	0.142
91–120	*LSM*	3.41^{GHIJKL}	3.07^{GHIJKL}	$3.31^{AHIJKLM}$	$3.32^{AHIJKLM}$	4.99^{GHIJKl}	5.10^{aGHIJK}
	SEM	0.321	0.288	0.310	0.267	0.143	0.119
121–150	*LSM*	4.28^{AGMnop}	3.74^{AGMnop}	3.79^{BH}	3.55^{BH}	4.81^{Al}	5.07^{aA}
	SEM	0.352	0.815	0.675	0.176	0.391	0.138
151–180	*LSM*	4.39^{BH}	4.16^{BH}	3.73^{CI}	3.57^{CI}	4.85^{BG}	5.01^{BG}
	SEM	0.889	0.748	0.377	0.2441	0.124	0.169
181–210	*LSM*	4.80^{CIn}	4.28^{CIa}	3.74^{DJ}	3.56^{DJ}	4.78^{CH}	4.99^{CH}
	SEM	0.252	0.703	0.435	0.222	0.145	0.144
211–240	*LSM*	4.72^{DJo}	4.37^{DJo}	3.60^{EKn}	3.54^{EKn}	4.75^{DI}	5.01^{DI}
	SEM	0.986	0.612	0.330	0.224	0.162	0.140
241–270	*LSM*	4.81^{EKM}	4.49^{EKM}	3.77^{FL}	3.69^{FL}	4.68^{EJ}	5.01^{EJ}
	SEM	0.984	0.634	0.306	0.242	0.290	0.106
271–305	*LSM*	4.58^{FLp}	4.44^{FLp}	3.87^{GMn}	3.74^{GMn}	4.80^{FK}	4.98^{FK}
	SEM	0.673	0.662	0.193	0.328	0.118	0.136

Means in the same column marked with the same letters differ significantly at: lowercase – $P \leq 0.05$; capitals – $P \leq 0.01$.

oxidative stress were severely negative energy balance and lower levels of milk production. Results of our study enable concluding that that younger animals were more exposed to free radicals and oxidative stress.

Superoxide dismutase (SOD) and glutathione peroxidase (GPx) represent the main forms of the intracellular antioxidant defense (Puppel et al. 2015). Tüzün et al. (2002) showed that GPx may be considered as an indicator of oxidative stress. Additionally, Bernabucci et al. (2005) reported that an increase of plasma GPx activity might reflect an altered oxidative status in the pre- and post-calving periods. The activity of GluRed was at a similar level during the first 200 days of lactation in both analyzed groups of cows. In the case of primiparous cows, the study showed a decrease in the activity of both Gpx and GluRed after 210 days of lactation. Additionally, levels of antioxidant enzymes (GluRed and Gpx) were lower in blood plasma of the primiparous compared with the multiparous cows over the entire lactation period (Table 2). Konvičná et al. (2015) demonstrated a lower concentration of Gpx in the final stage of lactation, which was also confirmed in our study. The reported differences between the analyzed groups of cows might have induced an imbalance between production of reactive oxygen metabolites and reduction of antioxidants, as well as

TABLE 2. Antioxidant capacity of blood plasma and milk of primiparous (PC) and multiparous (MC) cows

Days in lactation		MDA (nM/mL)		GluRed (U/L)		Gpx (U/L)		SOD (U/L)		TASp (mmol/L)		TASm (mmol/L)	
		PC	MC	PC	MC	PC	MC	PC	MC	PC	MC	PC	MC
61–90	*LSM*	28.01^{a}	30.14^{a}	80.22^{A}	84.16^{A}	332.12^{a}	508.58^{a}	245.93	220.98	2.14	1.22	1.21^{abC}	0.96^{abC}
	SEM	0.459	0.401	0.010	0.803	0.085	0.251	0.877	0.172	0.013	0.017	0.054	0.035
91–120	*LSM*	34.65	32.63	87.63^{a}	85.83^{b}	324.67^{bcEF}	464.10^{bcAB}	284.09	242.20	2.23	1.35	1.04^{d}	1.20^{d}
	SEM	0.394	0.467	0.298	0.466	0.507	0.817	0.436	0.334	0.042	0.020	0.017	0.060
121–150	*LSM*	30.95	31.07	82.59^{b}	87.32^{c}	393.52^{d}	505.90^{d}	260.94	255.42	2.18	1.36	1.06^{e}	1.24^{e}
	SEM	0.412	0.693	0.634	0.9948	0.290	0.112	0.601	0.239	0.071	0.071	0.033	0.043
151–180	*LSM*	32.19	30.85	85.17	94.65	451.65^{b}	540.16^{b}	231.89	258.00	2.10	1.51	1.05	1.30
	SEM	0.973	0.355	0.645	0.416	0.935	0.498	0.284	0.526	0.082	0.083	0.004	0.019
181–210	*LSM*	33.52^{a}	34.20^{a}	88.90	100.86	493.01^{adE}	599.01^{adA}	235.82	249.56	2.22	1.37	1.10^{a}	1.38^{a}
	SEM	0.639	0.110	0.433	0.129	0.845	0.473	0.837	0.596	0.053	0.082	0.093	0.027
211–240	*LSM*	28.43	34.84	86.67	102.19	411.43^{F}	600.57^{B}	249.35	250.81	1.94	1.29	1.25^{Cde}	1.46^{dCe}
	SEM	0.879	0.458	0.545	0.501	0.736	0.516	0.006	0.375	0.040	0.051	0.054	0.075
241–270	*LSM*	28.35	33.18	80.74	98.82	395.01	555.63	246.05	256.59	1.80	1.42	1.14^{b}	1.41^{b}
	SEM	0.442	0.649	0.850	0.598	0.535	0.287	0.283	0.352	0.029	0.037	0.026	0.045
271–305	*LSM*	29.64	31.11	80.13^{abA}	95.39^{Abc}	336.20^{c}	586.32^{c}	241.79	262.98	2.03	1.33	1.18	1.32
	SEM	0.631	0.313	0.448	0.122	0.516	0.806	0.688	0.199	0.066	0.055	0.096	0.035

MDA – malondialdehyde; GluRed – glutathione reductase; Gpx – glutathione peroxidase; SOD – superoxide dismutase, TASp – total antioxidant status in blood, TASm – total antioxidant status in milk.

Means in the same column marked with the same letters differ significantly at: lowercase – $P \leq 0.05$; capitals – $P \leq 0.01$.

lipid peroxidation process. Based on the obtained results, it can be concluded that that younger animals were more exposed to free radicals and oxidative stress.

Concentration of MDA was at a similar level in both groups (Table 2). Its highest value was determined in primiparous milk after 90 days in lactation, immediately after the peak of lactation. Also Bernabucci et al. (2005) demonstrated that cows after calving showed a decrease of plasma and erythrocyte SOD, and an increase of MDA. In addition, as demonstrated by the study, the lower level of MDA was related with a higher concentration of antioxidant enzymes. Our results were also consistent with findings reported by Castillo et al. (2005).

The study demonstrated that the total antioxidant status (TAS) in blood plasma of primiparous cows between 90th and 210th day of lactation was at a similar level. However, in the later period, a significantly decrease has been reported in the antioxidant capacity, probably due to reduction in the supply of exogenous antioxidants. In the case of multiparous cow, we showed that TAS plasma was at a similar level during the whole analyzed lactation period. Its lower level was determined only in the third month probably because of the post-natal period. The same tendencis was found in TAS measured in milk. Castillo et al. (2003) compared the level of TAS in high (35 L/day) and low (20 L/day) yielding cows, and demonstrated small differences between these groups.

The study showed a significant correlation between MDA concentrations and individual components (Table 3). As shown by study results, the high concentrations of MDA was associated with lowered level: TASs, TASm, GluRed and Gpx.

CONCLUSION

The study has demonstrated that the age of cows plays an important role in oxidative stress induction. In all cases, indicators of oxidative stress were higher in the plasma of primiparous

TABLE 3. Pearson correlations

	GluRed	Gpx	SOD	TASp	TASm	MDA
GluRed	1	0.420**	NS	0.750**	–0.402**	–0.315**
Gpx	0.420**	1	–0.221**	NS	–0.220**	–0.380**
SOD	NS	–0.221**	1	–0.342**	–0.597**	0.618**
TASp	0.750**	NS	–0.342**	1	–0.312**	–0.520**
TASm	–0.402**	–0.220**	–0.597**	–0.312**	1	–0.420**
MDA	–0.315**	–0.380**	0.618**	–0.520**	–0.420**	1

GluRed – glutathione reductase; Gpx – glutathione peroxidase; SOD – superoxide dismutase; MDA – malondialdehyde; TASm – total antioxidant status determined in milk; TASp – Total antioxidant status determined in blood plasma.

** The correlation significant at the 0.01 level (two-sided); * The correlation significant at the 0.05 level (two-sided); NS – not significant.

than multiparous cows. In both groups, oxidative stress has increased slightly in the last months of lactation compared to the previous months. The lowest level of oxidative stress was observed in the months after the peak (after 90 days) to about 250 days of lactation. Oxidative homeostasis was stable between 90th and 250th day of lactation.

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Streszczenie: *Zależność między wiekiem a stresem oksydacyjnym u wysokowydajnych krów rasy polskiej holsztyńsko-fryzyjskiej po szczycie laktacji.* Zdolność antyoksydacyjna to zdolność organizmu do usuwania reaktywnych form tlenu, które powodują stres oksydacyjny w organizmie. Istnieje wiele czynników, które mogą narazić krowy na ataki wolnych rodników. Najwyższy poziom stresu oksydacyjnego pojawia się w okresie porodu i w szczycie laktacji. Celem tego doświadczenia było wykazanie związku między wiekiem a poziomem stresu oksydacyjnym u wysokowydajnych krów rasy polskiej holsztyńsko-fryzyjskiej (PHF) po szczycie laktacji. Do eksperymentu wybrano 70 krów PHF według: wieku (35 pierwiastek i 35 wieloródek w drugiej laktacji) i fazy laktacji (po szczycie laktacji, między 61. a 90. dniem laktacji). Próbki mleka i krwi pobierano w miesięcznych odstępach, od 61.–90. do średnio 271. dnia laktacji. Badania wykazały istotny wpływ wieku krów i fazy laktacji na kształtowanie się markerów stresu oksydacyjnego. Pierwiastki charakteryzowały się niższym poziomem GluRed i Gpx. Najniższy poziom stresu oksydacyjnego wykazano w miesiącach po szczycie i do około 250. dnia laktacji. Na podstawie uzyskanych wyników można stwierdzić, że młodsze zwierzęta były bardziej narażone na działanie wolnych rodników i stres oksydacyjny.

Słowa kluczowe: enzymy oksydacyjne, krowa mleczna, rasa holsztyńsko-fryzyjska, mleko, krew, wiek

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Effect of dried pumpkin (*Cucurbita maxima* D.) supplementation on growth performance, serum biochemistry and parameters of antioxidant status of rats

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Abstract: *Effect of dried pumpkin* (Cucurbita maxima D.) *supplementation on growth performance, serum biochemistry and parameters antioxidant status of rats*. The aim of the studies was to determine the effect of dried pumpkin, used in the diets for rats on parameters of growth, nutrient metabolism and antioxidant status of the animals. The experiment was carried out for 7 weeks with 30 growing male Wistar rats. The animals were classified into three groups, 10 individuals in each group, with the initial body weight of 108 g. The control group (G-0) was fed the semi-synthetic mixture without dried pumpkin additive whereas the experimental groups received the mixture with 5% (G-5) and 10% (G-10) additive of the dried pumpkin, Ambar variety. The dry substance was obtained from disintegrated fruits, deprived of seed nests, dried at temperature of 60°C. During the experiment weight gains and feed intake were controlled. After termination of the experiment, the rats were killed by anaesthesia; the blood samples were collected and biochemical indices and indicators of antioxidant status were determined. The dietary treatments had no effects on animal growth and feed utilization. In the animals receiving dried pumpkin in their diets (G-5, G-10) significantly lower level of glucose concentration in serum was found. In group G-0, the higher concentration of triacylglycerols in relation to group G-10 was recorded. Also, the concentration of total cholesterol in group G-0 was higher in comparison to groups G-5 and G-10. In group G-0, VLDL concentration was also higher in relation to group G-10. In group G-10 compared to groups G-5 and G-0, the higher activity of glutathione peroxidise (GPx) was recorded. Total antioxidant status (TAS) was higher in group G-10 in comparison to groups G-0 and G-5. The effect of the administered diet on indicators of the degree of lipid oxidation was also found. In group G-10 compared to group G-0, thiobarbituric acid reactive substances (TBARS) concentration was lower.

Key words: rats, dried pumpkin, serum biochemistry, antioxidant status

INTRODUCTION

New varieties of pumpkin in relation to the traditional ones are characterized by higher dry matter content, reaching up to ca. 20% and relatively high participation of carotenoids (Daničlenko 2000, Murkovic et al. 2002, Korzeniewska et al. 2004). Fruits of pumpkin contain a lot of cellulose and pectins, and, also starch, glucose, fructose and saccharose. They are also rich in minerals – mainly potassium, calcium, phosphorus and magnesium and vitamins A, E and C and vitamins from B group (Daničlenko 2000, Korzeniewska et al. 2004, Nawirska et al. 2008).

The higher dry matter content in pumpkin fruits enables their easier drying and makes the process more effective. It increases the possibilities of utilizing the dried pumpkin in production of mixtures and feed for farm animals and pets.

High content of carotenoids in the pumpkin fruits is very important from the viewpoint of dietetic value of the discussed fruits. Nawirska-Olszańska (2011) informs that the content of carotenoids in the flesh of the pumpkin varies, in average, 2–10 mg/100 g DM. In Ambar variety, the level of carotenoids amounted to 42.41 mg/100 g DM, including 24.24 mg of beta-carotene in 100 g/DM. Owing to its polyene structure, carotenoids absorb light and neutralize free radicals, i.a singlet oxygen and organic radicals. Carotenoids reveal also stimulating effect on immunological system (Caili et al. 2006, Krzysik et al. 2007, Yang et al. 2007).

A high participation of pumpkin in the diet *via* the contained polysaccharides, including a high level of pectins, affects the increase of insulin level in the blood circulation, a phenomenon resulting in lowering the glucose content. The effect of structural carbohydrates, as present in the pumpkin, on decrease of the cholesterol level and that of triglycerides in blood, was also recorded (Zhang 2004, Kim 2005, Wikiera et al. 2014).

Owing to its chemical composition, dry pumpkin may be a valuable component of feed rations and mixtures, prepared for animals, including pets. The application of dried pumpkin in the diet should positively affect the utilization of diet's nutrients and enhance the antioxidative potential of the organism.

The aim of the study was the examination of the influence of dried pumpkin applied in the diet for rats on the growth of animals, indicators of metabolic changes as well as the oxidative stress parameters of the organism.

MATERIAL AND METHODS

Animals and nutrition

The study was carried out following the procedures approved by the local ethical commission for experiments with animals. The experiment was conducted with 30 growing Wistar rats with the initial body weight of 106 ±8 g. The animals were randomly assigned to three experimental groups, 10 rats each. All were fed semi-purified AIN-93G-based diets (Reeves 1997). Rats from control group (G-0) were fed AIN-93G-based diets without pumpkin.

The experimental groups received the mixture, containing 5% (G-5) and 10% (G-10) of dried pumpkin, Amber variety. The material destined for drying was obtained from ripened fruits, deprived of seed nests. The disintegrated pumpkins were dried at temperature of 60°C and then, added to experimental mixtures. All the mixtures were equalized in respect of crude protein, crude fat and crude fiber content. The additional quantity of water-soluble carbohydrates and of bioactive compounds (mainly carotenoids), assimilated by the animals in the diets with dehydrated pumpkin was the differentiating factor.

The composition and nutritive value of the mixtures is given in Table 1.

The rats were housed in individual cages with 12-hour light–dark cycle, temperature of 22°C and humidity of 50–60%. The experiment lasted for

TABLE 1. Composition and nutritive value of experimental diets

Item	Group		
	C-0	G-5	G-10
	g/kg diet		
Dried pumpkin*	–	50	100
Casein	197	190	183
Starch	674	637	600
Cellulose	40	35	30
Rapeseed oil	40	39	38
**AIN-93G Mineral Mix	35	35	35
***AIN-93-VX Vitamin Mix	10	10	10
Choline chloride	2	2	2
Methionine	2	2	2
In kg of mixture			
Dry matter (g)	904	911	906
Crude fiber (g)	40	40	40
Crude protein (g)	161	162	162
Crude fat (g)	40	40	40
Pectin (g)	–	17	36
Beta-carotene (mg)	–	18	34

C-0 control diet; G-5, diet with 5% dried pumpkin; G-10, diet with 10% dried pumpkin.

*Chemical composition of 1 kg of dried pumpkin: dry matter – 910 g, crude protein – 102 g, crude fat – 18 g, crude fiber – 101 g, pectin – 374 g, beta-carotene – 341 mg, luteine – 76 mg, total polyphenol content (TPC) – 3476 mg; **AIN-93G Mineral Mix (No 94046); ***AIN-93-VX Vitamin Mix (No 94047).

7 weeks. The animals had a free access to feed and water. During the experiment feed intake was controlled every day and weight gains once per week.

Collection and preparation of blood

The rats were subject to fasting for 12 h before the experiment termination. After anesthesia (overdose of ketamine – 50 mg/kg of body weight) the blood samples were collected from the left cardiac ventricle to plastic test tubes without and with an anticoagulant. To obtain serum and plasma blood was centrifuged (1,500 rpm for 10 min). The samples were frozen (–70°C) and stored until analyzed.

Analyses

Analyses of three samples of dry pumpkin and three respective diets were performed. On this basis, the average content of the ingredients was determined. The chemical composition of the dried pumpkin and diets was determined according to AOAC methods (2005). The pectin were determined according to Morris method (Pijanowski et al. 1993).

The analysis of carotenoids separation and contents was made by applying the HPLC system (Dionex) equipped with a CoulArray electrochemical detector (ESA Inc). The separation was conducted on a Hypersil BDS 150 × 4.6 mm, 5 µm column (Sigma-Aldrich) at a mobile phase flow rate of 1.2 ml/min. The mobile phase consisted of a methanol and isopropanol mixture (98 : 2). The conditions of electrochemical detection were: four electrodes with potentials 400, 500, 600, and 750 mV. The chromatograms were processed by identifying the pigments on the basis of standards and areas of chromatographic peaks, taking into account their retention times as well as the ratio of the peak area for the dominating electrode to that of neighbouring electrodes. The TPC was determined according to Fisk et al. (2006), applying Folin–Ciocalteau's reagent, using gallic acid (GA) as a standard for calibration curve. The results were read at 765 nm after 1 h in a Tecan Infinite M200 analyser.

Glucose, total protein, albumin, urea, ALT, AST, ALP, total cholesterol, VLDL cholesterol, HDL cholesterol and triacylglycerides (TAG) were determined in blood serum by the spectrometric method using VITROS analyzer in a system EKTAchem DT-60-II with module, DT, DTE, DTSC, using sets of slides of Johnson & Johnson Clinical Diagnostics.

Glutathione peroxidase (GPx) activity was measured in blood by the modified Kraus and Gather (1980) method. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is converted to the reduced form with a contaminant oxidation of NADPH to $NADP^+$. The absorbance was measured at wavelength 340 nm (Rancel RS 505, Randox, Crumlin, UK). The activity of PGx was expressed in U/ml blood, with 1 U corresponding to oxidation of 1 µmol NADPH/min.

The total antioxidative activity of blood plasma (TAS) was determined by the colorimetric method using kits by Randox Laboratories Ltd. and expressed in mmol/l. The results were read out with a Cobra Mira biochemical analyzer (Roche) at a wavelength of 600 nm (Smart at al. 1996).

Malondialdehyde (MDA), the most abundant product of all lipid peroxidation products, was measured in serum using thiobarbituric acid (TBA) according to the Uchiyama and Mihara (1978) technique. Absorbance was measured at a wavelength of 535 nm with a Tecan Infinite M200 analyser (Tecan Group Ltd., Switzerland). The results represent the concentration of thiobarbituric acid reactive substances (TBARS) in the samples.

Statistical analysis

The obtained results were elaborated statistically using a one-way analysis of variance with the least square method. Account was taken of the effect of feeding on the analyzed parameters. The tables contain mean values of parameters and standard errors of the means. Computations were made with Statgraphics 6.0 Plus Statistical Package.

RESULTS AND DISCUSSION

No significant differences in the growth indicators between animals from various groups were reported. With animals on diets with dry pumpkin, there was only a slight tendency towards a higher final

mass and a higher daily gain. This tendency was particularly visible with the group on a diet with 10% dry pumpkin content – group G-10 (Table 2).

In case of the animals, receiving dried pumpkin in their diets (G-5, G-10), significantly lower concentration of serum glucose as compared to the control group (G-0) ($P \leq 0.01$) was found. The application of dried pumpkin in the diets of the rats had an influence on indicators of lipid changes. In the control group, the higher concentration of triacylglycerols in relation to group G-10 ($P \leq 0.05$) was recorded. Also, the concentration of total cholesterol in control group (G-0) was significantly higher in comparison to group G-5 ($P \leq 0.05$) and G-10 ($P \leq 0.01$). In the control group, VLDL cholesterol concentration was also significantly higher ($P \leq 0.01$) in relation to group G-10 (Table 3).

TABLE 2. Parameters of growth performance of rats fed the diets containing dried pumpkin

Item	Group			*SE*	*P*-value
	G-0	G-5	G-10		
Initial body weight (g)	105.8	106.5	106.2	6.079	NS
Final body weight (g)	283.3	286.7	291.5	9.361	NS
Average daily gain (g)	4.27	4.30	4.41	0.173	NS
Feed convertion ratio (g/g)	4.23	4.42	4.29	0.152	NS

C-0 – control diet; G-5 – diet with 5% dried pumpkin; G-10 – diet with 10% dried pumpkin.

TABLE 3. Biochemical parameters in serum of rats fed the diet containing dried pumpkin

Item	Group			*SE*	*P*-value
	G-0	G-5	G-10		
Glucose (mmol/l)	9.81^{B}	8.09^{A}	7.84^{A}	0.388	≤ 0.01
Albumin (g/l)	33.72	31.94	31.35	0.854	NS
Total protein (g/l)	59.81	56.10	56.02	1.339	NS
Urea (g/l)	4.66	5.07	5.16	0.210	NS
ALT (U/l)	9.90	10.41	6.78	1.173	NS
AST (U/l)	47.04	48.74	48.36	3.998	NS
ALP (U/l)	219.34	190.02	187.84	15.406	NS
Triacylglycerols (TAG) (mmol/l)	1.77^{b}	1.53^{ab}	1.44^{a}	0.104	≤ 0.05
Total cholesterol (mmol/l)	1.94^{Aa}	1.71^{b}	1.66^{abB}	0.062	≤ 0.01
HDL cholesterol (mmol/l)	1.13	1.16	1.14	0.056	NS
VLDL cholesterol (mmol/l)	0.92a	0.77^{ab}	0.72^{b}	0.055	≤ 0.05

C-0 – control diet; G-5 – diet with 5% dried pumpkin; G-10 – diet with 10% dried pumpkin.

AB – differences between the selected rows ($P \leq 0.01$); ab – differences between the selected rows ($P \leq 0.05$).

The results, obtained in our studies have been also confirmed in the studies of other authors. Hypoglycemic properties of pumpkin pulp in the studies on rabbits and rats were confirmed by Fu et al. (2006). The decrease of the level of glucose, total cholesterol and triglycerides in blood serum of the rabbits, receiving dried pumpkin in their diets was also found by Zhang (1998). Yoshinari et al. (2009) employed 1% of lyophilisate obtained from pumpkin paste concentrate in the diets for adult Wistar and GK (Goto-Kakizaki) rats and found, i.a, lower level of glucose and total cholesterol in the group, receiving pumpkin lyophilisate.

Dried pumpkin is a rich source of water-soluble carbohydrates and pectin. The intake of the mentioned compounds by the animals, receiving dried pumpkin in their diets may be related to the lowered level of glucose and total cholesterol, triglycerides and VLDL in serum as compared to the control group (Table 1, Zhang 2004). The discussed compounds – *via* decrease of gastric and intestinal mobility – decrease the contact of glucose and mucous membrane and its absorption is worsened (Wikiera et al. 2014). Pectin and water-soluble carbohydrates cause also the increase in thickness and viscosity of mucus, covering intestinal mucous membrane what limits also absorption of glucose and affect lowering of its level in blood (Kim 2005).

The ability of creating gel layer in gastrointestinal tract limits also the process of lipolysis and deestrification of cholesterol; it decreases also absorption of the resulting products (Gulfi et al. 2006). Pectin in the diets increase also excretion of bile acids in feces what, in consequence, leads to decrease of cholesterol concentration in blood (Wikiera et al. 2014).

In group G-10 as compared to group G-5 and the control group (G-0) significantly higher activity of glutathione peroxidase was found ($P \leq 0.01$). The reductase was recorded on the same level in all groups. The diet with the highest content of dried pumpkin increased the total antioxidative potential of the body. In group G-10, TAS was highly significantly higher ($P \leq 0.01$) as compared to the group G-0 and group G-5. Also, the effect of administered diet on indicators of the degree of lipid oxidation was found. In group G-10 compared to control group, significantly lower concentration of compounds, reacting with thiobarbituric acid (TBARS) was recorded (Table 4).

TABLE 4. Parameters of the antioxidant status in blood serum and plasma of rats fed the diet containing dehydrated pumpkin

Item	Group			*SE*	*P*-value
	G-0	G-5	G-10		
Glutathione peroxidase – GPx (U/ml)	0.65^{B}	0.64^{B}	0.74^{A}	0.018	≤ 0.01
Glutathione reductase – GR (U/ml)	23.13	23.34	24.88	1.206	NS
TAS (mmol/l)	1.24^{B}	1.26^{B}	1.35^{A}	0.022	≤ 0.01
TBARS (nmol/g)	0.41^{a}	0.36^{ab}	0.34^{b}	0.018	≤ 0.05

C-0 – control diet; G-5 – diet with 5% dried pumpkin; G-10 – diet with 10% dried pumpkin; AB – differences between the selected rows ($P \leq 0.01$); ab – differences between the selected rows ($P \leq 0.05$).

Dried pumpkin, as employed in diets G-5 and G-10 is a rich source of carotenoids, including beta-carotene (Table 1). Owing to its structure and effect, beta-carotene is one of the most important natural antioxidants for biological systems (Caili et al. 2006, Krzysik et al. 2007). Therefore, its presence in the diet and its intake by the animals increase also antioxidant potential of animal. It is also reflected in the results of our studies where in the rats, receiving dried pumpkin, TAS was significantly higher as compared to the control group. The highest TAS was found in the animals, receiving highest content of dried pumpkin in their diets. It should be mentioned that apart from beta-carotene, dried pumpkin supplied also other carotenoids and compounds with antioxidative effect, e.g. flavonoids (Table 1).

In the studies, a positive effect of beta-carotene on the increase of antioxidative enzymes' activity was found (Zamora et al. 1991, Iyama et al. 1996). The higher level of GPx in group G-10 as compared to the remaining groups may be, therefore, the effect of higher intake of beta-carotene and other carotenoids in the diet. In the studies of Dang (2004), the stimulation of activity of antioxidative enzymes in the mice which received pumpkin extract, was recorded.

Owing to their antioxidative activity, beta-carotene and other bioactive compounds may limit the degree of oxidation of lipids in animal bodies. The effect of beta-carotene includes, i.a, inhibition of lipid oxidation in liposomes. It results in lower TBARS concentration in liver or serum. Such effect was found in our studies in the rats, receiving dehydrated pumpkin in the diets. The limitation of the degree of lipid oxidation in case of introducing antioxidants to the diet was also recorded by other researchers in the studies, conducted on humans and animals (Furusho et al. 2002, Actis-Goretta et al. 2004).

CONCLUSION

The application of dried pumpkin in the diets for rats had an influence on decrease of the level of glucose, triacylglycerols, total cholesterol and VLDL cholesterol in blood serum of the rats. The application of the mentioned dried substance in the diets increased also antioxidative potential of the rats. The results of the studies indicate that the dried pumpkin may be interesting and valuable component of feed for animals, including feed with a dietetic effect.

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Streszczenie: *Wpływ suszu z dyni (*Cucurbita maxima *D.) zastosowanego w dietach na wzrost, wskaźniki biochemiczne oraz wskaźniki statusu antyoksydacyjnego szczurów.* Celem badań było określenie wpływu suszu z dyni zastosowanego w dietach dla szczurów na wzrost zwierząt, wskaźniki przemian metabolicznych oraz status antyoksydacyjny organizmu. Doświadczenie przeprowadzono przez 7 tygodni na 30 rosnących szczurach samcach Wistar. Zwierzęta zostały podzielone na trzy grupy po 10 osobników o początkowej masie ciała 108 g. Grupa kontrolna (G-0) była żywiona mieszanką półsyntetyczną bez dodatku suszu z dyni, a grupy doświadczalne otrzymywały mieszankę z udziałem 5% (G-5) i 10% (G-10) suszu z dyni ambar. Susz uzyskano z rozdrobnionych owoców, pozbawionych gniazd nasiennych, suszonych w temperaturze 60°C. W trakcie trwania badań kontrolowano przyrosty masy ciała zwierząt oraz pobranie mieszanek. Po zakończeniu doświadczenia szczury poddano eutanazji i pobrano od nich krew, w której oznaczono wskaźniki biochemiczne w surowicy oraz wskaźniki potencjału antyoksydacyjnego. Nie stwierdzono wpływu diety na wzrost zwierząt i wykorzystanie paszy. U szczurów otrzymujących w dietach susz z dyni stwierdzono niższe stężenie glukozy w surowicy w porównaniu do zwierząt z grupy kontrolnej (G-0). W grupie G-0 występowało wyższe stężenie trójglicerydów w stosunku do grupy G-10. Stężenie cholesterolu całkowitego w grupie G-0 było także wyższe w stosunku do grup G-5 i G-10. W grupie G-0 wyższe było także stężenie cholesterolu VLDL w stosunku do grupy G-10. W grupie G-10 w porównaniu do grup G-5 oraz G-0 stwierdzono istotnie wyższe stężenie peroksydazy glutationowej (GPx). W grupie G-10 TAS był na wyższym poziomie w porównaniu do grup G-0 oraz G-5. Stwierdzono również wpływ podawanej diety na wskaźniki stopnia utleniania lipidów. W grupie G-10 w porównaniu z grupą kontrolną stwierdzono niższe stężenie kwasu tiobarbiturowego (TBARS).

Słowa kluczowe: szczury, susz z dyni, wskaźniki biochemiczne krwi, status antyoksydacyjny

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Use of hop cones in growing beef cattle nutrition

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Abstract: *Use of hop cones in growing beef cattle nutrition.* The objective of this study was to determine the effect of the supplementation of bull diets with hop cones on growing bull performance, the concentrations of blood analytes, including liver enzymes. Twenty-four growing bulls of Slovene autochthonous Cika breed (body weight 373 kg; age 329 days) were randomly allocated to six pens (four animals per pen). Before the start of experiment all animals received the same basic TMR which was afterwards either not supplemented (control; two pens) or supplemented with 50 g per animal (H50; 6 g of hop DM/kg diet DM; two pens) or 100 g of hop cones per animal (H100; 11 g DM/kg diet DM; two pens) daily. Bulls were weighed at the start of the experiment and then again after 30 and 60 days of experiment and average daily gain (ADG), dry matter intake (DMI) and feed to gain ratio (F : G) were calculated. At each weighing day, the blood samples were taken from each bull and plasma glucose and serum non-esterified fatty acids (NEFA), β-hydroxy butyrate (BHBA), urea, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyl transferase (GGT) were determined. The inclusion of hop cones in the diet did not have any effects on DMI, ADG or F : G and did not change BHBA, urea and GGT concentrations. Level of ALT was lower ($P = 0.025$) after 60 days of the experiment compared to control group. In treatments H50 and H100 blood glucose concentrations increased ($P < 0.05$) after 30 and 60 days of the experiment, while in treatment H100 NEFA concentration decreased ($P = 0.022$) after 60 days of the experiment. These findings suggest that hop cones and their constituents provoke changes in energy metabolism in ruminants.

Key words: cattle, hop cones, growth performance, blood metabolite, liver enzymes

INTRODUCTION

Female inflorescences (cones) of hops (*Humulus lupulus* L.) have been used in beer manufacturing in many parts of the world from the Middle ages (Behre 1999). Hop cones give to the beer not only the bitter flavour but also increase its resistance to microbial spoilage, as they possess antimicrobial properties against many species of bacteria (Sakamoto and Konings 2003, Siragusa et al. 2008). Hop cones contain yellow lupulin glands that contain condensed tannins and secondary metabolites such as phenolic acids, flavonoid glycosides, resin and essential oil compounds that all contribute to their antimicrobial properties, the greatest attributed to heat labile α-acids and more stable β-acids in the resins (Siragusa et al. 2008, Van Cleemput et al. 2009).

Besides their use in brewing, the use of hop cones increases in human and herbal medicine (Van Cleemput et al. 2009). Research on rats, mice and humans showed that hop cones and their constituents (e.g. xanthohumol, iso-α-acids, polyphenols) decrease

plasma glucose, non-esterified fatty acid (NEFA), triacylglyceride and urea concentrations and prevent lipid accumulation in the body, causing the decrease in body weight (Nagasako-Akazome et al. 2007, Obara et al. 2009, Everard et al. 2012, Sumiyoshi et al. 2013). These positive properties of hop cones and its constituents together with world hop production, which exceeds its use in brewing and herbal medicine, give the possibility to their use also for other purposes, such as feed additive for farm animals. The information on the use of hop cones and their constituents as feed additives for ruminants is scarce. The results of *in vitro* experiments with hop cones and its constituents on *in vitro* rumen fermentation and degradability (Narvaez et al. 2011, Narvaez et al. 2013, Lavrenčič et al. 2013, 2015) and on rumen microorganisms (Flythe 2009, Flythe and Aiken 2010) were very promising. Use of hop cones decreased *in vitro* dry matter and protein degradability and changed the short-chain fatty acid (SCFA) profile by increasing the proportion of propionic acid. However, these findings were not confirmed in performance trials on steers and finishing heifers (Uwituze et al. 2010, Wang et al. 2010, Axmann et al. 2015), probably because the used amounts of hop cones were too small.

The objective of the present experiment was to assess the effect of hop cones (cv. Aurora) in quantities, which gave positive results in *in vitro* experiments, on animal performance, energy and protein status and enzymes, indicating tissue damage in growing bulls of Slovenian autochthonous Cika breed.

MATERIAL AND METHODS

Animals, diets and feeding

All procedures with animals were performed according to the legislation on animal experimentation in Slovenia at the time experiment was performed. Twenty-four growing bulls of Slovene autochthonous Cika breed (370.8 ±6.8 kg BW; age 329 ±9 days) were randomly allocated to six pens (four animals per pen). Total mixed ration (TMR) was calculated according to German metabolizable energy and crude protein requirements (DLG 1997) for maintenance and 1.200 g of average daily gain (ADG) on the basis of chemically analysed TMR ingredients. Before the start of experiment all animals received the same basic TMR which was afterwards either not supplemented (control) or supplemented with 50 g per animal (H50; 6 g of hop DM/kg diet DM; two pens) or 100 g of hop cones per animal (H100; 11 g DM/kg diet DM; two pens) daily (Table 1).

Dry hop cones cv. Aurora (*Humulus lupulus* L.; 914 g DM/kg) contained (g/kg DM) 179 CP, 196 EE, 416 NDF, 76 ash and 24.0 cohumolon, 90.9 n + adhumulon, 24.1 colupulon, 24.5 n + adlupulon, 115.0 α-acids, 48.5 β-acids, 209.0 co-α-acids, 500.0 co-β-acids and 4.6 xanthohumol. The ratio between α- and β-acids was 2.37 : 1. Animals had *ad libitum* access to feed and water at all time. Diet was delivered once a day at 8:00. An *ad libitum* feed intake was assured by adjusting the amount of feed delivered to each experimental pen in order to obtain approximately a recovery of 3% feed refusals (as-fed basis) 24 h after delivery. Feed deliveries

TABLE 1. Ingredients and chemical composition of the growing bull basic TMR diet (control) and groups supplemented with 50 g (H50) or 100 g (H100) of hop cones daily

Item	Basic TMR	H50	H100
Ingredients (g DM/kg diet DM)			
Maize silage	398	395	393
Grass silage	398	395	393
Maize grain	171	171	170
Sunflower meal	22	22	22
Mineral-vitamin mix	11	11	11
Hop cones (cv. Aurora)	–	6	11
Diet chemical composition			
DM (g/kg)	506	508	509
CP (g/kg DM)	109	109	109
NDF (g/kg DM)	392	390	388
EE (g/kg DM)	27	28	29
NFC (g/kg DM)*	413	411	408
ME (MJ/kg DM)	10.5	10.5	10.4
Hop β-acids (mg/kg DM)	–	291	534

Mineral-vitamin mix contained (per kg): Ca (180 g), P (30 g), Mg (40 g), Na (80 g), Zn (12 g), Mn (4 g), Cu (1 g), Se (30 mg), vitamin A (500,000 IU), vitamin D_3 (100,000 IU), Vitamin E (3,000 mg).

* Non-fibre carbohydrates calculated as 1,000 – (NDF + CP + EE + ash).

and refusals were recorded and sampled twice a week on a pen basis. Dry matter contents of delivered feed and feed refusal were used to calculate the dry matter of feed (DMI). Twice a month the samples of delivered feed were analysed to check the concentrations of nutrients offered to animals.

Bulls were weighed at the start of the experiment (SDay 0) and then again after 30 (SDay 30) and 60 days (SDay 60) of the experiment before the feeding. Average daily gain (ADG; g/day) and feed to gain ratio (F : G; kg DM intake/kg of BWG) were calculated in the periods between start of experiment and day 30, between day 30 and day 60 of the experiment and in the whole experiment period (from day 0 to day 60).

Blood analyses

At each weighing (at SDays 0, 30 and 60) the blood samples were taken immediately after feed delivery into four test tubes (BD vacutainers) from each bull by jugular vene-puncture. Two vacutainers were used to obtain blood plasma (containing Li-heparin), while other two were used to obtain blood serum. Serum was obtained by leaving the tubes containing blood undisturbed at room temperature

allowing them to clot. After 15 to 30 min the clots were removed by centrifugation for 10 min at 1,500 rpm in a refrigerated centrifuge (4°C). Plasma was obtained by centrifugation of the heparinized blood sample for 10 min at 1,500 rpm in a refrigerated centrifuge (4°C). The resulting supernatants (plasma and serum) were transferred into 1.0 mL polypropylene tubes and stored in a freezer at –20°C during the night. Next morning the samples of blood serum and plasma were transported in isolated box containing dry ice to Istituto Zooprofilattico Sperimentale delle Venezie (Udine, Italy) where the contents of glucose in plasma and non-esterified fatty acids (NEFA), β-hydroxy butyrate (BHBA), urea and enzymes alanine aminotransferaze (ALT), aspartate aminotransferase (AST) and γ-glutamyl transferase (GGT) in serum were determined according to the methods of Cozzi et al. (2011), Marchesini et al. (2013) and Brscic et al. (2015).

Statistical analyses

The data were analyzed by using the MIXED procedure of the SAS/STAT software package ver. 9.4. The experimental units for data regarding body weight, daily gain and blood analytes were individual bulls, whereas for data regarding feed intake and feed conversion were individual pens and both were considered as a random effect. To establish if the animals were put in the groups randomly, we checked for all the traits the significance of the treatment at the beginning of the experiment (SDay 0). Body weight after 30 and 60 days of experiment and average daily gain in different periods were analyzed using a model with the fixed effect of treatment and body weight at SDay 0 as a covariate. For the feed consumption and feed to gain ratio only fixed effect of the treatment was included in the model. For the blood analytes, treatment was included as a fixed effect and the pretreatment values (at SDay 0) of corresponding blood analytes as a covariate in the analysis. Post hoc comparison of the least square means was performed using a Tukey multiple test correction. Significance was declared at $P \leq 0.05$ and tendency at $0.05 \leq P \leq 0.10$.

RESULTS AND DISCUSSION

Body weight and concentrations of blood analytes did not differ among treatments at the beginning of the experiment (Table 2), except serum urea, exhibiting the tendency of lower values in the control group. Serum urea concentrations were, on contrary to other blood analytes, which were within physiological ranges for particular animal age, weight and category (Jazbec 1990), well below the normal range of 1.7 to 7.3 mmol/L (Jazbec 1990, Knowles et al. 2000).

Serum urea concentration depends on protein contents in the diet, on protein degradation in the rumen and on the supply of energy to rumen microbes. As the amount of protein in the present experiment was supplied according to DLG (1997) standards to obtain at least 1.200 g of ADG, the low urea contents could be the consequence mainly of its low rumen degradability, distinctive of maize grain, maize silage and Slovenian grass silages.

Body weight, ADG, DMI and F to G ratio of bulls after 30 and 60 days from the beginning of experiment are presented in Tables 3 and 4.

TABLE 2. Average body weights and blood analytes in Cika bulls divided into treatment groups at the beginning of the experiment (LSMEANS ±*SEE*)

Item	Treatment			*P*-value
	control	H50	H100	
BW (kg)	377 ±12	383 ±12	358 ±12	0.317
Glucose (mmol/L)	5.0 ±0.1	5.2 ±0.1	5.0 ±0.1	0.575
NEFA (mmol/L)	0.13 ±0.01	0.13 ±0.01	0.13 ±0.01	0.753
BHBA (mmol/L)	0.31 ±0.03	0.35 ±0.03	0.36 ±0.03	0.647
Urea (mmol/L)	0.5 ±0.1	0.9 ±0.1	0.8 ±0.1	0.052
AST (U/L)	73 ±3	71 ±3	75 ±3	0.777
GGT (U/L)	14 ±1	14 ±1	16 ±1	0.482
ALT (U/L)	27 ±1	27 ±1	26 ±1	0.818

Treatments: diets supplemented with hop cones (0 (control), 50 g/day (H50) or 100 (H100) g/day).

TABLE 3. Body weights (BW) and average daily gains of Cika bulls fed diets supplemented with 0 (control), 50 g (H50) or 100 g of hop cones (H100) daily (LSMEANS ±*SEE*)

Item	Treatment			*P*-value	
	control	H50	H100	BW at SDay 0	treatment
BW (kg) at day 30	412 ±3	405 ±3	408 ±3	< 0.001	0.212
BW (kg) at day 60	451 ±4	442 ±4	446 ±4	< 0.001	0.362
ADG (g/day)					
Period 0–30 days	1358 ±92	1122 ±94	1222 ±95	0.212	0.228
Period 30–60 days	1380 ±71	1321 ±72	1343 ±73	0.839	0.470
Period 0–60 days	1369 ±73	1220 ±74	1282 ±75	0.362	0.262

BW at SDay 0 – body weight at the beginning of the experiment.

TABLE 4. Dry matter intakes (DMI) and feed to gain ratio (F : G) of Cika bulls fed diets supplemented with 0 (control), 50 g (H50) or 100 g of hop cones (H100) daily (LSMEANS ±*SEE*)

Item	Treatment			*P*-value
	control	H50	H100	
DMI (kg/day)				
Period 0–30 days	8.17 ±0.61	7.78 ±0.61	7.56 ±0.61	0.784
Period 30–60 days	8.76 ±0.42	7.87 ±0.42	7.68 ±0.42	0.225
Period 0–60 days	8.46 ±0.51	7.83 ±0.51	7.62 ±0.51	0.517
F : G				
Period 0–30 days	5.92 ±0.38	6.22 ±0.38	6.98 ±0.38	0.211
Period 30–60 days	6.84 ±0.21	6.28 ±0.21	6.22 ±0.21	0.145
Period 0–60 days	6.36 ±0.26	6.25 ±0.26	6.20 ±0.26	0.911

In available literature in only a few papers the effects of ruminant diet supplementation with hop cones and their extracts on ruminant performance have been evaluated. Axmann et al. (2015), who offered to heifers diets with hop cones to achieve 4.9, 7.7 and 15.8 mg of β-acids/kg diet DM and Uwituze et al. (2010), who supplemented the steer diets with hop cones to achieve 1, 8, 16, 24 and 30 mg of β-acids/kg diet DM, did not notice any effect on animal performance. Similar lack of effect on animal performance was found also by Wang et al. (2010), who fed steers with growing diet containing 10, 20 and 40 mg of β-acids/kg diet DM and later with finishing diet containing 20, 40 and 80 mg of β-acids/kg diet DM. Although the concentrations of hop β-acids used in the present experiment were at least 3.6 times greater than in above mentioned experiments (Table 1), they did not affect ADG, DMI and F to G ratio (Tables 3 and 4).

These results were not expected as hop cone acids were reported to possess ionophore activity, similar to monensin (Behr and Vogel 2009, Axmann 2015). Ionophores decrease the activity of Gram-positive bacteria, which produce acetate and butyrate and increase the activity of Gram-negative bacteria, which produce propionate (Russell 1996, Ipharraguerre and Clark 2003). The results of Narvaez et al. (2011 and 2013) and Lavrenčič et al. (2015) are in partial accordance with these statements as supplementation of the diet with hop cones decreased the amounts of acetic and butyric acid produced *in vitro* but did not increase the amount of propionate produced *in vitro*. Thus, the acetic to propionic acid ratio was narrower due to lower acetic acid production and not because of greater propionic acid production, suggesting the inhibition of fibre-utilizing bacteria and unaltered fermentation of rapidly fermentable carbohydrates (Lavrenčič et al. 2015).

Blood analytes glucose, NEFA and BHBA are frequently used to access the energy status of the ruminant animal. In comparison with the control, supplementation of diets with hop cones increased the glucose concentration in treatments H50 and H100 at SDay 30 ($P = 0.028$) and SDay 60 ($P < 0.001$). Glucose concentration in ruminants depends on gluconeogenesis and the main contributor to it is propionic acid, from which between 60 to 74% glucose is formed in the liver (De Koster and Opsomer 2013). Supplementing the diet with hop cones did not increase propionic acid production *in vitro* (Lavrenčič et al. 2015), suggesting that the increase of blood glucose concentrations might be a consequence of improved efficiency of hepatic glucose synthesis or decreased uptake of blood glucose by animal organs and tissues. However, increased blood glucose concentration might also promote the secretion of insulin, which reduce lipid mobilization, thereby decreasing the concentration of blood NEFA. NEFA has a high diagnostic value, as it is very sensitive to changes in energy balance (Cozzi et al. 2011). In the present experiment supplementation of diet with 100 g of hop cones daily (H100) decreased NEFA concentration at SDay 60 ($P = 0.022$), indicating lower lipolysis in these animals (Table 5).

Shimura et al. (2005) also found that isohumulones, which are important

TABLE 5. Blood analyte concentrations in growing bulls receiving diets supplemented with 0 (control), 50 g (H50) or 100 g of hop cones (H100) daily (LSMEANS ±*SEE*)

Item	SDay	Treatment			*P*-value	
		control	H50	H100	pre--treatment value	treatment
Glucose (mmol/L)	30	5.4^{b} ±0.1	5.6^{ab} ±0.1	5.8^{a} ±0.1	0.002	0.028
	60	5.1^{b} ±0.1	5.6^{a} ±0.1	5.7^{a} ±0.1	0.011	< 0.001
NEFA (mmol/L)	30	0.20 ±0.01	0.20 ±0.01	0.19 ±0.01	0.012	0.349
	60	0.25^{a} ±0.01	0.26^{a} ±0.01	0.21^{b} ±0.01	0.124	0.022
BHBA (mmol/L)	30	0.43 ±0.03	0.41 ±0.03	0.39 ±0.03	0.004	0.583
	60	0.41 ±0.02	0.37 ±0.02	0.38 ±0.02	< 0.001	0.486
Urea (mmol/L)	30	1.1 ±0.1	1.4 ±0.1	1.2 ±0.1	0.031	0.307
	60	0.9 ±0.1	1.2 ±0.1	1.0 ±0.1	0.125	0.120
AST (U/L)	30	86 ±3	78 ±3	77 ±3	< 0.001	0.065
	60	81 ±4	79 ±4	72 ±4	0.001	0.097
GGT (U/L)	30	16 ±1	17 ±1	16 ±1	< 0.001	0.112
	60	16 ±1	15 ±1	16 ±1	< 0.001	0.391
ALT (U/L)	30	29 ±1	28 ±1	27 ±1	< 0.001	0.242
	60	31^{a} ±1	28^{b} ±1	27^{b} ±1	< 0.001	0.025

Values in rows with different letter differ significantly ($P < 0.05$).

constituents of hop cone bitter acids, decreased blood NEFA concentration in mice. The increase of blood glucose concentration could arise also from the digestion and metabolism of glucose precursors other than propionic acid. Lavrenčič et al. (2013) determined the lower *in vitro* dry matter degradability of diets supplemented with hop cones, while the whole tract DM digestibility did not differ between treatments. This suggests that glucose could derive from enhanced rumen by-passed starch digestion in small intestines, what would result in more efficient utilization of exogenous glucose in ruminants (Arieli et al. 2001). In addition, Flythe (2009) found that rumen hyper-ammonia producing bacteria are inhibited by hop cones and their acids. This effect was confirmed by Lavrenčič et al. (2013) who determined lower *in vitro* crude protein degradability of ruminant diets supplemented with hop cones, whereas total tract crude protein digestibility remained unchanged, suggesting that some amino acids (e.g. alanine) could contribute to gluconeogenesis in the liver.

Supplementation of ruminant diets with hop cones reduced *in vitro* production of butyrate (Lavrenčič et al. 2015), suggesting that supplementation of diets with hop cones could affect butyrate conversion to BHBA by the rumen epithelium (Niwińska et al. 2017) and thereby decrease the entry of BHBA into systemic circulation. However, it seems that this conversion due to the diet supplementation with hop cones did not take place in present experiment

as the BHBA concentration remained unchanged between control and animals supplemented with 50 or 100 g of hop cones daily (treatments H50 and H100, respectively).

Serum urea concentration is one of the indicators of protein status in the animal. Serum urea concentration depends on the energy status, as its concentration in blood can increase when energy intake is restricted despite diet low crude protein contents. Low blood urea could be also a consequence of the more efficient conversion of nitrogen to amino acids, leading to the faster growth rate in young animals (Otto et al. 2000). However, main reasons for low serum urea contents are the low quantity of consumed crude protein and its low degradability. The required levels of crude protein in diets for growing Cika bulls were calculated in accordance with DLG (1997) standards, where only the amount of protein in the diet is considered. However, considering INRA standards (Jarrige 1989) for growing bulls it became evident that the low serum urea determined in the present experiment could be a consequence of the inadequate amount of protein in the diet and that INRA recommendations seem to be more appropriate. Inconsistencies in serum urea levels were also found when monesin, which is chemically similar to the hop cone β-acids, was supplemented to cattle. In the experiment of Yang et al. (2010) monesin increased the urea serum concentration, while it had no effect in the experiment of Mwenya et al. (2005).

High activities of liver enzymes AST, ALT and GGT are most often related to acute and chronic liver diseases and give the possibility to estimate the degree of cell damages (Jazbec 1990, Đoković et al. 2010). Increased AST activity in the serum is a sensitive marker of liver damage, even if the damage is of a subclinical nature, while increased GGT activity is a sign of hepatobiliary system diseases. By determining their activities the probable negative effects of hop cones on soft tissues could be determined. Activities of all three enzymes were within physiological levels from 35 to 85 U/L for AST, up to 27 U/L for GGT and up to 50 U/L for ALT during the entire duration of the experiment (Jazbec 1990, Table 5). Supplementation of diets with hop cones (H50 and H100) did not change the serum activities of GGT, while AST levels tended to be lower after 30 ($P = 0.065$) and 60 days ($P = 0.097$) of the experiment (Table 5). On contrary, ALT activity was lower ($P = 0.025$) only when bulls consumed hop cones for 60 days. Duff et al. (1994) and Demarco et al. (2014) observed that monensin, which is chemically similar to hop β-acids, did not affect AST and GGT activities in beef cattle. However, it could be possible that when the amounts of hop cones and/or their constituents are consumed in excess, they could have detrimental effects on performance and liver enzymes similar to that observed for monensin on feedlot cattle and dairy cows (Geor and Robinson 1985, Gonzales et al. 2005).

CONCLUSIONS

The inclusion of hop cones in a maize and grass silage-based diets for growing bulls at levels up to 11 g of hop cone DM/kg diet DM (534 mg of β-acids/kg diet DM) did not have any effect on feed utilization and performance of the animal. Supplementation of diets with

hop cones increased plasma glucose and decreased serum NEFA concentrations indicating that the use of hop cones provoked the changes in energy metabolism in ruminants. Furthermore, the supplementation of bull diets with 50 g or 100 g of hop cones daily decreased blood concentration of ALT after 60 days of treatment. Further research is needed to determine if changes in blood analyte concentrations are a long-term effect of hop cone supplementation of beef cattle diets.

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Streszczenie: *Wykorzystanie szyszek chmielowych w żywieniu bydła mięsnego*. Celem doświadczenia było określenie wpływu zastosowanej suplementacji w postaci szyszek chmielu na kształtowanie się przyrostów, jak również poziomu wybranych parametrów krwi, w tym enzymów wątrobowych. W doświadczeniu wykorzystano 24 buhaje słoweńskiej autochtonicznej rasy Cika (masa ciała 373 kg, wiek 329 dni), które losowo przydzielono do sześciu kojców (4 szt./kojec). Buhaje z grupy kontrolnej otrzymywały TMR, który następnie był suplementowany 50 g szyszek chmielowych na zwierzę (H50; 6 g suchej masy chmielowej/kg) lub 100 g szyszek chmielowych na zwierzę (H100; 11 g suchej masy chmielowej/kg). Buhaje były ważone trzykrotnie: na początku eksperymentu, a następnie ponownie po 30. i 60. dniach doświadczenia. Na podstawie uzyskanych pomiarów obliczono średnie przyrosty dzienne (ADG), pobranie suchej masy (DMI) i wskaźnik wykorzystania paszy (F : G). Próbki krwi pobierane były trzykrotnie, analogicznie jak w przypadku pomiarów masy ciała. Oznaczono koncentrację glukozy w osoczu oraz poziom niezestryfikowanych kwasów tłuszczowych (NEFA), kwasu β-hydroksymasłowego (BHBA), mocznika, aminotransferazy alaninowej (ALT), aminotransferazy asparaginianowej (AST) i γ-transferazy glutamylowej (GGT) w surowicy. Badanie wykazały, że suplementacja szyszkami chmielowymi nie wpłynęła na DMI, ADG oraz F : G. Ponadto nie wykazano zależności między zastosowaną suplementacją a poziomem BHBA, mocznikiem i GGT w osoczu. Jedynie w przypadku ALT po 60 dniach suplementacji wykazano niższy poziom (P = 0,025) w grupie doświadczalnej w porównaniu z grupą kontrolną. W przypadku dawek H50 i H100 wykazano podwyższenie stężenia glukozy we krwi ($P < 0,05$) po 30 i 60 dniach suplementacji. Odmienna zależność została wykazana w przypadku NEFA, w grupie H100 wykazano obniżenie koncentracji ($P = 0,022$) po 60 dniach doświadczenia. Suplementacja dawki podstawowej szyszkami chmielu wpływa w istotny sposób na metabolizm energetyczny przeżuwaczy.

Słowa kluczowe: bydło, szyszki chmielowe, przyrosty, metabolity krwi, enzymy wątrobowe

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Epibionts of ornamental freshwater shrimps bred in Taiwan

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Abstract: *Epibionts of ornamental freshwater shrimps bred in Taiwan.* One of the major problems in breeding *Neocaridina davidi* in Taiwanese aquaculture ponds are epibionts found on the body of ornamental shrimp. These organisms affect shrimp wellbeing by causing distress which leads directly to shrimp weakness, loss of colour and even casualties. They can also be observed in imported shrimps which put in danger individuals bred in Europe, mostly characterised by high level of inbreeding and sensitivity to pathogens. Microscopic analyses indicated presence of six freshwater shrimp epibionts. Some of them showing parasitic lifestyle (*Cladogonium ogishimae*, *Saprolegnia* sp., *Scutariella japonica*), others (phyla Ciliophora and Rotifera) may indicate level of organic matter in water. To allow an effective treatment and control of the spread of parasites, all of their preferred locations on shrimp body observer in this study should be checked and become a vital part of diagnostic methods. Researches on ornamental freshwater shrimps' epibionts are important to achieve success in shrimp breeding as well as to effectively monitor epibiont populations globally, especially that in some regions they may become potentially invasive organisms to the native crustaceans.

Key words: epibionts, parasites, aquarium, shrimps, aquaculture

INTRODUCTION

Freshwater shrimps belong to the most common crustaceans kept in aquarium. Their small sizes, intensive colouration and large diversity of possible patterns make them valuable to breeders and became reason of rising quantity of colour varieties available in global aquarium trade (Hung et al. 1993, Jayachandran and Raji 2005, Heerbrandt and Lin 2006, Barbier 2010).

Colour intensity, the most important feature of these pet animals, is affected by multiple factors including wellbeing that depends on shrimp health and environment conditions. Shrimp quality is verified by professional judges during valued international contests, where colour intensity and wellbeing of the pets are especially high-priced (Maciaszek 2016).

Growing market of aquarium shrimp causes establishment of new aquaculture farms adapted to producing mass quantities of low-cost crustaceans. Shallow, concrete-based ponds filled with rainwater are also much cheaper in keeping than aquarium farms. In case of Taiwanese breeders, aquaculture ponds usually do not have any additional filtration as they are exposed to wind blows which results in high rates of organic matter dispersed in water. Each year one pond may produce thousands of crustaceans which then are exported mainly to Europe to find their final destination in European aquariums (Maciaszek 2016).

Unfortunately, aquaculture ponds create suitable conditions for development of other, potentially undesirable organisms called epibionts. These organisms may affect shrimp wellbeing by causing distress which leads directly to shrimp weakness, loss of colour and even casualties. They can be also observed in imported shrimp which can put in danger individuals bred in Europe, characterised by high level of inbreeding and sensitivity to pathogens. Lack of effective treatment due to the relatively small knowledge of parasites found in freshwater shrimp farms may result in escalating of the problem. Except observations made by Patoka et. al (2015) current available literature on shrimps' parasites is almost completely restricted to marine species (Johnson 1989, Lightner and Redman 1998, Chakraborti and Bandyapadhyay 2011). Therefore, the aim of this study was to estimate seasonal changes in population of common freshwater shrimp epibionts as well as to identify species and their preferred locations on shrimp body.

MATERIAL AND METHODS

Present study was conducted in two seasons: spring (May) and autumn (October) during the period of 2012–2015. Live *Neocaridina davidi* (Bouvier 1904) adults bred in aquaculture ponds (Crimson Taiwan, New Taipei City, Taiwan) were collected in trials of 600 shrimps per season and transported in groups of 200 individuals to minimize possible effect on water parameters changes and casualties in result. Shrimp were transported inside styro boxes in 10-litre plastic aquarium fish bags half-filled with pond water and half with oxygen under pressure. Day-long (24 h) airplane imports in constant temperature of 20°C were performed once per season. Imported shrimp were taken under observation in Kumak Shrimp – aquarium shrimp farm (Konstancin-Jeziorna, Poland).

After initial acclimatization in 10-litre quarantine tanks equipped with air pump sponge filtration only (each), shrimps were checked for epibionts presence in four determined locations preferred by epibionts:

- location A – rostrum, antennas and antennules;
- location B – gills;
- location C – chelipeds and pereiopods;
- location D – pleopods, uropods and telson.

Studies were conducted using camera Canon 600D (Canon Inc., Japan) to allow an accurate examination a plastic Petri dish was used to keep shrimp inside the aquarium (trapping shrimp inside the dish and pressing against side wall until it calmed down). Additional observations and species identification were made with binocular Nikon SMZ1000 (Nikon Corporation, Japan) in the Department of Ichthyobiology, Fisheries and Biotechnology in Aquaculture of Warsaw University of Life Sciences – SGGW (Warsaw, Poland). Epibiont identification was made using available literature and identification keys (Matjašič 1980, Shiel 1995, Foissner and Berger 1996, Niwa and Ohtaka 2006, Diéguez-Uribeondo et al. 2007).

Results were statistically summarized with PQStat ver. 1.6.4.121. Epibionts quantity in different locations was analyzed with χ^2 test of independence. Comparisons between epibionts were

examined with multiple χ^2 test with use of Benferroni correction. Equivalence of epibionts' distribution in different locations was analyzed with χ^2 test for compatibility. Differences between quantities of epibionts and seasons were examined using two-way analysis of variance (ANOVA) for repeated measures. Dependent factor (repeated measure) was season (spring and summer) and independent factor (grouping factor) was epibiont. Post-hoc analyses in epibionts were examined with Tukey's test while differences between seasons were analyzed using contrast method. Value of *P* level used as statistically significant was $P < 0.05$ when statistically highly significant value was $P < 0.01$.

RESULTS AND DISCUSSION

Microscopic analyses indicated presence of six freshwater shrimps' epibionts: *Saprolegnia* sp. (Nees von Esenbeck 1823), *Scutariella japonica* (Matjašič 1980), *Vorticella* sp. (van Leeuvenhoek 1702), *Stentor* sp. (Ehrenberg 1831), representatives of phylum Rotifera (Cuvier 1817) as well as *Cladogonium ogishimae* (Hirose and Akiyama 1971, Matsuyama-Serisawa et al. 2014, Imai et al. 2017) that was not reported outside of Japan before. Location preferences of each epibiont species in representative group of 1,200 shrimps (600 per season) imported in 2013 were presented in Table 1. The biggest diversity of identified epibionts was

TABLE 1. Selected epibionts observed in examined parts of shrimp's body

Location		*Cladogonium ogishimae*	Rotifera	*Saprolegnia* sp.	*Scutariella japonica*	*Stentor* sp.	*Vorticella* sp.
A	*n*	18	799	19	877	708	319
	%	2.4	46.4	6.8	39.4	51.4	14.9
B	*n*	7	398	24	988	4	12
	%	0.9	23.1	8.6	44.4	0.3	0.6
C	*n*	358	310	106	176	303	905
	%	47.9	18.0	37.9	7.9	22.0	42.2
D	*n*	364	216	131	183	362	907
	%	48.7	12.5	46.8	8.2	26.3	42.3
χ^2 test of independence		$\chi^2 = 40.25$, $df = 15$, $P < 0.0001$					
Cladogonium ogishimae			< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Rotifera		< 0.0001		< 0.0001	< 0.0001	< 0.0001	< 0.0001
Saprolegnia sp.		< 0.0001	< 0.0001		< 0.0001	< 0.0001	< 0.0001
Scutariella japonica		< 0.0001	< 0.0001	< 0.0001		< 0.0001	< 0.0001
Stentor sp.		< 0.0001	< 0.0001	< 0.0001	< 0.0001		< 0.0001
Vorticella sp.		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	
χ^2 test for compatibility		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

n – quantity of shrimps settled by 1 epibiont observed in group of 1,200 individuals; % – percentage quantity of 1 epibiont observed in examined parts of shrimp's body.

observed in locations C and D (Table 1). These locations were occupied mostly by: *Cladogonium ogishimae* (47.9 and 48.7%, respectively), *Scutariella japonica* (37.9 and 46.8%, respectively) and *Vorticella* sp. (42.2 and 42.3%, respectively). Location A was taken in vast majority by rotifers (46.4%). Comparing to other examined epibionts, location B was particularly preferred by *Scutariella japonica* (44.4%) and rotifers (23.1%). Observations affirmed possible parasitic lifestyle of *Saprolegnia* sp. (Fig. 1) and *Cladogonium ogishimae* (Matsuyama-Serisawa et al. 2014) that were observed especially on the pleopods responsible for incubating host's eggs. Epibionts belonging to phylum Ciliophora were recorded in all examined locations except gills. This confirms ciliates usage of shrimp movable body parts only as a way of getting plankton which is their source of food (Psenner 1995). The ciliates also used additional surface created by *Cladogonium ogishimae* structures (Fig. 2). Similar preferences were observed for rotifers (Fig. 3) and scutariellids (Fig. 4) which used mainly location A as an opportunity for catching microorganisms. Parasitic behaviour of *Scutariella japonica* was clearly visible especially on gills where it layed eggs causing destruction to the host body structures (Fah and Christianus 2013, Klotz et al. 2013).

Statistically highly significant ($\chi^2 = 40.25$, $df = 15$, $P < 0.0001$) difference was examined in distribution depended on epibiont. Multiple comparisons indicated highly significant differences ($P < 0.0001$) between each pair of examined epibionts. The same result ($P < 0.0001$) was obtained using

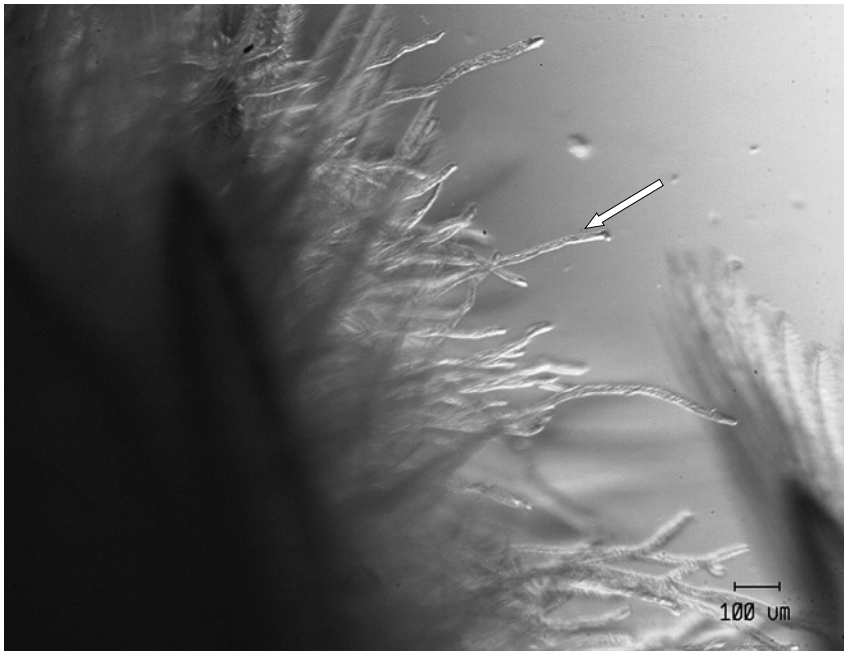

FIGURE 1. *Saprolegnia* sp. (white arrow) detected on pleopods of *Neocaridina davidi*

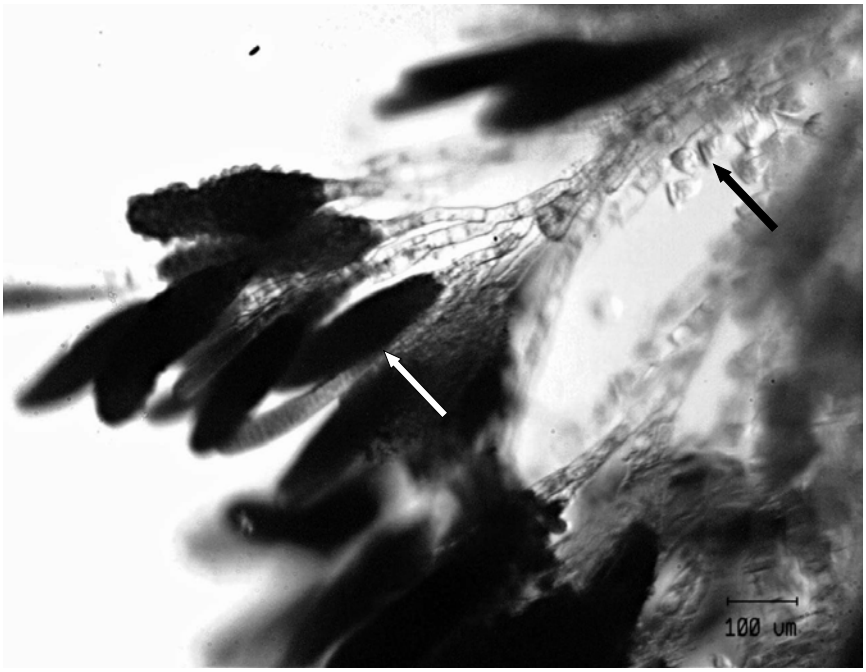

FIGURE 2. *Cladogonium ogishimae* observed on *Neocaridina davidi* pleopods. Structures of this species may be used by other organisms such us *Vorticella* sp. (black arrow)

FIGURE 3. Rotifera representatives (black arrow) observed in rostrum region

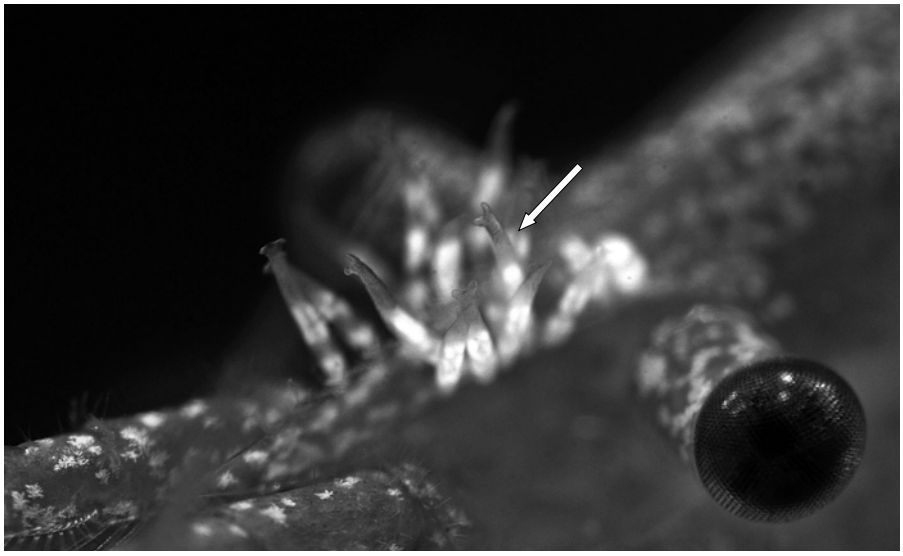

FIGURE 4. *Scutariella japonica* adults (white arrow) saddle in *Neocaridina davidi* rostrum

Bonferroni correction, what explains specific distribution of preferred location for each examined epibiont. Hypothesis involving equivalence of epibiont distribution was verified and in each case statistically highly significant ($P < 0.0001$) incompatibility with normal distribution was obtained. Epibiont location was not random, all examined epibionts preferred some locations.

Results obtained in four-year period indicates decreasing presence of most shrimps' epibionts (*Cladogonium ogishimae*, Rotifera, *Saprolegnia* sp., *Stentor* sp., *Vorticella* sp.) bred in aquaculture ponds what is clearly visible in changes of trial average (Table 2). It could be the effect of increasing, but still insufficient knowledge of shrimp parasites as well as possible improvement of ponds filtration quality. Although records achieved for individuals infected by *Scutariella japonica* (min. 69.5%) (Table 2) compared to Niwa and Ohtaka (2006) results (58%) indicates increasing quantities of this species in the aquarium trade. Observation shows *Scutariella japonica* can use shrimp as its own mobile base (for example by attaching itself to the rostrum) to obtain organic material from the water column as well as living its parasitic lifestyle using shrimp as its host (for example attaching to the gills). Thanks to this ability to adapt *Scutariella japonica* can be dangerous to the aquarium shrimp keeping as it can easily infect not only weakened individuals but also a healthy ones.

Statistically significant differences [$F(5;18) = 3.85$, $P = 0.0151$] between examined epibionts were observed. Post-hoc Tukey's test figured out that significant differences occurred between *Cladogonium ogishimae* and *Scutariella japonica*. Statistically highly significant [$F(1; 15) = 17.03$, $P = 0.0006$] were found between seasons. Epibionts quantity found on shrimps was bigger

TABLE 2. Selected epibionts observed in examined parts of shrimp's body

Years of observations	Scientific name												Trial average
	Cladogonium ogishimae		Rotifera		*Saprolegnia* sp.		*Scutariella japonica*		*Stentor* sp.		*Vorticella* sp.		
	spring	autumn	spring	autumn	spring	autumn	spring	autumn	spring	autumn	spring	autumn	
2012	125.3	57.0	193.0	114.0	189.0	112.3	143.0	139.0	143.0	138.7	200.0	192.3	145.55
2013	79.3	42.0	160.7	105.7	35.7	8.0	175.0	154.3	175.7	196.7	60.3	105.7	108.26
2014	25.0	11.7	159.0	119.0	17.3	9.0	185.3	155.0	19.0	11.7	145.0	132.0	82.42
2015	10.0	2.0	155.3	132.0	8.0	4.7	200.0	182.3	14.3	3.3	157.0	109.7	81.55
$\bar{x}$	59.90	28.18	167.00	117.68	62.50	33.50	175.83	157.65	88.00	87.60	140.58	134.93	
SD	52.79	25.68	17.48	11.01	85.12	52.57	24.17	18.02	83.48	95.54	58.49	39.96	
Median	52.15	26.85	159.85	116.50	26.50	8.50	180.15	154.65	81.00	75.20	151.00	120.85	
ANOVA	scientific name $F(5;18) = 3.85$, $P = 0.0151$, seasons $F(1; 15) = 17.03$, $P = 0.0006$, interaction $F(5; 18) = 1.86$, $P = 0.1524$												
Contrast	$P = 0.0281$		$P = 0.0016$		$P = 0.0425$		$P = 0.1880$		$P = 0.9763$		$P = 0.6756$		

in spring than autumn. Higher temperatures during springtime cause less water oxygenation and speeds up shrimps metabolism which is the main reason for increasing rates of toxic nitrogenous compounds which are responsible for shrimps weakening (Jiang et al. 2000, Figueroa-Lucero et al. 2012). Interaction between epibionts and season was not statistically significant [$F(5; 18) = 1.86$, $P = 0.1524$]. Differences between seasons are comparable to all examined epibionts. Contrast analysis indicated significant difference between seasons in *Cladogonium ogishimae* ($P = 0.0281$) and *Saprolegnia* sp. ($P = 0.0425$) as well as highly significant in Rotifera ($P = 0.0016$). No significant differences were found in *Scutariella japonica* ($P = 0.1880$), *Stentor* sp. ($P = 0.9763$) and *Vorticella* sp. ($P = 0.6756$).

CONCLUSIONS

Aquaculture ponds create favorable conditions for *Neocaridina davidi* shrimps' epibionts development, especially ectoparasites. Decreasing quantity of most epibionts in the four-year period is most probably a result of breeders increasing awareness of other organisms present in ponds and related to them possible shrimp casualties. Some of those organisms have parasitic lifestyle (*Cladogonium ogishimae*, *Saprolegnia* sp., *Scutariella japonica*), others (phyla Ciliophora and Rotifera) may be useful as indicators of level of organic matter in water. To allow an effective treatment and control of the spread of parasite, all of their preferred locations on shrimp body observed in this study should be checked and become a vital part of diagnostic methods and should be assisted by assuring of right transport conditions, post import acclimatization, optimal filtration and water parameters.

This work contains initial studies on shrimps' epibionts. Further researches on ornamental freshwater shrimps' epibionts are important to achieve success in shrimp breeding as well as to effectively monitor epibiont populations globally, especially that in some regions they may become potentially invasive organisms to the native crustaceans.

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Streszczenie: *Epibionty krewetek akwariowych hodowanych na Tajwanie*. Epibionty są jednymi z głównych problemów w hodowli krewetek *Neocaridina davidi* w stawach hodowlanych na Tajwanie. Ich obecność wpływa negatywnie na dobrostan krewetek poprzez wywoływanie stresu, osłabienia, upadków oraz utraty ubarwienia.

Epbionty stwierdza się również na krewetkach pochodzących z importu, co jest szczególnie niebezpieczne dla osobników hodowanych w Europie charakteryzujących się wysokim poziomem chowu wsobnego oraz słabą odpornością na patogeny. Analiza mikroskopowa wykazała obecność sześciu gatunków symbiontów krewetek słodkowodnych. Niektóre z nich prowadzą pasożytniczy tryb życia (*Cladogonium ogishimae*, *Saprolegnia* sp., *Scutariella* sp.), inne (typ Ciliophora oraz typ Rotifera) mogą być wykorzystane jako wskaźniki ilości materii organicznej w wodzie. Wykazane w obserwacjach miejsca ciała krewetek preferowane przez pasożyty powinny stanowić nieodłączną część metod ich diagnostyki pozwalającej na efektywne leczenie. Badania na epibiontach krewetek akwariowych są szczególnie istotne dla sukcesywnej hodowli tych skorupiaków, a także dla prowadzenia efektywnego monitoringu populacji epibiontów, które w niektórych regionach świata mogą stać się gatunkami potencjalnie inwazyjnymi dla naturalnie występujących skorupiaków.

Słowa kluczowe: epibionty, pasożyty, akwarium, krewetki, akwakultura

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Aggressive behaviors in domestic cats (*Felis catus*)

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Abstract: *Aggressive behaviors in domestic cats (*Felis catus*)*. Behavioral issues of cats include: furniture scratching, aggression, anxiety, over-stimulation, exaggerated vocalizations and excreting outside the litter box. Among these, aggression – both passive and active – is the most commonly encountered problem. Aggressive behavior is a complex phenomenon, dependent on both genetic and environmental factors. Among the factors leading to agonistic behavior two categories are distinguished: psychobiological factors (which include biochemical and physiological processes, disposition and mood, emotional reactions, motor actions and vegetative reactions) and environmental factors (such as incorrect socialization, unfriendly surroundings or irresponsible animal owners). The most widespread type of aggression in cats reared in groups is linked to the desire to gain and maintain their territory. Another type of agonistic behavior is one born out of fear, exhibited by cats in a crisis situation once there is no escape route, and the animal is certain it has to fight to survive. This behavior differs from others in that aggression is here the last resort and not the first response to a disturbing situation. Another source of aggression may be anxiety caused by a sudden change in the environment, the presence of people and other animals. An interesting type of aggression linked to the natural hunting sequence of cats is aggression during play, which especially affects cats during adolescence. While working with an aggressive animal, a caregiver has a range of different mitigating and calming measures at hand, but their proper selection requires experience and cooperation with a veterinarian and a behaviorist).

Key words: domestic cats, aggression, behavior

INTRODUCTION

Aggressive behavior is a complex phenomenon, dependent on both genetic and environmental factors. Among the factors conducive to aggression two main categories are distinguished. The first group are psychobiological factors which include biochemical and physiological processes, disposition and mood, emotional reactions, motor actions and vegetative reactions. The second category are environmental factors, such as incorrect socialization, unfriendly surroundings or irresponsible animal owners (Petrynka et al. 2004). All behaviors that result in any other individual being forced to maintain their distance in psychological, physical or social sense can be considered aggressive (Eibl-Eibesfeld 1990). O'Hearem (2009) defines aggression as "attacks, attempted attacks or threats to attack". This definition emphasizes that aggression is the behavior of one living creature towards another, and that this behavior does not necessarily have to involve the desire to do harm.

Research on the evolution of mammals shows that cats – despite being the most popular domestic animals in the world (Turner and Bateson 2000) – still remain wild in nature and full of secrets. Domestic cats, despite thousands of

years of living together with humans, continue to be great hunters, have not become dependent on food supplied by people and are famous for their love of freedom.

The results of multiple studies conducted show that cats, in contrast to dogs, have been only partially domesticated (Overall et al. 2005, Warren 2014). The DNA comparison of domesticated and wild cats (*Felis silvestris lybica*) shows that the only differences exist in the genes responsible for coat color, submission instinct and attachment to humans (Warren 2014). Slight changes were noted also in some anatomic features – e.g. decrease in size of both the pituitary gland and the adrenal gland (Fogle 2008). Incomplete domestication of cats, their independence and self-sufficiency historically gave rise to suspicion, lack of understanding and sometimes even hatred among the human population (Turner and Bateson 2000). Each feline kept at home needs an individual approach, empathy and for their needs to be met by the caregiver. Lack of opportunity to express natural instinct and behaviors can lead to anxiety and aggravation of behavioral problems (Da Graca Pereira et al. 2014).

Behavioral issues of cats include: furniture scratching, aggression, anxiety, over-stimulation, exaggerated vocalizations and excretion outside the litter box (Jongman 2007). Among these, aggression – both passive and active – is the most commonly encountered problem (Strickler and Shull 2014). The objectives of this study were to identify different type of aggression, and to obtain descriptive information on methods used to prevent the occurrence of aggression.

TYPES OF AGGRESSION

Instances of active aggression are difficult to overlook. Aggressive cats hiss, spit, growl, and ultimately attack. Openly agonistic behavior is very often planned – the aggressor is capable of waiting patiently and attacking the other cat when the animal is least prepared to defend itself. Unfortunately, passive forms of agonistic behavior are often dismissed and ignored. These behaviors consist in one animal observing the other cat with a look that prevents the latter from approaching the food bowl, litter box or its bed. A cat that is a victim of passive aggression will increasingly withdraw from active life. Subjected to continuous state of tension and stress, the feline may eventually fall into apathy. The most common diseases caused by stress are diseases of the urinary tract and diabetes. Furthermore, such a frustrated cat may begin to manifest different stereotypes or compulsive behaviors: excessive licking of fur, often even to topical baldness (McCobb et al. 2005).

Threats and aggression can be either offensive or defensive. Offensive aggression occurs when a subject is feeling the need to be assertive in a certain situation – e.g. when facing another cat or guarding an object. Cat who is feeling assertive will likely have ears facing forward, fur standing and tail stiffing towards the ground. Animal being offensive will stare directly at its target with constricted pupils, possibly moving it, most likely growling or yowling. Defensive aggression occurs when an animal is attempting to protect itself from an attack it believes it cannot escape. Defensive postures include crouching with the legs pulled

in under the body, laying the ears back, tucking the tail, and possibly rolling slightly to the side (Case 2009).

Levine (2005) points out that the domestic cat (*Felis catus*) belongs to the most aggressive species in the world. Aggressive behaviors increase the chance of survival of a given individual, and ensure safe upbringing of the young. All felines are, through their genetic makeup conditioned to aggressive behaviors. This characteristic sometimes does not find sufficient understanding among owners, and the cats end up on the streets, are relinquished to shelters, temporary homes and in the worst case are subjected to euthanasia. However, with correct identification of the source of aggression by a good behaviorist/animal psychologist the undesirable behaviors of the felines can in fact be mastered, and specific preventive actions can be taken for the future (Crowell-Davis et al. 2011, Moesta et al. 2011).

The most widespread type of aggression in cats kept in groups is aggression linked to their desire to gain and maintain territory (Houpt 1998). Such agonistic behavior may be more pronounced if another animal is introduced into the home (Hart and Hart 2014). It is a priority for every cat to have their own territory. The territory of a free-roaming cat consists of several zones (or fields) that overlap each other. One section of the territory is known as the cat's home range – this is where the cat hunts for food and explores. The cat's home range (sometimes called family range) can be shared with other individuals, although its central part belongs only to the single animal. The other parts of the territory are in fact "no man's land", i.e. their area is occupied by other species of animals, including dogs (Ellis 2009). In cats kept in homes, following this division of the territory is not so simple, thus cats have to settle for a substitute that is effectively confined by the home's four walls. There is a linear correlation between the number of cats living in a given space and the frequency of instances of aggressive behavior (Hart and Hart 2014).

A special type of **territorial aggression** is aggression directed at an individual that has been away from home for a long time. It is a set of agonistic behaviors when a cat is facing a familiar member of the social group, and sometimes even a well-liked one, but for some reason that animal is "unrecognizable". Such practice is often observed between cats after one returns from a veterinarian, from an exhibition or after being used as a stud cat (Kmecová et al. 2003). Behaviors associated with territorial aggression begin with mutual evaluation of opponents, exchange of scents, establishing eye contact and assumption of appropriate body posture. If none of the individuals retreats after such show, this leads to aggression and a fight occurs. Cats generally avoid fighting. Instead, they will rely on vocal and postural threats to challenge foes and appeasement to back down from stronger opponents. This safety mechanism is common among predatory species. Not only because it is dangerous to fight, but it is also time and energy consuming (Crowell-Davis et al. 2003).

When fights between cats are frequent, and are associated with serious bodily injury, the agonistic cats should be isolated from one another. In such a situation, an artificial barrier should be

created that would separate the cats, or the animals should be kept in separate enclosures or rooms equipped with a litter box, access to water and food (Bradshaw 2014). Similarly as in the case of introducing a new individual to a feline social group, caretakers should allow the cats to get acquainted once again and let them build a new model for their relationship. It is important to ensure the cats can still indirectly exchange scents during the period of isolation (Levine et al. 2005). It is recommended to release the felines alternately for a longer time. After some time, one should start letting the animals out at the same time, preferably during the feeding time. This will allow them to associate the presence of the other individual with a positive signal. However, it should be remembered that the distance between the cats at the first meetings after being separated should be large enough that they cannot communicate with each other (Moesta et al. 2011). In case of any symptoms of aggression emerging once more, re-isolation is necessary (Bradshaw 2014).

In the case of just incidental, short conflicts, it is better to abstain from intervention. Cats living in one place must establish a hierarchy for themselves. It is also worth remembering that social groups formed by cats are based around dynamically developing relationships, which means that small clashes or conflicts within them are normal (Biegańska-Hendryk 2017). It may happen that aggression between two animals intensifies at a specific time of the day (Bradshaw 2014), or takes place only in a very specific location. In such situations, the best solution is to separate the cats in question during this most turbulent time of day, and not to allow them into places that trigger unwanted behaviors.

In some cases, a cat with highly developed territorial tendencies will attack people who came to visit the owner. If a cat exhibits such behavior, the only method to deal with the issue is to isolate the cat whenever guests are present, and to not allow it to behave aggressively. It is recommended that the cat be slowly accustomed to unknown people visiting the house. The animal should be on a leash or in a transporter during such initial visits. The guest can also offer the animal a treat to consolidate a positive association with the visit in the feline mind (Bradshaw 2014). Subtype of aggressive behavior connected with territoriality is **maternal aggression**. Aggressive behaviour directed at other animals is common and expected from female cat because she has to protect their young at all times.

Another type of aggression, often confused with territorial aggression, is **aggression stemming from fear**. This type of behavior is exhibited by cats in a crisis situation once there is no escape route, and the animal is certain it has to fight to survive. This behavior differs from other types of aggression listed in that the combative behavior is here the last resort and not the first response to a disturbing situation. The source of aggression may be anxiety caused by some sudden change in the environment (Levine et al. 2005), the presence of people (Crowell-Davis et al. 2011) and other animals. Recognition of this type of aggression is possible due to close observation of the animal's body language. A frightened cat hisses and spits, sits in

a crouched position, puffs up its fur and lays down its ears flat against the head (Mertens 1991, Schwartz 2005). Working with such a cat, one should never discipline it for anxiety behavior. During an attack of fear, it is recommended to stay at a safe distance from the feline in question. Ignoring the animal and leaving it alone will allow it to quickly calm down and make a less panicked, more realistic threat assessment (Schwartz 2005). It is worth trying to get the animal used to the stimulus causing fear. Such desensitization occurs when a cat is exposed to a fear-inciting stimulus, but at such intensity that the reaction does not occur (Crowell-Davis et al. 1997). When the animal is calm and relaxed when exposed to a given intensity of the aversive stimulus, its intensity can be gradually increased – step by step, with care not to introduce too strong a stimulus that would cause fear and make the animal retreat. With this approach, both the processes of habituation and instrumental (operant) extinguishing – jointly responsible for reducing the intensity of the reaction – run in parallel (O'Hearem 2009). If a cat is very stressed, after consultation with a veterinarian it is recommended to use anxiolytic and sedative drugs (Hart and Hart 2014).

The next category of aggressive behavior encountered by cat owners is **redirected aggression**. It often occurs when a cat cannot turn its anger, excitement or fear at the real source of its arousal, for example a strange cat passing by the window outside. Because the "real" enemy is unobtainable, the cat will redirect the attack at another cat that is within reach. Cats unload their frustration in a similar manner – a situation in which cat A, scolded by cat B, turns instead on cat C, an individual that is submissive and withdrawn (the lowest in the hierarchy) is quite common (Bradshaw 2014). Importantly, the object of such redirected aggression caused by frustration may be another cat, another animal (e.g. a dog) or even a human.

Yet another type of aggression is **play-associated aggression**, often exhibiting elements that normally form part of the cat's hunting sequence. It may happen that cats deliberately attack human feet and hands like they would a mouse. The most agonistic, predatory play is observed in young cats between the onset of sexual maturation and the age of two years, in the so-called period of psychological adolescence (Curtis et al. 2003). Such behavior may be influenced by various factors, including too early separation from the dam and siblings. Young kittens should stay together with their dam in a family unit until about 12 weeks of age (Senczi et al. 2016). Living in a group, kittens are able to acquire skills useful in the future during hunting and social interactions (Crowell-Davis 2007). As they play together under the watchful eye of their mother, young cats learn how to use teeth and claws so as not to harm their siblings (Curtis et al. 2003). Another reason for aggressive play may be inappropriate behavior of the caregiver. Sudden, violent movements are not advisable, as they can provoke a cat to attack (Mertens 1991). It should be remembered that poorly designed session of play that does not end with completion of the hunting sequence can increase the cat's frustration, and thus lead to aggressive behavior. It is best to use appropriate toys whey playing with

cats, ones attached to strings or poles, known as rods, to keep the animal away from the owner's hands (Crowell-Davis 2007). If the cat has already learned to (play) hunt the caretakers, their task will be to create unpleasant associations with the act of aggression using a loud sound, a spray-bottle or a diffuser. In difficult cases, consultation with a behaviorist and a veterinarian is recommended to establish a treatment program for the animal (Amat et al. 2009).

Aggressive behaviors may also occur as a reaction to disease or pain (Camps et al. 2015). If a cat suffers from pain, even the normal, casual touch of the owner might be very unpleasant – and the animal will instinctively respond with aggression. If the diagnosis of the underlying cause is quick and the disease is curable, aggression disappears together with the disease symptoms. When diagnosing aggressive behaviors, it should be remembered that health issues may also exacerbate aggression the primary cause of which is not related to health. Accurate veterinary examination is an important element in developing the treatment optimal for a given animal.

SUMMARY

Aggressive behaviors of felines are a complex issue, as they may be caused be a number of factors occurring simultaneously and overlapping. An important role in preventing the development of a habit of aggression in cats is played by cat–human communication and mutual understanding. It is advisable to use specific, unchanging signals and to use stimuli that engage all senses of the animal (Hart and Hart 2014). Importantly, all caregivers should acquire and broaden their knowledge about function and significance of different feline sensory organs, as well as body language and behavior of the domestic cat. With this foundation, they should be able to understand the needs of a given animal and create a stronger relationship (Salman et al. 2000). When working with an aggressive animal, a caregiver has a range of different mitigating and calming measures, but their proper selection requires experience and cooperation with a veterinarian and a behaviorist (Biegańska-Hendryk 2017). Lack of sufficient knowledge about the causes of aggression in animals may lead to the problem getting worse.

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Streszczenie: *Zachowania agresywne u kota domowego (*Felis catus*)*. Wśród problemów behawioralnych kotów wymienia się: drapanie mebli, agresję, stany lękowe, nadmierne pobudzenie, przesadną wokalizację i wydalanie poza kuwetą, z których to właśnie agresja, zarówno bierna, jak i czynna są spotykane najczęściej. Zachowania agresywne są zjawiskiem złożonym, uzależnionym zarówno od czynników genetycznych, jak i środowiskowych. Wśród czynników prowadzących do agresji można wyróżnić czynniki psychobiologiczne, do których zaliczyć można: przebieg procesów fizjologicznych, usposobienie i nastrój, reakcje emocjonalne, akty motoryczne oraz reakcje wegetatywne, oraz czynniki środowiskowe, takie jak: błędna socjalizacja, nieprzyjazne otoczenie czy nieodpowiedzialni właściciele zwierząt. Najczęstszym typem agresji u kotów utrzymywanych w grupach, jest chęć zdobycia i utrzymania swojego terytorium. Innym typem agresji, jest agresja ze strachu którą przejawia kot „przyparty do muru", gdy nie widzi już możliwości ucieczki, i w swoim mniemaniu walczy o życie. Ten sposób zachowania różni się od innych tym, że jest on ostatnim, a nie pierwszym elementem odpowiedzi na niepokojącą sytuację. Źródłem agresji może być lęk spowodowany nagłą zmianą w otoczeniu, obecnością ludzi i innych zwierząt. Ciekawym typem agresji, powiązanej z łańcuchem łowieckim, jest agresja podczas zabawy, która dotyczy zwłaszcza kotów w okresie dorastania. W przypadku pracy ze zwierzęciem agresywnym opiekun ma do dyspozycji wiele różnych środków łagodzących i uspakajających, jednak ich prawidłowy dobór wymaga doświadczenia i współpracy z lekarzem weterynarii i behawiorystą.

Słowa kluczowe: kot domowy, agresja, behawior

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Characteristics of alpaca wool from farmed animals located on different continents

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Abstract: *Characteristics of alpaca wool from farmed animals located on different continents***.** Interest in breeding alpacas around the world is related to the acquisition of valuable fibre from these animals. The characteristics of alpaca wool are influenced by both genetic and environmental factors. The aim of the study was to compare chosen traits of Huacaya alpaca wool from three farms located in Australia, Africa and Europe. The wool samples were collected from 30 alpacas, 10 from each farm the assessed wool characteristics (fibre diameter, level of medullation and staple length) was determined. The following fibre types have been identified: with continuous medulla, with partial medulla and no-medullated. Similar results were obtained with respect to the fibre diameter, staple length, the level of medullation and even distribution of the type of medullated fibres in all tested farms.

Key words: alpaca wool, fibre diameter, medullation, geographical location

INTRODUCTION

Alpaca as the smallest species of camelids, occurring in South America, constantly expands its expansion to other continents and countries. The world population of those animals reaches 4 million. Most alpacas occur in the Altiplano region, which includes Peru, Bolivia and Chile in South America (Czaplicki 2012). Due to the great adaptability of alpacas resulting from the resistance of the organism to climatic conditions, low requirements of life and ease of maintenance and breeding, they can now be found all over the globe.

In the 1980s of the last century alpacas were successfully introduced from Chile and Peru to Australia (McGregor and Butler 2004), New Zealand (Wuliji 2000), the United States, Canada, and from Australia to Europe (Aylan-Parker and McGregor 2002).

The main reason for the spread of these animals is their fibre. Of the four South American camel species, only Alpaca characterizes with uniform wool, although showing a relatively high variability of thickness depending on the fibre location on the animal's body. Alpaca fibre is considered extremely delicate and characterized by excellent thermal insulation values (McGregor and Butler 2004, Lupton et al. 2006). The latter feature may be related to the presence of large amounts of medullated fibres (a distinctive feature of alpaca), even in very thin hair. On the other hand, a high degree of medullation can contribute to a reduction of the comfort factor as wool and of the final

products (McGregor 2006). The aim of the breeding work in alpaca flocks in Australia is the alignment of fibre thickness and elimination of fibre medullation by means of appropriate evaluation and selection methods. Unfortunately in other regions of occurrence of these animals this work can be disturbed by environmental conditions.

The main features of alpaca wool including the fibre thickness, length and large variety of colors are primarily affected by the genotype. However, these features may also be influenced by biological and climatic factors. According to some authors, the quality of wool produced by these animals can be shaped by environmental factors such as temperature differences, rainfall and altitude, which implies a differentiation of nutritional conditions (Braga et al. 2007, Montes et al. 2008).

The aim of the research was to compare selected traits of Huacaya alpaca wool from herds kept on farms located on different continents.

MATERIAL AND METHOD

The research material was the wool of Huacaya alpacas from three farms located in: Australia (Farm 1), Africa (Farm 2) and Europe (Farm 3). Australian farm was located in the southern part of the continent, in New South Wales near Canberra in Argyle Park. The Australian area characterized with the following climatic conditions: altitude – 520 m above sea level; average rainfall – 632 mm; average temperature +14°C (max +20°C, min +7.2°C). In Africa, alpacas were kept on a small farm on the west coast (Western Cape) in the Cape Town area: 230 m above sea level; 50 mm average annual precipitation; +17°C average temperature (max +22°C, min +11.5°C). European alpaca wool samples originated from a breeding center in Germany in Landebergen, in North Westphalia (51.6 m above sea level; 725 mm precipitation; +12.5°C average temperature).

Wool sample with a one-year shearing was collected from 30 alpacas, both females and males, 10 from each farm. All animals were between two and five years old and the sex ratio was as follows: Australian farm – 6♀, 4♂; African – 6♀, 4♂; European – 4♀, 6♂.

The wool samples were taken on the mid-side on the 10th rib midway between the back line and belly. These samples were used to determine the fibre diameter (FD), standard deviation of FD, coefficient of variation of FD and incidence of medullated fibres (Med) as a percentage of the number of measured fibers. In addition, the staple length (SL) was determined.

Fibre diameter was measured using projection microscope method at 500× magnification in accordance with PN-72/P-04900 standard. In each sample a minimum of 600 fibres was measured. The percentage of medullated fibres in all samples was estimated together with the diameter measurement. The following fibres: with continuous medulla, with partial medulla and no-medullated have been identified. In total, over 18,000 fibres were analyzed.

The staple length was measured to an accuracy of 0.5 cm, butt to tip staple length without disturbing its structure (without stretching and straightening of the crimps).

The obtained values have been processed statistically through a single-factor ANOVA using SPSS 23.0.

RESULTS AND DISCUSSION

The average fibre diameter in the alpaca wool from farms located in Australia, Africa and Europe was 20.20, 22.78 and 24.14 μm, respectively. It should be noted that differences in average diameter, standard deviation of fiber diameter (FD) and coefficient of variation of FD between alpaca wool from farms located on different continents were not statistically significant (Table 1).

The wool of the tested alpacas, both from Farm 1 and Farm 2, was thinner and more aligned than the Huacaya breed wool originating in Peru examined by Gutierrez et al. (2009) and Cervantes et al. (2010). In studies, the authors determined the fiber diameter and coefficient of variation in diameter of Peruvian alpaca wool as 23.0 μm and 24% and 23.07 μm and 23.31% respectively. Compared to the present study, Valbonesi et al. (2010) reported even greater values of fibre diameter and coefficient of variation (27.41 μm and 36.65%), for the fibres of three Peruvian domesticated camel species. McGregor and Butler (2004) determined thickness of Australian alpaca wool as 29,1 μm. In turn, Wuliji et al. (2000) using different methods of fiber diameter measurement in alpaca from New Zealand, obtained the value of this parameter in the range from 28 to 31.9 μm. In the examined alpacas from the United States and Poland, the wool thickness was 27.85 and 27.4 μm respectively (Lupton et al. 2006, Czaplicki 2012).

The differing values of the examined parameter obtained by the above-mentioned authors may result from differences in the number of tested samples, age differentiation of animals and the use of different test methods. According to Cortez (1984), the production of thinner wool is associated with an increase in altitude. In this study and in the research presented by Braga et al. (2007) the authors did not observe this relationship.

The average thickness of males' wool was lower compared to the females wool (21.27 vs 23.34 μm), but the differences were not statistically significant (Table 2). The lack of correlation between the wool thickness and sex was confirmed in studies conducted by Frank et al. (2006) and Lupton et al. (2006). The thinner fibres in Huacaya males' wool

TABLE 1. The effect of geographical conditions on the characteristics of alpaca wool

Item	Farm 1		Farm 2		Farm 3		*P*-value
	$\bar{x}$	*SD*	$\bar{x}$	*SD*	$\bar{x}$	*SD*	
Fibre diameter (μm)	20.20	3.00	22.78	3.55	24.14	6.64	0.180
Standard deviation of FD (μm)	4.40	1.31	4.82	0.93	4.40	0.90	0.600
Coefficient of variation of FD (%)	21.55	4.61	21.61	5.24	19.29	5.83	0.536
Medullation percentage (%)	53.56	21.89	51.73	13.66	52.84	25.30	0.914
Staple length (cm)	10.36	1.86	9.27	1.77	10.49	2.78	0.403

TABLE 2. The effect of sex on the characteristics of alpaca wool

Item	Males		Females		*P*-value
	$\bar{x}$	*SD*	$\bar{x}$	*SD*	
Fibre diameter (μm)	21.27	3.54	23.34	5.64	0.247
Standard deviation of FD (μm)	4.61	0.99	4.48	1.12	0.735
Coefficient of variation of FD (%)	21.93	4.73	19.84	5.52	0.277
Medullation percentage (%)	54.25	25.36	44.42	15.11	0.751
Staple length (cm)	10.54	1.98	9.61	2.32	0.251

maintained in the Huancavelica region, as in the present study, were also found by Montes et al. (2008). Opposite results were obtained by McGregor (2006) indicating that males produce thicker and more rough wool than females.

A detailed analysis of the fibre diameter variation did not show differentiation in this range depending on the place of keeping the tested alpacas (Fig. 1). Curves of variation indicate that all the analyzed wool samples can be classified as uniform wool, which is consistent with the widespread recognition (McColl et al. 2004, Hoffman 2006). Although alpaca wool does not have a clear division into thin and no-medullated and thicker with the medulla fibres, the occurrence of medullation in these animals has been confirmed in many studies (Aylan-Parker and McGregor 2002, Lupton et al. 2006, McGregor 2006).

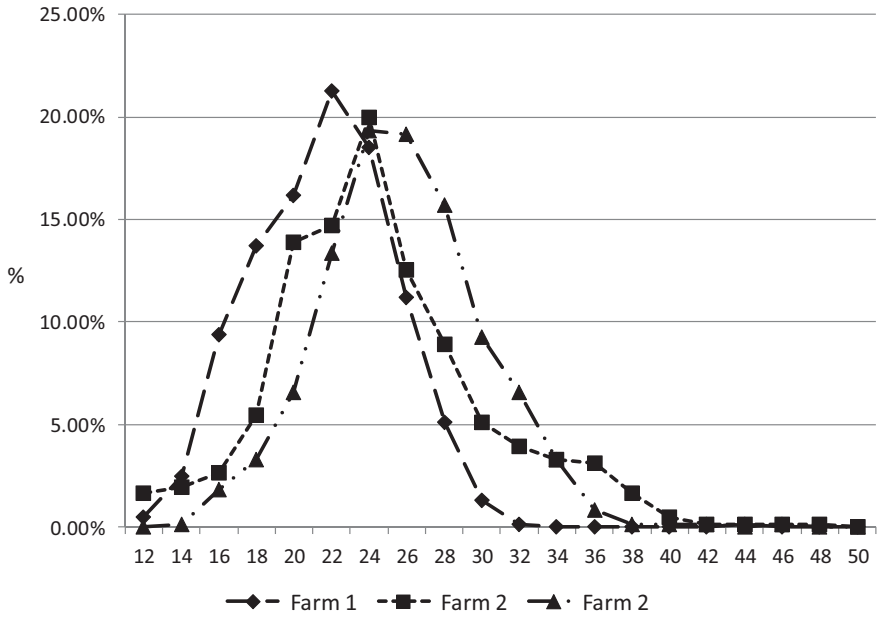

FIGURE 1. The variation of fibre diameter in alpaca wool from studied farms

The level of medullation in the wool samples from alpacas kept in Australia, Africa and Europe was similar ($P \geq 0.05$) – Table 1. The share of no-medullated fibres, with partial medulla and with continuous medulla in all tested flocks, remained at a similar level (Fig. 2). The most common type of medullation was partially medullated fibres, which accounted for about 40% of all fibres in the tested samples. Occurrence of the medullation in this species may be due to adaptation to the conditions of the South American continent, which is the place of origin of these animals. The high daily temperature in the mountains could have contributed to the development of a defense mechanism in the form of a medulla even in thin fibres, which supports the thermoregulation in alpacas. Although the amount of fibres with the medulla increased with their thickness, its presence has already been recorded in fibres with a diameter of 14–20 μm (Fig. 3). The medulla in very thin fibres, were also found by Radzik-Rant et al. (1994) in the wool of native Indian breeds of sheep kept in the Thar desert area.

In presented study, more than 50% of medullated fibres were found in 67% of examined alpacas. A detailed analysis of the medullation level indicated a high individual variability of this feature. Some fleeces were characterized by more than 70% and even 85% of no-medullated fibres, as well as fleeces with the medullation measuring more than 96%. Large individual variability of the medullation in alpacas wool has also been reported by Lupton et al. (2006). The most common type of fibres within the partial medullated fibres, were those with a regular partial medulla (Fig. 4). In addition, within this fibre type, the fibres with almost continuous medulla and with

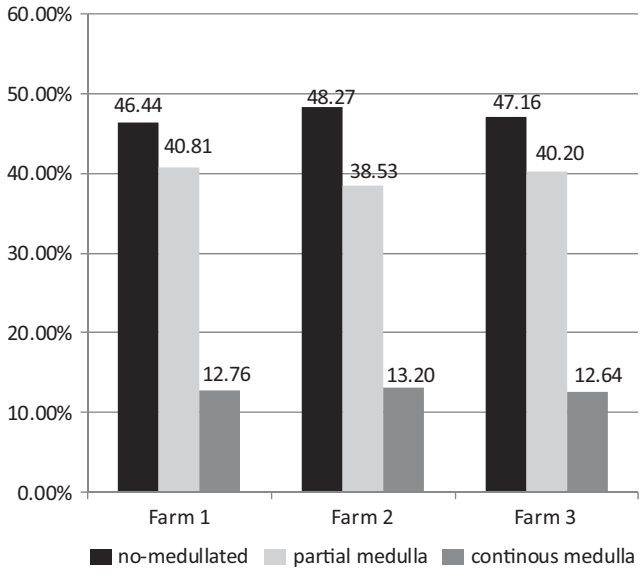

FIGURE 2. The distribution of different types of fibres in alpaca wool from studied farms

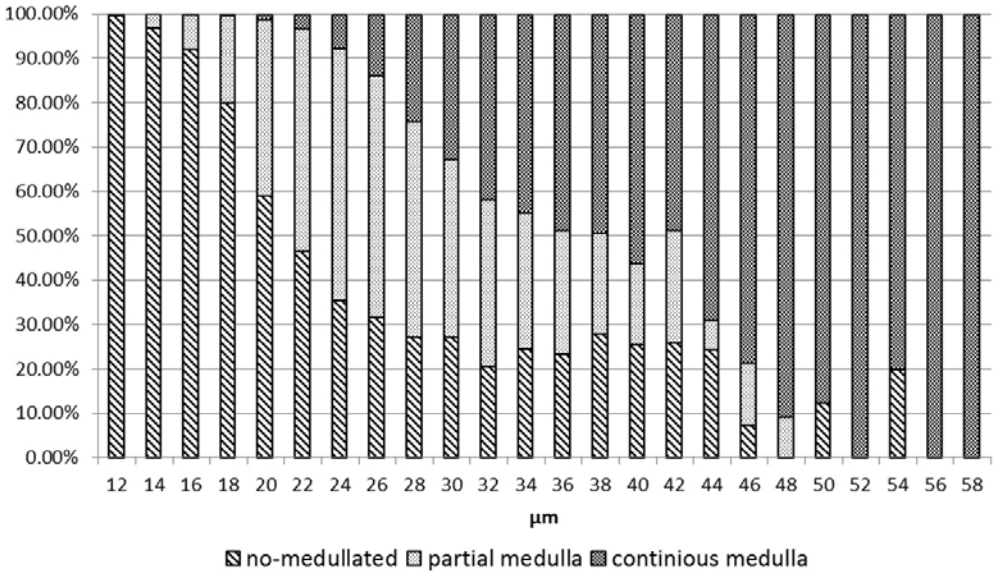

FIGURE 3. The diameter of different fibre types

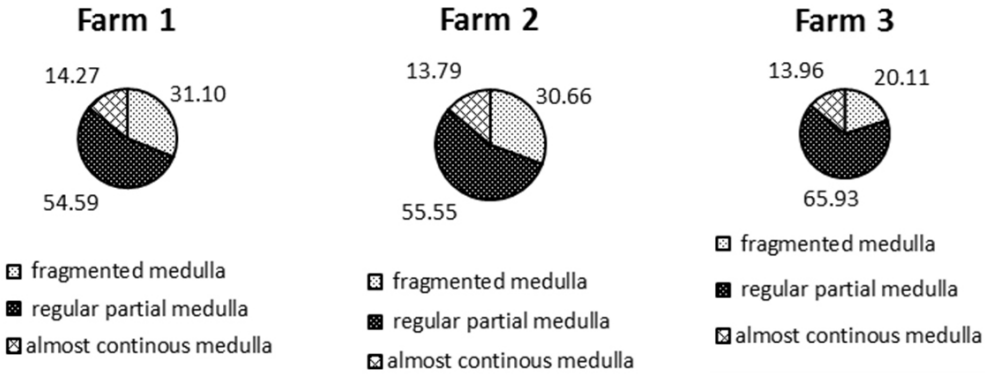

FIGURE 4. The percentage of fibres with different types of partial medulla in alpaca wool from studied farms (%)

fragmented medulla have also been identified. The latter were among the least common. Attention should be drawn to the high convergence of the distribution of the different types of medulla in all tested flocks, despite the differences resulting from the geographical location of the farms.

Although differences in the level of medullation depending on the sex were not confirmed statistically, more medullated fibres were recorded in the male fleeces as compared to the examined females (Table 2).

The wool of the tested alpacas did not differ ($P \geq 0.05$) in terms of staple length

despite the different environmental conditions connected with farms location on different continents (Table 1). Large similarity in this feature was shown by the alpacas kept in Europe and Australia, while alpaca staples from Africa were slightly shorter, with an average length of 9.27 cm. The results are consistent with the results obtained by Wuliji et al. (2000) and McGregor (2002) in New Zealand alpacas. The length interval for the tested samples ranged from 6.1 to 15.1 cm and did not exceed the range determined for alpaca wool (5–16 cm) by Hoffman (2006). Value less than 7.6 cm, which excludes processing worsted system, was noted only for two samples, which accounted for only 6% of the material tested. In the study on Australian alpaca wool, conducted by McGregor (2006), the number of too short fleeces was about 13%.

In the examined males, the staple length showed (statistically insignificant) higher values than in females (Table 2). Lupton et al. (2006) also indicated that males usually produce longer wool compared to females.

CONCLUSION

In summary, it can be concluded that the wool of the examined alpacas did not differ by average fibre diameter, coefficient of variation of fibre diameter as well as length of staples despite the different located of the farms. Extremely interesting are the results of the analysis of the medullation level, which showed similarity even with regard to the distribution of the type of medullated fibres in the tested flocks. The medullation of these wools seems to be independent of the breeding work, climatic conditions and thus nutritional conditions.

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Streszczenie: *Charakterystyka porównawcza wybranych cech wełny alpak pochodzących z farm położonych na różnych kontynentach*. Zainteresowanie hodowlą alpak na całym świecie jest związane z pozyskiwaniem od tych zwierząt cennego włókna. Na cechy wełny alpak mają wpływ zarówno czynniki genetyczne, jak i warunki środowiskowe. Celem przeprowadzonych badań było porównanie wybranych cech wełny alpak rasy huacaya pochodzących z trzech farm położonych w Australii, Afryce i Europie. Próby wełny pobrano od 30 alpak, po 10 z każdej fermy. Określono w nich grubość włókien, długość zespołów oraz stopień rdzenistości. Grubość włókien mierzono metodą mikroprojekcyjną. Jednocześnie z pomiarem grubości określono zawartość włókien z obecnością rdzenia. Identyfikowano włókna z ciągłym i przerywanym rdzeniem oraz włókna bezrdzeniowe. Uzyskano podobne rezultaty we wszystkich badanych fermach w odniesieniu do grubości wełny, jej długości, jak i przede wszystkim stopnia rdzenistości, a nawet rozkładu rodzaju włókien rdzeniowych.

Słowa kluczowe: wełna alpak, grubość włókien, rdzenistość, położenie geograficzne

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Parasites in dogs – prevention and control according to the questionnaire analysis

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Abstract: *Parasites in dogs – prevention and control according to the questionnaire analysis.* The aim of the study was to determine the extent of knowledge of dog owners on the incidence of parasitic diseases, their negative consequences and preventive measures. The research material was based on data from 162 anonymous questionnaires. The study was conducted in the period between July and October 2015, among owners of dogs living in different regions of Poland. Analysis of the results showed that most respondents were aware of the dangers posed by the development of parasitic diseases in their animals, however the extent of their knowledge was insufficient and required further deepening. Unfortunately, 6% of owners do not use any form of antiparasitic prophylaxis. Less than half of the respondents declared that they regularly pick up their dog's waste. More than half of the surveyed owners were not aware of the type and programme of antiparasitic prevention used by the vets, or the necessary changes in the dog's pharmacological treatment. The largest group of owners (32% of respondents) used the prevention of ectoparasites twice a year. Nearly half of the respondents used antiparasitic drops as a precaution against ectoparasitic diseases, while 33% used antiparasitic collars. 40% of respondents seeking to increase the effectiveness of protection against parasitic infestations applied two different forms of treatment simultaneously. The results of the questionnaire surveys indicated, according to specialists recommendations, that there was insufficient frequency of using antiparasitic treatment against endoparasites. Single deworming was performed by 41% of respondents while the smallest group of respondents (12%) did it three times.

Key words: parasites, dogs, prevention, combating parasites

INTRODUCTION

Interaction with animals has a positive effect on the human health and well-being. The role of dogs in human-animal relationships is quite special. They give psychological and physiological support working as "therapists", guides for the blind, rescue and police dogs. They have a positive effect on human physiological functions by normalizing blood pressure, cholesterol levels (Beck and Meyers 1996), and psychological and psychosocial wellbeing (Robertson and Thompson 2002).

Unfortunately, the presence of a dog also poses a potential threat to the health of the owners and causes many diseases. Viral, bacterial, fungal or parasitic infections can be transmitted to humans (Robertson and Thompson 2002). Animals become carriers of parasites no matter which environment they live in

– whether they are kept at home or outdoors (Uspensky and Loffe-Uspensky 2002). It has been proven that reducing the risk of exposure to pathogens in domestic dogs does not reduce the incidence of infections (Kowalska 2012). Appropriate prophylaxis, monitoring of any behavioural changes in dogs and testing for faecal parasites contributes to reducing the risk of developing parasitic diseases and ensuring effective protection of animals and thereby their owners.

It is important to be aware of the importance of picking up dog waste, due to the fact that they represent a potential epidemiological risk. Microorganisms found in the faeces carry the risk of infectious and parasitic diseases such as toxocariasis, toxoplasmosis, plateletosis, typhus, campylobacteriosis, salmonellosis and yersiniosis.

Eggs of nematodes and tapeworms are extremely resistant to adverse environmental conditions and can survive in the soil for up to several years. Removing faeces from places frequented by dogs is equivalent to reducing probability of parasite infection (Kowalska 2011).

In the case of parasitic infection the symptoms manifest themselves shortly after the dog's contact with the parasite or after longer incubation periods. The illness can disappear after some time, go into chronic form, sometimes asymptomatic, or even lead to the host's death (Buczek 2010). The intensification of the symptoms shortly after the parasite's invasion is characteristic for the so-called primary parasitic diseases. In the case of secondary parasitic diseases the symptoms begin when the balance between the parasite and the host is destroyed. The disease progresses gradually, which is why it is difficult to diagnose a parasitic infection shortly after it occurred.

The aim of the study was to determine the dog owner's knowledge concerning the incidence of parasitic diseases, their negative consequences and prevention.

MATERIAL AND METHODS

The research material was based on the respondents' statements taken from the questionnaires, which were carried out to determine the scope and level of knowledge of dog owners on the subject of parasitic diseases, prophylaxis and parasite control. The study was conducted from July to October 2015. The anonymous questionnaire consisted of 32 questions – 4 open and 28 closed. Responses were given by 162 people from different parts of Poland, 114 of them were sent *via* an Internet portal. Among the 162 respondents 118 were women. The majority of the respondents were 25–45 years old (34%) and over 45 (35%), with the smallest group being under 18 (5%). Over half of the respondents (53%) had higher education, 43% had graduated from secondary school, and 5% had a primary education. Most of the respondents were owners of crossbreds (52%), with males predominating (57%). Young dogs up to the age of five dominated within both purebreds and crossbreds. The least numerous was a group of dogs over 15 years old. Medium sized animals, weighing between 10 and 25 kg, constituted 41% of the analysed population, small dogs weighing less than 10 kg – 34%, big weighing from 25 to 40 kg – 22% and giant dogs weighing over 40 kg – 3%.

RESULTS AND DISCUSSION

Almost all respondents (94%) were aware of the fact that parasitic diseases can transmit easily between animals of both the same and different species, and that they can pose a risk to humans. This fact should be considered extremely optimistic when compared to Steinka's research (2016). The author's assessment of animal owners' knowledge in the field of hygiene and the health effects of interactions with domestic animals has shown that 91% of respondents did not believe that it was possible for humans to be infected by domestic animals, and less than 4% of respondents have considered it possible. According to their opinion animals can be a source of bacteria transmission (78% of respondents), fungi (15%) or both of these micro-organisms (27%). A small percentage of respondents defined animals as a potential source of worms yet no one perceived them as a source of viruses and protozoa. According to the author, owners probably considered the deworming procedure as a method of releasing their animals from the presence of these organisms.

As Grajek and Woźniak-Holecka (2014) reported, 9% of the 300 surveyed dog owners in urban areas were aware of the occurrence of zoonotic diseases. However, in response to that fact 65% of the respondents included the information that contact with dogs causes fungal infections.

In the present study the majority of the respondents (62%) owned more than one dog. In this group 69% had two, 21% three, 5% four, 2% five, 2% seven, and 1% ten dogs. As reported by Roliński (2008) and Niemand (2011), the increasing number of animals was equivalent to a higher risk of parasitic infestation, especially in the case of a common place of residence. Survey and serological research carried out by Zielicka-Hardy et al. (2012) on the presence of specific anti-Toxocara IgG antibodies within members of the Polish Hunting Association showed that infection was detected in 33% of hunters. The incidence of antibodies was higher in samples from hunters living in rural areas (95%), and the probability of infection increased with the number of dogs. The number of infected persons was higher among people who had more than two dogs and those who dewormed their dogs less than once a year.

According to Gliński and Kostro (2013), keeping animals at home increases the possibility of people being infected with parasitic diseases carried by animals and it is exactly how over 68% of the people surveyed is keeping their dogs. Raś-Noryńska et al. (2011) have studied faecal samples of children up to 17 years old that have been treated in the Provincial Children's Hospital in Olsztyn. The survey attached to each trial specified the issue of the presence of animals in the household. The obtained result has indicated the lack of correlation between permanent contact with animals in the house and the frequency of parasitic invasion.

The fact that 95% of the surveyed dog owners posessed the knowledge concerning the effects of neglecting the anti-parasitic prophylaxis in animals and 96% took preventive measures to protect themselves against parasites should be considered very important and optimistic. According to the studies of Grajek

and Woźniak-Holecka (2014), only 61% of respondents who were dog owners in urban areas have performed a deworming treatment at least twice a year, and less than 80% of the surveyed population protected dogs from ectoparasites.

In order to protect and eradicate ectoparasites in dogs, the owners can use external preparations applied directly on the skin, such as collars, spot-ons (drops), aerosols or *per os* (Roliński 2008, Zawiślak et al. 2011, Bowman 2012). Studies showed (Fig. 1) that the most used products were spot-ons (47%) and collars (33%). The aerosol formulations were the least popular (7%). In an effort to increase the effectiveness of the antiparasitic protection of their animals, almost 40% of the respondents used two formulations in different forms.

In turn, the results of a survey carried out by Lonc et al. (2016) in order to assess the knowledge of cat and dog owners from the Wroclaw agglomeration in the field of tick prevention pointed out that 61.3% of respondents protected their animals against ticks and the drops were the most frequently used form of application (60.1% of respondents). The collars were used by 32.9% of respondents, and 2.2% used preparations in the form of aerosols. In spite of undertaking safety measures, infestations were still being observed. Owners who did not use any form of treatment observed tick infestation more often (82.7%) than those who protected their animals from external parasites (69.4%). The probable reason, according to the authors, could have been not complying with the recommendations regarding the application of the preparations (incorrect frequency, loss of the collar's protective properties due to contact with water).

The choice of a particular formulation is usually dictated by its simplicity of use and effectiveness. In the case of drops and spray agents the duration of the protection period is determined by the type of active substance used, its release rate and absorption. Most of these preparations protect dogs for one month, however, the increased contact with water shortens the protection to about three weeks. The active substance released into the animal's body from the antiparasitic collar protects against infestation for up to 7–8 months.

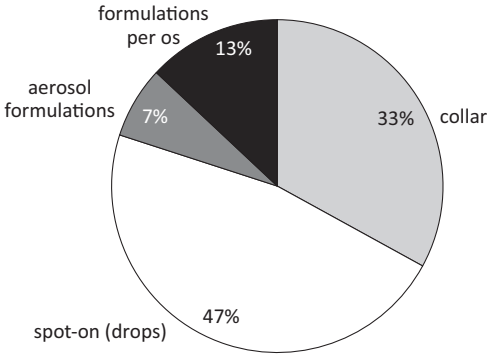

FIGURE 1. Types of prevention against ectoparasites

The effectiveness of antiparasitic prophylaxis depends on the systematic use and the frequency of use, determined by the period of protection a given method guarantees. The most representative group among the respondents (32%) was the owners who used prophylaxis against ectoparasites twice a year (Fig. 2).

In the studies of Grajek and Woźniak-Holecka (2014), 65% of respondents were protecting dogs against fleas and ticks seasonally – from spring to autumn, 14% continued securing also after the season, and 21% did not secure at all.

In the case of ticks, lice, fleas or other external parasite infestations, despite the use of therapeutic agents to eliminate the source of infection, there is a risk of recurrence. The residual effect of the drug, which accompanies the use of the latest therapeutic formulations, ensures the prolonged duration of the active substance in the body. This explains the use of such measures for preventive purposes (Niemand 2011).

The increased tick activity in recent years should encourage the rigorous use of prophylaxis against babesiosis which is caused by *Babesia canis*, a protist of Babesiidae family that can be found in tick salivary glands (Niemand 2011, Solano-Gallego and Baneth 2011). Infections with this parasite can cause haemolytic anaemia, drowsiness, fever, vomiting, haematuria, apathy, decreased appetite, stomach problems, enlarged spleen and liver, and pallor or redness of mucous membranes. The disease can take the form of a mild infection or cause haemolytic complications that can lead to paralysis and even death following kidney damage and uraemia. Constant proliferation of protozoa presented in red blood cells causes the tearing of the walls of the dog's erythrocytes, decreasing the number of red blood cells, leading to anaemia and dangerous haemolytic diseases (Buczek 2010, Dziubek 2014).

Dogs may be the final hosts of a large number of internal parasites, including not only aschelminthes and flatworms but also protozoa. From among the intestinal protozoa, the most common cause of parasitoses in dogs are *Babesia* spp., *Giardia intestinalis* (*G. duodenalis*, *G. lamblia*), *Toxoplasma gondii*, *Cystoisospora canis*, *Cryptosporidium* spp., *Leishmania infantum* and *Hammondia* spp. (Bajer and Bednarska 2007).

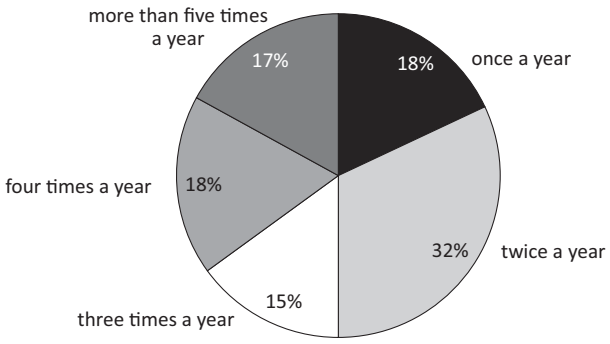

FIGURE 2. Frequency of prevention against ectoparasites

It is estimated that the percentage of domestic dogs in Poland that are infected with *Giardia* is 57% and with *Cryptosporidium* – 27%. The scale of the problem is illustrated by the fact that approximately 80% of the *Cryptosporidium* oocysts and *Giardia* cysts, which develop in the organisms of infected animals and are excreted into the environment with faeces, where they remain invasive for many months (Rożej et al. 2008).

Protozoa of *Giardia* and *Cryptosporidium* cause debilitating, watery diarrhoea, have influence on weight loss and lack of appetite (Hadaś and Derda 2014). The course of giardiasis, both in humans and animals, may be asymptomatic. Chronically infected and asymptomatic hosts may be reservoirs of invasion for several years (Gundłach and Sadzikowski 2004).

In the light of the threats described above, the results of the survey indicate that the frequency of treatments against endoparasites is insufficient. The most numerous group of respondents (41%) of respondents dewormed their animals once a year (Fig. 3). Only 12% of respondents administered three doses of the preparations.

In the studies of Grajek and Woźniak-Holecka (2014) more than half of the respondents coming from urban areas (61%) dewormed their dogs twice a year. Only 8% declared a complete lack of deworming activities, 10% did them once a year, and 21% more often than twice a year. According to Grajek and Woźniak-Holecka's (2014) own research, 92% of dog owners of Silesia region have dewormed dogs at least once a year. The similar regularity has been shown by the results of surveys conducted by Gawor and Marczyńska (2015) on farms in the Mazovian and Lublin Voivodeships. Exactly 94.1% of respondents have performed the deworming of dogs at least once a year.

According to Klockiewicz (2004a), when it is not possible to regularly conduct analyses of the faeces, which reflect the current level of parasitic infection, it is advisable to perform the deworming four times a year. Basing on the knowledge of the developing cycle of the most common parasites it can be concluded that a one or two-time deworming does not ensure complete protection of the animal.

The basic principle is to give appropriate, dose-compatible medicine. Active

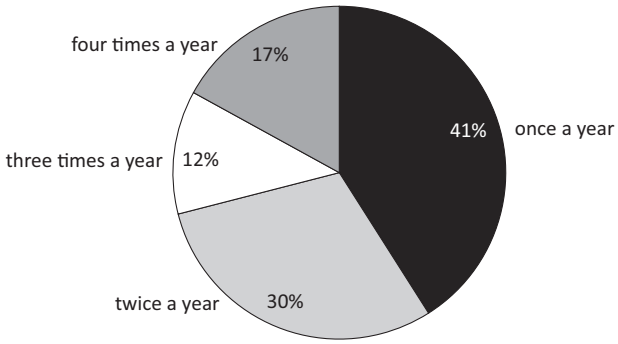

FIGURE 3. The frequency of dog deworming

substances that are part of market available medicines are: benzimidazoles, imidazothiazoles, tetrahydropyrimidines, macrocyclic lactones, organophosphorus compounds, piperazine and its derivatives, synthetic organic compounds, and diamidine derivatives. As Niemand (2011) has shown, most of these substances cause paralysis of the nervous-muscle system of the parasite, acting on receptors, inhibiting enzyme activity, and limiting access to essential nutrients. It is very difficult to create an effective, universal formulation, taking into account the huge variety of parasites. In the assessment of veterinary parasitologists, regardless of the availability of effectiveness of anthelmintics, animals still play an important role in the spreading of parasites, especially the *Toxocara* nematodes (Deplazes et al. 2011).

The larvae of the parasites can be found in the faeces of sick dogs, which poses a threat to healthy organisms. For this reason, clearing-up of canine faeces, as well as testing it for the presence of parasites is extremely important for antiparasitic prophylaxis (Hadaś and Derda 2014).

According to Gawor and Marczyńska (2015), not all preparations have an ovicidal effect. The dead or paralysed female nematodes excreted after administering the medicine have eggs that are capable to develop. For this reason, it is advisable to utilise the faeces for at least two days after deworming. Man infected with *Toxocara* plays a role of a paratenic (reservoir) host, where the parasite remains in the form of larvae in the internal organs.

Roundworms found in carnivorous animals (Gawor and Marczyńska 2015) pose another zoonotic threat. An intrauterine and lactogenic invasion causes that 16-day-old puppies already excrete worm eggs in the faeces (the infection rate of several-week-old puppies reaches 70–100%). As Schnieder et al. (2011) have shown, the survival time of roundworms may last up to several years, leading to their accumulation in the external environment. Investigation of the degree of soil and sand pollution by invasive geohelmint forms in urban and rural areas in Poland have shown a significant level of soil contamination by eggs of intestinal parasites among which the *Toxocara* spp. was most frequently observed. The analysis of the soil from courtyards, playgrounds and squares has shown the presence of *Toxocara* spp. in 7.2–28.6% of samples from different regions of Poland (Mizgajska and Luty 1998, Petryszak and Nosal 2003, Rokicki et al. 2007, Ronkiewicz et al. 2007, Gawor et al. 2008, Borecka et al. 2010, Błaszkowska et al. 2013, Bartosik et al. 2017). Studzińska et al. (2015) have shown that 54.3% of soil samples from urban areas were contaminated by parasitic eggs. Contamination was reported in 40% of sandpits and as much as 70% of residential alleys. According to Borecka and Kłapeć (2015), the incidence of toxocarosis in people, which is caused by a nematode from the *Toxocara* group, is a result of the severity of dog's infection and the contamination of soil with their faeces. As a component of the toxocarosis prevention the author suggests the need to introduce a legislation prohibiting the introduction of dogs into parks and playgrounds and to force owners to clean animal waste in public areas. The results

have shown that backyards and public playgrounds are places with the highest levels of *Toxocara* spp. (Gundłach et al. 1996, Borecka 2001, Gawor i Borecka 2004, Rokicki et al. 2007, Kłapeć and Stroczyńska-Sikorska 2009). In this study, only 40% of respondents reported clearing-up the dogs' waste, and only a quarter of the dogs have had their faeces tested for the presence of parasites.

Gawor and Marczyńska (2015) conducted surveys in agricultural holdings in the Masovian and Lublin Voivodeships to assess the level of parents' knowledge about the risk of zoonotic parasites to the children's health and the range of activities undertaken to ensure proper hygiene conditions (deworming, clearing-up of faeces, securing the sandboxes). For most of the respondents, the clearing--up of animal faeces had only aesthetic aspect. Only 9.8% treated such activities as a form of removal of the potential source of eggs of *Toxocara* spp. from the environment.

The season in which the owners of animals performed the deworming most often was spring – 40% of respondents, autumn – 27%, summer – 22% and winter – 11%. Bartosik et al. (2017) showed an increase in parasitic invasion during autumn and winter. The authors point out the need to increase the intensity of dog deworming during that period.

Many owners have chosen the antiparasitic preparation themselves. The lack of knowledge and experience in this area could have negative consequences for this procedure. According to Klockiewicz (2004b), an inadequately chosen formulation or an insufficient dose can aggravate the symptoms and worsen the condition of the animal. In addition to the ineffectiveness of deworming, such action can promote the development of parasitic resistance. The alternate use of therapeutic agents prevents the parasites from becoming resistant to the active substance of the preparation. Ensuring the effectiveness of the procedure is important due to the fact that even though the toxic effect of chemotherapeutics is stronger on the parasite's organism than on the animal, each use can have dangerous effects also for the host (Roliński 2008, Bowman 2012).

Information obtained from the survey indicated that 89% of the owners used the services of the same vet, who gave advice of dates and method of deworming to almost 86% of owners mentioned above. Similar results were obtained by Grajek and Woźniak-Holecka (2014). The authors stated that 80.5% of respondents had declared that they had received comprehensive answers from their vets. They had also obtained advice how to avoid zoonosis.

On average 11% of the respondents changed their vet every few months, once or twice a year, and sometimes consulted various specialists as a result of moving house or a variety of emerging problems.

The owner and the veterinarian should decide on the method of deworming. The owner informs of his or her financial possibilities and the frequency of performing the treatment. The doctor chooses the medicine, dose and frequency of use. However, as many as 56% of respondents were unable to answer why the veterinarian chose a certain type of deworming treatments and 58% had no knowledge of how adequate

deworming programme was. Only 41% of the respondents were aware of the changes made by the veterinarian in their preparations, although they claimed that it happened less than once a year. The remaining 59% of owners were not interested in what formulations were applied to their dogs, and 34% of people in this group could not tell whether the dog was weighed prior to application.

Advanced molecular and bioinformatic technologies can enrich knowledge of parasite pathways. Studies on the genetic diversity of protozoa occurring in wild and domestic canines characterize the dynamics of the development of parasitic infections in the animal population. Determination of the protozoal parasite population structure is an important component of the study defining the transmission of the pathogen, immunogenicity and pathogenesis of the disease (Sibley et al. 2009). It also becomes the basis for the development of effective control strategies that underpin veterinary protection and public health (Aroch et al. 2015). The radical transformations of the climate, landscape and ecosystem in recent years have contributed to changes in the transmission of parasitic diseases and the zoonotic pathogens can "return" to human populations. Pathogens adapt to new places and hosts, and thus remain a new form of danger. This causes the need to identify particular species of parasites, their life forms and ways of parasitism in order to plan and develop control strategies against pathogen (Otranto et al. 2015a, Otranto et al. 2015b, Bartosik et al. 2017). It seems that every animal owner should possess at least a part of the combined knowledge of microbiologists, physicians, veterinarians, parasitologists, biologists and epidemiologists since it is crucial for a better understanding of the aetiology of parasitic diseases.

CONCLUSIONS

Based on the analysis of the results of the survey, it was concluded that:

1. The vast majority of respondents were aware of the dangers posed by the development of parasites in dogs, although this knowledge turned out to be incomplete and needed to be deepened. On average 6% of owners did not use any form of antiparasitic prophylaxis.

2. Less than half of the respondents who found it reasonable to pick up dog faeces admitted doing it regularly.

3. More than half of the surveyed owners did not show any interest in the type of preventive medicine used by the vets, the programme and the necessary changes in the pharmacological agents applied.

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Streszczenie: *Pasożyty u psów – profilaktyka i zwalczanie w świetle badań ankietowych.* Celem przeprowadzonych badań była próba określenia zakresu wiedzy właścicieli psów na temat występowania chorób pasożytniczych, ich negatywnych następstw oraz działań prewencyjnych. Materiał badawczy stanowiły dane pochodzące ze 162 anonimowych ankiet. Badania przeprowadzono w okresie od lipca do października 2015 roku wśród właścicieli psów zamieszkujących różne rejony Polski. Analiza wyników wykazała, że większość respondentów wiedziała na temat zagrożeń wynikających z rozwoju chorób pasożytniczych u posiadanych zwierząt, zakres tej wiedzy był jednak niewystarczający i wymagał pogłębienia. Niestety 6% właścicieli nie stosowało żadnej formy profilaktyki przeciwpasożytniczej. Mniej niż połowa respondentów zadeklarowała, że regularnie sprząta odchody swoich podopiecznych. Ponad połowa ankietowanych właścicieli nie miała informacji na temat rodzaju i programu profilaktyki przeciwpasożytniczej stosowanej przez lekarza weterynarii oraz o wprowadzaniu

koniecznych zmian w aplikowanych psom środkach farmakologicznych. Najliczniejsza grupa właścicieli (32% ankietowanych) stosowała profilaktykę przeciw ektopasożytom dwukrotnie w ciągu roku. Prawie połowa respondentów jako prewencję chorób wywoływanych przez ektopasożyty stosowała preparaty w formie kropli, 33% używała obroży przeciwpasożytniczych. Dążąc do zwiększenia skuteczności ochrony przed infestacją pasożytami, 40% respondentów stosowało jednocześnie dwa preparaty w różnych formach. Wyniki przeprowadzonych badań ankietowych wskazują na niedostateczną częstotliwość stosowania zabiegów zwalczających endopasożyty. Jednokrotne odrobaczanie psów było wykonywane przez 41% ankietowanych, a t rzykrotne przez najmniej liczną grupę respondentów (12%).

Słowa kluczowe: pasożyty, psy, profilaktyka, zwalczanie

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Twenty years of the European bison Lowland line *Bison bonasus bonasus* conservation in captivity

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Abstract: *Twenty years of the Lowland European bison* Lowland line Bison bonasus bonasus *conservation in captivity.* The aim of the work was to track back changes taking place in the Lowland European bison population kept in captivity in years 1995–2015. The material for the study was data from European Bison Pedigree Book, collected from the years 1924–2015. The parameters such as inbreeding coefficient, kinship coefficient, contribution of founder genes and completeness of pedigree information were analyzed as well as the demographic structure of the population. Over the past 20 years, a steady increase in the population size has been observed. The share of different countries in Lowland wisent breeding also changed. The study showed that the accuracy of pedigree information decreased significantly between 1995 and 2015. In 1995 169 from 342 animals living in captivity had full pedigree information. In 2005 it was 76 from 320 animals. Within the population being alive in 2015, only 62 form 577 (10%) of all animals from Lowland line have full pedigree information, that leads to the founders of the population. An increase in the average values of inbreeding coefficient in the population and mean kinship was also observed.

Key words: European bison, *Bison bonasus bonasus*, population genetics, captive breeding

INTRODUCTION

Efforts to rescue the European bison *Bison bonasus* from extinction began on 2 June 1923 with the speech of Jan Sztolcman at the International Congress of Nature Conservation in Paris, and are continued to this day. Throughout all these years the work of many experts contributed to rebuilding the captive population and restoration of the European bison to the nature.

After the extinction in the wild at the begging of the 20th century, the European bison *Bison bonasus* population was saved from absolute extinction with only 54 individuals which survived in captivity. Later analyses have shown that only 17 actual founders of currently living population remained, representing 12 unique genotypes (Olech 2009). Only 7 from those 12 ancestors are the founders of the Lowland line. With so few animals in the breeding stock it was impossible to avoid mating related individuals. This caused the largest danger for the population – the loss of genetic diversity.

Maintaining genetic diversity is the biggest concern considering many endangered species. The loss of genetic diversity is directly associated with the mating of relatives. As many studies have already shown, the increase of inbreeding and subsequent probability of occurrence of inbreeding depression

adversely affect the survival chances of the population. Inbreeding depression is manifested, i.a, by the decrease in reproductive success and the deterioration of the overall fitness of individuals. In the longer term, it also has a negative impact on the response of the population to environmental changes (Ralls et al. 1979, Frankham et al. 2002, Reed and Frankham 2003).

European bison population continuously increases its population size. In 2016, the world population, including representatives of both breeding lines Lowland (LB) and Lowland-Caucasian (LC) in captive, semi-free and free ranging herds, reached 6,573 individuals. Free ranging herds counted 4,472 individuals, of which 2,793 were representatives of the Lowland line. Among the animals living in captivity prevail representatives of LC. Among 1706 European bison there are 1,234 animals from Lowland--Caucasian line (Raczyński 2016). The conservation strategy for the European bison from 2007, formulated goals for further conservation of the species. One of those goals is the preservation of genetic variability within the Lowland line. Actions to be taken include: evaluating of the genetic value of the individuals and planning mattings based on this information, as well as international exchange of individuals between herds. The basis for the success of these plans is good cooperation between the breeders and the coordinator. All captive herds should be treated as one population.

The aim of the study was to investigate the demographic and genetic changes within the captive population of European bison *Bison bonasus bonasus* Lowland line, over the last 20 years.

MATERIAL AND METHODS

The basis for the analysis was the pedigree data on the Lowland line population. The data obtained from the European Bison Pedigree Book from the years 1924–2015, concerned 3,215 individuals. Genetic analysis of the population was done using the Endog program (Gutiérrez et al. 2010). The average inbreeding coefficient, percentage of completion pedigree and kinship coefficient were calculated. Inbreeding coefficient reflects the probability that an individual has two identical alleles by descent. It is computed according to Meuwissen and Luo (1992). The kinship of two individuals reveals the probability that two alleles taken at random, one from each, will be identical by descent. Kinship (coancestry) of two individuals indicates the inbreeding of their offspring. The mean kinship coefficient for an individual is the average of kinship values for that individual with every individual in the population, including itself. Mean kinship value for the whole population was calculated as an average of mean kinship coefficient for all individuals in the population (Frankham et al. 2002) and it informs how closely are related animals in the population. The percentage of completion pedigree was calculated as a sum of the share of founders genes in the pedigree of each individual. If such sum is equal to one, it means that the individual has full pedigree information. The share of founder genes is calculated on the basis of pedigree data. Each descendant obtains 50% of the founders alleles from sire and 50% from dam and such proportion continues

through all generations. In the study compared were the above-mentioned parameters in populations of years 1995, 2005 and 2015. Only individuals with full pedigree information (leading to the founders) were included in the estimation of the chosen parameters. In 1995 there were 169 animals, in 2005, 76 and in 2015 only 62 such animals. The demographic analysis concerned all individuals living in years: 1995 (342 individuals), 2005 (320 individuals) and 2015 (581 individuals), according to the European Bison Pedigree Book. Changes in the structure of captive herds and the participation of different countries in wisents breeding were also analysed.

RESULTS AND DISCUSSION

Demography

The length of animal's life depends on many factors, usually in captivity it is longer than in the wild. Female bison only exceptionally live longer than 25 years, and males longer than 20 (Krasińska and Krasiński 2004). Period of reproductive activity of females usually starts at age of three years, so the first calf is born in the fourth year of life. Females often give birth until the old age. Males reach maturity around the third year of life. However in the wild, because of competition with dominant bulls, they become reproductively active later, when they are about 7 years old. In captivity, the beginning of mating depends on the breeder decision. Spermatogenesis usually disappears after 12th year of life (Krasińska and Krasiński 2004, Olech 2008, Olech and Perzanowski 2014).

The percentage of males (between 3 and 12 years old) and females (older than 3 years) being in reproductive age, was similar in populations living in years 2005, and 2015. Higher proportion of animals that could reproduce was observed in the year 1995. There were 68.8% of such females in 1995, 63.72% in 2005 and 65.92% in 2015. For males, there were 57.25, 46.55 and 47% of all males in 1995, 2005, and 2015 respectively.

Sex ratio (number of females to number of males, in reproductive age) were 2.23 : 1 in 1995, 2.60 : 1 in 2005 and 2.50 : 1 in 2015. The proportion of the young (animals younger than three years) in the population was: 31.00% in 1995, 38.44% in 2005 and 38.05% in 2015. Sex ratio in this group of animals was: 1.5 : 1 in 1995; 1.3 : 1 in 2005, and 1.1 : 1 in 2015 (Fig. 1).

Over the whole analysed period of 20 years, from 1995 to 2015, observed was a general increase in the size of captive European bison population. However initially, between the years 1995–2005 there was a slight decrease of this population. In 1995 registered were 342 animals, in 2005 – 320, and in 2015 the population reached 578 individuals. Unfortunately during that time, there was a decrease of the pedigree information accuracy. This is an effect of introduction of individuals from the wild, with unknown pedigree, into the captive herds. This resulted in reduction of the proportion of individuals with a well-known pedigree. Moreover, only full pedigree information (from founders to the individual) allows for sufficiently accurate estimation of the inbreeding coefficient and coancestry between individuals in the population.

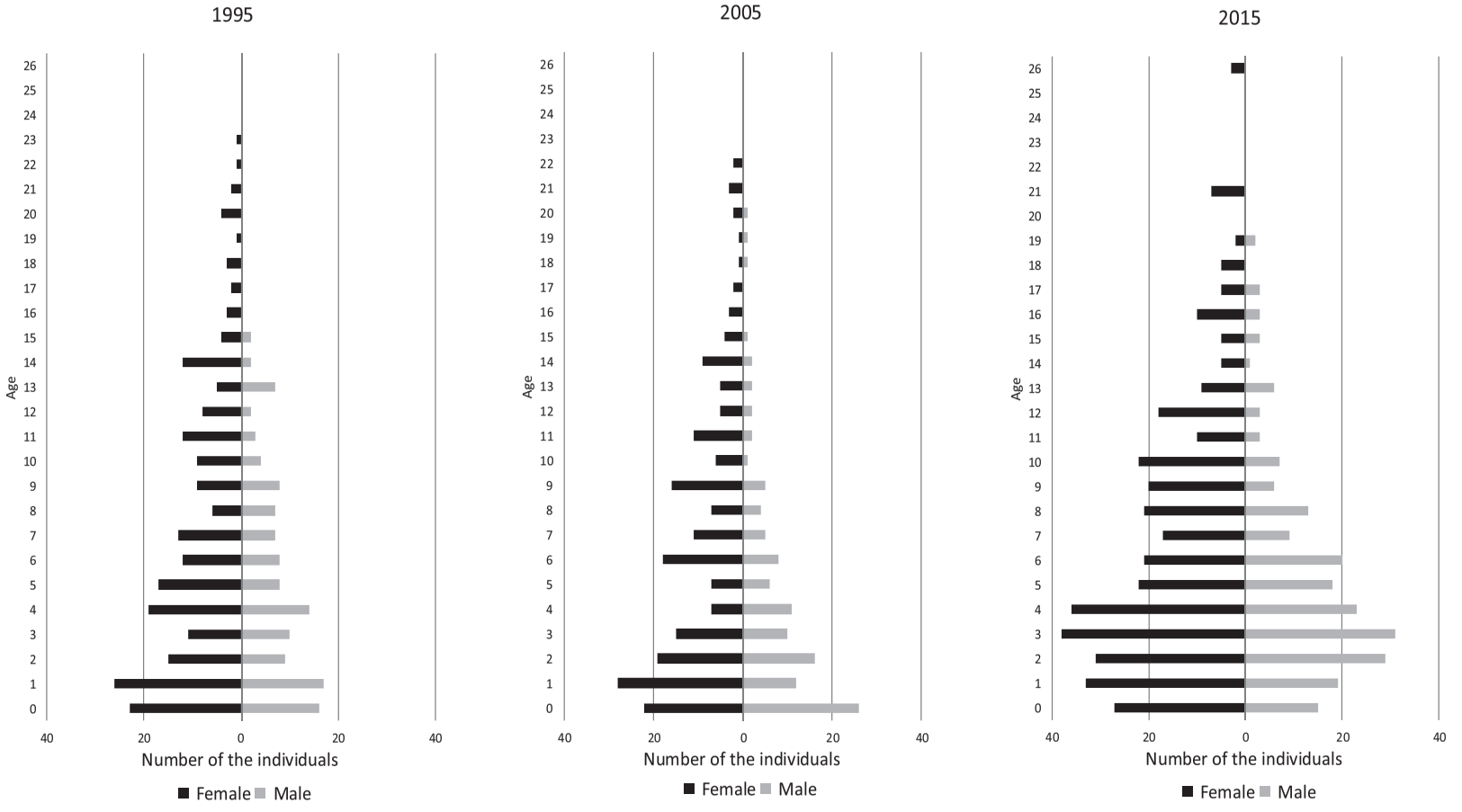

FIGURE 1. Age and sex structure of the European bison population in captivity in years 1995, 2005 and 2015. Respective population numbers were: 342, 320 581

In analysed years the percentage of individuals with full-known pedigree was respectively equal to: 49.42, 29.79 and 10.55% (Fig. 2).

The proportion of individuals in free-ranging herds and in the captive herds kept changing over the years. Some of the animals remained in the captive herds only for some time and then they were transferred to semi-free or free-ranging herds in various countries. In 1995, from the free-ranging herd of Białowieska Primeval Forest, introduced into captive breeding were only 19 individuals. In 2005, 22 animals were transferred to the captive population, and in 2015 – 38 animals. The largest number, 53 individuals from free-ranging herds, were introduced to the captive population in 2007. Descendants of these animals have either none or very incomplete pedigree information. That causes the overall decrease in the accuracy of the pedigree data. We know exactly which animals were the founders of the free-ranging herd of Białowieska Primeval Forest: 20 females and 10 males (Grzegrzółka et al. 2004). Basing upon this information, we can supplement with a certain degree of probability the pedigree data for individuals transferred to the captive population. This may significantly improve the level of pedigree knowledge on captive individuals, but since it still would be only a simulation, chosen parameters calculated in this way may be not precise.

According to Olech and Perzanowski (2016) between 2000 and 2015, the number of herds (both Lowland and Lowland-Caucasian line) increased by 50%. The number of semi-free herds grew up, and the number of herds where both genetic lines were kept simultaneously, was reduced. Such trend is positive because it helps to avoid introducing the genetic material of the Lowland line

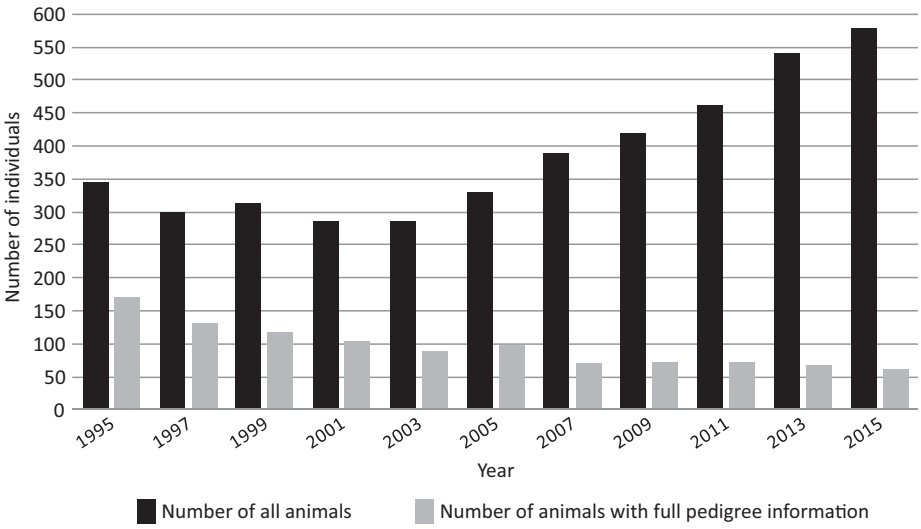

FIGURE 2. The number of all individuals belonging to the Lowland line (including Pszczyna line) of the captive part of European bison population, and individuals with full pedigree information in the years from 1995 to 2015

to the Lowland-Caucasian line. It is also optimistic that during these years the percentage of the smallest herds (up to 5 individuals) decreased, and the percentage of large herds – over 20 individuals, increased. Our analysis shown that animals belonging to Lowland line are a minority among all captive wisents – only 27.67% by the end of 2016. Currently the Polish population represents a significant part of all LB bison, and is undoubtedly the largest population of this line living in one country. Over the years, the number of countries other than Poland, which took part in Lowland line breeding, has grown steadily (Fig. 3). In 1995 (Raczyński 1995–2016) the animals of the Lowland line were kept in 11 countries apart from Poland, but only 9 out of 29 (31%) of foreign herds kept only wisents of LB line. Most of the foreign herds consisted of LC animals with only single LC individuals. In time those proportions have changed. For example, the German herd in Nürnberg in 1995 had 6 wisents, of which only 2 were representatives of the Lowland line. In 2005, there were 4 wisents, all from Lowland line. In 2015 there were already 8 animals also only from the Lowland line. Also, the herd in Springe (Germany) in 1995 consisted of 6 animals from Lowland line and 24 animals from Lowland-Caucasian line. In 2005 there were 29 LC animals, and in 2015 two separate herds with both LB (13 animals) and LC (19 animals) lines. In 1995 Polish population was 53% of all animals living in captivity. There were 18 captive herds in Poland. In 2005, 16 countries besides Poland bred Lowland line. Only 13 out

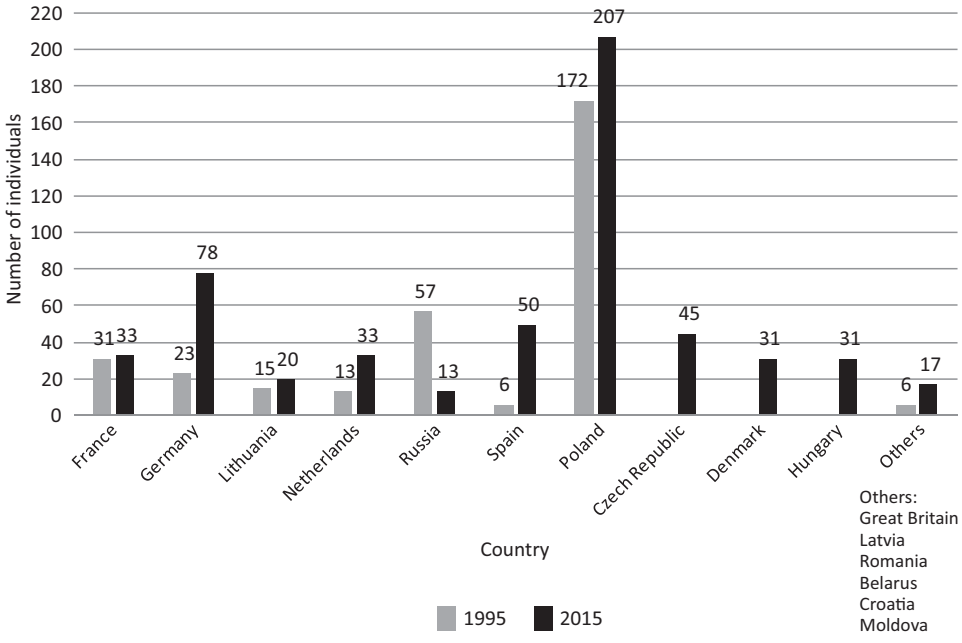

FIGURE 3. Distribution of captive European bison of Lowland line in 1995 and 2015 among European countries

of 35 (37%) breeding centers maintained only the LB animals. In Poland, there were 17 herds which members accounted to 53% of all LB wisents kept in captivity. In 2015, wisents of the Lowland line were maintained in 15 countries, including Poland. Foreign herds with only Lowland line wisents represented 75% of all herds. The Polish population in 2015 accounted for 36% of all captive LB animals. We can observe a positive change in the development of foreign breeding places, and a decrease of herds where both breeding lines remain mixed. Some of the herds has changed their status from captive to free or semi-free ranging. For example, the herd in Cherga (Russia) in 1995 was regarded as a captive one but in 2015 it became a semi-free herd, because it was not possible to obtain the pedigree data for such large group of animals with uncontrolled mating.

Pedigree analysis

There is a significant difference in the inbreeding coefficient between individuals living in 1995, 2005 and 2015. Over the 20 years of this population history, there is a significant increase in the average value of inbreeding coefficient among the individuals with full pedigree information living in subsequent years. Significant increase of coancestry among individuals in the population are also observed (Table 1) and decrease in completeness of pedigree data (Table 2). In 1995 average inbreeding coefficient equalled to 0.4299 (computed for 169 individuals with full pedigree information, including 26 from Pszczyna line). In 10 years it has grown to 0.4869 (computed for 76 individuals with full pedigree information, including 15 from Pszczyna line). Between years 2005–2015 this was

TABLE 1. Changes of analysed parameters in years 1995, 2005 and 2015 in Lowland line of captive *E. bison* population, determined for animals with full pedigree information, including Pszczyna line

Item		1995			2005			2015		
Number of individuals		169			76			62		
Statistics		$\bar{x}$	*SD*	dominant	$\bar{x}$	*SD*	dominant	$\bar{x}$	*SD*	dominant
Mean kinship		0.3816	0.098	0.3225	0.4201	0.0568	0.486	0.5318	0.0923	0.5849
Inbreeding coefficient		0.4299	0.1024	0.5209	0.4869	0.1139	0.3517	0.5704	0.103	0.6034
Contribution of founders genes	45	0.5707	0.0979	0.6953	0.5826	0.1087	0.6836	0.6478	0.0902	0.6948
	42	0.3007	0.0261	0.3047	0.2919	0.0192	0.3047	0.2997	0.0136	0.3047
	87	0.0278	0.0242	0	0.0205	0.0211	0	0.0084	0.0163	0
	89	0.0278	0.0242	0	0.0205	0.0211	0	0.0084	0.0163	0
	15	0.0182	0.0205	0	0.0211	0.0216	0	0.0089	0.0176	0
	16	0.0365	0.0409	0	0.0422	0.0431	0	0.0178	0.0353	0
	147	0.0182	0.0205	0	0.0211	0.0216	0	0.0089	0.0176	0

Parameters were calculated for individuals being alive in analysed years.

TABLE 2. Changes in population size and completeness of pedigree data in years 1995, 2005 and 2015 in the captive part of the Lowland line of European bison

Item	1995	2005	2015
Number of individuals (M, F)	342 (124, 218)	320 (116, 204)	577 (215, 362)
Average percentage of known pedigree	67.43	61.65	57.31
Number of individuals with known 100% of pedigree (% of the population)	169 (49.42)	76 (23.75)	62 (10.75)

an increase of 0.0835, from 0.4869 in 2005 to 0.5704 in 2015 (only 62 animals with full pedigree information, including 32 from Pszczyna line). Therefore, in the past 20 years (1995–2015), the average value of inbreeding coefficient increased by 0.1405 (32.68%). Data given in Table 1 represent relatively high standard deviation. Although these values apply to individuals with full pedigree information from Lowland line (including Pszczyna line), living in analysed years, some of those animals could be included in the analyses more than once.

Contribution of the seven founders to the contemporary genetic pool of European bison was never equal. The most represented in the population are genes of the male 45 PLEBEJER and female 42 PLANTA. This dominance has been already proved in many papers (Olech 2003, Krasińska and Krasiński 2004, Tokarska 2010). Equally represented in the population are genes of the founders 15 BEGRÜNDER and 147 BISMARCK. The 87 BILL and 89 BILMA have the same, the lowest representation in the gene pool. Over the years 1995–2015 the share of male 45 PLEBEJER increased while the share of genes 42 PLANTA, 87 BILL and 89 BILMA decreased. The representation of individuals 15 BEGRÜNDER, 16 PLAVIA and 147 BISMARCK increased by year 2005, but decreased again in 2015 (Table 1). Genes of these three founders were transferred only by female 524 BESTE (Krasiński 1994), which inherited 25% genes from male 15, 50% from female 16 and 25% from male 147. That is why males 15 and 147 have always the same representation in the population and female 16 is represented twice stronger.

All individuals living in analysed years were included in Table 2.

A large part of individuals with full pedigree information in the 2015 population (51.61%), were representatives of the Pszczyna line. It is a highly inbred line separate within the Lowland line. It derives only from two founders, male 45 PLEBEJER and female 42 PLANTA. For many years, the herd in Pszczyna was isolated and today it becomes an interesting research object (Krasińska and Krasiński 2004, Pigan and Wójtowicz 2015). Such high proportion of representatives of this herd among a captive part of the Lowland line could result in a serious bias in the calculation of the level of inbreeding coefficient. Estimation of average inbreeding performed for the entire Lowland line, including wisents from Pszczyna, does not reflect the true increase of inbreeding in the remaining part of the population, that is managed to minimize the rate of inbreeding.

Generally in subsequent years, the number of wisents with 100% known pedigree declines (Table 3). Average value of inbreeding coefficient among the individuals from Pszczyna line is higher than for the whole Lowland line (Fig. 4). However among animals from Pszczyna line, changes in the inbreeding coefficient over the last 20 years are lower and remain within the range from 0.55 to 0.63. During the same time, the average value of inbreeding coefficient,

TABLE 3. Individuals with full pedigree information for which the average inbreeding coefficient was calculated in 1995–2015

Line	1995	1997	1999	2001	2003	2005	2007	2009	2011	2013	2015
Lowland	143	107	102	95	77	61	52	47	47	40	30
Pszczyna	26	24	16	8	12	15	17	24	24	27	32

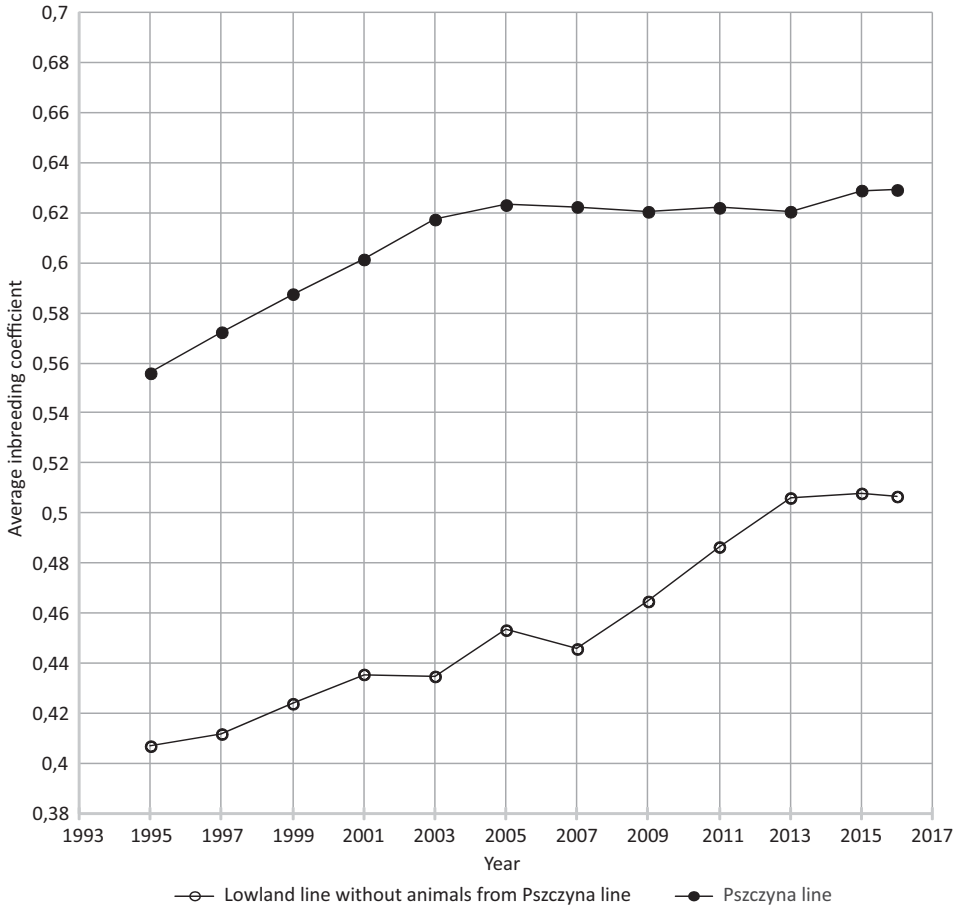

FIGURE 4. A comparison of average values of inbreeding coefficient among wisents belonging to Lowland line living between 1995 and 2016

among the individuals from Lowland line, ranged between 0.41 and 0.51. The highest and constant increase of inbreeding was observed between the years 2007–2013. The value of inbreeding coefficient has grown then from 0.45 in 2007 to 0.5 in 2013. All individuals from Lowland line born in this period of time, with full pedigree information, were offspring of the male 9368 PLISZAJ (Pszczyna line) and five females from Lowland line. Coancestry between these animals is high (from 0.6016 with female 8714 PODPINIA; to 0.6628 with female 9148 POTOWA), and that is why the average inbreeding coefficient was growing so quickly. In these years, the male 9997 PLAWAN born in Pszczyna in 2003, with full pedigree information, could be used for reproduction. Coancestry coefficients between him and five, already mentioned, females were lower (0.5864 with female 8714 PODPINIA; 0.6231 with female 9367 POLUCJA) than between these females and male 9368 PLISZAJ. However the male 9997 PLAWAN, died in 2012 in Karolew, leaving no offspring. Six individuals born between 2013––2015 (five born in Pszczyna and one in Jabłonowo) are the offspring of the male 11053 PLUDAR and five other females. Coancestry between these animals is lower than in previously described matings. It is coefficient takes values from 0.5669 (with female 8714 PODPINIA) to 0.5948 (with female 11771 POTYNKA).

CONCLUSIONS

An increase of inbreeding in a small population that has gone through a very radical reduction in numbers is unavoidable. An importance of preservation of genetic variability is raised almost in every study on European bison conservation (Strategy 2007, Olech 2008, Perzanowski 2016). Therefore every European bison herd should actively participate in the exchange of individuals. In the years 1995–2015, there is a positive change in the structure of *E. bison* herds of the Lowland line. There are more of large herds (more than 5 individuals). Also the number of such herds outside of Poland has increased. Exchange of animals among breeding centers in various countries can be observed. Individuals suitable for further breeding should be selected on the basis of their inbreeding coefficient, and more importantly – the mean kinship value. However the correct estimation of these parameters is possible only on the basis of complete pedigree information leading to founders of contemporary population (Olech 2008). The observed decrease in pedigree completeness may be an impediment to the use of these criteria for the selection of genetically valuable individuals. If individuals with gaps in pedigree information are considered for further breeding, it should be remembered that the parameters calculated on the basis of incomplete pedigree are underestimated. Therefore the only solution in such cases remain methods based on molecular genetics.

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Streszczenie: *Dwadzieścia lat ochrony linii nizinnej żubra* Bison bonasus bonasus *w niewoli.* Celem pracy było prześledzenie zmian zachodzących w populacji żubrów linii nizinnej utrzymywanych w niewoli w latach 1995–2015. Materiał do badania stanowiły dane z księgi rodowodowej żubrów zebrane z lat 1924–2015. Analizie podlegały takie parametry, jak: współczynnik pokrewieństwa, współczynnik kinship, udział genów założycieli oraz kompletność informacji rodowodowej. Analizie podlegała także struktura demograficzna populacji. W ciągu ostatnich 20 lat obserwowany jest stały wzrost liczebności populacji. Zmienił się także udział poszczególnych krajów w hodowli żubrów linii nizinnej. Wykazano, że w latach 1995–2015 znacząco spadła dokładność informacji rodowodowej. W 1995 roku 169 z 342 zwierząt utrzymywanych w niewoli miało pełną informację rodowodową. W 2005 roku było to już jedynie 76 z 320 zwierząt. W populacji żyjącej w 2015 roku jedynie 62 z 577 (10%) zwierząt miało pełną informację rodowodową, taką która prowadzi do założycieli populacji. Obserwowany jest także wzrost średniego współczynnika inbredu i średniej pokrewieństwa w populacji.

Słowa kluczowe: żubr, *Bison bonasus bonasus*, genetyka populacji, hodowla w niewoli

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Management of hunting animals population as breeding work. Part I: Impact of hunting and breeding work on animal conditions

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Abstract: *Management of hunting animals population as breeding work. Part I: Impact of hunting and breeding work on animal conditions.* The paper shows that rational management of game populations is a set of breeding practices. These mainly involve creation of appropriate conditions that will be most beneficial for normal development and reproduction of animals. However, game breeding is considerably more difficult and hunters face problems that differ from those encountered by breeders of domesticated animals. This part is focused on hunting work that can determine and primarily improve the ontogenic quality of animals. Unlike in livestock breeding, the size of the home range and living conditions of game animals can be improved by enrichment of the feed and shelter base, regulation of the population size by culling weak/diseased individuals, and minimization of stress factors. The results confirming the impact of the hunting and breeding treatments are illustrated in a population of roe deer.

Key words: breeding, hunting management, ontogenic quality

INTRODUCTION

The term "breeding" is usually used with reference to livestock animals. As specified by the Act of 2007 on organization of breeding and reproduction of farm animals, breeding is a set of practices targeted at improvement of the hereditary traits (genotype) of livestock, which comprises assessment of utility and breeding values of farm animals, selection, and choice of individuals for mating in normal breeding conditions. Hunting management is a breeding practice but it is more difficult than farm breeding, since game animals cannot be directly influenced and controlled by humans. Game breeding does not involve typical breeding treatments, there are no breeding registers or books, and the animal mating and reproduction are not controlled. Nevertheless, man can improve the ontogenic quality of game inhabiting a given area in a specific way, e.g. by regulation of the population size of species through reduction or selective culling. The impact on the density of game animals is of great importance in natural hunting grounds, as it facilitates management of habitat, thereby reducing stress associated with excessive density. The basis of game breeding is to provide an adequate living space and an appropriate sex and age structure in animal populations.

THE MAIN COMPONENTS OF GAME MANAGEMENT

Rational hunting management consists mainly in creation of appropriate conditions that will be most beneficial and ensure normal development and reproduction of game animals to great extent. The protection and management of hunting animal resources are regulated by the Hunting Law (1995). The most important statutory targets of hunting management in relation to wild animals include:

- protection, preservation of diversity and rational exploitation of hunting animal populations;
- protection and modelling of the natural environment aimed at improvement of the living conditions;
- maintenance of an appropriate population size of hunting animal species – regulation of the number of animals and maintenance of environmental balance.

This indicates that the two major objectives of hunting management are associated with modelling of an appropriate game living habitat. For persistence, individual species need specific ecological living conditions, primarily related to the nutritional and lifestyle requirements. An appropriate environment for deer (Cervidae) is provided by forest and field habitats. The amount and quality of feed available to animals varies between different regions. The feed base provided by the groundcover and understorey in mixed forest complexes growing on rich soils differs from that available in dry pine forests. Therefore, the basic analysis of the possibilities and principles of game management in a specific region should involve determination of the species for which the area will offer optimal conditions and the mode of providing the species with adequate living conditions. Another step should consist in determination of the appropriate density of animals, which is the allowable number of animals of a given species per area of land that can inhabit a hunting complex, with the provision that the damage caused by these animals to cultivated fields or forests will be economically tolerable, i.e. until there is a need for application of technical methods of protection (Haber et al. 1977).

Hunting mammals classified as the so-called big game are herbivores (except for the omnivorous wild boar). A detailed analysis of deer feed has shown that, depending on the season of the year, shoots, buds, leaves, needles and bark of trees and forest shrubs account for 35–75% of the total feed intake. Besides nutrition, animals need appropriate shelter conditions to reproduce, raise their offspring, and avoid danger, e.g. posed by predators. Foresters and hunters are able to shape and improve the animal living conditions. Enrichment of the feed base is necessary mainly in forest hunting grounds, as field habitats offer sufficient amounts of available food. In field areas comprising large-scale cultivations, creation of shelters is more necessary (Haber et al. 1977).

Breeding and hunting practices that can bring long-term benefits to game animals include enriching the species composition to tree stands, management of mid-forest meadows, and establishment of hunting plots. Reconstruction of the tree stand by planting undergrowth or understorey species serves not only animals but also forests. Forest

monocultures usually offer scanty feed and shelter bases. Reestablishment of the tree stand by introduction of climax species yields a plant community with greater resistance to diseases and weather conditions, thereby improving the living conditions of animals, which become an integral part of the habitat. Additionally, more rapid methods can be employed, e.g. enhancement of the productivity of mid-forest meadows by regulation of water relations, appropriate fertilization, planting appropriate species, and proper management. Foresters and hunters engaged in wild animal breeding can diversify the composition of animals' diet by establishment of special feed and browse plots, i.e. areas with sown or planted vegetation serving animals as stump feed. Particularly important is the introduction of winter crops in order to provide food rich in nutrients and water in the most demanding period, i.e. winter. This method not only increases and diversifies the natural feed base but also stops animals within tree stands, thus limiting damage to forest and field crops. The so-called feed-shelter areas among the hunting plots deserve attention. The basic goal of these plots is to provide animals with sufficient protection (Haber et al. 1977). When animals are provided safety in a given area, it is advisable to introduce plant species that will serve as their food. One of the methods to achieve this breeding goal may be the introduction of mid-field tree planting in the agricultural landscape. The method proved to be effective in single-species tree stands, in which understoreys or biocoenotic foci were introduced. Given the specificity and distinctness of agricultural ecosystems, the biocoenotic role of balks, mid-field refugia, or roadside shrub and tree plantings should be taken into account and exploited (Sporek and Sporek 2016). These small, forested areas surrounded by cultivated fields with intensive agriculture constitute flora and fauna refugia and "emergency stores" for species, which can restore their population in favourable circumstances. With their multi-layered vegetation system, they constitute the most effective shelters for free-living animals and provide sites of rest, birth, migration, and offspring rearing (Sporek 2002). Additionally, they provide a shelter at the time of human activity associated with conducting agrotechnical treatments and crop harvesting. Mid-field tree plantings are therefore one of the species-richest refugia in the agricultural landscape (Sporek 2009).

When there is a thick and persistent snow-cover, particularly when additionally covered by ice, winter crops, heather patches, or berry shrubs can be uncovered and paths can be created without startling game animals. This will enable the animals to obtain food and move around the refugium, thus preventing unnecessary losses of energy while searching and digging for food. Besides appropriate shelter and feed conditions, game animals should be provided with access to water. By taking care of natural waterholes or properly constructed artificial reservoirs, hunters can make water available to wild-living animals (Haber et al. 1977).

In this way, man can manage, to some extent, the habitat, i.e. an area where the animal's daily activities take place. It has its temporal and spatial dimension (Dzięciołowski 1994). By way of

comparison, the home ranges of wild-living animals correspond to livestock's cowsheds or piggeries, and enrichment of the feed and shelter base and management of the habitat as an element of targeted wild animal breeding is one of the most important practices, as indicated by the examples described above.

Additionally, the feeding of game animals must be rational. The practice of feeding animals in the hunting management has different importance than in the case of livestock animals. Game animals have adapted to the changing climate and prepare to adverse conditions by intensive feeding and fat storage in autumn, development of a denser and warmer hair coat, or migration into more favourable areas. Nevertheless, by feeding wild animals, i.e. enriching rather than replacing their natural feed base, man can contribute to improvement of their health. There are conflicting opinions on the question whether feeding wild animals is beneficial and indispensable or rather harmful and unnecessary; yet, this practice is widely applied in many regions of the world (Gill 1986, Putman and Staines 2004). In the case of game animals, the reasons why such practices are implemented are specified in the literature as (1) maintenance or an increase in the body weight and improvement of the wintering conditions; (2) enhancement of the effectiveness of reproduction and rearing; (3) increased winter survival rates; (4) maintenance of a high density of hunting animals and improvement of the trophy quality; and (5) reduction of damage in forestry and agriculture (Calenge et al. 2004, Geisser and Reyer 2004, Putman and Staines 2004, Sahlsten et al. 2010). In some cases, feeding may have a positive effect in view of the risk of infection with some diseases, as it improves the resistance of animals otherwise weakened by stress, age, or drought (De Vos et al. 2001), as many parasites and disease vectors tend to attack undernourished individuals (Cunningham-Rundles et al. 2005).

Both practices described above, i.e. establishment of home ranges and feeding, are specific elements of zoohygiene in the conventional livestock breeding. Game animals inhabit not only forest complexes but also fields. The structure of agricultural crops is constantly changing, and there is an increasing proportion of large-scale maize and wheat cultivation. In such monocultures, some animal species find not only food but also good protection conditions. Continuous human interference in the environment and mild winters contribute to a steady increase in the animal population size. Hence, the third purpose of hunting, i.e. management of an appropriate population size in a specified area, should be borne in mind. However, the intended regulation of the number of game animals through culling should be carried out taking into account the variability of the dynamics of the population present in the habitat on a local scale as well as the heterogeneity of the environment, diversity of forest ecosystems, microclimate, and presence and density of developments.

All deviations, i.e. excessive or insufficient density, substantially change the reproduction rate and pose stress to animals. This information is of great importance for the design of optimal harvesting of animals (Krupka 1989). As written by Krawczyński in his handbook for foresters and hunters (1947):

"the uncritical accusation of unnecessary killing of innocent animals on hunts is ridiculous, as game animals were created to be used; yet, there is a need for the awareness that animal reproduction and hygiene depends on rational and appropriate culling, either by hunting or breeding-selective practice". Without proper selective culling "roe deer undergo degeneration and bucks exhibit regress of antlers". In the case of elks, which "wander over long distances with no return (…), at a certain maximum population size in a given area, a further increase in the number of the animals is unnoticeable".

The regulation of the game population size by culling should proceed in an analogous way as livestock culling. These practices should be aimed at retaining the healthiest and fittest animals in the hunting grounds to ensure the best genes in the subsequent generations. The principle is to eliminate any deviations from the breeding target from the population. It is mandatory that diseased animals as well as those that clearly do not fulfil the body and antler weight criteria and the oldest individuals should be eliminated (Dzięciołowski 1994). Besides the selective culling, the practice of the so-called sanitary culling is employed in the case of occurrence of a disease or its threat (The act on the protection of animal health and the control of infectious animal diseases of 2004). The primary and fundamental task will always be to maintain populations of free-living animals in good health. An optimal population size in a given area prevents transmission of diseases. Growing numbers of wild animals can contribute to the spread of vectors of parasites into new areas in the natural environment where they have not been present before. Infections with new species of parasites are a very dangerous phenomenon and can lead to falls of a large number of animals (Burliński et al. 2011).

In game breeding, excessive density of animals should be prevented, as it results in constant stress associated with a shortage of food and proper shelters, which may lead to deterioration of immunity, increased prevalence of diseases, weakness, and mortality (in winter). Overpopulated herds with excessive numbers of females and juvenile animals exhibit new appetitive (e.g. imitative, consummatory) behaviour influencing the health status. Overpopulation in large ungulates has a negative impact on the function of forest ecosystems and causes local damage to forest crop cultivation, thus exacerbating the economic hunting-related problem of damage (Szukiel 1994).

Such elements as density, habitat quality, genetic structure, climate etc. are regarded to be key predictors of the body size in even-toed ungulates. It is highly important that managers of nature should pay particular attention to the dependence of animal body size on living conditions and, in the case of hunting animals, to the hunting ground and harvesting sizes (Zannese et al. 2006). One of the parameters of an appropriate density of wild ungulates in the habitat is the ontogenic quality reflected in e.g. body weight and size, antler quality, and reserves of adipose tissue (Czyżowski et al. 2008). At an appropriate quality of hunting grounds and controlled harvesting, the animal fitness can be improved and the population size can even be restored, as in the case of the red deer and elk in Poland.

Well-nourished animal females give birth to healthier and stronger offspring in the subsequent generation, while males develop stronger antlers and pass on better genes. Therefore, the body or carcass weight is one of the most important indicators of the ontogenic quality of animals. It depends on many factors, e.g. the species, sex, age, or physiological status of the animal (Bobek et al. 1984). In many game species, body weight varies not only throughout their lifetime but also in the different seasons of the year. The differences can be induced by various external factors such as weather conditions and anthropopressure or can be associated with the behaviour and physiological condition of the animal (oestrus, pregnancy, lactation etc.). The effects of these factors can be modified be regulation of the appropriate density of individual animal species in a given area.

The status of the native game species, i.e. the red deer, roe deer, or elk, in Poland underwent substantial changes in the past. This was often the result of human activity, which exerted a direct and indirect impact on the population size and distribution of wild-living animals.

IMPACT OF BREEDING-HUNTING PRACTICES ON THE ROE DEER POPULATION

The European roe deer (*Capreolus capreolus* L.) is the most numerous representative of the Cervidae family in Poland and Europe. The species is characterised by high plasticity and, hence, can live in different habitats. The roe deer thrives in open areas, treeless areas and arable fields, lowland and mountain areas, and large and small forest complexes (Pielowski 1999).

The roe deer, which is the least stress--resistant species of all Polish cervids (Reimoser 2012), exhibits population variability on a local scale, in a relatively small area, and within a short time. Animal falls are frequently noted in the case of food shortages and adverse weather conditions. However, there have been no large fluctuations in the population size of this species in recent years. Thanks to feeding, appropriate regulation of the population size in culling practice, and minimisation of stress by frequent harvesting of this species in individual hunts, the population size of roe deer it did not undergo a drastic decline. Slight fluctuations numbers of this species have not led to the need to undertake actions to rebuild populations.

At the turn of the 20th and 21st centuries, the population size of roe deer in Poland exhibited an upward trend. However, the population of this species has recently stabilised at approximately 800 thousand individuals (797 thousand in spring 2015). Roe deer harvesting per unit area, i.e. the density of these animals in individual regions of the country, is more levelled than in the case of other Cervidae (Panek and Budny 2015).

The examples mentioned below present the variability of selected morphological features of European roe deer related to improvement of the ontogenic quality of certain populations resulting from the biological traits of the species and implemented breeding-hunting practices. The investigations have shown a varied ontogenic quality of bucks in Poland determined from the body and antler

weight (Chrzanowski 1977, Fruziński et al. 1982, Dziedzic 1991, Żurkowski and Chartanowicz 1998, Drozd et al. 2000). As specified by Bergmann rule, the mean body mass increases from 12 kg in southern Europe and 16–20 kg in Poland to ca. 30 kg in northern Sweden (Pielowski 1999, Brzuski et al. 1997).

The investigations conducted in Poland confirm this rule, and the weight of adult animals is often similar or higher than the upper limit of this range. In a small area near Kraków, the mean carcass weight (without the head) was 15.1 kg in the second year, 17.3 kg in the third year, and 17.9 kg over the fourth year of animals' life. Another parameter analysed was the mean skull weight, which was in the range of 316.4–407.1 g in roe deer from Miechów Upland and Proszowice Plateau and 272.7–328 g in this species from the Jurassic Landscape Parks. An analogous trend was observed for of the antler weight in males. In individuals in the third and in the fourth year of life, the heaviest antlers were developed by individuals from Miechów Upland and Proszowice Plateau (mean 361.9 g), whereas the lowest and shortest antlers were noted in the Jurassic Landscape Parks (approx. 340 g and by 2.9–2.6 cm shorter) (Wajdzik et al. 2007).

As shown by these examples, the environment managed by humans has a major impact on animal body weight and, hence, their ontogenic quality (Dziedzic 1991). The bucks characterised by the highest body weight and the best parameters analysed represent a field ecotype from the area north of Kraków (Miechów Upland and Proszowice Plateau), in contrast to the roe deer living in the Jurassic Landscape Parks. Animals from field ecosystems are fitter and heavier on average by ca. 1.2 kg than forest animals (Fruziński et al. 1982, Pielowski 1993, Brzuski et al. 1997, Wajdzik and Jamrozy 2001).

Comparison of the mean carcass weight of the roe deer harvested in the around of Kraków showed that it was similar to the values reported from West Volhynia, Roztocze, Opolszczyzna, Piska Primeval Forest, Lubelszczyzna, or surroundings of Poznań. The mean weight buck carcass from Opolszczyzna, Piska Primeval Forest, and Poznań (forest ecotype) was 12.8 kg, 14.9 kg, and 16.3 kg in two-year-olds, three-year-olds, and over four-year-olds, respectively. In turn, these values reported from West Volhynia, Roztocze, and Lubelszczyzna were 15.3 kg, 17.3 kg, and 18.9 kg, respectively (Szczerbiński et al. 1972, Dziedzic 1991, Chartanowicz et al. 1992, Żurkowski and Chartanowicz 1998, Wajdzik and Jamrozy 2001). The animals living in eastern Poland are characterised by the best ontogenic quality in the country.

It has been proved that the resistance to seasonal changes in the availability of food resources is reflected by the size of the body and, simultaneously, the size of the skeleton. In particular, the length of the mandible is an important indicator in many Cervidae species. Larger mandibles have been found in animals living in better conditions. The greatest increase in the mandible length is observed between birth and the first year of life, and up to four years, when the length can increase by approx. 1 cm per year. This suggests that environmental conditions in which a young animal lives have a great impact on its final size and fitness. The mandible length was also compared with the density of roe deer in the southern part

of Belluno (Italy). It was found that the density value decreased from the north southwards of this region and was 0.44 and 0.33 animals per 1 km^2, respectively. The harvesting rate declined throughout the study period in the southern, which may have contributed to an increased density and a slight reduction of the mandible length in subsequent generations. In turn, the same harvesting rate was maintained in the north and the length of the bone in fawns was constant or increased minimally (Zannese et al. 2006). This has evidenced that, besides the living environment, animal density has a great impact on animal fitness and the regulation of the animal population size may be successfully carried out by man. Additionally, one parameter can be used to assess whether the management of Cervidae populations in a given area is conducted properly.

CONCLUSIONS

There are considerable effects of human interference on the populations of free-living animals. This study has demonstrated that hunting can bring positive effects in the management of the spatial, sex, and age structure of animals, thereby determining the ontogenic quality of individuals. Due to the possibility of reduction of the population size, e.g. by culling, the density of individuals in a given area can be regulated, which results in development of stronger antlers by males or giving birth to healthier offspring by females. Furthermore, the side effects (e.g. economic) of the density regulation practice include reduction of damage to hunting grounds and minimisation of road collisions. Nevertheless, it should be borne in mind that the regulation of the animal population size should be carried out based on knowledge of the quality of the biotope, number of inhabiting animals, and the number of natural enemies of the animals, since optimal densities may vary between ecosystems, depending on the species and habitat.

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Streszczenie: *Gospodarowanie populacjami zwierząt łownych jako hodowla. Część I: Wpływ prac łowiecko-hodowlanych na kondycję zwierząt.* W pracy wykazano, że racjonalne gospodarowanie populacjami zwierząt łownych to zespół zabiegów hodowlanych. To przede wszystkim tworzenie odpowiednich warunków, najbardziej korzystnych, odpowiadających w możliwie największym stopniu właściwemu rozwojowi i rozmnażaniu się zwierzyny. Hodowla zwierząt łownych jednak jest o wiele trudniejsza, a myśliwi napotykają się na problemy zupełnie inne niż hodowcy zwierząt udomowionych. W części tej zwrócono uwagę na prace łowieckie, dzięki którym można kształtować, a przede wszystkim poprawiać kondycję osobniczą zwierząt. Porównując hodowlę zwierząt dzikich do udomowionych, areały osobnicze zwierząt dzikich można poprawiać poprzez wzbogacanie bazy żerowej i osłonowej, regulowanie liczebności poprzez odstrzał osobników słabych/chorych czy też minimalizowanie czynników stresogennych. Wyniki potwierdzające wpływ zabiegów łowiecko-hodowlanych przedstawiono na populacji sarny.

Słowa kluczowe: hodowla, gospodarka łowiecka, kondycja osobnicza

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Influence of silver and copper nanoparticles on *Staphylococcus aureus* biofilm formation

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Abstract: *Influence of silver and copper nanoparticles on* Staphylococcus aureus *biofilm formation.* The purpose of this study was to investigate the effect of silver and copper nanoparticles on biofilm formation by *Staphylococcus aureus*. The bacteria cells were treated with silver and copper nanoparticles in increasing concentrations (5, 12.5, 25 μg/mL). Characteristics of nanoparticles was defined by measurement of zeta potential, size distribution and TEM analysis. Biofilm formation by *S. aureus* was identified by crystal violet staining and SEM analysis. The results showed that both nanoparticles inhibited the ability to form biofilm by *S. aureus*. However, in all used concentrations, silver nanoparticles had stronger effect than copper nanoparticles.

Key words: *Staphylococcus aureus*, biofilm, copper nanoparticle, silver nanoparticle

INTRODUCTION

Biofilm is a complex multicellular structure formed by bacteria and other microorganisms. The biofilm structure is surrounded by a layer of extracellular matrix composed of extracellular polymeric substances produced by microorganisms. Cells in biofilms are adhering closely to each other and also to biological or abiotic surfaces (Stoodley et al. 2002, Hall-Stoodley et al. 2004). This structure may be formed by one or more bacterial species and the cells in biofilm are physiologically different from the planktonic cells of the same microorganism (Stoodley et al. 2002). Nowadays biofilms generated by bacteria are considered to be an important factor in the development of chronic endodontic (Choi et al. 2018) and skin diseases (Sonesson et al. 2017), as wells as neoplasms including gallbladder cancer (Di Domenico et al. 2017). Furthermore, close adhesion to each other and the surface make bacteria highly resistant to bactericides and antibiotics (LewisOscar et al. 2015), leading to chronic and acute illnesses (Habash et al. 2017).

Nanoparticles which are within the area of interest of nanobiotechnology are used in many fields of science (Pulit et al. 2011), including medicine (Sawosz Chwalibog et al. 2014) Nanoparticles are the particles in size from 1 to 100 nm, which have unique properties when compared to their parent material. This results from a different distribution of electrons on their surface, and therefore they are characterized by greater reactivity (Sawosz Chwalibog et al. 2014).

Many of the metallic nanoparticles showed a proven antibacterial effect. The most popular are nanoparticles of: platinum (Pt-NPs), copper (Cu-NPs), zinc (Zn-NPs), gold (Au-NPs) and silver (Ag-NPs) (Pulit et. al. 2011). Pt-NPs, Ag-NPs and Au-NPs had harmful effects on both yeast and bacterial strains for example *Staphylococcus aureus* (Chwalibog et al. 2010), while Zn-NPs generate reactive oxygen species (ROS) which damage the cell membrane of bacteria (Vimbela et al. 2017).

Many studies sought to establish a mechanism of action of antibacterial activity exhibited by silver in both colloidal and ionic form. Loss of membrane functionality resulting from interaction between released Ag^+ ions and the cell membrane, as well as extensive cell membrane damage caused by the formation of ROS, ultimately causes damage to the cell due to oxidative stress (Belluco et al. 2016). It has been proven that Ag-NPs inhibited growth of different types of microorganisms such as bacteria (*Pseudomonas aeruginosa*, *Staphylococcus aureus*), yeasts (*Saccharomyces cerevisiae*) and algae (*Chlorella protothecoides*) (Dorobantu et al. 2015). It has been shown that they can bind to the cell wall of bacteria and then penetrate it causing damage leading to changes in cell membrane permeability and cell death (Prabhu and Poulose 2012). In addition, Ag-NPs may be the cause of free radicals formation (Kim et al. 2007). They can also be a source of free silver ions that inhibit crucial bacterial enzymes leading to the death of Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria (Yoshinobu et al. 2003). Habash et al. (2017) suggested that Ag-NPs enhanced tobramycin's activity against the biofilm-forming *Pseudomonas aeruginosa* (Habash et al. 2017). Moreover, Ag-NPs inhibited *Klebsiella pneumoniae* biofilm formation. It was also proven that Gram-negative bacteria are more sensitive to Ag-NPs than Gram-positive (Qayyum et al. 2017). Qayyum et al. (2017) showed 80 and 75% inhibition of *E. coli* and *S. mutans* biofilm formation, respectively.

Nanoparticles of Cu-NPs are also widely used. Chatzimitakos and Stalikas (2016) showed that Cu-NPs disturbed some of the metabolic pathways of the Gram-positive strains such as *S. aureus* and Gram-negative strains such as *Escherichia coli*. Cu-NPs had also an inhibiting effect on *Pseudomonas aeruginosa* (LewisOscar et al. 2015) and *Listeria monocytogenes* (Ghasemian et al. 2015) biofilms. The same effect was observed against biofilm formation in Cooling Water Systems (Ogawa et al. 2016). In addition, *in vitro* analysis showed more than 60% inhibition of biofilm formation by *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Aeromonas hydrophila* in presence of Cu-NPs (Chari et al. 2017). Beside the interference with metabolic pathways, Cu-NPs caused the damage of the bacterial cell membrane (Vimbela et al. 2017). Cu-NPs had also the negative effect on fungi such as *Aspergillus flavus* (Essa and Khallaf 2016).

The objective of this study was to determine the effect of Ag-NPs and Cu-NPs on *S. aureus* biofilm by measurement the cell viability and scanning electron microscope analysis (SEM). Characterization of the nanoparticles was obtained by survey of zeta potential, size distribution and also by determina-

tion of the shape and size of individual nanoparticles by transmission electron microscope (TEM).

MATERIAL AND METHODS

Bacteria culture

Bacteria strain *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 25923™) was obtained from LGC Standard (Łomianki, Poland). The strain was stored in 20% glycerol solution at –20°C. In next stage, cells were thawed and washed with distilled water to remove glycerol and next transferred to nutrient broth medium (Bio-Rad, Warsaw, Poland), sterilized in an autoclave (Classic 2100, Prestige Medical, Chesterfield, UK). Bacterial cultivation was conducted in strain specific conditions (aerobic atmosphere in 37°C) in incubator shaker (SI500, Stuart, Stafford, UK) with shaking speed set on 70 rpm for 24 h.

Preparation and characterization of copper and silver nanoparticles

Nanoparticles of Ag-NPs and Cu-NPs were obtained from Nano-Tech (Warsaw, Poland) and they were produced by electric nonexplosive patented method (Polish Patent 3883399) from high purity metals (99.9999%) and high purity demineralized water. The suitable amount of the powder of nanoparticles was suspended in ultrapure water to receive a concentration of 50 μg/mL.

Size distribution and zeta potential of nanoparticles in water (50 μg/mL) were measured by the dynamic light scattering and electrophoretic method using a Zetasizer Nano ZS, model ZEN3500 (Malvern Instruments, Malvern, UK). Each sample was measured in three replicates after 120 s of equilibration at 25°C.

Nanoparticles TEM analysis

The shape and size of individual nano_particles was determined by TEM. Amount of 10 μL of Ag-NPs and Cu-NPs suspension in ultrapure water (50 μg/mL) were placed onto formvar--coated copper grids and allowed to dry. Dried grids were placed in TEM and observed with the JEM-2000EX TEM at 80 keV (JEOL, Tokyo, Japan). The Morada 11 Mgpx camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany) was used to capture the images.

Biofilm formation assay

Staphylococccus aureus cells stored in a refrigerator were used to set up a night culture of bacteria. Amount of 100 μL bacterial suspension were transferred to a flask with 10 mL of sterile nutrient broth (Bio-Rad, Warsaw, Poland) and placed in 37°C in Incubator Shaker (SI500, Stuart, Stafford, UK).

Amount of 10, 25 and 50 μl Cu-NPs and Ag-NPs suspensions were applied in triplicate on a 96-well plate. Then the plate was allowed to dry for 24 h on Mini-Shaker (PSU-2T, Biosan, Riga, Latvia) placed in the laminar flow chamber. After drying, 100 μL of the overnight bacterial culture suspension were added into the wells. Final concentrations of the nanoparticles were 5, 12.5, 25 μg/mL. The plate was placed in the incubator for 48 h. Simultaneously, wells containing no nanoparticle suspensions were prepared. Afterwards, planktonic bacterial cells

were removed by rinsing three times with distilled water. Subsequently, 200 µL of methanol (POCH, Gliwice, Poland) were added to the wells and incubated at room temperature for 15 min. After incubation, the alcohol was removed and the plates were allowed to dry. Then 200 µL of crystal violet staining (Sigma-Aldrich, Munich, Germany) were added to determine the number of biofilm forming bacteria. After 15 min incubation, the dye was rinsed under running tap water and the plates were left to dry. Amount of 200 µL of 33% acetic acid (POCH, Gliwice, Poland) solution was added to the dried wells. Afterwards, the absorbance of the solutions in each well was measured at 570 nm using a microplate reader (Infinite M200, Tecan, Durham, NC,USA).

The amount of biofilm-forming bacterial cells was expressed as an optical density (OD), which is correlated to the number of bacterial cells in biofilm.

Biofilm SEM analysis

Amount of 2 mL of bacterial culture (10^6 CFU/ml) were incubated on sterile cover glass coated with Ag-NPs and Cu-NPs, or untreated bacteria were deposited on the surface of a cover glass without nanoparticles and incubated for 24 h at 37°C inside Petri dish. All samples were dried and covered with a gold. Finally, the samples were imaged with SEM (FEI Quanta 200, Tokyo, Japan) at an acceleration voltage of 15 kV.

Statistical analysis

Obtained data were analyzed using one-way ANOVA with STATGRAPHICS® Plus 4.1. Differences with $P < 0.05$ were considered statistically significant. Results were presented as means with standard deviations.

RESULTS AND DISCUSSION

The analysis of size distribution of Cu--NPs and Ag-NPs (the table) showed that both materials occurred as aggregates. An average diameter of the aggregates of Cu-NPs was 839.7 nm while average diameter of Ag-NPs – 211.1.nm. This results showed that Cu-NPs form more prominent aggregates than Ag-NPs.

The zeta potential measurements (the table) presented that both Ag-NPs and Cu-NPs had a negative potential but were not stable. The zeta potential for Cu-NPs and Ag-NPs was –14.3 and –25.6 mV, respectively, and it indicates that Ag-NPs has less ability to generate aggregates and therefore greater stability and better quality.

TABLE. The size distribution and values of zeta potential of copper nanoparticles (Cu-NPs) and silver nanoparticles (Ag-NPs) in concentration 50 µg/mL

Tested material	Size range; average size of aggregates (d.nm)	Average zeta potential (mV)
Cu-NPs	3–15; 839.7	–14.3
Ag-NPs	10–40; 211.1	–25.6

Size of individual nanoparticles was determined on the basis of TEM images and it was 10–40 nm for Ag-NPs and 2–15 for Cu-NPs (Fig. 1), what confirmed that only Cu-NPs were forming aggregates.

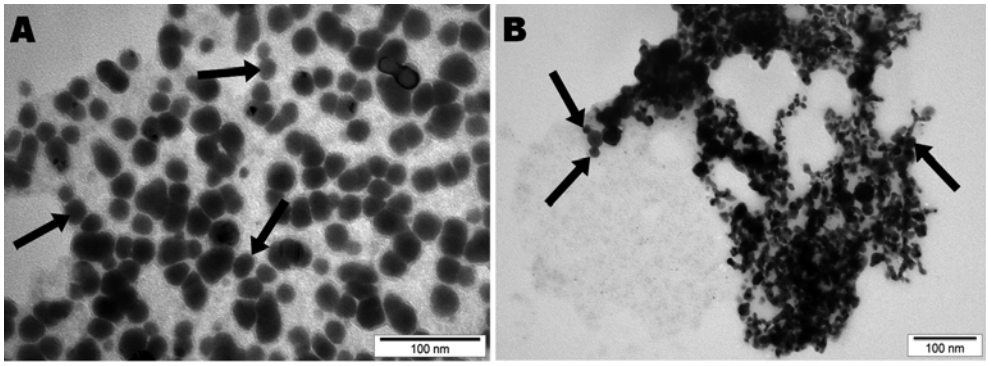

FIGURE 1. A transmission electron microscopy (TEM) image of A – Ag-NPs, B – Cu-NPs, in concentration 50 µg/mL. Nanoparticles were indicated by arrows

Previous preliminary studies proved the toxic effects of Ag-NPs on a various microorganisms. Qayyum et al. (2017) showed that Gram-positive bacteria are more resistant to Ag-NPs than Gram--negative. They designated a minimum inhibitory concentration of 16 and 8 µg/mL, respectively. In our study we showed that Ag-NPs in concentration 5 µg/mL suspension inhibit the formation of *S. aureus* biofilm by more than 70% (Fig. 2). The most significant inhibition of biofilm formation was recorded for the highest concentration of Ag-NPs suspension (25 µg/mL). The inhibitory activity may be due to the ability of Ag-NPs to damage the cell membrane (Prabhu and Poulose 2012) or to generate ROS (Kim et al. 2007). This effect has also been observed for other Gram-positive bacteria including *S. mutans* (Qayyum et al. 2017). We indicated that using the lowest concentration of Ag-NPs caused a significant decrease in

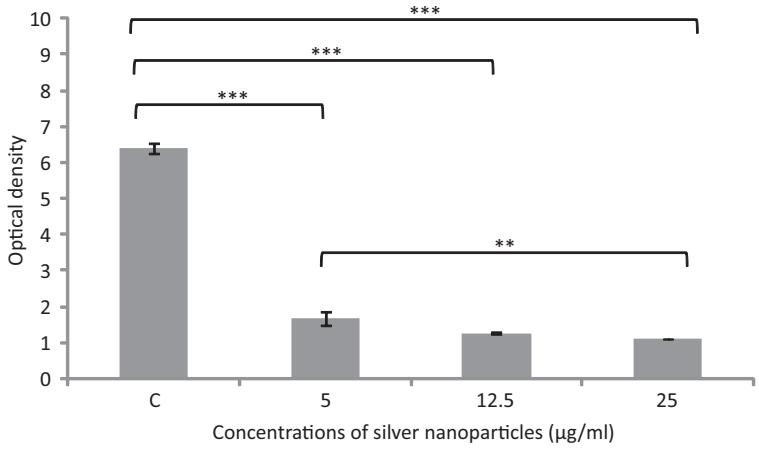

FIGURE 2. Effect of silver nanoparticles on *S. aureus* biofilm formation: C – control, 5, 12.5, 25 – concentration of solution of silver nanoparticles. Statistically significant differences were indicated as: $^{**}P < 0.01$, $^{***}P < 0.001$

the number of biofilm forming bacteria. We observed statistically significant differences between control group and all tested concentrations and the effect caused by the concentration of 25 µg/mL was the most prominent. It suggests that the relationship between the concentration of Ag-NPs and *S. aureus* ability to biofilm formation is not linear.

Nanoparticles of Cu-NPs have shown an antibacterial activity (Pulit et al. 2011). Essa and Khallaf (2016) showed that Cu--NPs had a better effect on Gram-positive than Gram-negative bacteria in concentration 50 µg/mL. In addition, Cu-NPs influenced *S. aureus* cells by induction of a negative effect on some bacterial metabolic pathways (Chatzimitakos and Stalikas 2016). In our study, Cu-NPs also had an observable and statistically significant inhibitory effects on *S. aureus* bacteria in all tested concentrations. The most prominent effect was observed at concentration 25 µg/mL of Cu-NPs – almost 70% inhibition of *S. aureus* biofilm formation. It can be related with ability of Cu-NPs to damage bacterial cell membrane (Vimbela et al. 2017). This effect of Cu-NPs was also observed against *Listeria monocytogenes* biofilm formation (Ghasemian et al. 2015). We observed a directly proportional effects between biofilm formation and the concentration of nanoparticles (Fig. 3). We indicated that there is a noticeable inhibitory effect on *S. aureus* biofilm formation which is also dependent on used concentration of Cu-NPs and there are statistically significant differences between tested groups.

Biofilm SEM analysis has shown that both nanoparticles inhibit *S. aureus* ability to form biofilm. In control group large number of bacteria cells were forming compact biofilm structure, in contrast to experimental groups (Fig. 4). In group with Ag-NPs we observed less number of bacteria cells comparing to Cu-NPs, which is in accordance to Ruparelia et al. studies (2008). In addition, in our study

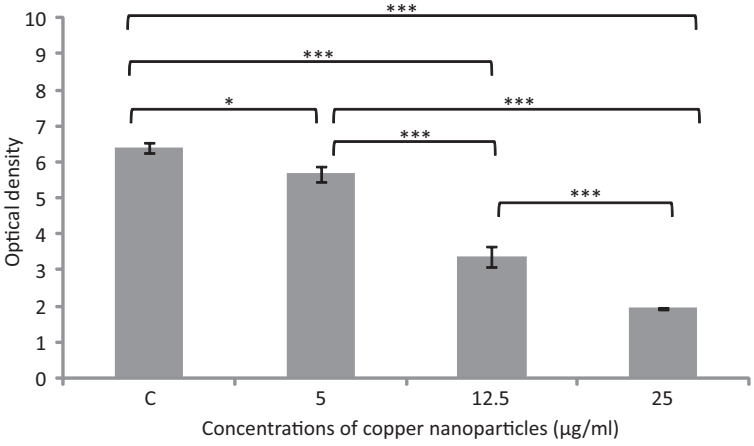

FIGURE 3. Effect of copper nanoparticles on *S. aureus* biofilm formation: C – control, 5, 12.5, 25 – concentration of solution of copper nanoparticles. Statistically significant differences were indicated as: **P* < 0.05, ****P* < 0.001

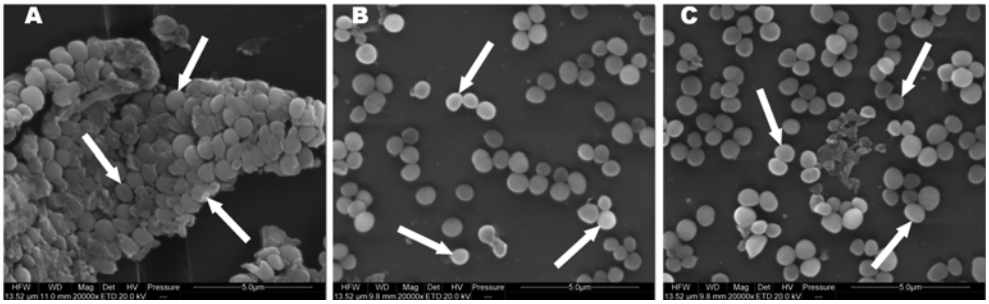

FIGURE 4. A scanning electron microscopy (SEM) image of *S. aureus* biofilm formation: A – control, B – Ag-NPs, C – Cu-NPs. Nanoparticles were in concentration 25 µg/mL. Bacteria cells were indicated by arrows

showed that Ag-NPs have better anti-biofilm activity than Cu-NPs.

We proved that both Ag-NPs and Cu-NPs decreased the number of biofilm forming bacteria *S. aureus*. However, Ag-NPs had a stronger effect on bacteria than Cu-NPs at all tested concentrations (5, 12.5, 25 µg/mL). We obtained similar effect of biofilm formation inhibition for the lowest used concentration of Ag-NPs (5 µg/mL) and the highest concentration of Cu-NPs (25 µg/mL).

Even though the effect of Ag-NPs was noticeable between control and tested groups, the differences between tested concentrations were not so prominent as it was observed for Cu-NPs. It can be related to different mechanism of action of this nanoparticles or results from differences in size distribution. Therefore, further studies are necessary to explain this obtained effect.

CONCLUSION

In our study, we showed that both Cu-NPs and Ag-NPs inhibit biofilm formation by *Staphylococcus aureus*. The effect of Ag-NPs was stronger than that of Cu-NPs, without significant differences between used concentrations of Ag-NPs. Our results indicate the potential use of Cu-NPs and Ag-NPs in medicine as a coating to prevent biofilm formation on surgical instruments or endoprostheses. In addition, such coatings could be used in the food and pharmaceutical industries where it is important to harness microbiological purity. However, due to the lower efficiency of Cu-NPs, application of higher concentrations is required.

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Streszczenie: *Wpływ nanocząstek srebra i miedzi na tworzenie biofilmu przez* Staphylococcus aureus. Celem pracy było zbadanie wpływu nanocząstek srebra i miedzi na formowanie biofilmu przez gronkowca złocistego. Komórki bakteryjne zostały potraktowane wzrastającymi stężeniami (5; 12,5; 25 μg/mL) nanocząstek srebra i miedzi. Charakterystyki nanocząstek dokonano przez pomiar potencjału zeta, rozkładu wielkości oraz analizy z wykorzystaniem TEM. Tworzenie biofilmu przez *S. aureus* zostało określone przez barwienie fioletem krystalicznym. Wyniki wykazały, że zarówno nanocząstki srebra, jak i miedzi hamują zdolność do tworzenia biofilmu przez *S. aureus* i analizę z wykorzystaniem SEM. Nanocząstki srebra mają jednak silniejszy efekt we wszystkich zastosowanych stężeniach od działania nanocząsteczek miedzi.

Słowa kluczowe: gronkowiec złocisty, biofilm, nanocząstki srebra, nanocząstki miedzi

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