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**Veterinary**

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# AN ANALYSIS OF CONSUMER ATTITUDES AND PREFERENCES CONCERNING PORK PURCHASES

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Key words: pork, meat selection criteria, consumers preferences.

Abstract

The aim of this study was to analyze consumer behaviors and preferences related to the consumption of pork, and to identify the main criteria that influence pork purchases. The study involved a Google survey questionnaire which was filled out online by 100 respondents, which the respondent individually marked the answers to the questions. The survey was anonymous. The respondents were city dwellers as well as residents of villages situated in the proximity of urban areas. The vast majority of the respondents consumed pork, and the content of intramuscular fat was the main factor limiting pork consumption. Despite the above, most respondents were of the opinion that fat improves meat palatability. Most respondents consumed pork on account of its flavor and nutritional value. The key factors influencing consumer preferences during pork purchase were freshness, absence of visible intramuscular fat (marbling) and absence of drip loss.

## Introduction

Pork accounts for 55–57% of meat consumed in Poland. In 2007–2011, pork consumption was stable at around 42.5 kg/person/year, and it decreased to 38.5/person/year in 2013 *(Krajowy Ośrodek…* 2017). According to the Institute of Agricultural and Food Economics – National Research Institute, pork consumption increased to 40.1 kg/person in 2016, whereas per capita consumption of beef was estimated at only 1.9 kg and poultry consumption – at 29.5 kg (*Rolpetrol. Giełda…* 2017). These data clearly

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indicate that pork is the preferred type of meat in Poland, and that periodic decreases in pork consumption result from changes in consumption preferences, consumer expectations and food promotion campaigns.

Pork plays an important role in the human diet. It is a rich source of nutrients, including complete protein and highly available micronutrients such as selenium, iron and zinc. Pork is also abundant in group B vitamins, vitamins A, D and E, as well as bioactive compounds (taurine, carnosine, ubiquinone, creatine, etc.) (KOŁODZIEJ-SKALSKA et al. 2016). The nutritional value and popularity of pork are considerably influenced by the content of intramuscular fat and the fatty acid profile (FLIS et al. 2010, FIEDOROWICZ et al. 2016).

Consumers purchase meat based on its sensory attributes and expected health benefits. The eating quality of pork is influenced by parameters such as color, tenderness (marbling) and pH which determine the acidity and water-holding capacity of meat. The color of meat is influenced by pH in 50% (COLE et al. 2005). The consumers pay special attention to the color of meat, because its changes determine the freshness of meat (JAWORSKA et al. 2006). The pink-red color of pork can be attributed to its myoglobin content which ranges from 0.7 to 1.1 mg $g^{-1}$. The myoglobin content of pork is 4-fold lower than in beef, but twice higher than in broiler chicken meat (BLICHARSKI et al. 2013).

Producers are making attempts to decrease the fat content of pork to cater to the growing demand for lean foods. However, these efforts compromise the quality and palatability of pork (MCAFEE et al. 2010). Intramuscular fat is responsible for marbling, but it also increases the tenderness and juiciness of meat and improves its taste and aroma (ŻAK 2010). Meat with a high content of intramuscular fat is also characterized by lower drip loss and desirable physicochemical properties, including water-holding capacity.

The main factor which discourages consumers from buying pork is its cholesterol content and, consequently, the conviction that pork does not deliver health benefits because it increases the risk of cardiovascular diseases and atherosclerosis (BIESALSKI 2005). The cholesterol content of pork ranges from 40 to 85 mg/100 g (BLICHARSKI et al. 2013). Lifestyle diseases are not caused by cholesterol in itself, but by its oxidation products which have mutagenic and carcinogenic properties (CROSS et al. 2007). The risk of heart and cardiovascular diseases increases with the consumption of diets rich in saturated fatty acids and diets with an unhealthy ratio of n-6 to n-3 fatty acids (HU 2001, HENDERSON et al. 2008). Factors that influence a product's nutritional value, eating quality and processing suitability, including pig breed, genotype, feeding regime, pre-slaughter handling and carcass processing, have to be taken into account in the process

of developing new meat products (WOOD et al. 2004, CZARNIECKA-SKUBINA et al. 2007, RAJ et al. 2010, MILCZAREK, and OSEK 2017).

For the average consumer, the quality of products is characterized by a number of distinguishing factors, which they pay attention to when buying pork (KOSICKA-GĘBSKA, GĘBSKI 2014). The process of choosing pork by the consumer depends primarily on the visually appraised traits of meat often connected to the place of purchase and purchase conditions (POŁOM, BARYŁKO-PIKIELNA 2004).

The aim of this study was to analyze consumer behaviors and preferences related to the consumption of pork, and to identify the main criteria that influence pork purchases.

## Materials and Methods

The study involved a Google survey questionnaire which was filled out online by 100 respondents. The survey was anonymous. It contained 12 closed-ended questions and 5 demographic questions relating to the respondents' age, gender, place of residence, education and professional status (Table 1).

Table 1

Responder demographics ($n$ = 100)

| Specification | | Share [%] |
|---|---|---|
| Gender | female | 59 |
| | male | 41 |
| Age (years) | 20–30 | 22 |
| | 31–40 | 20 |
| | 41–50 | 18 |
| | 51–60 | 20 |
| | > 60 | 20 |
| Education | primary school | 12 |
| | secondary school | 26 |
| | vocational school | 28 |
| | university | 35 |
| Place of residence | rural area | 41.8 |
| | urban area | 58.2 |
| Professional status | student | 9.3 |
| | blue collar worker | 36.1 |
| | white collar worker | 38.1 |
| | disability/ old-age pensioner | 17.5 |

Closed-ended questions concerning consumer behaviors and attitudes included the following types of questions:

– alternative questions where the respondent chooses one of the mutually exclusive answers (yes-no),

– single choice questions (disjunctive) and multiple choice questions (conjunctive),

– questions that filter out respondents who are not relevant to the target sample,

– scaling questions where the evaluated attitudes and preferences are ranked from the least to the most important.

The survey was conducted between 10 October and 27 November 2017 in the Regions of Warmia and Mazury, Kujawy, Wielkopolska, Śląsk, Mazowsze and Pomorze (Poland). The respondents were city dwellers as well as residents of villages situated in the proximity of urban areas. Women accounted for 59% and men – for 41% of the respondents. There were approximately 20 respondents in every age group. The majority of the respondents had university (35%), vocational (28%), secondary school (26%) and primary school (12%) education. The three latter categories were represented mainly by blue collar workers residing in rural areas.

The survey was preceded by a pilot study which was conducted in June 2017 on 20 randomly selected respondents from the Region of Warmia and Mazury and Kujawy. The study targeted respondents who fulfilled age and residential criteria. The pilot study was carried out to determine whether the questionnaire was comprehensible for the respondents. The results were used to modify the questionnaire for the needs of the survey. Selected questions were provided with explanations to guarantee that they were correctly interpreted by the respondents.

The questionnaires were analyzed and incomplete forms were eliminated from the study. The respondents who gave incomplete answers were replaced with new targeted subjects. The results were processed and presented graphically in Microsoft Excel.

The Shapiro-Wilka test was used to determine the impact of demographic factors such as: place of residence and gender of respondents on the choice of pork for consumption and to determine the link between the place of residence of respondents consuming pork and opinions regarding the selection of fatty pork, at the significance level $\alpha = 0.05$ (STATISTICA software ver. 10 PL).

## Results and Discussion

In the first question, the respondents were asked whether they consumed pork. Only 11% of 100 respondents did not eat pork, of which 9% were female and 2% were male. Most of the subjects who did not consume pork were city dwellers (9%) – Figure 1. In relation to the above question, the questionnaire was analyzed using the Shapiro-Wilk test to determine the impact of demographic factors, such as: place of residence and gender of respondents.

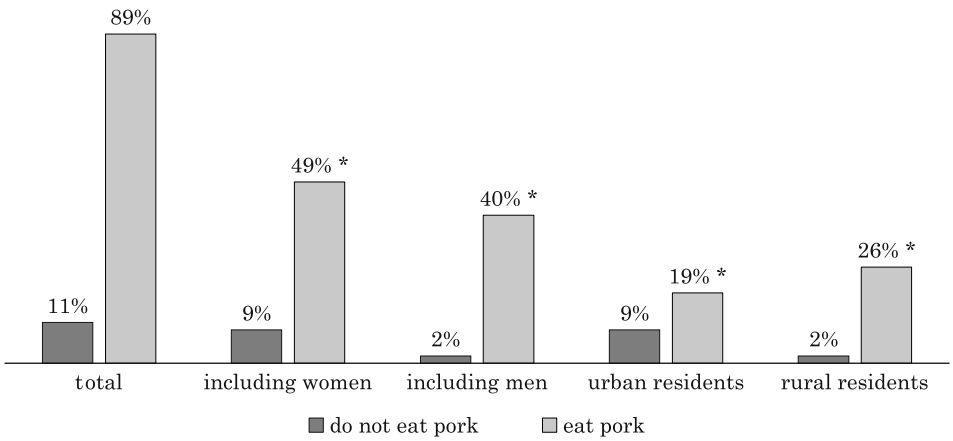


Fig. 1. The impact of demographic factors on the consumption of pork meat ($n$ = 89)
* Statistically significant value at $P \leq 0.05$

Based on the obtained data, it was found that there is a statistically significant relationship between the answers given to the question in the survey and the place of residence and the gender of respondents. The main reason for not eating pork was its health reasons and medical advice and this answer was given by 18.2% of the respondents in the above 60 age group and by 9.1% of the respondents aged 41–50 and 51–60 (Figure 2).

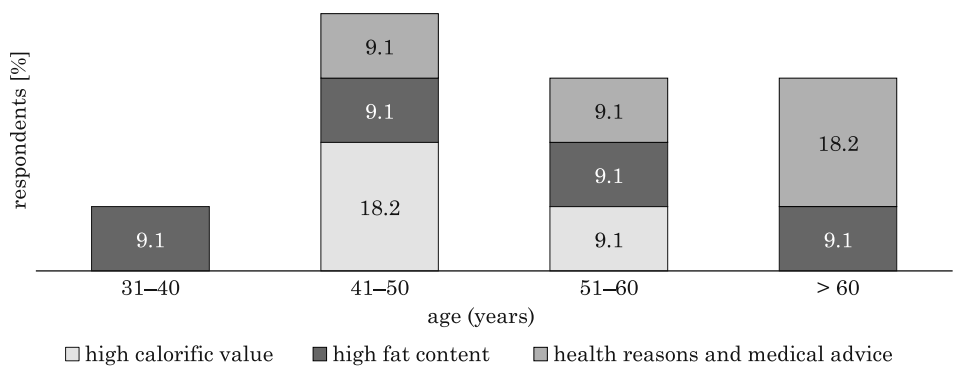


Fig. 2. Reasons for not consuming pork ($n$ = 11)

As a reason for this fact, respondents pointed to high cholesterol in pork and liver problems. The remaining questions were answered by respondents who consumed pork (89%), of which 49% were female and 40% were male. Pork was more frequently consumed by rural residents (26%) than by city dwellers (19%) – Figure 1.

According to a survey carried out by the Institute of Agricultural Sciences in Zurich in 1996, pork is most frequently consumed on account of its taste which gives a high perception of eating pleasure (ŻAK 2010). In a study conducted by SALEJDA et al. (2013), 39.6% of the respondents chose pork on account of its high nutritional value. Similar results were noted in the present study (Figure 3). Consumers were shown to consume pork mainly due to their taste (53.9%) and nutritional value (14.6%).

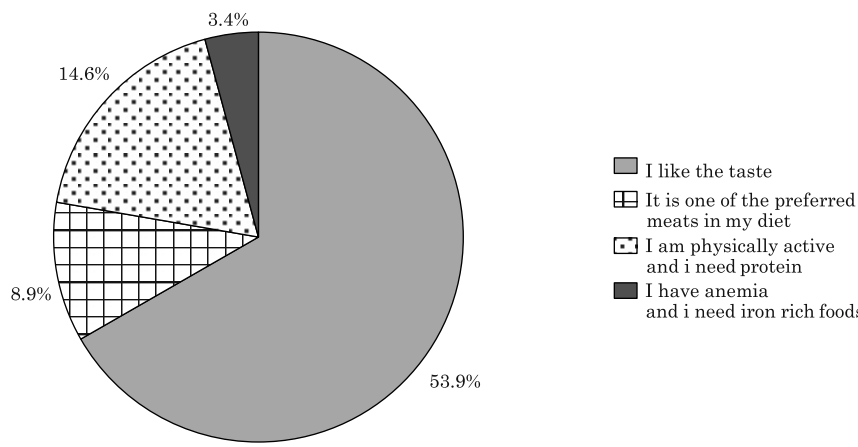


Fig. 3. Factors which contribute to pork consumption ($n$ = 89)

The fact that consumers are twice less likely to opt for pork as a rich source of nutrition can be attributed to health campaigns which encourage consumers to limit their pork consumption due to the risk of lifestyle diseases (KOŁODZIEJ-SKALSKA et al. 2016).

Knowledge of consumer preferences and opinions is very important in the food production process. According to JAWORSKA et al. (2006), ANTOSIK (2014), more than half of consumers pay attention to the quality of meat, mainly the content of visible intramuscular fat (around 17%), in their purchasing choices. The growing popularity of low-fat products stems from health concerns, but also limited knowledge about the health benefits of pork. In our study (Figure 4), female respondents were far more likely (85.71%) to pay attention to the fat content of meat than male subjects. The majority of respondents who had a preference for lean meat were city residents (50%), and only 33,33% of rural residents belonged to this group.

The percentage of consumers who disregarded the fat content of purchased meat was twice higher in rural areas (11.67%) than in cities.

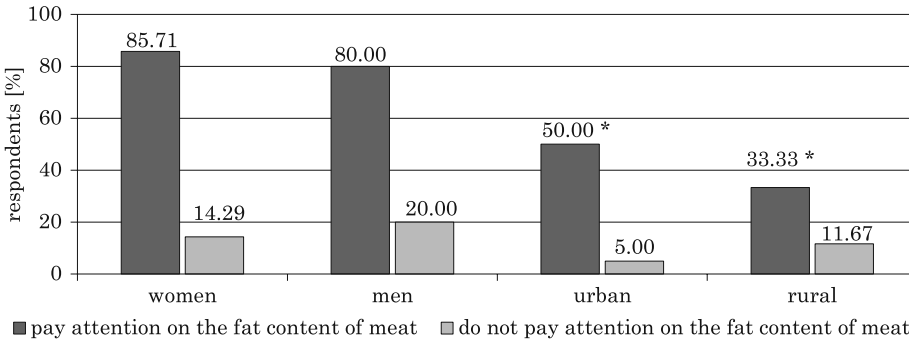


Fig. 4. Distribution of answers regarding the importance of fat content during meat purchases, given by respondents from different gender and residential groups ($n$ = 89)
* Statistically significant value at $P \leq 0.05$

The Shapiro-Wilk test was used to determine the degree of connection between the place of residence of respondents consuming pork and opinions regarding the choice of fat meat. It was shown that there is a relationship between the answers given to the question in the questionnaire and the respondents' place of residence (at $\alpha = 0.05$). Thus, there are statistically significant differences between the answers to this question among the residents of villages and cities.

In the next question, the respondents were asked to indicate whether they agreed with the presented statements (by giving yes or no answers) – Figure 5. More than half of the polled subjects (55%), including 33.8%

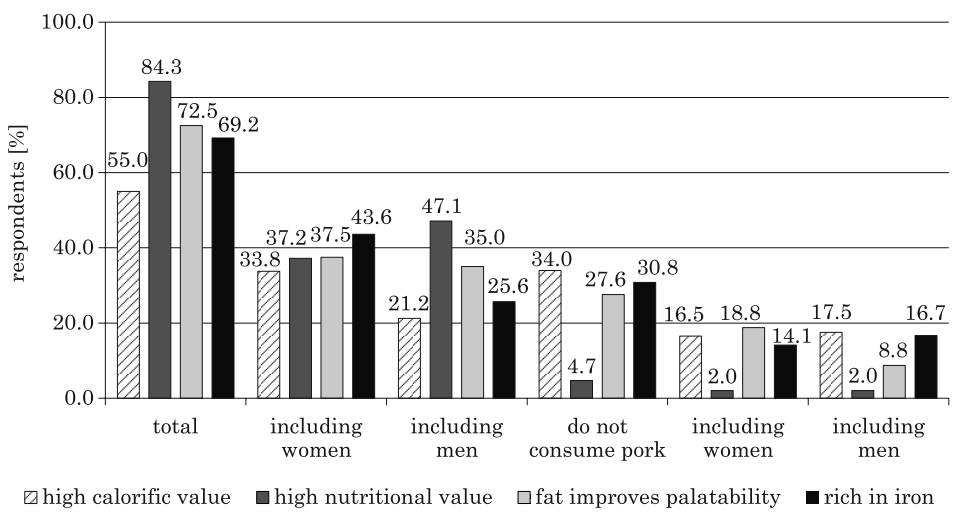


Fig. 5. Distribution of answers regarding pork consumption in gender groups ($n$ = 89)

women and only 21.2% men, were of the opinion that pork was high in calories. A total of 84.3 of the respondents were aware that pork has high nutritional value and is a rich source of complete protein. The above was an important selection criterion for 72.5% of respondents, including as much as 47.1% of men. Only 27.6% of the polled subjects did not agree with the statement that fat improves the palatability of meat. The above answer was given by 18.8% women who were convinced that low-fat foods delivered greater health benefits.

The answers given by the respondents when asked whether high fat content limited their pork purchases are presented in Figure 6. Only 47.2% of pork consumers were more likely to select lean cuts. City dwellers accounted for 36% of the respondents in the above group, whereas 16.9% of rural residents were not bothered by the high fat content of pork, and 10.1% of rural residents did not pay attention to the fat content of purchased pork. Fat content was a more important criterion for female (32.6%) than male (14.6%) respondents.

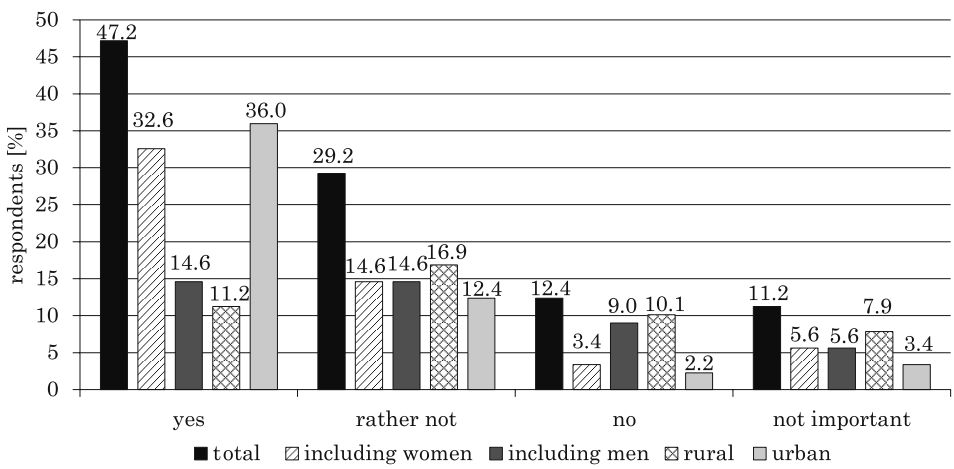


Fig. 6. Distribution of answers regarding the importance of fat content during meat purchases, given by respondents from different gender and residential groups ($n$ = 89)

The results of this study (Figure 7) revealed that 21.8% of the respondents older than 60 years regarded pork as a high calorie food despite the fact that most of them were of the opinion that fat increases the flavor of pork (16.3%). The average consumer pays attention to several quality indicators when purchasing pork (KOSICKA-GĘBSKA, GĘBSKI 2014). The high iron content of pork was more often recognized by younger respondents of reproductive age (Figure 7), mostly women (43.6%) who were of the opinion that pork is a rich source of this mineral. Respondents older than 60 years tend to observe dietary guidelines and restrict their intake of foods

with a high fat content and high calorific value. These consumers are aware that high-fat foods increase the risk of cardiovascular diseases, and that seniors have lower energy requirements (KOŁODZIEJ-SKALSKA et al. 2016).

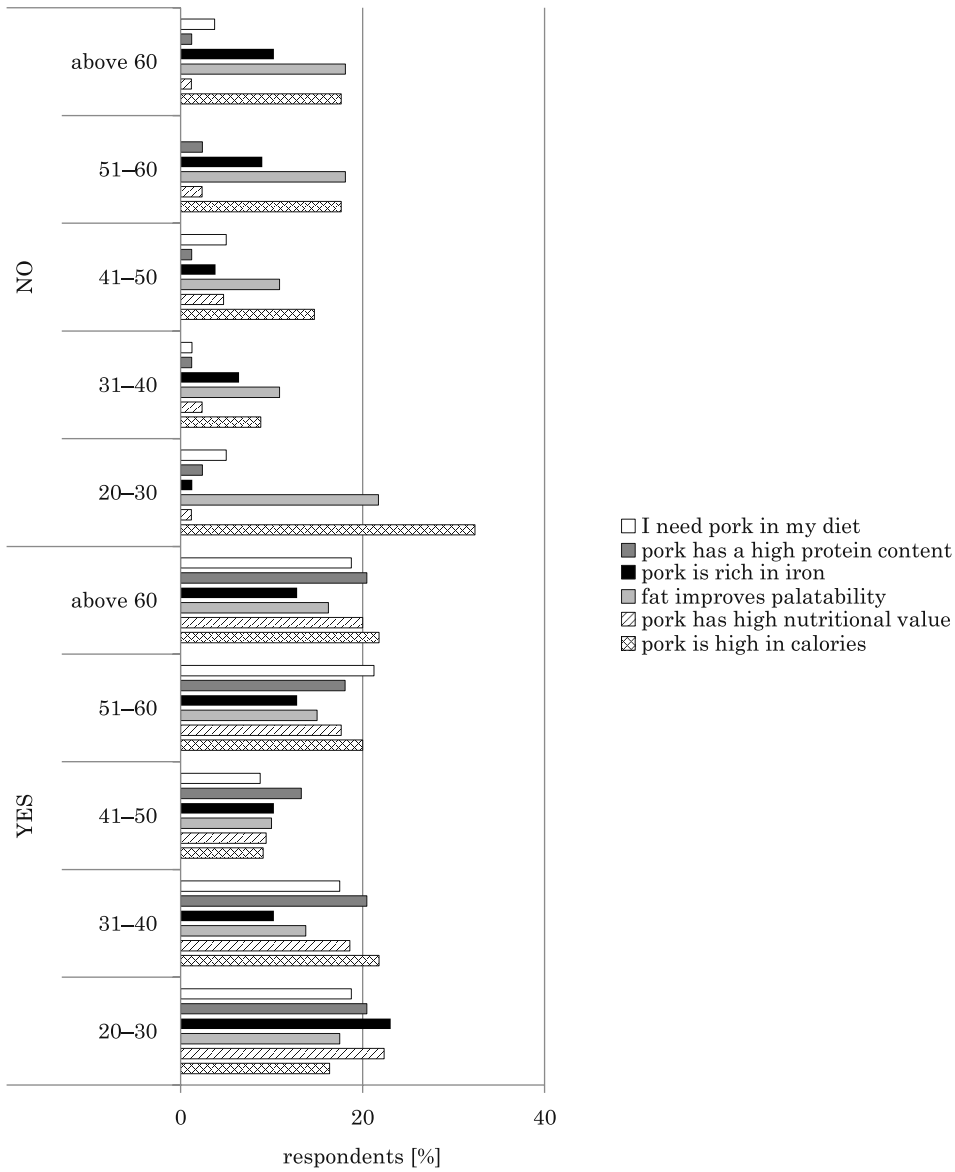


Fig. 7. Distribution of statements regarding pork given by respondents from different age groups ($n$ = 89)

Explain: Answer given by respondents to specified questions ,,YES" or ,,NO"

In the scaling questions, the respondents were asked to evaluate the importance of the key criteria which influence consumer perceptions of pork quality on a scale of 1 to 5 (1 – least important, 5 – most important) – Figure 8. According to the surveyed subjects, the most important criterion in the selection of pork was: freshness (64%), aroma (47%), lean content (42%), color (38%), hardness and fat content (35%). The second most important criterion was: drip loss (22%), lean content (21%) and fat content (17%).

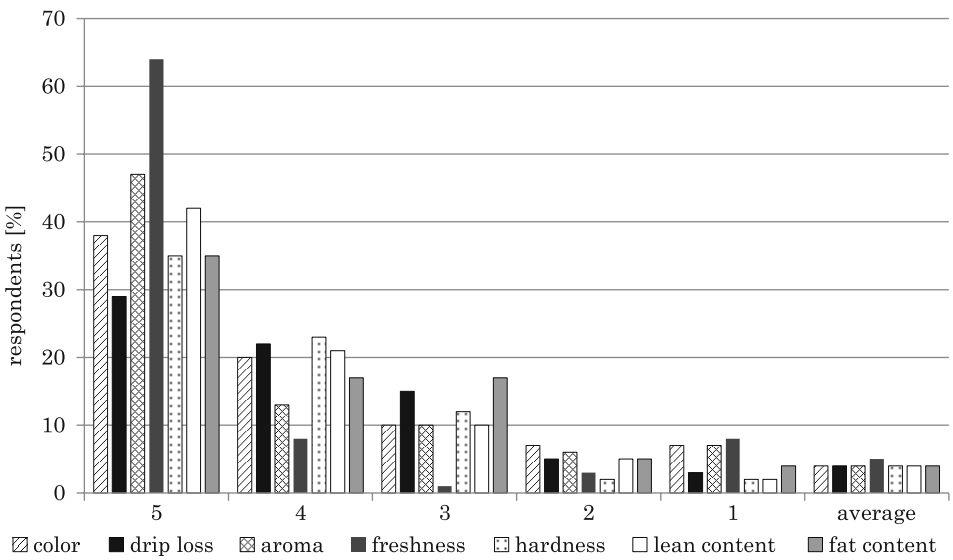


Fig. 8. The influence (1 – least important, 5 – most important) of selected attributes on consumer perceptions of pork ($n$ = 89)

These results indicate that freshness, absence of intramuscular fat (marbling) and absence of drip loss are the key determinants that influence the respondents' perceptions of pork quality. According to ANTOSIK (2014) (citing HUFF-LONERGAN, LONERGAN 2007), consumer acceptance of packaged meat with visible drip loss is low. ANTOSIK (2014) also pointed to a correlation between high drip loss and a low content of intramuscular fat. In a study by KOSICKA-GĘBSKA, GĘBSKI (2014), fat content was the second most important attribute determining the choice of pork after overall appearance (25% of indications). The results of the present study are consistent with the findings of other authors.

## Conclusions

1. It has been shown that the vast majority of respondents eat pork meat. Fat content was the main factor limiting the surveyed subjects' pork consumption. Despite the above, most respondents were of the opinion that fat improves the palatability of meat.

2. More than half of the respondents, mostly women, were of the opinion that pork has high calorific value. Despite the above, the polled subjects were aware that pork has high nutritional value and is a good source of complete protein.

3. Respondents older than 60 years tend to observe dietary guidelines and restrict their intake of foods with high fat content and high calorific value.

4. The attributes that were most likely to influence the respondents' pork purchasing decisions were freshness, absence of visible intramuscular fat (marbling) and absence of drip loss.

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# PHYSIOLOGICAL-BIOCHEMICAL PROPERTIES OF LEGUME SEEDS SUBJECTED TO LONG-TERM STORAGE

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Key words: natural seed ageing, vigour and viability, protein, catalase and guaiacol peroxidase activity.

Abstract

Preservation of high physiological-biochemical seed quality is of paramount importance for seed storage. The effect of prolonged storage (30 years at the temperature +20ºC and -14ºC) of legume seeds (three cultivars of lupin and three cultivars of faba bean) was studied in this paper. The impact on seed vigour, viability, protein content and profile, and the activity of catalase and guaiacol peroxidase was analysed. Seeds stored at -14ºC germinated at 73.3% to 100%, whereas seeds stored at +20ºC completely lost germinability. Seeds of faba bean cultivars (*Vicia faba* subsp. *minor* 'Nadwiślański' and 'Stego'), and yellow lupin (*Lupinus albus* L. 'Iryd') had the highest viability. Since seed storage at +20ºC caused degradation of proteins with higher molecular weights, low molecular weight peptides predominated, in seeds subjected to such treatment, whereas seeds subjected to cold storage remained rich in high molecular weight proteins. Seed storage at room temperature also caused decreased catalase activity. The activity of this enzyme in seeds stored at -14ºC was 3.00 U, whereas in seeds stored at room temperature it dropped to 0.23 U.

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## Introduction

Seed storability depends on plant species. Seeds can be divided into orthodox, recalcitrant, and intermediate, depending on their tolerance to desiccation. Seeds that develop tolerance to drying and can withstand low water content (usually below 5%) retaining high vigour and viability are considered orthodox (PAMMENTER and BERJAK 2000). Such seeds acquire tolerance to desiccation by accumulating storage compounds (some carbohydrates and proteins) and adjusting cell metabolism – inhibiting respiration and modifying organelle differentiation (NIEDZWIEDZ-SIEGIEN et al. 2004). Recalcitrant seeds, on the other hand, reach maturity while they remain hydrated and they lose germinability if subjected to drying (WOJTYLA et al. 2006). The generally narrow life span of recalcitrant seeds makes their long-term storage a difficult task whereas, for orthodox seeds low water content and low temperature are known as the key factors favouring seed storability (RAJJOU et al. 2008). Despite generally good storability of orthodox seeds, they also eventually lose germination capacity as a result of long-term stora ge. The deterioration of seed quality during storage is a consequence of physiological-biochemical processes, e.g. changes in enzyme activities, decreased contents of carbohydrates, proteins and nucleic acids. The cell repair mechanisms are not efficient enough to make up for these changes (KAEWNAREE et al. 2011).

Previous studies of seed ageing have mostly used accelerated ageing tests (EKSI and DEMIR 2011, MATTHEWS et al. 2010, OHLSON et al. 2010, PROCHAZKOVA and BEZDECKOVA 2009, AL-MASKRI et al. 2003). Reports on long-term seed storage experiments and the impact of such treatments on seed viability are scarceVarious species of *Picea* seeds were analysed after 35 years of storage at −20ºC and were determined to have 60% viability (SIMPSON et al. 2004). In addition to the germination capacity, seed water content and the storage temperature were recorded (FOURAR-BELAIFA et al. 2011, RAO et al. 2006). Analyses of biogenic amine content and profile, proteins and soluble carbohydrates in legume seeds have also been carried out (DOBIESZ et al. 2017, DOBIESZ and PIOTROWICZ-CIEŚLAK 2017).

The objective of this paper was to determine the vigour and viability of legume seeds (lupin and faba bean) stored for 30 years at the temperatures of -14°C and +20°C. Moreover, the contents of proteins, their SDS--PAGE profiles and activity of catalase and guaiacol peroxidase in stored seeds were determined.

## Materials and Methods

### Biological materials

The experiments were carried out on seeds of two yellow lupin cultivars (*Lupinus luteus* L. 'Iryd' and 'Manru'), one white lupin cultivar (*Lupinus albus* L.'Hetman') and three faba bean cultivars (*Vicia faba* subsp. *minor* 'Stego', 'Nadwiślański' and 'Dino'. The experiments were started in 1988 and the seeds were stored for 30 years at -14°C and +20°C in air-tightly closed glass jars with a capacity of 1 l. The containers were filled with seeds to ¾ of their volume.

### Seed vigour and viability

Seed vigour and viability were determined according to ISTA (2016) recommendations. To assess the viability, seeds were germinated for seven days on germination paper (Anchor Paper. USA) in a growth chamber (Sanyo incubator) at +20°C with 12-hour light provided by fluorescent tubes (840 lumens, Philips N.V., the Netherlands) and 12-hour darkness. Moreover, the fresh and dry mass of seedlings were determined after seven days of germination. The electroconductivity of seed leachates (exudates) was measured using a pH 211 meter (Hanna Instruments). For this purpose, the seeds were soaked for 24 hours in MQ water with initial electroconductivity of 0.03 μS.

### Protein contents and SDS-PAGE separations

Proteins were isolated from defatted seeds (20 mg) in 0.5 ml tris(hydroxymethyl)aminomethane, pH 8, containing 0.01 M $\beta$-mercaptoethanol. The extraction was carried out for one hour and the extract was centrifuged at RCF 11 000 g and a temperature of 4°C. The total protein content of the extracts was determined with the Bradford method (Bradford 1976). The analysed protein fraction was dissolved in a buffer containing Tris-HCl (0.0625 M, pH 6.8), SDS (2%), glycerol (10%) and 2-mercaptoethanol (5%), to reach a final protein concentration of 2 mg $ml^{-1}$. The samples were heated for 5 min in a water bath at 100°C. They were then cooled and loaded onto a 10% polyacrylamide gel (7.0 cm × 10.0 cm) and subjected to SDS-PAGE in a Mini PROTEAN Tetra System (Bio-Rad). The separation was performed at 200 V for 40 min. After electrophoresis, the gels were stained with colloidal Coomassie Brilliant Blue G-250 (Sigma Aldrich). The gel images were digitized with a Gel Doc EZ Imager (Bio-Rad) scanner and analysed with ImageLab (Bio-Rad) software.

### Guaiacol peroxidase activity assay

Seeds (100 mg) were homogenized with a porcelain pestle and mortar at 4ºC in 1 ml isolation buffer (0.01 M Tris-HCl (Sigma-Aldrich), 8.75% polyvinylpyrrolidone (Sigma-Aldrich), 0.1 M KCl (PPH Stanlab), 0.28% Triton X-100 (Sigma Aldrich)). The extract was centrifuged for 30 min at 2800 g, 4ºC. The protein content in the extracts was determined with the BRADFORD method (1976). The activity of guaiacol peroxidase was determined with a Cecil Aurius Series CE 2021 spectrophotometer (Cecil Instruments Ltd.). The extract (50 ul) and 25 ul of 0.06% $H_2O_2$ (Chempur) were added to 2 ml of the reaction mixture containing 0.1 M $KH_2PO_4$ (Chempur), and 100 µl 1% guaiacol (Sigma-Aldrich). The rate of absorption increase was measured at the wavelength $\lambda$ = 470 nm at room temperature and the oxidation of 1 µmole $H_2O_2$ in one minute was assumed as one unit of peroxidase activity (RYDZYŃSKI et al. 2017).

### Catalase activity assay

Seeds (100 mg) were homogenized in 1 ml phosphate buffer which contained 2% polyvinylpyrrolidone (Sigma-Aldrich), 0.02 mM EDTA (Sigma-Aldrich) and 10 ml $l^{-1}$ Triton X-100 (Sigma-Aldrich). The extracts were centrifuged for 20 min at RCF 12 000 g, 4ºC. The protein content in the supernatant was determined with the BRADFORD (1976) method. Catalase activity was measured using a Cecil Aurius Series CE 2021 spectrophotometer (Cecil Instruments Ltd.). The reaction mixture contained phosphate buffer (50 mM, pH 7) and $H_2O_2$ (15 mM). Changes in absorbance at the 240 nm wavelength were followed for 10 min at room temperature and the oxidation of 1 µmole $H_2O_2$ in one minute was assumed as one unit of catalase.

## Results and discussion

### Seed viability and vigour

Seed age is an important factor strongly affecting germination capacity and the ability to produce good root and stem growth (BRUTOVSKÁ et al. 2013). Seeds that had been stored for 30 years at -14ºC germinated at the level 73.3% – 100%. The highest germination capacity was found for faba bean 'Nadwiślański', 'Stego', and yellow lupin 'Iryd' seeds. However, the lowest germination capacity (73.3%) was observed in white lupin 'Hetman'. Seeds stored at +20ºC did not germinate at all (Table 1, 2).

Table 1

Vigour and viability, germination [%], root and stem length [cm], seedlings fresh and dry mass [g], water content [%] and electroconductivity [μS $g^{-1}$ fresh mass], of yellow lupin (*Lupinus luteus* L.) seeds 'Manru' and 'Iryd' and white lupin (*Lupinus albus* L.) seeds 'Hetman' stored for 30 years at + 20°C and -14°C

| Specification | | Lupinus luteus | | | | Lupinus albus | |
|---|---|---|---|---|---|---|---|
| | | 'Manru' | | 'Iryd' | | 'Hetman' | |
| | | +20°C | -14°C | +20°C | -14°C | +20°C | -14°C |
| Germination [%] | | 0 | 93.33±2.23 | 0 | 100 | 0 | 73.33 |
| Length [cm] | root | 0 | 8.571±2.742 | 0 | 7.733±2.868 | 0 | 11.512±6.046 |
| | stem | 0 | 4.514±1.148 | 0 | 2.887±1.712 | 0 | 4.195±1.897 |
| Seedling fresh mass [g] | root | 0 | 0.095±0.036 | 0 | 0.082±0.047 | 0 | 0.189±0.088 |
| | stem | 0 | 0.301±0.084 | 0 | 0.282±0.96 | 0 | 0.503±0.231 |
| Seedling dry mass [g] | root | 0 | 0.006±0.002 | 0 | 0.007±0.002 | 0 | 0.009±0.006 |
| | stem | 0 | 0.013±0.003 | 0 | 0.015±0.007 | 0 | 0.036±0.019 |
| Water content [%] | root | 0 | 92.81±1.734 | 0 | 88.981±3.315 | 0 | 95.501±2.128 |
| | stem | 0 | 95.327±0.793 | 0 | 94.585±2.694 | 0 | 92.786±2.079 |
| Electroconductivity [μS $g^{-1}$] | | 217.01 ±23.7 | 184.3±4.1 | 165.16 ±4.81 | 30.41±2.7 | 201.31 ±3.83 | 56.81±2.57 |

Table 2

Vigour and viability viability, germination [%], root and stem length [cm], seedlings fresh and dry mass [g], water content [%] and electroconductivity [μS $g^{-1}$ fresh mass], of faba bean (*Vicia faba* var. *minor*) seeds 'Stego', 'Nadwiślański' and 'Dino' stored for 30 years at +20°C and -14°C

| Specification | | *Vicia faba* var. *minor* | | | | | |
|---|---|---|---|---|---|---|---|
| | | 'Stego' | | 'Nadwiślański' | | 'Dino' | |
| | | +20°C | -14°C | +20°C | -14°C | +20°C | -14°C |
| Germination [%] | | 0 | 93.333 | 0 | 100 | 0 | 100 |
| Length [cm] | root | 0 | 7.323±4.321 | 0 | 8.44±3.526 | 0 | 7.1±3.088 |
| | stem | 0 | 2.125±1.149 | 0 | 2.793±1.456 | 0 | 2.513±1.282 |
| Seedling fresh mass [g] | root | 0 | 0.205±0.091 | 0 | 0.28±0.077 | 0 | 0.239±0.043 |
| | stem | 0 | 0.205±0.101 | 0 | 0.24±0.099 | 0 | 0.215±0.088 |
| Seedling dry mass [g] | root | 0 | 0.011±0.007 | 0 | 0.032±0.017 | 0 | 0.019±0.009 |
| | stem | 0 | 0.007±0.005 | 0 | 0.017±0.006 | 0 | 0.016±0.007 |
| Water content [%] | root | 0 | 95.635±2.154 | 0 | 89.074±5.329 | 0 | 91.574±4.934 |
| | stem | 0 | 97.247±1.444 | 0 | 93.799±2.565 | 0 | 92.302±7.106 |
| Electroconductivity [μS $g^{-1}$] | | 181.43 ±1.67 | 31.96±0.83 | 160.60 ±7.29 | 32.28±1.03 | 152.56 ±0.68 | 32.60±1.79 |

Seedling vigour assessments were based on mean root and shoot length. Mean root length (84.4 mm) was significantly higher than shoot length (31.7 mm). The longest roots (115 mm) were formed by white lupin 'Hetman' seedlings and the shortest roots (71 mm) by faba bean 'Dino'. The mean fresh mass of the roots (151 mg) was approx. twice smaller than the mean fresh mass of the stems (291 mg). Stems with the highest fresh mass were formed by white lupin 'Hetman' (503 mg), and roots with the lowest fresh mass (82 mg) developed in yellow lupin 'Iryd'. The highest dry mass of stem (36 mg) was recorded in white lupin 'Hetman', while the lowest dry mass of root (6 mg) was observed in yellow lupin 'Manru' seedlings. There were significant differences in water content across varieties (ALVES et al. 2017, CHATTERJEE and NAGARAJAN 2006, CHAUGHULE et al. 2005, OBROUCHEVA 2017, VERTUCCI and LEOPOLD, 1987).

The highest water content (97.2%) was observed in faba bean 'Stego' stems, while the lowest water content (89%) occurred in roots of yellow lupin 'Iryd'. Seed leachate electroconductivity was on average three times higher in seeds stored at +20°C, compared to those stored at -14°C. The mean exudate electroconductivity in seeds stored at +20°C was 179.67 $\mu S\ g^{-1}$, while in seeds stored at -14°C it was 59.41 $\mu S\ g^{-1}$. The highest exudate electroconductivity (217.01 $\mu S\ g^{-1}$) was measured in seeds of yellow lupin 'Manru', stored at +20°C, whereas the lowest electroconductivity (30.41 $\mu S\ g^{-1}$) was found in seeds of yellow lupin 'Iryd', stored at -14°C. The electroconductivity of leachates of faba bean 'Stego' seeds was nearly six times higher than in seeds of the same variety stored at -14°C. The lowest difference in electroconductivity between seeds stored at +20°C and those stored at -14°C (1.25 x drop) was observed in yellow lupin 'Manru'. Seed ageing under dry storage results from the gradual loss of integrity of cell membranes which are important barriers enabling undisturbed concurrent proceeding of various biochemical and physiological reactions. The impairments of cell membranes result in significant increases of their conductivity leading to the leakage of ions, amino acids and sugars (DEMIDCHIK et al. 2014, OUYANG et al. 2002).

## One-dimensional electrophoresis

The patterns of polypeptide bands in fractions extracted from seeds stored at -14°C or +20 °C differed significantly in band numbers and intensities across the analysed cultivars. However, the differences between cultivars were small. Most polypeptides had similar molecular weights. Analysis of polypeptide bands in one dimensional electrophoresis was successfully applied to characterize protein profiles of different wheat cultivars. It

was found that protein profiles of stored seeds can be useful markers for studies of genetic variation and classification of cultivars increasing the efficiency of wheat breeding (SHUAIB et al. 2007). Using SDS-PAGE analyses VOIGT (1993) studied the presence of albumins and globulins in seeds.

The patterns of protein bands in SDS-PAGE were also analysed in seeds of various cultivars of chili peppers (*Capsicum* L.). The occurrence of 21 protein polypeptides was demonstrated with molecular masses 18.6 to 72.0 kDa (Figures 1, 2 and 3). The study revealed considerable differences between the genotypes. The variation of protein profiles suggested that selected genotypes could be good candidates for crop improvement by hybridization programs (KUMAR and TATA 2010).

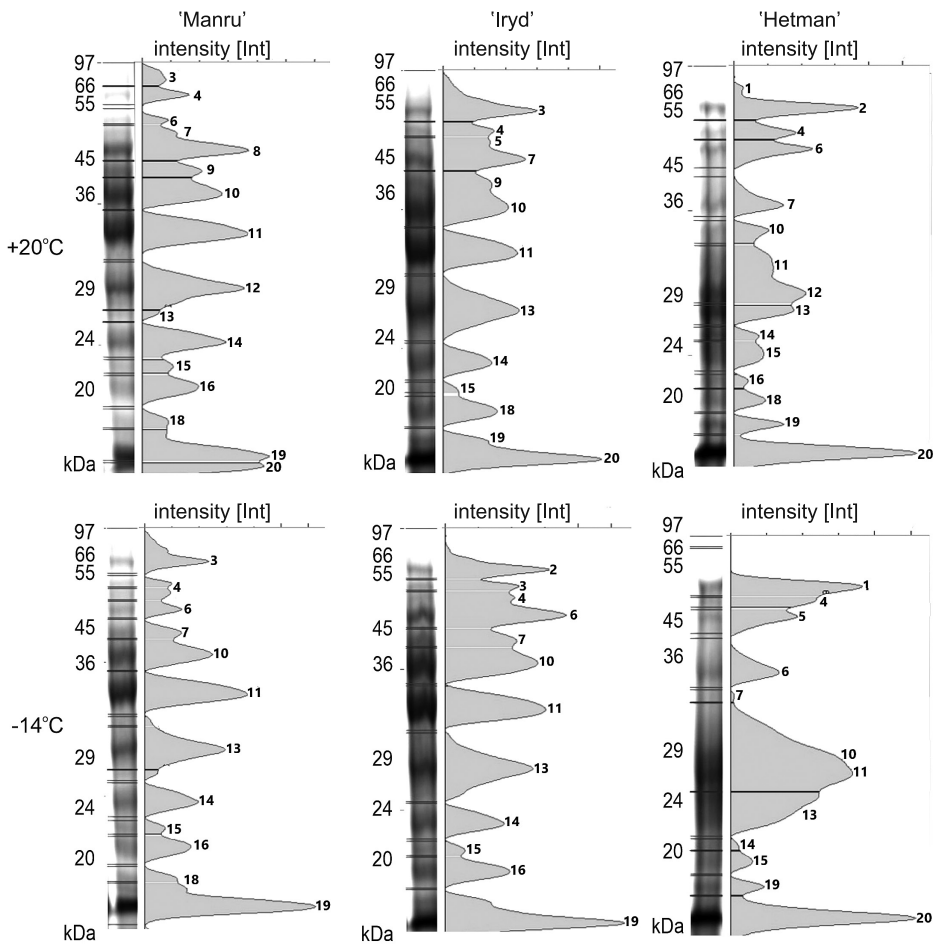


Fig. 1. SDS-PAGE electrophorograms and densitograms of lupin seed proteins. The seeds were stored at +20°C and –14°C during the 30-years period. Peptides sizes (kDa) and band numbers are given for each separation lane

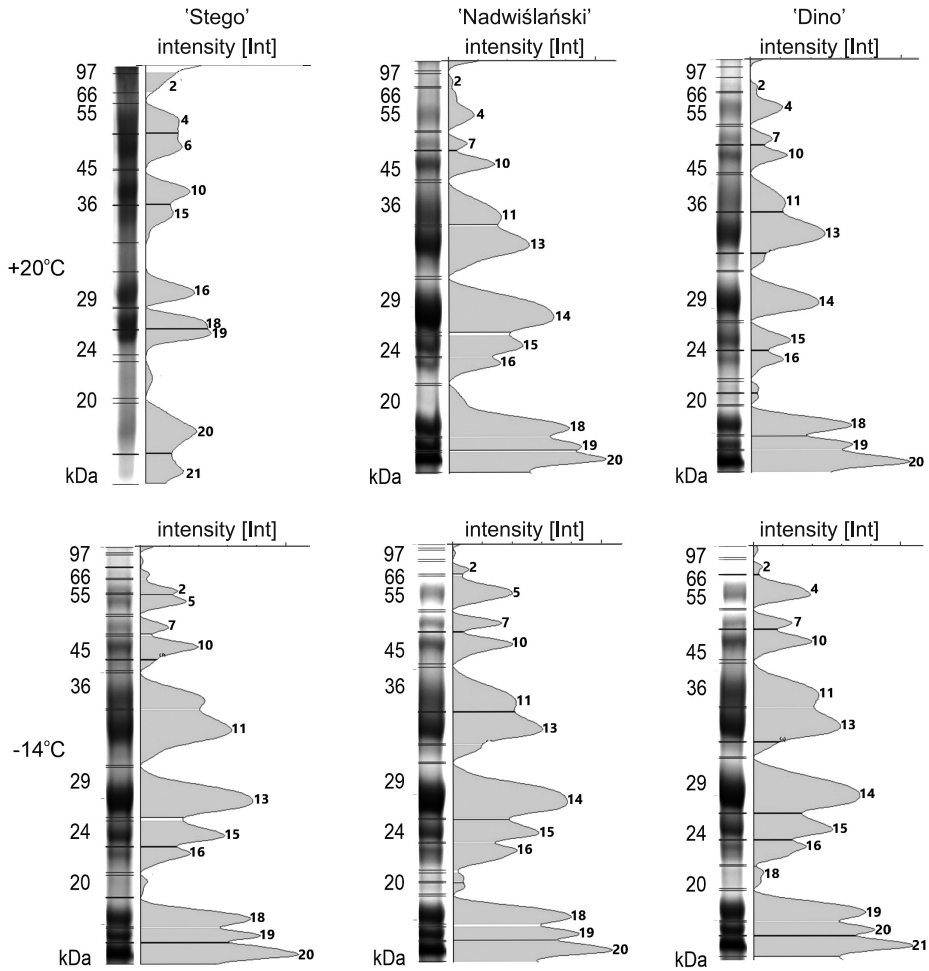


Fig. 2. SDS-PAGE electrophorograms and densitograms of faba bean seed proteins. The seeds were stored at +20°C and –14°C during the 30-years period. Peptides sizes (kDa) and band numbers are given for each separation lane

Seeds stored at -14°C were characterised by higher intensities of most polypeptide bands, compared to seeds stored at +20°C. This was particularly visible with bands of polypeptides with molecular masses 66 and 55 kDa (Figures 1 and 2). However, the intensities of bands corresponding to lower molecular mass polypeptides were higher in seeds stored at +20°C. The highest numbers of polypeptide bands were observed in seeds of yellow lupin ' Manru' and white lupin 'Hetman', stored at +20°C. Additionally, the band corresponding to a polypeptide with molecular mass 21.5 kDa was lacking in seeds of all studied cultivars of yellow lupin and white lupin

when the seeds were stored at -14 ºC (Figure 1). The faba bean seeds stored at +20ºC contained a protein with a similar but slightly higher mass 20.1 kDa (Figure 2).

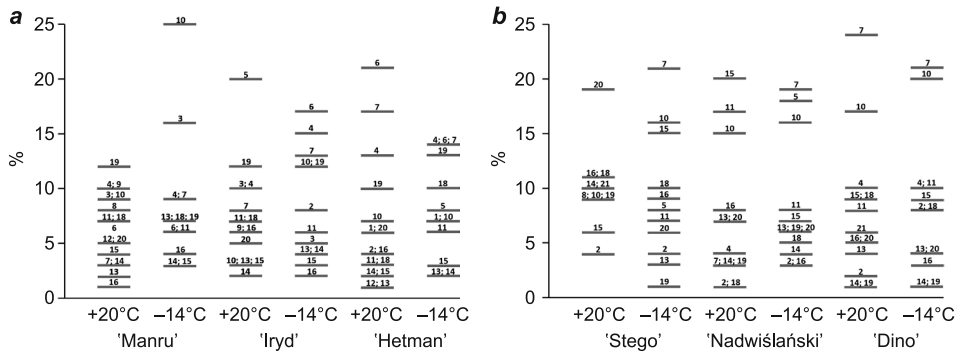


Fig. 3. Percent intensities of the polypeptide protein bands after SDS-PAGE separation of proteins. The numbers given above each band denote band location within each separation line (the lowest numbers correspond to the bands located closest to the sample wells); the percent values within each separation lane sum up to 100%. The seeds of lupin (*a*) and faba bean (*b*) were stored at different temperatures (+20 ºC, –14 ºC) during the 30-years period

Increased numbers and intensities of low molecular weight polypeptides in seeds stored at +20ºC suggest severe degradation of proteins during seed storage at such high temperature.

## Enzyme activities

Seed enzyme activities differed depending on storage conditions. Temperature and ambient humidity are considered key factors in this regard (BALDOS et al. 2014).

Dehydration of plant tissues results in production of reactive oxygen species, which may interact with one another and start various detrimental oxidative reactions. The antioxidation defence is an important component of plant adaptation to desiccation, although it is not easy to precisely describe all its physiological contexts. The enzymes involved in antioxidation defence system show different susceptibility to seed ageing and are also affected plant species and seed storage conditions. The mechanisms of protection against reactive oxygen species involve free radicals, superoxide dismutase, catalase and the members of the ascorbate-glutathione cycle, such as glutathione reductase and antioxidative compounds, including reduced glutathione, ascorbate and α-tocopherol (BERNAL-LUGO and LEOPOLD 1992).

In seeds subjected to storage, changes in antioxidative enzyme activities were observed, e.g. catalase and peroxidases (AYYAPPAN et al. 2010). In drying seeds, catalases prevent damages resulting from dehydration. Hydrogen peroxide is probably involved in the regulation of catalase gene expression and signal transduction. In stored cucumber seeds a temporary increase of catalase activity was observed, which suggests the induction of antioxidative and anti-ageing protection system (AYYAPPAN et al. 2010). The decrease of catalase activity, observed later, was probably caused by insufficient biosynthesis of this enzyme as a result of seed ageing. Heat shock and oxidative stress contribute to catalase inactivation and prevent biosynthesis of new molecules of this enzyme (HERTWIG et al. 1992).

A significant decrease of peroxidase activity was observed in cucumber seeds subjected to accelerated ageing (AYYAPPAN et al. 2010). The decline of viability of stored wheat seeds is accompanied by decreases in activities of catalase, superoxide dismutase, and an increase in glutathione reductase activity (LEHNER et al. 2008).

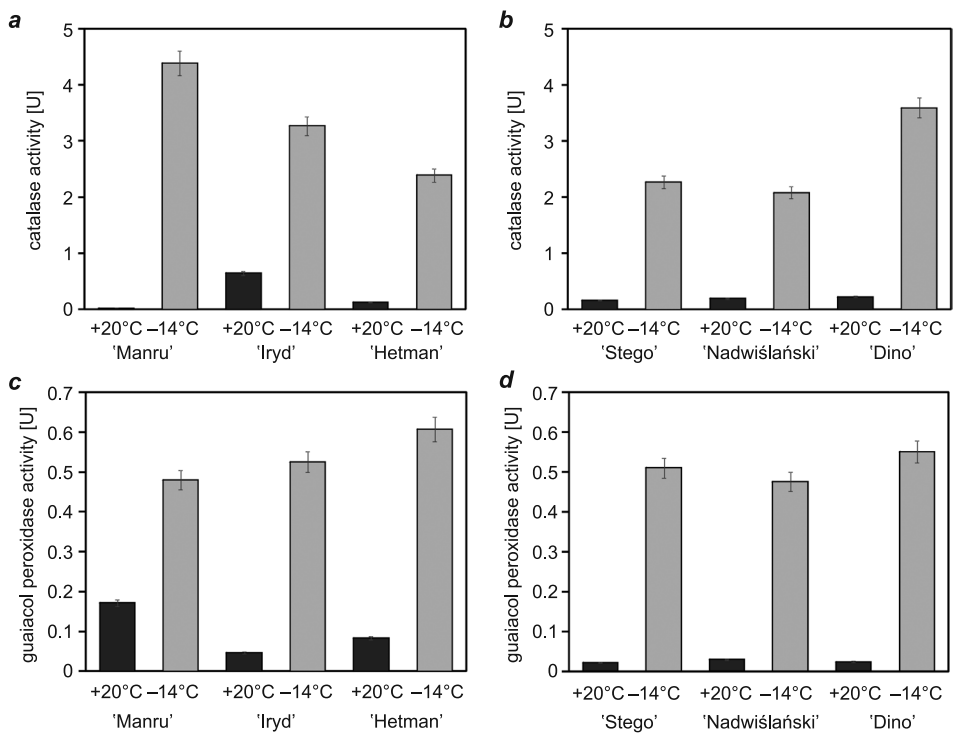


Fig. 4. Activity of catalase in lupin (*a*) and faba bean (*b*) seeds and guaiacol peroxidase activity in lubin (*c*) and faba bean (*d*) seeds (U one unit of enzyme activity corresponds to the oxidation of 1 µmole $H_2O_2$ for 1 min). The seeds were stored at +20 °C and –14 °C during the 30-years period

A few antioxidative enzymes of mung bean (*Vigna radiata*) – glutathione reductase, catalase and ascorbate peroxidase – were found highly sensitive to Maillard reaction (MURTY et al. 2003). In stored seeds, the Maillard reaction can result in chemical modifications of macromolecules, leading to a gradual decrease of seed ability to metabolically counteract the damages caused by free radicals during storage and germination. These changes cause decreasing seed viability and eventually seed death (MURTHY et al. 2003).

The decreases in enzyme activities can be caused by denaturation of the peptide part of the enzyme. It was shown in this paper, that a considerable decrease in enzyme activities occurs in seeds stored at +20°C in comparison to seeds stored at -14°C (Figure 4). Both the highest and lowest activities of catalase were noted in seeds of yellow lupin 'Manru'. In seeds stored at +20°C, the activity of this enzyme was 0.23 U, whereas in seeds stored at -14°C it was 3.00 U. The lowest activity of guaiacol peroxidase (0.021 U) was found in faba bean 'Stego' seeds stored at +20°C, while the highest activity of this enzyme (0.61 U) was detected in white lupin 'Hetman' seeds stored at -14°C. Mean activity of the guaiacol peroxidase in seeds stored at +20°C was 0.06 U and in seed stored at -14°C it was 0.525 U.

## Conclusions

1. During 30 years of storage at -14°C the studied legume seeds retained high vigour and viability.
2. Seed storage at +20°C for 30 years resulted in degradation of high molecular weight proteins.
3. Activities of catalase and guaiacol peroxidase in seeds subjected to 30 years storage at +20°C were lower than in seeds stored for 30 years at -14°C.

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# THE DIFFERENCES OF THE ELEMENTS CONTENT IN *RHIZOPHORA MUCRONATA* LEAVES FROM ASAHAN REGENCY, NORTH SUMATRA, INDONESIA*

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Abstract

Mangroves have a good mineral absorption system against change in environmental conditions, i.e., through the storage in leaves. This study aimed to determine the relationship of mineral contents to differences in the color levels of *R. mucronata* mangrove leaves. Theresults showed that the growth of leaves from young to old revealed decreased potassium content, while calcium and magnesium increased significantly. The order of microelementsfrom the largest to the smallest for green leaves or young leaves is Mn > Fe > Zn > Cu > B > Si, and for yellow leaves or old leaves is Mn > Fe > Zn > Cu > B > Si. The correlation of elements in mangrove leaves showed that potassium (0.78), copper (0.96), manganese (0.96), and zinc (0.95). The elements of copper and manganase that have a high correlation.

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## Introduction

Mangrove forests in North Sumatra, Indonesia are widespread on the east coast and mainly found in Asahan Regency. The mangrove environment is influenced by biotic and abiotic factors in controlling nutrients available for plants (BEREZINA et al. 2017, FRIESS and WATSON 2017). Mangroves provide several ecological functions for organisms (GOESSENS et al. 2014, ARIYANTO et al. 2018a, ARIYANTO 2019). Mangrove forests in the tropical region are highly productive, marked with the presence of nitrogen, phosphorus, and elements (micro, meso, and macro). Several factors play a role in determining the pattern of use and storage of elements in forest ecosystems, including soil fertility, species composition, and age of vegetation.

Mangroves require various elements to support their growth. Plants have a range of different mechanisms to protect themselves from the absorption of toxic elements and to limit their transportation in plants (ALMEIDA et al. 2006). The pattern of translocation of elements in plants is highly dependent on the level of ion mobility and specific metabolic needs (MORRISSEY and GUERINOT 2009, FERNÁNDEZ and BROWN 2013). Mangroves through leaf organs have a function as storage of elements (ISHII et al. 2014, LECHTHALER et al. 2016).

Mangroves can absorb nutrients in coastal waters, which are reflected in nutrient status (GRITCAN et al. 2016) and elemental content (MAATHUIS 2009, ARIYANTO et al. 2018b). Elements consist of macro, meso, and microelements. Macroelements are nitrogen (N), phosphorus (P), and potassium (K). Meso elements are magnesium (Mg), calcium (Ca), and sulfur (S). Microelements are iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu), molybdenum (Mo) and silicon (Si). PILON-SMITS et al. (2009) reported that plants require elements to increase the resistance to biotic pressures such as pathogens and herbivores, and abiotic pressures such as drought, salinity, and poisoning or nutritional deficiencies. The availability of these elements also has an effect on growth and reproduction productivity primary and secondary metabolism for cell defense, and from signal transduction to gene regulation, energy metabolism, and hormonal perception (HÄNSCH and MENDEL 2009, VATANSEVER et al. 2016, ARIYANTO et al. 2018c). This study aimed determine the relationship of element concentration to leaf colour difference with maturity *R. mucronata*.

## Material and Methods

### Sample Analysis

This research was conducted in February – May 2019. Leaf samples of *R. mucronata* were taken from Asahan Regency, North Sumatra, Indonesia (03°02′09″S dan 099° 51′31″E.). The leaves were taken 50 g. The research data analysis was carried out at the environmental Productivity Laboratory, IPB University.

Leaf samples (2 g) were dissolved in 10 mL perchloric acid and incubated at 100°C for 5 min. The processed samples were then treated with 10 mL of concentrated $HNO_3$, and it was followed by 10 ml concentrated $H_2SO_4$. Acid treatment was carried out at 100°C for 5 min each in the hot plate. Finally, the sample was evaporated and diluted with 10% HCl. The diluted samples were then filtered through washed acid-free filter papers. The main mineral elements (K, Mg, Ca) and trace elements (Fe, Zn, Mn, B, Cu, Mo, and Si) were determined in the Atomic Absorption Spectrophotometer, while total nitrogen and total phosphorus were determined using spectrophotometry (PANSU and JACQUES 2003, APHA 2012).

### Statistical Analysis

Data were expressed as mean ± SEM. The principal component analysis (PCA) was used to determine the analysis of the relationship between leaf color and the element's concentration. The principal component analysis displayed data in graphical form, data matrix, which consists of leaf color (row) and concentration variable (column). This analysis was performed using the Xlstat 2018 program.

## Results

### Element N, P, K

The level of macro-elements consisting of nitrogen (N) and phosphorus (P) showed different levels of contents. The nitrogen content had a higher concentration value compared to phosphorus. The higher concentration of nitrogen showed that the dominant macro element found in the leaf was nitrogen. Figure 1 shows the differences in percentage N & P between green leaves and yellow leaves.

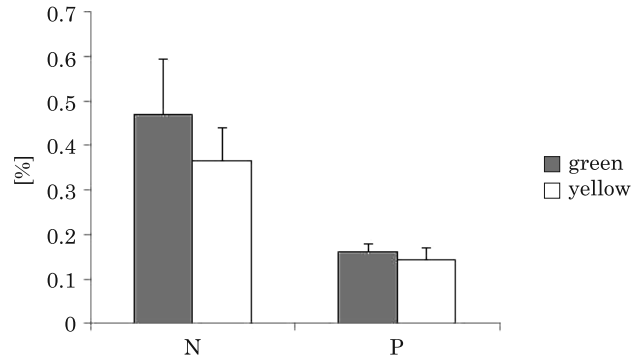


Fig. 1. The differences between green leaves and yellow leaves (N, P) in *R. mucronata* in Asahan Regency, North Sumatera, Indonesia

Figure 2 shows the potassium (K) content had a different concentration at the leaf color level. The green leaf had a higher concentration the yellow leaves. The results howed that *R. mucronata* had supported of N and P as expressed by the concentration of these elements in leaves.

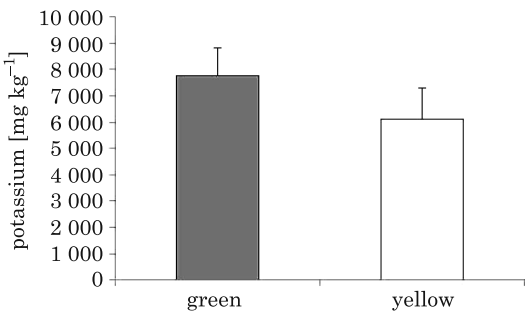


Fig. 2. The potassium (K) content of green and yellow leaves in *R. mucronata* in Asahan Regency, North Sumatera, Indonesia

## Element Mg, Ca

Atomic absorption spectrophotometry analysis showed differences in calcium and magnesium contents in *R. mucronata* leaves. Figure 3 shows the difference in the content of Mg and Ca in *R. mucronata* mangrove leaves based on the leaf color level. The green leaf has 134 Mg content (61,153.663 ± 4,277.63 mg kg$^{-1}$) and Ca content (36,087.147 ± 4,193.55 mg kg$^{-1}$) which are higher than those in the yellow leaves. The more leaves experience aging, the less Mg and Ca contents.

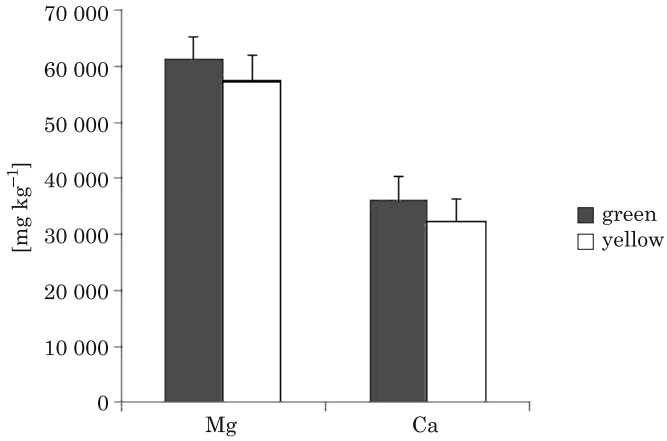


Fig. 3 The Mg and Ca content of green and yellow leaves in *R. mucronata* in Asahan Regency, North Sumatera, Indonesia

## Element Fe, Mn, Zn, B, Cu, Mo, Si

Table 1 shows that the content of microelements consisting of Fe, Mn, Zn, B, Cu, Mo, Si concentrations had differences based on the color of *R. mucronata* leaves. Overall, green leaves showed a higher content of microelements than yellow leaves. The descending order of microelements concentrations in green leaves is Mn > Fe > Zn > Cu > B > Si and in yellow leaves is Mn > Fe > Zn > Cu > B > Si. The concentration of elements in green leaves towards yellow leaves of *R. mucronata* shows changes along with the aging of leaves.

Table 1

Content of Fe, Mn, Zn, B, Cu, Mo, Si [mg kg$^{-1}$] in the leaves of *R. mucronata* in Asahan Regency, North Sumatra, Indonesia

| Microelements | Green leaves | Yellow leaves |
|---|---|---|
| Boron (B) | 4.67 ± 2.43 | 4.51± 0.46 |
| Copper (Cu) | 4.72±1.19 | 6.99±3.44 |
| Manganese (Mn) | 460.34±15.87 | 391.44±124.76 |
| Zinc (Zn) | 11.75±7.06 | 4.67±1.06 |
| Iron (Fe) | 16.83±9.65 | 14.06±4.511 |
| Silica (Si) | 0.43±0.07 | 0.40±0.06 |

### The relationship between leaf color conditions and elemental content

Figure 4 shows a PCA analysis of the relationship between the color differences of *R. mucronata* leaves that has three eigenvalues. The analysis of the first main component had an eigenvalue of 4.36 (variance 39.69%), the second had 3.62 (variant 33.61%), and the third had 1.52 (variance 10.54%). The three eigenvalues describe the data diversity of 87.13% (cumulative % / total variance). The loading factor illustrates the relationship of the effect of different colors of *R. mucronata* mangrove leaves to produce PCA 1 including potassium (0.78), copper (0.96), manganese (0.96), and zinc (0.95); while PCA 2 produces total P (0.97), magnesium (0.96), calcium (0.73), and iron (0.72); and PCA 3 involving boron (0.88).

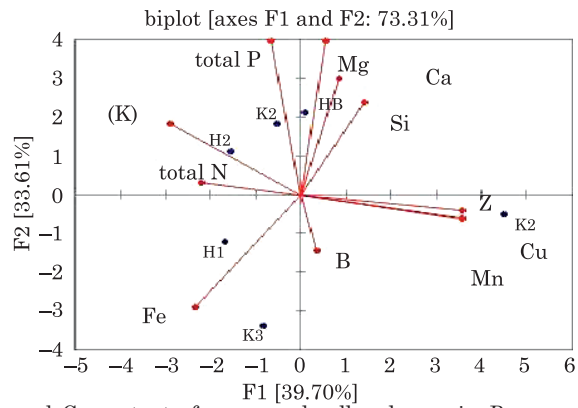


Fig. 4. The Mg and Ca content of green and yellow leaves in *R. mucronata* in Asahan Regency, North Sumatera, Indonesia

## Discussion

Phosphorus has an important role in the nucleotide chain, dark photosynthetic reaction, respiration, other metabolisms, and membrane-forming phospholipids. The nitrogen content had a higher concentration value compared to phosphorus (Figure 1). The P content of *R. mucronata*'s leaves was 0.06% and N was 0.6% (ANANDA et al. 2007) When the leaf is aging, the concentration of nitrogen, phosphorus, and potassium decreases (LIN and WANG 2001). High levels of calcium and phosphorus can function as a good supplement (COOPOOSAMY and MAGWA 2007). MEDINA et al. (2015) reported that the N concentration was significantly higher in *R. mangle* for all leaf stages. In the case of *Rhizophora*, N and P concentrations were higher than the values reported for mangrove forests in the

Caribbean. Wang mentioned that seasonal changes could affect the content of elements in the leaves. LOVELOCK et al. (2007) showed that the nutrient content of mangrove leaves (nitrogen and phosphorus contents) correlates well with the level of nutrient addition and natural variability in the concentration of nutrients in the environment. The green leaf had a higher concentration than the yellow leaves in Potassium (Figure 2). Potassium (K) is an important element due to its role in regulating the opening and closing of stomata (ANDRES et al. 2014). Stomata help to maintain water balance, and pumps of potassium ion support this process. Potassium is the third most common limiting factor for plant growth. The development of leaves from young to old tends to decrease potassium content (ZHENG et al. 1999). Figure 3 showed that the green leaf has high Mg and Ca content. ADHIKARI et al. (2018) reported that the Ca and Mg contents were mostly found and in high amounts, i.e., 6,412.8 mg $kg^{-1}$ and 8,640 mg $kg^{-1}$. These results showed lower contents when compared to this study. TANOI and KOBAYASHI (2015) found that (Mg) decreases when the leaves are old or mature. Also, another study reported that decreased Mg content could lead to the performance of enzyme and metabolic functions (HERMANS et al. 2013, Guo et al. 2016). TRÄNKNER et al. (2018) revealed that Mg plays an important role in the process of photosynthesis.

This study describes that the Mn content was higher than the other contents. Mn is a major contributor to various photosynthetic processes (MILLALEO et al. 2010). The concentration of Mn in this study is the most abundant in microelements. This finding has similarities in average concentration in the range of mangrove ecosystems (LEWIS et al. 2011, BAYEN 2012). The content of Cu and Zn in this study showed a higher content than the study reported by SARI et al. (2018) i.e., Cu 0.0865 mg $kg^{-1}$ and Zn 1.6385 mg $kg^{-1}$. Comparison in this study also showed a higher content of Zn, Mn, and Fe than the study conducted by DUDANI et. al. (2017) i.e., the content of Zn 0.275 ± 0.049, Mn 0.269 ± 0.067, and Fe 3.36 ± 0.240 [mg $kg^{-1}$]. PAHALAWATTAARACHCHI et al. (2009) also revealed that mature leaves have a higher content than young leaves, i.e., Zn (8.36), and Cu (3.23). HOSSAIN et al. (2001) reported that Cu content in the leaves of *Rhizophora mucronata* was 2.58 mg $kg^{-1}$. ADHIKARI et al. (2018) also reported that Cu and Zn contents were 10.49 and 3.2 mg $kg^{-1}$, respectively.

Comparisons also occur if Fe is compared to Zn and Cu in Table 1. Fe had a higher content than Zn and Cu. This research has the same results of the study conducted by AL HAGIBI et al. (2018) that found higher Fe content than Zn and Cu in *R. mucronata* mangrove leaves from Yemen. This study revealed that Silicon (Si) had the lowest concentration compared to the other microelements, which is in the range of 0.40 – 0.43 mg $kg^{-1}$.

The results of this study showed a lower value compared to a study conducted by ZHANG et al. (2013), which obtained 0.51 mg $kg^{-1}$. SAHEBI et al. (2015) mentioned that Si plays an important role in reducing plant susceptibility to various biotic and abiotic pressures.

In Figure 4, the elements found in *R. mucronata* mangrove show the value of relationships in several elements. Elements of Cu, Mn, and Zn were elements that dominate the element contents in *R. mucronata* mangrove leaves. The content is higher and inversely proportional to the content of elements K and Fe. These results indicated that the difference in absorption of elements in various parts of the mangrove is influenced by several factors, i.e., season, tissue age, morphology, and physiology (LEWIS et al. 2011). Cu and Zn act as important elements of micronutrients, which are involved in enzymes. The presence of Cu and Zn in high levels of concentration can cause damage to metabolic processes at the cellular level, inhibit enzyme reactions and anti-oxidative processes, delay growth, and cause the death of biota (VATANSEVER et al. 2016). MEJÍAS (2013) mentioned that mangrove ecosystems could absorb elements through the root and translocate them to other parts of the plant. Excessive Mn concentrations in plant tissues can change various processes, such as enzyme activity, absorption, and translocation that cause oxidative stress (DUCIC and POLLE 2005). The concentration of Mn depends on plant species and genotypes in a species (FERNANDO and LYNCH 2015).

This research showed element that high correclation i.e., potassium (K), copper, manganese (Mn) and zinc. Potassium (K) is an essential nutrient that affects most of the biochemical and physiological processes that influence plant growth and metabolism (WANG et al. 2013). K concentrations in green leaves were weakly but positively correlated with growth rates (FELLER et al. 2009). Copper is required for chlorophyll formation, cata lyzes various processes in plant metabolism. Manganese acts mainly as part of the enzymatic system in plants, activates several important metabolic reactions, has a direct effect on photosynthesis helping in the synthesis of chlorophyll, accelerates germination and maturity and increases the availability of phosphorus and calcium. Manganese is an essential element for plants, intervening in several metabolic processes, mainly in photosynthesis and as an enzyme antioxidant-cofactor (MILLALEO et al. 2010). The Zn plays very important role in plant metabolism by influencing the activities of hydrogenase and carbonic anhydrase, stabilization of ribosomal fractions and synthesis of cytochrome. Plant enzymes activated by Zn are involved in carbohydrate metabolism, maintenance of the integrity of cellular membranes, protein synthesis, regulation of auxin synthesis and pollen formation. The regulation and maintenance of the gene expression

required for the tolerance of environmental stresses in plants are Zn dependent (CAKMAK 2000)

## Conclusions

Development of leaves from young to old tends to decrease the potassium content, while calcium and magnesium increased well. In general, the elements found in *R. mucronata* mangrove showed the value of relationships in several elements. Elements of Cu, Mn, and Zn were elements that dominate the element contents in *R. mucronata* mangrove leaves. These contents were higher and inversely proportional to the content of potassium and iron.

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# DIVERSITY AND DISTRIBUTION PATTERN OF AIRBORNE FUNGI IN GREATER TUNB, ABU-MUSA, AND SIRRI ISLANDS, PERSIAN GULF, IRAN

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Key words: airborne fungi, Persian Gulf Islands, Polymerase chain reaction (PCR).

Abstract

Airborne fungi play an important role in the cause of different adverse effects on humans and animals health because of their pathogenicity, allergic reactions and toxicity. The Persian Gulf Islands have a warm and humid climate and there is no information regarding airborne fungi in these regions. Therefore, this study aimed to determine the mycoflora of environments of Iranian islands, namely Greater Tunb, Abu Musa, and Sirri. In this study, a total of 90 air samples were prepared by settle plate method using Sabouraud dextrose agar with Chloramphenicol. The fungal species were identified the bases of morphology, molecular and sequencing techniques. A total of 231 fungal isolates, including 16 genera were detected. *Cladosporium* was determined as the predominant genus (32.46%); *Aspergillus* (20.34%), sterile mycelia (9.08%), *Alternaria* (8.23%) and *Penicillium* (4.75%) were revealed as the common fungal

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spores. In our study, dematiaceous fungi had a high incidence with the frequency of ~56% of total isolated fungi that reason may be due to the resistance of spores in colored fungus against UV rays of the sun. Although different airborne fungi isolated in this study does not necessarily imply a cause and effect relationship with illness, but the results provide a better perception of the diversity and distribution pattern of airborne fungi, which may be beneficial for physicians, allergists, as well as epidemiologists.

## Introduction

Fungi as a heterogeneous group of microorganisms have a high distribution in the environment including soil, plants, animals, water, and air (GONÇALVES et al. 2017). There are many biological contaminants in the air including fungi, pollens, viruses, and bacteria (AYANBIMPE et al. 2012). Fungi are the most important and most abundant group of organisms in the air because they are present in all habitats and can originate from various sources such as plants, animals and soil (SINDT et al. 2016). Among different airborne biological contaminants in the environment, fungi play an important role in the cause of different adverse effects on humans and animals health (SHAMS-GHAHFAROKHI et al. 2014, SHARMA et al. 2015). World concerns about the air quality and its health become more prominent due to the vital need of air for the survival of life on earth (GHIASIAN et al. 2016, LEUNG 2015). Airborne fungal spores can act as a cause of allergies and asthma, respiratory infections, primarily irritations and toxic effects. Many of airborne fungi can also act as etiologic agents of keratomycosis, otomycosis, chronic bronchitis, emphysema, and onychomycosis. Besides, air-transmitting diseases caused by airborne fungi such as histoplasmosis, invasive aspergillosis, acute allergic alveolitis are life--threatening to immunocompromised hosts (GHIASIAN et al. 2016, IRGA and TORPY 2016, KHAN and KARUPPAYIL 2012, RUDERT and PORTNOY 2017). Fungal spores are almost always present in the air, but their quality and quantity vary according to the geographical situation, climate, time of day and the presence of spore sources in the environment (NICHOLAS et al. 2015, RUZER and HARLEY 2012). Knowledge of airborne fungi in each region is important for the ecological diagnosis and specific treatment of allergic manifestations induced by inhaled allergens. Up to now, numerous studies have been conducted on the presence and diversity of airborne fungal spores in different occupational and residential areas of the world (AKGÜL et al. 2016, GONÇALVES et al. 2017, ILOND and NWEKE 2016, MORENO-SARMIENTO et al. 2016, TONG et al. 2017). There are also several studies from various parts of Iran and so on demonstrated a wide array of fungi in air samples from environments (GHIASIAN et al. 2016, HEDAYATI

et al. 2005, PAKSHIR et al. 2015, Sepahvand et al. 2013, SHAMS-GHAHFAROKHI et al. 2014, Soleimani et al. 2013). The Persian Gulf Islands have a warm and humid climate and there is no information regarding airborne fungi in these regions. With this background in mind, the current study was conducted with the aim of determination of the diversity and distribution patterns of airborne fungi in Greater Tunb, Abu-Musa, and Sirri islands using morphological and molecular (DNA sequence analysis) methods.

## Materials and Methods

### Geographical characteristics of the studied islands

Greater Tunb, Abu Musa, and Sirri islands are located at the Persian Gulf in the most southern part of Iran (Figure 1). These three islands are considered as part of Hormozgan province. The Greater Tunb (10.3 $km^2$ wide) has a longitude and latitude of 55° 28–55° 34 and 26° 34–26° 30 respectively. Abu Musa Island (12 $km^2$ wide) has a longitude and latitude of 54° 26–55° 19 and 25° 51–26° 19, respectively. Furthermore, Sirri Island has situated 76 km from Bandar-e Lengeh and 50 km west of Abu Musa Island. This island is almost 5.6 km long with a width of about 3 km. It covers an area of 17.3 $km^2$. All three islands have a warm and humid climate (AFSHARI et al. 2017).


Fig. 1. Map of Persian Gulf, showing relative positions of the three islands of Greater Tunb, Abu Musa, and Sirri

## Sample collection and cultivation

This descriptive study was conducted in the second half of 2011 in three Iranian islands of Greater Tunb, Abu-Musa, and Sirri. A total of 90 air samples (i.e., 30 samples from each island) were prepared using settle plate method according to HOEKSTRA et al. 2000 and ANDON et al 2006. In this method, the plates to diameter of 9 cm containing Sabouraud's dextrose agar medium (Merck, Germany) plus chloramphenicol (0.1 mg $mL^{-1}$; Sigma-Aldrich, USA) were placed at the height of about one meter from the floor, for 30 minutes. After collection of samples, the plates were taken to the laboratory of mycology for isolation and identification of fungi. The plates were incubated at 28°C in the dark for 3–4 weeks and control and evaluated daily for fungal growth (RIPPON 1988). Different types of colonies were subculture on Sabouraud's dextrose agar and tested by slide Riddle method. Fungal isolates were identified according to their microscopic and macroscopic morphological criteria according to standard procedures (RIPPON 1988).

## Molecular identification of unknown isolates

The unknown fungal isolates were identified through performing DNA sequence analysis. DNA was extracted according to LEE 1990. The quality and quantity of the extracted DNA were evaluated using electrophoresis and NanoDrop, respectively. The ITS1-5.8S-ITS2 rDNA was amplified using ITS1 and ITS4 as forward and reverse primers (WHITE et al. 1990).

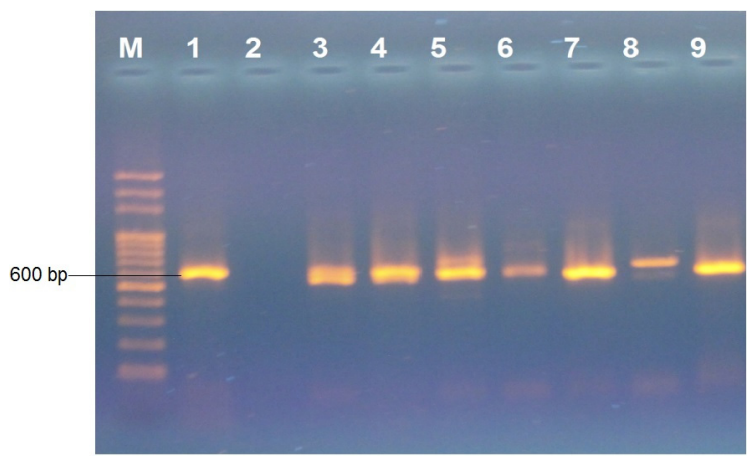


Fig. 2. Agarose gel electrophoresis and polymerase chain reaction products from number of isolates: M – 100 bp DNA ladder; lane 1 – positive control (*A. flavus*); lane 2 – negative control; lane 3–5 – *Neocamarosporium chichastianum*; lane 6 – *Stemphylium solani*; lane 7 – *Chaetomium subaffine;* lane 8 – *Embellisia phragmospora*; lane 9 – *Phoma tropica*

The PCR was performed in a total volume of 25 µL in each tube containing 12.5 µL Master mix (buffer, dNTP, Taq DNA polymerase, 2 mM $MgCl_2$; Amplicon III, Denmark), 1 µL of the template DNA, 1 µL of each primer (20 pmol final concentration of each the primer), and 9.5 µL distilled water. The PCR conditions were as follows. initial denaturation at 94°C for 5 min, 35 cycles with denaturation at 94°C for 30 sec, annealing at 56°C for 45 sec, extension at 72°C for 45 sec, and a final extension step of 7 min at 72°C. Amplicons were visualized by electrophoresis in 1% agarose gels using the ethidium bromide (Figure 2) and the PCR products were sent for sequencing (Macrogen, South Korea) in both directions. The sequence results were processed by using the web-based blasting program, basic local alignment search tool (BLAST), at the NCBI site (http://www.ncbi.nlm.nih.gov/BLAST), and the data were compared with the NCBI/Genebank database.

## Results

In the present study, distribution and diversity of airborne fungi in three Iranian islands of Greater Tunb, Abu Musa, and Sirri were evaluated. A total of 90 air samples were prepared by settle plate method using of Sabouraud Dextrose Agar Medium with Chloramphenicol. The fungal species were identified the bases of morphology, molecular and sequencing techniques. A total of 231 fungal isolates, including at least 16 genera were detected. Overall, 90% of the samples obtained from the studied islands were contaminated with fungi; this percentage was more than 93% in some islands such as Siri and less than 90% in Abu Musa Island. The dominant species were members of *Cladosporium* species (32.46%). The frequency of these species in Great Tunb, Abu Musa and Siri were 34%, 26.79% and 33.3%, respectively. Other species by frequency include *Aspergillus* species (20.34%), sterile mycelia (9.08%), *Alternaria* species (8.23% ), *Penicillium* species (4.75%), *Chaetomium* species (3.91%), yeasts (3.91%), *Fusarium* species (3.02%), *Aureobasidium pullulans* (3.02%), *Exophiala* species (1.74%), *Mucor* species (1.74%), *Stemphylium solani* (1.74%), *Neoscytalidium dimidiatum*, *Neocamarosporium chichastianum*, *Ulocladium* species and *Rhizopus* species (1.3%), *Phoma tropica* and *Embellisia phragmospora* (0.44%).The diversity and frequency of fungi isolated from Greater Tunb were higher than the other two islands. The number and frequency of fungal isolates in each island is represented in Table 1 and Table 2.

Table 1
Frequency and percentage of positive samples of airborne fungi isolated from Greater Tunb, Abu Musa, and Sirri islands, Persian Gulf, Iran

| Specification | Islands | | | Total frequency [%] |
|---|---|---|---|---|
| | Greater Tunb | Abu Musa | Sirri | – |
| Number of samples examined | 30 | 30 | 30 | 90 |
| Number of positive samples | 27 | 26 | 28 | 81 |
| Percentage of positive samples | 90 | 86.67 | 93.33 | 90 |

Table 2
Frequency and distribution of airborne fungi isolated from Greater Tunb, Abu Musa, and Sirri islands, Persian Gulf, Iran

| Specification | Islands | | | | | | Total frequency | |
|---|---|---|---|---|---|---|---|---|
| Species isolated | Greater Tunb | | Abu Musa | | Sirri | | | |
| | No. | % | No. | % | No. | % | No. | % |
| *Alternaria* species | 9 | 7.83 | 6 | 10.71 | 4 | 6.67 | 19 | 8.23 |
| *Aspergillus flavus* | 4 | 3.48 | 5 | 8.93 | 3 | 5 | 12 | 5.19 |
| *Aspergillus niger* | 4 | 3.48 | 4 | 7.14 | 5 | 8.33 | 13 | 5.63 |
| *Aspergillus* species | 6 | 5.22 | 8 | 14.29 | 8 | 13.33 | 22 | 9.52 |
| *Aureobasidium pullulans* | 3 | 2.6 | 1 | 1.79 | 3 | 5 | 7 | 3.02 |
| *Chaetomium globosum* | 2 | 1.74 | 0 | 0 | 0 | 0 | 2 | 0.87 |
| *Chaetomium subaffine* | 1 | 0.87 | 0 | 0 | 0 | 0 | 1 | 0.44 |
| *Chaetomium* species | 3 | 2.6 | 1 | 1.79 | 2 | 3.33 | 6 | 2.6 |
| *Cladosporium* species | 40 | 34.79 | 15 | 26.79 | 20 | 33.33 | 75 | 32.46 |
| *Embellisia phragmospora* | 1 | 0.87 | 0 | 0 | 0 | 0 | 1 | 0.44 |
| *Exophiala* species | 3 | 2.6 | 0 | 0 | 1 | 1.67 | 4 | 1.74 |
| *Neoscytalidium dimidiatum* | 2 | 1.74 | 1 | 1.79 | 0 | 0 | 3 | 1.3 |
| *Fusarium* species | 4 | 3.48 | 1 | 1.79 | 2 | 3.33 | 7 | 3.02 |
| *Mucor* species | 2 | 1.74 | 0 | 0 | 2 | 3.33 | 4 | 1.74 |
| *Neocamarosporium chichastianum* | 2 | 1.74 | 1 | 1.79 | 0 | 0 | 3 | 1.3 |
| *Penicillium* species | 6 | 5.22 | 3 | 5.35 | 2 | 3.33 | 11 | 4.75 |
| *Phoma tropica* | 1 | 0.87 | 0 | 0 | 0 | 0 | 1 | 0.44 |
| *Rhizopus* species | 2 | 1.74 | 1 | 1.79 | 0 | 0 | 3 | 1.3 |
| *Stemphylium solani* | 4 | 3.48 | 0 | 0 | 0 | 0 | 4 | 1.74 |
| Sterile mycelium | 10 | 8.7 | 7 | 12.5 | 4 | 6.67 | 21 | 9.08 |
| *Ulocladium* species | 2 | 1.74 | 0 | 0 | 1 | 1.67 | 3 | 1.3 |
| *Unknown yeasts* | 4 | 3.48 | 2 | 3.57 | 3 | 5 | 9 | 3.9 |
| Total | 115 | 100 | 56 | 100 | 60 | 100 | 231 | 100 |

## Discussion

Airborne fungi can result in several adverse health effects such as asthma, rhinitis, allergic sinusitis, toxic reactions, allergic bronchopulmonary mycoses, etc. (BAXI et al. 2016). It has been reported that more than 180 genera of airborne fungi with worldwide distribution are associated with allergies and human and animal infections and the most common species are likely to belong to the genera *Cladosporium*, *Penicillium*, *Alternaria* and *Aspergillus* (HORNER et al. 2004). In the present study, the most dominant fungal genera isolated from the air samples were *Cladosporium* (32.46%), *Aspergillus* (20.34%), sterile mycelia (9.08%), *Alternaria* (8.23%) and *Penicillium* (4.75%). As mentioned, the species of *Cladosporium* and *Aspergillus* were widely distributed in the air accounting for approximately 53% of total isolated fungi. In several previous studies of airborne fungal spores in different geographic areas of the world, it has been demonstrated that the most prevalent fungi belonged to the genera of *Cladosporium* and *Aspergillus*. In the study carried out by AKGÜL et al. (2016) on the determination of airborne fungal spores of Gaziantep, Turkey, *Cladosporium* (56.48%) was determined as the predominant genus. In another study was conducted on distribution of airborne fungi in indoor and outdoor environments at a French hospital, *Cladosporium* species were the dominant genus (55%) In outdoor samples, while in the clinical units, *Penicillium* species (23 to 25%) were the most frequently recovered airborne fungi (SAUTOUR et al. 2009). In a study on mycoflora of the outdoor air environment in Abraka, Nigeria, *Aspergillus* (23.47%) was reported as the most dominant species (ILONDU et al. 2016). CHARAYA and NARUKA (2016), reported the genera of *Aspergillus*, *Alternaria*, and *Cladosporium* as the most prevalent airborne fungi in India. Several studies have been conducted in Iran and various fungi are reported as dominant air mycoflora. Airborne Fungi in Indoor and Outdoor of Asthmatic Patients' Home, of Sari in Iran was evaluated by HEDAYATI et al. (2005). They reported the genera *Cladosporium*, *Aspergillus*, *Penicillium*, and *Alternaria* as the most prevalent fungi. GHIASIAN et al. (2016), conducted a study on airborne fungi in Qazvin that the most commonly found fungi in order of frequency were *Cladosporium*, *Penicillium*, and *Aspergillus* genera. In the study carried out by Shams-GHAHFAROKH et al. (2014) on the distribution of airborne fungi in an outdoor environment in Tehran, *Aspergillus* was the most predominant fungus followed by *Cladosporium*, *Penicillium*, and *Alternaria*. These results are similar to those found in our study. The reasons why the dominant airborne fungi from these genera may be due to this fact that *Aspergillus*, *Penicillium* and *Cladosporium* species produce small and

light spores in large numbers that generally suspended in air for periods ranging from a few hours to several days, whereas other fungal genera produce fewer, larger and heavier spores which tend to have faster settling (VONBERG and GASTMEIER 2006). High temperature and humidity were shown to be suitable for *Cladosporium* species sporulation (EBNER et al. 1989). Considering the hot and humid climate of islands investigated in this study, the high frequency of *Cladosporium* species in the air of these regions may be justifiable. *Cladosporium* is of particular clinical importance because it possesses a high allergenic potential and its spores can trigger the development of symptoms such as rhinosinusitis, dry cough or bronchial asthma in susceptible persons (DENNING et al. 2014, SINDT et al. 2016). *Aspergillus* genus also is one the most common types of molds in the environment. HEDAYATI et al. (2005), stated that the high frequency of *Aspergillus* in the kitchen may be due to their thermo tolerant ability. Some members of *Aspergillus* group are the causative agents of allergy in atopic individuals and some diseases, including otomycosis, keratomycosis, onychomycosis, and mycetoma. Besides, some species are relevant as mycotoxin producers (NIELSEN 2003, PASQUALOTTO 2010). Following the *Cladosporium* and *Aspergillus* species, Mycelia sterilia (9.08%) had the highest frequency in the air of studied areas. The high frequency of Mycelia sterilia isolated in this study is in accordance with our previous study on soil-borne fungi of Greater Tunb, Abu-Musa and Sirri Islands (NOSRATABADI et al 2017). In our research, dematiaceous fungi including *Cladosporium*, *Alternaria*, *Chaetomium* and etc. had a high frequency versus hyaline hyphomycetes with the frequency of ~56% of the total isolated fungi. The high frequency of dematiaceous fungi isolated in this study is in accordance with results of another study conducted on air of Qeshm Island, that authors reported the most abundant fungi were black and colored fungi such as *Alternaria* (63.86%) and *Cladosporium* (11.81%). These authors stated that dematiaceous fungi have melanin pigment in their cell membrane structure and the role of melanin is the protection of fungus against harmful effects of the sun's ultraviolet rays (BARATI et al. 2009). In this study, fungi such as *Embellisia phragmospora*, *Neocamarosporium chichastianum*, *Chaetomium subaffine*, *C. globosum*, *Phoma tropica* and *Stemphylium solani* were identified by using of molecular and sequencing methods. All of these fungi are from black fungi and this can be a confirmation of resistance of colored fungi in the hot and humid weather of studied islands. *E. phragmospora* in classification is placed in the Ascomycota phylum, Dothideomycetes class, Pleosporales order and Pleosporaceae family. This fungus is a saprophyte and pathogenic plant that was previously known as atypical *Helminthosporium* (LAWRENCE et al. 2012). *N. chi-*

*chastianum* is also a new species that was introduced in 2014 (CROUS et al. 2014). This fungus was isolated from Urmia Lake for the first time in Iran. *Neocamarosporium* species detected as saprobes, and *N. chichastianum* isolated from soil and saline environments (PAPIZADEH et al. 2017).

## Conclusion

In general, results of the present study showed that different groups of airborne fungi are present as real contaminants of air of Greater Tunb, Abu-Musa and Sirri Islands. These fungi especially those involved in the etiology of fungal diseases and allergies such as *Cladosporium*, *Aspergillus*, *Alternaria* and *Penicillium* species must be considered as major threats for public health. In this study, dematiaceous fungi including *Cladosporium*, *Alternaria*, *Chaetomium* and etc. had a high frequency versus hyaline hyphomycetes that reason may be due to the resistance of spores in colored fungus against UV rays of the sun. The results of this study provide a better perception of the diversity and distribution pattern of airborne fungi, which may be important for physicians, allergists, as well as epidemiologists.

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## Conflicts of interest

The authors have no conflicts of interest to disclose.

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# EFFECT OF SUPPLEMENTATION OF AMARANTH MEAL ON BLOOD PLASMA CHOLESTEROL IN RAINBOW TROUT

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Abstract

The aim of the study was the assessment of the effect of amaranth meal on serum cholesterol in rainbow trout *Oncorhynchus mykiss*. Two experimental feeds contained 10.0% and 20.0% of amaranth meal and control feed were prepared. All diets were formulated to be isoenergetic and isonitrogenous on a digestible nutrient basis. Initial body wet weight of experimental trout ($n$ = 180) was 106.5 ± 28.4g and mean length of 22.0 ± 2.2 cm. Feed was offered in ration 1.3% of fish biomass of respective group for 56 days. Blood cholesterol levels were measured in 1st, 2nd, 4th and 8th week of the experiment.

Obtained results has shown that amaranth addition to trout feed causes temporary fluctuation of cholesterol level.

## Introduction

Cholesterol is a necessary constituent for eukaryotic cell growth and development. The biosynthesis of cholesterol provides crucial building blocks for cell membranogenesis and membrane fluid regulation (RUSSEL 1992). Moreover, cholesterol serves as a precursor of many physiologically active compounds, such as sex hormones, bile acids and vitamin D (GOEDEKE and FERNÁNDEZ-HERNANDO 2012). Thus, cholesterol plays an important role in maintaining the physiological functions of fish. At present, knowledge on cholesterol metabolism in fish is limited (NRC 2011).

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Feeds, traditionally formulated with fish meal (FM), provide at least 1g of cholesterol per kg of feed (TOCHER et al. 2008). According to DENG et al. (2013), the replacement of FM with high levels of plant proteins induces the hypocholesterolemia in fish. This may be related to both insufficient dietary cholesterol and decrease in endogenous cholesterol synthesis in rainbow trout. Cholesterol metabolism is affected by *de novo* cholesterol synthesis in the liver (endogenous cholesterol) and dietary cholesterol content (exogenous cholesterol) (MAITA et al. 2006) or its precursors.

Amaranth (*Amaranthus cruentus*) is a rich source of squalene (ESCUDERO et al. 2004). The squalene content in *Amarantus cruentus* ranged from 2.26% to 5.67% (BERGANZA et al. 2003). Squalene is a precursor in the cholesterol biosynthetic pathway (SPANOVA and DAUM 2011). POCZYCZYŃSKI et al. (2014) and NIEWIADOMSKI et al. (2016) observed higher growth and no negative effect on health status, when amaranth oil and meal respectively were used as ingredients of feeds for rainbow trout. High level of squalene in amaranth seeds may prevent hypocholesterolemia in fish fed feeds containing high level of plant ingredients. The aim of the study was to evaluate the influence of amaranth meal on the cholesterol level in the serum of rainbow trout *Oncorhynchus mykiss*.

## Materials and methods

### Experimental design

The experimental design was reviewed by the Local Ethics Committee and was released from the preparation of the application. Rainbow trout with initial mean body weight of 106.5 ± 28.4g and mean total length of 22.0 ± 2.2 cm were used in the experiment. Fish ($n$ = 180) were randomly distributed in 9 tanks (3 groups in triplicates: control and two experimental) and acclimated for 14 days to the experimental conditions. Each tank volume was 1 $m^3$ and all tanks were a part of the recirculated aquaculture system (RAS). Fish were exposed to a natural light regime of approximately 8 LD and 16 DD. Water quality parameters were measured every day (7.00 am) and the mean values were as follow (mean ± SD): dissolved oxygen 8.05 ± 0.20 mg $dm^{-3}$, temperature 16.05 ± 0.25°C, pH 7.75 ± 0.25, total ammonia < 0.005 mg $dm^{-3}$, nitrate < 0.20 mg $dm^{-3}$, nitrite < 0.001 mg $dm^{-3}$ and phosphates < 0.005 mg $dm^{-3}$. Before the experiment, all fish were caught, immediately anaesthetized with propofol (7 mg $dm^{-3}$) (GOMUŁKA et al. 2014) measured and weighted. Fish were fed by automatic feed dispenser trough 8h a day. Fish were sampled after 8 weeks of feeding. During the experiment, daily feed intake was 1.3% of the biomass of fish body.

## Diet Formulation and Preparation

Fish were fed with 2 experimental diets containing different levels of amaranth flour 10.0% (AF10) and 20.0% (AF20) and control feed (CF).

Table 1

Ingredients and chemical composition of the diets

| Formulation [g $kg^{-1}$] | CF | AF10 | AF20 |
|---|---|---|---|
| Fishmeal[a] | 30.00 | 30.00 | 30.00 |
| Soybean protein concentrate[b] | 25.00 | 24.01 | 23.25 |
| Amaranth meal[c] | 0.00 | 10.00 | 20.00 |
| Wheat flour[d] | 32.30 | 23.20 | 14.05 |
| Cod-liver oil[e] | 5.00 | 5.00 | 5.00 |
| Soybean oil[f] | 5.00 | 5.00 | 5.00 |
| Premix[ghi] | 2.70 | 2.70 | 2.70 |
| Chemical composition [g $100g^{-1}$] | | | |
| Dry matter | 97.64 | 97.16 | 97.11 |
| Crude protein | 44.45 | 43.45 | 43.53 |
| Crude fat | 14.08 | 14.67 | 15.25 |
| Crude fibre | 1.82 | 2.08 | 2.53 |
| NFE | 28.07 | 27.76 | 26.57 |
| Crude ash | 9.22 | 9.20 | 9.33 |
| Squalene | 0.10 | 0.12 | 0.14 |
| Gross energy [MJ $kg^{-1}$] | 15.12 | 15.12 | 15.12 |
| Essential amino acids [g 16 $gN^{-1}$] | | | |
| Arginine | 2.61 | 3.39 | 4.18 |
| Histidine | 0.91 | 1.15 | 1.39 |
| Isoleucine | 2.36 | 2.73 | 3.10 |
| Leucine | 3.90 | 4.46 | 5.02 |
| Lysine | 3.31 | 3.51 | 3.72 |
| Methionine | 1.74 | 2.07 | 2.39 |
| Phenylalanine | 3.07 | 3.84 | 4.60 |
| Threonine | 2.03 | 2.27 | 2.50 |
| Tryptophan | 0.60 | 0.71 | 0.82 |
| Valine | 2.66 | 3.04 | 3.42 |

[a]FF SKAGEN, Danemark, [b]HP 300 HAMLET, Danemark, [c]SZARŁAT, Poland, [d]Castello Poland, [e]Mollers, Norway, [f]Artex, Poland, [g]Dolfos, Poland, [h]Vitamin premix (IU $kg^{-1}$ or mg $kg^{-1}$ dry diet): Witamin A – 70 000 UI $kg^{-1}$; Witamin D – 200 000 UI $kg^{-1}$; Witamin E – 17 500; Witamin K – 867; Witamin C (ascorbic acid phosphate) – 28 500; Witamin $B_1$ – 1067; Witamin $B_2$ – 2000; Witamin $B_5$ – 5334; Witamin $B_6$ – 1334; Witamin $B_{12}$ – 4; Biotin – 200, Niacin – 12 000, Folic acid – 800; Inositol – 20 000; Choline chloride – 120 000; Betaine – 75 000. [i]Mineral premix (mg $kg^{-1}$ dry diet): Ferrum ($FeSO_4$* $H_2O$ ) – 4 334; Iodine (KI) – 734; Copper ($CuSO_4$* $5H_2O$) – 267; Mangan (MnO) – 734; Zinc ($ZnSO_4$* $H_2O$) – 1250 and Zinc (ZnO) – 750; Selen – ($Na_2SeO_4$) – 34 mg

Squalene consisted of 10%, 12% and 14% of crude fat in CF, AF10 and AF20 respectively. The diets were formulated to meet or exceed NRC (2011) requirements for all indispensable amino acids and other nutrients. Formulation and composition of diets are presented in Table 1. All diets were formulated to be isoenergetic (15.12 MJ $kg^{-1}$) and isonitrogenous on a digestible nutrient basis.

All the dry ingredients were ground in a hammer mill, sieved through a 0.8-mm screen, mixed, preconditioned and extruded in a twin screw extruder (METALCHEM, Poland) with 2.0 mm die. Extruder processing parameters were controlled during feed production: temperature in conditioner outlet – 100°C, temperature in second segment 110°C; temperature in endplate – 120°C, pressure at endplate – 15 bar, revolution of screws – 105–125 rpm, revolution of cutter – 50 rpm.

## Chemical analysis

The content of the basic chemical components in experimental diets (dry matter, crude protein, crude fat and crude ash) were determined in accordance to standard methods (AOAC 2016). Dry matter was determined by drying in an oven at 105°C for 24 h. Total protein was determined by Kjeldahl's method and crude fat by Soxhlet's method. Crude ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h.

## Growth measurements

Growth performance was determined as follow:

Specific growth rate (SGR) = [(ln final weight – ln initial weight) / time (days) × 100];

Feed conversion ratio (FCR) = (feed intake / wet weight gain);

Hepato-somatic index (HSI) = (liver weight / body weight) × 100

## Blood plasma cholesterol

Blood samples were centrifuged at 12000 g for 30 s. Blood plasma concentration of cholesterol (CHOL) was determined with VetTest Chemistry Analyzer (Idex x Lab., USA). Blood cholesterol level was measured in randomly sampled 15 fish from each group after the 1st, 2nd, 4th and 8th week of the experiment.

## Statistical analysis

Normality of data distribution was tested by Shapiro-Wilk test and variance homogeneity by Leven's test. When above assumptions were met,

differences between means were analysed using ANOVA and *post hoc* Tuckey's test. For the others, Kruskal-Wallis ANOVA and Mann-Whitney test were used. Results were analyzed with Statistica 13.0 (Statsoft, USA) software at significance level $p \leq 0.05$.

## Results

No significant difference was found in the blood cholesterol level between experimental groups when compared in the same day of experiment ($p > 0.05$). However comparison of results within one group at different weeks of the experiment revealed significant differences in blood cholesterol in AF10 and AF20 groups (Table 2). Statistically significant drop of blood plasma cholesterol level occurred after 28 days of feeding in both above groups (Figure 1).

Table 2

Cholesterol plasma level of *Oncorhynchus mykiss* fed experimental diets formulated with amaranth meal. Results are presented as mean ± SD (range). Values in columns marked with the same upper index are not significantly different ($p \leq 0.05$)

| CHOL [mmol $dm^{-3}$] | CF | AF10 | AF20 |
|---|---|---|---|
| 1 week | $3.87^{a}$ ± 0.35 (3.41–4.21) | $3.99^{a}$ ± 0.92 (2.84–5.05) | $3.68^{ab}$ ± 0.87 (2.46–4.47) |
| 2 week | $3.91^{a}$ ± 0.28 (3.72 –4.32) | $4.66^{ab}$ ± 0.19 (4.39–4.84) | $4.40^{a}$ ± 0.50 (3.93–4.91) |
| 4 week | $3.96^{a}$ ± 0.83 (3.15–4.97) | $3.80^{a}$ ± 0.89 (2.89–4.78) | $2.99^{b}$ ± 0.19 (2.74–3.19) |
| 8 week | $4.73^{a}$ ± 1.50 (2.34–9.89) | $5.12^{b}$ ± 1.36 (1.70–8.55) | $4.61^{a}$ ± 1.11 (2.57–7.29) |

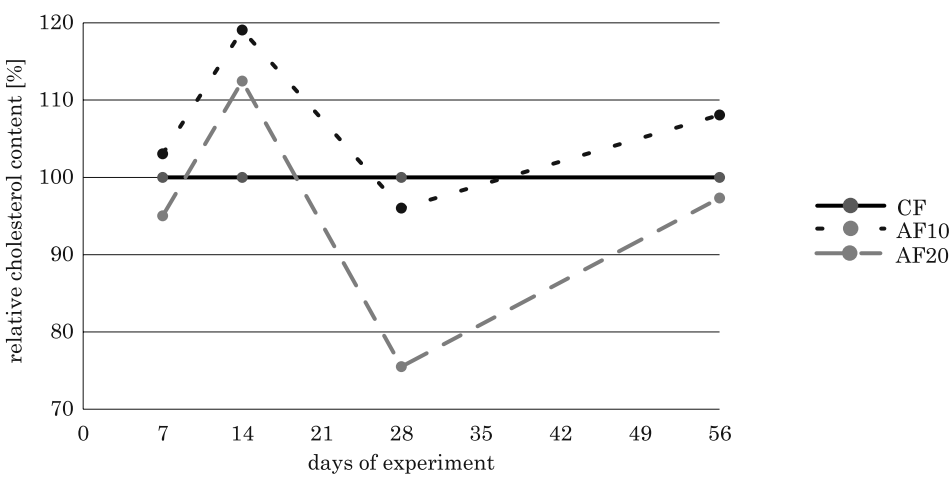


Fig. 1. Changes in blood plasma cholesterol level in rainbow trout fed experimental diets formulated with amaranth meal in relation to cholesterol content in control group

Growth indices of experimental fish are presented in Table 3. Increase of body weight and length was not significantly different between all experimental groups. SGR for CF (1.13) was lower than those obtained for AF10 (1.15) and AF20 (1.23). HSI recorded in CF (1.61%) was higher than those in experimental group AF10 (1.59) and AF20 (1.52). Significantly different FCR was observed when CF (1.25) and AF20 (1.10) groups were compared.

Table 3
Growth performance and feed efficiency of rainbow trout feed on experimental diets for 60 days. Values are presented as mean ± SD (range). Number indexes show columns with significantly different results ($p \leq 0.05$)

| Specification | CF | AF10 | AF20 |
|---|---|---|---|
| Initial total length [cm fish$^{-1}$] | $21.79^{a} \pm 1.75$ | $22.31^{a} \pm 2.08$ | $21.43^{a} \pm 2.50$ |
| Final total length [cm fish$^{-1}$] | $25.33^{a} \pm 2.44$ | $25.98^{a} \pm 2.51$ | $25.56^{a} \pm 2.46$ |
| Initial weight [g fish$^{-1}$] | $99.67^{a} \pm 20.80$ | $106.71^{a} \pm 25.05$ | $97.26^{a} \pm 28.62$ |
| Final weight [g fish$^{-1}$] | $191.36^{a} \pm 64.11$ | $211.32^{a} \pm 64.83$ | $206.38^{a} \pm 66.18$ |
| SGR [%/d] | $1.13^{a} \pm 0.32$ | $1.15^{a} \pm 0.24$ | $1.23^{a} \pm 0.32$ |
| FCR [1] | $1.25^{a} \pm 0.14$ | $1.18^{a} \pm 0.02$ | $1.10^{b} \pm 0.06$ |
| HSI [%] | $1.61^{a} \pm 0.40$ | $1.59^{a} \pm 0.35$ | $1.52^{a} \pm 0.33$ |

## Discussion

Plasma cholesterol level is a result of several factors influencing its metabolism in the fish body. The key element in controlling its level is the type of diet used (MAITA et al. 2006). The main source of exogenous cholesterol in the diet are animal components. Components of plant origin have trace amounts of cholesterol, so a diet with the predominance of these components prompts the body to increase the synthesis of endogenous cholesterol. What is more, vegetable flours contain also anti-nutritional substances which limit the level of absorption of exogenous cholesterol from feed (FRANCIS et al. 2001 ) Among them, saponins and phytosterols (KROGDAHL et al. 2010) seems to be the most important. Saponins form complexes with cholesterol limiting its absorption (MALINOV et al. 1977). Phytosterols compete with cholesterol in the formation of micelles in the intestinal lumen inhibiting its absorption (CARDEN et al. 2015) and thus lowering its level in the blood (LAW 2000). The meal of amaranth contains saponins (ESCUDERO et al. 2004), however, their content in amaranth grains is at a very low level of 0.09–0.1% (OLESZEK et al. 1999), as well as phytosterols – 2% (MARTIROSYAN et al. 2007) in oil from amaranth. So their contribution to reducing cholesterol absorption seems to be negligible.

Another component used in the studied feed formulas containing phytosterols is wheat flour. The highest concentration of anti-nutritional substances occurs mainly in seed sprouts of this cereal (LAIRON et al. 1987, BOREL et al. 1989). Research carried out by OSTLUND et al. (2003) showed that the phytosterols present in wheat are biologically active and play an important role in reducing the absorption of cholesterol, but do not completely block the absorption. The share of phytosterols in mature cereals, depending on the type of grains, is rather negligible from 670–959 $\mu g^{-1}$ g (winter) and 797–949 $\mu g^{-1}$ g (spring – summer) (NURMI et al. 2008). One can conclude that amount of substances that actively limit absorption of cholesterol is negligible in experimental feeds.

Extending the time of exposure of rainbow trout to fodder with the addition of protein of vegetable origin most often causes a decrease in blood cholesterol level (IWASHITA et al. 2008, YAMAMOTO et al. 2010). The addition of amaranth meal did not cause significant differences in the level of cholesterol in the blood of the fish at the end of the experiment, although in the experimental groups a higher level was observed than in the control group. The cholesterol content in the experimental groups (4.61–5.12 mmol $dm^{-3}$) was also higher than those noted for soy components in rainbow trout studies, e.g. for soy flour (2.04–2.32) (IWASHITA et al. 2008), fermented soy flour (2.74–2.96, 2.93 –3.41) (YAMAMOTO et al 2010, 2012), modified soy flour (3.22–4.09) (MURASHITA et al. 2013) in a similar period of the experiment. According to the authors, the reason for this phenomenon is the limited absorption of cholesterol resulting from the presence of significant amounts of both phytosterols and saponins in the soy components. In order to eliminate this effect, a soy protein concentrate containing negligible amounts of phytosterols and saponins was used in the recipes of experimental feeds.

The cholesterol increase in blood plasma of experimental fish after 2 weeks of feeding was probably caused by the biosynthesis of cholesterol from the squalene present in amaranth meal. The increase of squalene content to 0.12% and 0.14% of feed caused an increase in blood cholesterol concentration of the studied fish. A similar dependence and increase in blood cholesterol level was observed by MENDONÇA et al. (2009) giving hamsters the feed containing amaranth oil rich in squalene.

The results of the studies in which cholesterol was given in feed, suggested a buffer system in fish that protects against high cholesterol (DENG et al. 2013). According to the authors, this system inhibits the production of endogenous cholesterol in the case of exogenous cholesterol excess. A fall in the blood cholesterol level of trout fed with fodder with the addition of amaranth meal may indicate decrease of endogenous cholesterol production after 4 weeks of feeding.

Biosynthesis of cholesterol is regulated at two key stages, including hydroxymethylglutaryl-COenzyme A reductase (HMGCR) activity and squalene monodoxidase activity (SMO) (GOEDEKE et al. 2012). The increase in the amount of cholesterol in the blood causes the inhibition of HMGCR (BOTHAM et al. 2015). The activity of SMO is controlled by squalene excess. We believe that feeding fodder containing more than 0.10% of squalene resulted in a decrease in the activity of these enzymes and a decrease in blood cholesterol. However, after further 4 weeks, the cholesterol content in the blood plasma increased again to the level observed at the beginning of the experiment. These observed cholesterol fluctuations reflect the action of the system controlling the cholesterol level in the body of trout.

The use of vegetable meal in the diet of salmonids very often causes an increase in FCR value (DREW et al. 2005, COLLINS et al. 2012). The addition of amaranth meal to experimental feed for rainbow trout has had no negative effect on growth parameters and the most advantageous FCR. The better FCR was also observed by NIEWIADOMSKI et al. (2016) who used the amaranth meal addition at the level of 5–10% in feeds for rainbow trout and obtained FCR values 1.12–1.00, respectively. SGR and HSI values although not significantly different has shown good tendency; increasing SGR and decreasing HSI with the increase of amaranth meal content.

## Conclusions

Obtained results has shown that amaranth addition to trout feed causes temporary fluctuation of cholesterol level. Blood plasma cholesterol level was stabilized within 4 weeks following significant drop. We speculate that increased level of squalene in experimental feeds was the main reason of cholesterol fluctuation. Influence of higher squalene levels in fish feed should be studied in future research.

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# PHYSICOCHEMICAL CHARACTERISTICS AND ANTIOXIDANT ACTIVITIES OF JAPANESE QUAIL (*COTURNIX COTURNIX JAPONICA*) EGG YOLK OIL (QEYO) EXTRACTED USING TWO DIFFERENT METHODS

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Key words: japanese quail, egg yolk oil, extraction methods, mineral content, physicochemical characteristics, antioxidants.

Abstract

In this study, the physico-chemical characteristics, mineral, phenolic contents and antioxidant activities of QEYO were evaluated. The oil was extracted using gentle heating (GH) and *n*-hexane (NH) methods, extracts were subjected to proximate, physicochemical and mineral analysis using atomic absorption spectrophotometry (AAS).Total phenolic contents (TPC) was quantified using Folin-Ciocalteau method; while the antioxidant activity were measured using the 1,1-diphenyl-2 picryl hydrazyl (DPPH) – radical scavenging, ferric reducing antioxidant activity (FRAP) and total flavonoid contents. Physicochemical screening showed the presence of saponins, peroxides, ash, moisture and iodine, with little anthraquinones and cardiac glycosides. Sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), and phosphorus (P), were obtained in both fractions. Oil extracted by GH had the higher TPC and FRAP values ($p < 0.05$), while the NH fraction was higher in (TFC) ($p < 0.05$). QEYO showed antioxidant activities and can be used for the management of different diseases related to oxidative stress.

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## Introduction

The nutritional, vitamins and minerals content of quail eggs are much higher than those offered by other eggs. They are especially rich in the essential amino acids (methionine, lysine, and phenylalanine), and are rich sources of antioxidants, minerals, vitamins, and nutrients (WUNJUNTUK et al. 2016). The egg have been claimed to improve metabolism, combat stress, helps in the treatment of obesity, asthma and various forms of allergies. (LALWANI 2011, TUNSARINGKARN et al. 2013). Regular consumption of quail eggs helps fight against many diseases as it is a natural combatant against digestive tract disorders such as stomach ulcers, strengthen the immune system, promote memory health increase brain activity and stabilize nervous system (TRUFFIER 1978). Recently, (LESNIEROWSKI and STANGIERSKI 2018) reported that, eggs are not only a highly nutritious food, but also a rich source of diverse bioactive components also including nutraceutical. Quail pickled egg have been reported to offer such nutritious, ready-to-eat product to the consumers in Egypt (BAYOMY et al. 2017). Because of their extraordinary nutritional and medicinal properties, they are being used with more and more success in Europe and America as well as in the far east (DOWARAH and SETHI 2014). Egg yolk oil, also called ovum oil is derived mostly from the yolk of chicken eggs, although it can be derived from goose, duck and other avian eggs. It consists mainly of triglycerides with traces of lecithin, cholesterol, xanthophylls such as lutein and zeaxanthin and immunoglobulins (WALKER and EMERSON 1964). Oil extracted from hens and duck egg has anti-inflammatory and analgesic effects (HANDE 1983). Solvent extraction of egg oil has been reported to be the simplest and inexpensive method of extraction of egg yolk (KOVALCUKS and DUMA 2014a, WU et al. 2016), and the use of hexane in a combination of 70/30 in ratio with 2-propanol tends to produce more oil in term of yield and $\beta$-carotene content, Furthermore, hexane has been identified to be the most popular solvent for lipid extraction in food application (KOVALCUKS and DUMA 2014b). On the other-hand, egg oil extracted using the gentle heating method was reported to have the best anti- inflammatory effects (MAHMOUDI et al. 2013). Therefore this study was design to compare the two inexpensive extraction methods of yolk oil from quail egg, with the aim of comparing them in term of yield, mineral and antioxidant content for recommendation for use in cosmeceutical and drug industries.

## Materials and Methods

### Quail Egg Material

The eggs were purchased from a local market in Abuja Nigeria. They were identified and authenticated by an Avian Pathologist from the Department of Veterinary Pathology, Usmanu Danfodiyo University, Sokoto, Nigeria.

### Preparation of Quail egg powder

Two Hundred and forty (240) quail eggs yolk were manually separated from the egg white as described by (MAHMOUDI et al. 2013) sundried in a beaker at 31–37°C and subsequently blended till a powdery form was obtained. Eight hundred gram (800 g) of the powder was divided into two equal parts for the extraction of the oil. The extraction of the Quail egg yolk oil was performed using the *n*-hexane method of extraction (WARREN et al. 1988) and gentle heating method (MAHMOUDI et al. 2013).

### Quail Egg yolk oil Extraction using *n*-hexane

The extraction was performed in the Pharmacology and Toxicology Department, Faculty of Pharmaceutical Science, UDUS, using the Soxhlet extractor (Konte, USA). Four hundred grams (400 g) of the powdered egg sample was poured into a porous thimble and placed in a Soxhlet extractor, using 150 $cm^3$ of N-hexane (with boiling point of 40–60°C) as extracting solvent for 6 hours. The oil was obtained after the solvent was removed under reduced temperature and pressure and refluxing at 70°C to remove the excess solvent from the extracted oil. The oil was then stored in freezer at – 2°C for subsequent studies (WARRA et al. 2011b, WARREN et al. 1988).

### Gentle heating extraction of Quail Egg yolk oil

Egg yolk oil was prepared from 400 g of egg powder. Yolk powder was directly heated for about 4 hours gently and filtered with cheesecloth size 50, (Vertical x Horizontal thread 28 x 2) cm filter. The filtrate was used as yolk oil prepared by direct heating (MAHMOUDI et al. 2013).

### Oil yield (OY) determination [%]

Solvent that was freed from the oil obtained after extraction was placed over a water bath at 70°C for 30 mins and the volume of oil was recorded and expressed as oil content [%] as calculated below:

$$\%\ yield = \left(wt \text{ of } \frac{oil}{wt} \text{ of egg yolk powder}\right) \cdot 100$$

(PRITCHARD and ROSSELL 1991)

### Preliminary physicochemical, proximate and mineral analysis of Quail Egg Yolk Oils
### Moisture content (MC) determination

Phytochemical screening of Quail Egg Yolk oil extracted through the gentle heating and *n*-Hexane extraction methods was carried out as follows.

A crucible was washed and dried in the oven, after cooling in the desiccator and weighed ($W_1$). 1.16 g of the sample was carefully weighed in the crucible and the weight was taken as ($W_2$). The crucible containing the sample was then placed in an oven at temperature of 105°C for 1 hour. It was cooled and weighed. The crucible was then introduced into the oven again and process of cooling and weighing continued at intervals until a constant weight was obtained ($W_3$) (COX and PEARSON 1962).

Percentage moisture content was calculated using the formula:

$$\%\ \text{MC}\ [g] = \left(W_2 - \frac{W_3}{W^2} - W^1\right) \cdot 100$$

where:
$W_1$ – weight of empty crucible [g]
$W_2$ – weight of crucible with extract [g]
$W_3$ – weight of crucible after heating [g]

### Ash content (AC) determination

A porcelain crucible was washed and dried in an oven then cooled in a desiccator and weighed. 0.53 g of the sample was carefully weighed in the crucible containing the sample and was heated gently on a Bunsen burner until the smoke ceased. It was then transferred to a muffle furnace and heated at a temperature of 550–570°C for 2 hours to burn all organic

matter. The crucible was taken out of the muffle furnace after a white residue was observed and placed in a dessicator to cool and weighed (COX and PEARSON 1962).

$$\%\, AC[g] = \left(wt \text{ of } \frac{\text{ash}}{\text{wt}} \text{of sample}\right) \cdot 100$$

**Relative density (RD) determination**

A specific density bottle was washed, dried and weighed ($W_1$). It was filled with distilled water and weighed ($W_2$). The water was poured off and the bottle was dried to its previous constant weight and then filled with the oil sample and weighed ($W_3$) (COX and PEARSON 1962).

$$\text{RD} = (W_2 - \frac{W}{W_1} - W_3)$$

**Iodine value (IV) determination**

Iodine monochloride (13.6 g) was dissolved in 8243 ml glacial acetic acid and cooled. 25 ml of this solution was then titrated against 0.1 N sodium thiosulphate. Another portion of 200 ml of glacial acetic acid was added to 3ml of bromide, and 5 ml of this solution was added to 10 ml of 15% potassium iodide solution and titrated against 0.1 N sodium thiosuphate and mixed well.

Oil samples (0.5 g) was weighed into an iodine flask and dissolved in 10 mL of chloroform this was followed by 25 ml of Hanus solution. The flask was stopped and kept in the dark cup board for 30 minutes at room temperature; 10 ml of 10% of potassium iodide solution and 100 ml of freshly boiled and cooled water added. This was titrated against 0.1 ml Sodium thiosulfate solution using starch as an indicator. A blank titration was also conducted under the same conditions without the sample (COX and PEARSON 1962).

$$\text{IV} = (\frac{(B - S)\ \cdot N\ 12.69}{\text{g}} \text{of sample})$$

where:
$B$ – mL thiosulphate for blank
$S$ – mL thiosulphate for the sample
$N$ – normality of thiosulphate solution.

### Peroxide value (PV) determination

One gram (1 g) of the oil sample was transferred into 20 ml flask and 1 g of powdered potassium iodide (KI) and a solvent mixture (2:1 of glacial acetic and trichloromethane) were then added. The solution was then placed on a water bath for a few minutes for complete dissolution. 20 ml of 50% potassium iodide was then introduced into the conical flask and the sample titrated with 0.1 M $Na_2S_2O_3$. The indicator was a regular starch solution. Blank experiment was similarly performed (COX and PEARSON 1962).

$$\mathrm{PV} = \left(\frac{S \cdot N}{g} sample\right) \cdot 100$$

where:
$S$ – mL $Na_2S_2O_3$ Test–Blank)
$N$ – normality of $Na_2S_2O_3$.

### Saponification value (SV) determination

The oil sample at 5.0 g was weighed in to 200 ml conical flask and 25 ml of 0.5 M of ethanolic potassium hydroxide solution was added. The flask was configured to a condensing set-up and heated on a water-bath for 1 hour with frequent shaking and the content was allowed to cool. The solution was then titrated with warm 0.5 M hydrochloric acid using 1% phenolphthalein indicator. PEARSON 1991, Equivalent titration was performed for the blank and generated values were employed for computation according to the following relation

$$\mathrm{SV} = A - B \cdot 28.05/Q$$

where:
$A$ – volume of 0.5 M of hydrochloric acid used in the blank titration,
$B$ – volume of 0.5 M of hydrochloric acid used in the sample titration,
$Q$ – weight in grams of the oil sample 28.05 = conversion factor

### Cardiac Glycosides (PG)

One mL of the sample was extracted with 10 $cm^3$ of 70% ethanol and filtered. From the filtrate, 8 $cm^3$ was transferred into 100 $cm^3$ volumetric flask and the volume made to the mark with distilled water and filtrate. 8 $cm^3$ of 12.5% lead acetate solution was added to 8 $cm^3$ of the filtrate. The

mixture was shaken thoroughly and transferred into a 100 $cm^3$ volumetric flask and made to the mark with distilled water and filtered. A 50 $cm^3$ of the filtrate was then pipetted into 100 $cm^3$ flask and 8 $cm^3$ of 4.5% $Na_2HPO_4$ solution and added. This mixture was filtered twice and 10 $cm^3$ of Baljet's reagent was added to the filtrate.

A blank experiment was also prepared by excluding the sample from the mixture, but all other conditions were kept constant. The mixture was allowed to stand for an hour for the colour to develop and the intensity of the colour was measured using Spectrophotometer at 495 nm (COX and PEARSON 1962). Percentage of glycoside was calculated as follows:

$$\text{PG}\,[\%] = A \cdot 100/70$$

### Anthraquinones

Two mL of the oil extract was boiled with 5 ml of 10% hydrochloric acid for 3 minutes. 5 ml of chloroform was added. 5 drops of 10% ammonia was further added. A rose pink coloration indicates a positive result (HARBORNE 1998). Absorbance of the resulting colour was then measured using 1 cm cuvette.

### Determination of Mineral Elements by Wet Digest Method

The elements were extracted from the oil by the wet digest method (TAIYE and ASIBEY-BERKO 2001). The digested sample was analysed for the elemental composition using Atomic Absorption Spectrophotometer (AAS). Na, K, Mg, and Ca were determined and the concentrations of the elements were presented in $mgL^{-1}$.

### Determination of effect of oil extract On 2,2-diphenyl-1 picryl hydrazyl (DPPH) radical

The extract (0.02 g) was dissolved in 20 mls of 95% methanol and stirred using a stirrer, then 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1.0 ml from mixture was poured into 5-sets of test tubes labelled (*A* to *E*) and one test tube was used as control which contains 1.0 ml of distilled water labelled (F). 1 ml of DPPH regent was added into each of test tubes labelled (*A* to *F*). The absorbance of tests and that of control were measured spetrophotometrically at 517 wavelength (HATANO et al. 1998)

### Estimation of total phenolic content using Folin–Ciocalteu's reagent

The extract (0.1 g) was dissolved in 50 ml of distilled water after which solution was mixed, 2 mls of mixture was poured into 3 sets of test tubes. 2 mls of 17% $Na_2CO_3$ into each of test tube. One test tube was used as control. Then 0.5 ml of Folin–Ciocalteu's reagent was added into test tube after which the sample were centrifuged using centrifuge machine at 4,000 revolution/minute. The supernatant was collected in fresh 2 ml micro-tubes absorbance of test and controlled was measured at 765 nm wavelength.

### Determination of ferric reducing antioxidants assay (FRAP)

The ferric reducing antioxidant power of the oil extract was determined in this study by reacting the extract (1 ml) with (5 ml) of distilled water, then 1 M HCl (1.5 ml) and 1% potassium ferricyanide (1.5 ml) was added. Then sodium dodecyl sulphate (SDS, 0.5 ml) and 0.5 ml of 0.2% ferric chloride ($FeCl_3$) were added, and the entire solution was incubated at 50 for 20 min to complete the reaction. Gallic acid was used as the standard and the absorbance was measured using a spectrophotometer at 750 nm, the results were expressed as mg GAE/g d.w. sample.

### Determination of flavonoids using precipitation method

A weighed sample of 5 grams quail egg oil was hydrolysed by boiling in 100 mls of hydrochloric acid solution for about 35 minutes. The hydro--lysate was filtered to recover the extract (filtrate). The filtrate was treated with ethyl acetate drop wise twice until in excess. The precipitated flavonoid was recovered by filtration using a weighed filter paper after drying in an oven at 100°C for 30 minutes; it was cooled in a desiccator and reweighed. The difference in weighed gave the weighed flavonoid which was expressed as a percentage of the weighed of sample analysed.

### Statistical Analysis

The data obtained from the study were analysed using INVIVOSAT statistical software and data was expressed as mean ± standard error of mean (SEM), while significant difference was determine using Student *T*-Test, and values less than 0.05 were considered to be statistically significant.

## Results

### Preliminary Physicochemical Properties of QEO

The results of the preliminary physicochemical screening show the presence of saponins, peroxides, ash, moisture and iodine, with very small amounts of anthraquinones and cardiac glycosides The saponins, and Iodine content are relatively higher in the oil extracted using the gentle heating method, while the peroxide value, moisture content and the yield of the oil sample is higher in oil extracted using the *n*-hexane solvent as shown in Table 1.

Table 1

Preliminary physicochemical screening results of QEO

| QEO/Parameter | QEO (GH) | QEO (NH) |
|---|---|---|
| Saponification value [mg KOH/g] | 237.9* | 230.5 |
| Peroxide value [mq $H_2O_2$] | 7.0 | 10.0* |
| Iodine value [g$I_2$/100 g] | 21.83* | 18.82 |
| Ash content [%] | 1.89 | 1.42 |
| Moisture content [%] | 13.04 | 19.83* |
| Cardiac glycoside [g/100 g] | 0.093 | 0.078 |
| Oil yield [%] | 15.9 | 35* |
| Relative density [g] | 0.84 | 0.83 |
| Anthraquinone [mg $g^{-1}$] | 0.012 | 0.015 |

QEO – quail egg yolk oil NH – *N*-Hexane GH – gentle heating
* Values significantly higher when compared between the two extraction methods ($p < 0.05$)

### Mineral Content of QEO

The results for the preliminary mineral screening shows the presence of sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), and phosphorus (P), in the quail egg yolk oil for both *n*-Hexane and gentle heating extraction processes. Calcium and potassium level are higher in oil extracted using the gentle heating method while sodium, magnesium and phosphorus level are higher in the oil extracted using the *n*-hexane solvent as shown in Table 2.

Table 2

Preliminary mineral screening results of QEO

| QEO/parameter [mg $L^{-1}$] | Na | K | Ca | Mg | P |
|---|---|---|---|---|---|
| QEO(GH) [mg $L^{-1}$] | 12.5 | 130.0* | 0.90* | 0.60 | 4.65 |
| QEO(NH) [mg $L^{-1}$] | 25.0* | 92.5 | 0.55 | 1.95* | 6.41* |

Note: QEOGH – quail egg yolk oil gentle heating; QEONH – quail egg yolk oil; NH – N-Hexane; Na – sodium; K – potassium; Ca – calcium; Mg – magnesium; P – phosphorus

* Values are significantly higher when comparing the values between the two extraction methods ($p < 0.05$)

## Total phenolic content (TPC)

### 2,2-diphenyl-1,1-picrylhydrazyl (DPPH)

The TPC results showed that, quail egg samples extracted using the gentle heat method showed higher total phenolic content with high absorbance level compared to the egg samples extracted using the *n*-hexane extraction ($P > 0.05$) as shown in Figure 1.

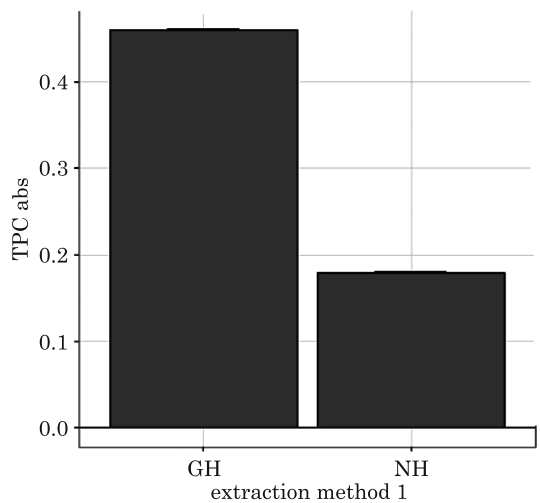


Fig. 1. Showing the mean ± SEM total phenolic content of quail egg oil extracted using the gentle heat and *n*-hexane methods;

* Indicate a significant difference between the two methods of extraction ($p < 0.05$)

The DPPH antioxidant results between the two group shows that, DPPH scavenging activities is higher in egg samples extracted using the gentle heat method compared to *n*-hexane extraction method although not significant ($P > 0.05$) as shown in Figure 2.

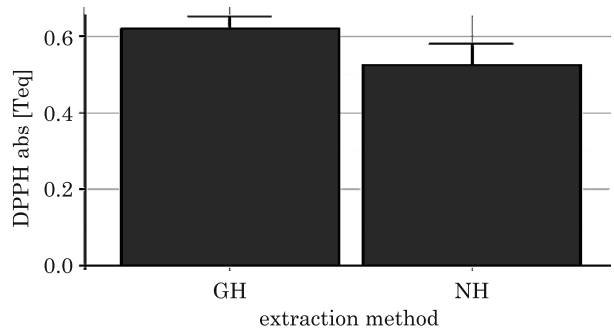


Fig. 2. Showing mean ± SEM DPPH scavenging activity using gentle heat and *n*-hexane extraction methods. No significant difference when comparing the absorbance level between the two methods of extraction ($p > 0.05$)

## Ferric reducing antioxidants power (FRAP)

The Ferric reducing antioxidant results between the two group shows that, FRAP is higher in egg samples extracted using the gentle heat method compared to n-hexane extraction method. ($P > 0.05$) as shown in Figure 3.

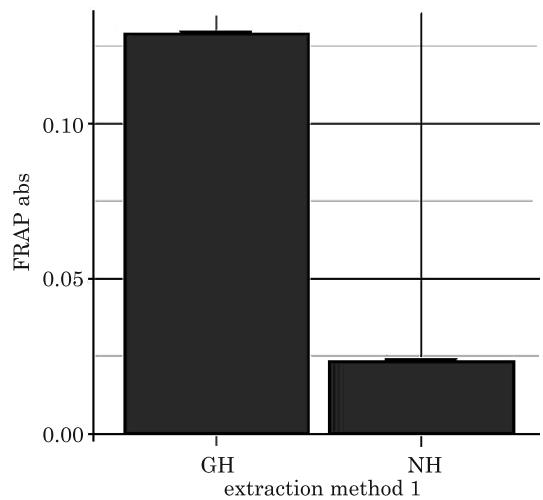


Fig. 3. Showing the mean ± SEM Ferric reducing antioxidants property of quail egg oil extracted using the gentle heat and *n*-hexane method of extraction

***** Indicate a significant difference between the two methods of extraction ($p > 0.05$).

### Total flavonoid contents (TFC)

The TFC results showed that, quail egg samples extracted using the gentle heat method showed lower total flavonoid content with low absorbance level compared to the egg samples extracted using the *n*-hexane extraction ($P > 0.05$) as shown in Figure 4.

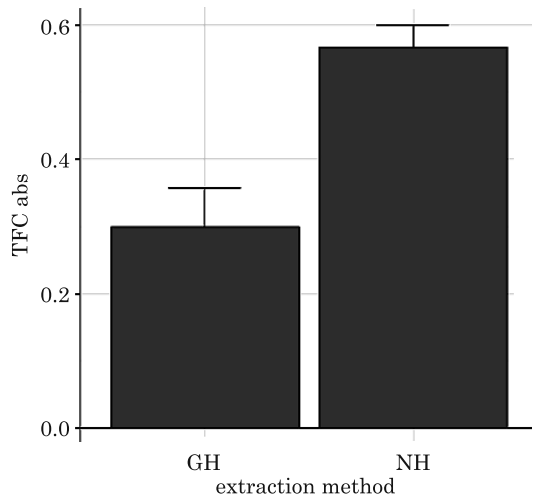


Fig. 4. Showing the mean ± SEM total flavonoid content of quail egg oil extracted using the gentle heat and n-hexane methods of extraction;
* Indicate a significant difference between the two methods of extraction ($p < 0.05$)

## Discussion

Oils are known for their use in various medicinal purposes and sometime as adjuvants or co-therapeutic agents. Extraction of oil from quail egg and the quantification of its bioactive compounds with the study of its antioxidant properties have not been reported in the literature. The percentage yield of crude oil obtained in this study was higher in the fraction extracted using *n*-hexane (35%), previous study by (LARSEN and FRONING 1981) also reported a high yield of the oil using *n*-hexane. KOVALCUKS and DUMA 2014a reported a yield of 28.90±0.27% and 26.37±1.04% with 2-propanol/hexane and ethanol/chloroform solvent combination. Although, the *n*-hexane solvent extraction gives the high yield than the gentle heating method (15.9%), previous research reported high concentration of residual solvents which limits the usage of egg oil which were extracted using solvents in food or in cosmetics (KOVALCUKS 2014). Preliminary mineral element screening of Quail egg yolk oil (QEYO) extracted with the gentle

heating and *N*-Hexane methods of extraction revealed the presence of sodium (Na), potassium (K), calcium (Ca), magnesium (Mg) and phosphorus (P). A significant concentrations of potassium was obtained in the two oil extracts (130 mg $L^{-1}$ for GH fraction and 93 m $L^{-1}$ for the NH extract) which was higher when compared to what was obtained in raw quail egg (63.0 mg $L^{-1}$) by (OLUWAFEMI and UDEH 2016) . The preliminary physicochemical analysis showed the presence of saponins in the oil. QEOGH and QEONH had an abundance of saponins with 237.90 mg KOH $g^{-1}$ and 230.57 mg KOH $g^{-1}$ respectively. Saponification value of cotton seed was estimated to be 199.42 ± 0.53 mg KOH $g^{-1}$ while neem seed oil is 213 mg KOH $g^{-1}$, groundnut oil is 168 mg KOH $g^{-1}$ while moringa oil is 155.68 mg KOH $g^{-1}$ , coconut oil however has a higher saponins content of 253.2 mg KOH $g^{-1}$ (AFOLAYAN et al. 2014, WARRA et al. 2011a).

Anthraquinone content of QEO was 0.0012 mg in QEOGH and 0.0015 mg in QEO (NH). Anthraquinones are known to have anti-inflammatory and purgative and emetic effects. They also possess antihypertensive effects and have been shown to inhibit angiotensin converting enzyme (ACE) in rats at a dose of 169.93 microgram $L^{-1}$ (HYUN et al. 2009).

Total phenolic contents and antioxidants activity of quail egg oil was best demonstrated in the samples extracted using the gentle heating method therefore, the findings of the present study revealed that extraction method plays an important role in determining the total phenolic contents and antioxidant activity of quail egg oil. Extraction methods has a tremendous effect on the yield of bioactive compounds and antioxidant activities of some products (SANI et al. 2012).

The gentle heat method used in this work has higher total phenolic content relatively compared to *n*-hexane method of oil extraction; the result is in accordance with previous studies which shows that efficiency antioxidants phenolics is lowest in hexane extracts of cashew shoot (RAZALI et al. 2008). Similarly, the result shows that quail egg oil extracted using gentle heat method has higher antioxidant activity for DPPH and FRAP antioxidants relatively compared to *n*-hexane method of extraction, the anti-inflammatory effects observed in the previous studies using the gentle heating extraction method might be linked to the presence of these antioxidants. Previous research showed that, hexane extract of *Limonium delicatulum* had the lowest anti-radical activity while ethanol had the highest (MEDINI et al. 2014). This might be the reason why gentle heat oil extract had higher DPPH and FRAP antioxidant activities compared to oil extract of *n*-hexane.

However, present study revealed that *n*-hexane method of extraction had higher total flavonoid content compared to gentle heat method of

extraction. Drastic reduction in flavonoid content of broccoli-based bars treated with heat was reported by (BARAKAT and ROHN 2014).

The DPPH antioxidant potential of quail egg oil extracted using gentle heating method of extraction is higher than *n*-hexane method but was not statistically significant ($p > 0.05$). The result showed that the total phenolic content and ferric reducing antioxidants activity were significantly higher ($P < 0.05$) in quail egg oil extracted using gentle heat method but statistically, quail egg oil using *n*-hexane extraction method has significantly higher total flavonoid content relatively compared to gentle heat method of extraction. The higher antioxidant potential of quail egg oil obtained in this study was not surprising due to the higher content of vitamin E (tocopherol, 5920.0 μg 100 $g^{-1}$) obtained in the raw yolk reported by (TUNSARINGKARN et al. 2013).

## Conclusion

The results of the present study showed that, *n*-hexane have the higher yield in term of extraction, but the fraction of the oil extracted using the gentle heating method portrays a higher antioxidants and other chemical constituents, and this might be the reasons for its reported anti-inflammatory effect. High yield of saponins, anthraquinones and other bioactives which are higher compared to those extracted in other products gives the quail egg an upper hand to be utilized as source of antioxidant and other bioactives for further utilization in cosmeceutical and pharmaceutical industries.

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# ANTHROPOGENIC TRANSFORMATIONS OF RIVER VALLEY'S VEGETATION AND THEIR IMPACT ON PERCEPTION OF ECOSYSTEM SERVICES BY INHABITANTS. A CASE STUDY FROM THE KŁODNICA VALLEY (SILESIAN UPLAND, POLAND)

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Abstract

Over 200 years of anthropopressure had an impact on the abiotic and biotic environment of the Kłodnica valley, as well as on ecosystem services and the standard of living of its inhabitants. Vegetation of semi-natural section of the Kłodnica valley (Katowice) was created by species-rich and species diverse phytocoenoses of nitrophilous fringes (*Urtico-Calystegietum sepium*, *Alliaria petiolata-Valeriana sambucifolia* community), wet (*Alopecuretum pratensis*) and fresh (*Festuca rubra* community) meadows. The probability of flood was low. Anthropogenic section of the Kłodnica valley (Gliwice) was dominated by poor in species and low diverse communities of invasive (*Impatiens parviflora*, *Reynoutria japonica*, *Aster novi-belgii-Parthenocissus inserta*) and expansive plants (*Urtico-Aegopodietum podagrariae*, *Bromus inermis* community). The probability of flood was high. Provisioning (fresh water), regulating (climate, flood regulation), as well as cultural (aesthetic value of landscape and plant cover, recreation) services were degraded and in the opinion of the residents the valley needs revitalization.

## Introduction

The development of human settlements has been inseparably connected with river valleys. The rivers have provided many important ecosystem services, including provisioning (e.g. food, water, timber, fiber), regulating (climate changes, floods, diseases, waste, water quality), cultural (e.g. recreational, aesthetic, spiritual benefits), as well as supporting (soil formation, photosynthesis, nutrient circulation) services (Millennium Ecosystem Assessment 2005). The intensification of urbanization and industrialization, which had begun in the 19$^{th}$ century, was accompanied by an increase in negative human interference in the river valleys. Some hydrotechnical works, including the straightening of riverbed, the artificial construction of flood embankments, melioration and drainage, have caused that riverine vegetation to be severely transformed or even completely destroyed. In addition, a considerable amounts of municipal and industrial wastewaters were discharged into the river, causing their pollution. The consequence of ecosystem degradation was the deterioration or even complete loss their ecosystem services (Bender et al. 2012, Everard and Moggridge 2012, Januchta-Szostak 2012, Sender and Maslanko 2018).

Vegetation seems to be one of the most sensitive component of the environment, that can be treated as indicator of changes in both biotic and abiotic conditions of the riverine ecosystem (Kowalska 2012). The anthropogenic disturbances of riverside ecosystems led to the synanthropization of existing vegetation. This is often manifested by growth in the number of communities, the spread of natural hemerophilous communities, the disappearance of the original species combinations, the creation of new, heterogeneous combinations of species, the emergence of poor in species and less diverse communities, and the lack of a dynamic balance expressed by many stages of degradation, regeneration and pioneer communities (Sudnik-Wójcikowska and Koźniewska 1988). Floodplain vegetation provides many ecosystem services, such as protection against soil erosion, water retention, macro- and microelements circulation, river self-purification, carbon sequestration, improvement of ionization and air circulation. In addition, it also has certain aesthetic value and serves as a place for recreation and tourism. One of the most significant consequences of strong transformations of riverine phytocoenoses is an increase in flood risks in heavy urbanized catchments (Hanczaruk et al. 2016, Hanczaruk and Kompała-Bąba 2016, 2017, Hanczaruk and Kulik-Knapik 2017, Kompała-Bąba and Hanczaruk 2017).

Kłodnica is an model example of river valley, which has been subjected to strong anthropopressure since the middle of the 19th century (ABSALON et al. 2007). From springs, located in Katowice, river flows through densely populated and industrialized towns of the Upper Silesia Industrial Region, such as Katowice, Ruda Śląska, Zabrze and Gliwice. It is transformed mainly by mining and processing of hard coal (DRĄG 2012). Below Gliwice, until its mouth in Kędzierzyn-Koźle, Kłodnica flows mainly through agricultural lands (OLKOWSKA et. al. 2017).

The aims of the study were:

– to recognize the diversity and distribution of the current vegetation of the Kłodnica valley being under different impact of anthropogenic transformations,

– to assess the flood risk in the investigated section of the Kłodnica valley,

– to assess the perception of ecosystem services provided by the Kłodnica valley by inhabitants, as well as the standard of living of the residents.

## Material and Methods

### Research sections of the Kłodnica valley

In order to show the influence of anthropogenic disturbances on the Kłodnica valley, two sections were selected, that differ in the structure of land use, the degree of the riverbed regulation, population density and the degree of synanthropization of vegetation. Their length was about 1.5 km.

The semi-natural section was located in Katowice (N 50°13'44", E 18°55'00) – Figure 1). The width of the Kłodnica valley reaches 100 meters. Kłodnica flows through unregulated or slightly regulated channel. The valley has numerous oxbow lakes and its area is characterized by the use of forest and agricultural land. The population density is 242 inhabitants/km$^2$, that valley being covered by a large dispersion of single-family buildings (CABAŁA and GREŃ 2002, *Otwarty Regionalny*... 2016a,b).

The anthropogenic section was located in Gliwice (N 50°17'20", E 18°41'06") – Figure 1). The development of industry and urbanization was accompanied by the regulation of the riverbed and concreting of its banks, as well as by removal of thickets and riparian forests, which naturally occurred along the Kłodnica valley from the beginning of the 18th century. Currently, densely populated areas (2081 inhabitants/km$^2$) are found near the Kłodnica valley in Gliwice. The landscape of the valley is dominated by compact urban and industrial buildings, didactic buildings of the

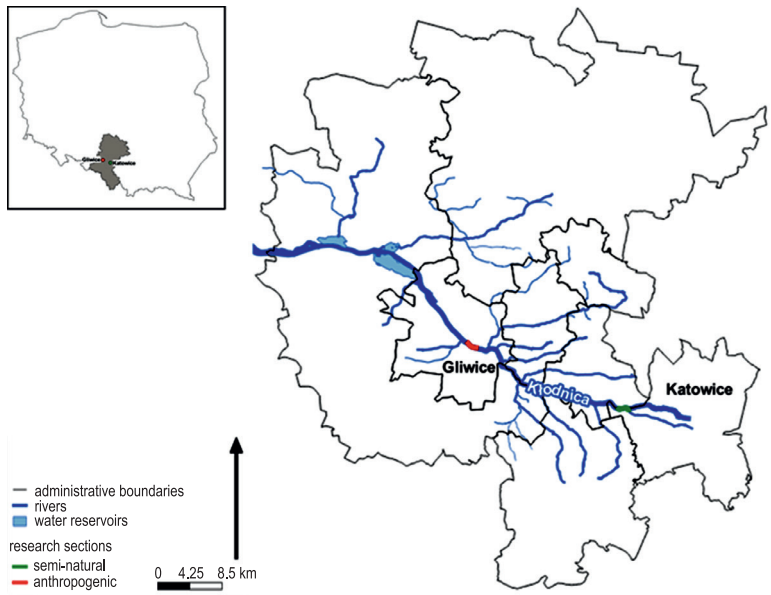


Fig. 1. Location of the research section on the background of the Kłodnica catchment map

Silesian University of Technology and sport, as well as recreational infrastructure. In the vicinity of the valley the city park occurs (*Otwarty Regionalny*... 2016a,b, HANCZARUK and KOMPAŁA-BĄBA 2017).

## Vegetation sampling

In the vegetation seasons 2014–2018, 151 phytosociological relevés were made along the studied sections of the Kłodnica valley using the BRAUN-BLANQUET (1964) method in order to recognize the diversity of present vegetation. Relevés had an area of 25 $m^2$. The plant nomenclature follows MIREK et al. (2002). The invasive alien plant species were taken after TOKARSKA-GUZIK et al. (2012).

## Classification of current vegetation of the Kłodnica valley

Before further analyses, the quantitative-qualitative Braun-Blanquet scale used for vegetation sampling was transformed into percentage values according to the rules: *r* – 1%, + –2%, 1–3%, 2–13%, 3–38%, 4–68%, 5–88%. The data were classified using the unweighted pair group method with arithmetic mean (UPGMA) and the Bray-Curtis distance (DZWONKO 2007). The calculations were made for log transformed data. In order to assess the affinity of species to distinguished vegetation units phi coeffi-

cient of association as a measure of fidelity was used (fidelity values in the range from -1 to 1 were multiply by 100). Each vegetation units was described by diagnostic, constant and dominant species. As a diagnostic species of particular vegetation unit were considered taxa, for which the phi coefficient was higher than 20. Constant species were taxa with frequency above 40% and dominant ones with a coverage of more than 25% in at least 15% of phytosociological relevés (TICHÝ 2002, TICHÝ and HOLT 2006, TICHÝ et al. 2010).

### Species richness and species diversity

The distinguished plant communities were compared in relation to species richness and species diversity. For these purpose, for each community the mean number of species (no.) and mean value of Shannon-Wiener diversity index (*H'*) were calculated in JUICE 7.0 program taking into account abundance of species (TICHÝ and HOLT 2006).

### Flood risk assessment

Area threatened by flooding was based on the range of the previous floods that have taken place since the beginning of the 20$^{th}$ century. Available historical data, photographic documentation, expertises and flood risk maps were used (RZĘTAŁA 2000, ABSALON et al. 2001, ABSALON et al. 2007, SCHMIDT 2009, ZAWARTKA 2012, *Centrum Ratownictwa Gliwice* 2015, Krajowy Zarząd Gospodarki Wodnej 2015).

### Questionnaire survey

In June 2017 a direct questionnaire survey was conducted on the actual state of the Kłodnica valley in Gliwice and the necessity of its revitalization, as well as on ecological awareness of the inhabitants and their perception of ecosystem services provided by the river. The research population was 100 residents of Gliwice, including students living in a student dormitories and rented flats. The data was collected on socio-demographical attributes of interviewers related to their gender, age, level of education, current status of work and place of residence (district). At the beginning of the survey, the respondents were informed in details about its purpose. The structure of survey and the way of answering particular questions were also briefly explained. Respondents were asked 11 questions. There were 6 open questions and 5 closed questions.

# Results

## Classification of current vegetation of the Kłodnica valley

The classification of the vegetation based only on the floristic criterion enabled 23 plant communities to be distinguished. They contained from 2 to 18 phytosociological relevés. Along the semi-natural section of the Kłodnica valley 18 plant communities constituted by 139 vascular plant species were recorded. In contrast, along the anthropogenic section 13 plant communities created by 86 species were identified. 10 plant communities occurred only in the semi-natural section, 5 communities – exclusively in the anthropogenic section, and 8 communities were common to both studied sections of the Kłodnica valley. The descriptions of plant communities was given below (Table 1).

Table 1

Diagnostic (Dg), constant (C) and dominant (Dm) species for the plant communities that were distinguished.

| Plant communities | Research section | Diagnostic, constant and dominant species |
|---|---|---|
| U-A | SA | Dg: *Aegopodium podagraria* (41.9), *Ballota nigra* (31.2); C: *A. podagraria* (85), *Urtica dioica* (42); Dm: *A. podagraria* (100) |
| U-C | SA | Dg: *Urtica dioica* (28.0), *Galium aparine* (20.1); C: *U. dioica* (78), *G. aparine* (43); Dm: *U. dioica* (89) |
| Sc | *A* | Dg: *Solidago canadensis* (54.7), *Humulus lupulus* (30.0); C: *S. canadensis* (85); Dm: *S. canadensis* (100), *H. lupulus* (33), *Aegopodium podagraria* (33) |
| Ip-Gp | *A* | Dg: *Impatiens parviflora* (66.2), *Galeopsis pubescens* (34.8); C: *I. parviflora* (63); Dm: *I. parviflora* (60), *G. pubescens* (40) |
| G-Ch | SA | Dg: *Chelidonium majus* (63.6), *Geum urbanum* (25.8); C: *C. majus* (84); Dm: *C. majus* (100) |
| Ap-Vs | *S* | Dg: *Alliaria petiolata* (54.4), *Valeriana sambucifolia* (27.2); C: *A. petiolata* (81), *Urtica dioica* (43); Dm: *A. petiolata* (100) |
| Ig | *S* | Dg: *Impatiens glandulifera* (45.4), *Veronica chamaedrys* (28.0), *Equisetum palustre* (25.7), *Myosotis palustris* (23.4), *Rubus idaeus* (21.5); C: *I. glandulifera* (47); Dm: *I. glandulifera* (50), *Anthriscus sylvestris* (50) |
| Ap | *S* | Dg: *Alopecurus pratensis* (83.0), *Ranunculus repens* (24.0), *Cirsium arvense* (23.3), *Holcus lanatus* (23.0); C: *A. pratensis* (82); Dm: *A. pratensis* (100) |
| Phr | SA | Dg: *Phragmites australis* (66.7); C: *P. australis* (84); Dm: *P. australis* (100) |

| | | |
|---|---|---|
| Sg | SA | Dg: *Solidago gigantea* (45.8); C: *S. gigantea* (86); Dm: *S. gigantea* (100) |
| Bi | A | Dg: *Bromus inermis* (58.3); C: *B. inermis* (88); Dm: *B. inermis* (100) |
| Ae | SA | Dg: –; C: *Arrhenatherum elatius* (67); Dm: *A. elatius* (50), *Eupatorium cannabinum* (50) |
| Hm | *S* | Dg: *Heracleum mantegazzianum* (84.2); C: *H. mantegazzianum* (85); Dm: *H. mantegazzianum* (100), *Scrophularia nodosa* (50) |
| Rj | SA | Dg: *Reynoutria japonica* (95.2), *Convolvulus arvensis* (22.3), *Calystegia sepium* (21.7); C: *R. japonica* (96); Dm: *R. japonica* (100) |
| Ht | *A* | Dg: *Helianthus tuberosus* (79.6), *Chenopodium album* (36.6), *Leontodon hispidus* (36.6), *Plantago media* (36.6), *Agropyron repens* (34.9); C: *H. tuberosus* (72), *A. repens* (47); Dm: *H. tuberosus* (75), *A. repens* (25) |
| Anb-Pi | A | Dg: *Aster novi-belgii* (76.1), *Parthenocissus inserta* (57.3), *Rubus caesius* (32.2); C: *A. novi-belgii* (73), *R. caesius* (49), *P. inserta* (43); Dm: *A. novi-belgii* (80), *P. inserta* (40) |
| Ce | SA An | Dg: *Calamagrostis epigejos* (60.0); C: *C. epigejos* (82); Dm: *C. epigejos* (100) |
| Dc | *S* | Dg: *Deschampsia caespitosa* (75.5); C: *D. caespitosa* (81); Dm: *D. caespitosa* (100) |
| Fr | *S* | Dg: *Festuca rubra* (56.8), *Rumex acetosa* (47.4), *Viola tricolor* (43.9), *Cardaminopsis halleri* (32.3), *Luzula campestris* (29.4), *Achillea millefolium* (23.7), *Ranunculus acris* (21.1), *Galium mollugo* (20.1); C: *F. rubra* (88); Dm: *F. rubra* (100) |
| Ss | *S* | Dg: *Scirpus sylvaticus* (56.3); C: *S. sylvaticus* (84); Dm: *S. sylvaticus* (100) |
| Phal | *S* | Dg: *Phalaris arundinacea* (64.6); C: *P. arundinacea* (90); Dm: *P. arundinacea* (100) |
| Ip-Gm | *S* | Dg: *Iris pseudacorus* (71.9), *Glyceria maxima* (69.3), *Caltha palustris* (37.7), *Lotus uliginosus* (25.3), *Juncus effusus* (20.3); C: *I. pseudacorus* (61), *G. maxima* (52); Dm: *I. pseudacorus* (57), *G. maxima* (43) |
| Lm | *S* | Dg: *Lemna minor* (85.0), *Lemna trisulca* (53.6); C: *L. minor* (82); Dm: *L. minor* (85) |

Explanations: research section: *S* – semi-natural, *A* – anthropogenic; abbreviations of the names of plant communities means: (U-A) *Urtico-Aegopodietum podagrariae*, (U-C) *Urtico-Calystegietum sepium*, (Sc) *Solidago canadensis*, (Ip-Gp) *Impatiens parviflora-Galeopsis pubescens*, (G-Ch) *Geo urbani-Chelidonietum maji*, (Ap-Vs) *Alliaria petiolata-Valeriana sambucifolia*, (Ig) *Impatiens glandulifera*, (Ap) *Alopecurus pratensis*, (Phr) *Phragmitetum australis*, (Sg) *Solidago gigantea*, (Bi) *Bromus inermis*, (Ae) *Arrhenatheretum elatioris*, (Hm) *Heracleum mantegazzianum*, (Rj) *Reynoutria japonica*, (Ht) *Helianthus tuberosus*, (Anb-Pi) *Aster novi-belgii-Parthenocissus inserta*, (Ce) *Calamagrostis epigejos*, (Dc) *Deschampsia caespitosa*, (Fr) *Festuca rubra*, (Ss) *Scirpetum sylvatici*, (Phal) *Phalaridetum arundinaceae*, (Ip-Gm) *Iris pseudacorus-Glyceria maxima*, (Lm) *Lemnetum minoris*.

1. *Urtico-Aegopodietum podagrariae*: medium-sized patches (50 $m^2$) of nitrophilous fringe community with *Aegopodium podagraria* or *Ballota nigra* were recorded in shady, slightly inclined (0–20°) places, as well as at the top and in the middle part of the slopes of the semi-natural and anthropogenic section of the Kłodnica valley. The average height of the herb layer is 105 cm.

2. *Urtico-Calystegietum sepium*: medium-sized patches (50 $m^2$) nitrophilous "weil" community with *Urtica dioica* and *Galium aparine* occur on flat or slightly inclined slopes (0–20°), shaded places, at the top and in the middle part of the embankments of the semi-natural and anthropogenic section of the Kłodnica valley. The average height of the herb layer is 142 cm.

3. *Solidago canadensis* community: small-sized (30 $m^2$) patches dominated by *Solidago canadensis* and *Humulus lupulus* were recorded on open sites on slightly inclined (0–15°) embankments of anthropogenic section of the Kłodnica valley. The average height of the herb layer is 140 cm.

4. *Impatiens parviflora-Galeopsis pubescens* community: large patches (125 $m^2$) of the nitrophilous fringe community with *Impatiens parviflora* and *Galeopsis pubescens* were developed under the canopy of trees, in depressions and in the flattening along the anthropogenic sections of the Kłodnica valley. The average height of the herb layer is 59 cm.

5. *Geo urbani-Chelidonietum maji*: small-sized patches (25 $m^2$) of nitrophilous fringe community with *Chelidonium majus* and *Geum urbanum* occur on shaded and flat sites along the semi-natural and anthropogenic section of the Kłodnica valley. The average height of the herb layer is 45 cm.

6. *Alliaria petiolata-Valeriana sambucifolia* community: medium-sized (96 $m^2$) patches of the *Alliaria petiolata* and *Valeriana sambucifolia* nitrophilous fringe community were recorded under the canopy of trees, at the top and in the middle part of embankments of the semi-natural section of the Kłodnica valley on a 0–20° slope inclined. The average height of the herb layer is 120 cm.

7. *Impatiens glandulifera* community: small-sized (40 $m^2$) patches with a dominance of *Impatiens glandulifera* occurs in slightly inclined ($0–15^0$) shaded sites, at the top and in the middle part of the waterside of the semi-natural section of the Kłodnica valley. The average height of the herb layer is 130 cm.

8. *Alopecuretum pratensis*: large patches (130 $m^2$) of the meadow community with such diagnostic species as *Alopecurus pratensis*, *Ranunculus repens*, *Cirsium arvense* and *Holcus lanatus* developed on open and waterlogged sites on the floodplain terrace of the semi-natural section of the Kłodnica valley. The average height of the herb layer is 126 cm.

9. *Phragmitetum australis*: small in size (25 $m^2$) patches of a rush community with *Phragmites australis* were recorded on floodplain terrace of the semi-natural section of the Kłodnica valley, as well as nearby the riverbed of the anthropogenic section of the Kłodnica valley. The average height of the herb layer is 160 cm.

10. *Solidago gigantea* community: an aggregation of *Solidago gigantea*, the average height of the herb layer is 164 cm. Compact, large-sized (150 $m^2$) patches of the community occur on open places with inclination of 0–20° on the riverside of the semi-natural and anthropogenic section of the Kłodnica valley.

11. *Bromus inermis* community: a ruderal community with a dominance of *Bromus inermis*, the average height of the herb layer is 127 cm. Medium-sized (90 $m^2$) patches of the community developed at the top and in the middle part of the embankments of the anthropogenic section of the Kłodnica valley, slopes inclined at an angle 2–20°.

12. *Arrhenatheretum elatioris*: small in size (15 $m^2$) patches of a fresh meadow with a dominance of *Arrhenatherum elatius* were recorded at the top of embankments of semi-natural and anthropogenic section of the Kłodnica valley, slightly inclined 5–20°. The average height of the herb layer is 115 cm.

13. *Heracleum mantegazzianum* community: the height of the herb layer is up to 400 cm. Small (15 $m^2$) patches of the community were noted in shaded and slightly sloped (5°) sites, nearby the riverbed along the semi-natural section of the Kłodnica valley.

14. *Reynoutria japonica* community: compact large-sized (105 $m^2$) aggregations of invasive species *Reynoutria japonica* phytocoenoses occur on open places on the floodplain terrace of the semi-natural section of the Kłodnica valley, as well as along the pathways and at the top of embankments of the anthropogenic section of the Kłodnica valley with a slope 0–15°. The average height of the herb layer is 260 cm.

15. *Helianthus tuberosus* community: small (25 $m^2$) patches of the *Helianthus tuberosus* community were recorded on flat sites along the anthropogenic section of the Kłodnica valley. The average height of the herb layer is 193 cm

16. *Aster novi-belgii-Parthenocisus inserta* community: large-sized (300 $m^2$) patches of *Aster novi-belgii*, *Parthenocissus inserta* and *Rubus caesius* phytocoenoses developed along embankments of the anthropogenic section of the Kłodnica valley, in open sites with a slope 5–15°. The average height of the herb layer is 110 cm.

17. *Calamagrostis epigejos* community: patches of this grassy community occupy large areas (120 $m^2$) on the floodplain terrace of semi-natural section of the Kłodnica valley, as well as at the top of riverside of the anthropogenic section of the Kłodnica valley with a slope 10–15°. The average height of the herb layer is 152 cm.

18. *Deschampsia caespitosa* community: large patches (110 $m^2$) of this wet meadow community were recorded in open and often heavily moiste-

ned sites on the floodplain terrace of the semi-natural section of the Kłodnica valley. The average height of the herb layer 45 cm.

19. *Festuca rubra* community: large patches (200 $m^2$) of dry meadow phytocoenoses occur in open places, on less moistened soils on the floodplain terrace of the semi-natural section of the Kłodnica valley. The average height of the herb layer is 50 cm.

20. *Scirpetum sylvatici*: small in size patches of this wet meadow community (30 $m^2$) were recorded in open and frequently moistened sites near the riverbed and depressions periodically filled with water on the floodplain terrace of the semi-natural section of the Kłodnica valley. The average height of the herb layer is 70 cm.

21. *Phalaridetum arundinaceae*: medium-sized rush community (62 $m^2$) developed in water depressions, as well as in wet sites on the floodplain terrace of the semi-natural section of the Kłodnica valley. The average height of the herb layer is 168 cm.

22. *Iris pseudacorus-Glyceria maxima* community: medium-sized (52 $m^2$) patches of the rush community were recorded in depressions filled with water and shaded by trees on the semi-natural section of the Kłodnica valley. The average height of the herb layer is 130 cm.

23. *Lemnetum minoris*: large-sized (100 $m^2$) patches of aquatic community with *Lemna minor* and *L. trisulca* phytocoenoses developed on the surface of water that filled local depressions in the area of the semi-natural section of the Kłodnica valley.

## Species richness and species diversity

Plant communities recorded along the semi-natural section of the Kłodnica valley were richer in species and more diverse than phytocoenoses that occurred along anthropogenic section. The exception are aggregative patches of aquatic community – *Lemnetum minoris*, created only by 2 species – *Lemna minor* and *L. trisulca* (Figure 2 and Figure 3).

Along the semi-natural section of the Kłodnica valley developed the species-rich and diverse communities of wet (*Alopecuretum pratensis*: no. = 18.25, $H' = 2.15$) and fresh (*Festuca rubra* community: no. = 14.33, $H' = 1.87$) meadows, as well as phytocoenoses of nitrophilous fringe communities (*Urtico-Calystegietum sepium*: no. = 15.20, $H' = 2.04$; *Alliaria petiolata-Valeriana sambucifolia* community: no. = 14.86, $H' = 1.92$). It is worth paying attention, that the most rich in species (no. = 19.50) and the most diverse ($H' = 2.25$) was community built by an invasive species – *Impatiens glandulifera*. However, it is a community with the physiognomy of the nitrophilous fringe, in which encroaching of *Impatiens glandulifera*

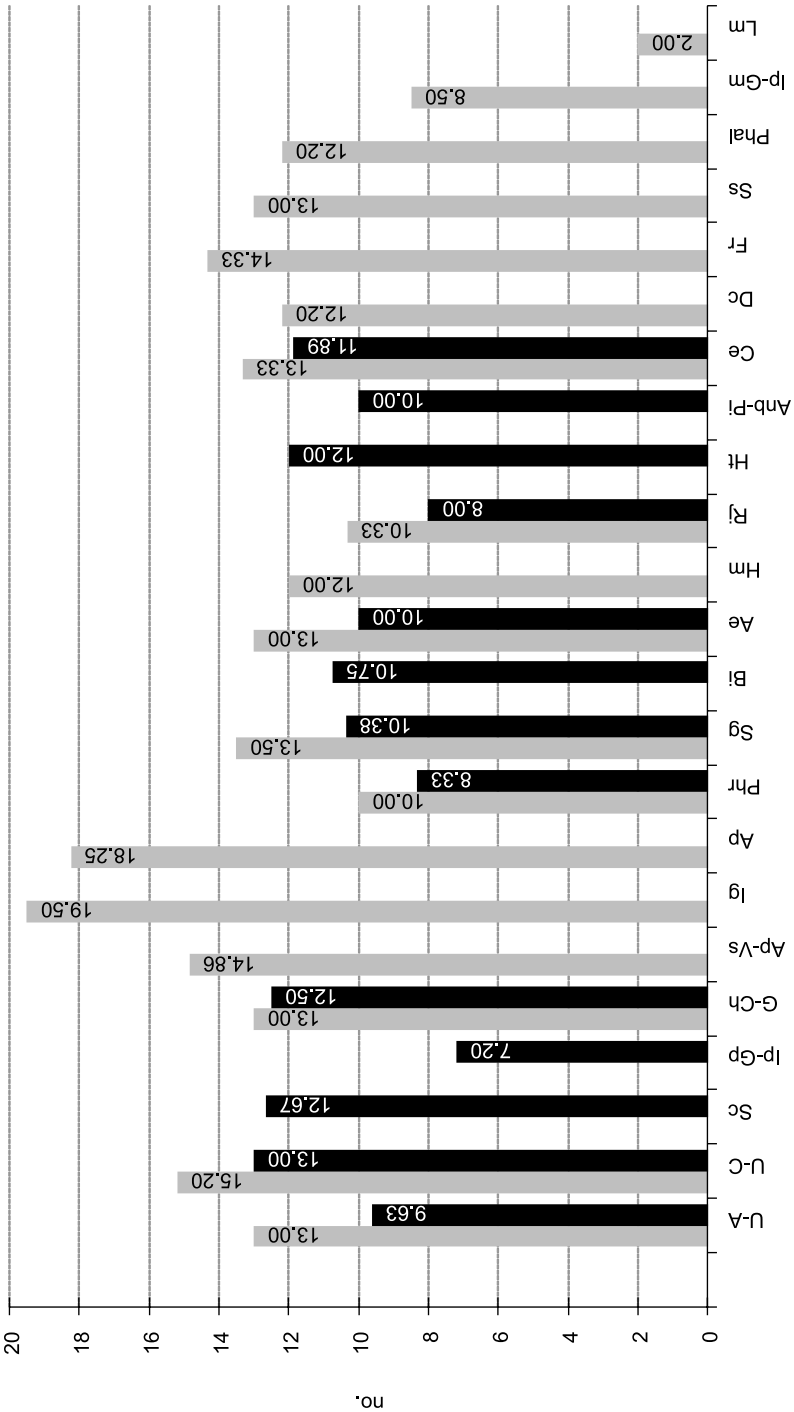


Fig. 2. Comparison species richness of the vegetation of two section of the Klodnica valley being under different levels of anthropogenic transformations

Explanations: no. – means the average number of species. See Table 1 for explanations of the abbreviations for the names of the plant communities

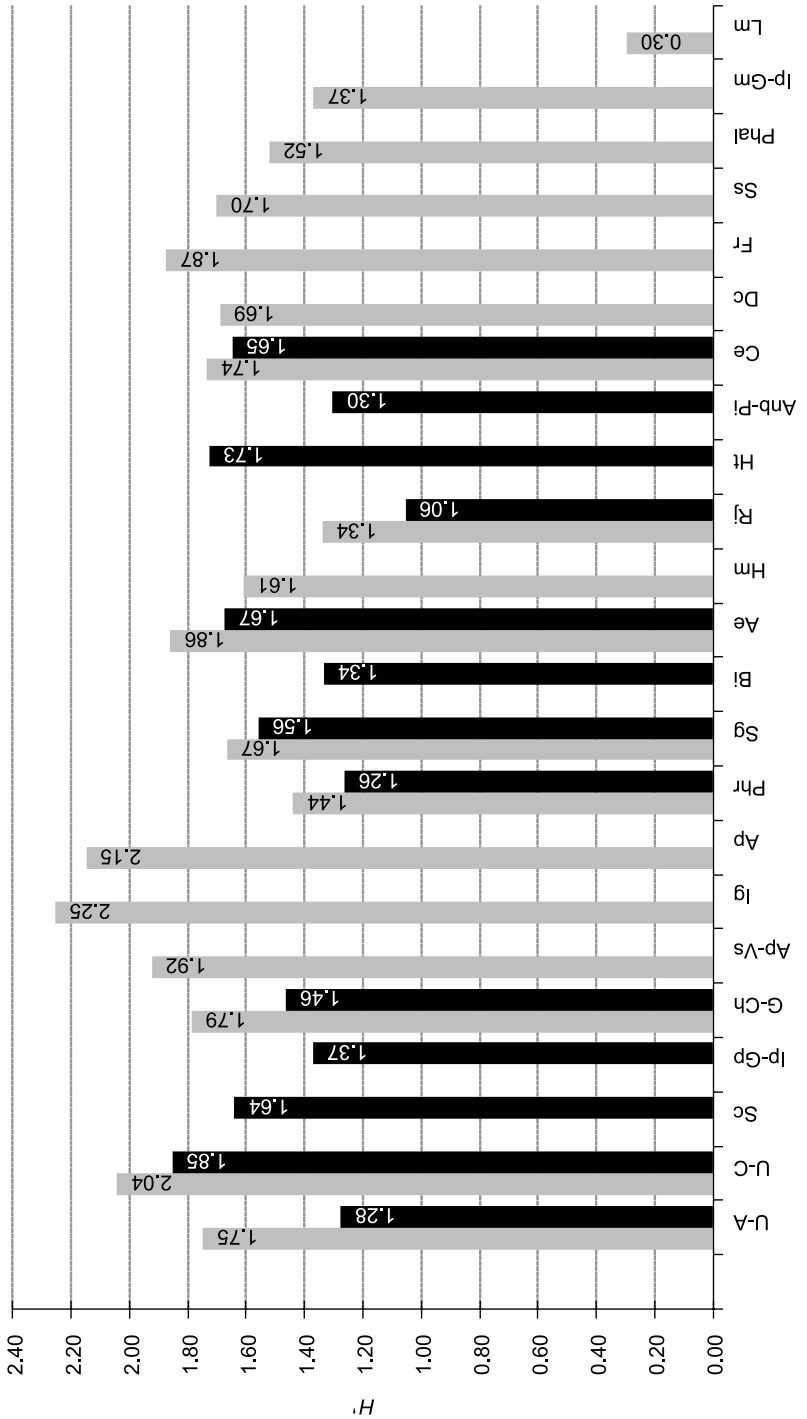


Fig. 3. Comparison of species diversity of the vegetation of two section of the Klodnica valley being under different levels of anthropogenic transformations

*H* – the average value of Shannon-Wiener diversity index. See Table 1 for explanations of the abbreviations for the names of the plant communities

is observed. In the species composition of those phytocoenoses occur nitrophilous (*Anthriscus sylvestris*, *Rubus idaeus*) and meadow (*Veronica chamaedrys*, *Equisetum palustre*, *Myosotis palustris*) plants (Table 1, Figure 2 and Figure 3).

The poorest in species and the least diverse were aggregation of invasive species – *Impatiens parviflora* (no. = 7.20, $H'$ = 1.37), *Reynoutria japonica* (no. = 8.00, $H'$ = 1.06), *Aster novi-belgii* and *Parthenocissus inserta* (no. = 10.00, $H'$ = 1.30), as well as *Urtico-Aegopodietum podagrariae* phytocoenoses (no. = 9.63, $H'$ = 1.28) and grass-like community with *Bromus inermis* (no. = 10.75, $H'$ = 1.34), which recorded through the anthropogenic section of the Kłodnica valley. Lower number of species and lower diversity were also characterized rushes with dominance of expansive grass, such as *Phragmitetum australis* (semi-natural section: no. = 10.00, $H'$ = 1.44; anthropogenic section: no. = 8.33, $H'$ = 1.26) and community with *Iris pseudacorus* and *Glyceria maxima* (no. = 8.50, $H'$ = 1.37) – Figure 2 and Figure 3.

## Flood risk assessment

For the area of Panewniki (Katowice district), where the semi-natural research section is located, there is no information about flood events. On the basis of data presented on the maps of flood risks by the National Water Management it can be detected, that along the semi-natural section of the Kłodnica valley the probability of flood occurrence is low and amounts to once a 500 years (Q 0.2%), and the flood risk area is limited to green fields in the immediate vicinity of the riverbed – Figure 4.

In contrast, in the anthropogenic section, 10 flood events have been recorded since the beginning of regular observation of the water level in Gliwice in 1911. The floods took place in 1913, 1915, 1925, 1930, 1940, 1970, 1972, 1985, 1997 and 2010. The largest flood took part at the turn May and June 1940. The water level in Kłodnica was 505 cm, and the flow was 121.5 $m^3 s^{-1}$. For comparison, in July 1997 the water level was 360 cm and flow rate 88.1 $m^3 s^{-1}$, while during the last flood in May 2010 water level was 384 cm and flow 105 $m^3 s^{-1}$ (Figure 5). The frequency of floods in the last 108 years makes it possible to predict, that the probability of flood occurrence is high and amounts to once a 10 years (Q 10%). The area at risk of flooding in Sośnica, Baildon, Ligota Zabrska and Politechnika (Gliwice districts) is 1.8 $km^2$ at the height of flood wave ≥ 350 cm. When the level of flood wave reaches ≥ 505 cm, Śródmieście is also at risk of flooding, and the flood risk area is 2.1 $km^2$. Within the area of above-mentioned districts threatened by flooding, apart from dense residential and industrial

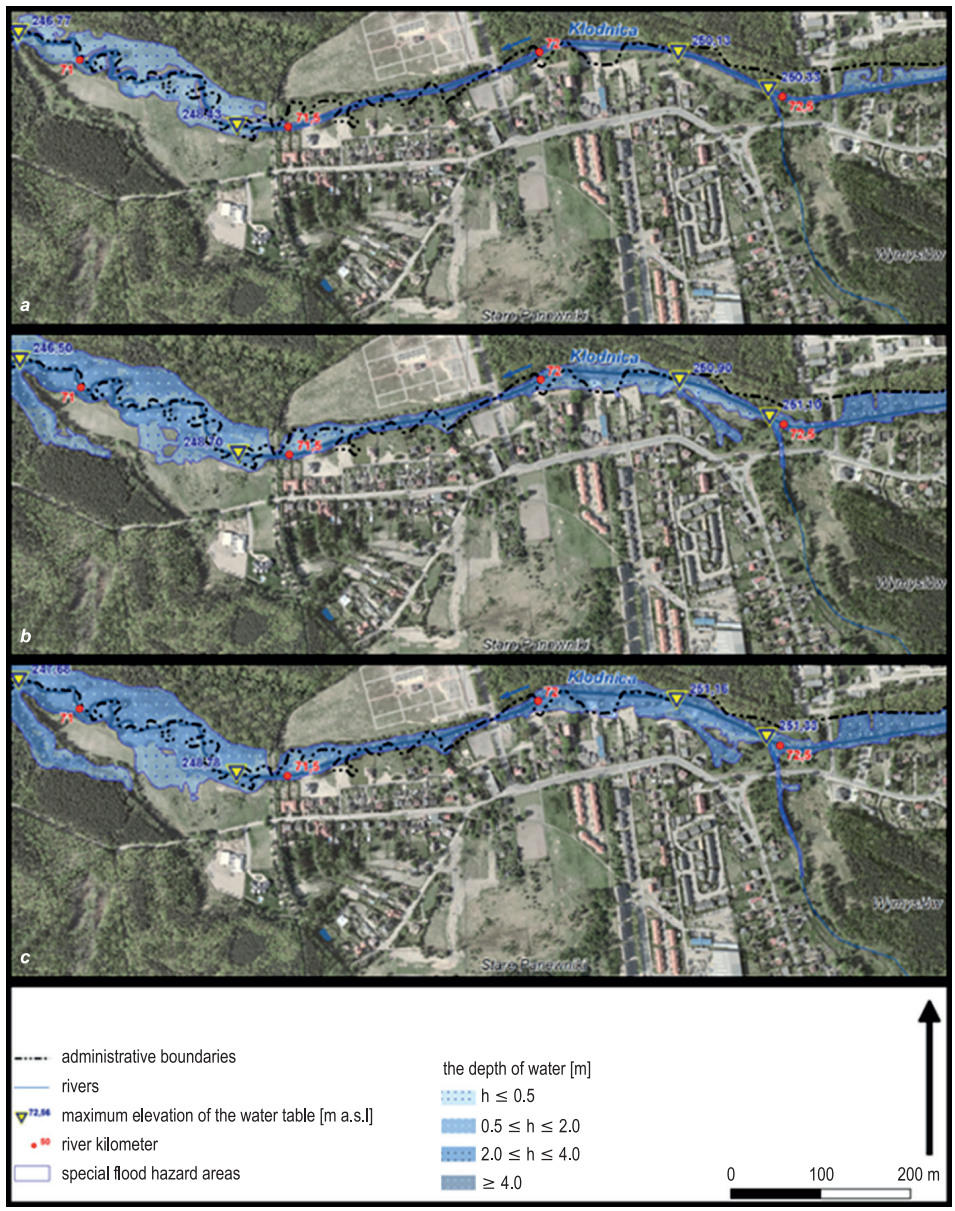


Fig. 4. Flood risk map of Katowice-Panewniki (semi-natural section):
*a* – areas, in which the probability of flood occurrence is low and amounts to once a 500 years (Q 0.2%); *b* – areas, in which the probability of flood occurrence is medium and amounts to once a 100 years (1%); *c* – areas, in which the probability of flood occurrence is high and amounts to once a 10 years (Q 10%)
Source: Krajowy Zarząd Gospodarki Wodnej (2015)

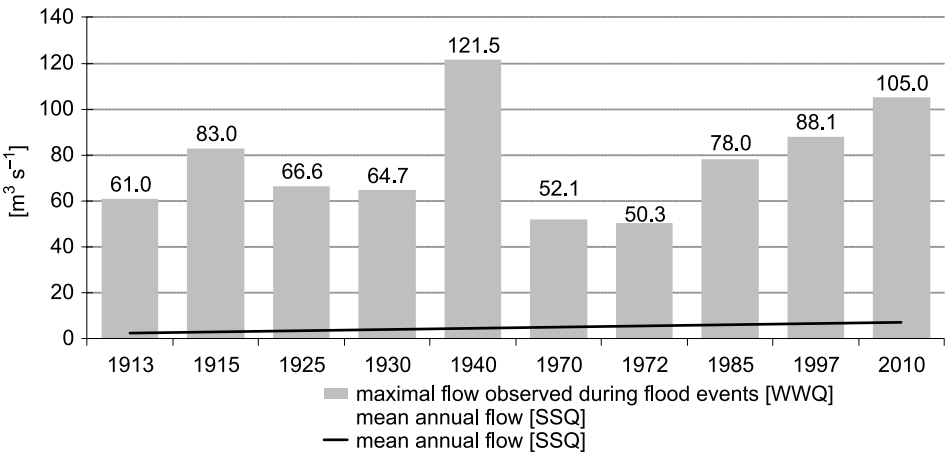


Fig. 5. Maximal flows (WWQ) observed during flood events in a period 1913–2010 in Gliwice (anthropogenic section)

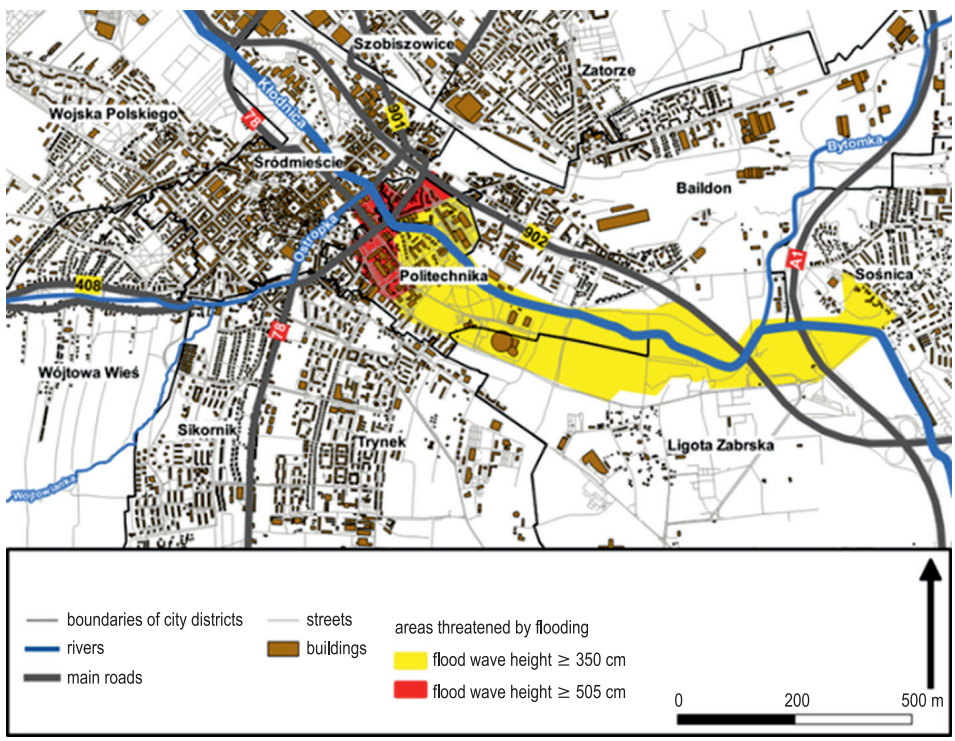


Fig. 6. Flood risk map of Gliwice (anthropogenic section)

buildings and road networks, there are also important facilities, such as the Chrobry city park, large Entertainment-Sports Hall „Gliwice", faculties of the Silesian University of Technology, Science and Technology Park

"Technopark Gliwice", primary and secondary schools, the Municipal Headquarters of the State Fire Service and the County Sanitary-Epidemiological Station (Figure 6).

## Questionnaire survey

In the group of 100 respondents, 58% were females and 42% were males. The age structure was dominated by respondents in the age group from 20 to 25 years (23%). They were followed by respondents aged 26–39 (19%), 16–19 (18%), 40–60 (16%), 6–15 (14%) and above 60 years (10%) respectively. The largest number of respondents were those with primary education (23%), basic vocational education (22%) and higher education (22%). The share of the upper (19%) and lower (14%) secondary school leavers was lower. Taking into account the current labour status employed (37%) and pupils (32%) prevailed over students (16%), retired (11%) and unemployed (4%). The vast majority (76%) of respondents lived in districts threatened by flooding, while 24% lived in other districts of the city of Gliwice.

The majority of the respondents visited the Kłodnica valley daily (40%) or 1–3 times a week (31%). Interviewees indicated walking with the dog (28%), walking (25%), jogging/running (12%) and playing with children (9%) as the purpose of the visit. Respondents generally rated the landscape of the Kłodnica valley, as monotonous (39%) and disturbed (33%). Positive connotations with the valley, such as wild (16%), diverse (7%) and near-natural (5%) were generally less frequent (Figure 7).

The interviewees rated the valley landscape (83%), river water quality (100%), plant cover (78%) and infrastructure (77%) as unattractive or rather unattractive. Cultural and historical values assessed as attractive or rather attractive (51%). In the case of tourist values of the Kłodnica valley, the share of positive (attractive or rather attractive, 46%), as well as negative opinions (unattractive or rather unattractive, 46%) was similar (Figure 8). Almost half of the respondents (46%) were not able to show elements increasing the attractiveness of the Kłodnica valley. Others interviewees appreciated the advantages of the Kłodnica valley, such as "place for walking" (24%), "place for rest and recreation" (17%) and "green place near the city center" (13%). According to the respondents, the main features decreasing the attractiveness of the Kłodnica valley are often floods (39%), unpleasant smell (14%), pollution of the river (10%), monotonous vegetation (10%), lack of recreational infrastructure (9%) and riverbed regulation (6%). The majority of interviewees completely agree (51%) or mostly agree (25%) with the necessity of revitalization of the Kłodnica valley (Figure 9).

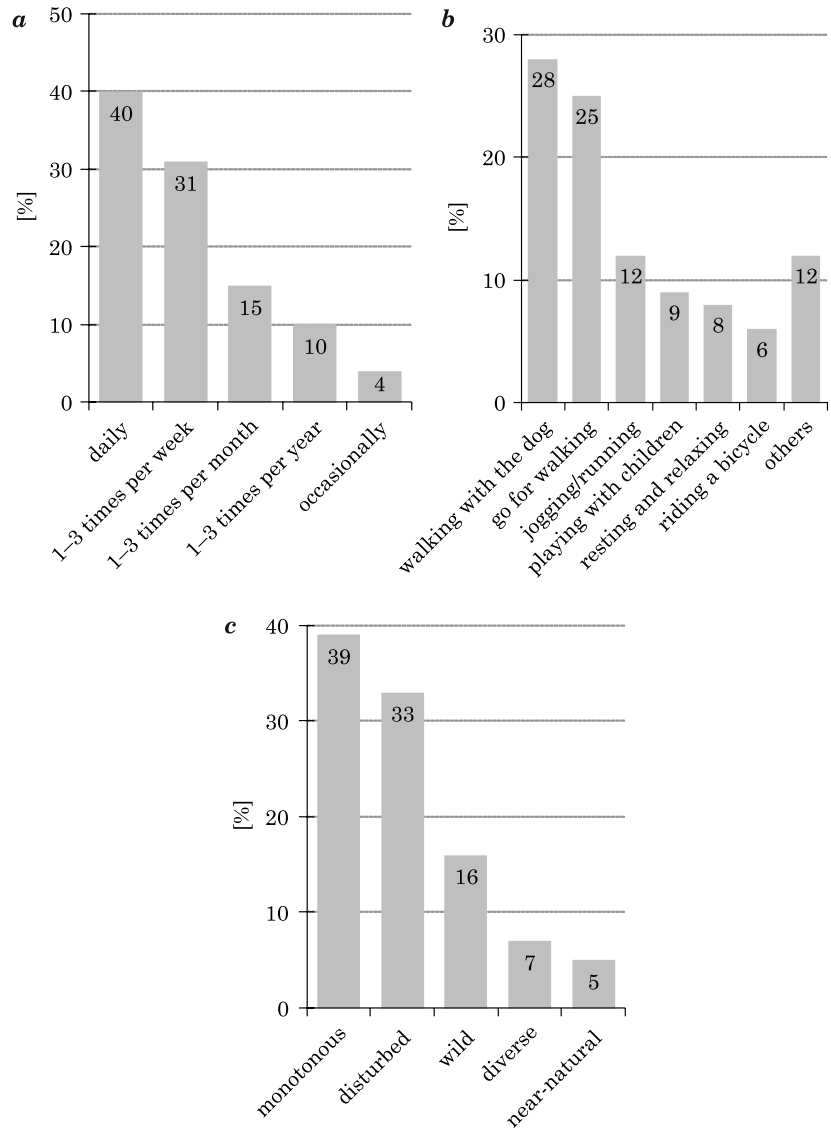


Fig. 7. Frequency (*a*), motivation (*b*) of visit and (*c*) connotations with the Kłodnica valley in Gliwice (anthropogenic section)

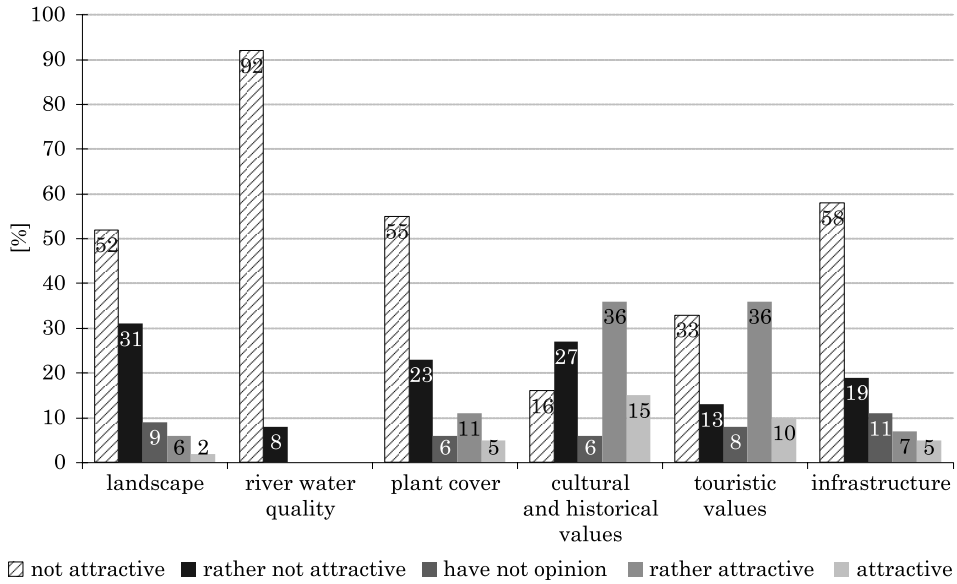


Fig. 8. Evaluation of the attractiveness of the Kłodnica valley in Gliwice (anthropogenic section)

More than half (58–95%) of the respondents could recognize 8 out of 11 plant species, that occur abundantly in the Kłodnica valley (Figure 10). Only 28% of the interviewees correctly found out, which of the presented plants were native species in comparison with the invasive ones. 26% of the respondents did not know the negative effects caused by invasive species. Others interviewees most often mentioned monotypization of vegetation (22%) and the decrease in the attractiveness of the landscape (17%). Only few respondents mentioned such effects as loss of the biodiversity (14%), displacement of native species by alien ones (10%), disturbances in ecosystem functioning (7%) and economic losses (4%). The concept of ecosystem services was known only to 7% of respondents (Figure 11).

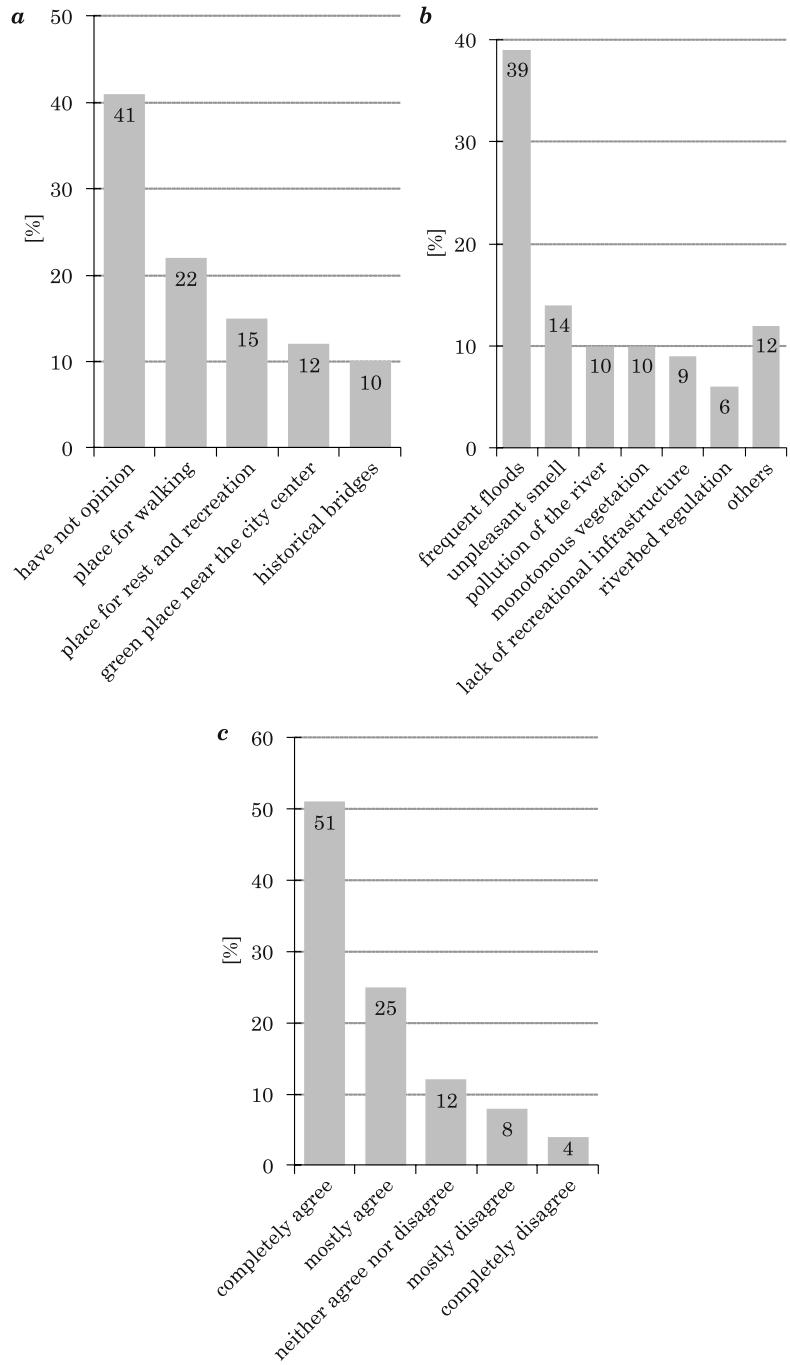


Fig. 9. Features increasing (*a*) and decreasing (*b*) the attractiveness of the Kłodnica valley in Gliwice (anthropogenic section). The necessity of revitalization of the valley (*c*)

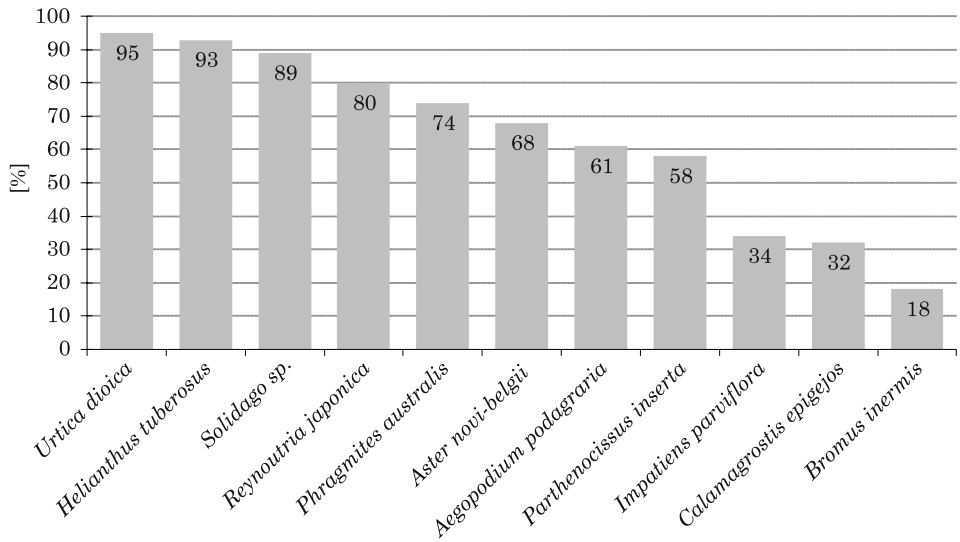


Fig. 10. Knowledge about plant species occurring abundantly in the Kłodnica valley in Gliwice (anthropogenic section)

## Discussion

The vegetation of the semi-natural section of the Kłodnica valley in Katowice was represented by 18 plant communities and 139 vascular plants species, mainly of native origin. In comparison to other Polish river valleys, the vegetation cover of semi-natural section of the Kłodnica valley is characterized by moderate diversity. In the Zgłowiączka valley near the village of Janiszewo and Zgłowiączka in the Kuyavian-Pomeranian Voivodeship 76 phytocoenoses, built by 324 plant species were distinguished (WAROT et al. 2001). ŁASKA (2009) found 8 plant communities, created by 172 plant taxa in the Płoska valley near Supraśl in the Podlaskie Voivodship. GAMRAT et al. (2010) recorded 15 phytocoenoses and 102 plant species in Ina valley near Sowno in West Pomeranian Voivodeship. KRYSZAK and KRYSZAK (2010) identified 6 plant communities and 115 taxa of vascular plants in Główna valley in the vicinity of Wierzenica in Greater Poland Voivodeship. Rich in species and diversified phytocoenoses of wet and fresh meadow, as well as nitrophilous fringe communities developed along the semi-natural section of the Kłodnica valley. The highest number of species and species diversity characterized *Impatiens glandulifera* community. Results of studies devoted to the impact of *Impatiens glandulifera* on diversity of vegetation are contradictory (TOKARSKA-GUZIK et. al 2015).

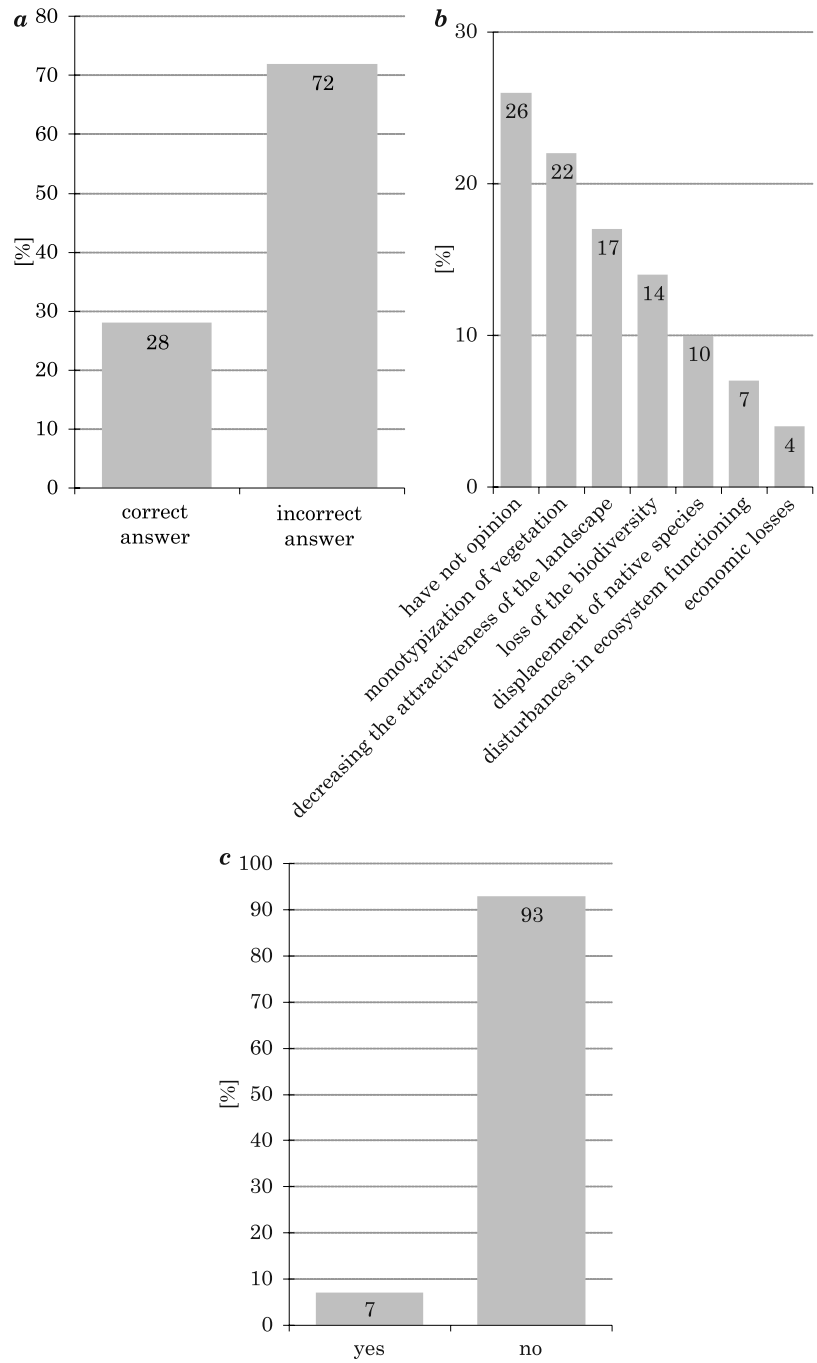


Fig. 11. Recognition of native and invasive plant (*a*), knowledge of the effects caused by invasive species (*b*) and the concept of ecosystem services (*c*)

On the one hand, in the initial stage of invasion it is possible, that species richness and species diversity of invaded vegetation increased, whereas in later stages of invasion decreased (HULME and BREMMER 2005, JAROSZEWICZ 2011). According to HEJDA and PYŠEK (2006), the impact of *Impatiens glandulifera* may depend on the density of the population. In our case *Impatiens glandulifera* does not occur abundantly. This plant community is co-created mainly by nitrophilous and meadow species. Low degree of anthropogenic disturbances of vegetation caused the semi-natural section of the Kłodnica valley is a place of a high natural and landscape value (MATUSIAK and WOJTCZAK 2006). A well-developed plant cover is also important in reducing of flood risk, which has been indicated along the semi-natural section of the Kłodnica valley as low. Vegetation plays an important role in rainwater retention and limits the surface runoff. In the period of elevated water levels in the river, the valley which is almost 100 m in width, densely overgrown by permanent grassland serves as a "dry polder". Period water retention enables to reduce the flow rate and effectively cut off the flood wave (BEDNARCZYK et al. 2006, JANKOWSKA-HUFLEJT 2006, JANUCHTA-SZOSTAK 2012, 2017, Krajowy Zarząd Gospodarki Wodnej 2015, MIODUSZEWSKI 2016, JANKOWSKI 2017, PRUS et al. 2018).

The contemporary landscape of the anthropogenic section of the Kłodnica valley has been created as a result of over 200 years of human economic activity (ABSALON et al. 2007). Willow shrubs (*Salicetum triandro-viminalis*) and willow-poplar forests (*Salicetum albo-fragilis*, *Populetum albae*), which naturally occurred in the valley, were cut down during regulatory works, including the riverbed straightening and the construction of embankments (HANCZARUK et al. 2016). Kłodnica waters received significant amounts of municipal and domestic sewage, as well as salty groundwater from hard coal mines (BARBUSIŃSKI and NOCOŃ 2011). In the place of riparian vegetation, 13 secondary non-forest plant communities, created by 86 species of vascular plants were developed. Among them were poor in species and low diverse aggregations of invasive alien plants (*Impatiens parviflora*, *Reynoutria japonica*, *Aster novi-belgii*, *Parthenocissus inserta*) and phytocoenoses with dominance of expansive species (*Aegopodietum podagraria*, *Bromus inermis*) have a negative influence on the landscape values of the valley. Strong modification of hydrogeological conditions, as well as destruction of the natural plant cover resulted in a twofold acceleration of the average annual flow (SSQ from 3.0 $m^3 s^{-1}$ before regulation to 6.41 $m^3 s^{-1}$ after regulation) and water damming of the river (RZĘTAŁA 2000, ABSALON et al. 2001, JANUCHTA-SZOSTAK 2012, ZAWARTKA 2012). Since 1911, 10 flood events have been recorded in the immediate vicinity of the anthropogenic section of the Kłodnica valley

(ABSALON et al. 2007, Centrum Ratownictwa Gliwice 2015). The frequency of floods indicates, that the likelihood of floods is high. The spatial range of the previous floods allows to estimate, that over 2 $km^2$ of densely populated areas in the downtown districts of Gliwice is at risk of flooding (ABSALON et al. 2007, SCHMIDT 2009, Centrum Ratownictwa Gliwice 2015). The losses caused by the last flood in municipal infrastructure in 2010 amounted to 13.1 million of PLN, and the costs associated with removing the floods amounted to 0.9 million PLN (PALIGA and KNURA 2013).

An questionnaire survey showed, that for the inhabitants of Gliwice, the Kłodnica valley is an important space for leisure and recreation needs. Similar conclusions were drawn by BRÓDKA and MIEDZIŃSKA (2015) for the Warta valley in Poznań, and SENDER and MASLANKO (2018) for Bystrzyca valley in Lublin. The majority of the respondents do not know the concept of invasive alien plants and ecosystem services. Therefore, it is important to undertake actions aimed at increasing the ecological awareness of local communities in the field of biodiversity, biological invasions, as well as the importance of riverside ecosystems and the value of ecosystem services provided by them. The usage of online social media (e.g. Facebook, Instagram, Twitter, Google+, etc.) will allow to transfer knowledge about the environment to a broader audience (JANUCHTA-SZOSTAK 2014, PIETRZAK-ZAWADKA and LEWOŃ 2018). However, the majority of interviewees often visit the Kłodnica valley and perceive the anthropogenic disorders of the Kłodnica valley and their consequences. Most of them describe the Kłodnica valley as monotonous or disturbed and rated the valley landscape, water quality in the river, plant cover as unattractive or rather unattractive. Majority of respondents had a problem with showing the elements increasing the attractiveness of the Kłodnica valley. Similar results was obtained BERNAT (2013) for the Bystrzyca valley in Lublin. Respondents often mentioned flood events, pollution of the river and associated with them unpleasant smell followed by monotonous vegetation and lack of recreational infrastructure as the main factors reducing attractiveness of the Kłodnica valley. Comparison with others Polish river valley shows, that high flood risk is the significant problem of the Kłodnica valley. The list of elements decreasing the attractiveness of the Bystrzyca valley in Lublin, includes water pollution and bad quality of water, and in the case of Warta valley in Poznań – pollution of water, insufficient care for the valley and lack of recreational infrastructure (BRÓDKA and MIEDZIŃSKA 2015, SENDER and MASLANKO 2018). As in other European cities, the inhabitants of Gliwice see the need for re-integration of the city with the river and indicate the necessity of revitalization of the Kłodnica valley (SZWED 2011).

Examples of other European cities (Duisburg, Oberhausen, Bottrop, Essen, Gelsenkirchen, Bochum, Dortmund and Bonn), where comprehensive revitalization projects of river valleys (e.g. Emscher Valley – one of the main river in Ruhr Area) have been implemented in recent years, providing valuable solutions and experience in various aspects of revitalization (Wieruszewski 1999, Pancewicz 2007, Zöpel 2011, Bernat 2013, Auer and Lavier 2013, Latkowska 2014). In 2014 in Ostrava, the project "Revitalization of the Ostravice River" was launched. This includes the revitalization of riverbed, some plantings of riverbanks, the creation of a new cycle and walking paths and small architectural elements, the adaptation of the current pier for water sports, as well as the construction a workout area (Lampartová and Schneider 2014, Pajurková 2017). The assumptions of the green infrastructure concept were successfully applied during the revitalization of the Ślepiotka valley (the western tributary of the Kłodnica river) in the Upper Silesia. Within the framework of the international project REURIS (Revitalization of Urban River Spaces) in the years 2010–2011, a 350-metre long section of Ślepiotka located along heavily urbanized areas of Ochojec (Katowice district) was revitalized. The cost of the project was 0.4 mln euro. Concrete riverbeds were replaced by natural material, such as boulders and wood. Invasive alien plant species were eliminated and natural habitat of river valley, as riparian and broadleaved forest and wetlands were restored. In addition, a wild flower meadow was created on an area of about 1 ha. The wooden amphitheatre at the entrance to the valley increases the cultural and landscape values of the Ślepiotka valley. The educational trail, as well as educational boards (riverine forests, species typical of forests, old varieties of fruit trees) arrange along it, contribute to building ecological awareness of the residents (*Śląski Związek*... 2011, Bender et al. 2012, Lange and Nissen 2012, Gieroszka et al. 2014, Janiszek 2015).

Revitalization of river valleys is a long-term and an multi-faced process, in which knowledge about ecosystem functioning, as well as existing socio-economic conditions should be applied (Bernat 2013, Gieroszka et al. 2014). The objective should be to improve current state of the environment (reduction of solid, liquid and gaseous wastes flowing into rivers, restoration or reconstruction of vegetation in the coastal zone, improvement of abiotic conditions important for water biocenoses, as well as restoration of connections with other river valleys) (Przewoźniak 2005). The final effect of properly carried out ecological revitalization should be the improvement of riverside ecosystems functioning and provision of many ecosystem services, such as decreasing the flood risk, improvement of local microclimatic conditions, as well as creation of friendly for inhabitants

riverside spaces – places of rest, recreation and observation of nature (JANUCHTA-SZOSTAK 2012, LANGE and NISSEN 2012, JANISZEK 2015).

## Conclusions

The field research carried out on the diversity of vegetation of the Kłodnica valley being under influence on long-lasting anthropopressure, as well as the results of studies on the perception of ecosystem services by the city residents indicate the need to continue and extend the field research to further sections (e.g. some streams flowing into Kłodnica, such as Bytomka, Ostropka, Wójtowianka) in terms of undertaken of revitalization activities of river valley, as well as to monitor the existing ecosystem services. The gain knowledge could enable to plan further investments in the river valley, which cannot contribute to the further weakening or even disappearance of some ecosystem services.

Research on the identification of ecosystem services would provide more complete data on the impairment of ecosystem services and enable to develop a better system of their evaluation.

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# AWARENESS OF THE CITTASLOW NETWORK AMONG STUDENTS IN OLSZTYN AND GDAŃSK CITIES

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Abstract

This study is based on an analysis of a questionnaire answered by 400 students which aimed at demonstrating the awareness of the notion of Cittaslow. The questions pertained to issues connected with environmental protection, knowledge of national heritage, participation in cultural events, and ideas how to promote Cittaslow-networked towns better. The results show that the awareness of the Cittaslow movement among students is poor. Most respondents had not heard of Cittaslow cities before the survey. According to the distinguished subgroup of students living in Cittaslow cities, the most severe environmental problem was the air pollution caused by incineration of prohibited fuel materials in household furnaces. The respondents suggest that it would be desirable to build alternative energy installations, mainly solar power facilities, in or near Cittaslow cities.

## Introduction

The increasingly rapid pace of life, with such manifestations as the lack of free time, noise, unhealthy fast food, intense sensations, loud advertisements, mass tourism, etc., has been met with opposition on behalf of residents of small Italian towns, situated far from big cities that attract swarms of visitors every year. In 1986, an organisation called Slow Food

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was established in the city of Bra, in Piemont. Today, this organisation has grown over 160 countries and comprises 100 thousand members (*Slow Food*… 2019 ).

In 1998, the Slow Food organisation held a meeting with mayors of several Italian cities, such as Bra, Greve in Chianti, Orvieto and Positano, which led to the creation of an international network of cities, called Cittaslow (referred to as Slow Cities or Cittaslow network). The name Cittaslow is a merger of the Italian noun 'città' meaning 'a city' and the English adjective 'slow'. The full name of the organisation is Rete internazionale delle città del buon vivere, which means 'International network of good life cities'. At present, the organisation associates 252 cities from 30 countries. Most member cities are in Italy (89), with the second most numerous group of slow cities being in Poland (30) (*Cittaslow*… 2019).

Since the foundation of the Cittaslow network, the member cities have been cooperating with one another, organising international and national meetings, festivals, study trips. By being Slow Cities, they are seen as tourist destinations. The Cittaslow cities are also an object of research interest. Empirical studies in this field, mostly based on surveys conducted among residents of these cities and among tourists, are increasingly more frequent. However, no such questionnaire has been addressed to students, which has motivated us to undertake this study, with an aim to explore the state of awareness in this societal group, and particularly university students in Olsztyn, the capital of the region of Warmia and Mazury, which has the highest number of Cittaslow towns in Poland; for comparison, the same questionaire was answered by university students from Gdańsk, the capital city of the Province of Pomerania, where only one Slow City is located and one City (Nowy Staw) is applying for membership in the organization (*Cittaslow. Aktualności*… 2019). Research results can be a valuable clue for city authorities that promote a good and peaceful rhythm of life. They can be used to better promote the city and contribute to achieving better results from belonging to the Network.

Studies into the phenomenon of Cittaslow towns, despite the short time elapsing since the foundation of the network, have rich history and literature (Radstrom 2011, *Alternatywne modele*… 2017). The underlying cause is deep interest in the achievement of the goals which this movement, already comprising 30 cities in Poland (in 2018), has set. Other than the obvious aims, such as the development of small towns (Blazy 2016, Mazur-Belzyt 2018), improvement of the quality of living of their residents (Meyer and Knox 2009) and protection of the natural environment as well as proecological education (Domeradzki and Tyburski 2011), the dominant objectives combine expectations of the Cittaslow towns' inhabi-

tants and the development of tourism are included (KÜÇÜKALTAN and PIRNAR 2016). Examples are taking care of the urban landscape, restoration of historical buildings, or broadly speaking revitalisation of the urban space (POCZOBUT 2010), building local identity (JASZCZAK and ANTOLAK 2015, PINK and SERVON 2015), creating and promoting an offer of cultural and recreational events as well as promoting local crafts and cuisine (MIELE and MURDOCH 2002). The aspect of tourism for the Cittaslow network (SUKIENNIK 2014) is obviosuly associated with sustainable development (EKINCI 2014, CENGIZ et al. 2017), local growth (GRUSZECKA-TIEŚLUK 2013, ZADĘCKA 2018), economy and revenue increase (ZAWADZKA 2017).

Conducting our study into students' awareness, we posed questions about knowledge, opinions and attitudes and about actions. Awareness is composed of these three levels. Furthermore, there is individual and societal awareness. The latter is divided into historical awareness, ecological (environmental), legal, civic and other types of awareness (LEWANDOWSKA 2007). In our research, the facts (knowledge), i.e. the first level of awareness, will consist of the information concerning the notion of Cittaslow, naming the cities which belong to the network, knowledge of the cities' historic buildings and cultural events which take place there. The second layer of awareness consists of opinions, attitudes and values. Consequently, there were questions about the existing and potential promotional activities as well as problems connected with the natural environment. We asked our respondents to express their opinion whether it would be advisable to locate alternative energy generation facilities near the Cittaslow cities. Apart from a simple choice (yes, no, I do not know), students were also asked to select the type(s) of alternative sources of energy that would be the most beneficial, and to justify their choice. When asking about activities, our intention was to separate knowledge from the actual involvement in the idea of 'good living cities'. We asked about participation in cultural events in Cittaslow cities, the time of year when students stay in these cities, about buying regional food, and about the need they feel to enlarge the area of green places.

To direct the research objectives, we set a task to find out to what extent students were aware of the guidelines contained in the 'Manifest of Slow Cities for the new humanism of living'. The manifest's content is being implemented by: 1) performing the environmental policy whose aim is to sustain and develop characteristics of the urban surroundings and tissue; 2) executing the infrastructural policy, whose aim is to raise the value of a given territory rather than occupying it; 3) promoting the production and use of food products made by natural technologies and in an eco-friendly manner; 5) protection of local production, rooted in the culture

and tradition, which contributes to the promotion of the whole region, 6) supporting the culture of hospitality as a moment of true connectiveness with a given community and its specific traits; promoting among residents the awareness of living in one of the Slow Cities (*Cittaslow. Vademecum* (2013), p. 5). The manifest is concordant with 'Guidelines for Excellence', contained in Attachment C to the Charter of the Association of Cittaslow Cities, which – by virtue of being a more detailed document – served to help us analyse the replies to our questionnaire (*Cittaslow. Vademecum* (2013), p. 19–21).

## Materials and Methods

The study was conducted in April and May 2018, among 400 students in Olsztyn, the University of Warmia and Mazury (the UWM), and in Gdańsk, Gdańsk University (GU) and the University of Physical Education and Sports (AWFiS). In Olsztyn (the UWM), the questionnaire was addressed to students of the following courses: Tourism and Recreation, History (major in Tourism), International Affairs, Journalism and Social Communication. Students from Gdańsk studied the following subjects: Geography, Landscape Knowledge and History Tourism (UG), and Tourism and Recreation (AWFiS). Such selection of respondents seemed optimal for the attainment of the goal, which was to collect data for our analysis of the awareness of the presence and functioning of Slow Cities. As for the place of residence, most respondents fell in almost two equal parts (Table 1).

Table 1

Place of residence of respondents

| Voivodships | Percentage of respondents |
|---|---|
| Warmińsko-mazurskie | 39 |
| Pomorskie | 37 |
| Mazowieckie | 7 |
| Podlaskie | 6 |
| Kujawsko-pomorskie | 2 |
| Others | 9 |

The questionnaire was answered by 65% of women and 35% of men, of which 98% were young people, aged up to 30 years, while 2% were between 30 and 50 years old, and only one person was over 50 years of age.

The chosen research tool was an auditorium questionnaire, completed by students in the presence of the lecturer, who is one of the co-authors of this analysis. The questionnaire contained 17 questions, closed and open ones. There were also multiple-choice questions, as well as questions where given answers had to be ordered with respect to their importance. The questions will be discussed below in several subject-related blocks. The following analyses comprise the entire research population, without any division according to the age, place of residence or the course of studies pursued by the respondents, as such divisions would make the article less readable. On the other hand, we wished to refer the current results to some earlier studies and to scientific references. We are in possession of the source material, which will enable us to carry out an in-depth analysis at a later time.

## Results and Discussion

### The notion and the location of Cittaslow cities according to the students

More than half of the respondents (57%) did not know anything about the idea of Cittaslow (question 1). However, those who were familiar with it, were able to characterise Slow Cities correctly (question 2), e.g.

– cities were living is slower, people are not in a rush, they use renewable energy resources, sort out rubbish;

– cittaslow is directed towards 'a slower pace of life', living in a Slow City is well managed, there are many opportunities for relaxation, e.g. bicycle paths, a slow city is people friendly.

– a Slow City is well organised, for example is has a well-developed tourism infrastructure, it is people-friendly;

– a city with limited motor traffic in the centre, with high quality public spaces;

– a slow city, focused on regional values, local resources;

– a city growing more slowly and less dynamically than major urban cen tres;

– smaller towns with high natural and cultural assets;

– the network of Good Living Cities, slow, calm, in agreement with culture;

– Cittaslow is against globalisation and is dedicated to the development of small towns.

Despite the correct answers explaining the notion, some students did not understand the specific character of Cittaslow cities and translated

this term literally. This conclusion can be drawn from examples of cities given by respondents as Slow Cities (question 3). The list included Warsaw, Kraków, Gdańsk, Lublin, Białystok, Zielona Góra, Katowice, Poznań and Wrocław, as well as some European or global cities like Copenhagen, London, New York, Sevastopol, Amsterdam, Stockholm, Barcelona, or Florence. Knowledge of the concept of the city of Cittaslow among students is relatively low, unlike among the inhabitants of these cities. In a study conducted in 2017, among 100 inhabitants from 10 Polish member cities, as much as 90% had knowledge about the city's membership of the Good Life Cities network (BATYK and WOŹNIAK 2017).

The first Polish city to join the Cittaslow network was Reszel (2004). Today, 19 cities from the Province of Warmia and Mazury are members of the network: Barczewo, Bartoszyce, Biskupiec, Bisztynek, Działdowo, Dobre Miasto, Gołdap, Górowo Iławeckie, Jeziorany, Lidzbark Warmiński, Lidzbark Welski, Lubawa, Nidzica, Nowe Miasto Lubawskie, Orneta, Olsztynek, Pasym, Reszel, Ryn, Sępopol. Two member cities, Głubczyce and Prudnik, are in the Province of Opole. There are also single cities in other Polish provinces, such as Kalety (Province of Silesia), Murowana Goślina (Province of Wielkopolska), Nowy Dwór Gdański (Province of Pomerania), Rejowiec Fabryczny (Province of Lublin), Rzgów (Province of Łódź) and Sianów (Province of West Pomerania) – Figure 1.


Fig. 1. Location of Polish Cittaslow cities: colour gray – the area of the Warmian-Masurian voivodeship; 19 – Cittaslow cities in the province Warmia-Mazury: Barczewo, Bartoszyce, Biskupiec, Bisztynek, Działdowo, Dobre Miasto, Gołdap, Górowo Iławeckie, Jeziorany, Lidzbark Warmiński, Lidzbark Welski, Lubawa, Nidzica, Nowe Miasto Lubawskie, Orneta, Olsztynek, Pasym, Reszel, Ryn, Sępopol

Let us now look at the respondents indicating Slow Cities correctly. Of the 377 persons who named the Cittaslow cities known to them, 157 persons lived in the Province of Warmia and Mazury. This had some influence on their answers. The city most often mentioned was Lidzbark Warmiński (49 persons), which is now the seat of the National Association of Cittaslow Cities. Similar results were obtained in surveys among urban residents (BATYK and WOŹNIAK 2017). In addition to Lidzbark Warmiński, students further mentioned Dobre Miasto (39 persons), Olsztynek and Biskupiec (27 persons each) – Figure 2. Although 146 of the respondents

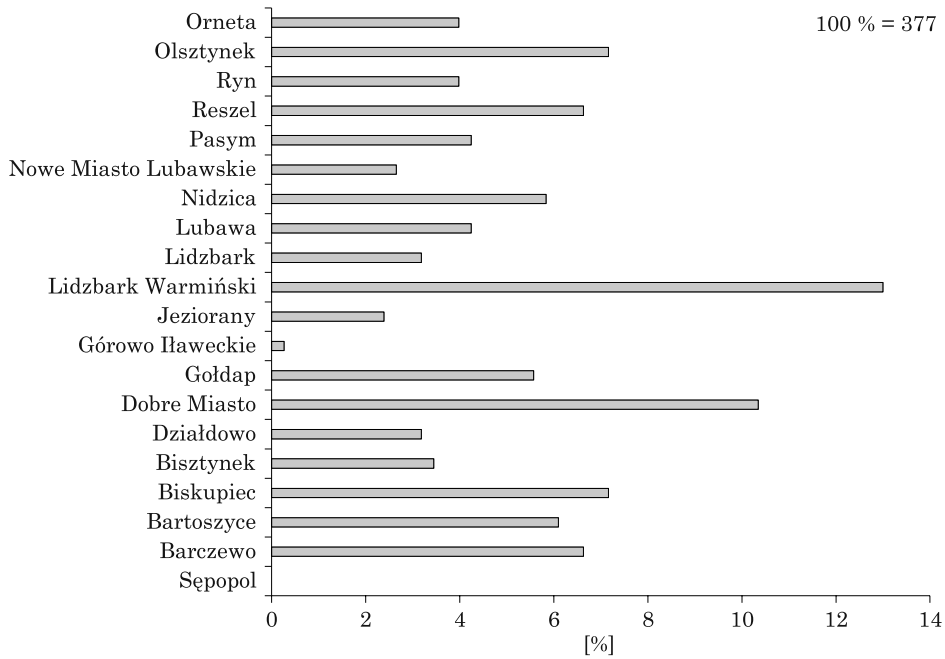


Fig. 2. Knowledge of Cittaslow cities in the province Warmia-Mazury

come from the Province of Pomerania, only 12 mentioned Nowy Dwór Gdański. Other cities which belong to the Polish network of Slow Cities, that is Prudnik and Murowana Goślina were named by 3 persons each, Kalety – two indications, and Głubczyce, Rejowiec Fabryczny and Sianów – 1 indication each.

## Requirements for excellence in the Cittaslow movement

The Cittaslow Charter contains Attachment C, which defines Requirements for Excellence. It consists of eight blocks, which in total comprise 63 points. Some of these points were referred to when requesting the

respondent students to state which Slow Cities they knew. As many as 63% (220 out of 349 answering this question) admitted that they had never been in a Slow City (question 4). Hence, their other replies in the questionniare were based on their intuition more than on experience. The remaining 37% of the respondents were asked to write their impressions of a Slow City they knew. The aim was to verify whether visitors (or inhabitants) noticed any changes induced by the implementation of the statutory tasks of Cittaslow member cities (question 5).

In compliance with Attachment C to the Cittaslow International Charter, one of the requirements for excellence is quality of urban life, which finds its manifestation in 'Plans for interventions concerning the restoration of historic urban centres and/other assets having cultural and historic value', and in 'Plans for international signposting of historic centres on tourist trails'. Slow Cities should therefore make efforts to restore and revitalise their old centers and historic monuments there. This is one of the most easily noticeable effects of cities joining the network. This result was observed by the students, who stated that the city centers had been renovated entirely (93% answers), and there were signposts and notice boards in and around the old quarters informing about historic buildings, tourist attractions, cultural heritage, etc. All tourist trails passing through the cities were also signposted (88% indications).

In the block of requirements for excellence called 'Agriculture, tourism and artisan policies' and another one defined as 'Quality of urban life policies' there are several points regarding production of local and traditional products, for example: 'Protection of handmade and labelled artisan production', 'Increasing the value of working techniques and traditional crafts', 'Creation of spaces for the commercialization of local products'. In our study, the opinions about local products, their production and distribution, were almost evenly distributed, as 55% of the respondents noticed such activities and 45% stated that local products were not noticeable in Slow Cities.

Slow Cities are obliged to promote regional and local food. This is expressed in provisions of Attachment C to the Cittaslow Charter, namely: 'Use of local products, if possible organic, in comunal public restaurants (school canteens etc.)', 'Education of flavours and promoting the use of local products, if possible organic in the catering industry and private consumption', 'Support for Cittaslow campaigns and activities', and 'Collaboration with other organisations promoting natural and traditional food'. Questions 15 and 16 of our survey tested this aspect. The respondents were asked about buying local food products, and if the answer was 'no' or 'I don't know', they were requested to explain why they did not buy local food. 56% answered that would like to buy such products, but 44% did not

because of high prices (58%). Others pointed to the lack or shortage of points of sale distributing local food (37%), while few (5%) indicate specific taste of local food which they did not enjoy.

The 'Infrastructure policies' block contains such points as 'Plans for safe urban traffic', and 'Efficient cycle paths leading to schools and public buildings'. Although the Slow Cities known to our respondents had grids of bicycle lanes (84%), motor traffic was not banned in historic quarters of most of the Cittaslow member cities (75%).

The cities had green areas (96% indications) with service inftastructure, including street furniture, benches, lamp posts, litter bins and playgrounds. This is also set as an objective in the Cittaslow Charter, where the 'Guidelines for Excellence' in the section 'Agriculture, tourism and and artisan policies' suggest 'Promoting municipal parks and school playgrounds, including cultivation of local plants with tranditional methods'. We returned to the problem of expanding the total area of green spaces in Slow Cities, including parks, flower beds or tree stands, in our question 17. 60% of the students agreed that having more greenery was necessary. Those who admitted to not knowing or not visiting Slow Cities had no opinion on this matter (36%).

The idea of Slow Cities presumes a slower pace of life, more peaceful lifestyle and less stress, which is supposed to give rise to more friendly relationships between people as well as better well-being and more cheer among residents. 56% of our respondents said they had noticed such qualities among Cittaslow inhabitants. This is indicated in Attachment C to the Cittaslow Charter, where 'Hospitality' is included among the 'Guidelines for Excellence'.

## Historic buildings, culture and promotion

In the subsequent part of the questionnaire, the respondents were asked to name historic buildings or other tourists attractions which were worth seeing in Slow Cities (question 6). This was an open question, and the suggestions were highly varied. In total, 37 elements were mentioned, of which castles (28%), basilicas and churches (145), museums (11%), Old Town squares (8%) as well as fortified walls and towers (7%) were indicated most often. Fewer persons pointed to townhalls, a skansen, a water tower, monuments and statues, a synagogue, a glassworks, graveyards, townhouses, monuments of nature. Single respondents wrote about bathing beaches, piers, amusement park, nature reserve, fort, old brewery, festival, railway bridge, spa, graduation towers, orangery, Old Prussian 'baba' stones, a mill, military barracks, or a palace. Thus, most often stu-

dents mentioned such monuments that are typical for historical cities, not necessarily in the Cittaslow Network. However, single indications suggest that the respondent had in mind a monument or attraction in a specific city, e.g. orangery (located only in Lidzbark Warmiński), spa and graduation towers (only in Gołdap), Prussian babes (in Bartoszyce), historic brewery (in Nidzica ), a glassworks and an open-air museum (in Olsztynek), and a synagogue (in Barczewo). In questions 7 and 8, the respondents were asked to name cultural events which take place in Cittaslow cities, and to state whether they had taken part in any such event. Cultural events are mentioned in Attachment C to the Cittaslow Charter, namely in 'Preservation and promotion of local cultural events'. Unfortunately, as many as 86% of the students had never attended any local cultural event. Those who were unable to name specific events wrote about concerts, festivities, Christmas feasts, harvest festivals, artisan and craftsmen's fairs, street runs. Specific events most often included Days and Nights of the City (here, providing the name of a specific city) with 12 indications, or the Lidzbark Nights of Humous and Satire, indicated by five persons. The Feliks Nowowiejski Choir Music festival in Barczewo, the Cepelinai Feast in Gołdap, and the Winter Chivalric Tournament in Nidzica were indicated 3 times each. The following events were indicated twice each: The Spit Cake Sękacz Feast, the Jazz Festival, the Herbal Festival in Olsztynek. Single persons also recalled the Festival of Pierogi in Biskupiec, The Kaziuki Wilniki Festival, the Yotvingian Run, the Procession of the Three Magi in Dobre Miasto, the Copernicus Run in Lidzbark Warmiński.

A high percentage of indications (85%) was earned by the problem of lacking sufficient information about Slow Cities (question 9). We asked students to give their ideas how Cittaslow towns could be promoted (question 10). Because the study involved a group of adolescents, whose main source of information is the Internet, they often suggested using this medium for promotional activities. The Internet was indicated most often (74 persons) as a very good and effective form of the dissemination of knowledge about the Cittaslow network. In addition, 6 persons mentioned Facebook, and 12 other students suggested websites and social media in general. 29 persons indicated mass media, sometimes specifying social media. Mass media also include television, and 26 persons suggested TV commercials, 11 indications were to use radio commercials, and 6 persons proposed adverstisements in the press. One student had an idea of a column in a local newspaper dedicated to the presentation of Slow Cities. Twelve persons suggested printing and distributing leaflets. Two persons proposed placing information about Slow Cities in tourist guidebooks (which is already done).

A relatively large group of students (11 persons) said that education about the Cittaslow network in schools (from primary to secondary schools) and in higher education institutions was important. Some respondents recommended placing information boards and hoarding boards in centers of Slow Cities and along roads entering such cities (10 indications). Others wrote that travel agencies should offer special tours on the Trail of Slow Cities. Other ideas are adverts on public transport vehicles, e.g. in trains, organising more cultural events, and promoting Cittaslow cities in other Polish cities.

Students wrote:

– 'I am the one who knows very little about such cities, so it is necessary to disseminate knowledge and to promote Slow Cities through commercials and adverts, so as to draw people's attention to these locations'.

– 'representatives of the Cittaslow network from time to time should give lectures at universities or in schools'.

– 'Slow Cities should be promoted on television, radio and the Internet (interesting interviews, promotional video clips) because these media stay in memory the longest.'

– 'Trips should be organised to such cities; it would help to place signs, for example saying that a given city belongs to the Cittaslow network'.

– 'Commercial spots promoting Slow Cities should be given more attention, as they can show the charm, natural values and cultural assets of these locations'.

– 'Tours offered by travel agencies, commercials on Internet wedsites dedicated to travel/tourism'.

Although the students empahsised the need to inform visitors that they were staying in a Slow City, 61% of our respondents said that the Cittaslow cities had proper signposting. Promotion of the Cittaslow notion is strongly underlined in the Charter of the Association. This is confirmed by several guidelines from Attachment C, such as 'Preparation of 'slow' trails in the city (brochures, wedsites, etc.)', 'Constant provision of information about the importance of the Cittaslow movement to residents of Slow Cities', 'Supporting promotional campaigns of Slow Cities', 'Using the Cittaslow logo on promotional materials (websites, letterhead paper, visiting careds, etc.)'.

## Protection of the natural environment

Another set of questions pertained to issues of environmental protection and healthy lifestyle. Power supply and environmental policy are both very important for Slow Cities. This objective is defined in Attachment C

to the Cittaslow Charter, under such headings as 'A wastewater treatment plant to treat municipal or industrial wastewater', 'Plan to save energy in the city, and in particular by using alternative energy resources (renewable energy resources, hydrogen, small hydropower plants) and using the process of incineration of solid waste and biomass to generate energy'. Question 12 referred to environmental problems the respondents may have seen in Slow Cities they had visited. They were asked to put in order several possible issues. The most severe one, in their opinion, was the problem of waste management (33%), followed by air pollution (26%), noise (23%) and pollution of surface water bodies (17%). The obtained results show that students are quite interested in environmental protection problems. This is comparable to the research obtained in 2017 by BATYK and WOŹNIAK (2019). The analysed groups of students believed that waste was a more serious environmental problem than air pollution. This is contrary to some tracking research (*Trackingowe badania…* 2018) where the Polish population more often pointed to air contamination as an environmental issue than to the problem of rubbish. A possible explanation is that Cittaslow towns need to improve their system of waste management. More intensive environmental contamination is seen in specific seasons of the year. Thus, question no 11 was about the season of the year when the respondents visited Slow Cities. Most pointed to the summer (34%) or spring (29%), while fewer would in the winter (19%) or autumn (18%). The persons who chose the answer 'I stay in good life cities outside the summer season' are the ones who live there (100%). They all decided that the most noxious environmental problem was the wintertime air pollution caused by illegal incineration of waste materials in household furnaces. The severity of this problem is confirmed by analyses carried out by the WIOŚ Provincial Inspectorate for Environmental Protection (KOBUS and al. 2017), where the main source of high levels of major air pollution parameters in Poland are individual heating facilities in private flats and houses. This is contrary to the common opinion expressed by Polish citizens in tracking studies (*Trackingowe badania…* 2018)

Regardless of the year when the questionnaire was completed, the respondents most frequently pointed to emissions from large power consuming facilities, such as industrial plants, as the main source of air pollution.

Other problems which the respondents identified as causing degradation of the natural environment were: the lack of public toilets, excessive amount of advertisements, diminishing area of public green spaces, and their excessive landscaping. The presence of municipal green areas and their proper management are fundamental to the protection of biological

diversity in towns. For instance, a lawn seeded with a monoculture of plants, regularly mowed, never allowed to produce flowers or fruits, sprayed with herbicides, etc., fails to provide food or shelter to most insects or birds. Hence, in places not exposed to much walking it is worth replacing a lawn with a flower meadow (TRZASKOWSKA 2015). A meadow will attract many insects, such as honeybees, bumblebees, butterflies, beetles, as well as small insectivorous animals, including amphibians, reptiles, birds or mammals (OCKINGER et al. 2009).

Poland has committed herself to fulfil the requirements associated with the goal of reaching 20% of renewable energy resources in the national electric power generation budget by the year 2020. The students asked to complete our questionnaire are aware that some power generation facilities using alternative sources of energy need to be built in or near Cittaslow cities. 68% of the respondents agreed with it, while only 2% were against and 30% had no opinion (question 13). To gain deeper understanding, we asked: 'If there was a choice, what installations from the list below would you choose to have built in or near Cittaslow cities?' (question 14). We suggested three types of power plants: solar, wind and hydropower facilities, but we also left an option to write own ideas and justification. Nearly half of the respondents (46%) chose solar power plants, almost a third opted for wind farms (29%), while a quarter chose hydropower plants (24%). The results are in accord with the opinions about renewable energy resources expressed by a group of 88 Polish citizens interviewed by DROSIŃSKA et al. (2015). Solar, wind and water power are most often mentioned as the first three sources of energy best for the environment. The respondents were also asked to name which sources of energy they could accept in the vicinity of their home.

## Conclusion

The study demonstrated little interest among student in the problems of Cittaslow cities. Few had heard about this network of cities, and often guesed the meaning of the concept. They were unable to give names of Slow Cities correctly, be it in Poland or in their own province of the country. Most had never been in any of these cities, and could not describe them, name historic buildings situated there or cultural events which take place in these cities. Those students who knew Cittaslow cities most often concluded that living there is more peaceful and at a slower pace, that historic centres of such cities are restored and revitalised, that there are bicyle paths there, and that it is possible to buy local food. They gave spe-

cific examples of historic buildings and cultural events, and were able to associate them with specific locations. However, all students had interesting ideas for promotional activities, mostly indicating the use of electronic and traditional media, but interestingly they also suggested spreading the knolwedge about the Cittaslow network in schools and universities.

The environmental issue most often indicated by the students as occurring in Cittaslow cities is the problem of waste. The distinguished groups of students living in Slow Cities, however, pointed to a more serious problem of air pollution caused by burning forbidden materials in household furnaces. The respondents suggest the need to build in or near Slow Cities some installations using alternative sources of energy. The most preferred renewable energy source was solar power plants.

The respondents themselves suggested how to make students' awareness increase. They proposed to include the topic of Cittaslow cities in the subject of lectures at universities or high schools, or to organize thematic meetings and lectures from time to time. Therefore, especially UWM research workers can benefit from these applications, because in the province of Warmia and Mazury is the most Cittaslow cities in Poland.

It seems that many students heard about Cities of Good Living for the first time when filling in our questionnaire. Their astonishment at discovering the Cittaslow network and realising that it was worth finding more about it was a source of satisfaction for the research authors and a confirmation that such studies are necessary and should be repeated regularly.

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# USE OF ULTRASONOGRAPHY FOR DETERMINATION OF CISTERN SIZE IN DIFFERENT GENOTYPES OF DAIRY SHEEP*

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Key words: sheep, udder, ultrasonography, milkability, stripping milk, mammary gland cistern.

Abstract

A total of 263 Improved Valachian (IV), Tsigai (*T*), Lacaune (LC) and crossbred lactating ewes were used to study milk cistern anatomy in dairy sheep bred in Slovakia, to compare two methods of ultrasound udder scanning and to evaluate relations between cistern size and milkability. Milkability traits recording, external measurements, linear assessments and ultrasonic scanning of sheep udders were done. Sums of both cistern cross-section areas were computed on the basis of two methods of udder ultrasonography – from the side of udder (SCA) and from below in a water bath (BCA). Between BCA and milk yield was found out slightly lower correlation ($r$ = 0.48) than between SCA and milk yield ($r$ = 0.53).

## Introduction

Sheep milking has long and plentiful tradition in Slovakia. Nevertheless machine milking have been introduced more widely into dairy sheep husbandry only in the last two decades. The introduction of machine mil-

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king evokes the requirement to pay more attention on morphological and functional characteristics of sheep udders. One of the most interesting udder morphological characteristics from the machine milkability point of view is the size of glandular cistern (*Sinus lactiferus pars glandularis*), as the "cisternal milk" is available for milking before the oxytocine ejection, the large-cisterned animals being in general more efficient producers of milk and more tolerant to long milking intervals (WILDE et al. 1996).

There are large differences in the proportion of total milk stored within the cistern among ruminant dairy species. Specialized dairy cows store less than 30% of the total milk yield volume in the mammary gland cisterns (AYADI et al. 2003). Percentages of cisternal milk in sheep vary from 25% to 75% according to the breed but they are greater than 50% in most dairy sheep breeds (CAJA et al. 1999, ROVAI et al. 2000). *In vivo* scanning of the udder internal structures could be done by ultrasonography. Cisterns filled by milk are detectable very well as anechogenic structures in ultrasound scans. Different methods of sheep udder ultrasonography were proposed by BRUCKMAIER and BLUM (1992) and RUBERTE et al. (1994). The methods were used for cisternal measurements by BRUCKMAIER et al. (1997), CAJA et al. (1999), ROVAI et al. (2000), NUDDA et al. (2000), MAKOVICKÝ et al. 2015.

This investigation was aimed on the study of milk cistern anatomy in dairy sheep breeds and crossbreeds in Slovakia, the comparison of two methods of ultrasound udder scanning and the evaluation of relations between cistern size and milkability traits in dairy sheep.

## Materials and Methods

Investigations were performed in the experimental flock of Research Institute of Animal Production in Nitra. Totally 263 lactating ewes of Tsigai (*T*; $n = 47$), Improved Valachian (IV; $n = 61$), Lacaune (LC; $n = 52$) and various crossbreeds between them ($n = 103$) were used. Six control days were organized in different stages of lactation and some animals were investigated repeatedly, so totally 590 sets of measurements were done. Control milkings were done in 1 x 24 side by side milking parlor, using vacuum level 40 kPa and pulsation rate 120 pulses/min. Ewes were milked without udder prestimulation and milk flow was recorded in 10 s intervals. After 60 s of machine milking and if no milk flow was detected for 20 s the machine milking with manual udder massage was performed. Then milk emission curves were constructed and total milk yield, machine milk yield, machine stripping milk yield and percentage of stripping milk

from total milk yield were computed. Subsequently 12 hours after control milking the external measurements (6 traits: using tape – udder length; using ruler – udder width, udder height, cistern height, teat length; using protractor – teat angle), linear assessments (7 traits: udder depth, cistern height, teats placement, teat length, udder attachment, udder cleft and overall subjective assessment of udder shape from the point of view of machine milking) and ultrasonic scanning of udders were done. Ultrasonography was carried out from the side of udder according to methodology of RUBERTE et al. (1994) and from below in a water bath as described by BRUCKMAIER and BLUM (1992). Scans were made with a digital ultrasound scanner Medison SonoVet2000 using a linear probe L2-5/170 CD. Acoustic coupling agent (Kerolan, Aveflor Kopidlno) was used to attach the probe to the skin in a case of scanning from the udder side. Images were recorded on memory card and later processed by the use of computer program Zodop32. Sums of cross section areas of both cisterns measured from the side (SCA) and from below (BCA) were measured. The statistical analysis of variance in the data set was performed using the GLM procedure of SAS. The model equation used for the data adjustment considered the effects of the day of control milking (fixed effect – 6 levels), breed or crossbreed combination fixed effect – 7 levels), parity (fixed effect – 3 levels), interaction between breed and parity and covariables days in milk (DIM) and square of DIM ($DIM^2$). The CORR procedure SAS was used for the computing of partial correlation coefficients on residuals after the data adjustment by the above mentioned model equation.

## Results and Discussion

In Table 1 the F-values of systematic effects obtained by analysis of variance of cistern areas are presented. Both measurements, from side (SCA) and from below (BCA), were significantly affected by all effects considered in model equation, anyway the effect of breed or crossbred combination was the strongest in both cases. Determination coefficients of used model equations of variance analysis were $R_2 = 0.531$ for BCA and $R_2 = 0.512$ for SCA. The least squares means and standard errors for the effect of breed or crossbred combination are listed in Table 2. There were statistically significant differences in cistern size and milk yield between purebred Improved Valachians (IV), Tsigai (*T*) and Lacaune (LC). LC imported to Slovakia in order to improve milk production had the highest cistern size and the highest milk yield and also hybrids between IV or *T* and LC had higher cisterns and milk yield than purebred animals of native

breeds. On the other hand purebred LC had significantly the highest stripping milk yield. Average percentage of stripping milk from total milk yield was 40.9% in LC while in other genotypes varied from 22.8% to 28.2%.

Table 1

Analysis of variance of sums of cistern cross-section areas measured from side (SCA) and from below (BCA)

| Parameters | BCA | – | SCA | – |
|---|---|---|---|---|
| Effects | F-value | $P > F$ | F-value | $P > F$ |
| Control milking | 5.02 | 0.0019 | 4.98 | 0.0020 |
| Breed or crossbred combination | 67.25 | <0.0001 | 53.49 | <0.0001 |
| Parity | 7.08 | 0.0009 | 3.91 | 0.0205 |
| Breed-parity interaction | 2.15 | 0.0086 | 2.10 | 0.0104 |
| Days in milk (DIM) | 5.71 | 0.0172 | 19.81 | <0.0001 |
| $DIM^2$ | 5.90 | 0.0154 | 15.85 | <0.0001 |
| – | $R_2 = 0.531$ | – | $R_2 = 0.512$ | – |

$DIM^2$ – squere of days in milk – in course to account of non-linearity of the relation between observed trait and day of lactation

Table 2

Effect of breed or crossbred combination on cistern size and milkability traits in sheep (LS-means±SE)

| Breed | BCA $cm^2$ | SCA $cm^2$ | Total milk yield ml/milking | Stripping yield ml/milking |
|---|---|---|---|---|
| Improved Valachian (IV) | 30.52±1.27*b* | 38.35±1.29 *b* | 412.3±13.8*b* | 105.5±8.4*a* |
| Tsigai (*T*) | 25.13±1.07*c* | 30.08±1.09*c* | 293.1±11.5*d* | 79.3±6.8*b* |
| Lacaune (LC) | 58.55±1.19*d* | 60.98±1.21*d* | 544.9±12.8*a* | 222.9±7.5*d* |
| IV x LC | 43.04±2.42*a* | 46.80±2.46*a* | 510.2±26.1*a* | 134.3±15.3*a* |
| (IV x LC) x LC | 38.22±1.61*a* | 44.43±1.63*a* | 502.8±17.3*a* | 130.4±10.1*a* |
| *T* x LC | 37.81±1.72*a* | 41.91±1.74*ab* | 424.1±18.4 *bc* | 119.7±10.8a |
| (*T* x LC) x LC | 42.67±4.81*a* | 45.37±4.88*ab* | 468.8±51.8*ac* | 107.1±30.2*ab* |

*a*, *b*, *c* – values with the same letters in the same column do not differ significantly ($P < 0.05$)

The sums of both cistern cross-section areas were higher for measurements from side (30.08–60.98 $cm^2$) than from below (25.13–58.55 $cm^2$) in all genotypes. Higher relative differences in cistern areas between scanning from below and from side was detected in native breeds (IV – 26.6%; *T* – 19.7%) while in LC the difference was only 4.2%. BRUCKMAIER et al. (1997) refer about total cisternal cross sections obtained by udder ultrasonography from below 33 ± 7$cm^2$ for LC.

However in response to oxytocin injection alveolar milk was ejected causing enlargement of the cisternal area by 45 ± 8%. CAJA et al. (1999) detected in Ripollesa ewes 4 hours after milking average cistern area 5.6 ± 0.5 $cm^2$ measured by ultrasonography from side of udder. Partial phenotypic correlations between BCA or SCA and other morphological and functional udder characteristics in purebred IV, *T*, LC and in all genotypes together are presented in Table 3. Correlations between cistern areas and

Table 3

Correlations between cistern areas and other udder traits in sheep

| Breed | Improved Valachian BCA | SCA | Tsigai BCA | SCA | Lacaune BCA | SCA | All genotypes BCA | SCA |
|---|---|---|---|---|---|---|---|---|
| Ultrasound measurements SCA | 0.71 | – | 0.76 | – | 0.84 | – | 0.79 | – |
| Linear scoring of udders | | | | | | | | |
| Udder depth | 0.53 | 0.49 | 0.48 | 0.41 | 0.55 | 0.59 | 0.61 | 0.48 |
| Cistern height | 0.31 | 0.14 | 0.38 | 0.23 | 0.25 | 0.09 | 0.27 | 0.11 |
| Teat placement | 0.23 | 0.04 | 0.32 | 0.15 | 0.21 | 0.02 | 0.22 | 0.06 |
| Teat length | -0.08 | -0.06 | 0.21 | 0.12 | 0.26 | 0.17 | 0.13 | 0.09 |
| Udder attachment | 0.24 | 0.35 | -0.06 | 0.11 | 0.12 | 0.16 | 0.11 | 0.18 |
| Udder cleft | 0.23 | 0.36 | 0.26 | 0.31 | -0.04 | 0.06 | 0.10 | 0.18 |
| Udder shape | 0.47 | 0.46 | 0.42 | 0.43 | 0.16 | 0.26 | 0.27 | 0.32 |
| External udder measurements | | | | | | | | |
| Udder length | 0.57 | 0.44 | 0.53 | 0.46 | 0.60 | 0.55 | 0.55 | 0.50 |
| Udder width | 0.43 | 0.50 | 0.40 | 0.38 | 0.40 | 0.47 | 0.41 | 0.45 |
| Udder height | 0.60 | 0.44 | 0.45 | 0.38 | 0.58 | 0.54 | 0.55 | 0.49 |
| Cistern height | 0.33 | 0.17 | 0.45 | 0.31 | 0.29 | 0.20 | 0.32 | 0.19 |
| Teat length | -0.10 | -0.09 | 0.04 | 0.03 | 0.21 | 0.06 | 0.03 | 0.00 |
| Teat angle | 0.14 | 0.01 | 0.24 | 0.05 | 0.15 | 0.06 | 0.17 | 0.03 |
| Milkability traits | | | | | | | | |
| Milk yield for 30 s | 0.48 | 0.48 | 0.55 | 0.50 | 0.04 | 0.21 | 0.23 | 0.29 |
| Machine milk yield | 0.47 | 0.50 | 0.49 | 0.50 | 0.09 | 0.21 | 0.28 | 0.37 |
| Total milk yield | 0.53 | 0.58 | 0.49 | 0.52 | 0.45 | 0.53 | 0.48 | 0.53 |
| Stripping yield | 0.25 | 0.31 | 0.19 | 0.24 | 0.48 | 0.44 | 0.38 | 0.35 |
| % of stripp. Yield | -0.07 | -0.06 | -0.15 | -0.11 | 0.23 | 0.11 | 0.06 | -0.02 |

external udder size represented by udder height, udder width and udder length were moderate ($r$ = 0.38–0.61). Correlations between cistern areas and total milk yield were slightly higher in a case of measurements from

side ($r$ = 0.52–0.58) than from below ($r$ = 0.45–0.53). CAJA et al. (1999) found out similar correlation between SCA and milk yield $r$ = 0.46. For purebred LC was characteristic low correlation between machine milk yield and cistern size and on the contrary higher correlation between stripping yield and cistern size. These facts resulted in slightly positive correlations between cistern size and percentage of stripped milk in LC, while in IV and *T* these correlations were rather negative. Also correlations between cistern size and linear score for the udder shape from the point of view of machine milking were in LC much lower ($r$ = 0.16–0.26) than in IV ($r$ = 0.46–0.47) or *T* ($r$ = 0.42–0.43). Many LC ewes had baggy udders with big cisterns and horizontally placed teats. This fact was also documented by MILERSKI et al. (2006). Big part of cistern volume was located below the orifice into the teat canal and therefore part of cisternal milk could be reached rather by stripping than by machine milking. In this investigation the average percentage of stripping milk yield from total milk yield was 40.9%. Also MARGETÍN et al. (2013) found higher LSM for percentage of stripping milk in LC ewes 37.8% (SE 1.4%) compared to IV 24.8% (SE 1.5%) and TS 27.9% (SE 1.3%). TANČÍN et al. (2011) refere LSM 33.3% (SE 3.04%) for percentage of stripping milk in LC. FERNÁNDEZ et al. (1997) found out high positive genetic correlation between milk yield and udder depth (rg = 0.82) and negative genetic correlation between milk yield and linear assessment of udder shape (rg = -0.26). These correlations showed that selection for milk yield could produce worse udder morphology.

## Conclusions

The results show that the use of Lacaune sheep breed in Slovakia for genetic improvement of native dairy sheep breeds or for creation of synthetic line will lead to improving of milk production, but on the other hand could turn to the worse udder morphology with negative impact on some aspects of milkability. Taking this fact into account the use of udder morphology traits in breeding programs for dairy sheep would be reasonable. Ultrasonography of udders, both from below and from side, could be used for cistern size evaluation. Correlations between cistern size and milkability traits could be utilized in breeding. Nevertheless some breed specificities have to be considered.

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# THE SAFETY OF VETERINARY IMMUNOLOGICAL PRODUCTS IN EUROPE

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Abstract

Vaccination is a standard procedure that stimulates the immune response against microbes. In regards to animals, an indication for vaccination is usually to protect their health as well as economic factors. The purpose of immunoprophylaxis is to reduce the susceptibility of animals to infections by stimulating immunity. Safety and effectiveness of vaccines is dependent among others, on its manufacture process and qualitative composition, with particular emphasis on adjuvants. As public health and animal health are interrelated, it is an important issue. This review article is based on an analysis of the current situation related with veterinary vaccines safety on the European market.

## Introduction

Currently in human and veterinary medicine, a lot of attention is paid to proper nutrition and prophylaxis as a way to improve health outcomes. This strength is mostly referred to as immunity – the ability to fight infection. At the same time, animal owners and breeders are concerned about whether the use of vaccines is safe and beneficial. Stimulating the immune response against microbes by vaccination is the most effective method for protecting against infections (BASTOLA 2017, NICHOLSON 2016). Vaccines were used long before the mechanisms of immune protection became known. Today, vaccination is a standard procedure in animal husbandry due to the fact that in the long term, it not only prevents disease but also

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saves production costs. Continued research in the field of veterinary immunology, adaptation of new technologies has provided molecularly defined, genetically engineered, vector vaccines and others (BULL 2015). There are many well-established types of veterinary vaccines on the market successful against viral, bacterial, protozoan and multicellular pathogens. The development of efficacious vaccines has substantially reduced the impact of the human and animal viral diseases on public health (KNIGHT-JONES et al. 2014).

## Immunity

The ability to fight infection is referred to as immunity. The collection of cells, tissues and molecules that mediate resistance to infections is called the immune system. Coordinate reaction of these cells and molecules to infectious microbes is an immune response. Immunity may be induced in an organism by infection or vaccination (active immunity) or conferred by transfer of antibodies or lymphocytes from an actively immunised organism (passive immunity). The purpose of immunoprophylaxis is to reduce the susceptibility of animals to infections by stimulating immunity. This can be accomplished by the use of biological and synthetic immune-boosting vaccines (ABBAS and LICHTMAN 2011, SCHUNK and MACALLUM 2005).

Innate immunity is often considered separate from acquired immune responses. Several distinct activities mediate innate immune defense including: epithelial barriers, antimicrobial serum proteins such as complement, natural antibodies produced by B1 lymphocytes, the activity of cells such us neutrophils, macrophages, dendric cells, natural killer (NK) cells that can lyse virus-infected cells also interferon (IFN), apoptosis and small RNA molecules that interfere with virus replication (MACLACHLAN and DUBOVI 2010). Adaptive immunity includes humoral and cellular components. Humoral immunity is mediated principally by antibodies released from B lymphocytes. In addition, dendric cells, macrophages, NK cells and cytokines are all critical to adaptive immune responses. Adaptive immunity is antigen-specific and takes at least several days to develop. This type of immunity is mediated by lymphocytes that possess surface receptors that are specific to each pathogen. Adaptive immunity stimulates long-term memory after infection, meaning that protective immune responses can be quickly reactivated on re-exposure of the organism to the same pathogen (MACLACHLAN and DUBOVI 2010, SHULTZ 1991).

## Importance

Vaccination is the most effective way of preventing viral diseases. This concept is considered to have been widely introduced in 1798 by Edward Jenner to protect humans from smallpox. Nearly a century later, the concept was shown by Louis Pasteur and could be used to prevent rabies. In 1950s, a very important segment of vaccine production was developed – live attenuated and inactivated virus vaccines (WEISS and ESPARZA 2015). In Table 1, we can observe the dynamics of some infectious diseases and population data in Europe. Analysing the number of born infants and the number of surviving infants, significant improvement is noticeable. In year 1980: born – 20 197 000, surviving – 13 140 000 and in year 2016: born – 11 183 000, surviving – 11 085 000 (Table 1).

Table 1

Immunization profile in European region

| *Population data in thousands | 2016 | 2015 | 2014 | 2013 | 2012 | 2000 | 1990 | 1980 |
|---|---|---|---|---|---|---|---|---|
| Total population | 916'315 | 913'134 | 909'962 | 906'789 | 903'592 | 868'538 | 847'107 | 797'828 |
| Live births | 11'183 | 11'255 | 11'307 | 11'336 | 11'341 | 10'267 | 19'086 | 20'197 |
| Surviving infants | 11'085 | 11'153 | 11'201 | 11'227 | 11'227 | 10'083 | 12'413 | 13'140 |
| Pop. less than 5 years | 56'618 | 56'815 | 56'274 | 56'500 | 56'098 | 51'302 | 64'231 | 63'920 |
| Pop. less than 5 years | 163'867 | 162'671 | 161'338 | 160'041 | 158'876 | 172'741 | 192'275 | 192'437 |
| Female 15–49 years | 213'964 | 215'146 | 216'623 | 218'162 | 219'667 | 221'464 | 208'502 | 197'358 |
| Number of reported cases | | | | | | | | |
| Japanese encephalitis | 0 | 1 | 1 | 0 | 0 | – | – | – |
| Measles | 4'363 | 25'965 | 14'176 | 26'346 | 27'379 | 37'421 | 234'827 | 851'849 |
| Mumps | 20'874 | 10'027 | 10'807 | 35'303 | 39'072 | 243'344 | – | – |
| Pertussis | 69'490 | 43'615 | 43'858 | 28'170 | 57'539 | 53'675 | 129'735 | 90'546 |
| Polio | 0 | 2 | 0 | 0 | 0 | 0 | 370 | 549 |
| Rubella | 1'471 | 655 | 653 | 39'391 | 30'579 | 621'039 | – | – |
| Rubella (CSR) | 6 | 14 | 28 | 49 | 62 | 47 | – | – |
| Tetanus (neonatal) | 0 | 1 | 1 | 0 | 0 | 27 | 69 | 26 |
| Tetanus (total) | 137 | 122 | 68 | 105 | 208 | 412 | 879 | 1'715 |
| Yellow fever | 1 | 0 | 0 | 0 | 0 | 0 | – | – |

Source: World Health Organisation (WHO), http://www.who.int/immunization/monitoring_surveillance/data/en/, access: 16.03.2018.

Currently, modern 'new generation' vaccines are also being produced through various forms of recombinant DNA and related technologies. The majority of large-scale production for use in animals continue to include either a live-attenuated or inactivated virus (MACLACHLAN and DUBOVI 2010).

Live-attenuated virus vaccines can be produces from naturally occurring attenuated viruses, introduced by Jenner in 1798 for the control of human smallpox, utilised cowpox virus, a natural pathogen of cow. The same principle has been applied to other diseases – like Marek's disease, where a vaccine was produced using related herpesvirus of turkeys or the protection of piglets against porcine rotavirus using a vaccine derived from bovine rotavirus (MACLACHLAN and DUBOVI 2010). Live vaccines can also be prepared by attenuation of viruses by serial passage in cultured cells. The cells may be homologous, or more commonly, heterologous host origin. During the accumulate passage in culture cells, viruses typically accumulate nucleotide in their genome, which in turn leads to attenuation. Another possibility is attenuation of viruses by serial passage in heterologous host. For example, rinderpest and classical swine fever viruses were each adopted to grow in rabbits. After serial passage viruses become sufficiently attenuated to be used as vaccines. Viruses can be also passaged in embryonated eggs. Live vaccines can be produced by a selection of cold-adapted mutants and reassortants (MACLACHLAN and DUBOVI 2010). Live viral vaccines have played a successful role in disease control and eradication. Eradication of rinderpest virus from the globe is believed to have been dependent on the use of the "Plowright" vaccine (MEEUSEN et al. 2007).

Inactivated virus vaccines are usually made from a virulent virus; chemical or physical agents are used to destroy infectivity while maintaining immunogenicity. Such vaccines are considered to be safe. The most commonly used inactivated agents are formaldehyde, $\beta$-propiolactone and ethylenimine (MACLACHLAN and DUBOVI 2010). Inactivated vaccines are generally more stable and do not post risk of reversion to virulence compared to live vaccines, but they can be less protective due to the inability to activate cytotoxic T cells (MEEUSEN et al. 2007). Consequently, inactivated vaccines generally require strong adjuvants and sometimes more than one injection to induce the required level of immunity. Although inactivated vaccines are considered to be safe, adjuvanted vaccines post a greater risk of causing autoimmune disease, allergic disorders and vaccine injection site sarcomas (MEEUSEN et al. 2007).

Another type of vaccines is those produced by recombinant DNA methods and related technologies. It is a relatively recent technology of vaccine production. This immunological product can be produced by attenuation of viruses by gene deletion or site-directed mutagenesis. Gene

deletion is especially feasible with large DNA viruses that carry a significant number of genes that are not essential for replication in cultured cells. The ability to identify and selectively delete genes from a pathogen has allowed the development of "marker vaccines", that combined with suitable diagnostic assays allow to differentiate animals infected from vaccinated. It is possible by differentiation of antibody responses induced by the vaccine from those induced during infection with a wild-type virus. Such Differentiating Infected from Vaccinated Animals (DIVA) vaccines are available for several infectious diseases including infectious bovine rhinotracheitis (IBR), Aujeszky's disease, classical swine fever (CSF) and food-and-mouth disease (FMD) (MEEUSEN et al. 2007).

Naked DNA can be used for immunisation of animals. DNA vaccination involves immunisation with a plasmid encoding an antigen of the pathogen. The plasmid is transfected into host cells via direct injection, or injection with electroporation or gene gun. The gene of interest then undergoes transcription and translation by host cellular machinery, resulting in the production of an antigenic protein that can induce cellular and humoral immune responses. This type of DNA vaccines promotes induction of cytotoxic T cells after intracellular expression of the antigens. They are temperature stable and safe to transport, which can be important for farms located in remote areas or for wildlife vaccines that need to remain in the open area for a prolonged period of time (MEEUSEN et al. 2007, REEDING and WERNER 2009).

Veterinary bacterial vaccines are also of a great value in a prophylaxis against bacterial diseases. There are three general categories of bacterial vaccines – a live vaccine containing a bacteria that can replicate in the host, thereby functioning as an immunogen without causing its natural disease. A subunit or inactivated vaccine is an immunogen that can replicate in the host. Another type is a DNA-based vaccine, it is taken up by cells, in which it directs the synthesis of bacterial vaccine antigens (ELLIS and BRODEUR 2003). Control of zoonotic diseases have had a major role in reduction of some diseases like rabies, rinderpest, brucellosis and others among people (ROTH 2011).

## Safety

The most common failures in the effectiveness of vaccination of animals can be linked to: the vaccine losing immunogenicity due to the expiration date or incorrect storage, the vaccine having been administered via a route other than that indicated by the manufacturer, the animal having been vaccinated during the incubation period of the disease, vaccination

having been induced by the transition of a latent form into an overt form of the disease, the animal being vaccinated in a state of immunosuppression, the animal being in a state of reduced immunological reactivity or hypersensitivity to the components of the vaccine, the vaccinated animal being weakened and poorly nourished or under the influence of strong stress, vaccinated animal having a high level of passive immunity provided by the mother, the protective action of the vaccine being overcome by large doses of the pathogen or very virulent strains (KOSTRO et al. 2015).

Safety and effectiveness of vaccines is also dependent on its manufacture process and qualitative composition, with particular emphasis on adjuvants enhancement of the specific immune response. When developing a composition of an immunological veterinary medicinal product, a key element is to choose the right type of adjuvant that will provide induced immunological response, without adverse reactions. In large-scale breeding, to improve protection, adjuvants must be stable and safe so that they do not decrease the growth and reproduction rate. What is more, adjuvant can reduce number of immunization needed, help to reduce the amount of vaccine material or allow vaccination against a selected disease of several species of animals (GERDTS 2015, PARKER 2009).

Adjuvants can act in a variety of ways: as a specific delivery vehicle, targeting a molecule or acing as a depot at the site of injection, to representing a specific danger signal that induces a very particular type of immune response (VIDYASHANKARA et al. 2015). Most often, adjuvants cause some sort of tissue injury, which in effect can lead to engagement of the immune system and its specialised mechanisms, triggering stimulation and activation of innate and adaptive immunity (GERDTS 2015). Sometimes such activation can result in apoptosis or necrosis at the site of injection, local inflammation manifesting in redness, pain, swelling and more rarely, granulomas or sterile abscesses. It can be perceived as a negative value in connection with visual aspects (animal trade, exhibitions, etc.). Adjuvants can also form a depot at the site of injection, which allows slow release of the antigen and more effective antigen uptake. However, this effect is not necessarily beneficial for all adjuvants and in some cases, transport to the draining lymph node seems to promote antigen-presentation more effectively (GERDTS 2015). Nonspecific adverse effects of vaccines and their adjuvants may also include: fever, arthritis, uveitis, anorexia, soreness and lethargy. An overdose of IL-2, a cytokine used as adjuvant may also increase the probability of autoimmune reactions. (SPICKLER and ROTH 2003, PETROVSKY 2016). A good adjuvant can allow the reduction of the dose or of the antigenic concentration, increasing stability and decreasing the price of vaccine (AUCOUTURIER et al. 2001, AROUS et al. 2013).

The most commonly used adjuvants for commercial animal vaccines are: mineral salts, emulsions (oil-in-water, water-in-oil and water-in-oil--in-water), nanoparticles and microparticles, cytokines, saponins, liposomes and archaeosomes, nonionic block copolymers, derivatized polysaccharides, carrier proteins, complement derivatives, bacterial products and their derivatives, toll like receptor (TLR) ligants and small molecules, immune-stimulating complexes (ICOMs), combination adjuvants, e.g.: FCA (water-in-oil emulsion with mycobacteria) (SPICKLER and ROTH 2003).

## UE regulations

Legal requirements regarding the safety of vaccines on the EU veterinary market are defined by various directives and regulations. Marketing authorization holders have to comply with those legislations in areas such as pharmacovigilance, applying to vary a marketing authorisation, submitting product data to European Medicines Agency (EMA) and reporting product defects or recalls. European regulatory requirements for veterinary medicinal products are governed by Directive 2001/82/EC, as amended (Directive 2001/82/EC), Commission Regulation (EU) No. 712/2012 of 3 August 2012 amending Regulation EC No. 1234/2008 concerning the examination of variations to the terms of marketing authorisations for medicinal products for human use and veterinary medicinal products (Commission Regulation (EU) No. 712/2012), Commission Regulation (EU) No. 37/2010 of 22 December 2009 on pharmacologically active substances and their classification in relation to maximum residue limits in foodstuffs of animal origin (Commission Regulation (EU) No 37/2010) and Regulation (EC) No. 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down community procedures for the establishment of maximum residue limits of pharmacologically active substances in foodstuffs of animal origin and repealing Council Regulation (EEC) No. 2377/90 and amending Directive 2001/82/ EC of the European Parliament and of the Council and Regulation (EC) No. 726/2004 of the European Parliament and of the Council (Council Regulation (EC) No 470/2009).

After the authorisation for vaccine is granted, before it can be placed on the market, a specific batch has to be tested. Manufacturers incorporate a batch release safety test into their quality assurance monitoring protocols to meet their internal quality standards and to confirm governmental regulatory requirements. These tests are conducted by vaccinating target species animals or laboratory animals with single or multiple doses of the test batch and observing vaccinated animals for sign of local or sys-

temic adverse reactions. These tests serve as a broad-spectrum bioassay to assess the biological properties of each batch of vaccine approved for the market (GIFFORD et al. 2011). Batch release safety tests are an important part of a comprehensive quality assurance monitoring system. Relevant articles of Directive 2001/82/EC, as amended by Directive 2004/28/EC provide guidelines for the Official Batch Protocol Review Certificate (OBPR) and for Official Control Authority Batch Release of Immunological Veterinary Medicinal Products (OCABR). The procedures and guidelines for running OCABR/OBPR apply equally in all Member States (Council of Europe; EDQM).

Veterinary pharmacovigilance concerns monitoring, evaluating and improving the safety of veterinary medicines, with particular reference to adverse events in animals and human beings related to the use of these medicines. The pharmacovigilance system in the European Union (EU) operates with the management and involvement of national competent authorities, the European Commission and the European Medicines Agency (EMA), in collaboration with the marketing-authorisation holders for the medicines (European Medicines Agency; EMA). Continued increase of the number of reports in the central EU database allows for better monitoring and enables the authorities to provide better feedback to the veterinarians on safe and effective use of veterinary medicinal products in the EU. Summary statistics on adverse event reports for centrally authorised products by target species, including reports in humans (reports received between 1 January 2016 and 31 December 2016) are presented in the Table 2.

Table 2

Summary statistics on reports for centrally authorised products by target species

| Species | Number of adverse event reports | Number of affected animals |
|---|---|---|
| Dogs | 11 657 | 12 312 |
| Cats | 3072 | 3 499 |
| Cattle | 1429 | 52 926 |
| Pigs | 615 | 320 550 |
| Rabbit | 582 | 6 025 |
| Horse | 250 | 399 |
| Sheep | 73 | 875 |
| Chicken | 27 | 875 |
| Goat | 15 | 927 766 |
| Others* | 139 | 148 161 |
| Human | 554 | 554 |
| Total | 18 413 | 1 475 139 |

*"Other" species include duck, ferret and guinea pig amongst others
Source: European Medicines Agency (EMA).

EMA has developed a number of tools and measures over time, to promote access and availability of veterinary vaccines to the EU market. These tools include: major species and minor species (MUMS) limited markets policy for immunological, scientific advice, Innovation Task Force and Ad Hoc Expert Group on Veterinary Novel Therapies (ADVENT), accelerated assessment, authorisation under exceptional circumstances, contribution to the Disease Control Tools project (DISCONTROOLS) survey, reduced fees for vaccines against epizootic diseases under certain circumstances, with a multi-strain dossier approach (European Medicines Agency; EMA).

## Conclusion

Although there are different types of vaccines and the overall manufacturing procedures for immunological products are standard in the medical industry, there are still important differences between vaccination practices in human and veterinary medicine. First off, all the economic constraints are generally of less importance in human medicine. Veterinary vaccines include about 23% of the worldwide market for animal products. The European veterinary vaccines market is poised to attain a Compound Annual Growth Rate (CAGR) of 4.97% from 2015 to 2020 (PETTERS 2018). The major goals of veterinary vaccines are to improve the health and welfare of companion animals as well as a cost-effective production of livestock and epidemic prevention of zoonotic diseases. Veterinary vaccines can be an efficient tool in reducing the need to use antibiotics in animal husbandry. There is also the issue of safety of immunological products and adverse reactions. Nevertheless, veterinary vaccines have had a significant impact on public health, through increasing safety of the products of animal origin and prevention of infectious diseases transmission between domestic and wild animals, and in an animal-to-human relation.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of the article.

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# A RESTING AND DYNAMIC ENDOSCOPY AS DIAGNOSTIC TOOLS IN DECREASING TRAINING CAPACITY IN HORSES – A REVIEW

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Key words: effort, respiratory, larynx, overground endoscopy, horse.

Abstract

During the effort, when the need for oxygen increase, the respiratory frequency, heart rate, and cardiac output increase and a pool of erythrocytes from the spleen are ejected. Therefore, the evaluation of training capacity based on the changes in the heart rate and the blood-picture. Among the most common cause of decreasing training capacity and eliminating horses from sports training, the upper respiratory tract (RT) diseases are the second. The dynamic obstructions of upper RT such as recurrent laryngeal neuropathy (RLN) and dorsal displacement of the soft plate (DDSP) case of stenosis, occurring when the soft tissues in pharynx collapse due to the changing pressures in RT. Horses suspected of lowering sports performance due to upper RT disorders are examined using endoscopy: resting endoscopy, the high-speed treadmill endoscopy and the newest one, overground endoscopy are conducted. However, the accuracy of diagnosing dynamic changes in the pharynx increase, when the dynamic endoscopy is conducted. The overground endoscopy is considered the best diagnostic tool to detect and confirm RLN, DDSP and other dynamic upper RT disorders. It is significant in the aspect of pre-purchase examination, in particular in racehorses in which the frequency of occurring of these disorders is high.

## Physiological background

Respiratory tract (RT) is an instrument for gas exchange. About 40–60 l of air per minute goes through horse's respiratory tracts when the horse is breathing during the rest. It can get to 40 times more air during intensive exertion – Table 1 – (DUCHARME et al. 1994, DUCHARME et al. 1999).

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Table 1
A comparison of respiratory parameters in healthy horses at rest and during strenuous exercise

| Respiratory parameter | Rest | Strenuous exercise |
|---|---|---|
| Breathing frequency [breaths $min^{-1}$]* | 10 to 15 | 120 to 150 |
| Tidal volume [l]* | 3 to 6 | 14 to 20 |
| Minute ventilation [l $min^{-1}$]* | 40 to 60 | 1500 to 2000 |
| Peak inspiratory pharyngeal pressure [cm $H_2O$]** | | -20 to -26 |
| Peak expiratory pharyngeal pressure [cm $H_2O$]** | | 10 to 20 |
| $pO_2$ [mm Hg]*** | 105 to ~110 | 69 to ~74 |

Data from: *AERTS et al. (2008), **DUCHARME et al. (1994), ***DUCHARME et al. (1999)

Beside gas exchange, RT also ensures proper pH in the organism (SANCHEZ et al. 2005). Each disorder that causes shrinking of the light of respiratory tracts will result in higher blood pressure and lower breathing efficiency. That is why, in order to maintain proper aerobic and training capacity, it is important to keep high larynx's throughput which allows efficient ventilation. During the exertion, lactates are accumulated and metabolic acidosis appears which can be partially compensated by the respiratory system. Despite the fact that horses have a very high tolerance when it comes to working in low-oxygen conditions, they do have their limits. In this case, measuring $pO_2$, $pCO_2$ and blood pH, before, immediately after and 30 minutes after finishing the training are also reliable for defining the training efficiency (BUTLER et al. 1993). Therefore, diseases of RT may affect this compensation processes leading to decreased training capacity. It is worth to mention that such diseases, after the orthopedic injuries, are the second most common reason for which horses are put away from the training (VAN ERK-WESTERGREN et al. 2013).

## An indicators of training capacity

During the effort, the need for oxygen increases significantly. The organism tries to cover it up by increasing respiratory frequency, heart rate, and cardiac output as well as ejecting a pool of erythrocytes from the spleen to the bloodstream. Physical effort causes endogenous stress which leads to changes in the red cell system, changes in the concentration of lactic acid in blood and tissues and causes oxidative stress (ART and LEKEUX 2005). The previously mentioned changes are used in assessing exercise capacity of horses. To determine changes in the heart rate, pulse measurement is done right before the exertion and 15 minutes after. Hor-

se's resting heart rate approximates from 20 to 48 beats per minute and in aerobic conditions, it goes up to 150 beats per minute. After 15 minutes the exercise is over, it should vary between 52 and 64 beats per minute if the training was done properly (AERTS et al. 2008). Nowadays, Holter ECG is used to monitor heartbeat which allows measuring myoelectric heart activity before, during and after the exertion. Additionally, hematological tests can be done to assess the exercise capacity, of which the best indicators are HGB (hemoglobin) and HCT (hematocrit) that provide information about oxygen volume in blood. After the exertion, HCT increases significantly because of spleen contraction and dehydration and should get back to normal level in about 30 minutes after the training. What is interesting, in well-trained horses and horses with outstanding sports predispositions high level of HGB and HCT can be observed (BURLIKOWSKA et al. 2015). Among biochemical tests, total protein (TP), lactic acid (LA), aspartate transaminase (AST) and creatine kinase (CK) concentrations give the information about the level of training and regenerative capabilities of the horse. TP indicates dehydration level caused by a noticeable increase in body temperature during exercise, whereas LA is a measure of working in low-oxygen conditions. Resting concentration of LA oscillates between 0.8–1.0 mmol $l^{-1}$, but after the race, it can raise up even to 30.0 mmol $l^{-1}$ (AERTS et al. 2008). The significant increase in LA concentration may be observed only immediately after intense effort so that is why we might not spot in the next samples. Finally, the concentration of AST and CK indicates the muscles' damage degree (BUTLER et al. 1993).

## The respiratory causes of decreasing training capacity

The recurrent laryngeal neuropathy (RLN) and dorsal displacement of the soft plate (DDSP) are the most commonly diagnosed disorders of upper RT. Both are the case of stenosis, occurring when the soft tissues in pharynx collapse due to the changing pressures in RT – Table 1 – (FRANKLIN 2008a). The shrinking of the diameter of upper RT changes partial pressure of blood gases, with a decrease of $pO_2$ and an increase of $pCO_2$ in arterial blood, and decreases training capacity (DURANDO et al. 2002). In both cases, the abnormal respiratory noises from upper RT occur, similar to other disorders such as subepiglottis cysts (SEC) or laryngeal dysplasia (LD) (FRANKLIN 2008a). In case of LD, the rostral displacement of palato-pharyngeal arch and right laryngeal haemiplegia or arytenoid cartilages deformations occur and the additional diagnostic tools such as endoscopy, ultrasonography, and MRI are necessary for proper diagnosis (GARRETT et al. 2009). Howe-

ver, recent studies have shown, that resting endoscopy is an insufficient tool for accurate differentiation of upper RT diseases (ELLIOT and CHEETHAM 2018, PARENTE 2018).

## Recurrent laryngeal neuropathy

RLN is the most common form of laryngeal hemiplegia, when the demyelination of the recurrent laryngeal nerve, the longest nerve in a horse body, occurs (DUPUIS et al. 2012). Nerve damage causes muscles dysfunctions, especially the cricoarytenoideus muscle which is responsible for the abduction of arytenoids cartilages (RHEE et al. 2009). The following are mentioned among the most common reasons of RL: injuries, fungal infections of the guttural pouches, intoxications, mineral deficiencies and idiopathic causes (DART et al. 2005). They lead to progressive degenerative changes, laryngeal muscles atrophy, lack of abduction of arytenoid cartilage or limpness, and collapse of vocal folds when the pressure inside the respiratory tract is increased. As a consequence, a decrease of breathing efficiency appears along with turbulent airflow and characteristic wheezing sound (STICK and DERKSEN 2010).

RLN is more common in large breeds, with higher frequency in males than in females (GARRETT et al. 2009). DUPUIS et al. (2012) identified two regions of the horse genome responsible for predispositions for this neuropathy. The base of diagnosis includes 4-grade Havemayer scale (COLLINS et al. 2009), or rather 5-grade and 6-grade classification (DIXON et al. 2001), describing the degree of larynx impairment. At 1$^{st}$ grade, both arythenoid cartilage and vocal folds are symmetrical while resting as well as in the move. At 2$^{nd}$ grade, slight asymmetry of cartilage movement and vocal folds are visible; when a horse is in motion, with complete abduction. The 3$^{rd}$ grade is characterized by asynchronic and incomplete abduction, whereas the 4$^{th}$ grade by total larynx collapse. Such horses are qualified for laryngoplastic surgery (DIXON et al. 2001, COLLINS et al. 2009). Proper gradation becomes more difficult when the partial paralysis of larynx occurs and it collapses only in motion. Then the resting endoscopy examination turns out to be insufficient and dynamic endoscopy, on a treadmill or overground, is recommended (WOODIE 2011).

## Dorsal displacement of the soft plate

Physiologically, the palate is located below epiglottis and separates airways from alimentary tract. DDSP consists in periodic or permanent

displacement of the caudal part of soft palate above the epiglottis cartilage which causes disorder in airflow to larynx and trachea as well as appearing of abnormal breath sounds. The characteristic sound is caused by fluctuating caudal part of the soft palate when breathing out (FRANKLIN et al. 2003).

In a case of the intermittent DDSP, decreasing training capacity during exertion and dysphagia in resting occurs. The less advanced disorders consist only in lifting or fluctuation of the soft palate (ALLEN et al. 2007, BARAKZAI and DIXON 2011). Among the reasons of DDSP, too long palate, palate laxity caused by atony, diminished epiglottis, pharynx inflammation but also RLN or myopathy of pharynx muscles were reported (FRANKLIN et al. 2003). According to another hypothesis, a curb in horse's mouth may trigger a swallow reaction and an increase of saliva secretion which may lead to palate displacement. Palate displacement over the epiglottis can happen during intensive exertion when the pressure in pharynx and trachea is high (FRANKLIN et al. 2002).

During diagnosis, the abnormal breath sounds, rattling and loss of training capability occur. When the soft palate is displaced, slowdown during movement may appear. DDSP episodes cause significant reduction of airflow when breathing out and an increase in the minute ventilation (FRANKLIN et al. 2002). Among horses with DDSP during rest, 76% show intermittent DDSP also during the exertion. However, in most cases, horses that were diagnosed with DDSP would not have any visible symptoms in resting endoscopy. It is a second major case, when the dynamic endoscopy, on a treadmill or overground, is recommended (PARENTE 2018).

## Available diagnostic tools

The evaluation of training capacity is based on the changes in the heart rate and the blood-picture. In research conducted in South Africa, in group of horses with abnormal respiratory sounds on larynx and trachea, and decreasing training capacity, pharynx and larynx disorders were observed in 77% of examined horses and in 48%, more than one disorder occurred (MIRAZO et al. 2014). Horses suspected of lowering sports performance due to upper RT disorders were, so far, examined using resting endoscopy. Resting endoscopy performed before and right after the training reveals structural disorders of RT or the diseases that show symptoms in rest, such as mucus inflammation, polyps, cysts, guttural pouches mycosis or permanent dorsal displacement of the soft palate, epiglottis entrapment or inflammations of trachea and presence of exudates (BARAKZAI and DIXON 2011, WOODIE 2011).

## Resting endoscopy

Running tests right after the exertion, when the horse is still breathing fast and deep, increases the chance of diagnosing dynamic changes in the pharynx. However it often happens that horses, immediately after the endoscope is inserted, are able to correct the epiglottis position and arytenoid cartilages and vocal folds come back to their resting position which can give false negative results. Another method for diagnosing dynamic disorders of pharynx and larynx is closing the horse's nostrils and stimulating swallowing. It leads to increased pressure in the respiratory tract and imitates exertion. It allows to diagnose epiglottis entrapment and DDSP but the effectiveness of this method is questionable. An application of lobeline was also done to cause hyperventilation and increased the number of breaths in order to simulate to the number reached during exertion. Unfortunately, it was not possible to reach maximum values (FRANKLIN et al. 2008b).

## Dynamic endoscopy on the high-speed treadmill

The high-speed treadmill was brought to use which allowed the examination during movement. It gives the opportunity to diagnose changes that can be seen only during the maximum effort. In MELKOVA et al. (2016) research, it was observed that 66% of examined horses had upper RT disorders where 8 of them were diagnosed with DDSP, 4 others with RLH of $2^{nd}$, $3^{th}$, and $4^{th}$ degrees. In the same research, it was noted that horses can suffer from more than one upper RT disease and two of the examined horses had simultaneously DDSP and exercise-induced pulmonary hemorrhage.

Other research on the treadmill made it possible to diagnose 71% (LEUTTON and LUMSDEN 2015) and 89% (DORE and KANNEGIETER 1995) of examined horses. DORE and KANNEGIETER (1995) confirmed the diagnosis from resting endoscopy in 19 horses and denied in 21 horses. LEUTTON and LUMSDEN (2015) observed soft palate disorders, vocal folds, and folds of arytenoid cartilages collapse and demonstrated that excessive abduction of arytenoid cartilages may have an influence on the development of additional disorders of pharynx and larynx. On the other hand, TAN et al. (2005), during horses examination on the treadmill, proved that 49% of horses, which showed no symptoms in resting endoscopy, showed changes in dynamic endoscopy. The most common disorder occurring in 105 horses, which is over 50%, was a limping of aryepiglottic folds (TAN et al. 2005). Also, BARAKZAI and DIXON (2011) showed differences between diagnostics

in resting and dynamic endoscopy. They examined 281 horses and confirmed DDSP in 47 horses during dynamic endoscopy, while only 25% demonstrated symptoms during resting endoscopy.

Dynamic endoscopy (Figure 1) gave the opportunity for recognizing axial deviation of ery-epiglottic folds (ADAF) during the effort which is caused by the instability of soft palate. It consists in subsiding of membrane part of folds into the larynx, changing its diameter and leading to abnormal sound during inhalation (AHERN and PARENTE 2008). This disorder occurs rarely and is diagnosed even more rarely because of low popularity of dynamic endoscopy. In KING'S et al. (2001) research, in a group of 871 horses, only 52 demonstrated the symptoms of ADAF in dynamic endoscopy.

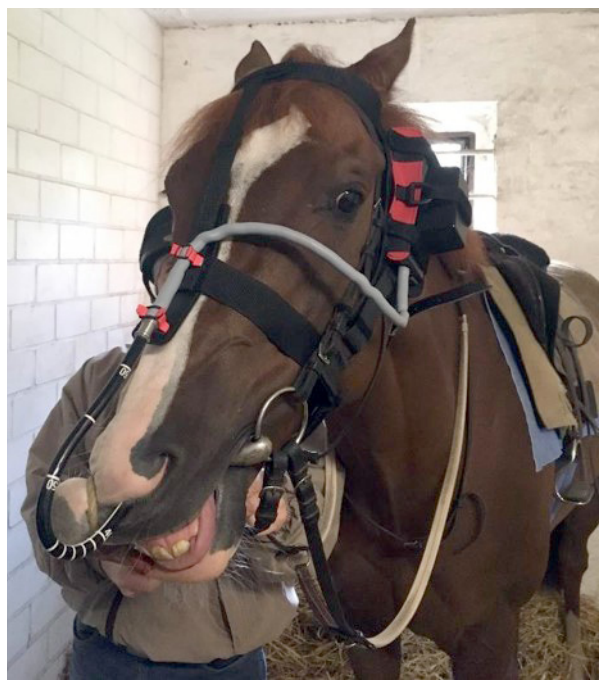

Fig. 1. Head mounted overground laryngoscope

## Dynamic overground endoscopy

Dynamic endoscopy on the high-speed treadmill has many advantages according to standardization of examination conditions however, it causes many technical difficulties (MELKOVA et al. 2016). There is a need for a special place, equipment, and staff for operating and controlling. Also, the examination requires the horse to be transported to the clinic, what

generates additional costs. It was observed that horses on the treadmill have different length and frequency of steps, presenting different values of exercise capacity parameters compared to standard overground training. Also, the indicators of training capacity such as heart rate and the concentration of lactates were significantly lower after examination on the high--speed treadmill in comparison to overground. Therefore, the treadmill examination doesn't reflect changes during the exertion, especially during natural training with the influence of rider and the neck flexion. STICK and DERKSEN (2010) observed that horses with DDSP often do not show any symptoms on the high-speed treadmill, what can be directly caused by lack of reins tension and different head and neck position. DAVIDSON et al. (2011) proved that the likelihood of diagnosing a dynamic disorder of upper RT increases 3.5 times with the neck flexion during endoscopy examination what is essential especially in dressage horses.

Fig. 2. Overground laryngoscopy examination

The overground endoscopy (Figure 2) is the most technologically advanced examination that allows to adapt the endoscope for in-the-field testing purposes. Mobile endoscopes use the miniaturized endoscopic system, mounted on a special halter. Nowadays, the overground endoscopic systems allow obtaining high-quality video signal almost regardless of the distance from the examined horse to the receiver. In comparison to the

first examinations on the lounge, when the horse could not achieve the maximum level of effort (FRANKLIN et al. 2008b, ALLEN and FRANKLIN 2010), a freely moved overground endoscopy allows evaluating the horse in intensive training, essential for symptoms to appear. ELLIOT and CHEETHAM (2018) demonstrated that in the exercise endoscopy, subgrades of LRT are worsening and proportion of complete or partial paralysis is increasing. Overground endoscopy gave the opportunity for examination in everyday, natural environment, during the standard training with harness (HACKETT and LEISE 2018). It was proved that the symptoms of DDSP in race horses can be seen only when the horse is getting close to the finish line (MIRAZO et al. 2014). It allows also to evaluate influence of the rider and the level of head and neck position for the pharynx diameter and airflow (VAN ERCK-WESTERGREN et al. 2013). It is often not possible to diagnose the upper RT disorder during the first dynamic endoscopy because the moment of obstruction may be omitted (ALLEN and FRANKLIN 2010).

## Conclusions

Numerous researches prove that overground endoscopy is the best diagnostic method to detect and confirm RLN, DDSP and other dynamic upper RT disorders. It is significant in the aspect of pre-purchase examination, in particular in racehorses in which the frequency of occurring of these disorders is high. Moreover, endoscopy reduced the cost of dynamic upper airway disorders examinations, what is worth attention due to the need to repeat the examination. An overground endoscopy is a valuable tool in the diagnosis of pure performance in show and racing horses.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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