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SEKCE 1

Hygiena a technologie potravin

Bacillus cereus group bacteria in catering establishments: The environment as a factor of transmission

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Summary
Microbiological contamination is one of the main parameters that must be assessed to assure the safety of foods produced by a catering system. The occurrence of B. cereus sensu lato (B. cereus s. l.) bacteria in the food processing environment is an important risk factor for B. cereus intoxications and infections. Therefore, the prevalence of B. cereus s. l. in a total of 238 environmental swabs from six catering establishments was investigated to determine the possible contamination routes of heat-treated foods. B. cereus s. l. was detected in 52.1% of the swab samples. The detection rates for the B. cereus s. l. nheABC complex, hblDAC complex, cytK and ces virulence genes among the isolates were 98.4, 34.6, 31.5 and 4.7%, respectively. All the isolates (100%) carried at least one or more enterotoxin genes, confirming the high pathogenic potential of B. cereus s. l. bacteria. Hence, from the viewpoint of prevention of diseases caused by B. cereus, it is necessary to identify sources of contamination in the food processing chain.

Keywords*: food safety; hygiene; contamination; enterotoxin; mass catering*

Introduction

The *B. cereus* group, also known as *B. cereus sensu lato (B. cereus s. l.)*, is composed of gram-positive, rod-shaped, spore-forming aerobic bacteria that exist widely in the environment (Zhuang et al., 2019). The group constitutes of eight major species, i.e., *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. anthracis*, *B. thuringiensis*, *B. cereus sensu stricto (B. cereus s. s.)*, *B. cytotoxicus* and *B. toyonensis*, as well as several newly described members (Tuipulotu et al., 2020). *B. cereus s. s.* (usually referred to as *B. cereus*) is an opportunistic pathogen capable of causing a range of diseases, most prominently foodborne disease due to the production of enterotoxins (diarrheal toxins) or emetic toxin (cereulide) (Fiedler et al., 2019). The emetic toxin gene is found in a specific subgroup of *B. cereus s. s.*, while the enterotoxin genes and other virulence factors are broadly distributed among the members of the *B. cereus* group (Messelhäußer and Ehling-Schulz, 2018).

Bacillus cereus is the 3rd most frequent bacterial agent responsible for foodborne outbreaks in Europe, right after *Salmonella* spp. and *Campylobacter* spp. (EFSA, 2021). In 2019, 26 % of cases involved in strong- and weak-evidence foodborne outbreaks became exposed to contaminated foods in a school or kindergarten and canteen or workplace catering (EFSA, 2021). *B. cereus* represents one of the major pathogens in mass catering, as its elimination is not guaranteed neither by pasteurization nor sanitation procedures (Senesi and Ghelardi, 2010).

Therefore, the objective of this study was to assess the incidence of *Bacillus cereus s. l.* in the processing environments in school and workplace canteens in the Czech Republic and provide a thorough description of the virulence genes distribution patterns in the *B. cereus s. l.* isolates along with the contribution of the facility environment on the spread of the bacteria.

Material and methods

The study was conducted on 3 public schools and 3 staff canteens in the Czech Republic. A total of 238 swab samples were collected by swabbing areas of different types of food contact

and handling surfaces (work surfaces and table desks, cutting boards, cutlery, kitchen sinks and taps and equipment controls and handles) in the food preparation facilities. The swab samples were collected using 3M™ Cellulose Sponge-Sticks with 10 mL buffered peptone water (3M, Saint Paul, USA) following the manufacturer's protocol after completing work on a given section. The collected samples were transported to the laboratory at a temperature of 4 ± 1 °C.

The samples were further investigated qualitatively for the presence of *B. cereus s. l.* bacteria according to ISO 7932 with certain modifications. The species identification of *B. cereus s. s.* was performed based on detection of the *gyr*B gene encoding DNA gyrase subunit B (Yamada et al., 1999). All *B. cereus s. l.* isolates were screened for the presence of toxin genes *nhe*ABC, *hbl*CDA, *cyt*K and *ces* encoding for non-haemolytic enterotoxin (Nhe), haemolysin BL (Hbl), cytotoxin K (CytK) and cereulide (Ces) production using specific primers (Ghelardi et al., 2002; Guinebretiere et al., 2002; Rowan et al., 2003; Horwood et al., 2004; Guinebretiere et al., 2006).

Species identification of suspected isolates of *B. cereus s. l.* that were not identified as *B. cereus s. s.* by PCR was performed using the MALDI-TOF MS. As a sample preparation technique, the direct transfer method was used with 1 µl of 70% formic acid overlay. All spectra in the mass-to-charge ratio (m/z) range of 2,000–20,000 were analysed using MALDI BioTyper software, database version 3.1 (Bruker Daltonics, Billerica, USA).

Results and Discussion

In this study, 124 of the 238 collected samples (52.1%) were positive for *B. cereus s. l.* occurrence. The level of handling surfaces and equipment contamination was higher than in case of Bogdanovičová et al., (2019), who reported *B. cereus* presence in 39.6% of the food service environmental swab samples. The *nhe*ABC and *hbl*DAC gene clusters were found in 98.4% and 34.6% of the *B. cereus s. l.* isolates in our study, respectively. Moreover, 31.5% of the isolates possessed the *cyt*K gene, whereas the *ces* gene was the least frequently detected toxin gene, present in just 4.7% of the isolates. Generally, 1% or less of isolates from food or the environment carry the *ces* gene (Ramarao et al., 2020).

Table 1: Toxin gene profiles of *Bacillus cereus s. l.* isolates obtained from processing environments of six catering establishments.

Toxin gene profile[a]	Toxin gene combination				Number of isolates[b]
	nhe	*hbl*	*cyt*K	*ces*	
A	+	+	+	-	27 (21.3)
B	+	-	+	+	0 (0.0)
C	+	+	-	-	15 (11.8)
D	+	-	+	-	12 (9.4)
E	+	-	-	+	6 (4.7)
F	+	-	-	-	65 (51.2)
G	-	-	+	-	0 (0.0)
H	-	+	-	-	1 (0.8)
I	-	+	+	-	1 (0.8)

[a] Toxin gene profile classification (A-G) according to Ehling-Schulz et al., (2006).
[b] Number of isolates with toxin gene combination /number of all isolates (% relative frequency).

We observed 25 different virulence gene distribution spectra. The most abundant genetic profile based on Ehling-Schulz et al. (2006) classification (Table 1), present in 51.2% of the isolates, harbored one or more virulence genes of the *nhe*ABC operon. Overall, 127 (100%) isolates carried at least one or more enterotoxin genes. The pathogenic potential of *B. cereus* is extremely variable, with strains being harmless and others lethal. Therefore, the

characterization of *B. cereus* potential of pathogenicity is a major challenge for the agri–food industries (Ramarao et al., 2020).

Conclusion

The results of the study suggests the need for strengthening the standard of hygiene and the procedures for food manufacturing and preparation in mass catering facilities, as well as the HACCP plans for such establishments.

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Patogenní mikroorganismy jako potencionální riziko v ready-to-eat potravinách

Pathogenic microorganisms as a potential risk in ready-to-eat foods

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Summary
Our study is focused on determination of selected pathogenic microorganisms in ready-to-eat foods. The study is mainly focused on the determination and identification of major pathogens such as Listeria monocytogenes, Salmonella spp. and Staphylococcus aureus which can cause serious alimentary diseases The study also includes the determination of Helicobacter pylori because food can play an important role in its spread to the human population. A culture method will be used to determine bacteria. Biochemical tests and molecular-biological methods will be used for subsequent confirmation.

Keywords: *rady-to-eat foods; RTE foods; pathogenic microorganisms; food safety*

Úvod

Ready-to-eat potraviny (RTE potraviny) jsou definovány jako potraviny určené k přímé spotřebě, bez nutnosti tepelného ošetření či jiné úpravy. Do kategorie RTE potravin je zahrnuta velká a různorodá škála potravin, které mohou být syrové, vařené, chlazené, ale i mražené. RTE potraviny jsou konzumenty oblíbené zejména pro časovou úsporu, která je s jejich konzumací spojená, cenovou dostupnost, rozmanitost a organoleptické vlastnosti (Mengistu et al., 2020). Pro bakterie představují potraviny vhodné vehikulum při přenosu infekce na člověka (Akinnibosun et al., 2015). Vzhledem k chybějící tepelné úpravě RTE potravin před jejich konzumací, představují tyto potraviny zvýšené potencionální zdravotní riziko pro konzumenty. Riziko spočívá především v možné kontaminaci RTE potravin patogenními mikroorganismy během jejich výroby, manipulace a skladování (Mengistu et al., 2020). Mikrobiální bezpečnost RTE potravin rovněž ovlivňuje kvalita a vhodnost vstupních surovin, pH a aktivita vody.

V ready-to-eat potravinách se mohou vyskytovat významné patogenní mikroorganismy, jako jsou *Listeria monocytogenes, Salmonella* spp. nebo *Staphylococcus aureus* (Kotzekidou, 2016). *L. monocytogenes* a *Salmonella* spp. patří k významným původcům alimentárních onemocnění na světě (Westrell et al., 2009). *Listeria monocytogenes* je život ohrožující patogenní bakterie způsobující onemocnění listeriózu. Zvláště nebezpečná je listerióza pro těhotné ženy a imunosupresivní jedince. Mortalita uváděná u toho onemocnění je vysoká, dosahuje až 20 % (Huang et al., 2012). V případě humánní listeriózy jsou RTE potraviny považovány za nejvýznamnější zdroj bakterie *Listeria monocytogenes* (Westrell et al., 2009).

RTE potraviny mohou být rovněž významným zdrojem patogenní bakterie *H. pylori* (Quaglia and Dambrosio, 2018). Odhaduje se, že více než 50 % světové populace je v současnosti infikováno bakterií *Helicobacter pylori* (Kao et al., 2016). Způsoby přenosu *H. pylori* nejsou doposud přesně známy, ale ukazuje se, že potraviny můžou hrát významnou roli v šíření tohoto patogenu v lidské populaci (Vale and Vítor, 2010).

Evropská legislativa uvádí mikrobiologická kritéria pro některé druhy RTE potravin (Nařízení komise č. 2073/2005). Jsou v ní uvedeny požadavky týkající se přítomnosti *Listeria monocytogenes, Salmonella, E. coli* a shigatoxin produkující *E. coli.*

Materiál a metodika

Vzorky RTE potravin budou zakoupeny v běžné tržní síti na území České republiky. Výběr vzorků bude zahrnovat různé typy RTE potravin zejména živočišného původu (masné, rybí a mléčné výrobky). Ke stanovení patogenních bakterií budou využity jak klasické kultivační metody, tak i metody molekulárně-biologické. *Salmonella* spp. bude stanovena dle ČSN EN ISO 6579, *Listeria monocytogenes* dle ČSN EN ISO 11290-1 a *S. aureus* dle ČSN EN ISO 6888-1.

Následně bude u získaných izolátů provedena konfirmace pomocí biochemických testů a u vybraných izolátů pomocí polymerázové řetězové reakce (PCR). PCR bude využita pro detekci druhově specifických genů a vybraných genů kódující faktory virulence (geny kódující stafylokokové enterotoxiny, toxin syndromu toxického šoku a gen rezistence k methicilinu). Detekce *H. pylori* bude stanovena pomocí nested PCR. DNA *H. pylori* bude izolována přímo z vyšetřovaných potravin pomocí kitu DNeasy mericon Food Kit (QIAGEN®).

Výsledky

Tato studie bude provedena v roce 2021. Předpokládaným výsledkem je získání ucelených informací a dat o kvalitě a mikrobiálním profilu ready-to-eat potravin dostupných v tržní síti. Hlavním přínosem projektu bude rozšíření poznatků o mikrobiologické jakosti RTE potravin z hlediska přítomnosti vybraných patogenů.

Poděkování

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Melon seed milk: production and storage stability

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Summary
This study aimed to produce and check the stability of melon seed milk stored at the refrigeration temperature for 7 days. Among the tested parameters, only DPPH assay showed significant change from 9.16% to 11.53% of free radical inhibition. Values for total polyphenolic compounds, FRAP (ferric reducing ability of plasma) and malondialdehyde right after production were 0.28 mg GAE/g, 2.50 µmol Trolox/g and 0.80 µg/g respectively. No significant ($p>0.05$) change was obtained for them during 7 days of refrigerated storage. It was concluded that from melon seeds, that mainly represent discarded byproducts, stable vegetable milk alternatives can be made.

Keywords*: melon seed kernels, vegetable milk, melon seed milk*

Introduction

Consumers' preference today is going towards natural and healthy food. Environmental aspects also gain in importance and thus finding the ways of use of food production byproducts. There is also interest in vegetable alternatives of milk and milk products, mainly due to allergies (lactose intolerance) or diet preferences (vegan diet). Some of the vegetable milk products such as soy, almond, rice and coconut milk are widely produced and consumed already. On the other hand, milk that can be obtained from pumpkin or melon seed were only part of a few studies (Bastıoğlu et al., 2016).

Melon crop industry creates byproducts that are mainly discarded as a waste. Part of this waste (around 10% of fruit mass) are seeds that are rich in nutrients and bioactive substances (Rabadán et al., 2020; Mallek-Ayadi et al., 2018). 80% of melon seed composition goes to oil, proteins and minerals (45%, 35% and 5% respectively) (Petkova and Antova, 2015). Present polyphenolic compounds and flavonoids are suggesting antioxidant potential (de Cunha et al., 2020). Melon seed milk, reported in studies, consisting of 3.6% proteins, 4% fat and with no lactose present can be considered as a healthy and nutritional beverage that can be used by lactose intolerant consumers or vegans (Bastıoğlu et al., 2016).

The high pH value and water activity of beverages makes them more susceptible to spoilage. This fact shortens their shelf life even at refrigerated storage (Akubor and Ogbadu 2003). Akubor et al. (2002) studied microbial activity on melon seed milk and revealed doubling the bacterial count during 2 days storage at 10°C.

This study aimed to investigate stability of obtained melon seed milk during 7 days of refrigerated storage, by controlling the antioxidant activity, total polyphenolic content and lipid oxidation markers.

Materials and methods

For creation of melon seed milk, dried melon seed kernels (Natco brand, India) were used. After being milled in a small coffee mill, seeds are mixed with water in proportion seeds/water 1:2.5 and then compressed through a fine sieve. The tests on parameters MDA (malondialdehyde), TPC (total phenolic content), FRAP (ferric reducing ability of plasma) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assay right after production and on third and seventh day of refrigerated storage at 5°C. Analysis of mentioned parameters were done

according to work of Antonic and Dordevic, (2020). Statistical analysis was done using the SPSS statistical software (IBM Corporation).

Results and discussion

The results obtained for parameters DPPH, FRAP, MDA and TPC are presented in Table 1. Values obtained for DPPH free radical inhibition significantly ($p<0.05$) increased from 9.16% (Day 0) to 11.53% (Day 7). Literature reports in studies including melon seed extract show values of 48.5% of DPPH inhibition (Ibrahim and El-Masry, 2016), which means that antioxidants were only partially transferred to prepared milk. This increase could be explained by possible release of certain antioxidants in milk matrix during storage. On the other hand, FRAP and TPC values that are the main indicators of antioxidant activity showed decreasing trend during storage. Though, no significant ($p>0.05$) difference was obtained between values in samples on Day 0 and Day 7 for both parameters. MDA is one of the markers of lipid degradation (Custodio-Mendoza et al., 2019). MDA values increased from 0.80 μg/g (Day 0) to 0.93 μg/g (Day 7), but no significant ($p>0.05$) difference was obtained. This means that obtained melon seed milk kept the stability of lipids during 7 days storage.

Table 1. Measured parameters in melon seed milk during storage

Parameter	Day 0	Day 3 at 5 °C	Day 7 at 5 °C
DPPH (%)	9.16 ± 1.24^{a}	9.79 ± 2.73	11.53 ± 1.27^{b}
FRAP (μmol Trolox/g)	2.50 ± 0.05	2.33 ± 0.56	2.19 ± 0.39
MDA (μg/g)	0.80 ± 0.14	0.88 ± 0.26	0.93 ± 0.15
TPC (mg GAE/g) *	0.28 ± 0.05	0.26 ± 0.05	0.23 ± 0.02

Lowercase letters (a and b) indicate statistically significant differences ($p < 0.05$) between the columns. *GAE - gallic acid equivalent.

The value of TPC in melon seed milk (0.28 mg GAE/g) is lower than the one reported for the melon seeds (1.50 mg GAE/g) in the work of Vella et al. (2018). The studies including other seeds and their milk confirm that loss of antioxidant activity can be up to 84% in produced milk. The reason for this is mainly because polyphenols and other antioxidants are mainly situated in skins of the seeds, and those mainly remain on sieves in milk production. Hydrophobic polyphenols may not be transferred to the milk as well (Aydar et al., 2020).
The oxidative stability is an important factor that determines sensory quality and shelf-life of the products (Naziri et al., 2017). The milk obtained from melon seeds showed good oxidative and antioxidant stability. This is in line with the study of Naziri et al. (2017), where vegetable milks made of maize germ, hazelnut and sesame seed, showed only slight decrease in tocopherols, known as antioxidants.

Conclusions

Melon seeds that are mainly discarded as waste contain a significant amount of nutrients and bioactive substances. They can be used in production of vegetable milk alternatives that can be interesting to the consumers with different diet preferences. Obtained vegetable milk showed statistically insignificant change in antioxidant potential and lipid oxidation stability.

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Textural and sensory changes of biscuits fortified by natural substances with antioxidant effect

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Summary
Biscuits are a very popular fine pastry, commonly consumed by a wide population. Due to a higher fat content, long shelf-life and temperature, at which are stored, can cause oxidation of contained fat. The production process and not only the type of package is not enough barrier to slow down oxidation processes. For this reason, chemical antioxidants are often used in the recipe, but they are not well perceived by consumers. Previous studies have confirmed the appropriateness of the use of natural raw materials rich in antioxidant substances. However, their addition affects the texture, colour and overall consumer acceptance as well. Measured values of hardness, and fracturability show, that there was a statistically significant shift of these values in the case of the addition of mint, cloves, grape flour and cinnamon, already at a dose of 1%. However, the sensory evaluation confirmed acceptability of fortified products for consumers.

Key words: *biscuits, texture, polyphenols, spices, herbs, sensory, antioxidants*

Introduction

Many natural materials such as spices and herbs contain antioxidants that can slow down the rancidity of fats. Their proper use in foods can improve the nutritional profile of these foodstuffs. The use of these substances has recently expanded considerably, and a number of related studies have been carried out (Ibrahium et al., 2013). The antioxidant activity of herbs and spices is mainly due to the content of phenolic diterpenes, flavonoids, volatile oils and phenylpropanoids (Dhartiben et. al, 2016). However, the use of these natural substances in foods subsequently affects taste and "visual appearance", which are decisive for product appeal, sensory quality, aesthetics, expected safety, willingness to accept the product, taste appeal and influence selection (Paakki et al., 2019). If consumers' expectations regarding texture, colour and sensory parameters are not met, there will be no interest in the product and it will not be sold (Bajaj et al., 2006; Reddy et al., 2005). The previous study confirmed the positive effect of the addition of mint, cinnamon clove and grape flour, which were added in different amount, in terms of increasing the number of polyphenols and the antioxidant capacity of biscuits (Kral et al., 2020). The aim of this study was to confirm whether biscuits enriched in this way are still attractive to consumers.

Material and methods

The biscuits were prepared from the following ingredients: wheat flour (150g), Baking fat (45 g; Hera), powdered sugar (150g), egg yolk (15g). Biscuits were fortified by powdered clove (*Eugenia caryophyllata*), powdered cinnamon (*Cinnamomum verum),* mint leaves crushed (*Mentha piperita L.)* and grape seed flour (*Vitis vinifera*), at dosage 1%, 3%, 5% a 10%. Determination of texture included measurement of hardness and fracturability. A TA.XT. PLUS Texture Analyzer (Stable Micro Systems, Godalming, UK) was used for measurement. Sensory characteristics of the products were assessed by a panel of 15 trained evaluators. The parameters colour, consistency, smell, taste, sweetness, overall impression and the price that the evaluators would be willing to pay for the product were evaluated.

Results and discussion

Texture: The measured results of hardness are shown figure 1, fracturability in figure 2. The results show a statistically significant reduction ($p < 0.05$) of both parameters in all cases of the addition of spices and herbs. These parameters are reduced by the addition of 1% and other changes are not statistically significant ($p > 0.05$). This is consistent with other studies (Antonic et.al., 2021, Samohvalova et al., 2016). Only in the case of mint, with the addition of 10 percent, there is an increase again. It can be due to presence of leave particles in the place of measurement.

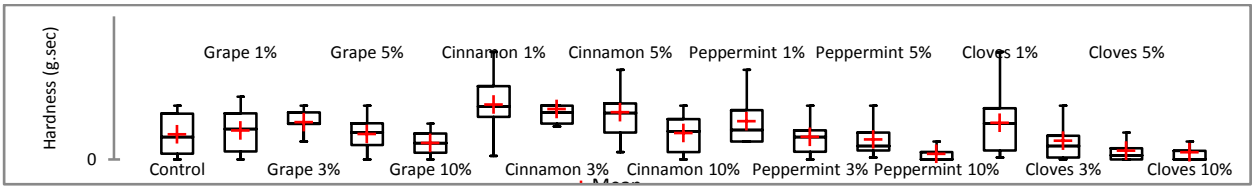

Figure 1: Hardness of biscuits

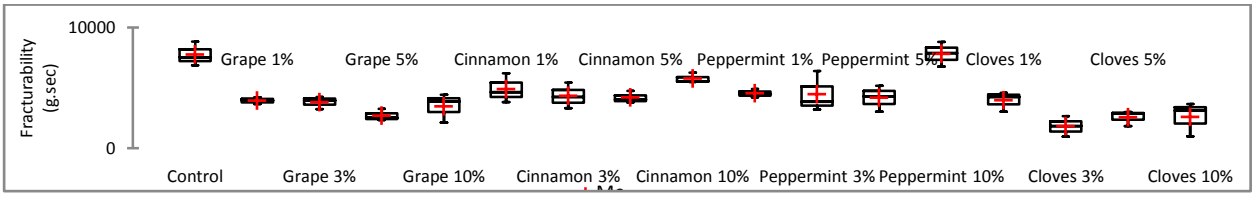

Figure 2: Fracturability of biscuits

Sensory: Within the sensory evaluation were assessed colour, consistency, smell, taste, sweetness, and the price that the evaluators would be willing to pay for the product. These parameters contributed to the determination of overall impression. The results of samples with grape seed didn't show significant ($p < 0.05$) differences in the overall acceptability with increasing addition. This is confirmed by previous study (Antonic et.al., 2021). However, for the samples with cloves, mint and cinnamon, significant ($p > 0.05$) changes occurred with increasing addition. The changes in perception are relatively small, except of clove, when with the addition of 10% the decrease is already significant. Results are shown in table 3.

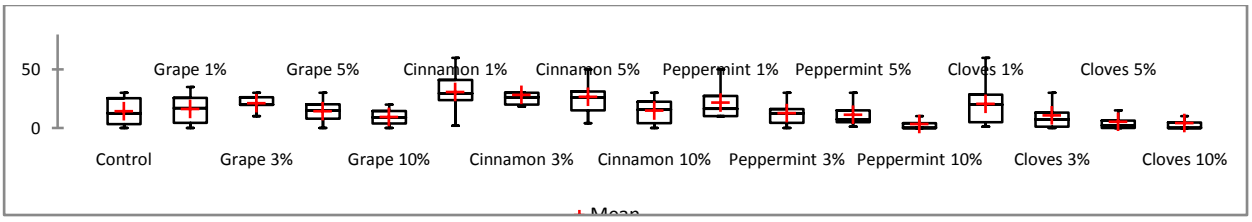

Figure 3: Boxplot of overall impression of biscuits (red cross mean value)

Conclusion

In previous studies, it has been confirmed that the addition of suitable spices to biscuits can transfer polyphenols to the product and thus increase the antioxidant capacity. This study confirmed that while a well-chosen type of spice and its dosage affects the texture of the product, the overall consumer acceptability does not change much with the appropriate dosage. Dosage of 10% appears too high, but at lower dosages a compromise can be found for all test substances between increasing antioxidant capacity and overall consumer acceptability. The resulting findings can be used in the creation of recipes.

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Escherichia coli a *Staphylococcus aureus* v parených syroch

Escherichia coli and *Staphylococcus aureus* in pasta-filata cheeses

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Summary

In this work, the effects of different combinations of lactic acid bacteria (LAB) on the growth of coagulase-positive staphylococci (CPS) and Escherichia coli were evaluated during ripening of 24 curd cheeses, and their subsequent behaviour during the manufacture and storage of pasta-filata cheeses was characterized. Three groups of cheeses were prepared in total: first, CC - control cheeses from raw milk without LAB addition; further PM - pasteurized milk cheeses with LAB, CPS and E. coli intentional inoculation; and finally, RM - raw milk cheeses with LAB added. The aim was to compare the effect of LAB from starter culture, and in combination with native LAB, and to evaluate the LAB effect as a group, and further to suggest the culture with the best inhibitory potential. Regarding the culture used, the best microbiological inhibitory effect in 28-day-old cheeses was reached by the combination of Fresco culture with Lacticaseibacillus rhamnosus GG, and the best sensory properties were judged to be those for cheeses manufactured with Culture A. A moderate negative effect of storage on overall sensory acceptance was noted, according to the final evaluation of overall acceptability of pasta-filata cheeses. The most satisfactory overall acceptability after 28 days of storage at 6 °C was reached for cheese with the addition of culture A.

Keywords: *raw milk cheese; Staphylococcus aureus; Escherichia coli; steaming process*

Úvod

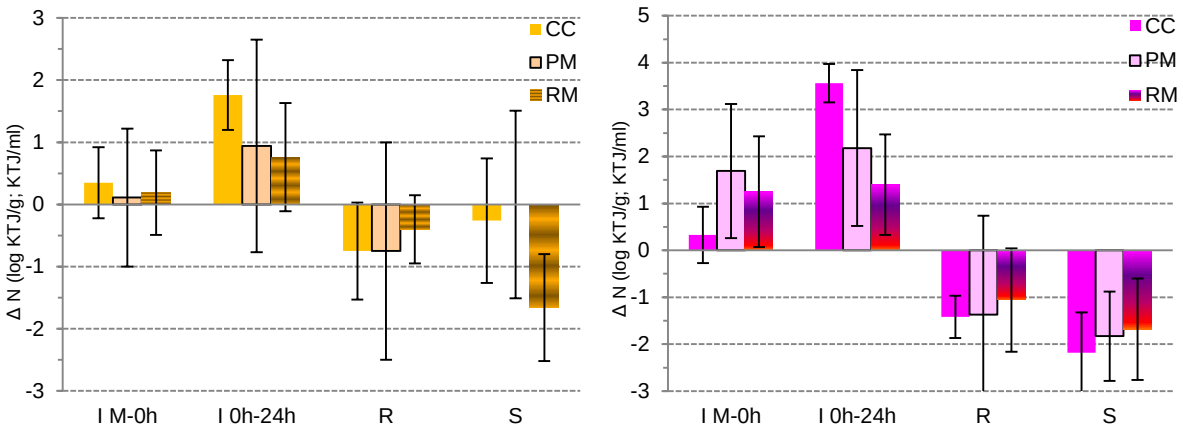
Parené syry tvoria rozmanitú skupinu syrov, pričom v mnohých krajinách sa vyrábajú zo surového mlieka. Napriek mnohým nesporným benefitom konzumácie syrov, bolo v histórii zaznamenaných niekoľko ochorení súvisiacich s ich konzumáciou. Ich pôvodcami boli, okrem *Salmonella* spp. a *Listeria monocytogenes*, aj *E. coli* a *S. aureus*, pričom ich zdrojom bolo surové, nedostatočne pasterizované alebo postpasterizačné kontaminované mlieko (Little a kol., 2008). *S. aureus* patrí pre svoju schopnosť tvoriť celý rad termostabilných enterotoxínov a dobrý rast v mlieku k najsledovanejším mikrobiologickým ukazovateľom v ručne a strojovo nadojenom mlieku, ale aj v remeselne vyrobenom syre (Normano a kol., 2007). Počas výroby syrov je *S. aureus* koncentrovaný v zrazenine a jeho obsah v mladom syre je priamo závislý od jeho počtov v mlieku (Asperger a Zangerl, 2003). Z hľadiska konzumácie potravín je však najproblematickejšia jeho schopnosť tvoriť enterotoxíny, ktoré prežívajú aj proces pasterizácie a aj vtedy, ak sú bunky usmrtené (Medveďová a kol., 2017). *E. coli* je trvalým obyvateľom ľudského a zvieracieho črevného traktu a je rozšírená všade v prírode. Nachádzať sa môže aj tam, kde sa nepredpokladá priame fekálne znečistenie, napríklad v syroch alebo mäsových výrobkoch (Valík a Prachar, 2009). Pri výrobe syrov s nízko-dohrievanou syrovinou fermentáciou zvyškovej laktózy vyvoláva ich skoré nadúvanie (Burdová a Lauková, 2001).

Cieľ práce
Popísanie dynamiky rastu *S. aureus* (ďalej len STA) a *E. coli* (ďalej len EC) v laboratórne pripravených syroch vyrobených zo surového alebo pasterizovaného mlieka v závislosti od prítomnosti zákysových kultúr baktérií mliečneho kysnutia.

Metodika
Parené syry boli vyrobené z mlieka, buď surového alebo pasterizovaného (pri 68 °C počas 30 minút). Do mlieka sme pridali potrebné množstvo syridla, a prípadne zákysovej kultúry (Fresco kultúra alebo kultúra A) a probiotických kmeňov baktérií mliečneho kysnutia (ďalej len BMK). Koagulácia prebiehala pri 30 °C počas 30 minút, potom bol syr nakrájaný pomocou syrárskej harfy a znovu zahriaty na 45 °C. Po výrobe syrovej hrudky nasledovalo primárne zrenie syra, ktoré trvalo 6 hodín pri laboratórnej teplote a následne 18 hodín pri 21 °C. Posledné kroky boli parenie (pri 63 °C po dobu 5 až 10 minút), formovanie nití a ich solenie v 10 % roztoku NaCl. Parené syry „Nite“ sa skladovali 28 dní pri 6 °C a v pravidelných intervaloch sa odoberali vzorky na mikrobiologickú analýzu.

Výsledky a diskusia
V laboratóriu sme vyrobili 24 syrov, ktoré sme zaradili do 3 skupín: 1. skupina – CC, bez prídavku BMK; 2. skupina – PM, s prídavkom BMK, EC a STA; 3. skupina – RM, s prídavkom rôznych kultúr BMK. Pri porovnaní zmien mikrobiálnych počtov v procese výrob a skladovania u kontrolných syrov a syrov vyrobených zo surového mlieka, s prídavkom zákysových kultúr nastal v obidvoch prípadoch nárast laktokokov, laktobacilov aj sledovaných skupín patogénnych mikroorganizmov počas prvých 24h. Procesom parenia došlo k prirodzenej redukcii počtov koagulázo-pozitívnych stafylokokov (ďalej len KPS) a EC v prípade kontrolných syrov o 1 a 1,5 log KTJ/g, v poradí. V druhom prípade došlo taktiež k redukcii počtov, o 0,5 a 1 log KTJ/g, v poradí. Následne počas skladovania nastal výraznejší pokles počtov patogénnych druhov v prípade syrov s prídavkom BMK, pričom v porovnaní s prvým dňom skladovania boli počty nižšie o takmer 2 log KTJ/g.
Rovnako bolo cieľom aj sledovanie zmien v počtoch KPS. Na začiatku výroby syra sme zaznamenali najvyšší nárast ich počtov v kontrolných syroch. Počas 24h fermentácie syrov sme zaznamenali nárast o viac ako 1 log KTJ/g rovnako aj u syrov bez prídavku BMK. Procesom parenia došlo k prirodzenej redukcii ich počtov u všetkých troch skupín syrov, pričom v prípade syrov s prídavkom zákysových kultúr bol pozorovaný pokles KPS len o 0,4 log KTJ/g. Naopak v kontrolných syroch, bez prídavku BMK parením došlo k poklesu počtov KPS o 0,75 log KTJ/g. Nasledujúcim skladovaním sme pozorovali výrazné zníženie počtov KPS v syroch s prídavkom BMK, pričom vo všetkých syroch s prídavkom BMK boli počty KPS v súlade s limitom EÚ. Na druhej strane, v syroch bez prídavku BMK, sme síce zaznamenali priemerný pokles o 0,2 log, ale na konci doby skladovania sa priemerná hodnota KPS pohybovala na úrovni 3,8 ±1,22 log KTJ/g, teda jednak výrazne vyššia, ako v syroch s prídavkom kultúr a zároveň v niektorých prípadoch vyššie, ako povolený limit.
Rovnako sme sledovali aj zmeny v počtoch EC. Na začiatku výroby syra sme zaznamenali najvyšší nárast jej počtov v syroch vyrobených z pasterizovaného mlieka a surového mlieka s prídavkom kultúr. Avšak počas 24h fermentácie syrov sme zaznamenali najvyšší nárast, o viac ako 3 log KTJ/g u syrov bez prídavku BMK, v dôsledku čoho sa počty EC pred parením pohybovali na úrovni 6 log KTJ/g v kontrolnej skupine syrov, resp. na úrovni 4 log KTJ/g v prípade syrov s prídavkom zákysových kultúr. Podobne, ako v prípade STA, aj v prípade EC nastala procesom parenia prirodzená redukcia počtov u všetkých troch skupín syrov. Následným skladovaním syrov sme pozorovali ďalší pokles počtov EC vo všetkých troch skupinách syrov, vďaka čomu sa na konci doby skladovania syrov bez prídavku kultúr

pohybovali na úrovni 2 ±2,2 log KTJ/g, kým v syroch s prídavkom kultúr BMK bola jej denzita v intervale 0-2,8 log KTJ/g.



Obr. 1: Grafické zobrazenie zmien počtov KPS (vľavo) a *E. coli* (vpravo) počas výroby zo surového mlieka M (I M -0h), kysnutia (I M -0h-24h), parenia (R) a skladovania (S) parených syrov

Záver

Záverom môžeme skonštatovať, že zníženie hodnoty *pH* viedlo k inhibícii rastu patogénnych baktérií rodu *Staphylococcus* a *Escherichia*. Pre dosiahnutie výroby mikrobiologicky bezpečných syrov je dôležité dodržiavať zásady správnej výrobnej a hygienickej praxe, a rovnako je potrebné na výrobu syrov používať kvalitné a bezpečné suroviny. Dôraz treba klásť aj na prídavok dostatočného množstva metabolicky aktívnych kyslomliečnych kultúr, z dôvodu produkcie antimikrobiálnych látok, znižovania hodnoty *pH* a predovšetkým kompetície o živiny.

Poďakovanie

Táto práca bola podporená projektmi VEGA 1-0532-18 a APVV 15-0006.

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Charakteristika karagenanových a chitosanových obalů s přídavkem rostlinných extraktů

Characterization of carrageenan and chitosan edible packaging with the addition of natural extracts

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Summary

The main aim of this study was to produce edible packaging based on chitosan and carrageenan with the addition of blue tea (Clitoria ternatea) and red cabbage (Brassica oleracea). The packaging were analyzed by FRAP method (ferric reducing antioxidant power) for the determination of antioxidant acivity and by measuring of thickness and breaking strain by texturometer. The best antioxidant activity was found in samples $CH_{LMČ}$ (1,46 ± 0,06 µmol Trolox/g) and $CH_{LČZ}$ (1,26 ± 0,04 µmol Trolox/g). The breaking strain was highest in sample CH_L (127,66 ± 10,85 %) and lowest in sample KAR (96,43 ± 2,89 %). The highest impact on studied properties of prepared edible films not have the addition of natural extracts, but the basic matrices used for films production. In future studies the higher addition of extracts from Clitoria ternatea same as Brassica oleracea and their impact on prepared edible carrageenan and chitosan films should be studied.

Keywords: *antioxidant activity; textural properties; Brassica oleracea; Clitoria ternatea*

Úvod

Jedlé obaly jsou v posledních letech významnou součástí výzkumu, jelikož jsou vyrobeny z přírodních biopolymerů a mohou tak fungovat jako náhrada syntetických obalů potravin. Také se do základní matrice mohou přidávat další přírodní látky jako například rostlinné extrakty a připravené obaly tak mohou i prodlužovat trvanlivost balené potraviny nebo detekovat kažení na základě změny barvy obalu (Benbettaïeb *et al.*, 2019).

Modrý čaj (*Clitoria ternatea*) (MČ) patří mezi jedlé květy a je charakteristická vznikající intenzivně modrou barvou díky přítomnosti anthokyanů, které mění barvu v závislosti na pH prostředí ve kterém se nachází a také vykazují antioxidační aktivitu. Obdobně jako modrý čaj je na anthokyany bohaté i červené zelí *Brassica oleracea* (ČZ) (Vo *et al*., 2019; Ahmad *et al*., 2020).

Cílem bylo zjistit vliv přídavku vodných extraktů modrého čaje a červeného zelí na antioxidační vlastnosti chitosanových a karagenanových obalů a také jejich vliv na tloušťku a pružnost získaných obalů.

Materiál a metody

Obaly byly vyrobeny z vodných extraktů červeného zelí (*Brassica oleracea var. capitata f. Rubra*) a modrého čaje (*Clitoria ternatea*), kdy základní matrici tvořil κ-carrageenan (KAR) nebo nízkomolekulární chitosan (CH_L), ke kterým byly následně přidávány jednotlivé extrakty tak, aby jejich konečná koncentrace ve film-formujícím roztoku byla 20%. Film-formující roztoky byly následně ponechány uschnout v Petriho miskách do formy obalu a použity pro další labratorní analýzy.

U vzniklých obalů byla analyzována tloušťka za použití mikrometru, pružnost obalů za použití texturometru a take antioxidační aktivita, která byla provedena metodou FRAP (ferric reducing antioxidant power), kdy jako reagent byl použit octanový pufr, TPTZ a $FeCl_3$ a po 8

minutách inkubace byla změřena absorbance při 593 nm (Behbahani et al., 2017). Výsledky byly statisticky zpracovány pomocí ANOVA testu za použití programu IBM SPSS.

Výsledky a diskuze

Shrnuté výsledky stanovení antioxidační aktivity jsou uvedeny v Tabulce 1. V případě porovnání chitosanových a karagenanových obalů je hlavním zjištěním, že jsou vždy statisticky významně odlišné ($p < 0,05$). Antioxidační aktivita tedy závisí i na použití základní matrice. Stejně tak je i zajímavým poznatkem, že antioxidační aktivita byla zjištěna i u vzorků bez přídavku extraktů, kdy tyto vlastnosti jsou způsobeny u karagenanu přítomností a počtem sulfátových skupin (Benbettaïeb *et al.*, 2019) a u chitosanu přítomností dusíku na pozici C_2, který je schopen vychytávat celou řadu volných radikálů (Park *et al.*, 2004). Když se však porovná přídavek extraktů v rámci stejné matrice, pak bylo zjištěno, že u vzorků na bázi karagenanu nedošlo ke statisticky významnému zvýšení ($p > 0,05$) antioxidační vlastností, zatímco u vzorků na bázi chitosanu byl přídavek extraktu z modrého čaje i červeného zelí statisticky významně vyšší ($p < 0,05$) než u vzorku CH_L bez přídavku extraktu. Vyšší antioxidační aktivita u vzorků s extrakty je zapříčiněna přítomností anthokyanů v modrém čaji a červeném zelí, které mají antioxidační vlastnosti (Vo *et al.*, 2019; Ahmad *et al.*, 2020).

Tabulka 1: Výsledky antioxidační aktivity vyjádřené metodou FRAP

Vzorek	FRAP (µmol Trolox/g)
KAR	$0,52 \pm 0,02^{a}$
CH_L	$0,73 \pm 0,02^{b}$
$CH_{LMČ}$	$1,46 \pm 0,06^{cd}$
$KAR_{MČ}$	$0,84 \pm 0,08^{ab}$
$CH_{LČZ}$	$1,26 \pm 0,04^{c}$
$KAR_{ČZ}$	$0,85 \pm 0,08^{ab}$

*rozdílná písmena v horním indexu znázorňují statisticky významné rozdíly mezi řádky

Fyzikální vlastnosti filmů byly analyzovány za použití měření tloušťky a pružnosti (Tabulka 2). V případě tloušťky byla zjištěna vyšší hodnota u vzorků složených z chitosanu oproti vzorkům s karagenanem. Také přídavek extraktu měl vliv na zvýšení tloušťky. U vzorků z karagenanu ale nebyly vzorky $KAR_{MČ}$ a $CH_{LMČ}$ statisticky významně odlišné ($p > 0,05$) od vzorku KAR a obdobně tomu bylo i u chitosanových obalů. U pružnosti byly vyšší hodnoty zjištěny u chitosanových vzorků, avšak po statistickém vyhodnocení nebyl mezi žádným ze vzorků zjištěn statististicky významný rozdíl ($p > 0,05$). V dřívějších výzkumech bylo zjištěno, že vliv na texturní vlastnosti má i rozdílné pH, kdy pro výrobu chitosanových obalů byl použit kyselý roztok 1% mléčné kyseliny a pro přípravu karagenanových obalů destilovaná voda (Gennadios *et al.*, 1993; Gontard *et al.*, 1992).

Tabulka 2: Výsledky tloušťky filmů a pružnosti

Vzorek	Tloušťka (mm)	Pružnost (%)
KAR	$0,09 \pm 0,01^{a}$	$96,43 \pm 2,89$
CH_L	$0,17 \pm 0,05$	$127,66 \pm 10,85$
$CH_{LMČ}$	$0,19 \pm 0,03^{b}$	$107,80 \pm 8,63$
$KAR_{MČ}$	$0,10 \pm 0,01^{ac}$	$97,77 \pm 1,61$
$CH_{LČZ}$	$0,17 \pm 0,02^{b}$	$108,94 \pm 9,05$
$KAR_{ČZ}$	$0,10 \pm 0,01^{ac}$	$102,30 \pm 3,32$

*rozdílná písmena v horním indexu znázorňují statisticky významné rozdíly mezi řádky

Závěr

U zkoumaných vzorků obalů bylo zjištěno, že největší vliv na antioxidační a texturní vlastnosti má použití polysacharidu do základní matrice. Nejvyšší antioxidační aktivitu vykazoval vzorek $CH_{LMČ}$. U měření tloušťky byla nejvyšší hodnota zaznamenána u vzorku $CH_{LMČ}$ a u pružnosti bylo zjištěno, že pružnější jsou chitosanové obaly než obaly karagenanové. V rámci dalšího výzkumu mohou být zkoumány změny vlastností při přidání přírodních extraktů při vyšších koncentracích.

Poděkování

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Vliv přítomnosti pylu na bioaktivní vlastnosti medů

Effect of pollen on the bioactive honey properties

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Summary

There are numerous pollen grains in each honey. It can provide essential information on the botanical and geographical origin of honey. However, the presence of pollen can also be important in the evaluation of antioxidant properties. In this work, honey samples from hobby beekeepers from the 2020 season, with a known pollen profile, were evaluated for the content of total polyphenols and antioxidant activity. A statistically significant difference ($p < 0.05$) was found between the pollen (P) and filtered (BP) samples, both in terms of total polyphenols and in terms of antioxidant activity. Furthermore, a statistically highly significant correlation ($p < 0.01$) was found between the content of total polyphenols and antioxidant activity.

Key words: *96-well microplate method, total polyphenols, ABTS*

Úvod

Med patří mezi přírodní sladidla. Každý med obsahuje určitý podíl pylových zrn, zejména pokud byl získán z pylodárných druhů rostlin. Obsah pylu a konkrétní druh může pomoci při hodnocení botanického a geografického původu medu. Některé studie, které prokazují pozitivní účinky medu na zdraví konzumenta, spojují tyto účinky právě s obsahem pylu, který se v medu nachází. (Cianciosi et al., 2018, Giorgi et al., 2011). Chemické složení pylu závisí především na jeho botanickém původu. Hlavní složky obsažené v pylu jsou bílkoviny, aminokyseliny, lipidy a cukry. Minoritně zastoupené složky jsou rozmanitější, jedná se o flavonoidy, karotenoidy, vitamíny, minerály aj. (Yang et al.,2013). Právě tyto složky mohou ovlivnit antioxidační vlastnosti medu a tím i benefity jeho konzumace.

Cílem této práce bylo ověřit vliv přítomnosti pylových zrn na vybrané antioxidační vlastnosti medu.

Materiál a metodika

Medy byly sbírány přímo od včelařů a po vytočení skladovány při teplotě do 20 °C. Vzorky byly připraveny v duplikátech. Analyzovány byly vzorky standardních medů (P) a vzorky medů po membránové filtraci (BP) (n=20) s využitím vakuové filtrační soupravy (ThermoFisher, USA) s 25 mm filtry s póry o velikosti 3 μm (MF-Millipore, GER), Vzorky byly 4x zředěny.

Pro stanovení celkových polyfenolů byla použita spektrofotometrická metoda dle Folin-Ciocalteua, vycházející ze studie Zhang et al. (2006). Antioxidační kapacita (TAC) byla stanovena podle Re et al. (1999) spektrofotometricky. Obě metody byly přizpůsobeny pro mikrotitrační destičky a měření probíhalo pomocí spektrofotometru Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific Inc., USA).

Výsledky byly zpracovány a vyhodnoceny pomocí programů Unistat 6.0 (Unistat Ltd., GB) a XLSTAT 2021 (Addinsoft, USA). Pro vyhodnocení byl použit Pearsonův korelační koeficient a Kruskal–Wallis test.

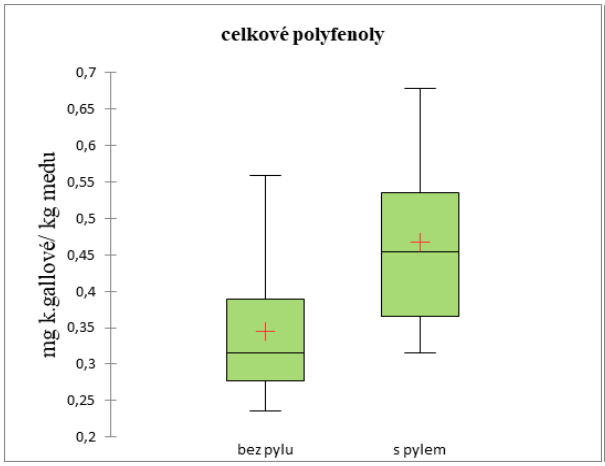
Výsledky a diskuse

Polyfenoly hrají zásadní roli v hodnocení medu jako potraviny s příznivými zdravotními účinky, jejich vysoký obsah je tedy žádoucí (Jibril et al., 2019, Giorgi et al., 2011). Celkový obsah polyfenolů v medu závisí zejména na vnějších faktorech, jako je doba sběru nektaru,

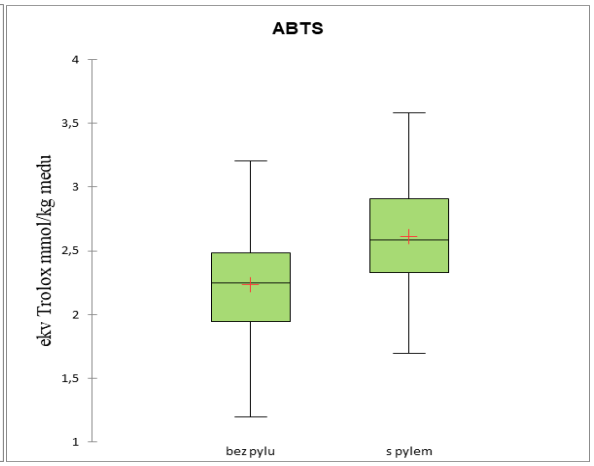
vytáčení a celkové klimatické podmínky. Tyto faktory způsobují také velkou diverzitu pylových zrn v medu. Například Kostic (2019) ve své studii prokázal vysoké koncentrace celkových polyfenolů i vysokou antioxidační aktivitu ve vzorku pylu izolovaném ze slunečnicového medu.

V grafu č. 1 lze vidět, že skupina vzorků s přirozeným obsahem pylu (P) měla celkový obsah polyfenolů výrazně vyšší než skupina vzorků bez pylu (BP). Mezi skupinami BP a P byl prokázán statisticky významný rozdíl *(p <0,05)*. Medián pro skupinu BP byl 0,315 mg k. gallové/kg medu, zatímco u skupiny P byl medián 0,454 mg k.gallové/kg medu.

Celková antioxidační aktivita vyjadřuje kapacitu vzorku eliminovat volné radikály. Stejně jako polyfenoly, je TAC u medu ovlivňována zeměpisnou a botanickou lokalitou, ze které med pochází. (Khalil et al.,2010). Rozdíly ve skupinách vzorků BP a P jsou patrné také z grafu č. 2. Při měření TAC metodou ABTS byla ve skupině vzorků BP hodnota mediánu 2,248 mmol T.ekv/kg medu, zatímco pro skupinu P to bylo 2,585 mmol T.ekv/kg. Mezi oběma skupinami byl prokázán statisticky významný rozdíl *(p <0,05)*.



Graf č.1: Celkové polyfenoly



Graf č.2: Celková antioxidační aktivita

Již v dřívějších studiích byla prokázána významná korelace mezi obsahem polyfenolických látek a TAC (Cianciosi et al., 2018, Khalil et al.,2010, Bertoncelj et al, 2007). I v naše práce potvrzuje, že obsah celkových polyfenolů s TAC koreluje (tabulka č. 1).

Tabulka č.1: Korelační koeficient použitých metod

	TP	***TAC***
TP		*0,6907**
TAC	*0,6907**	

*Vysvětlivky: Významnost *p<0,01*

Závěr

V této práci byl hodnocen vliv obsahu pylu na celkový obsah polyfenolů a antioxidační aktivitu. Ačkoliv může mít pyl v medu alergizující potenciál, jeho přítomnost v této potravině může zároveň zvyšovat jeho zdravotní benefity, a to z hlediska obsahu bioaktivních látek. Je zřejmé, vždy záleží na konkrétním druhu medu i jeho původu, avšak můžeme říci, že pyl je přínosná součást medu a pro zachování těchto vlastností by neměl být cíleně odstraňován.

Poděkování
Tato práce vznikla za podpory Ministerstva zemědělství v rámci projektu ZEMĚ číslo QK1920344.

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SEKCE 2

Chov zvířat, výživa zvířat a biochemie

In vitro anti-stafylokokový účinek kombinace gentamicinu a pyrithion zinku

In vitro anti-staphylococcal combinatory effect of gentamicin and zinc pyrithion

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Summary
The objective of the study was to evaluate antimicrobial interactions between gentamicin (GEN), an antibiotic commonly used in veterinary medicine, and zinc pyrithion (ZnP) against Staphylococcus aureus strains. In vitro antimicrobial effect of GEN and ZnP was evaluated towards 7 strains of S. aureus by broth chequerboard method. Results show synergistic effect against all MSSA strains only at one concentration of tested substances. In the case MRSA, the combinatory effect of tested substances was not tested due to insolubility at high concentrations.

Keywords: *Staphylococcus aureus; bacterial resistance; antibiotics; interactions*

Úvod

Mikrobiota kůže a sliznic zvířat sestává z řady mikrobiálních společenstev zahrnujících mimo jiné i bakterie rodu *Staphylococcus* spp. (Grice & Segre 2011). Oportunitní patogen *Staphylococcus aureus* je grampozitivní nesporulující mikroorganismus tvořící koky, který může v případě dysbiózy způsobovat závažné lokální či systémové infekce v organismu člověka či zvířete (Cogen a kol. 2010). Meticilin-rezistentní *S. aureus* (MRSA) patří mezi klinicky významné patogeny rezistentní na většinu antibiotik ze skupiny β-laktamů (Yang a kol. 2016). Kombinační terapie je jedním z řešení, jak bakteriální rezistenci předcházet.

Stále více jsou diskutovány látky přírodního charakteru, jakožto alternativy komerčních antibiotik, pro použití v chovech hospodářských zvířat. Například zinečnatý chelát N-hydroxy-2-pyridinethionu, pyrithion zinek (ZnP), který vznikl odvozením od aspergilové kyseliny, vykazuje široké spektrum antimikrobiální aktivity proti řadě plísní i bakteriálních patogenů (Shaw a kol. 1950). Lze předpokládat, že *in vitro* testováním ZnP v kombinaci s vybranými antibiotiky by se mohlo docílit synergického účinku a zajistit tak inhibici patogenních mikroorganismů pomocí významně nižší použité koncentrace, což byl cíl této studie.

Materiál a metody

Bakteriální kmeny a kultivační podmínky

K posouzení antibakteriálních vlastností GEN a ZnP bylo testováno sedm kmenů *S. aureus*, konkrétně šest meticilin-senzitivních kmenů SA (ATCC 29213, CCM 885, CCM 2022, CCM 2773, CCM 4516 a DSM 6732) a jeden meticilin-rezistentní kmen SA (ATCC 43300). Zdroje bakteriálních kmenů byly následující: CCM, Česká sbírka mikroorganismů (Brno, Česká republika) a ATCC, American Type Culture Collection (Manassas, USA). Všechny bakteriální kmeny byly kultivovány a udržovány ve vhodném růstovém médiu (Oxoid Inc., Basingstoke, Hampshire, UK) při teplotě 37 ˚C po dobu 24 hodin za aerobních podmínek.

Testované látky
GEN (100 %) a ZnP (100 %) byly zakoupeny od společnosti Sigma-Aldrich (Praha, ČR). Testované látky byly rozpuštěny v odpovídajících rozpouštědlech na počáteční koncentraci stanovenou podle tabulek CLSI (2015), čímž byly vytvořeny zásobní roztoky všech zkoušených substancí.

Stanovení minimálních a frakčních inhibičních koncentrací testovaných látek
Pomocí *in vitro* mikrodiluční bujónové metody v 96-ti jamkových mikrotitračních destičkách byly pomocí metodiky CLSI (2015), upravené dle poznatků Cos a kol. 2006, stanoveny minimální inhibiční koncentrace (MIK) testovaných látek vůči všem výše zmíněným kmenům *S. aureus*. Prostřednictvím mikrodiluční šachovnicové metody byl následně testován antibakteriální kombinační efekt kombinace ZnP a GEN. Mikrotitrační destičky byly inokulovány bakteriální suspenzí o konečné hustotě $5*10^5$ KTJ/ml a inkubovány při 37 °C po dobu 24 hod.

Nárůst organismů v médiu byl hodnocen měřením zákalu v jednotlivých jamkách pomocí Infinite® 200 PRO Microplate Reader (Tecan, Švýcarsko) při vlnové délce 405 nm. Účinky kombinací antibiotika a pyrithion zinku byly následně určeny podle hodnoty indexů frakčních inhibičních koncentrací (FIK), které byly vypočítány dle vzorce:

$$FIK_{AB} = \frac{MIK_A\ (kombinace)}{MIK_A\ (samostatná)} + \frac{MIK_B\ (kombinace)}{MIK_B\ (samostatná)}$$

Dle hodnot indexu FIK lze definovat tři různé způsoby interakce (Odds 2003, Kalan & Wright 2011): synergie: FIK ≤0,5; indeference: FIK 0,5≤4; antagonismus FIK >4.

Výsledky
Výsledné MIK, jakožto aritmetické průměry hodnot získané z experimentů, jsou uvedeny v Tabulce 1. Naměřené MIK ZnP se pohybovaly v rozsahu 0,25 - 1,00 µg/ml. U kmenů MSSA dosahovaly MIK GEN hodnot od 1,00 do 2,00 µg/ml. V případě MRSA byla MIK GEN stanovena >256 µg/ml. Vyšší koncentrace GEN nemohla být testována, a to z důvodu nerozpustnosti tohoto antibiotika při vyšších koncentracích. Kmen SA ATCC 43300 tedy nebyl v rámci kombinačního účinku testován.

Výsledný kombinační efekt ZnP s GEN proti kmenům *S. aureus* stanovený mikrodiluční metodou je uveden v Tabulce 1. U všech MSSA kmenů byl pozorován synergický efekt ZnP a GEN, nicméně pouze u jedné z koncentrací látek (0,25 µg/ml).

Tabulka 1: Výsledný kombinační efekt ZnP s GEN vůči kmenům *S. aureus*

	MIK testovaných látek (µg/ml):		ZnP + GEN, koncentrace (µg/ml):							
			0,5		0,25		0,125		0,0625	
Kmen *S. aureus*	MIK GEN	MIK ZnP	MIK GEN	FIK	MIK GEN	FIK	MIK GEN	FIK	MIK GEN	FIK
ATCC 29213	1	0,5	0,02	0,57	0,11	**0,46**	0,63	0,59	1,21	0,61

ATCC 43300	>256	0,5	-	-	-	-	-	-	-	-
CCM 885	1	1	0,03	0,51	0,18	**0,48**	0,42	0,62	0,67	0,955
CCM 2022	1	1	0,02	0,69	0,11	**0,45**	0,44	0,61	1	1,14
CCM 2773	2	0,5	0,02	0,85	0,09	**0,47**	0,54	0,52	1,58	1,15
CCM 4516	1	0,5	0,02	0,56	0,12	**0,47**	0,64	0,60	1,33	0,66
DSM 6732	2	1	0,09	0,56	0,35	**0,45**	1,44	0,96	2	1,23

Diskuze

V posledních letech nebyla provedena žádná komplexní studie kombinačního účinku ZnP s antibiotickými látkami. Je však známo, že pro úspěšnou implementaci kombinační terapie látek je důležitá znalost MIK, ale především znalost mechanismu účinku určující charakter konkrétní látky (Basri a kol. 2014).

ZnP se považuje za látku baktericidního charakteru, jejíž mechanismus účinku spočívá v narušení buněčné membrány prostřednictvím degradace fosfolipidů (Dinning a kol. 1998). Konvenční antibiotikum běžně využívané ve veterinární medicíně, GEN, patří stejně jako ZnP mezi substance se smrtícím účinkem na dělící se bakteriální buňky. Antibakteriální mechanismus účinku GEN spočívá ve vazbě na 30S ribozomální podjednotku, čímž indukuje inhibici syntézy proteinů, a bakteriální buňka následně zaniká (Yoshizawa 1998).

Vazbou aktivních hydroxylových či aminových skupin GEN mohou vznikat komplexy s kovy, což může omezit jeho biologickou aktivitu. Kombinace ZnP a GEN může zvýšit baktericidní aktivitu obou látek. Lze usuzovat, že mechanismus vedoucí ke zvýšené baktericidní aktivitě kombinace ZnP a GEN může souviset s produkcí reaktivních forem kyslíku ZnP pod vlivem GEN, což způsobí oxidativní stres uvnitř buňky (Wang a kol. 2016). Volné radikály mohou přímo působit na polynenasycené mastné kyseliny přítomné v membránách bakteriálních buněk a zahájit tak lipidovou peroxidaci. Primárním účinkem peroxidace lipidů je změna vlastností membrány, která může významně poškodit proteiny vázané na membránu (Humpries & Sweda 1998).

Ze zjištěných skutečností se lze domnívat, že kombinace baktericidních látek, ZnP a GEN, může vést k synergickému účinku.

Závěr

Tento výzkum prokázal, že kombinace ZnP a GEN vykazuje v rámci jedné koncentrace synergickou aktivitu. Společné použití těchto látek v příslušných koncentracích tak může značně eliminovat šíření rezistentních kmenů bakterií *S. aureus* jak v lidské populaci, tak v chovech zvířat.

Poděkování

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Biochemical parameters in saliva as a marker of workload in horses

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Summary
This study shows the results of salivary markers of 40 (20 recreational, 20 sport) horses of different age and breeds. Biochemical markers such as α-amylase and lactate were amended with lipid peroxidation to determine stress level during exercise. Positive correlation (p<0,01) was shown in α-amylase at recovery phase after workload in sport horses, but also in the beginning of exercise. Whereas recreation horse group needs more time to recover, it may be due to lack of adaptability of organism to higher demands. All of the used methods mutually showed statistically significant correlation (p<0.01) so evidently, they are useful markers of disruption of well-being in horses. Increase activity of hypothalamic-pituitary-adrenal axis that was launched, determined as increased α-amylase, potentially means that horses were expected higher demands that can fulfil. Our findings show that the methods are applicably for analysis of stress and disrupted welfare.

Key words: *equine; oxidative stress; welfare, non-invasive*

Introduction

Stress response in horses can be evaluated by different markers, such as change of behaviour, but also physiological changes. In current time, non-invasive sampling methods for stress evaluation are favoured (Lindner et al., 2000). Biochemical markers can define stress and excessive workload more accurately (Ayala et al., 2012; Contreras-Aguilar et al., 2018). For better understanding of poor physiology in stressful event or living condition in organism is possible to add the analysis of α-amylase, lactate and oxidative stress in horse saliva. The disruption of organism during excessive stressful events and exercise are defined on the level of damage of cell proteins, lipids and also nucleic acids due to effect of free radicals (Avellini et al., 1999; Wang et al., 2015). Oxidative stress is complex of disruption of organism by free radicals. In this study we can associate changes with excessive stress and inappropriate workload with lipid peroxidation. The aim of the study is use of non-invasive sampling methods to analyse stress by comparison of oxidative stress and commonly used biochemical biomarkers.

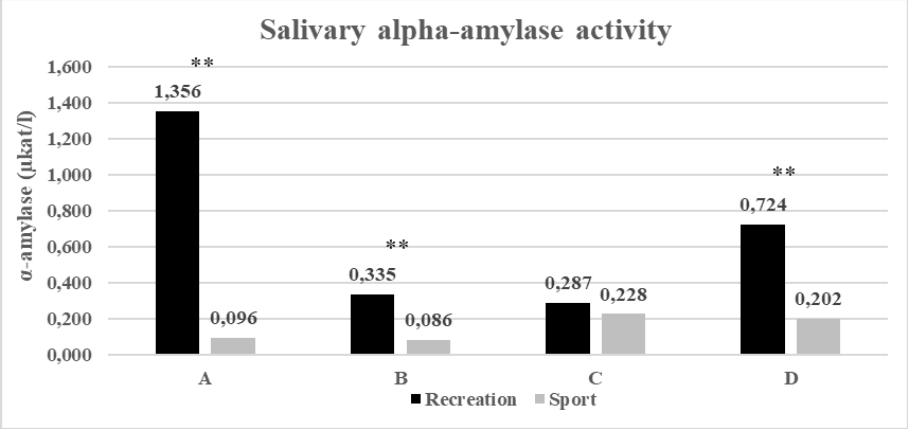
Materials and methods

Totally 40 horses (20 recreational and 20 sport horses) were used in this study: 22 mares and 18 geldings of various breeds, in age from 4 to 28, all of them was clinically healthy. All horses were stabled and fed a commercial horse pellets twice a day and hay and water were given ad libitum. Saliva were obtained from horses during their typical workload, such as recreation horses were ridden in the countryside for 1 hour and sport horses competed in show jumping competition. All samples were obtaining 15 minutes before riding (A), closely before riding (B), immediately after riding (C) and 15 minutes after riding (D). Saliva were collected by special collected device Salivette (Sarstedt, Germany). Subsequently the samples were centrifuged at 10.000 rot./10 min and stored at -80°C until analysis. Saliva were analysed for biochemical parameters such as α-amylase (sAA) and lactate by commercial kits BioVendor (BioVendor Laboratorní medicína, Brno) using biochemical analyser Thermo Scientific INDIKO (Thermo Fisher Scientific Inc., USA). Determination of oxidative stress via measuring of lipid peroxidation (TBARS) in blood (Okhawa et al., 1979) modified to saliva

and measured spectrophotometrically by Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific Inc., USA). All parameters were measured in duplicate. Results were statistically analysed (Unistat) and checked for normality using Shapiro-Wilk normality test. Tested biomarkers without normality distribution were consequently tested on non-parametric Tukey-HSD test. Pearson-Spearman-Kendall correlation was used for comparison of tested methods. Tested variables were considered as statistically significant on the level 0,01.

Results

Activity of sAA showed highly significant differences ($p<0.01$) in tested groups A, B and D between recreation and sport horse groups. Activity of sAA between recreation and sport horse in group C (immediately after exercise had no statistical significance ($p>0,01$) (Fig 1).



***Statistically significant differences (p<0.01) between the recreation and sport groups*

Figure 1: Comparison of salivary α-amylase activity in groups of recreation and sport groups of horses

Correlation of tested variables (sAA, lactate and TBARS) was statistically significant ($p<0.01$) in all used methods. Positive correlation was seen between sAA and lactate while between sAA was negative. Significant correlation of lactate was positive between sAA just as TBARS (Tab 1).

Table 1: Correlation coefficient of used methods (Pearson correlation test)

	AMYLASE	LACTATE	TBARS
AMYLASE		0,025**	-0,001**
LACTATE	0,025**		0,110**
TBARS	-0,001**	0,110**	

Discussion

Changes in various stress markers were detected in saliva due to the saliva components vary in excretion during stressful event (Rubio et al., 2009; Andriichuk et al., 2015). Relatively stable results of oxidative stress in sport horses can indicate that appropriate training of horse can be really beneficial (Avellini et al., 1999). Appropriate aerobic training can improve physiological condition (Stevanovic Jelka et al., 2013; Alandrovič et al., 2018), so it can be linked with lower levels of sAA in sport horse group in this study. Decreased level of response of hypothalamic-pituitary-adrenal axis might show us that experienced horses are less stress during higher workload (Marc et al., 2000) and the antioxidant mechanism is capable to fulfil higher demand of organism (Puzio et al., 2017) which correspond with our result. Even in recreation horse group was seen persisting higher level of sAA in all times of

sampling except C group. Possible meaning is that the horses are not trained sufficiently and it might indicate raised activity of hypothalamic–pituitary–adrenal axis (Marc et al., 2000; Contreras-Aguilar et al., 2018) compared to expectations of well-known events in horses.

Conclusion

The outcome of this study was that there is a great possibility to use saliva as non-invasive sample for biochemical assessment of stress and disrupted welfare. Selected parameters indicate that the appropriate training of horses is beneficial and when the optimal living condition and welfare are fulfilling, higher workload for horses is not inconvenient. These parameters can be amended by evaluation of behavioural changes of each individual to better understanding of good welfare of horses in future.

Acknowledgment

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Produkční účinnost diet na bázi lupinového šrotu z odslupkovaných lupinových semen ve výkrmu kuřat a kachen

Feed efficacy of lupine meal-based diets from dehulled white lupine seeds in chick and duck fattening

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Summary
This thesis presents the results of 50% and 100% replacement of soybean extraction meal with dehulled white lupine (Zulika variety) seed meal in feed mixtures for production performance and health of fattened chicks and ducks. The following chick performance parameters have been reached when using the dehulled form of lupine: for slaughter weight (day 35 of fattening) in control K0% 2.33 kg, in experimental groups P50% 2.42 kg and P100% 2.35 kg; with feed mixture conversions of 1.39 kg, 1.39 kg and 1.43 kg and an overall mortality of 7.5%, 5.0% and 2.5%. The following duck performance parameters have been reached when using the dehulled form of lupine: for slaughter weight (day 42 of fattening) in control K0% 3.22 kg, in experimental groups P50% 3.40 kg and P100% 3.47 kg; with feed mixture conversions of 1.95 kg, 2.04 kg and 1.98 kg and an overall mortality of 5.9%, 2.9% and 1.5%. The results of experiments provide evidence of a positive effect of lupine diets on the production performance and health of fattened chicks and ducks. The 50% replacement of soybean extraction meal with lupine meal appears to be optimal.

Keywords: *Feed mixtures; white lupine; broiler; duck; live mass; conversion; mortality*

Úvod

V současné době se ve výživě hospodářských zvířat hledají, nejen v ČR, ale i v rámci Evropy, alternativní zdroje tuzemských proteinových krmiv, které by v krmných směsích alespoň částečně nahradily dovozová proteinová krmiva, především sójové extrahované šroty, které jsou prozatím dominantní proteinovou komponentou pro výrobu krmných směsí. Tyto alternativní proteinové komponenty by měly kvantitativně i kvalitativně v krmných směsích nahradit částečně, případně úplně, sójové extrahované šroty, s cílem zachování vysoké užitkovosti, dobrého zdravotního stavu zvířat a vysoké kvality animálních produktů. Zájem o perspektivní využití lupinových semen ve výživě zvířat potvrzují i literární zdroje, např. Suchý et al. (2017), Geigerová et al. (2017) nebo Karel et al. (2017).

Materiál a metody

Dílčím cílem předložené práce bylo na základě biologického experimentálního sledování ověřit vliv náhrady sójového extrahovaného šrotu šrotem lupinovým. Byly testovány kompletní krmné směsi pro výkrm brojlerových kuřat a brojlerových kachen. Pro biologické testování byla sestavena kontrolní krmná směs (K0%) na bázi sójového extrahovaného šrotu a pokusné krmné směsi, kde byla provedena 50% (P50%) a 100% (P100%) náhrada hrubého proteinu sójového extrahovaného šrotu lupinovým. Kompletní krmné směsi (kontrolní i pokusné) měly stejné komponentní složení, vyjma výše uvedené náhrady. Pokusy byly realizovány v akreditované stáji FVHE s řízeným světelným, teplotním a krmně technologickým režimem, podle technologického návodu pro výkrm příslušného hybrida, tzn. u brojlerových kuřat - ROSS 308, u brojlerových kachen - Cherry Valley. Brojlerová kuřata byla sledována 1., 13., 28 a 35. den jejich věku; brojlerové kachny byly sledovány 1., 10., 21., 35. a 42. den jejich věku. Průběžně byla sledována živá hmotnost individuálním vážením, konverze krmných směsí a zdravotní stav zvířat jejich úhynem.

Výsledky byly zpracovány matematicko-statistickými metodami (program Unistat 5.6), za použití testu Tukey-HSD. Soubory jsou charakterizovány průměrnou hodnotou ($\overline{x}$) a směrodatnou odchylkou (± SD). Rozdíly byly testovány na hladině významnosti $P \leq 0,01$.

Výsledky a diskuse

○ ***Biologické sledování na brojlerových kuřatech***

Výsledky testace pokusných diet s 50% a 100% náhradou sójového extrahovaného šrotu lupinovým dokládají pozitivní vliv lupiny na živou hmotnost kuřat ve 35. dnu výkrmu, jak dokumentuje tabulka 1. Nejvyšší průměrnou živou hmotnost na konci výkrmu dosáhla pokusná kuřata, kterým byla podávána krmná směs s 50% náhradou sójového šrotu lupinovým. Přesto, že kuřata pokusných skupin dosáhla na konci výkrmu vyšší průměrnou živou hmotnost, ve srovnání s kontrolou, nebyly rozdíly mezi průměry skupin testovány jako statisticky významné. Námi dosažené výsledky jsou ve shodě Geigerová et al. (2017).

Tabulka 1: Průměrná živá hmotnost (kg) brojlerových kuřat ve 35. dnu výkrmu (n - počet, $\overline{x}$ - aritmetický průměr, SD - směrodatná odchylka).

Živá hmotnost	n	$\overline{x}$	SD
K 0%	74	**2,33**	0,335
P 50%	76	**2,42**	0,278
P 100%	78	**2,35**	0,266

Jako vysoce pozitivní lze hodnotit výsledky o úhynu kuřat v průběhu výkrmu, jak uvádí tabulka 2. U pokusných skupin byl zaznamenán nižší úhyn oproti kontrole. Dokonce s vyšší náhradou sójového šrotu lupinovým, výrazně klesal úhyn. Výše úhynu je jeden z významných faktorů, který ovlivňuje celkovou ekonomiku výkrmu.

Tabulka 2: Úhyn kuřat v průběhu výkrmu (1., 13., 28. a 35. den).

Úhyn	1.	úhyn	%	13.	úhyn	%	28.	úhyn	%	35.	úhyn	%
K0%	80	**0**	0,0	76	**4**	5,0	75	**5**	6,2	74	**6**	7,5
P50%	80	**0**	0,0	80	**0**	0,0	79	**1**	1,2	76	**4**	5,0
P100%	80	**0**	0,0	80	**0**	0,0	80	**0**	0,0	78	**2**	2,5

Výsledky sledování konverze krmných směsí u kuřat v průběhu výkrmu dokládají, že u pokusných skupin konverze krmných směsí u kuřat pokusných skupin byla buď stejná u skupiny s 50% náhradou, nebo jen nepatrně vyšší u skupiny se 100% náhradou, ve srovnání s kontrolou. Námi dosažené výsledky jsou ve shodě Suchý et al. (2017).

○ ***Biologické sledování na brojlerových kachnách***

Výsledky testace pokusných diet s 50% a 100% náhradou sójového šrotu lupinovým dokládají pozitivní vliv lupiny na jatečnou hmotnost kuřat ve 42. dnu výkrmu, jak dokumentuje tabulka 3. Výsledky prokázaly, že kachny pokusných skupin (P50% a P100%) dosáhly vyšší průměrnou hodnotu živé hmotnosti na konci výkrmu (42. den) ve srovnání s kachnami kontrolní skupiny (K0%). Mezi průměrnou hodnotou živé hmotnosti kachen kontrolní skupiny, jak dokumentuje tabulka 3, a průměrnými hodnotami živé hmotnosti kachen pokusných skupin byly rozdíly mezi průměry testovány jako vysoce významné ($P \leq 0,01$). Výsledky jsou dokladem pozitivního vlivu lupiny v dietě na růstovou intenzitu kachen v průběhu výkrmu. U pokusné skupiny se 100% náhradou (P100%), ve srovnání s 50% náhradou (P50%), byla dosažena nižší průměrná hmotnost kachen. Rozdíly mezi průměry pokusných skupin byly statisticky nevýznamné.

Tabulka 3: Průměrná živá hmotnost kachen (kg) ve 42. dnu výkrmu (n - počet, $\bar{x}$ - aritmetický průměr, SD - směrodatná odchylka, AB P ≤ 0,01).

Živá hmotnost	n	$\bar{x}$	SD
K 0%	64	**3,22^{B}**	0,279
P 50%	66	**3,40^{A}**	0,268
P 100%	67	**3,47^{A}**	0,279

Obdobné výsledky jako u kuřat byly potvrzeny i po podávání diet na bázi lupinového šrotu u kachen, a to, že s obsahem lupiny v dietě klesal úhyn v průběhu výkrmu i u kachen, jak uvádí tabulka 4. Nejvyšší úhyn byl potvrzen u kachen kontrolní skupiny (K0%), nižší u skupiny s 50% náhradou (P50%) a nejnižší u pokusné skupiny se 100% náhradou (P100%). Námi dosažené výsledky jsou ve shodě Karel et al. (2017).

Tabulka 4: Úhyn kachen v průběhu výkrmu (1., 10., 21., 35. a 42. den).

Úhyn	1.	úhyn	%	10.	úhyn	%	21.	úhyn	%	35.	úhyn	%	42.	úhyn	%
K0%	68	**0**	0,0	67	**1**	1,5	66	**2**	2,9	64	**4**	5,9	64	**4**	5,9
P50%	68	**0**	0,0	68	**0**	0,0	68	**0**	0,0	68	**0**	0,0	66	**2**	2,9
P100%	68	**0**	0,0	68	**0**	0,0	68	**0**	0,0	68	**0**	0,0	67	**1**	1,5

Výsledky sledování konverze krmných směsí u kachen v průběhu výkrmu dokládají, že u pokusných skupin kachen byla konverze krmných směsí jen nepatrně vyšší u skupiny se 100% i 50% náhradou, ve srovnání s kontrolou. Námi dosažené výsledky jsou ve shodě Karel et al. (2017).

Závěr

Obecně lze konstatovat, že na základě dosažených výsledků, lze doporučit využití lupin v krmných směsích určených pro výkrm kuřat a kachen. Jako optimální se jeví v krmných směsích 50% náhrada sójového šrotu lupinových. Pozitiva využití lupin v krmivech: snížení použití dovozových extrahovaných šrotů (v souladu se zemědělskou politikou); využití možnosti použití tuzemských proteinových krmiv ve výživě zvířat; současně pěstované odrůdy lupin nepatří mezi geneticky modifikované organismy; v krmných směsích zvyšují jejich produkční účinnost; zlepšují zdravotní stav zvířat, hodnoceno na základě nižších úhynů.

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Iron level in serum of dogs is not affected by diet, age, and sex

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Summary
The objective of this study was to evaluate impact of diet, age and sex on iron serum levels in dogs. Iron was measured in serum of 39 animals. Only clinically healthy dogs with no changes in biochemistry screening (ALT, ALP, AST, urea, creatinine, total protein, albumin) were included. Data about age, breed, sex and diet were obtained from owners. Mean serum iron level was 30.2 µmol/l (range 13.3 – 62.8 µmol/l), which is in agreement with previous studies. There was no statistically significant difference between sex, age or diet fed to the dogs.

Keywords: *nutrition; canis; commercial food, home-made food*

Introduction

Iron is a microelement essential for a dog. It serves as a cofactor of many enzymes. Also, it is important for binding and transport of oxygen, cellular respiration, transport of electrons, DNA synthesis, cell proliferation and differentiation, gen regulation, and more (Pantopoulos et al., 2012).

Most of the iron in dogs' body is contained as hemoglobin in erythrocytes. In serum, there is less than 0.1% of the total iron. Even so, iron depletion manifests with low serum levels. Apart from iron deficiency, we can encounter low serum levels in dogs with renal disease, inflammatory diseases, hypothyroidism or hypoproteinemia. Elevated levels can be caused by liver disease or corticoid therapy (Bohn, 2013). It is important to take all these factors into consideration.

The total body iron is regulated by its intake – by amount of iron absorbed in enterocytes. It is the only way, since there are no possibilities of controlling iron levels by its excretion (Bohn, 2013). Deficiency can be caused either by inadequate diet of chronical blood loss and can lead to anemia (Naigamwalla et al., 2012). In Europe, manufactures of diets for dogs follow Nutritional Guidelines set by The European Pet Food Industry Federation (FEDIAF) to ensure the diet covers sufficient intake of iron. The minimal recommended amount of iron is 4.17 mg/100 g DM (dry matter) of diet for MER (maintenance energy requirement) 95 kcal/kg^{0,75} and 3.60 mg/kg DM for MER 110 kcal/kg^{0,75} for adult dogs (FEDIAF, 2020). That means healthy dogs fed commercial diets should not develop iron deficiency. Situation can be different with dogs fed home-made food. Pedrinelli et al. (2019) analyzed 75 recipes for home-made food, meant for healthy adult dogs, and found that 56% (44 recipes) had iron content below FEDIAF recommendation. The objective of this study was to evaluate impact of diet, age, and sex on iron levels in serum of dogs.

Materials and methods

The blood samples were obtained by privet veterinarians from clinically healthy dogs who came to their practices for preventive blood tests, usually before undergoing surgery (most commonly sterilization) or as a part of geriatric or other screening. Part of the obtained blood samples (usually 1 ml) was collected into tube with no additives and after at least 20 minutes in room temperature centrifuged. Obtained serum was frozen at -80°C until analysis.

Fifty-one dogs were included in this study, and after biochemistry examinations (ALT, ALP, AST, urea, creatinine, total protein and albumin – data not included), dogs with elevation in any of these parameters were excluded from the study. Thirty-eight samples remained. The dogs were of various breeds and ages. The most common were mixed breed dogs (12), followed by Belgian Shepherd (3), and Labrador Retriever (3). Other breeds were represented by one or two dogs each only.

The owners of the dogs signed informed consent and filled anamnestic questionnaire, answering questions about age, sex, breed and diet. According information given by owners, dogs were divided into 3 groups – dogs fed more than 75% commercial dog diet (CD), dogs fed more than 75% home-made diet (HD), and dogs fed combination of both (CHD). All biochemistry parameters and serum iron were analyzed by photometry, using clinical chemistry analyzer Indiko^{TM}. For statistical analysis, one-factor analysis of variance ANOVA was used. We assessed the level of significance $P \leq 0.05$. The statistical analysis was performed by UNISTAT Version 6.0.

Table 1: Dogs included in the study

CD – fed more that 75% commercial diet, HD fed more that 75% home-made diet, CHD – fed combination of commercial and home-made diet

				age (years)			
	all dogs	**male**	**female**	**≤ 2**	**2 - 6**	**6 - 12**	**≥12**
all dogs	39	17	22	7	18	10	4
CD	18	9	9	3	7	7	1
HD	11	5	6	1	7	0	3
CHD	10	3	7	3	4	3	0

Results

The mean value of serum iron was 30.2 µmol/l (range 13.3 – 62.8 µmol/l) with median of 29.1 µmol/l. All results are summarized in table 2. There was no statistically significant difference between iron serum levels and age, sex and diet groups.

Table 2: Results – serum iron levels (µmol/l)

CD – fed more that 75% commercial diet, HD fed more that 75% home-made diet, CHD – fed combination of commercial and home-made diet, SD – standard deviation, n – number of dogs

		diet			**sex**		**age**			
	all dogs	**CD**	**HD**	**CHD**	**male**	**female**	**≤ 2**	**2 - 6**	**6 - 12**	**≥12**
mean	30.2	32.8	30.3	27.8	33.0	29.1	29.7	30.4	29.7	34.6
SD	10.1	9.6	11.3	8.5	11.8	8.0	15.4	8.4	8.5	14.1
range	13.3 – 62.8	20.0 - 62.8	13.3 - 53.9	20.4 - 50.7	13.3 - 62.8	20.0 - 50.7	13.3 - 62.8	20.4 - 50.7	20.7 - 43.8	20.0 -53.9
n	39	18	11	10	17	22	7	18	10	4

Discussion

The mean iron serum level measured in this study (30.2 µmol/l) is in agreement with results of previous studies – Vitale et al. (2019) found median of 29.1 µmol/l in serum of 50 healthy dogs and Zaldívar-López et al. (2014) found mean level 27.5 µmol/l in a group of 55 control dogs of various breeds. The range of serum iron was very wide, not only in all dogs, but also in respective groups of dogs. However, no statistically significant difference was found between dogs of different sex, age or fed different diets. This study was limited by a small

number of dogs (39) and also by the fact, that diet information was given by owners and so it can be inaccurate.

Conclusions

Type of diet (commercial, home-made or combination of both), age and sex seem to have no impact on serum levels of iron in dogs. However, more studies, including bigger number of dogs and more accurate information about diet composition are needed to verify our findings.

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SEKCE 3

Ochrana zvířat, welfare a etologie

Dairy cow longevity on selected farms of Holstein cattle

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Summary
Longevity of dairy cows is an essential indicator of animal health, welfare and a sustainability of milk production. Study showed that Holstein cows were culled at young age (approximately 4 years and 5 months) and most often in the first three lactations (72.49% of culled cows). Such results indicate a low utilization of the production potential of a number of cows that are included in the production herd on conventional farms. Prolonging the life of dairy cows should not be the goal of farmers, but it should be the result of successful prevention of involuntary culling on farms.

Keywords: *Holstein cows; age; lactation number; culling rate*

Introduction

Longevity is a reflection of animals' ability to cope with environmental conditions. The natural lifespan for cattle is up to 20 years. However, on dairy farms, life expectancy of cows is only 25% of the recorded maximum lifespan due to management of dairy farm (Hoffman and Valencak, 2020). Life expectancy is shortened by the high intensity of production, which is characteristic of Holstein breed on conventional farms. The life of a dairy cow includes two periods. The costly first period which is from birth to first parity and following productive period which lasts from the first lactation until culling or death of animal. Longevity is such an important part of any breeding goal because it affects the overall performance of animals and thus the achieved financial profit of farmer from dairy production (Fetrow et al., 2006). For that reason, it is considered economically the most important functional trait in cattle populations. Unfortunately, it is characterized by rather low heretability, so it is necessary for farmers to focus on many factors that affect life expectancy (Zavadilová et al., 2009).
Dairy cow longevity on farm is determined by culling rate and possible deaths. In any farm, culling is an essential part in managing herd productivity (Rilanto et al., 2020). Culling decisions are the result of internal and external factors affecting animals and their longevity. Internal risk factors include deteriorated health, reduced lactation, low fertility or poor body conformation. Herd size, availability of replacement heifers or economics of dairy production on farm belong to significant external factors (De Vries et al., 2010; Hoffman and Valencak, 2020; De Vries and Marcondes, 2020).
The aim of this study was to assess the longevity of Holstein cows on selected dairy farms in the Moravian-Silesian Region. It also aimed to evaluate the average annual culling rates on farms, length of productive life of cows and number of achieved lactations of culled cows.

Materials and Methods

Dairy cow longevity was studied in 3 conventional dairy farms of Holstein cattle in the period from 2019 to 2020. Data for analysis were obtained from farm records and PLEMDAT database. Average annual culling rates were evaluated according to the annual average herd size in individual farms and number of culled cows in a given year. For comparison between individual farms, average annual culling rates for the period 2019 to 2020 was used. Dairy cow longevity was assessed according to average age, median age, the lowest and the highest age of culled cows. For comparison between individual farms, average age of all culled cows of herd was used. Length of productive life was determined on the basis of the difference between the average age of culled cows and the average age of first calving. For comparison

between individual farms, average length of productive life of herd was used. Lactation number was evaluated according to number of lactation cycles achieved by cows. Cows with different achieved lactation numbers were compared within the each farm and further compared between the all monitored farms. The results were evaluated statistically using the program Unistat 6.5 for Excel. For the purposes of statistical comparison of average age in individual farms, F-test and unpaired two sample T-test were used. For the purposes of statistical comparison of the frequency of annual culling rates and the frequency of given lactation number in individual farms, a chi-square test was used to assess statistical significance in a 2×2 contingency table.

Results and Discussion

A total of 1,945 dairy cows (farm A: 816; farm B: 460; farm C: 669) were culled from production herds in the period from 2019 to 2020. The annual culling rates of the herd in published studies varied between 20% and 40% depending on the intensity of production and herd management. The results of this study showed statistically highly signifigant differences ($p < 0.01$) in the average annual culling rates (farm A: 38.70%; farm B: 25.19%; farm C: 18.96%). Fetrow et al. (2006) reported that an annual herd turnover of less than 30% is essential for the economic prosperity of farm. However, higher annual culling rate in farm A may be conditioned by the abundance of reared heifers, which are used for more frequent herd renewal. Culling rate is determined by the number of voluntary and involuntary culling. Voluntary culling is an important tool for improving the quality of herds. Involuntary culling points to mistakes in herd management and also the presence of disease. Causes for culling will be assessed in a more detailed study because they affect the lifespan of dairy cows and point to the level of health and welfare in herds.

Dairy cows are usually culled from the herd at the moment they become unprofitable for farmer due to production diseases, reproductive inefficiency or low milk production (Zavadilová et al., 2009). Available studies point to the fact that dairy cow longevity has declined worldwide. For example, in Poland the age decreased from 6.40 to 5.43 between 2006 and 2011 (Oler et al., 2012). The results of this study showed an even lower average lifespan of cows (farm A: 52.96 months; farm B: 54.09 months; farm C: 53.26 months). Average age in all farms was 4.44 years, while no statistically significant differences between farms ($p > 0.05$) were found. According to the breed standard, the age at first calving should be in the range of 23-27 months for Holstein cattle. The average age of first calving in our farms was 24 months. However, the results showed that cows were culled also at the age of 23 months which could be caused by calving difficulties. On the other hand, the highest age of culled cows was 186 months (15.5 years). Such a high age indicates the efforts of farmers to keep in herd animals with good production indicators which is favorable in terms of economics of milk production.

Length of productive life is described in many studies. De Vries and Marcondes (2020) reported that the average productive lifespan on intensive farms in dairy cows is 2.5 to 4 years. The average length of production life in the conventional farms in this study was also low (2.44 years). The significance of higher age in terms of welfare is uncertain because of increasing risk of production diseases in older dairy cows. Therefore, a long productive lifespan may be associated with animal suffering and thus is not necessarily a sign of good welfare.

In this study was also assessed the number of achieved lactations of culled cows. It is stated that the maximum milk production is reached by cow in the 4th to 6th lactation. Frelich et al. (2010) found out that mean number of lactations decreased between 2000 and 2007 from 2.7 to 2.5 in Holstein cows on low-input farms in the Šumava. The results of this study showed that most dairy cows were culled in all farms in the 2nd and 3rd lactation (47.66%). Fewer

dairy cows were culled in the 1st lactation (24.83%) and in the 4th and 5th lactation (23.86%). The least dairy cows were culled in 6th and higher lactation (3.65%). No statistically significant differences in given lactation number were found between farms ($p > 0.05$). De Vries et al. (2010) came to the same conclusion. In their study, 37% of cows were culled in the 1st parity and 46% within the 2nd and the 3rd parities. It turned out that a large part of young dairy cows are culled and slaughtered in the early stages of production life. The lower age is unfavorable in terms of the use of production potential of reared cows which could take effect firstly in 4th lactation. Oler et al. (2012) further pointed out that there is an increasing trend of culling in cows in the first lactation. This fact should be verified in a more detailed study because the culling in the 1st lactation causes significant financial losses (Fetrow et al., 2006).

Conclusion

It should be in the interest of each farmer to keep in the herd only dairy cows with a good level of health, fertility and milk production. It turned out that the annual culling rate may vary significantly between farms, which may be related to different levels of herd health or herd management. Holstein dairy cows are culled very young, but it is obvious that healthy and highly productive cows may remain on the farm for a very long time. However, most cows are culled in the 1st three lactations, which is unfavorable in terms of using their production potential. The main farmer´s task is not to prolong the life of cows, but to focus on preventing the causes of involuntary culling. This attitude may create space for voluntary culling and for improving the herd production quality. Such a herd can also reach a higher average age. Prolong the life is important only for productive cows if their production potential is used. Too long lifespan on farm is asocciated with the risk of production diseases, which are unfavorable in terms of economy but also in terms of animal welfare.

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Ovlivnění hematologických a biochemických parametrů pstruha duhového (*Oncorhynchus mykiss*) orální aplikací mikročástic polystyrenu

Impact of oral application of polystyrene microparticles on haematological and biochemical parameters of rainbow trout (*Oncorhynchus mykiss*)

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Summary:
The main task of this study was the evaluation of the polystyrene microplastic particles effects on selected haematological and biochemical parameters of rainbow trout (Oncorhynchus mykiss) juveniles. Tested fish were divided into 4 groups - control group without the addition of microparticles in their diet and groups exposed to three different microplastic concentrations for 6 weeks. The results showed a statistically significant decrease in the number of leukocytes, specially the lymphocytes, after oral intake of polystyrene microparticles if compared to the control. Regards the biochemical parameters, ammonia, total protein and ceruloplasmin decreased significantly in plasma samples if compared to control.

Keywords: *fish; microplastics; aquatic environment; pollution*

Úvod

Znečištění životního prostředí plastovým odpadem je celosvětovým problémem, který je způsoben nadměrným používáním těchto materiálů, špatným nakládáním s odpady a jejich odolností vůči vnějšímu prostředí. Z plastových částic větších rozměrů se mohou působením vnějších vlivů uvolňovat menší částice, tzv. mikroplasty (velikost < 5 mm), jejichž přítomnost byla prokázána v tělech mnoha různých organismů, a to napříč všemi trofickými úrovněmi vodního potravního řetězce (Crawford a Quinn, 2016).
Orální příjem těchto plastových polutantů může obecně způsobovat mechanické poškození nebo ucpání trávicího traktu vedoucímu k zamezení vstřebání živin. U částic menších rozměrů byla navíc prokázána schopnost jejich prostupu střevní stěnou s následným transportem do dalších tkání, což následně může negativně ovlivňovat životně důležité procesy organismu (Cooper a Corcoran, 2010).
V předložené studii byly použity mikročástice polystyrenu s průměrnou velikostí 52,5 ± 11,5 µm (EPRUI Biotech Co. Ltd, Čína), které byly přidány exponovaným skupinám do krmiva. Hlavním cílem experimentu bylo zjištění případného negativního vlivu polystyrenových mikročástic na vybrané hematologické a biochemické parametry pstruha duhového po 6-týdenní expozici.

Materiál a metodika

V experimentu bylo použito 128 juvenilních jedinců pstruha duhového, kteří byli rozděleni do 8 nádrží po 16 kusech s napojením na recirkulační systém. Nádrže byly každých 12 hodin

kontrolovány s doprovodným měřením vodních parametrů. Test probíhal v duplikátu – dvě nádrže byly vystaveny 0,5%, 2% a 5% koncentraci mikroplastů v krmivu. Dvě nádrže sloužily jako kontrolní bez přídavku polystyrenových mikročástic do krmiva.
V průběhu experimentu, který trval 6 týdnů, byly provedeny dva dílčí odběry – po 2 a 6 týdnech. Odběr krve byl proveden z ocasní žíly heparinizovanou injekční stříkačkou, následně byly ryby omráčeny tupým úderem do hlavy a usmrceny přetětím žaberních oblouků. U každého jedince byly zjištěny biometrické údaje a při pitevním vyšetření byly odebrány vzorky tkání pro následné analýzy. Získaná data byla předložena ke statistickému vyhodnocení, kdy byla porovnána kontrolní skupina se skupinou vystavenou polystyrenovým mikročásticím.
Hematologické vyšetření probíhalo dle metodiky Svobodova a kol. (2012). Biochemické parametry krevní plazmy byly analyzovány s využitím biochemického analyzátoru Konelab 20i a komerčně dodávaných kitů (Biovendor).
Statistické zpracování dat bylo provedeno s využitím programu Unistat for Excel 6.5 a pro zjištění konkrétních rozdílů mezi testovanými skupinami byla využita analýza rozptylu a Tukey-HSD test, případně vícevýběrový mediánový test. Testování bylo provedeno na hladině významnosti $p<0,05$ a $p<0,01$.

Výsledky a diskuze

Z celého spektra hematologických a biochemických parametrů bylo zjištěno statisticky vysoce významné ($p<0,01$) ovlivnění hladiny leukocytů, lymfocytů, amoniaku, celkového proteinu a aktivity ceruloplasminu při jejich porovnání s kontrolní skupinou (Tabulka 1).

Tabulka 1: Výsledky hematologické a biochemické analýzy pstruha duhového po expozici polystyrénovým mikročásticím (průměr ± SD)

Druhý týden	Kontrola	0,5 %	2%	5%
Leukocyty (G/l)	77,3 ± 19,4	67,7 ± 28,4	60,2 ± 20,0	64,7 ± 16,5
Lymfocyty (G/l)	72,9 ± 19,6	65,6 ± 27,4	57,8 ± 19,1	61,5 ± 15,0
Amoniak (µmol/l)	310,2 ± 79,2	**189,9 ± 41,5***	234,2 ± 68,1	**202, 6 ± 33,3***
Celkový protein (g/l)	37,0 ± 2,6	**32,3 ± 2,0***	**31,4 ± 2,7***	**33,5 ± 4,5***
Ceruloplasmin (ΔA/min × 10000)	111,7 ± 17,1	**90,4 ± 10,3***	**94,3 ± 19,2***	99,4 ± 12,2
Šestý týden	**Kontrola**	**0,5%**	**2%**	**5%**
Leukocyty (G/l)	71,0 ± 27,1	**34,6 ± 14,1***	49,7 ± 21,3	53,7 ± 9,4
Lymfocyty (G/l)	68,1 ± 24,8	**32,9 ± 14,5***	47,5 ± 21,3	50,3 ± 11,3
Amoniak (µmol/l)	310,4 ± 80,9	**233,8 ± 86,2***	**193,3 ± 36,8***	**182,0 ± 54,7***
Celkový protein (g/l)	38,5 ± 4,5	37,8 ± 4,5	39,0 ± 4,3	35,5 ± 6,1
Ceruloplasmin (ΔA/min × 10000)	132,0 ± 18,8	**110,6 ± 14,5***	**105,4 ± 15,2***	**106,9 ± 14,3***

Signifikantní změny mezi kontrolou a pokusnou skupinou na hladině významnosti *$p<0,01$

Pokles leukocytů a lymfocytů může být ukazatelem zvýšeného stresu ryb vystavených xenobiotikům či negativním vlivem na lymfatické tkáně u ryb vystavených působení mikroplastů. Tyto kontaminanty mohou být leukocyty fagocytovány. Imunitní systém podléhá interakci s těmito částicemi, což v důsledku může vést k uvolňovaní toxických látek blokující trávení (Espinosa a kol., 2017). Snížení hladiny amoniaku v plasmě může souviset se specializací střeva dravých ryb na vysoký příjem bílkovin, kdy metabolizace bílkovin ve střevech vede k následnému zvýšení hlavního degradačního produktu, tzn. amoniaku v krvi. Pokud je ovšem bílkovinná složka v krmivu nedostatečná, k čemuž pravděpodobně došlo v případě suplementace krmiva vysokým procentem polystyrenu, sníží se hladina amoniaku. Snížená využitelnost proteinu z potravy v důsledku poškození trávícího traktu je i možným

důvodem poklesu celkového proteinu, což může vést až k výživovým poruchám (Nematdoost Haghi a Banaee, 2017). Druhou možností poklesu hladiny amoniaku je snížení aktivity enzymu glutamátdehydrogenázy, která je zodpovědná za metabolismus dusíkatých látek v játrech (Ip a Chew, 2010). Pro ověření této hypotézey bude provedena analýza genové exprese uvedeného enzymu. Snížení aktivity ceruloplasminu může být způsobeno případným poškozením jater na molekulární úrovni, kde je tento plazmatický protein syntetizován (Hedayati a kol., 2016).

Závěr

Cílem práce bylo posouzení vlivu krmiva s přídavkem polystyrenových mikročástic na vybrané parametry pstruha duhového. Předložená studie potvrzuje, že tyto kontaminanty životního prostředí negativně ovlivňují zdraví ryb, což bylo prokázáno signifikantními změnami ve vybraných hematologických a biochemických parametrech. Pro komplexní zhodnocení budou výsledky doplněny histologickým vyšetřením, analýzou ukazatelů oxidativního stresu a zhodnocením genové exprese.

Poděkování

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Výskyt zubního kamene u ježků západních (*Erinaceus europaeus*) v záchranné stanici – pilotní studie

Occurence of tartar in European hedgehogs (*Erinaceus europaeus*) in rescue centre – pilot study

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Summary
The European hedgehog´s teeth are at the risk of formation of tartar in case of incorrect nutrition and other factors. Severe tartar buildup may cause pain in the oral cavity or even loss of teeth and thus reduce hedgehog´s ability to feed and, subsequently, survive. The results of this pilot study suggest that younger hedgehogs suffer from tartar buildup less than older individuals, however, the statistically significant difference was not proved due to the limited number of individuals monitored. Further studies are needed to confirm this hypothesis, as are the studies on the feed components responsible for tartar formation in both wild hedgehogs and those kept in rescue centres.

Keywords: *rehabilitation; weight; health problem; oral cavity; mortality*

Úvod

Ježek západní (*Erinaceus europaeus*) je savcem vyskytujícím se po celé Evropě (IUCN Red List, 2020). Ačkoli dle Mezinárodního svazu na ochranu přírody patří mezi druhy, které nejsou přímo ohrožené vyhynutím, i přesto některé státy hlásí pokles jejich počtu (Mathews et al., 2018). Na poklesu počtu ježků se podílí celá řada faktorů, často spojených s antropogenní činností (Rautio et al., 2016).

Ježci jsou u veřejnosti poměrně populární, příčinou může být jejich ochota žít v blízkosti lidských obydlí, parků a zahrad, které pro ně skýtají řadu výhod (Williams et al., 2018). Vzhledem k této skutečnosti jsou handicapovaní ježci veřejností snadno zpozorováni a často je jim z jejich strany poskytnuta pomoc. Jedna z forem této pomoci je také přikrmování ježků, oblíbené zejména v podzimním období, kdy se ježci chystají na zimní hibernaci, jejíž problematika je veřejnosti známa. Vzhledem k tomu, že ježci stanoviště s potravou opakovaně navštěvují (Gazzard and Baker, 2020), je pravděpodobné, že i potravu předkládanou lidmi budou opakovaně přijímat. O to důležitější je zvolit správnou formu těchto příkrmů, často podávaných ve formě psích či kočičích krmiv. Tato krmiva jsou někdy měkčí, než přirozená strava ježka, kterou tvoří zejména bezobratlí živočichové (Jones and Norbury, 2011). Je také známo, že starší ježci se specializují na konkrétní kořist, obvykle větší než jakou požírají v mládí (Dickamn, 2009). Bezobratlí živočichové, které dospělí ježci loví, mají často tvrdou schránku, která může sloužit jako prevence vzniku zubního kamene. V případě měkkých složek potravy však tento proces mizí (Sainsbury et al., 1996). Zubní kámen je častým problémem a je popisován jak u domácích, tak u volně žijících ježků (Mullineaux, 2016). Vzhledem k tomuto faktu je jakékoli jednání, podporující vznik zubního kamene, dalším faktorem vedoucím k oslabení populace ježků. Silný zubní kámen způsobuje bolest v dutině ústní, neochotu k přijímání potravy a v nejhorším případě vede ke ztrátě zubů.

Cílem této práce bylo zjistit, u jakých věkových (hmotnostních) kategorií se zubní kámen vyskytuje a v jaké míře. Hmotnostní kategorie byly využity vzhledem k tomu, že hmotnost je jedním z faktorů odrážejících věk ježků (Haigh et al., 2014).

Materiál a metodika

Do výzkumu bylo zahrnuto 12 ježků západních uhynulých v záchranné stanici DES OP v Plzni, do které byli přijati na podzim roku 2019. Byli rozděleni do 3 skupin dle hmotnosti, a to na zvířata vážící méně než 300 g, 300-700 g a nad 700 g. U jednotlivých ježků byla během pitvy zaznamenána přítomnost zubního kamene a jeho míra (žádný, mírný, výrazný). Ke statistickému zpracování byla použita metoda chí-kvadrát test s Yatesovou korekcí v rámci metodiky 2x2 kontingenčních tabulek a to v programu UNISTAT 6.5 for Excel (Unistat Ltd., London, UK). Hodnota $p < 0,05$ byla stanovena jako statisticky významná.

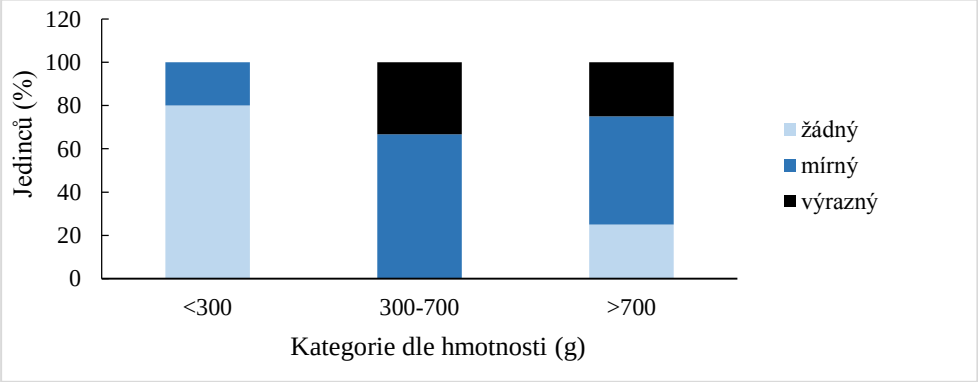
Výsledky a diskuze

Tabulka č. 1 udává, u jakého podílu ježků z konkrétních hmotnostních skupin byl přítomen zubní kámen. Graf č. 1 zobrazuje stupeň postižení zubním kamenem u jednotlivých hmotnostních skupin.

Tabulka č. 1. Výskyt zubního kamene u ježků západních dle hmotnosti

Hmotnost (g)	Počet ježků postižených zubním kamenem (%)
< 300	20[a]
300-700	100[a]
>700	75[a]

[a] stejné indexy značí statisticky nevýznamný rozdíl



Graf č. 1. Výskyt zubního kamene u ježků západních dle závažnosti postižení v jednotlivých kategoriích

Výskyt zubního kamene se mezi sledovanými hmotnostními kategoriemi statisticky významně nelišil ($p > 0,05$), výsledky statistického hodnocení však mohly být ovlivněny malým počtem zvířat. Pro posouzení vlivu věku/hmotnosti by bylo třeba provést analýzu u většího počtu jedinců ve všech kategoriích. Výsledky naší pilotní studie naznačují, že by věk mohl mít vliv na vznik zubního kamene. U ježků západních, kteří měli nejnižší tělesnou hmotnost (< 300 g), byl zubní kámen pozorován u nejmenšího podílu zvířat. Překvapující je, že i u mláďat na mléčné výživě se tento problém vyskytuje. Mohlo by to tedy potvrzovat názor, že ježci jsou vůči zubnímu kameni predisponováni již od útlého věku (Mullineaux, 2016). U starších zvířat byl zubní kámen přítomen téměř vždy a to i ve výraznějších vrstvách. Tento nález nepodporuje domněnku, že by příjem větších bezobratlých živočichů staršími ježky (Dickamn, 2009) mohl být prevencí proti vzniku zubního kamene. Naše předběžné výsledky naznačují, že vliv na vznik zubního kamene má genetická predispozice u těchto zvířat, kdy je zubní kámen formován už u nejmladších kategorií, a že by u starších jedinců přikrmování měkkou stravou mohlo vést k jeho zhoršení.

Závěr

Naše pilotní studie naznačuje, že zubním kamenem jsou sice postižena zejména starší zvířata, nicméně může se nacházet i u mláďat. Další rozsáhlejší studie mohou odhalit, jak velká část populace volně žijících ježků má tento problém, a rovněž potvrdit či vyvrátit domněnku predispozice ke vzniku zubního kamene již u mláďat. Je žádoucí sledovat také potravu přijímanou ježky, a to zejména s ohledem na přijímání měkkých krmiv, kterými jsou ježci přikrmováni, a zjistit, zda podporují tvorbu zubního kamene u těchto zvířat. Přestože v našem výzkumu nebyla potvrzena statistická významnost vlivu věku/hmotnosti na výskyt zubního kamene, byl potvrzen jeho výskyt u všech sledovaných věkových/hmotnostních kategorií. Vzhledem k dopadu výskytu zubního kamene na kvalitu života ježků je třeba se této problematice dále věnovat.

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The effect of sampling time on cortisol level in pig saliva

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Summary
In the European union, there is an increasing consideration on reduction of environmental stress in animals. In order to assess the stress in animals, it has been developed several methods using different types of samplings. With regard to present knowledge, animal species (domestic pig) and breeding technology we have decided to evaluate stress level by assessment of salivary cortisol. Multiple factors have impact on the level of salivary cortisol but there is still not many information about the best sampling time. So that is the reason why we have focused on cortisol levels dependency and sampling time. Samples were taken in the different time intervals after stress induction (10, 20, 30, 40, 50, 60, 90 and 120 minutes). The data showed that the best moment for sampling is 40 minutes after stress induction.

Keywords*: time; sampling; cortisol; pig; saliva*

Introduction

Recently, there is an increasing interest in reduction of environmental stress in animals. Unfortunately, there are some necessary interventions as a part of breeding technology which are difficult to replace and represent a cause of stress, e.g. castration, vaccination, handling, tail docking, tooth resection, tattooing etc. There are several methods using different types of sampling for animal stress assessment. We have decided to evaluate stress level by assessment of cortisol values in saliva samples. With regard to sampling method, we can say that samples used for evaluation are not clear saliva but mixture of all oral fluids. There are many factors as a part of oral fluids except saliva with impact on laboratory results (for example food residues, mouth cavity bacterial microflora, blood or the other contaminants) (Kivlighan and col., 2004; Whembolua and col., 2006). These factors can be reduced by starvation or mouthwash with water in human studies, but unfortunately in animal studies the reduction is not possible because it can be additional stress factor for animals which can affect the results (Whembolua and col., 2006). Magnano and col. (1989) found out that in samples taken from breastfed individuals the level of cortisol might be increased as a consequence of sample contamination by breast milk. Because the piglets used in our study were still breastfed so that is why we were taking the samples few minutes after finishing the breastfeeding and not during breastfeeding. Another factor affecting the cortisol level is the circadian rhythm, in pig saliva the basal concentration of cortisol is higher in the morning and lower in the evening (Griffith and col., 1991).

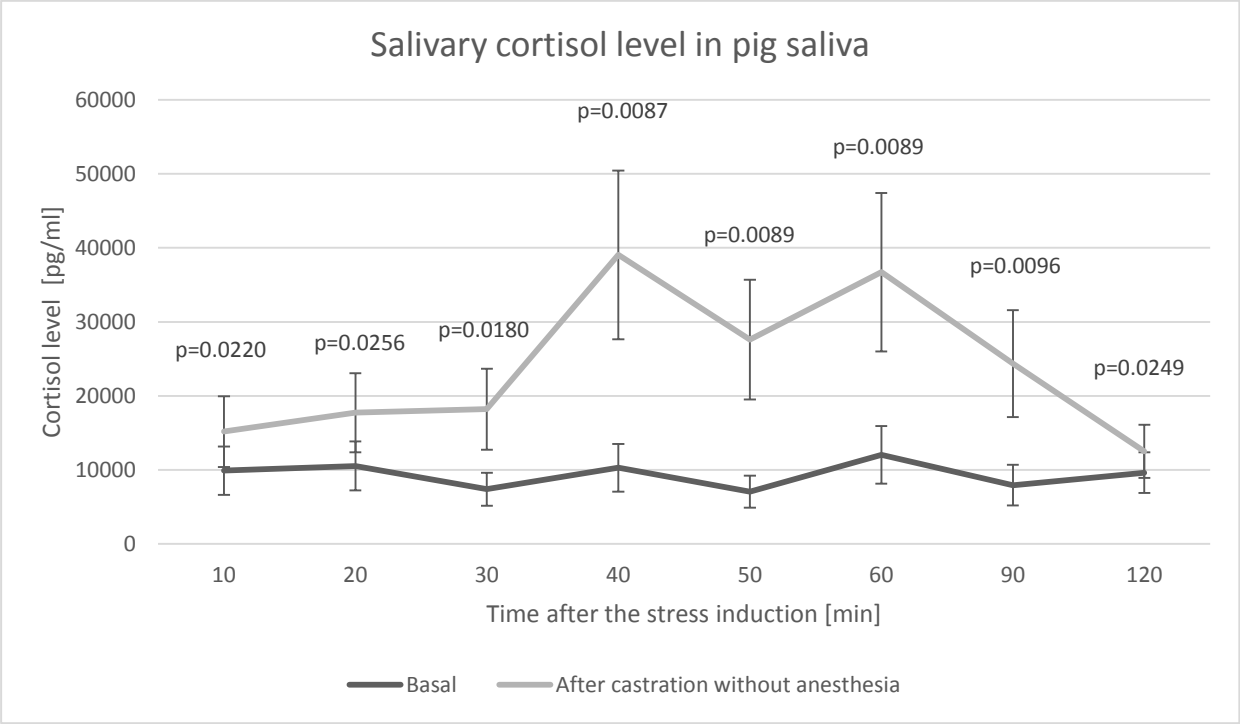
Material and methods

In our experiment we used 4 days old mix breed piglets (*Landrace x Czech improved white pig*). The castration without anaesthesia, tattooing and vaccination were sat as stress factors. These interventions are performed together during one handling. In this paper the used term for this procedures is „castration without anaesthesia“. Samples were taken by cotton swabs from the oral cavity. Samples were taken as a group sample – one group sample was taken from all piglet boys in one pen. All samples were taken as pair samples, it means before and after castration without anaesthesia. Samples were taken 10, 20, 30, 40, 50, 60, 90 and 120 minutes after castration without anaesthesia, respectively after giving piglet back to the pen. After sampling, the samples were centrifugated at 14 000 rpm, they were frozen by dry ice and transported to the freezer (- 80 °C). The experiment was ten times repeated. The

laboratory analysis was made with commercially available ELISA kits (Cortisol EIA kit) for cortisol assessment in saliva of all species. After laboratory analysis, statistical analysis in the UNISTAT for Excel with the Wilcoxon test was applied.

Results and discussion

Our study is focused on finding the effect of sampling time on cortisol values in pig saliva, more precisely on finding the most suitable time for sampling after the stress induction. In the published studies we can meet with different proposal of the best timing for samples taking, e.g. Cook and col., (1996) found out that the maximal salivary cortisol level is 5 minutes after the stress induction by the fixation with nose-snare. On the contrary Coutellier and col., (2007) determined that the highest level of cortisol in saliva is 4-5 hours after stress induction by the animal manipulation and regrouping. The important finding is that different salivary biomarkers appear to react differently following various types of stressors (Ott and col., 2014). The salivary cortisol level assessment is often used for stress assessment during animal handling, transport or regrouping (Coutellier and col., 2007; Merlot and col., 2004). For assessment of stress induced by castration, tooth resection or tail docking is more popular to use serum or plasma (Prunier and col., 2005; Backus and col., 2018; Shuterland and col., 2012). In this study we have decided to use the saliva samples for assessment of stress induced by castration without anaesthesia. The most statistically significant difference between the samples taken before and after stress induction is in 40 minutes (graph 1).



Graph 1: Salivary cortisol level in pig saliva

Conclusion

In our study we determined that the most statistically significant difference is in 40 minutes, it means that the best time for sampling in order to assess salivary cortisol level is in 40 minutes after stress induction by pig castration without anaesthesia. These findings will be further

applied in our following studies focused on assessment of salivary cortisol level after stress induction.

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Weaning as a stress factor for goat kids

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Summary
The aim of this study was to find out whether weaning affects the welfare of goat kids and thus represents a stressful situation. We used a non-invasive method of saliva sampling to determine the concentration of stress hormone cortisol. This method has many advantages over invasive blood sampling, but also over other non- invasive methods such as determination of cortisol concentration in urine, feces or fur. Our results show that weaning at 3 months of age is a stress factor for goat kids, because cortisol levels in saliva samples after weaning were statistically significantly increased compared to basal values.

Keywords: *goat; welfare; salivary cortisol*

Introduction

Many stressful situations can occur in dairy goats breeding. The goat kids can be exposed to stressful situations, such as transport, dehorning or weaning. It is known that stress has a negative impact not only on the growth of animals, but also on their production, reproduction or susceptibility to disease (Kumar et al., 2012). Weaning is defined as the complete physical separation of mother and young, and the nutritional transition from the feeding of milk to solid feeds (Lynch et al., 2019). In addition to separation from the mother, the kids also have to deal with a change of diet during weaning, where milk is gradually replaced by forage and concentrate or a grain-based diet (Magistrelli et al., 2012), which requires adaptation in rumen functions (Baldwin et al., 2004). Thus, weaning can result in period of growth stasis (Greenwood and Cafe, 2007) as a possible consequence of stress caused by poor coping with a new diet (Magistrelli et al., 2012). Therefore, weaning can be a multifactorial stressor, in which, nutritional, social, physical, and psychological stress are combined (Karakuş, 2014).

Material and Methods

A total of 10 kids of the white shorthair breed were selected from the DoRA organic farm in Ratibořice near Třebíč. This is an extensive goat breeding, which works on the principles of organic farming. Saliva samples were taken from these kids before and after weaning. Samples were taken using cotton swabs, which wiped the oral cavity of the kids. The samples were then centrifuged at 1400 rpm for 3 minutes and stored at -80 ° C until analysis.

For analysis, we used commercially available ELISA kits for the detection of cortisol in saliva in all animal species. The detection limits of this kit are 100 - 3200 pg / ml and the sensitivity is 17.3 pg/ml. The analysis was performed according to the enclosed instructions and at the end a quantitative calorimetric detection of cortisol was performed by a Varioskan reader at a wavelength of 450 nm. The results were determined by subtraction from a standard curve designed in ELISA Software free. The results obtained in this way were statistically processed using the program Unistat for Excel version 6.5 using the Wilcoxon test.

Differences were considered statistically significant when the probability of a null hypothesis was less than 0.05 (ie, $p < 0.05$) and highly significant when the probability of a null hypothesis was less than 0.01 (ie, $p < 0.01$). A result when the probability of a null hypothesis was greater than 0.05 (ie $p > 0.05$) was considered a statistically insignificant result.

Results and Discussion

Table 1.: Average cortisol levels in pg/ml and statistical evaluation

	Weaning	
	BASALS	**AFTER**
Mean	237,30	350,95
SE	130,45	85,17
SEM	41,25	26,93
Statistics	Wilcoxon	**0,0391**

Weaning on a goat farm DoRA is gradual. The kids get first used to their mothers going to the milking parlor twice a day. Then there is weaning and separation from the mother, which takes place at about 3 months of age.

Our results show that even a gradual weaning is stressful for the goat kids, because there was a statistically significant increase in cortisol levels after weaning. Zavy et al. (1992) found that cortisol concentration increased dramatically postweaning in beef calves and was similar to the increase observed in beef calves post-transport as well. In contrast, Magistrelli et al. (2012) found in their study that weaning at 48 days of age is not stressful for goat kids and thus has no effect on cortisol levels or weight gain. Tolu et al. (2016) found that social isolation for 5 minutes is more stressful for kids than weaning and also found that weaning cortisol levels are not affected by the environment.

Orgeur et al. (1999) evaluated the psychobiological consequences of two types of sudden weaning at 3 months of age in sheep. Plasma cortisol levels of lambs showed a greater increase when ewes and their lambs were totally separated than when they have allowed to visually and vocally communicate (partial separation).

Another study by Mohapatra et al. (2021) did not provide a statistically significant increase in plasma cortisol levels in weaned and unweaned lambs. However, they found that in the group of weaned lambs no playful behavior was observed compared to the unweaned group and also a greater vocalization was observed in weaned group, which indicates some degree of stress. Enriquez et al. (2011) noted that among the many behavioural changes taken as indicators of weaning stress, the most characteristic was the high frequency of vocalizations emitted by the calf.

Secondary factors that may affect weaning are sex, goat breed, or diet (Lu and Potchoiba, 1987). According to Morand-Fehr (1981) males are more sensitive to weaning shock than females, and Teh et al. (1984) report that Nubian goats are more sensitive to weaning shock than Alpine goats.

Conclusion

Our study confirms that even a gradual weaning of goat kids from the mother is stressful situation, which was also confirmed by cortisol levels, which increased statistically significantly after weaning ($p < 0.05$) compared to basal values. Although weaning is necessary all farmers should employ proper management practices in order to minimize stressful situations.

Acknowledgements
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Social isolation of laboratory rats causes a depressive-like behaviour

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Summary
The aim of this study was to assess effects of two types of housing on rats. One group of rats was housed individually and the other group was housed socially in pairs during the testing period. After 30 days, the animals were subjected to a test evaluating the degree of depressive behaviour, referred to as the forced swim test. The results of the study show that rats kept in isolation exhibited increased immobility in the forced swim test, i.e. behaviour considered to reflect a measure of behavioural despair. In contrast, rats kept in pairs exhibited higher level of activity, namely climbing. Socially housed rats put more effort in getting out and saving their lives. The study shows the negative impact of social isolation on mental health of rats.

Keywords: *laboratory rat; depression; cage; forced swim test; welfare*

Introduction

The environment and social interactions play an important role in housing of rats, they significantly affect their behaviour and brain development. Social housing is important because it promotes social behaviours such as social play, group sleeping or cooperation. If captive animals are not exposed to a social element that is inherently stimulating and is a prerequisite for positive effects, undesirable behavioural elements may appear and performance of natural behaviours is reduced (Renner, 1987). Exposure to severe or persistent social stress may result in the development of psychiatric disorders, namely anxiety and depression. These mood disorders are associated with structural changes in the neural architecture in the limbic areas of the brain that control emotions, mood, and cognition. In social mammals, isolation can be a strong stressor that may have negative effects on maintaining homeostasis, leading to disruption of neuroimmunoendocrine communication (Cacioppo et al., 2016). The hypothalamic-pituitary-adrenocortical axis is sensitive to social changes, and periods of social isolation may induce increased sympathetic tone and HPA activation (Steptoe et al., 2004).

The aim of this study was to expose laboratory rats to two types of housing (social and individual) for one month and subsequently to measure degree of behavioural despair with the use of the validated forced swim test, i.e. a depression-like behaviour was compared between the rats housed socially and individually.

Material and methods

Twenty Wistar male rats aged 6 weeks were randomly divided into two groups. The first group of rats (n = 10) was housed individually and the second group of rats (n = 10) was housed socially in pairs. All rats were housed in standard cages in the same room. A 12-hour light regime was maintained and the temperature ranged from 19.4 to 23.3 °C in the room where the rats were kept. After 30 days, all rats were subjected to the forced swim test. During the test, an animal was placed in a transparent cylindrical glass container filled with lukewarm water (the height of the water column was 30 cm) and its behaviour was continuously observed for 5 minutes. Subsequently, the duration of swimming, climbing and immobility behaviours was measured for each rat. A t-test was used to assess a difference between the means of the two groups.

Discussion

The aim of this study was to assess whether social isolation in 6-week-old rats has an effect on depressive and anxiety behaviours. Loneliness in adult rats has been shown to be a risk factor resulting in physical inactivity (Smith et al., 2017), indicating the occurrence of social deprivation and reduced spontaneous physical activity (Tsvirkun et al., 2012). In our study, laboratory rats were housed socially (in pairs) or individually without access to physical elements of enrichment for 30 days to exclude the possible effect of the environmental enrichment. During the experiment, contacts with humans were minimized, only weight of rats was recorded. On the last day of the 30-day period, the rats were exposed to a pre-test that preceded the validated test for depressive behaviour. The pre-test lasted 15 minutes and was not recorded on the camera system. Its purpose was to expose the animal to a hopeless situation.

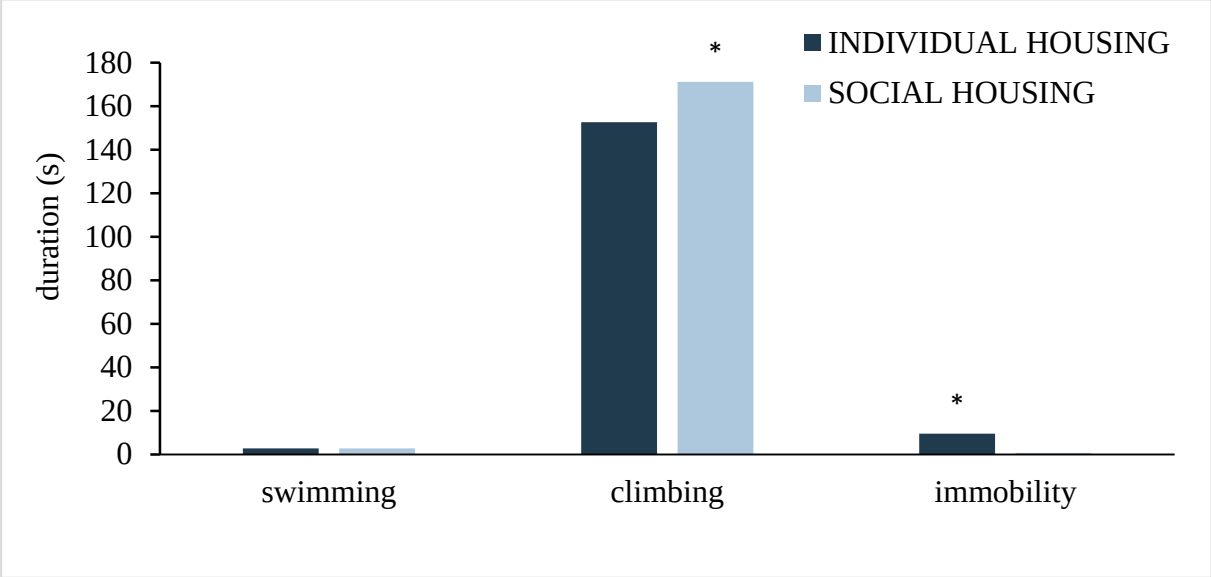

Figure 1: Duration of selected behavioural elements measured during the forced swim test in individually and socially housed rats (* $P < 0.05$)

The test is based on the principle that an animal exposed to such a situation first attempts to escape, which manifests behaviourally as vertical attempts to climb the wall. Socially housed rats exhibited climbing statistically significantly longer ($P < 0.05$) compared to individually housed rats. Swimming is defined as a horizontal movement on the water surface including diving. No difference ($P > 0.05$) was found between socially and individually housed rats in the duration of swimming. If the rats stop trying to escape, they adopt a passive relatively immobile position (immobility). No complete immobility was recorded in our experiment. For the purposes of this study, immobility was considered as behaviour aimed only at keeping the head above water, with no active exploration of the environment or attempts to climb the wall present. Immobility was more frequently ($P < 0.05$) observed in individually housed rats compared to rats housed in pairs. Rats kept in social isolation exhibited a higher incidence of immobility, suggesting that social isolation leads to disorders similar to depression and anxiety. Socially housed rats were willing to put more effort in getting out of the hopeless situation. Similar findings were made also in experiments performed on mice (Petit-Demouliere et al., 2004).

Conclusions

Social isolation has negative consequences on rats´ behaviour. Our study shows that individually housed rats exhibit a depressive-like behaviour potentially affecting their survival in life-threatening situations. The laboratory rats should be housed in groups not only considering their welfare but also in order to achieve reliable research results. The impact of depression on physical structures in the brain and thus the central control center of nervous system has been well documented and thus may affect results of a large variety of experiments performed on rats.

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Changes in sociability of shelter cats as welfare indicator

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Summary
Cat stress can be manifested by the negative response to a human-initiated interaction. In this study, we investigated changes in sociability of shelter cats by using a modified 5-point scale of Human-Approach-Test. A total of 158 cats were assessed during 26 shelter visits at two-week intervals. Most shelter cats (81%) showed a high sociability level (rated as friendly and very friendly) in the first assessment. The scores of cats with lower level of sociability (rated as neither friendly nor unfriendly, unfriendly and very unfriendly) tended to improve during the stay in the shelter. Assumedly, it was caused by habituation to the presence of humans or by stress reduction in general. Level of sociability was found to be a factor influencing cats' length of stay in the shelter. From the animal welfare perspective, the most vulnerable are cats with low sociability level that did not experience improvement despite of their long stay in the shelter. Shelter staff should reconsider keeping such individuals in the facility and if appropriate, choose to release them to the location of their capture.

Keywords: *feline; stray animal; human-approach-test; animal-based indicator*

Introduction

Cats are animals sensitive to changes; for most of them, placement in a shelter is a stressful experience (Bradshaw et al., 2012). Cats' stress is usually manifested by changes of behaviour (Stella et al., 2013). Behavioural indicators of worsened welfare include absence or negative response to a human-initiated interaction (Amat et al., 2016), particularly redirected aggression and some forms of affective aggression. While most of well-socialized cats experience a stress reduction within a few weeks (Rochlitz et al., 1998), unsocialized cats are at risk of welfare impairment when housed in the shelter longer than necessary. Various approaches are currently used to assess willingness of a cat to interact with human. Most of them are based on rating scales. The individual scores of scale usually represent specific behavioural manifestations of the cat when approaching or in direct physical contact with human (Kessler and Turner, 1999). The aim of this study was to assess the changes in the sociability of shelter cats during their stay in the shelter using a modified scale of the Human-Approach-Test.

Materials and Methods

Data collection was carried out in a private shelter with group-housed cats. The capacity of this facility is 25 cats, with an annual admission rate of about 200 cats. A modified 5-point scale of Human-Approach-Test by Kessler and Turner (1999) was used to evaluate the sociability of cats. A score of 1 represented the best possible outcome (cat is very friendly). The score of 2 represented the friendly cat, the score of 3 neither friendly nor unfriendly cat, the score of 4 unfriendly cat. The lowest sociability level of cats was represented by the score of 5 (cat is very unfriendly). The population of shelter cats was monitored regularly in two-week intervals for 12 months (from March 2019 to March 2020). The maximum number of assessments for an individual cat was 14, no cat was assessed more than 14 times. The evaluation of cats was always conducted at the same time (in the morning (9:00) and in the evening (18:00)). The mean scores of both assessments were calculated for each cat. The calculated value was rounded to the nearest whole number. A total of 158 cats were assessed, only cats older than 12 weeks of age were evaluated. The obtained data were analyzed using statistical software Unistat 6.5 for Excel (Unistat Ltd., UK). The Shapiro-Wilk test was used

to verify the normal distribution of the data (irregular distribution was detected). Differences in the number of animals in the monitored categories were analyzed by the $\chi 2$ test (2 x 2 contingency tables). Statistical significance among the cat's rated scores was tested by the nonparametric Mann-Whitney U test. The Spearman's rank correlation coefficient was used to verify the correlation between the length of stay of cats in the shelter and mean scores of cats' sociability. A value of $P \leq 0.05$ was considered significant.

Results and Discussion

One hundred females (63.3%) and 58 males (36.7%) older than 12 weeks were admitted to the shelter and assessed at least once. Most shelter cats were adopted during their stay in the shelter (n=123, 77.8%). The second most common outcome was death or euthanasia (n=20, 12.7%). Only one cat was returned to its original owner and another one was released to the capture location. For 13 cats (8.2%), their stay in the shelter was not terminated during the monitored period. The age of monitored cats ranged from 3 to 168 months.

Most cats showed a high level of sociability in the first assessment. Significantly more cats (P=0.000) were rated as friendly (the score of 1 or 2, n=128; 81%) than unfriendly (the score of 3, 4 or 5, n=30; 19%). In majority of unfriendly cats (n=19), the level of sociability improved during their stay in the shelter (the scores of those cats decreased). In 13 cats, the improvement of sociability level occurred within two weeks of the first assessment. The scores given in the first and the second assessments differed significantly (P=0.0107). This can indicate a stress reduction; most cats adjust to the shelter environment within 2 to 5 weeks (Rochlitz et al., 1998). The score of 6 unfriendly cats did not change during the entire stay in the shelter. Keeping non-socialised cats in a shelter for longer than necessary has significant consequences for them in terms of deteriorating living conditions (Slater et al., 2013). No permanent deterioration in the sociability scores was recorded in any of unfriendly cats, although the temporary deterioration in the sociability level during the shelter stay occurred in 2 cats. Among cats that were rated as friendly in the first assessment, a permanent deterioration was found in 3 cats. Temporary deterioration of sociability score occurred in 3 cats. A cat's negative experience with human could cause a temporary change in the sociability level. For example, cats can perceive veterinary treatment provided in the shelter as stressful and it can result in the temporary change in their response to humans. Figure 1 presents the development of the scores of friendly and unfriendly cats during their stay in the shelter.

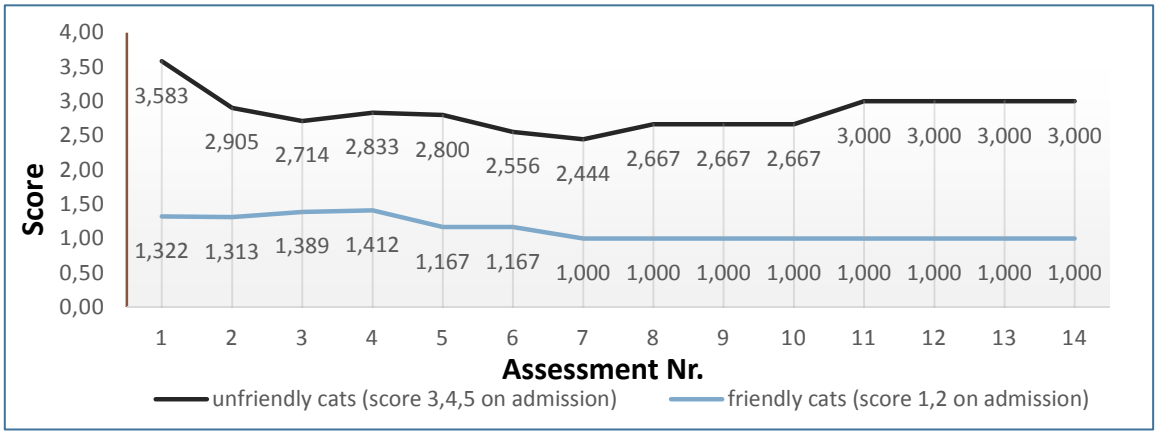

Figure 1: Changes in sociability score in friendly and unfriendly cats during the stay in the shelter.

In unfriendly cats, a significant difference (P=0.0001) was found between initial scores and scores given in the last assessment. The decrease in scores means the sociability of cats originally rated as unfriendly improved during their stay in the shelter. Unlike unfriendly cats, no significant difference (P=0.1720) was found when comparing the initial and last assessment scores of friendly cats.
A significant positive correlation was found between the cats' length of stay in the shelter and their sociability level (r_s=0.1864, *P*=0.0258). Unfriendly cats stayed in the shelter longer than friendly cats. This finding is in agreement with results of previous studies. As reported by Brown and Stephan (2020), the mean length of stay of interactive cats was approximately three times shorter than that of unapproachable cats (37 days and 119 days, respectively). The cat's temperament and appearance are factors influencing potential adopters' preferences (Fantuzzi et al., 2010).

Conclusion

Most shelter cats showed friendly behaviour towards human in the first assessment. The sociability scores of cats rated as unfriendly in the initial evaluation improved significantly during their stay in the shelter. Only 2 cats exhibited a significant deterioration in their sociability level during the stay. Unfriendly cats, whose attitude do not change in the shelter, are the most vulnerable category in terms of welfare impairment. Keeping such animals in the shelter should be reconsidered. Cats whose behaviour indicates that they have not been previously socialized towards humans will do better when released. Trap-neuter-return programme should be followed as an appropriate approach to prevent deteriorating living conditions of unsocialized cats.

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SEKCE 4

Veřejné a soudní veterinářství a toxikologie

Could mixtures of UV filters affect the embryonic development of zebrafish?

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Summary
The aim of this study was to evaluate whether the mixture of UV filters can affect embryonic development. Modified Fish Embryo Acute Test was used for this experiment. *Danio rerio* embryos were exposed to different range of UV filters mixtures concentration - benzophenone-3 (BP-3), octocrylene (OC), 4- methylbenzylidene camphor (4-MBC)**,** ethylhexyl methoxycinnamate (EHMC) and phenylbenzimidazole-5-sulfonic acid (PBSA) for 96 hours. Lethal and sublethal endpoints were recorded during 24-hour intervals. Results revealed statistically significant mortality after OC and 4-MBC exposure. Earlier hatching rate was recorded after exposure to OC and 4-MBC mixture, further for PBSA and BP-3 and EHMC mixture and for mixture of all five UVs.

Keywords: *personal care products; Danio rerio; mortality; hatching; embryonic development*

Introduction

UV filters (UVs) are used in common personal care products (shampoos, lotions, sunscreens, cream, *etc.*) and they are becoming an important micropollutant for our environment due to the fact of their increased consumption. These pollutants are present in the environment rather a mixture, but not as a single substance. It is necessary to study how these pollutants react with each other and how those can affect the non-target organisms. Recent studies have revealed impaired embryonic development and embryotoxicity caused by commonly used UVs. Benzophenone-3 (BP-3) can decreased the number of hatched embryos and caused tail and jaw deformation (Balász et al. 2016). Brain and liver development, haematopoiesis, formation of blood vessels and fat cell differentiation were disrupted after octocrylene (OC) exposure in *Danio rerio* (Blüthgen et al., 2014). 4- methylbenzylidene camphor (4-MBC) decreased heart and hatching rate and caused malformation in zebrafish embryos (Torres et al. 2016; Quintaneiro et al. 2019). Many papers refer the importance of testing the mixture toxicological effects instead of individual substances. Li et al. (2018) pointed to the adverse effect of a mixture of UVs (BP-3, ethylhexyl methoxycinnamate – EHMC and OC), even on the next generation of zebrafish, meaning decreasing of heart and hatching rate and increasing of embryo mortality. The aim of our study was to evaluate the effect of the UVs´ mixtures on the embryonic development of zebrafish.

Material and methods

Modified Fish Embryo Acute Toxicity Test (FET) - OECD guideline 236 was used for toxicological assessment of the mixture of UVs. We have selected for our tests a fertilized eggs of zebrafish embryo (*D. rerio*), maximally at the 16-cell stage with no development deviation. These eggs were exposed to the mixtures of common used UVs. As a solvents were used dimethylsulfoxid for solution of phenylbenzimidazole-5-sulfonic acid (PBSA), EHMC and BP-3; and ethanol for solution of 4-MBC and OC, in the total concentration 0.01% (10 µl in 100 ml). Selected combination of UVs depended on the solubility of individual UVs. Groups combination of OC and 4-MBC; PBSA, EHMC and BP-3; all 5 UVs were used for the experiment. Tested concentrations were 0.1; 10 and 100 µg/l, complemented by the control group without UVs. The lowest concentrations reflected the environmentally relevant concentrations of UVs in the surface water. Higher concentrations were chosen as a multiple

of the lowest one to determine the potential dose-dependent relationship. Stereomicroscope (StrereoBlue, Euromex) was used for checking egg quality and development determination. Zebrafish embryos were exposed to the different scale of UVs´ concentration in the 48-wells microwell plates, one embryo in each well, in total of 24 eggs for each tested group. The toxicological impact was assessed by the lethal endpoints (mortality) and development disorders (hatching rate, oedema *etc*.) whereas reported after 24, 48, 72 and 96 hours post fertilization (hpf).

Results

Statistically significant mortality was found in experimental group treated by 10 µg/l OC and 4-MBC combination after 48, 72 and 96 hpf ($p < 0.05$). Earlier hatching was recorded after 72 hpf exposure to 100 µg/l OC and 4-MBC combination ($p < 0.05$), further after exposure to 10 and 100 µg/l concentrations of PBSA, BP-3 and EHMC (both at $p < 0.05$) and also to mixture of all five UVs at concentrations 0.1 ($p < 0.01$) and 10 µg/l ($p < 0.05$) (Figure 1). Rare malformations were found in embryos exposed to mixture of PBSA, BP-3 and EHMC at the lowest test concentration at 48 hpf (4.2%) and 72 hpf (4.3%). In contrast to that, numerous malformations such as total deformation, curvature of the spine, yolk sac oedema, oedema of pericard, bent spine, undeveloped tail were revealed after OC and 4-MBC exposure, but still with no significant difference ($p > 0.05$) compared to the control group. Noteworthy, no malformations were observed after exposure to all five UVs during the whole experiment.

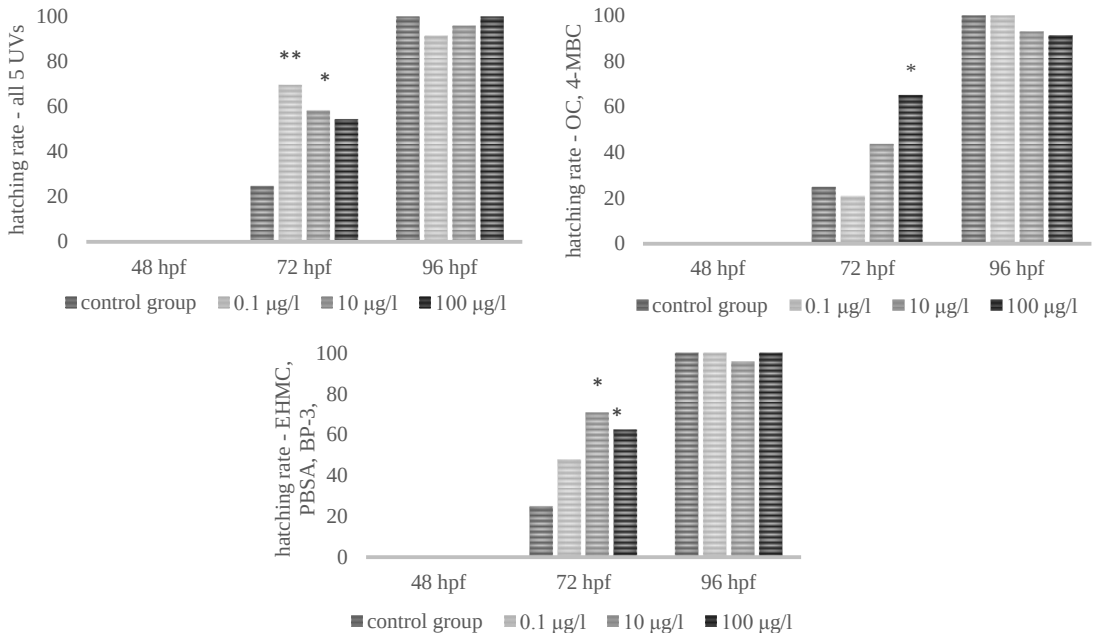

Figure 1: Results of hatching rate (%) of zebrafish embryos. An asterisk indicates a significant difference (* $p < 0.05$, ** $p < 0.01$) between control and experimental groups at the same time of exposure. 4-MBC (4-methylbenzylidene camphor), BP-3 (benzophenone-3), EHMC (ethylhexyl methoxycinnamate), OC (octocrylene), PBSA (2-phenylbenzimidazole-5-sulfonic acid).

Discussion

Many of UVs (BP-3, EHMC, OC) are not reported acute embryotoxicity in environmentally relevant concentration (Kaiser et al. 2012). In our experiment we have recorded statistically significant mortality in group treated by 4-MBC and OC combination after 48, 72 and 96 hpf. Experiment result shows that UVs in a mixture could present a significant toxicological risk

for zebrafish embryos. Hatching process disruption was found in our experiment, too. Earlier hatching was recorded in a group treated by OC and 4-MBC, as well as in PBSA, BP-3 and EHMC experimental group. Noteworthy, earlier hatching was proven after all five UVs´ exposure even in the environmental relevant concentration. In recent papers, hatching process disruption was revealed also after BP-3, EHMC and OC exposure (Balász et al. 2016; Li et al. 2018). We hypothesize such phenomenon could relate to the disruption of the embryonic membrane by UVs. Malformation in our experiment were not recorded as a statistically significant in any tested group, although some malformations were revealed after OC and 4-MBC exposure. These results are contrary to outcomes of Balász et al. (2016) experiment. They have proven yolk sac oedema, deformed jaw or dilated gut after BPs exposure. Nataraj et al. (2020) revealed lesions in muscle fibres and yolk sac after EHMC exposure. Based on our results, we suppose that once these substances are used in mixture they would have an additive toxic effect.

Conclusion

Considering the results of our experiment, it is obvious that mixtures of UVs present toxicological risk for zebrafish embryos. These mixtures of heterogeneous substances occur naturally in the environment and it is necessary to investigate how UVs affect organisms when they are presented together. The principle how the entire synergism or antagonism process works needs to be further investigated.

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Embryonální testy na dániu pruhovaném (*Danio rerio*) – vliv pesticidů

Embryonal tests on zebrafish (*Danio rerio*) – effect of pesticides

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Summary
Pesticides, industrial chemicals, pharmacologically active substances, and many other types of contaminants have been constantly entering the aquatic environment. Such a contamination may result in negative effects on both human and aquatic biota. In few past decades, water pollution caused by pesticides has become a commonly discussed issue. Residues of pesticides entering water environment can have a negative effect on fish and even their consuments because of their persistence in the aquatic environment and bioaccumulation potential. The aim of this study was to compare the effects of MCPA in its clear form and as a part of Bofix commercial herbicide on Danio rerio embryos in 96 hours long acute toxicity test with the MCPA concentrations of 0.1; 1; 10; 100 µg/l, and 1; 10; and 100 mg/l.
The results of the study show that the commercial preparation Bofix is more toxic than pure MCPA due to the content of additives. In the acute toxicity test, statistically significant changes were observed only at high concentrations that do not occur in nature.

Keywords: *MCPA; Bofix; herbicide; acute toxicity; water pollution*

Úvod

Povrchové vody jsou znečištěny rezidui různorodých látek, jako jsou například průmyslové chemikálie, látky používané k ochraně rostlin, léčiva či produkty osobní péče. Bylo prokázáno, že řada z těchto látek může následně negativně ovlivnit vývoj necílových vodních organismů i v nízkých, environmentálně-relevantních koncentracích. K posouzení toxických vlastností látek vyskytujících se ve vodním prostředí se používají testy toxicity na celé řadě vodních organismů, přičemž velmi důležité jsou testy na rybách, které stojí na vrcholu potravního řetězce vodního prostředí (Sehonová et al., 2016). Mezi nejčastěji využívané ryby používané k testům toxicity patří *Danio rerio, Oryzias latipes, Poecilia reticulata*, plůdek kapra obecného a pstruha duhového (Sehonová et al., 2016). Dánio pruhované (*D. rerio*) je obzvláště oblíbeným druhem, a to vzhledem ke snadnému chovu, velkému počtu jiker s průhledným obalem, dobře popsanému vývoji a také díky kompletně sekvenovanému genomu (Kanungo et al., 2014). Pesticidy jsou běžně používané přípravky v zemědělství a domácnostech především k ochraně rostlin a odstranění nežádoucích škůdců. Jejich rezidua vyskytující se v povrchových vodách však mohou negativně ovlivňovat necílové organismy vodního prostředí a vzhledem k jejich perzistenci a potenciálu bioakumulace i konzumenty (Rahman et al. 2021). Například Gaillard et al. (2016) monitorovali na severovýchodě Francie koncentrace pesticidů v povrchových vodách, přičemž byla zaznamenána koncentrace MCPA (kyselina (4-chlor-2-methylfenoxy)octová) v koncentraci 26,5 µg/l. V Austrálii byl proveden monitoring na pěti lokalitách v oblasti Melbourne, kde byly detekovány tyto herbicidy: MCPA, simazin, diuron a atrazin (Allinson et al., 2017). Za účelem posouzení negativního efektu hojně využívaného herbicidu MCPA na raná vývojová stádia ryb bylo cílem naší práce otestovat vliv pesticidního přípravku Bofix (MCPA v koncentraci 200 g/l, clopyralid 20 g/l a fluroxypyr 40 g/l) a čisté MCPA na embryonální vývoj dánia pruhovaného (*D. rerio*).

Materiál a metodika

Byl proveden akutní embryonální test toxicity podle metodiky OECD 236, ve kterém byly oplozené jikry dánia pruhovaného (*D. rerio*) exponovány účinkům herbicidního přípravku s účinnou látkou MCPA Bofixu a čisté MCPA. MCPA byla testována v koncentracích 0,1; 1; 10 a 100 µg/l; 1 000; 10 000 a 100 000 µg/l po dobu 96 hodin. V případě Bofixu byly roztoky připraveny tak, aby koncentrace MCPA v roztoku byla stejná jako u čisté MCPA. K ředění byla použita ředící voda připravená podle normy ISO 7346 (1996). Bofix je přípravek rozpustný ve vodě, MCPA je rozpustná v ethanolu. Z tohoto důvodu byly k testu vyžadovány dvě kontrolní skupiny, a to kontrolní skupina vystavená ředící vodě a kontrolní skupina vystavená ředící vodě s ethanolem. Maximální koncentrace etanolu byla ve všech testovaných roztocích 100 µg/l, což je v souladu s metodikou OECD 236. Embrya byla nasazena do 60–90 minut po oplození do 48 jamkových mikrotitračních destiček, v každé jamce bylo umístěno jedno embryo. V každé testované koncentraci bylo testováno 18 embryí, stejně tak v obou kontrolních skupinách (kontrola a kontrola s etanolem). Během pokusu byla embrya umístěna do růstové komory s konstantní teplotou 26 °C a fotoperiodou 12 hodin světlo a 12 hodin tma. Každých 24 hodin docházelo k výměně roztoků a zaznamenávání pozorovaných letálních i subletálních endpointů: koagulace embrya, absence tvorby somitů, nedokonalému oddělení ocasu od žloutkového váčku a nepřítomnost srdečního tepu, morfologické změny a čas líhnutí. Embryím byl po 48 hodinách měřen tep, kde se počítalo za kolik sekund udělá dané embryo 20 tepů. Výsledky byly hodnoceny v programu Unistat 5.6 for Excel. Statistická významnost byla hodnocena na hladině $p<0,05$ a $p<0,01$ a porovnána ke kontrole.

Výsledky a diskuze

Po 24hodinové (24 hours post fertilization, hpf) expozici MCPA v přípravku Bofix byla ve skupinách 10 a 100 mg/l pozorována 100 % mortalita ($p<0,01$). V čase 48 hpf byla pozorována 38,8% mortalita v koncentraci 1 mg/l ($p<0,01$). Naproti tomu, ve skupinách, kde byla testována pouze čistá MCPA (ne ve formulaci Bofix) nebyla pozorováno statistiky významné zvýšení mortality oproti kontrole ani v nejvyšší testované koncentraci 100 mg/l. Také Johansson et al. (2006) nepozoroval změny na pulcích skokana hnědého, který byl vystaven působením MCPA v koncentraci 0,75; 3 a 12 mg/l. Johansson et al. (2006) uvádí LC50 pro ryby 180 mg/l a pro obojživelníky 3,6 g/l. U přeživších jedinců byla v období 24–72 hpf pozorována rychlost líhnutí a v čase 48 hpf též srdeční tep. Rozdíly v líhnutí přeživších jedinců byly pozorovány pouze u skupin exponovaných čisté MCPA, kdy byla rychlost líhnutí embryí snížena v koncentracích 10 a 100 mg/l (skupiny exponované MCPA ve formulaci Bofix byly v těchto koncentracích již uhynulé). V čase 72 hpf bylo ve skupině 10 mg/l čisté MCPA pouze 33,3 % vylíhlých jedinců a v 100 mg/l nebylo vylíhnuto žádné přeživší embryo ($p<0.01$). Po ukončení testu v druhé nejvyšší testované koncentraci (10 mg/l) už však tento rozdíl pozorován nebyl, v nejvyšší testované koncentraci (100 mg/l) bylo vylíhnuto 23% ($p<0,01$). V koncentraci1 mg/l MCPA ve formulaci Bofix byly pozorovány značné vývojové malformace ($p<0,01$) u přeživších jedinců (jedenáct přeživších embryí). V čase 72 hpf byly pozorovány malformace u devíti jedinců z jedenácti (81,8%). V 96 hpf se zvýšil výskyt malformací na 100 %, tedy u všech přeživších jedinců byly pozorovány morfologické změny, jako edém srdce, krevní sraženiny, menší pigmentace. Ve skupině exponované koncentraci 100 mg/l čisté MCPA byla pozorována celá řada malformací ($p<0,01$). V této koncentraci bylo přeživších třináct jedinců, kde u jedenácti embryí (84,6 %) byly pozorovány edémy srdce, krevní sraženiny a deformace páteře. Lutnicka et al. (2018) testovali vliv MCPA v koncentraci 100 µg/l na juvenilním kapru obecném, u kaprů byly pozorovány změny v krevním diferenciálu a také mírné morfologické změny na ledvinách.

Závěr

Cílem práce bylo porovnání vlivu pesticidního přípravku Bofix (MCPA v koncentraci 200 g/l, clopyralid 20 g/l a fluroxypyr 40 g/l) a čisté MCPA na embryonální vývoj dánia pruhovaného (*D. rerio*) za účelem posouzení negativního efektu hojně využívaného herbicidu MCPA na raná vývojová stáda ryb. Z výsledků práce vyplývá, že komerční přípravek Bofix je z důvodu obsahu přídatných látek toxičtější než čistá MCPA. V akutním testu toxicity byly pozorovány statisticky významné změny pouze ve vysokých koncentracích, které se v přírodě nevyskytují. To však neznamená, že rezidua této látky v povrchových vodách nemají vliv na necílové organismy. Tyto organismy žijí v kontaminovaných vodách po celý svůj život a jsou vystaveny směsím různorodých látek. Rovněž je na základě výsledků předešlých prací pravděpodobné, že pokud by byly pozorovány další, citlivější, endpointy, jako je třeba změna exprese genů zodpovědných za vývoj orgánových soustav, byly by pozorovány odchylky i při nižších, environmentálně-relevantních koncentracích. Výsledky z akutních testů toxicity s vyššími koncentracemi testovaných však pomáhají odhalit mechanismus toxického účinku látek a pomohou zvolit na jaké endpointy se zaměřit při následných studiích.

Poděkování

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The influence of long-term exposure diploid and triploid population of *Danio rerio* to the estrogenic substances

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Summary
There is a wide range of chemical pollutants in the aquatic environment known for their ability to affect the hormonal system of aquatic organisms. The presence of vitellogenin in male zebrafish is a definite symptom indicative of the potential for such changes due to the action of estrogenic substances. In the present study, we focused on the influence of synthetic estrogen, ethinylestradiol (EE2), often used in oral contraceptive pills. We specifically focused on the different vitellogenin synthesis in diploid and triploid zebrafish and we found a significant difference ($p < 0.01$). Zebrafish, triploid form population consists only from male fish, therefore no histological examination is required to determine the sex of experimental animals.

Keywords*: ethinylestradiol; EE2; vitellogenin; zebrafish*

Introduction

For a long time, environmental pollution is one of a major topic of interest for many research groups. There are number of chemical pollutants in the aquatic environment that are suspected or known for their ability to affect the hormonal system of aquatic organisms (Šauer et al, 2018). Although many people attribute the elevated levels of hormones in the aquatic environment to the use and application of hormonal drugs, large amounts of these substances enter the environment in a completely natural way. About 30,000 kg of natural estrogens by humans and about 83,000 kg by livestock is discharges every year in EU and USA via urine and feces (Adeel et al., 2017). Throw wastewater treatment plant and the application of manure directly to soils, estrogens enters the ecosystem (Shrestha et al., 2012). Synthetic estrogens, used for example in birth control pills, significantly increase contamination of ecosystem. In wastewater treatment plant, the hormone elimination is in the range 0-90% and in some cases, the process instead of hormone elimination leads to an increase in their activity (based on conjugated and unconjugated form) (Adlercreutz et Järvenpää, 1982; Combalbert and Hernandez-Raquet, 2010; Shreshtha et al., 2012). In our experiment, we tested the toxicity of long term exposure of ethinylestradiol (EE2), a semisynthetic estrogen used in oral contraceptives, on diploid and all-male triploid zebrafish (*Danio rerio*).

Material and methods

Triploidy in zebrafish was induced by applying a temperature shock to fertilized embryos 2 minutes post fertilization (41 °C for 2 minutes) (Franěk et al., 2019). Swim-up larvae stage were assessed for the ploidy by flow cytometry.

The fish (total n = 120) were divided into four groups (diploid control, troploid control, diploid experimental, triploid experimental) and fish were placed into six-liter aquariums. Water was changed every second day. The concentration of EE2 in experimental aquariums was 10 ng/l, the solution was mixed every second day during water changing.

After 7 weeks, fish were euthanized (using MS222) and fish were divided into groups, based on its intended laboratory analysis. It was histology, RNA and vitellogenin synthesis examination. At the University of Veterinary Sciences Brno, we focused on determining synthesis of vitellogenin.

Zebrafish vitellogenin ELISA kit (Biosense Laboratories AS, Norway) was used for vitellogenin detection. Samples and standards were kept on ice during the whole procedure. Detection of vitellogenin was performed in whole-body homogenate. Samples were homogenized (25 Zh, 70 seconds – two cycles; TissueLyser II, Qiagen, USA), centrifuged (18 000 g, 30 minutes, 4°C; Microfuge, 22R Centrifuge, Beckman coulter, United States) and supernatants were removed. Samples were applied to the plates as described in the manufacturer´s instructions. The absorbance at 492 nm was read with microplate reader (Varioskan flash, Thermo-scientific, USA).

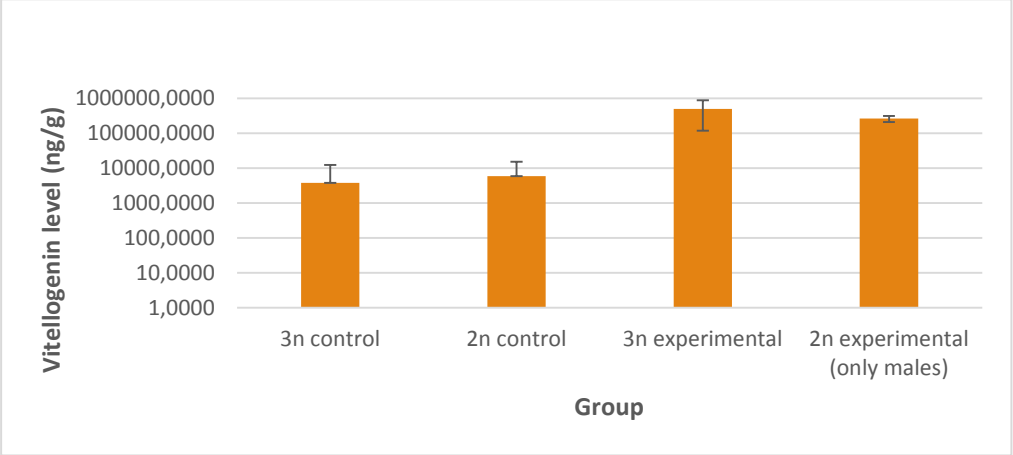
For the statistical analysis Escel software Unistat program 5.6. was used. Shapiro-Wilk normality test was used for criteria of normality data testing. For multiple comparisons for evaluation of the significance of the differences between all possible pair groups the Turkey-HSD test was used.

Results

During the experiment, behaviour, body deformities and appetite were monitored every day during the feeding period. During the whole experiment, fish showed interest in feed and no behaviour changes and morfological agnormalities were detected.

Average vitellogenin level in experimental fish is shown in Graph 1. Diploid females in control group showed a vitellogenin concentration range 4,937,949 – 28,111,698,093 ng/g, while in diploid and triploid males, vitellogenin concentration was below limit of detection, in most cases. There is a significant difference ($p < 0.01$) between the experimental 3n group and the other groups, in case only 2n males were included.

Graph 1: Average vitellogenin level



Discusion and conclusion

EE2, which has been recorded in waters across the globe, is a highly potent hormone that has been shown to have significant effects on aquatic organisms, even at low levels (Gomes et al., 2004). It can affect the endocrine system (Scala-Benuzzi et al, 2018), reduced number of spermatogonia (Piferrer and Donaldson, 1992) or alter gonadal development resulting in delayed sexual maturity in aquatic organisms (Andersen et al., 2003).

Our study confirm the ability of EE2 to induce vitellogenin synthesis in all-male triploid zebrafish and the presence of vitellogenin in body tissue is a definite symptom indicative of the potential for such changes due to the action of oestrogenic substances. It also shown, that the use of all-male triploid zebrafish population could serve as a suitable alternative for controlled testing of the effects of xenoestrogenes on male fish.

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SEKCE 5

Veterinární ekologie a choroby volně žijících zvířat

Causes of admission and outcomes of white-tailed eagles (*Haliaeetus albicilla*) in wildlife rescue centres in the Czech Republic

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Summary

This study documents the causes of admission and final outcomes of treatment of 68 white-tailed eagles (Haliaeetus albicilla; WTE) admitted to 20 rescue centres in the Czech Republic (CZ). The outcomes were expressed as the mortality rate (Mr = 35%), release rate (Rr = 38%) and permanent captivity rate (Cr = 27%). Mr was the highest in the trauma category (46%, 18/39). Rr was the highest in non-trauma (52%, 13/25) and orphaned individuals (50%, 2/4). The most frequent cause of admission was trauma (n = 39). Birds injured in fights had the highest release rate compared to other traumatic causes (60%, 3/5). Time until death (Td) and length of stay in the centre for the released individuals (Tr) were estimated. The median for these variables, and in cases of unknown trauma, was: Td = 5 and Tr = 58 days. The most frequent non-trauma causes of admission were weakness (n = 13) and toxicosis (n = 9). Birds admitted for weakness were mostly released (Rr = 62%, 8/13, median Tr = 24 days), whereas poisoned birds usually died (Mr = 67%, 6/9, median Td = 1 day). Only two poisoned birds could be released (median Tr = 47.5 days). A total of 37% of admissions of the WTE in our study were caused by anthropogenic factors. The research of morbidity factors points out important risk factors for this continuously threatened species in CZ.

Keywords: r*aptors; birds of prey; morbidity; rehabilitation; trauma; poisoning*

Introduction

The white-tailed eagle (WTE) (*Haliaeetus albicilla*) is classified as "critically endangered", according to annexes to Executive Regulation no. 395/1992 Coll of the Czech Republic. Adults from the breeding population in CZ are mostly sedentary (Cepák et al. 2008). After fledging, juveniles move far out in all directions and repeatedly use certain temporary settlement areas (Rymešová et al. 2021). They tend to group together, especially in winter at good foraging sites. In the Czech Republic, South Bohemia and South Moravia are important wintering sites for individuals from northern parts of Europe (Cepák et al. 2008). An increasing trend in WTE population size (Birdlife International 2021) along with an increasing public interest in birds of prey has led to the rise of ill or injured WTEs accepted for treatment in rescue stations (Müller et al. 2007). The aims of this study were to summarize causes of admission and final outcomes of WTEs treated in 20 Czech animal rescue stations over a 10-year period to identify the main morbidity risks with regard to age and seasons.

Materials and Methods

The study was performed using records from a database provided by wildlife rescue centres coordinated by the Czech Union for Nature Conservation during 2010-2020. The species, sex, estimated age, date and cause of admission, locality of finding and outcome were recorded for each individual. We divided these records by: 1) season: spring (March-May); summer (June-August); autumn (September-November); winter (December-February), and 2) age: first calendar year (1st CY, nestlings and juveniles), 2–4 CY (immatures), 5 CY and more (adults) according to Forsman (2016). For this study we categorised reasons for admission according to Molina-López (2013) into 1) trauma: collision with motor vehicles; collision with power lines; unknown collision; electrocution; fights; illegal activity and unknown trauma; 2) non-trauma: weakness; poisoning; entanglement and 3) orphaned nestling. The final outcomes were divided into three categories: 1) release to the wild; 2) euthanasia/death during care; 3)

staying permanently in captivity. These were expressed with the rates: mortality rate (Mr), release rate (Rr), and permanent captivity rate (Cr). Additional parameters such as time until death (Td); and length of stay in the centre for the released individuals (Tr) were estimated. Statistical analysis was conducted in R 3.6.3 (R Core Team 2019).

Results and Discussion

We analysed the records of 68 WTEs admitted alive to 20 rescue stations in the Czech Republic of these 26% were females, 31% were males, and 43% were undetermined. Adults represented the minority of admitted birds (25%, n = 17), compared to the first-year calendar group (37%, n = 25) and immatures (34%, n = 23). The number of admitted individuals varied between years (Mean ±SD = 6 ± 2.68, range 1 –10), with peaks in 2012 (n = 9), 2014 (n = 8) and 2016 (n = 10), 2019 (n = 9). WTEs were found significantly differently (X^2 = 10.941, df = 3, p = 0.012) throughout the year with peaks in spring (mostly immature and adult birds, but no significant difference in three age classes, X^2 = 3.5, df = 2, p > 0.05) and summer (primarily 1st CY group, X^2 = 15.308, p < 0.01, df = 2). An analysis comparing the causes and final outcomes showed the following rates: Mr = 35% (n = 24), Rr = 38% (n = 26), and Cr = 27% (n = 18). Mr was notably higher in the trauma category (46%, 18/39) compared to non-trauma (24%, 6/25). Rr was similar between the non-trauma category (52% 13/25) and orphans (50%, 2/4). Concerning age, Rr was higher for 1st CY group (56%, 14/25) and adult birds (50%, 8/16). Cr was the highest for the orphans (50%, 2/4) and similar between trauma (26%, 10/39) and non-trauma (24%, 6/25) categories. A total of 37% of WTE admission in our dataset was caused by anthropogenic factors (collision, electrocution, illegal traps, intoxication, or entanglement).

The most frequent causes of admission were trauma related (n = 39). Birds with unknown trauma had a median Td = 5 days (range= 0-120) and Tr = 58 days (range 25-88). Collision with motor vehicles dominated in 1st CY group in comparison with older birds, where collision with power lines dominated. Collision with vehicles may be associated with scavenging carcasses on roads and train tracks (Krone et al. 2003). Fights concerned adults (n = 4) and immature birds (n = 1) and had the best Rr compared to the other trauma causes (60%, 3/5). One individual had been trapped in an illegal snare and one had been shot. We assume that the unknown trauma category included cases related to anthropogenic factors as well (Krone et al. 2003, Müller et al. 2007). Trauma is one of the most common causes of admission of raptors to rescue stations throughout Europe (Müller et al. 2007, Molina-López 2013) and the seriousness of injuries had a significant effect on the outcomes across all raptor species (Maphala et al. 2021). The most frequent non-trauma causes were weakness (n = 13) and toxicosis (n = 9). The birds admitted for weakness were mostly released (Rr = 58%, 7/12, median Tr = 24 days, range = 8–153). Causes of weakness were usually not diagnosed, but 75% of cases concerned juveniles (n = 9) in the summer. The database states that some of them had digestive problems or were probably too inexperienced to hunt for prey. In one case, a parasitic infection was diagnosed. It can be assumed however, that the true prevalence of parasitic infection was higher (Krone 2000) and that weak, injured, old and young birds should all be routinely examined. The following poisons were found in a total of nine individuals: carbamate (n = 5), anticoagulant rodenticides (n = 1), botulism (n = 1) and non-identified poison (n = 2), and they were recorded mainly during the spring (n = 4) and winter (n = 4). Poisoned birds had a Mr = 67% 6/9), and median Td = 1 day. Only two of them were released (median Tr = 47.5 days). We did not encounter lead intoxication in our study, in contrast to results from Germany (Krone et al. 2003, Müller et al. 2007), which might mean

that there were no examinations of birds admitted for heavy metal poisoning. WTEs often ingest lead bullets or their fragments from carcasses (Krone et al. 2003). Lead toxicosis can be combined with trauma due to effect on motoric functions (Müller et al. 2007) and we suggest examining trauma-associated cases for heavy metals (via X-ray examination and lead blood test).

To check that rehabilitated animals can survive in the wild, they should be tracked using modern telemetry methods. Ten of 26 released WTEs were tracked by satellite telemetry (GPS / GSM loggers) and their behaviour and survival will be assessed in further research.

Conclusion

This study documents trauma as an important source of morbidity of WTE in the Czech Republic, followed by weakness and intoxication. In total, anthropogenic factors were responsible for 37% of admitted WTE cases in our study. The research of morbidity factors points out important risk factors for this continuously threatened species in CZ.

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Hibernation strategy: functions of bat-derived cell lines at low temperatures

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Summary
Heterothermy is bats' adaptive strategy of controlled regulation of their body temperature to cope with seasonal climatic extremes with unavailability of insect prey. During hibernation and daily torpor, body temperature significantly decreases. Therefore, it is essential that tissues and cells are adapted on these temperature changes. The aim of this study was to examine phagocytosis intensity, proliferation and morphology of a bat macrophage cell line at different temperatures simulating the bat body temperature during hibernation (8 °C), daily torpor (17.5 °C) and euthermy (37 °C) and survival of cells obtained from different organ under experimental temperature -20 °C in medium without externally added cryoprotectant. Bat cells are adapted for significantly decreased incubation ~ body temperature. Cells maintain viability and functions at low temperatures, but the proliferation rate and intensity of phagocytosis decrease compared to euthermic conditions. Cells at 8 and -20 °C stay round shaped, they do not adhere to the cultivation surface and form a few-number cell clusters. Cells of different tissues differ in their ability to survive an extreme decrease and a subsequent increase of the temperature. The lowest viability in vitro was observed in body core organ cells (liver), contrariwise cells close to the body surface (nerves) show high viability and a rapid regrowth. We conclude that bat cells are adapted to overcome low temperatures using hypometabolism and they start to function fully immediately after the transition to euthermia.

Key words: *thermoregulation; proliferation; cell morphology; phagocytosis; cell freezing*

Introduction

Heterothermic bats are adapted with their body functions to high seasonal variability. The benefits of mammalian hibernation or daily torpor are characterized by behavioural (Geiser and Ruf, 1995) and physiological changes at organismal, organ, cellular and molecular levels (Andrews, 2007; Carey et al., 2003). Cells and tissues at a temperature decrease use different mechanisms for protection. Water-soluble sugars (e.g. glucose), inorganic cations or amino acids have cold-protection effects (Karow, 1969). During hibernation, bats reduce metabolism up to approximately 1% of the euthermic state. However, all metabolic pathways are not reduced to the same extent (Geiser and Ruf, 1995). Immune functions are modulated by body temperature and during torpor bouts the immune system may not be fully competent to control infections (Bouma et al., 2010). Therefore, length of torpor bout and frequency of arousals must balance with the costs and benefits. If the torpor bout is too long, pathogenic agents may infect the organism without any response of the immune system. On the other hand, bats may deplete energy reserves for frequent arousals, resulting in death (Blažek et al., 2019).

Material and methods

Cell line: macrophages and nerve cells from *Myotis myotis* and liver and kidney cells from *Nyctalus noctula* were obtained and prepared as described previously (He et al., 2014). ***Proliferation assay and morphology***: 10 000 macrophage cells/per well were cultivated in 6-well plates with DMEM + 10% FBS + 1% ATB for 6 days at three different temperatures of

8, 17.5 and 37 °C. Cells were enumerated every day, stained with DAPI (Vector Laboratories, California US), visualized and photographed using a microscope Cytation 1.
Phagocytosis assay: 10 000 macrophage cells/well were incubated in 96-well plates in FluoroBrite DMEM at 37 °C overnight to allowing cells to attach to the plate surface. Then, the plates were relocated into test temperatures (8, 17.5, 37 °C) for 24 hours. Then, cells were treated by pHrodo™ Zymosan A BioParticles Phagocytosis (Thermo Fisher Scientific) in triplicate or stayed as control non-treated cells. Plates were incubated A: at three test temperatures (8, 17.5 and 37 °C) or B: at 37 °C. The level of phagocytosis was evaluated every 60 min (a total of 11 times) by fluorescence measurement at (Ex/Em) 485/528 nm using Cytation 1 imaging Multi-mode reader (BioTek, Vermont, USA).
Survival in -20 °C: cells (see above) in suspension (100 000 or 1 000 000 cells/ml/well) in 12-well culture plates, without any extra-added cryoprotectant were exposed to a freezing temperature (-20 °C) for 24 hours in 3 different DMEM media with various levels of glucose (24; 16 and < 1 mmol/L glucose) and counted after gradual thawing and also examined for viability using cultivation for the next 3 days at 37 °C.

Results and discussion
Proliferation of the cell culture is significantly influenced by the temperature of incubation. The highest number of cells and the rapidity of mitotic activity was observed at 37 °C (Fig. 1). At euthermic conditions, the metabolism is fully active, including proliferation and tissue remodelling (Carey et al., 2003). At a low temperature, proliferation rapidity decreases, but the cells retain their viability and adapt to the temperature change (Tamura et al., 2006). Shape modification (i.e. reduction of surface) can probably decrease energy/heat loss.

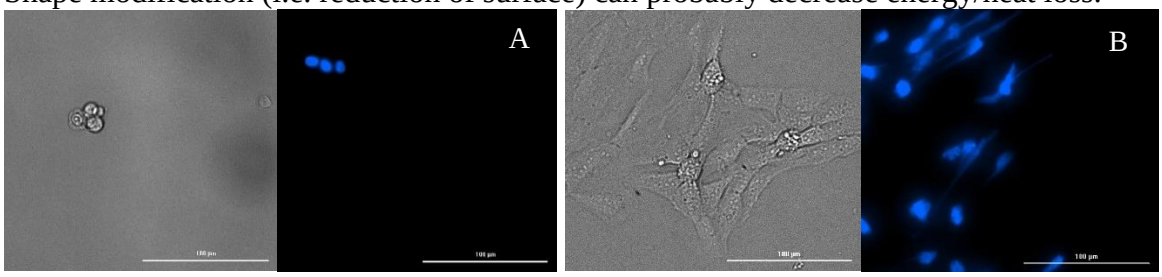

Figure 1. Morphology of macrophage cell line/nuclei of cells after 72 hours of incubation *in vitro* at 8 °C (A) and 37 °C (B). After incubation: 7 000 cells at 8 °C versus 43 000 cells at 37 °C.

Phagocytosis of zymosan particles by macrophages is significantly influenced by the actual temperature (Fig. 2a) during the phagocytic activity of cells, but not the pre-incubation temperature (Fig. 2b). It seems that macrophages can be ready to protect the organism against infectious agents immediately after torpor periods and partly during the torpor period. This is important because hibernating bats carry pathogenic agents which may proliferate when the animal becomes euthermic (Bouma et al., 2010)

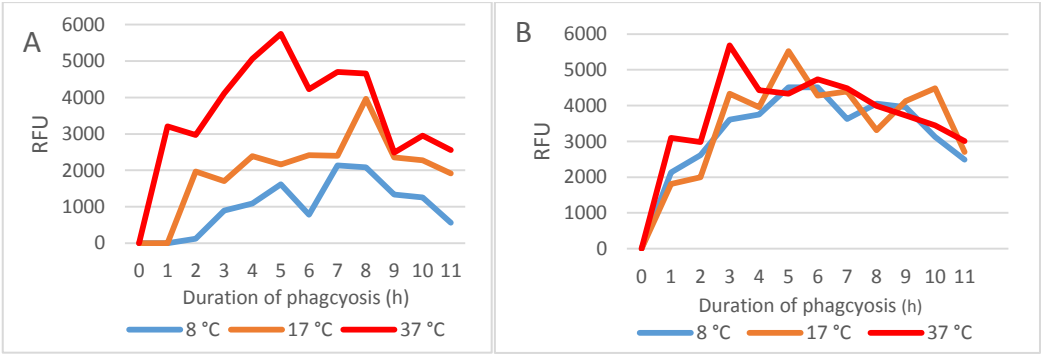

Figure 2. Phagocytosis intensity (relative fluorescence units) according to Phagocytosis assay A and B, respectively.

Cells of different tissues cultured *in vitro* differ in their ability to adapt and survive an extreme decrease and a subsequent increase of temperature. Generally, the glucose in medium helps to survive at low temperatures (Table 1), but we assume that the cells need a certain amount of glucose and higher levels have no added effect. However, cells in medium without glucose can also survive. There must be some other mechanisms that contribute to cell survival at low temperatures.

Table 1. Number of surviving cells obtained from different tissues after 24 hours at -20°C in DMEM medium without any added cryoprotectant with various glucose level.

	surviving neural cells			surviving macrophages			surviving kidney cells			surviving liver cells		
glucosis level (mmol/L)	<1	16	24	<1	16	24	<1	16	24	<1	16	24
100 000 cells	3 000	14 000	5 000	2 000	11 000	7 000	2 000	6 000	4 000	500	5 000	2 000
1 000 000 cells	85 000	257 000	206 000	54 000	89 000	86 000	31 000	73 000	43 000	3 000	54 000	36 000

Conclusion

Bat cells *in vitro* are adapted to overcome low temperatures. Cells retain their viability and functions at low temperatures, but the intensity of cell functions (mitotic activity, phagocytosis) decrease compared to euthermic conditions, corresponding to the metabolism at organismal level (Bouma et al., 2010; Carey et al., 2003). Full activity is re-established immediately after relocation into optimal conditions. Cells use their own adaptability (shape) as well as external factors (glucose) to overcome low temperature periods. Cells of different tissues differ in their ability to survive an extreme decrease and a subsequent increase of temperature. The lowest viability *in vitro* was observed in body core organ cells (liver), contrariwise, nerve cells show high viability and rapid regrowth.

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Reprodukční strategie samic netopýra rezavého (*Nyctalus noctula*)

Reproductive strategy in Noctules (*Nyctalus noctula*)

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Summary
The reproductive strategies of European insectivorous bats differ from mammalian standards due to the use of so-called delayed fertilization. Its phenology is influenced by environmental conditions and can vary in different latitudes, between years at the same locality and inter-individually within a colony. In pregnant Noctule females (Nyctalus noctula), we determined plasma progesterone levels by ELISA and evaluated embryo development by ultrasound. Females were randomly divided into two groups with different lengths of hibernation (control group versus experimental group with hibernation 7 days longer). The majority of females gave birth to a single pup (65.0 %), however, one female aborted twins two days before the first successful births by other females. Plasma progesterone levels were higher in females with twins in the first stage of pregnancy and also after births. In the middle stage, progesterone levels reached the highest values (app. 700 ng/ml) and did not differ between females with singletons and twins. In 6 cases, we supposed embryonic mortality or resorption of the fertilized egg. The length of pregnancy differed significantly between the two groups (48 versus 50 days). Longer hibernating females (experimental group) had a shorter pregnancy, but the size of their pups did not differ from the control group at the end of pregnancy.

Key words: *reproduction; progesterone; bat; gestation length; ultrasound*

Úvod

Životní strategie savců se obvykle liší v závislosti na velikosti jejich těla. Drobní savci mívají obecně početné vrhy malých mláďat, která rychle rostou a dospívají, ale žijí krátce. Naopak velcí savci rodí malý počet mláďat, jejichž dospívání je pomalé a tito jedinci jsou dlouhověcí. Netopýři jsou v tomto ohledu neobvyklí, neboť se jakožto malí savci vyznačují dlouhověkou životní strategií. Ačkoli většina druhů netopýrů váží méně než 100 g, samice rodí pouze jednou ročně jedno nebo dvě mláďata. Nicméně není výjimkou, že se některé druhy dožívají více než 30 let.

Z hlediska teorie optimální strategie je klíčovým faktorem pro samice kompromis mezi investicí dostupných energetických zdrojů do okamžité reprodukce, při níž může dojít k riziku úmrtí jak matky, tak mláděte a investicí do sebe sama, a tedy zvýšenou možností úspěšně se reprodukovat v budoucnosti. Tento takzvaný trade-off je o to důležitější u druhů, které obývají temperátní zónu, kde načasování reprodukce silně podléhá enviromentálním podmínkám (Willis 2017). Navíc u netopýrů panuje obecný předpoklad, že díky využívání denní strnulosti není doba březosti fixní, ale může se měnit dle aktuálních klimatických podmínek (Eisentraut 1936).

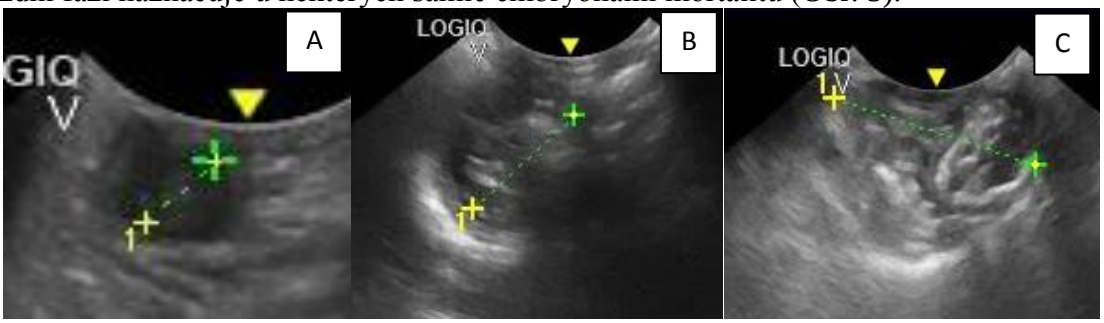
Netopýr rezavý je běžný palearktický druh, který se v České republice vyskytuje celoročně. Reprodukční strategie netopýra rezavého se vyznačuje využíváním tzv. opožděného oplodnění. Při páření, ke kterému dochází na podzim, si samice netopýrů před hibernací ukládají sperma ve žlázách dělohy. K ovulaci a samotnému oplodnění vajíčka, které jsou stimulovány hormonálně po změně tělesné teploty na konci hibernace, dochází ve střední Evropě obvykle koncem dubna (Gaisler et al. 1979). Samice v přirozených podmínkách rodí

koncem června až začátkem července 1 nebo 2 mláďata, což odpovídá délce březosti až 73 dní (Eisentraut 1936).
Důležitou roli v plodnosti savčích samic hraje tvorba a vylučování hormonů a jejich vzájemné ovlivňování. Jde o dráhu hypotalamus – hypofýza – pohlavní orgány. V různých fázích estrálního cyklu lze měřit koncentraci specifických hormonů v krvi (estrogeny, progesteron, folikulostimulační hormon apod.). Za udržení březosti je zodpovědný progesteron, který je zpočátku produkován žlutým tělískem, později jeho funkci přebírá také placenta. Množství progesteronu v krevní plazmě některých savců může odrážet počet embryí (Ranilla et al 1997).
Cílem našeho výzkumu bylo otestovat následující dvě hypotézy: 1. Délka březosti a termín porodu se bude lišit mezi samicemi s různou dobou výstupu z hibernace, a to přesně o dobu odpovídající rozdílné délce hibernace. 2. Hladiny progesteronu se budou lišit u samic, které budou mít jedno nebo dvě mláďata.

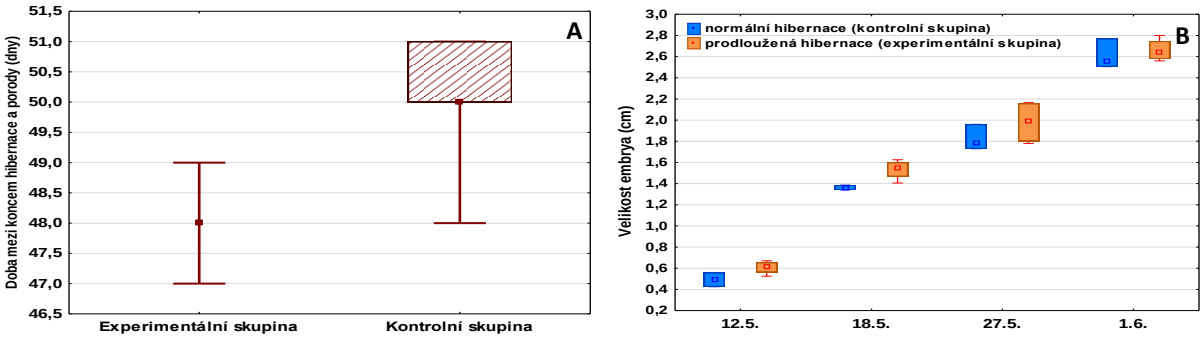
Materiál a metody
Při zateplování budovy polikliniky ve Velkém Týnci bylo zachráněno 29 jedinců netopýra rezavého, přičemž 7 z nich bylo možné vypustit. Celkem 22 samic bylo nutné přijmout do veterinární péče, přičemž dvě samice uhynuly. Dne 14. 4. 2020 byli jedinci změřeni, zváženi, individuálně označeni a náhodně rozděleni do dvou skupin s různým dnem ukončení hibernace. Kontrolní skupina A (zvířata číslo 2, 7, 10, 11, 15, 16, 17, 18, 19, 21, 22) byla umístěna do dřevěného boxu a držena při pokojové teplotě (22 °C) a pravidelně krmena. Experimentální skupina B (zvířata číslo 1, 3, 4, 5, 6, 8, 9, 12, 13, 14, 20) byla vrácena do umělého hibernakula (6 °C) na dobu 7 dní a poté umístěna do stejného dřevěného boxu spolu s jedinci skupiny A. Zvířata ve skupině A jsou tak považována za jedince s normální dobou hibernace, zvířata ve skupině B za jedince s prodlouženou dobou hibernace.
Ve 3 termínech reprezentujících počáteční fázi březosti (duben), kdy dochází k ovulaci a uhnízdění oplodněného vajíčka, pozdní fázi březosti (květen) a dobu po porodu (červen) byla samicím odebírána krev z cévy na křídelní bláně (propatagium). Místo odběru bylo vydezinfikováno etanolem a céva byla napíchnutá sterilní jehlou. Krev byla následně odebrána pomocí sterilní heparinizované špičky automatickou pipetou do připravené zkumavky. Po odběru byly vpichy ošetřeny tkáňovým lepidlem značky Surgibond. Odstředěná plasma byla uchovávána v mrazících boxech při teplotě -20 °C a následně byla zjišťována hladina progesteronu pomocí Progesteron ELISA kitu (Enzo Life Sciences Inc.). Vyšetření ultrazvukem (LOGIQ V2, GE Healtcare, USA) bylo prováděno ve veterinární ordinaci v týdenních intervalech od 18. 5. do 1. 6. 2020. Statistická analýza byla provedena pomocí programu Statistica for Windows 13.2 (StatSoft, Inc. USA).

Výsledky
Sonogramy ukazují postupně se vyvíjející plod samice č. 14 (Obr. 1A – C). Plod starý 21 dní měří 0,5 cm. Struktura je patrná jen jako tmavší váček kulovitého tvaru (Obr. 1A). Plod starý 27 dní již měří 1,64 cm a začíná vykazovat klasický tvar fetu (Obr. 1B). Plod starý 41 dní dosahuje délky 2,32 cm, jsou u něj již rozeznatelné základní struktury jako kraniální část s pohyblivými čelistmi, pumpující srdce, *chorda dorsalis* a osifikované antebrachium (Obr. 1C). Všechny samice porodily v rozmezí 47 až 51 dní. První předčasný porod nastal 1. 6. 2020 (2 dny před prvním úspěšným porodem), kdy samice porodila mrtvá dvojčata. Délka březosti se signifikantně lišila mezi skupinami A a B (Mann-Whitney test; $p < 0{,}001$) (Obr. 2A), nicméně velikost plodů se na konci březosti nelišila (Mann-Whitney test; $p = 0{,}480$) (Obr. 2B).
Hodnoty progesteronu vykazují 3 různé modely odpovídající různým reprodukčním strategiím samic. Samice, které porodily dvojčata, měly vyšší hladiny progesteronu na

začátku březosti a po porodu (Obr. 3 a 4) než samičky s jedním mládětem. V pozdní fázi březosti dosahovaly hladiny progesteronu nejvyšších hodnot, a nelišily se mezi samicemi s jedním nebo dvěma embryi. Vysoká hladina progesteronu na začátku březosti a její pokles v pozdní fázi naznačuje u některých samic embryonální mortalitu (Obr. 5).



Obr. 1 Sonogramy embryí netopýra rezavého ve věku 21 dní (A), 27 dní (B) a 41 dní (C).

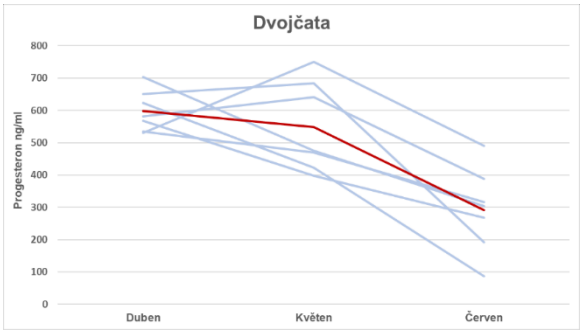


Obr. 2 Srovnání délky březosti (A) a velikostí embryí (B) mezi kontrolní a experimentální skupinou.

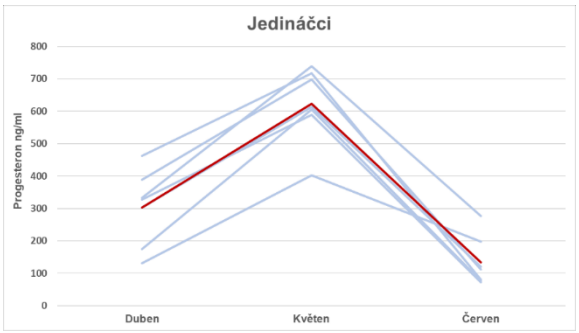
Diskuse

V mírném pásmu, kde jsou netopýři nuceni vyrovnat se s obdobím nedostatku potravy a zvýšených energetických nákladů, hraje načasování reprodukce zásadní roli. Samice, které vylétávají z hibernace, jsou v rané fázi březosti, přičemž v této době může být potravní nabídka až desetkrát nižší než v pozdním létě (Racey et al. 1987). K úspoře energie mohou využívat denní strnulost (torpor), která zároveň zpomaluje fyziologické procesy, včetně vývoje plodu. Samice tak musí přizpůsobit svoji termoregulaci optimálnímu vývoji plodu. Dzalová a Brigham (2013) u druhu *Eptesicus fuscus* potvrdili, že březí samice minimalizují dobu strávenou v denním torporu, aby zachovaly vývoj plodu. Samice v našem experimentu byly drženy při příznivých teplotních podmínkách s dostatkem potravy, proto pravděpodobně neupadaly do denní strnulosti a jejich doba březosti dosahovala v průměru pouze 49,2 dne podobně jako zjistil Asdell (1946). Některé práce (Racey 1969) prokázaly, že samice evropských druhů netopýrů, které upadly během březosti do torporu, rodily později přesně o dobu, kterou v torporu strávily. Naše výsledky naopak ukazují, že samice, které strávily v torporu o týden déle, rodily v průměru pouze o 4 dny později. Zkrátily teda dobu březosti o 3 dny, aby umožnily mláďatům delší dobu vývoje. Tomuto předpokladu odpovídá i studie u *Rhinolophus ferrumequinum* ukazující, že mláďata narozená dříve mají vyšší pravděpodobnost přežití (Ransome & McOwat 1994).

Netopýři jsou druhou nejdiverzifikovanější skupinou savců na světě s mnoha reprodukčními strategiemi. Naše data na základě hladin progesteronu naznačují, že samice netopýra rezavého dokáží manipulovat s počtem donošených embryí. Pouze u druhu *Noctilio albiventris* byl histologicky potvrzen zánik luteinizovaného folikulu (Rasweiler 1984). Například u lemurů, kteří také mohou využívat torpor, je počet mláďat ve vrhu ovlivněn množstvím dostupné potravy (Canale et al. 2012). Proto je důležité posuzovat rozmnožovací strategie heterotermů na úrovni jedince, nikoliv celé populace (Dammhahn et al. 2017).



Graf 3. Hladiny progesteronu samic *Nyctalus noctula*, které porodily dvojčata. Průměrné hodnoty za celou skupinu jsou označeny červeně



Obr 4. Hladiny progesteronu samic *Nyctalus noctula*, které porodily jedno mládě. Průměrné hodnoty za celou skupinu jsou označeny červeně.

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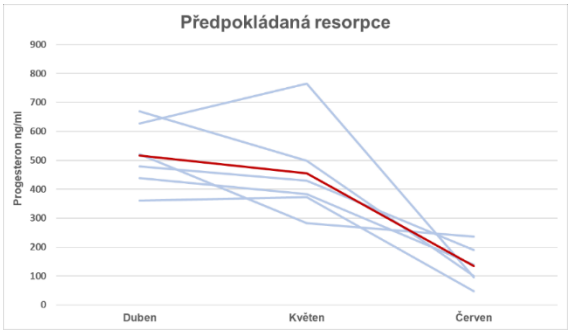

Obr 5. Hladiny progesteronu samic *Nyctalus noctula*, které porodily jedno mládě, ale vysoké hodnoty progesteronu v počáteční fázi naznačují embryonální mortalitu. Průměrné hodnoty za celou skupinu jsou označeny červeně.

Závěr

U netopýra rezavého nebyly dosud popsány hladiny progesteronu. V této studii jsme potvrdili dynamiku vývoje progesteronu u samic *Nyctalus noctula* s různou rozmnožovací strategií odrážející jejich reprodukční úspěšnost. Tyto informace jsou využitelné pro hodnocení vývoje populací netopýrů s ohledem na jejich ochranu.

Poděkování

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Distribution, prevalence and host species of Snake Fungal Disease in the Czech Republic

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Summary
New emerging diseases add to many other causes of the global decline of species diversity. Significant population declines of wild snakes caused by fungus Ophidiomyces ophiodiicola have been recorded since 2006 in the eastern United States. This parasitic fungus, the causative agent of "Snake fungal disease", was detected for the first time in continental Europe at the Brno reservoir in 2017. This study aimed to evaluate the presence of Ophidiomyces ophiodiicola in snakes in the Czech Republic and to determine its effect on the population of the critically endangered dice snake (Natrix tessellata) at the Brno reservoir.

Keywords: *Snake Fungal Disease; Ophidiomycosis; Natrix; Czechia*

Introduction
Snake Fungal Disease (SFD) is a dermatomycosis of snakes caused by the ascomycete *Ophidiomyces ophiodiicola*. SFD is considered a serious emerging disease that causes the mortality of wild snakes with an impact on their populations (Allender et al., 2015a; Lorch et al., 2015).

The first records of SFD are from a museum specimen collected in 2000 from southern Illinois (Allender et al., 2016). Beginning in 2006, the population declines of timber rattlesnake (*Crotalus horridus*) were reported in association with severe skin infections. Nowadays the disease is one of the main problems in the conservation of wild snakes in North America (Allender et al., 2011). The symptoms of the disease include most commonly yellow to brown crusts, scale discolouration, subcutaneous nodules, skin ulceration and increased ecdysis frequency. These skin lesions are the site of entry for secondary bacterial infection, and in these cases, the disease often results in a death of the animal (Lorch et al., 2015). SFD has now been recorded in snake families Boidae, Pythonidae, Acrochordidae, Colubridae, Elapidae and Viperidae (Tetzlaff et al., 2015; Allender et al., 2015b; Lorch et al., 2016).

In the Czech Republic, the pathogen was isolated from a sample of dice snake (*Natrix tessellata*) from the Brno reservoir (Franklinos et al., 2017). The effects of this disease on the population of wild snakes in the Czech Republic have not been recorded so far (Allain *et al., 2019).*

The work aimed to determine the host species, distribution and prevalence of SFD in snakes in the Czech Republic. Main effort was concentrated to describe the situation of *O. ophiodiicola* presence and impact at the Brno reservoir. On the collected material and data we tried to evaluate which variables (e.g. sex, physical condition, location) are linked to higher probability infection or manifestation of the disease.

Materials and Methods
The sampling was conducted from April to October 2020, and a small part of the samples was available from the previous year. For easier handling, we used special snake tongs. To prevent transmission of the infection, disposable gloves were used at all times when animals were

handled. To increase effectiveness of the search for the snakes we installed 30 artificial shelters along the shoreline of the reservoir. We collected data on body length, weight, age category, sex and presence of any clinical signs from each captured specimen. Every individual wasphoto documentated for later identification of recaptures. Samples for SFD detection were collected by sterile swabs moistened with deionized water. Ventral shields, crypts between scales and pathological lesions, if present, were swabbed. The samples were transported in tubes with silica gel and stored at -20 ° C for further processing.

DNA was isolated with simple and fast protocol including homogenization with zirconium beads and subsequent treatment with PrepMan buffer (Bohuski et al., 2015). To detect the pathogen, we used quantitative polymerase chain reaction (qPCR) method with a specific probe for *O. ophiodiicola* targeting the ITS region (4). We used 10× diluted DNA isolates for the reaction because concentrated PrepMan has a slight PCR-inhibitory effect. The reaction mixture contained 5,75 μl H_2O, 12,5 μl Roche Probes Master (2×), 0,5 μl Primer-F (20nM), 0,5 μl Primer-R (20nM), 0,25 μl SFD probe (20nM), 0,5 μl bovine serum albumin and 5 μl sample. Each sample was analysed in duplicate. The gBlock fragment of the linear dsDNA was used as a positive control and quantification standard. For qPCR, we used a LightCycler 480 and a program: initiation of 95 ° C for 3 minutes, then 50 cycles of denaturation of 95 ° C for 3 seconds and 60 ° C for 30 seconds of amplification. Fluorescence was measured at the end of each amplification step (Bohuski et al., 2015). Confidence intervals of prevalence were calculated in the QuantitativeParasitology program. The data on body length, weight, age category, sex and the presence of skin lesions were compared with qPCR results in MS Excel.

Results

In total, we collected 308 samples from all species of snakes living in the Czech Republic (*Coronella austriaca*, *Natrix natrix*, *N. tessellata*, *Vipera berus*, *Zamenis longissimus*). Using qPCR, we identified 51 positive individuals from both *Natrix* species. We detected a low signal in one individual of the species *C. austriaca*. The Brno reservoir was the main study locality, but we accidentally found *O. ophiodiicola* in *N. natrix* in the Krušné mountains. In the Podyjí National Park, we did not detect *O. ophiodiicola* in any of the 17 *N. tessellata* and 2 *Z. longissimus.* By studying the population of *N. tessellata* in the Brno reservoir, we recorded more males than females in a ratio of 1.4: 1. The average size of the sampled individuals was 592.4 mm and 76.7 g, with a range of min-max 170 - 980 mm and 4 - 950 g. We did not observe any significant differences in the prevalence of SFD between sexes. The intensity of infection in positive snakes was not correlated with their body size. The presence of skin lesions is not reliable for the diagnosis of the disease. The individuals without lesions often showed very low intensity of infection, while up to 54 percent of individuals with lesions were qPCR negative. We did not observe any dead individuals with pathognomonic symptoms of SFD.

Discussion and Conclusion

The cause of fungal dermatitis of snakes, *O. ophiodiicola*, is in the Czech Republic to a much greater extent than was previously known. However, its expansion is probably not throughout the Czech Republic and it is necessary to continue the study. Quite surprising is the high prevalence of the pathogen in the population of critically endangered dice snake *(N. tessellata*) in the Brno reservoir. We regularly found individuals with distinct lesions in this population. However, the disease in this area does not cause mass deaths as observed in the United States. Our results revealed the presence of the pathogen in two other species of snakes – *Natrix natrix*, *Coronella austriaca.*

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Molecular detection of *Rickettsia* sp. in ticks from wildlife animals living in six provinces of South Africa

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Summary
In total, 854 ticks of different species from six provinces of South Africa were screened for the presence of Rickettsia sp. by a single PCR assay targeting the gltA gene using the primers CS 78 and CS 323. The DNA of Rickettsia sp. was detected in 144 samples (17 %) with 18% in females, 13% in males, 26 % in nymphs (26%) and 18% in larvae. The highest prevalence of Rickettsia sp. was in Free state Province (50%) and in ticks collected in spring (22%) and autumn (19%). Positive samples will be sent for sequencing for accurate identification. The results of this study bring new knowledge about the prevalence of this pathogen in ticks in six provinces of South Africa and may be the basis for more extensive research in South Africa.

Keywords: *PCR; Tick-borne diseases; Wildlife animals; Rickettsiosis*

Introduction

Members of the genus *Rickettsia* are small, obligate intracellular, Gram-negative bacteria that are distributed throughout the world. The lifecycle of *Rickettsia* species involves arthropod vectors (ticks, fleas, mites, and lice) and vertebrate hosts including humans. The infection can be transmitted through arthropod bites and can cause health problems to the animals and humans, because it is widespread „tick-borne diseases" zoonoses. Human rickettsiosis may show clinical symptoms that range from fever, rash, myalgia, headaches meningitis, endocarditis, to lymphadenopathy/ lymphangitis (Dongyou, 2015).
The aim of the thesis was to detect *Rickettsia* sp. in ticks from South Africa.

Material and Methods

Ticks were collected during the years 2012-2019 in six provinces of South Africa including Limpopo, Mpumalanga, Free State, Northern Cape, North West, and Gauteng Province. Ticks were taken from dead animals (most often because of a collision with a car) e.g. from Serval, African Civet, Banded Mongoose, Lion, or more rare types like Bush Pig, Chacma Baboon, Hippopotamus or Puff Adder. In total 2003 ticks (154 females, 778 males, 454 nymphs and 617 larvae) were collected and divided into 854 samples. There were following species of ticks: *Amblyomma hebraeum, atum, marmoreum* and *tholloni; Rhipicephalus appendiculatus, fallus, simus, theileri, zambeziensis; Heamaphysalis spinulosa group, zumpti, elliptica; Hyalomma truncatum* and *Ornithodoros eboris*. The DNA from ticks was isolated by NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) to detect *Rickettsia* sp. by single PCR using primers CS 78 and CS 323 to amplify gene gltA (Labruna et al., 2004). Samples positive for *Rickettsia* sp. will be purified and confirmed by Sanger sequencing (Macrogen).

Results and Discussion

The total prevalence of *Rickettsia* sp. in ticks was 17%. The results and characteristics of ticks are summarized in Table 1.

Table 1: Detailed characteristics of ticks tested for *Rickettsia* sp.

Characteristics	**Total number**	**Positive (%)**
Gender		
Female	230	41 (18%)
Male	479	64 (13%)
Nymph	99	26 (26%)
Larvae	46	13 (18%)
Year		
2012	5	1 (20%)
2013	58	11 (19%)
2014	110	13 (12%)
2015	255	70 (27%)
2016	217	33 (15%)
2017	122	8 (7%)
2018	56	6 (11%)
2019	31	2 (6%)
Season		
Spring	218	48 (22%)
Summer	266	41 (15%)
Autumn	131	25 (19%)
Winter	163	21 (13%)
Unknown	76	9 (12%)
Locality		
Free State Province	4	2
Gauteng Province	13	0
Limpopo Province	617	102 (17%)
Mpumalanga Province	213	40 (19%)
North West Province	1	0
Northern Cape Province	1	0
Unknown	4	0
Species		
Amblyomma sp.	175	56 (32%)
Haemaphysalis sp.	392	7 (2%)
Hyalomma sp.	3	0
Ixodes sp.	5	2 (40%)
Ornithodoros eboris	1	1
Rhipicephalus sp.	278	78 (28%)
Total	854	144 (17%)

In this study, the prevalence of *Rickettsia* sp. was lower comparing to the prevalence (49%) in ticks from Tanzania (Kim et al., 2018) or to the prevalence (27% and 37%) obtained in two studies from South Africa (Mitshali et al., 2015; Mitshali et al., 2017). The prevalence was higher in females (18%) compared to males (13%), and higher in nymphs (26%) compared to the larvae (18%). Relatively high *Rickettsia* sp. prevalence (27%) was in year 2015 compared to 2016 (15%). The highest prevalence was in ticks collected in spring (22%) and autumn (19%). The low prevalence (13%) in winter season corresponds with the fact, that the highest number of ticks is occurring in spring and summer. The highest prevalence (50%) of *Rickettsia* sp. was in Free State Province that was similar with 52% detected in ticks from the same province (Mitshali et al., 2017). There were differences in other provinces: in North West and in Mpumalanga Province was 0% and 19% in ours study, compared to 38% and 0% in study of Mitshali et al. (2017). The prevalence of tick species *Amblyomma* (32%) was in the middle of the prevalence (16% and 51%) in ticks from Kenya (Macaluso et al., 2003) and ticks from Tanzania, respectively (Kim et al., 2018).

Conclusion

In this study, there are current data on the prevalence of *Rickettsia* sp. in ticks in South Africa. These results will be expanded with statistics and sent for sequencing for accurate identification.

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Detekce viru deformovaných křídel včely medonosné (*Apis mellifera* L.) v České republice

Detection of honey bee (*Apis mellifera* L.) infecting Deformed wing virus in Czech Republic

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Summary
Deformed wing virus (DWV) is a honey bee (Apis mellifera L.) infecting virus commonly present in colonies without obvious signs of ongoing infection. Characteristic symptoms of ouvert infection are limb and wing deformities, paralysis and high mortality of emerging honey bees. Due to the high mutation rate of +ssRNA viruses, DWV exists in a form of viral quasi-species, resulting in detection dificulties. In this study, two types of samples, adult honey bees and hive derbis were examined using two different detection methods (PCR and real-time PCR), each with few different pairs of primers. Detection rate of DWV was as high as 92,9% of all samples, with differences in detection success rates in different approaches. The most successful detection method was the use of real-time PCR targeting the helicase region of the virus. Additionally, honey bee samples were examined for the presence of two DWV quasi-species master variants, DWV-A and DWV-B. The results indicate that DWV-B is the dominant variant of DWV in the Czech Republic, while DWV-A mostly occurs in form of coinfection with DWV-B.

Keywords: *Apis mellifera; Deformed wing virus, molecular epidemiology, PCR*

Úvod

Virus deformovaných křídel (DWV) je celosvětově rozšířeným včelím virem. Mezi charakteristické příznaky symptomatické infekce patři deformace křídel a končetin, paralýza a vysoká mortalita nově vyvinutých včel. Virus je blízce asociovaný s parazitem *Varroa destructor* v kontextu kolapsu včelstva (de Miranda a Genersch, 2010). Jakožto jednořetězcový RNA virus podléhá často mutacím a existuje ve formě tzv. quasi-species – v důsledku mutací a přírodního výběru existuje vysoká genetická heterogenita s několika hlavními variantami. V současné době jsou rozeznávány tři hlavní varianty DWV: DWV-A, DWV-B, DWV-C (Mordecai et al., 2016). Genetická variabilita však může znesnadňovat detekci těchto virů, která je nejčastěji prováděna metodami molekulární biologie.

Materiál a metody

Na přítomnost DWV bylo vyšetřeno celkem 521 vzorků odebraných v roce 2020. Vzorky byly odebírány na různých geografických oblastech České republiky v rámci několika různých epidemiologických studií. Odebraný materiál zahrnoval především úlovou zimní měl (356 vzorků), dále také dospělé včely (165 vzorků). Z odebraného materiálu byly extrahovány nukleové kyseliny za použití TRIzol™ Reagent (ThermoFisher Scientific, USA). Nukleové kyseliny extrahované ze vzorků měli byly následně přečištěny, aby byly eliminovány látky inhibující PCR enzymy. Extrahované nukleové kyseliny byly přepsány do cDNA reverzní transkripcí. Pro přepis byla použita sada ProtoScript II First Strand cDNA Synthesis Kit (New English BioLabs, USA). Se syntetizovanou cDNA bylo provedeno PCR pro zmnožení cílových fragmentů za pomocí OneTaq® Quick-Load® 2X Master Mix with Standard Buffer (New England BioLabs, USA). V reakci byly použity dříve publikované primery, dále

označené jako DWV1, DWV2, DWV3 (Berényi et al., 2007). U včelích vzorků byly použity i dříve publikované primery pro určení dvou z hlavních podtypů viru, DWV-A a DWV-B (Bradford et al., 2017).

Dále byla s extrahovanými nukleovými kyselinami provedena real-time PCR s použitím sady Luna® Universal One-Step RT-qPCR kit (New England Biolabs, USA). Detekovány byly dva různé cíle virového genomu – oblast kódující RNA-dependentní RNA polymerázu (RdRP) a helikázu. V reakci byly použity dříve publikované komponenty (RdRP sonda a primery, dále v textu značené jako RdRP a Heli) (Forsgren et al., 2009) i komponenty vlastního designu (helikázová sonda).

Výsledky a diskuze

Z celkového počtu 521 vyšetřovaných vzorků se podařilo DWV detekovat alespoň jednou z použitých metod u 484 z nich (92,9 % vzorků), z toho ve 333 případech šlo o vzorky měli (93,54 % ze vzorků měli) a ve 151 o vzorky včel (91,56 % ze vzorků včel). Z primerů použitých při detekci viru z cDNA byl virus zachycen nejčastěji při použití DWV1, a to u 94 (26,4 %) vzorků měli a 110 (66,6 %) vzorků včel. Dále byl za použití primerů DWV2 virus detekován u 69 (19,38 %) vzorků měli a 61 (36,97 %) vzorků včel, v případě primerů DWV3 šlo o 63 (17,70 %) vzorků měli a 41 (24,85 %) vzorků včel. Přítomnost viru byla za použití těchto postupů indikována u celkem 102 vzorků měli (28,65 %) a 114 vzorků včel (69,09 %). Při detekci hlavních genetických skupin těchto virů u vzorků dospělých včel byla přítomnost varianty DVW-A zjištěna u 58 (35,15 %) vzorků, více zastoupená byla varianta DVW-B, kterou se podařilo detekovat u 101 (61,21 %) vzorků, celkem byla detekce úspěšná u 116 (70,30 %) vzorků dospělých včel. Ve 43 případech byly obě tyto varianty přítomny současně, což odpovídá 37,07 % vzorků pozitivních na DWV-A nebo DVW-B.

Vzhledem ke svojí citlivosti byla detekce pomocí real-time PCR častější. Pomocí primerů RdRP byla přítomnost viru zaznamenána u 240 (67,42 %) vzorků měli a 108 (65,46 %) vzorků včel, v případě primerů pro helikázovou oblast šlo o 304 (85,39 %) vzorků měli a 149 (90,3 %) vzorků včel. Alespoň jeden z cílů byl úspěšně identifikován u 321 (90,17 %) vzorků měli a 149 (90,30 %) vzorků dospělých včel.

V českých vzorcích z let 2006–2009 byl DWV nejčastěji detekovaným včelím virem. Jeho přítomnost byla prokázána u 31 % vyšetřených vzorků (Ryba et al., 2012). U českých vzorků z roku 2016 byl tento virus také detekován nejčastěji ze sledovaných virů; 39,62 % vyšetřovaných vzorků bylo DWV-pozitivních (Čukanová, 2019). V rámci těchto prací byla použita PCR. V rámci této práce bylo zjištěno vyšší procento zastoupení DWV-pozitivních vzorků, záchyt se však mezi jednotlivými primery značně lišil. Potvrzuje se proto, že pro virové quasi-species je vhodnější detekovat více cílů a maximalizovat tak možnost záchytu virus pozitivních vzorků. Real-time PCR procento DWV-pozitivních vzorků značně navýšila díky své vyšší citlivosti. Pomocí této procedury byl virus detekován v extrémně vysokém procentu vzorků, zejména za použití primerů a sondy cílících na oblast kódující helikázu. Tato oblast se na základě výsledků zdá být více konzervovaná, než oblast kódující RdRP.

V rámci této práce byly kromě běžně vyšetřovaných vzorků dospělých včel pro detekci využily i vzorky zimní měli. Odběr měli je jednoduchý a neinvazivní a vzhledem k úspěšným detekcím včelích virů DWV (v rámci této práce), ABPV, CBPV a SBPV (Čukanová et al., 2020) může představovat vhodnou alternativu k odběru včel za účelem virového screeningu.

Závěr

Virus deformovaných křídel se podařilo nejčastěji detekovat pomocí real-time PCR s primery a sondou navrženými pro oblast kódující helikázu. Detekce viru byla úspěšná v obou typech vyšetřovaných vzorků (dospělé včely, měl), a to s obdobnými procenty virus-pozitivních vzorků. DWV-B byl ve vzorcích dospělých včel z různých geografických oblastí České republiky častěji detekovanou variantou viru. Druhá varianta, DWV-A, byla detekována především při současné infekci DWV-B.

Poděkování

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Immune reaction of Koi carp and Amur wild carp to Koi herpesvirus infection

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Summary
Common carp is one of the most economically important freshwater fish in the world. Koi herpesvirus (KHV) causes serious disease affecting common carp and its breeds. In this study, we focused on revealing influence of immune reaction (IR) on diametrically different mortality between chosen breeds of common carp. We have chosen koi carp (koi), susceptible to KHV and its highly resistant wild variety Amur carp (AS) as model organisms. We measured levels of expression of selected genes related to antiviral IR, with focus on class I interferon (IFN-I) signalling. We detected overexpression of IFN-I gene in lymphatic organs of AS in early phase of infection. On the other hand, we observed increased expression of some IR related genes in later phase of infection in koi carp samples. Further, we found out in infected fish generally increased expression of *mx* gene co-inducing antiviral state in cell.

Keywords: *Cyprinid herpesvirus 3; qRT-PCR;class I interferon*

Introduction

Importance of aquaculture production increases every year with growing global population. Common carp (*Cyprinus carpio* Linnaeus, 1758) is the fifth most produced freshwater fish in the world (FAO, 2020). Common carp suffers from numerous diseases, of which viral diseases are probably the most serious. Koi herpesvirus (also Cyprinid herpesvirus 3; CyHV-3) is etiological agent causing serious disease (KHVD) of common carp. KHVD is typical with very high morbidity and mortality. KHVD is seasonal disease, which manifests itself at temperatures between 15-28°C. Except water temperature, susceptibility to KHV is influenced by numerous factors, such as age, breed, size and overall physical state of carp (Piackova *et al.*, 2013). To present, there is neither KHV treatment nor vaccine allowed in EU (Bergmann *et al.*, 2020; Pokorova *et al.*, 2005).
IFN-I signalling plays irreplaceable role in antiviral immune reaction (Magnadottir, 2010; Langevin *et al.*, 2013). Purpose of this study was to measure levels of expression of the most promising representatives of IFN-I, *e.g.*: *ifn a3*, member of class I IFNs, cellular receptor of dsDNA *tlr9* (Toll-like receptor 9), whose activation leads also to IFN-I production and *mx* (Myxoma resistance), which participate in set up of antiviral state in cell and may suppress or inhibits viral replication.

Material and methods

Two breeds of common carp were chosen as experimental organisms, highly KHV resistant breed- Amur wild carp (AS), and KHV susceptible breed- koi. Individuals of both carp breeds were divided into control and infected group. Carps from control groups were injected intraperitoneally with virus free media. Fish from infected groups were injected intraperitoneally with dosage 10^4 $TCID_{50}$/ml of KHV suspension. Both groups were kept in separated water tanks with the same temperature 23°C for 7 days. Further, samples of organs were taken at 3 and at 7 dpi (days post infection). Relative expression levels of IFN-I signalling pathway genes was measured using qRT-PCR (quantitative real-time polymerase

chain reaction). RNA for analysis of gene expression were isolated from organs using commercial kit (Qiagen, Germany) according to manufacturer's instructions. Further, cDNA (complementary DNA) was obtained using LunaScript RT SuperMix Kit (New England Biolabs, USA). Target cDNA was labelled by intercalating dye SYBR Green (Qiagen, Germany) and gene expression was measured at LightCycler 480 (Roche, Switzerland).

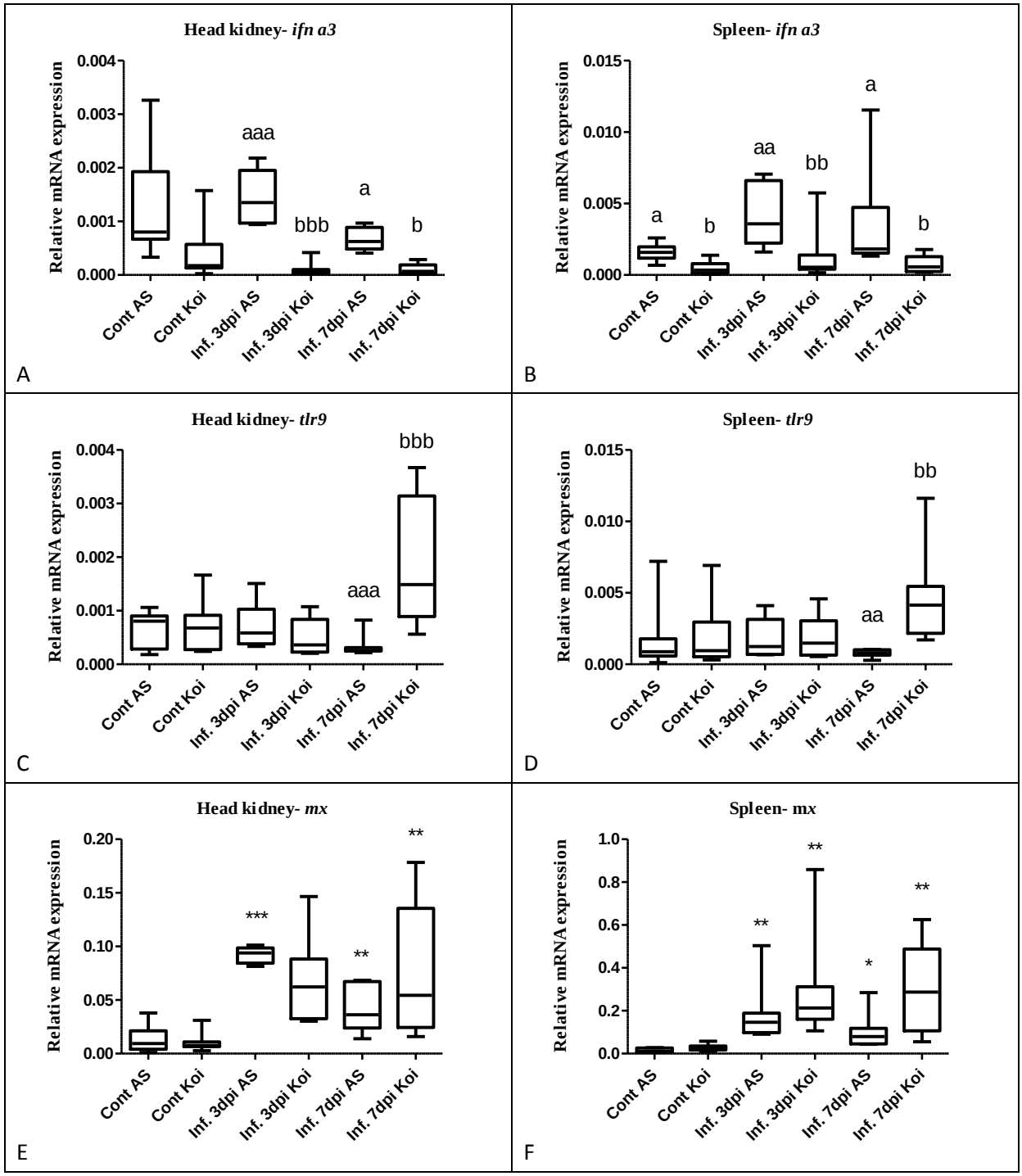

Figure 1: Relative mRNA expression of key genes of IFN class I signalling pathway in head kidney (A, C, E) and spleen (B, D, F) of koi carp and Amur wild carp (AS). A/b: comparing pairs AS:Koi; *: comparing infected group with control; a,b,*= p<0,05 aa,bb,** = p<0,01 aaa,bbb,*** = p<0,001.

Results and discussion

This study compared the expression levels of antiviral immunity related genes in two carp breeds with different susceptibility to KHV: AS and koi. Only the genes with significant changes in expression were chosen to be presented in this paper (Fig. 1).

In head kidney of AS, higher *ifn a3* expression was observed at 3 dpi. Moreover, higher expression even in AS control was revealed in spleen if compared to koi control. Nevertheless, difference between *ifn a3* expression levels decreased at 7 dpi. On the other hand, overexpression of intracellular receptor of xenogenous dsDNA, *tlr9*, was detected in koi comparing with AS at 7 dpi. Expression of gene *mx* gene was upregulated in both, koi carp and AS at 3 and 7 dpi.

According to obtained data, there was more rapid immune response to KHV infection in AS, as represented by *ifn a3* overexpression. Lover expression of *tlr9* in AS than in koi carp at 7 dpi may imply that immune system of AS suppressed KHV during first days after infection and there is no need to activate TLR9 signalling pathway in AS. In contrast, koi immune system responded to virus later, i.e. at 7 dpi.

Conclusion

Obtained data showed promising results revealing reason of AS's higher resistance to KHV. Nevertheless, further research is recommended to understand the origin of AS endurance to the virus.

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IncX plasmids and their transferability under induced stress

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Summary
Dissemination of genes encoding for antibiotic resistance is mediated predominantly by the conjugation of plasmids. Here we focus on the IncX1 and IncX2 group of plasmids carrying genes conferring plasmid-mediated quinolone resistance (PMQR), presenting trends of their transfer under induced stress of temperature change and antibiotic supplementation. In two representatives of IncX1 (pHP2) and IncX2 (p194) plasmids and their deletion mutants in qnrS gene (pHP2 ΔqnrS, p194 ΔqnrS), the influence of induced stress on conjugative transfer was investigated. A deletion of the qnr gene was observed to modify the frequency of plasmid transfer. In order to affect the transfer rate of wildtype plasmids, the influence of two stresses simultaneously was needed, whereas the single stress was sufficient to affect the IncX ΔqnrS plasmid transfer rate. This study showed the significant properties and behaviours of IncX plasmids carrying antibiotic resistance genes that are likely to play a role in their dissemination in bacterial populations.

Keywords*: IncX, PMQR, qnrS, plasmid conjugation, transfer rate, Escherichia coli, induced stress*

Introduction

The spread of antimicrobial resistance within the bacterial populations is predominantly mediated by conjugation and is known to be affected by antibiotic supplementation causing SOS response and overexpression of certain genes [1]. Conjugation rate can also be altered not only by antibiotics but by the physiological state of bacterial cells before conjugation. Therefore, the effect of temperature change to lower temperatures is assumed to have influence on the transfer rate [2]. In this study we monitor transfer tendencies of IncX1 and IncX2 plasmid sublineages widely distributed in non-related *Escherichia coli* from various sources and geographical areas [3]. The aim of this investigation was to further understand the factors which influence their successful transfer through a series of conjugation assays under various conditions.

Material and Methods

In order to determine the effect of environmental stress and occurrence of *qnrS* gene on frequency of plasmid transfer, the mating assays were conducted with the IncX plasmids and their *ΔqnrS* variants, therefore pHP2, pHP2 *ΔqnrS*, p194 and p194 *ΔqnrS* within donor *E. coli*. Donor mid-exponential phase culture (OD600 = 0.7 - 0.8) and overnight culture of recipient *E. coli* A15 (OD600 > 1.5), 500 µl of each, were mixed (ratio of cells 1:2) and centrifuged at 5.000 rpm for 2 min and the pellet was resuspended in 50 µl of LB broth. Mixed cultures were co-incubated on sterile 0.22 µm bacteriological filter on LB agar plate for 1 hour at 37 °C. Additionally, the plasmid transfer assays were conducted at 25 °C and 37 °C in combination with ciprofloxacin in concentration of 0, 0.001, 0.05, 0.5 and 2 µg/mL supplemented into the mating agar plates. Similarly, donor mid-exponential phase culture alone was simultaneously incubated and served as a control of donor growth. Donor cells were selected and colony counted on LB agar plates supplemented with ciprofloxacin (0.05 µg/mL). Transconjugants were selected and colony counted on LB agar plates supplemented with ciprofloxacin (0.05

µg/mL) and sodium azide (100 µg/mL). Mating assays were performed in triplicates. Transconjugant colonies (4 per sample) were confirmed by PCR assays for *qnrS* and *taxC* genes. Frequency of plasmid transfer was calculated as the number of transconjugants per the number of donors (T/D). For statistical analysis the conjugation frequencies were tested with Student's T-test, and a p value < 0.05 was considered statistically significant.

Results

Conjugation frequencies of pHP2 (IncX1) and p194 (IncX2) and their mutant variants (*qnrS* deletion - pHP2 *ΔqnrS* and p194 *ΔqnrS)*, were determined both without stress and under stress of temperature change and supplementation of various concentration of ciprofloxacin (Figure 1). Statistically significant differences in plasmid transfer were observed between IncX1 and IncX2 group. IncX1 plasmid demonstrated higher frequency of transfer compared to IncX2 plasmid.

Our study showed substantial differences in frequency tendencies under variable stress conditions between the individual IncX plasmid groups. The *qnrS* gene knockout alone did not significantly alter the plasmid transfer. A deletion of *qnrS* gene was observed to modify the frequency of plasmid transfer of both plasmids under influence of induced stress. In order to affect the transfer rate of wildtype IncX plasmids, the influence of both stresses simultaneously was needed, whereas the single stress was sufficient to affect the IncX *ΔqnrS* transfer rate.

Combined stress factors reduce wildtype IncX1 transfer rate while single stress factors alter the IncX1 *ΔqnrS* transfer rate. The frequency of IncX1 *ΔqnrS* transfer significantly increased with induced stress caused by supplementation of ciprofloxacin but, on the other hand, it was significantly reduced by temperature change to 25 °C. The frequency of wildtype IncX2 transfer increases with supplemented ciprofloxacin whereas the temperature change to 25 °C increased IncX2 *ΔqnrS* transfer. The transfer of wildtype IncX2 plasmid demonstrated exactly an opposite tendency compared to the wildtype IncX1 plasmid transfer, an increase in frequency at 25 °C with supplementation of ciprofloxacin.

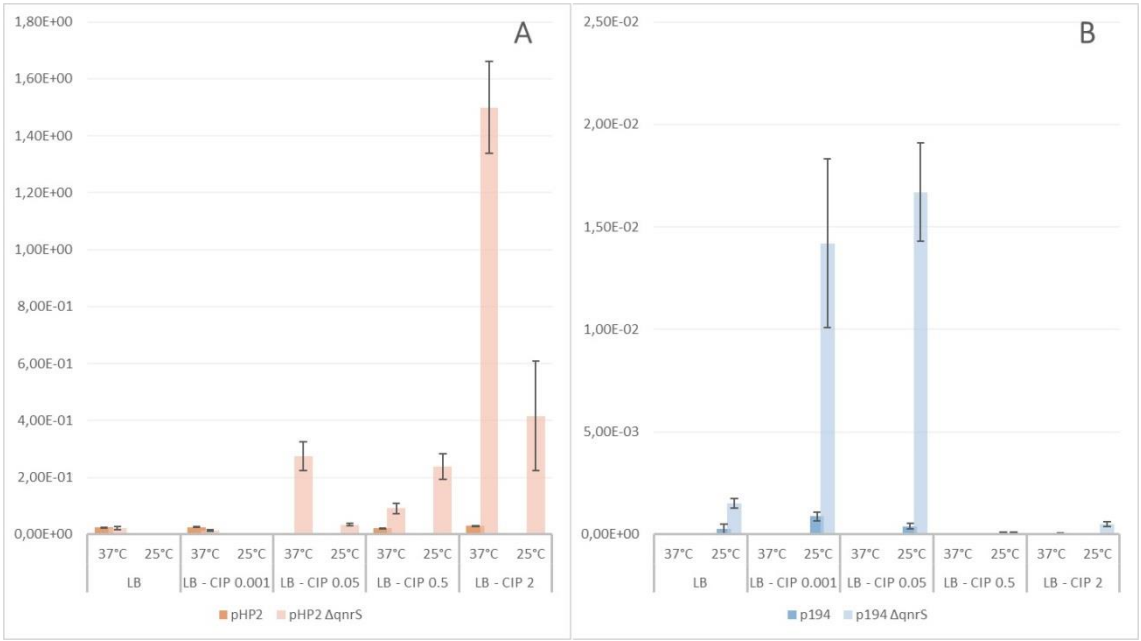

Figure 1 Influence of induced stress on IncX1 (A) and IncX2 (B) plasmid conjugative transfer. Frequency of transfer was calculated as the number of transconjugants per the number of donors (T/D). **LB** stands for Luria-Bertani agar. **CIP** represents the concentration (µg/mL) of supplemented ciprofloxacin into the media.

Discussion

This study demonstrated a substantial variability in frequency of plasmid transfer in IncX group. IncX1 group demonstrated higher frequency of plasmid transfer compared to IncX2 group. Lower rate of IncX2 plasmid transfer may also be the cause of lower occurrence of these plasmids in the environment however this statement requires further investigation to determine the cause. The cause of diverse frequency of conjugative transfer is still not fully elucidated, but we can speculate that one possibility could be an upregulation of transfer proteins [4, 5]. In a study by Lopatkin et al. [2], they applied a selection pressure or stress such as non-inhibitory levels of several antibiotics or temperature change to 25 °C to cells during conjugation. The selection pressure or induced stress also consequently did not play a role solely in conjugative transfer. We confirmed their statement and expanded it with more insights using combined induced stresses and the deletion of *qnrS* gene, thus achieved diverse results under specific conditions during conjugation. These findings indicated the stimulating effect of ciprofloxacin supplementation on the plasmid transfer that can be nullified by the carriage of single PMQR gene.

Conclusions

The influence of induced stress on conjugative transfer of IncX plasmids and their deletion mutants in *qnrS* gene was determined. A deletion of *qnr* gene was observed to modify the frequency of plasmid transfer of both plasmids under influence of induced stress. In order to affect the transfer rate of wildtype IncX plasmids, the influence of both stresses simultaneously was needed, whereas the single stress was sufficient to affect the IncX *ΔqnrS* plasmid transfer rate. These findings indicated the possible stimulating effect of ciprofloxacin supplementation on the plasmid transfer that can be neutralised by the carriage and effect of the *qnrS* gene. This study showed the significant properties and behaviour of IncX plasmids carrying antibiotic resistance genes that are likely to play a role in their dissemination and stability in bacterial populations.

Acknowledgement

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Fitness effect of CTX-M-15-encoding IncF plasmids on their native *Escherichia coli* ST131 hosts

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Summary
Dissemination of multi-drug resistant pathogenic bacteria is usually connected with plasmid-encoded genes. However, carriage of these plasmids commonly pose a fitness cost for its host bacteria. In this study, we aim to estimate the fitness cost of large IncF plasmids carrying $bla_{CTX\text{-}M\text{-}15}$ on the natural host Escherichia coli ST131 subclone H30Rx to study possible plasmid-host coevolution. Five representatives each carrying one IncF plasmid were selected. The plasmid of each isolate was eliminated using plasmid curing method. Whole genome sequencing was performed to obtain complete chromosome and plasmid sequences and to detect chromosomal mutations in plasmid-free strains. Competition assays were conducted using flow cytometry to determine relative fitness of plasmid-free strains. The study showed that IncF plasmids produce low to non-significant fitness cost in their natural host even in a non-selective environment pointing towards host-plasmid co-evolution.

Keywords*: ST131; IncF; plasmid curing; fitness; sequencing*

Introduction

Extraintestinal pathogenic *Escherichia coli* causing a wide range of infections represent a serious threat to public health [1]. Successful *E. coli* sequence type 131 (ST131) is spread world-wide among humans, wild and domestic animals and in the environment [2,3]. Dissemination of multi-drug resistant pathogenic bacteria is usually connected with plasmid-encoded genes providing a selective advantage. However, carriage of these plasmids usually impose a fitness cost for its host. In this study, we aim to estimate the fitness cost of large incompatibility group F (IncF) plasmids carrying $bla_{CTX\text{-}M\text{-}15}$ on the natural host *E. coli* ST131 subclone *H30*Rx in order to study possible plasmid-host coevolution.

Material and Methods

Five *E. coli* ST131 representatives of diverse origin, including humans, wastewater treatment plant effluent and a dog, each carrying one IncF plasmid were selected to study the fitness effects of IncF plasmids on their native host. These large plasmids were complex and carried ESBL gene $bla_{CTX\text{-}M\text{-}15}$ along with other antibiotic resistance genes, several toxin-antitoxin systems and two IncF plasmid replicons (RepFIA, RepFII). The transferability of IncF plasmids was determined by conjugation experiments. The IncF plasmid of each isolate was eliminated using plasmid curing method [4] based on the incompatibility with a small designed plasmid vector pMDP5_cureEC958. Obtained plasmid-free clones and wild-type isolates were subjected for short read sequencing on MiSeq (Illumina) to detect chromosomal mutations in plasmid-free strains. Additionally, long read sequencing using Sequel I platform (PacBio) was performed to obtain complete plasmid sequences of wild-type isolates for genomic comparison. Wild-type strains were marked with small non-transmissible plasmid vector pBGC carrying a gene for green fluorescent protein in order to distinguish two bacterial populations in competition assays. Plasmid-free strains were competed with the corresponding marked wild-type isolates for 22 hours in a non-selective environment.

Relative fitness of plasmid-free clones was determined using flow cytometry (NovoCyte) before and after competition. Data were normalised and statistically processed using Student's T-test where relative fitness with p value < 0.05 was evaluated as significant. Competition assays between wild-type isolates and plasmid-free clones with reintroduced IncF plasmid were conducted as a control.

Results

The size of wild-type CTX-M-15-encoding IncF plasmids selected for this study varied from 106.9 to 144.6 kb. The plasmids carried multiple genes for antimicrobial resistance and 4 to 5 toxin-antitoxin systems, as shown in Table 1. All of them proved non-conjugative with missing parts of *tra* region intermediating conjugal transfer proteins. Three out of five plasmids missed transcriptional regulator *traJ*. Two other plasmids harboured almost whole *tra* region but missed genes *traW* and *traU* responsible for pilus assembly and DNA transfer.

Table 1 Characteristics of wild-type IncF plasmids selected for this study

Plasmid ID	**Origin**	**Size [bp]**	**Toxin-antitoxin systems**					**ARGs**								
			*ccd*AB	*pem*KI	*vap*CB	*par*ED	*hok/sok*	$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$	$bla_{\text{OXA-1}}$	*aac(6')-Ib-cr*	*sul1*	*aadA5*	*mph*(A)	*dfrA17*	*tet*(A)
pM24	human	116543	■	■	■	■	■	■		■	■	■	■	■	■	
pM45	human	106909	■	■	■	■		■		■	■	■	■	■	■	■
pM70	human	126514	■	■	■	■	■	■	■			■	■	■	■	■
pDog168	dog	131080	■	■	■		■	■		■	■	■	■	■	■	■
pOV24	WWTP[1]	144582	■	■	■		■	■	■	■	■	■	■	■	■	■

[1]wastewater treatment plant effluent; coloured cells visualise the presence of a gene

Even though the plasmids contained toxin-antitoxin systems, each one of them was successfully eliminated. Small plasmid vector pMDP5_EC958 was designed for this purpose. The construct carried IncF replicons, *sacB* gene used for counterselection of plasmid-free clones without this vector and antitoxins to the toxins encoded in wild-type plasmids ensuring survival of the cell after the IncF plasmid elimination. Chromosomes of plasmid-free clones were subjected for sequencing and harboured zero to five non-synonymous mutations, mostly in protein coding sequences.

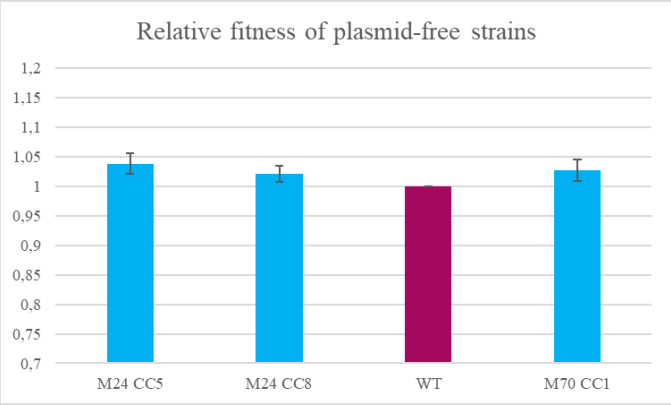

Figure 1 Relative fitness of plasmid-free strains with significant fitness changes in comparison to the corresponding wild-type isolates (M24, M70). Relative fitness of M24 cured clone 5 (CC5) was 1.03 ± 0.18 (p = 1.82 x 10^{-6}), in M24 CC8 1.02 ± 0.13 (p = 4 x 10^{-4}) and in M70 CC1 1.03 ± 0.19 (p = 1.5 x 10^{-3})

Relative fitness of plasmid-free clones was estimated in comparison to the corresponding wild-type isolates considering a background fitness of wild-types as 1. Plasmids of three isolates (pM45, pDog168 and pOV24) were producing **no significant** fitness cost and the two other plasmid-free isolates (pM24, pM70) showed **slightly increased** but significant relative fitness in comparison to their corresponding plasmid-free strains, as visualised in Figure 1. Plasmid-free clones with reintroduced wild-type IncF plasmid used as a control contained zero non-synonymous mutations in their chromosome. Relative fitness of these strains showed no significant fitness changes in comparison to their wild-types.

Discussion

This study represents a unique work focusing on a plasmid-host coevolution of pandemic subclone *H30*Rx of *E. coli* ST131. IncF plasmids are a complex and diverse plasmid group, including both conjugative and non-conjugative representatives [5]. Our fitness study is using the pCURE method for a construction of plasmid-free clones in order observe fitness effects of large IncF plasmids on their native host. As it was mentioned previously [6], plasmid curing is one of the best ways to study fitness effects of plasmids. The plasmid curing proved efficient in our study, as it was successful in all representatives. However, we observed non-synonymous mutations among the obtained plasmid-free clones in protein coding sequences which pinpoints the importance of sequencing after plasmid elimination to obtain valid results. In this study, we demonstrated that IncF plasmids produce a small or no fitness cost in their native host indicating that *E. coli* ST131 is well adapted to maintain large IncF plasmids.

Conclusions

Results obtained during this study demonstrate that subclone *H30*Rx of the world-wide spread *E. coli* ST131 is able to sustain naturally occurring IncF plasmids with a reduced fitness cost. This indicates that large IncF plasmids underwent through coevolution with its native host *E. coli* ST131 subclone *H30*Rx contributing to their rapid world-wide dissemination.

Acknowledgement

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Rekombinantní protein *Acanthamoeba polyphaga mimiviru* pro využití v sérologické diagnostice ryb

Recombinant protein of *Acanthamoeba polyphaga mimivirus* for serological diagnostic in fish

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Summary
The virus population in the aquatic ecosystem is largely composed of viruses belonging to phylum of viruses Nucleocytoviricota also known as the nucleocytoplasmatic large DNA viruses. These viruses correspond in size to bacterias and unlike other viruses can be observed with a light microscope. The aim of our research is to express recombinant protein of Acanthamoeba polyphaga mimivirus, for serological detection of mimiviruses in fish.

Keywords: *recombinant protein; Mimiviridae; giant virus; fish*

Úvod

Zásadní změnu na poli vnímání virů přinesl objev *Acanthamoeba polyphaga mimiviru* (APMV) v roce 2003, později hlavního zástupce virů nově vytvořené čeledi *Mimiviridae* (La Scola et al., 2003). Ten se se svým genomem tvořeným dvouřetězcovou DNA (dsDNA) o velikosti 1,2 Mb, genetickými a proteomickými složitostmi významně lišil od ostatních, v té době známých virů (Colson et. al, 2017). Díky tehdejšímu definování virů dle jejich submikroskopické velikosti a neschopnosti zadržet je na sterilizačním filtru, byla celá léta APMV považována za intracelulární parazitickou bakterii améb (La Scola et al., 2003; Claverie and Abergel, 2016).

Přirozeným hostitelem Mimivirů je *Acanthamoeba*, měňavkovitý prvok z říše *Amoebozoa,* ačkoliv existují také důkazy o replikaci mimiviru ve fagocytech obratlovců (Ghigo et al. 2008). Améby představují důležitou složku lidské i zvířecí mikrobioty a jsou extrémně odolné vůči změnám pH, vysokým teplotám a dezinfekčním prostředkům. Obývají nejrozmanitější typy prostředí – vodním ekosystémem počínaje (voda, odpadní voda, fontány), permafrostu, vzduchu a půdy konče.

V průběhu posledních několika let byli izolováni nejrůznější zástupci čeledi *Mimiviridae* jak v organismu bezobratlých živočichů, tak u lidí s pneumonií či jiným plicním onemocněním (La Scola et al., 2005; Zhang et al., 2016). Doposud však není objasněn vliv těchto virů na organismus živočichů. Podle nejnovějších studií (Axén et al., 2018; Rud et al., 2020) byl virus *Acipenser iridovirus-European* (AcIV-E) schopen infikovat jesetera bílého (*Acipenser transmontanus*), přičemž ryby vykazovaly neurologické příznaky jako je letargie, neschopnost udržet vzpřímenou polohu, nepravidelné plavání, a byl spojen s jejich vysokou úmrtností.

Ačkoliv je toto téma na našem území zcela neprobádané, předpokládáme, že v našich vodních ekosystémech jsou ryby hojně exponovány nejrůznějšími viry fytoplanktonu.

Cílem práce je vytvořit rekombinantní protein L725 *Acanthamoeba polyphaga mimiviru* pro využití v sérologické diagnostice ryb.

Materiál a metodika

Pro amplifikaci požadovaného inzertu viru *Acanthamoeba polyphaga mimiviru* byl použit izolát (GeneBank: AY653733.1) získaný z Aix-Marseille Université (prof. Bernard La Scola).

Kultivace APMV byla uskutečněna na prvocích *Acanthamoeba polyphaga* (Puschkarew) (ATCC ® 30871 ™) a *Acanthamoeba castellanii* (Douglas) (Page) (ATCC ® 30010 ™). Jako kultivační médium pro Acanthamoeby bylo použito ATCC médium 712 PYG (Proteose Peptone, Yeast Extract, Glukóza), pH 6,5.

Příprava DNA konstruktu
Specifické primery ohraničující čtecí rámec kódující L725 (bez start a stop kodonů) byly navrženy programem Geneious Prime v2021.1.1. Ty poté byly nasyntetizovány a použity při amplifikaci požadovaného DNA úseku.

- sekvence forward primeru: GCAAATAATTTGGTGCAACTTATCT
- sekvence reverse primeru: ATGAGCACAATTACATTTCTTGGAT

Polymerázová řetězová reakce (PCR) byla po úvodní denaturaci při 94 °C/4 min uskutečněna provedením 35 cyklů jednotlivých kroků:

- 94 °C/60 sec
- 53 °C/60 sec
- 72 °C/60 sec

Závěrečná extenze probíhající při teplotě 72 °C po dobu 30 sec umožnila následné klonování vzniklých fragmentů do plazmidového vektoru pENTR™ (Invitrogen) metodou TA klonování. Výsledná konstrukce byla transformována do kompetentních buněk *E. coli* (kmen TOP 10). Pro následnou expresi byl gen klonován do pDest™17 Gateway® (Invitrogen).

Exprese rekombinantního proteinu
Exprese rekombinantního proteinu L725 byla provedena v kultuře bakterie *E. coli* transformovanou rekombinantním plazmidem. Noční kultura bakterií byla zředěna 1:50 ve 2 l LB média suplementovanými 100 µl/ml ampicilinu a 50 µg/ml chloramfenikolu. Kultura byla inkubována při teplotě 37 °C a za stálého třepání (250 rpm) do dosažení OD_{600} 0,5 (denzitní hustota). Exprese rekombinantního proteinu byla indukována přidáním 1 mM isopropyl-β-D-thiogalactosidu (IPTG). Buňky byly následně stočeny při 4500 g po dobu 15 min a získaný pelet uchován při -20°C.
Pelet bakteriálních buněk byl resuspendován v lyzačním pufru (20 mM fosfátový pufr, 300 mM NaCl, 0,1 % Tween, 10 mM imidazol, 8 M močovina) a bakteriální buňky desintegrovány ultrazvukovými rázy (60 % amplitud/10 pulzů; 1 min na ledě pro zamezení prohřátí materiálu). Získaná inkluzní tělíska byla oddělena centrifugací. Rekombinantní protein byl následně purifikován metalochelatační afinitní chromatografií. Následná identifikace získaného rekombinantního proteinu byla provedena metodou Western blot za využití SDS-PAGE.

Výsledky a diskuse
Získané frakce proteinu L725 měly koncentraci 100-150 µg/ml. Získaný protein bude dále využit pro další studium mimivirů v našich vodních ekosystémech a jejich přímého vlivu na organismus vodních živočichů, který je doposud nejasný. První potenciálně potvrzené případy (Axén et al., 2018; Rud et al., 2020) infekce ryby mimivirem, konkrétně virem *Acipenser iridovirus-European,* proběhly u jesetera bílého (*Acipenser transmontanus*). Tento virus byl původně spjat s čeledí *Iridoviridae*, další skupinou obřích virů, ale podle nejnovějších studií má více molekulárních podobností s čeledí *Mimiviridae*. V tuto chvíli se čeká na překvalifikování zařazení viru Mezinárodním výborem pro taxonomii virů (ICTV), pravděpodobně však bude virus AcIV-E oficiálně přiřazen k čeledi *Mimiviridae*.

Závěr

Rekombinantní protein bude využit k sérologické diagnostice ryb. Vzhledem k nedostatečnému množství informací o těchto virech a jejich působení na organismus, bude tato studie pojata jako pilotní. Aktuálně nedisponujeme pozitivními ani negativními séry. V rámci dalšího výzkumu budou *Acanthamoeba polyphaga mimivirem* imunizovány experimentální ryby, a kromě případného získání pozitivního séra, budou provedeny i analýzy hematologických, biochemických a imunologických ukazatelů a patologické šetření.

Poděkování

Tato studie vznikla za podpory ERDF/ESF „PROFISH“ no. CZ.02.1.01./0.0/0.0/16_019/0000869 a Interní tvůrčí agentury FVL/Celer/ITA2020.

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Mapování B buněčných epitopů proteinu C-type Lectine viru Afrického moru prasat

Mapping B-cell epitopes of the Afican swine fever virus C-type Lectine protein

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Summary
The topic of this study was the African swine fever virus and the serological properties of the virus. The main methods used are the In silico analysis and serodiagnosis by PEPSCAN procedure. These methods are necessary to explore the proteins of the virus and their peptides, mainly B-cell epitopes. It is essential because researchers found out that the epitopes cause the immune reaction in the host system.

Keywords: *Pepscan; antigenic design of peptides; African swine fever*

Úvod

Africký mor prasat je závažné horečnaté onemocnění s haemorrhagickým průběhem. Původcem onemocnění je dsDNA virus z čeledi *Asfarviridae*. U domácích prasat dochází v případě nakažení až ke 100 % mortalitě. Z důvodu rychlého šíření viru z endemických oblasti do okolních částí světa je podrobnější zkoumání patogeneze této virové infekce a vývoj diagnostických metod zcela zásadní. Jedním z proteinů, který kóduje genom viru Afrického moru prasat, je hemaglutinující protein C-type Lectine. Význam v patogenezi tohoto proteinu není doposud plně objasněn, nicméně jeho delecí lze docílit oddálení virémie a diseminace viru do organismu. Cílem předloženého projektu byla identifikace B–buněčných epitopů na povrchu C-type Lectine molekuly.

Materiál a metodika

Použité peptidy

Peptidy pokrývající celou délku C-type Lectine proteinu byly syntetizovány (Genecust, Lucembursko), naředěny v koncentraci 1mg/ml v destilované vodě nebo DMSO. Složení peptidů je uvedeno v Tabulce 1.

Tabulka č. 1: Aminokyselinové složení peptidů

Označení peptidu	Aminokyselinové složení
Ctype1	MYFKKKYIGLIDKNCEKKILDDSSTIKICY
Ctype2	DDSSTIKICYILIGILIGTNMITLIYNFIF
Ctype3	MITLIYNFIFWDNYIKCYRNNDKMFYCPND
Ctype4	NDKMFYCPNDWVGYNNICYYFSNGSFSKNY
Ctype5	FSNGSFSKNYTAASNFCRQLNGTLANNDTN
Ctype6	NGTLANNDTNLLNLTKIYNNQSMYWVNNTV
Ctype7	YWVNNTVILRGDNKYSQKVNYTDLLFICGK

Použitá séra

K testování sérologické reaktivity peptidů byla použita ASFV pozitivní i negativní séra.
Pozitivní séra byla získána od divokých prasat z Polska (n=5), Španělska (n=5) a jako pozitivní kontrolní séra z diagnostických kitů (n=2). ASFV negativní séra (n=20) byla získána z chovů prasat z ČR.

Použité laboratorní vybavení

1. ELISA Hydroflex (TECAN, Švýcarsko)
2. Infinite M200 PRO (TECAN, Švýcarsko)

Použité metody

In silico analýza

Primární aminokyselinová sekvence C-type Lectine proteinu byla analyzována z hlediska umístění potenciální B buněčných epitopů prostřednictvím softwarového nástroje Immune Epitope Database (WWW.IEDB.org). Analýza byla provedena prostřednictvím algoritmů pro stanovení predikce lineárních epitopů, antigenicity a hydrophilicity.

Přítomnost lineárních B-epitopů byla dále experimentálně stanovena technikou „pepscan" s využitím syntetických peptidů pokrývajících celou délku analyzovaného proteinu. Jednotlivé peptidy byly syntetizovány (Genecust, Lucembursko) a využity jako antigeny v ELISA testu pro stanovení jejich reaktivity s ASFV pozitivními i negativními séry.

Peptidová ELISA

Sérodiagnostická metoda. Veškeré reagencie byly pipetované v množství 100µl/jamku mikrotitrační destičky. Jednotlivé peptidy byly naředěny na pracovní koncentraci 20µg/ml uhličitanovým roztokem pH9.6. Následovalo blokování destičky 2 % BSA v PBS. (1 h při pokojové teplotě).

Testované sérum bylo naředěno 1:100 roztokem TwPBS s 2 % BSA. Inkubace séra probíhala 1 h při pokojové teplotě. Konjugát (Anti-Pig IgG-Peroxidase) byl naředěn 1:30000 v roztoku 2 % BSA v TwPBS. Inkubace konjugátu probíhala 1 h při pokojové teplotě. Vizualizace reakce byla provedena inkubací se substrátem TMB-Complete (100µl/jamku, při pokojové teplotě 5-20 min) a následně byla zastavena 100µl zastavovacího roztoku a výsledky odečteny spektrofotometricky na vlnové délce 450nm.

Výsledky

Výsledky pepscan analýzy s ASFV pozitivními a negativními séry jsou znázorněny v Tabulce 2.

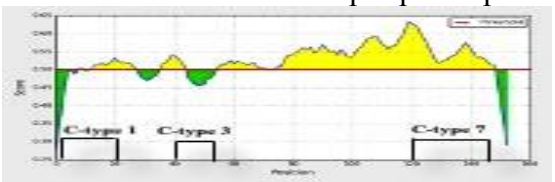
Tabulka 2. Reaktivita syntetických peptidů s kontrolními pozitivními i negativními séry.

Peptid č.	1	2	3	4	5	6	7
ASFV+ séra	+/-	-	+/-	-	-	-	+
ASFV- séra	-	-	-	-	-	-	-

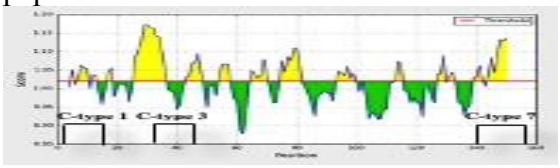
Výsledky in-silico analýzy

Výsledky této analýzy jsou znázorněny na obrázcích 1. - 4.

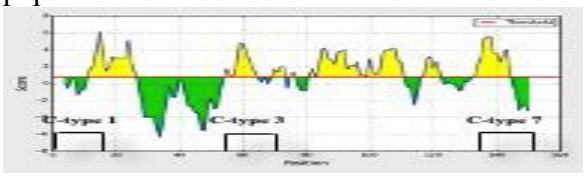
Obrázek 1. Predikce B-epitopů a porovnání s polohou sérologicky reaktivních peptidů.



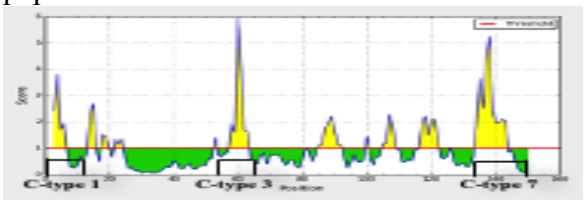
Obrázek 2. Predikce antigenních oblastí a porovnání s polohou sérologicky reaktivních peptidů.



Obrázek 3. Predikce hydrofilních oblastí a porovnání s polohou sérologicky reaktivních peptidů



Obrázek 4. Predikce povrchové dostupnosti a porovnání s polohou sérologicky reaktivních peptidů.



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Red Kites wintering in Austria, the Czech Republic and Slovakia: GPS tracking and direct field observations over 2020/2021

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Summary
The Red Kite (Milvus milvus) is a common raptor in the tripoint border area of Austria, the Czech Republic and Slovakia during the non-breeding season; however, the exact number of birds wintering in this area remains unknown. Using satellite telemetry, direct field censuses at nocturnal roosts and online avian platforms, we estimated the wintering population of Red Kites in this area over the winter of 2020/2021. Based on three censuses taking place at the beginning (28 November 2020), middle (9 January 2021) and end (5 February 2021) of winter, we counted 567, 560 and 537 Red Kites, respectively. A comparison of historical data and our own results suggests that the wintering population of Red Kites in the study area has increased substantially, most likely due to changes in climatic conditions and food availability and increased conservation efforts for endangered raptors.

Keywords: *Birds of prey; communal roosting; Milvus sp.; satellite telemetry; Central Europe*

Introduction

The Red Kite (*Milvus milvus*) is a medium-sized raptor whose European population, estimated at 60 000–70 000 individuals, appears to be increasing (BirdLife International 2020). Red Kites from Austria, the Czech Republic and Slovakia migrate south to their wintering grounds in southern Europe (ranging from the Iberian Peninsula to Greece), but some stay in their natal area over the winter period (Literák et al. 2019).

Single wintering individuals have been observed throughout Burgenland and the Lower Austrian Provinces in northeastern Austria since 1990 (Archive of BirdLife Austria, unpublished data), while the first observations of Red Kites at communal nocturnal roosts took place in a floodplain biotope near Bernhardsthal in 2002, with 39 individuals counted. The first records of Red Kites wintering in the Czech Republic were from the South Moravian region (southeastern part of the Republic) in the mid-1980s, with one individual observed in February 1984, one in December 1985 and another in January 1985 (Bejček et al. 1995). Since then, the number of wintering Red Kites at communal nocturnal roosts in the South Moravian region has increased, with a maximum of seven individuals for the winter of 1987/1988, 18 for 1991/1992 and 18 for 1992/1993 (Mrlík, 1990; Danko, 1994; Voříšek, 1995). Records from the 1970s suggest that Red Kites have been regularly overwintering in lowlands in the eastern part of Slovakia throughout the 20th Century (Hudec & Černý 1976). In the western part of Slovakia, the first records of communal nocturnal roosts were near Moravský Svätý Ján, with 32 individuals counted in 2006, 70 in 2009 and 90 in 2010 (David Horal, unpublished data). There is an increasing tendency of wintering population and number of Red Kites at communal nocturnal roosts in all three countries in the last two decades (David Horal, unpublished data).

Here, we make use of available data from online birdwatcher platforms covering our study area and combine this with data on wintering populations obtained from telemetry tracking and direct censusing at communal nocturnal roosting sites in order to obtain the most accurate results yet for Red Kite overwintering numbers in the Austrian, Czech Republic and Slovakian region.

Material and Methods

The Red Kite counting survey took place over the winter of 2020/2021 in Austria, the Czech Republic and Slovakia. For the purposes of this study, the wintering period was defined as from 15 November 2020 to 15 February 2021. In addition to telemetry data on 24 tagged Red Kites collected over the study period in the three countries, we used two other datasets to obtain the total number of wintering Red Kites: i) field observations at the communal roosts obtained while tracking of the tagged Red Kites, and ii) data from five online avian platforms (www.ornitho.at, www.birds.cz/avif, www.birding.sk, www.vtaky.sk, www.ebird.org). Field observations were conducted on 28 November 2020, 9 January 2021 and 5 February 2021, when all known communal roosts were simultaneously observed and Red Kites counted by a group of field workers from late afternoon to sunset. Data were obtained from the online avian platforms for the period two days before and two days after each field observation. Only those data obtained 20 km (i.e. the maximum foraging distance from the communal roost) or more from the observed nocturnal roosts were used in order to avoid double counting of the same individual.

Results

Including the 24 tagged Red Kites using the nocturnal roosts over the same period, we recorded a total of 567 Red Kites wintering in Austria, the Czech Republic and Slovakia at the start of winter, 560 during the middle of winter, and 537 at the end of winter 2020/2021. Of these, direct observations recorded 514 Kites in the first census, 531 in the second and 502 in the third. The online avian platforms provided observation records confirming presence of 381 Red Kites within the study area, with a further 117 Red Kites observed at distances greater than 20 km from the communal roosts during two days before and after three census periods (Census 1 = 53, 2 = 29, 3 = 35).

Discussion

Our results confirm an increasing trend in the numbers of wintering Red Kites in Austria, the Czech Republic and Slovakia. Red Kites appear to show a strong preference for the ±70 km tripoint border region of Austria, the Czech Republic and Slovakia as their wintering grounds. Lowland habitats in this Central European region include floodplain forests (suitable conditions for wintering) and relatively high breeding density of Red Kites (Keller et al. 2020). Global climate change (Huntley et al. 2007), high food availability (e.g. the Common Vole; Sunyer & Viñuela 1994) and an increasing conservation (e.g. projects PannonEagle LIFE LIFE15/NAT/HU/000902 and LIFE EUROKIE LIFE18/NAT/AT/000048) of this species could involve their population dynamic in winter in the study area.

Conclusion

Overall, tracking Red Kites tagged with satellite telemetry loggers proved to be a very useful tool for identifying the communal roosts of these highly social birds, enabling us to obtain a much-improved assessment of the total wintering population. Likewise, the use of observational data from online platforms allowed us to improve our estimates of the number of wintering birds away from the roosting sites. With the number of Red Kites observed at communal nocturnal roosts in the study area (90–95%), we recommend the monitoring of

these communal roosts used by highly social birds as an important tool for precise estimate of their population in the future.

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Weather-influenced water crossing behaviour of black kites (*Milvus migrans*) during migration

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Summary
From 2014 to 2020, 32 black kites from various European countries were tagged with telemetry devices and tracked to study their spatioteporal behaviour. Eleven birds which crossed the Mediterranean Sea and the Black Sea directly over large water bodies out of traditional migration routes over the Strait of Gibraltar, the Dardanelles/the Bosporus area and the east coast of the Black Sea were selected for this study. Ten birds attemped to cross the Mediterranean Sea and one attempted to cross the Black Sea. All black kites crossed the sea succesfully but in one case the bird failed. The maximum water crossing length was recorded at 542 km. The average speed of the birds crossing the sea included a broad range from 27.7. to 97 kph. There was a correlation between average speed and tail-wind speed during the crossing. We conclude that the most favourable conditions for black kites when crossing large water bodies such as the Mediterranean and Black seas were sunny to partly cloudy weather with tepmeratures over 20 °C, the speed of the tailwind of 1.1. - 10.8 m/s and the air pressure over the standard value of 1013 hPA.

Keywords: Raptors; telemetry; migration; *Milvus migrans*

Introduction

Circannual variations including the migration of black kiteds have been extensively reviewed and it is known that black kites are summer residents in Europe and winter mostly in sub-Saharan Africa with a few remaining to winter in the Mediterranean region (Ortlieb 1998; Panuccio et al. 2013; Literák et al. 2017; Ovčiariková et al. 2020). Since most of migrating black kites are reluctant to fly over large water bodies and cross transcontinental boundaries over the Strait of Gibraltar, the Bosporus Strait, the Dardanelle Strait and the Bab-Al-Mandeb Strait (Pannucio et al. 2013; Ovčiariková et al. 2020; Santos et al. 2020), in this study we characterize 11 black kites using unusual routes of migrating over large water bodies of the Mediterranean Sea and Black Sea from Europe to Africa and from Europe to Middle East. We reveal that black kites fly continually over water bodies for distances exceeding 250 km but in some cases their flight over large water bodies can be fatally unsuccessful.

Materials and methods

Eleven birds which crossed the Mediterranean Sea and Black Sea directly over large water bodies out of traditional routes over the Strait of Gibraltar, the Dardanelles/the Bosporus area, the Bab-al-Mandeb Strait and the east coast of Black Sea were selected for this study. Loggers equipped with solar panels (20g; Ecotone, Poland or Ornitela,Lithuania) were used to track the birds. The loggers function in GPS (Globall Position System) /GSM (Global System for

Mobile Communications) systems. The GPS positions of the birds were collected according to individual settings (usually one position fixed per 6 h) and were sent as SMS text messages by local mobile operators to the Ecotone and Ornitela Centers in Poland and Lithiuania, respectively, where they were saved and archived. Coordinates of bird positions were analysed using GIS (Geographic Information System) and the software ArcGIS 10. (Esri, Redlands, USA). The distances of the trajectories of direct water crossing flights were calculated from GPS data. We investigated time periods, directions of their migration routes over water bodies, water crossing lenght, the velocity of black kites flying over water and the weather conditions during their flight over large water bodies. Weather records were downloaded from the web portals www. timeanddate.com and www.ncdc.noaa.gov. We worked with data from the nearest coastal cities to the bird´s flight trajectories, in both Europe and Africa, and with weather data from Mediterranean Sea recorded by ferries which travelled in similiar directions to obtain reliable information about the weather conditions during the migration of individual birds over the sea. Pearson correlation test (MS Excel) was used to determine if there is any connection between the speed of tail-wind and average velocity of migrating birds.

Results

Ten birds attemped to cross the Mediterranean Sea and one attemped to cross the Black Sea (Table 1). All black kites crossed the sea successfully but in one case, the bird failed. This black kite (BK2) left mainland Greece to cross over the Mediterranean and died 66 km prior to reaching the Libyan coast where its body was later found by local co-worker. We recorded the maximum water crossing lenght of a black kite at 542 km. The average speed of birds crossing the sea was in a broad range from 27.7 to 97 kph. Weather conditions during these crossings are also provided in Table 1. There was a significant positive correlation between the average flight speed of the birds and tail-wind speed during the crossings (r = 0.9; df = 14; p = 0.05). We were able to record the altitude fluctuation while sea crossing of BK11 (equipped with Ornitela logger) (Table1). Rest of the studied birds were equipped with Ecotone loggers which lack the fuction of an altitude recording.

Table 1. Long flights of black kites over the sea and the weather conditions during the flights.

Black kite	Direction of the flight	The sea	Flight date	Estimated age when crossing the sea	Flight time (hour)	Average speed (kph)	Average altitude (m)	Total distance (km)	Weather	Temperature of air (°C) max./ min.	Wind speed (m/s)	Wind direction	Air pressure (hPa)
BK1	Italy (mainland)/Sicily	Tyrhennian Sea	2 Sep 2014	ND	7.7	64.4	-	495	Scattered clouds	27/23	9.7	NW	1007
	Sicily/Tunisia	Strait of Sicily	11 Sep 2014		9.0	30.4	-	274	Partly cloudy	32/22	3.6	N	1011
BK2	Greece (mainland)/(Libya)	Mediterranean Sea	19 Aug 2017	88 days	11.9	38.5	-	459*	Cloudy	33/22	2.2	NW	1013
BK3	Crete/Egypt	Mediterranean Sea	30 Aug 2017	92 days	13.6	32.7	-	445	Sunny	27/21	2.2	N	1011
BK4	France (mainland)/Corsica	Mediterranean Sea	11 Sep 2017	82 days	5.9	42.7	-	256	Sunny	28/13	5	NW	1006
	Sardinia/Alergia	Mediterranean Sea	17 Sep 2017	88 days	5.9	39.7	-	238	Sunny	27/14	1.7	NW	1020
BK5	Sardinia/Algeria	Mediterranean Sea	28 Sep 2017	95 days	8.6	28	-	240	Cloudy	27/15	1.1	W	1023
BK6	Morocco/Spain	Gulf of Cádiz	28 Apr 2019	ND	7.3	34.6	-	252	Cloudy	27/13	4.2	SE/ E	1012
BK7	Greece/Libya	Mediterranean Sea	23 Sep 2019	94 days	-	-	-	(750)	-	-	-	-	-
BK8	Croatia/Italy (mainland)	Adriatic Sea	17 Sep 2019	87 days	4.9	34	-	165	Partly cloudy	26/20	1.3	SW	1018
	Italy (mainland)/Sicily	Tyrhennian Sea	20 Sep 2019	90 days	6.8	40.6	-	276	Partly cloudy	28/24	2.8	N/W	1016
	Sicily/Tunisia	Strait of Sicily	17 Oct 2019	117 days	6.7	27.7	-	187	Partly cloudy	22/17	2.3	N/NW	1018
BK9	Crimea/Turkey	Black Sea	7 Sep 2019	92 days	8.6	33.5	-	288	Partly cloudy	26/15	1.6	N/W	1018
BK10	Greece (mainland)/Libya	Mediterranean Sea	17 Sep 2019	ND	4.1	97	-	395	Partly cloudy	27/22	10.8	N	1015
BK11	Croatia/Italy	Adriatic Sea	8 Sep 2020	66 days	6	40.5	436	243	Sunny	27/16	2.6	N/NE	1021
	Italy/Sicily	Tyrhenian Sea	10 Sep 2020	68 days	14.0	38.7	158	542	Sunny	30/20	2.5	N/SE	1013
	Sicily/Tunisia	Strait of Sicily	13 Sep 2020	71 days	7.4	36.6	190	271	Scattered clouds	27/21	4.8	NE	1018

Discussion

Raptors perform a soaring-gliding flight behaviour exploiting rising thermals and ridge lifts over land to reduce energetic expense. However, during migration when crossing large water bodies, thermal updrafts are weak, and birds mainly use flapping (powered) flight increasing both energy consumption and mortality risk (Agostini et al. 2015). We considered that our tagged black kites used also a flapping flight technique when crossing the water bodies of the Mediterranean Sea out of the Strait of Gibraltar, the Dardanelle Strait and the Bosporus Strait. Black kites crossing Strait of Gibraltar engaged in heavy wing flapping during Levanter winds or they exhibited less flapping amplitude when crossing during weak crosswinds (Santos et al. 2020). We were able to obtain data of the altitude fluctuation while sea crossing only for one bird (BK 11). We assume that this bird changed the direction along with the wind, rather than risk high energy cost of the flapping flight againts the crosswind even though it had doubled the final crossing lenghth and duration. Visible difference in altitude and speed fluctuation are noticeable after the change of flight direction, therefore the bird might have changed the direction to use upward currents to eliminate the energy cost of the powered flight rather than fly shorter distance againts the crosswind. We considered all routes over large water bodies as suboptimal for black kites and the are supposedly used only by a minority of black kites originating from Europe since for some of them, as we proveded, this route could be fatally unsuccessful. The ability to cross the Mediterranean Sea (and lately Sahara Desert) in black kites as well as other raptor species is likely the result of evolutionary permanent selection pressure.

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Plasmid-mediated colistin resistance in human and animal isolates of *Escherichia coli*: genomic surveillance

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Summary
Increasing occurrence of colistin-resistant bacteria represents a global concern since colistin is last-line antibiotic for the treatment of severe infections. Recent discovery of mobile colistin resistance mediated by mcr genes has raised extensive concerns worldwide. Within national surveillance of mcr genes in human clinical isolates in the Czech Republic a total of 1440 colistin-resistant isolates (MIC >2 mg/l) were examined. At the same time more than 400 samples from food and food animals at slaughter were collected and selected for resistant isolates on agar media with colistin. The presence of mcr-1 to mcr-9 was tested by multiplex polymerase chain reaction (PCR). Isolates carrying mcr genes were subjected to antibiotic susceptibility testing and whole-genome sequencing to determined their resistance phenotype and genomic profiles. Conjugation experiments were used to test the transferability of mcr-carrying plasmids. The results showed a low prevalence of mcr genes in clinical samples (69/1440; 4,7 %) compared to isolates from veterinary sector (52/400; 13 %). Most isolates showed multi-drug resistance phenotype, belonged to various sequence types and carried mcr genes on conjugative plasmids.

Keywords: *Escherichia coli; plasmid-mediated colistin resistance; mcr genes*

Introduction

By reducing the effect of existing antimicrobials, so-called reserve antibiotics for the treatment of life-threatening infections have come to the fore. These include colistin (polymyxin E), which acts on a wide range of Gram-negative bacteria. In the human field, colistin has so far been of unique use in the treatment of systemic infections caused by multi-drug resistant bacteria. In contrast, in the veterinary field, colistin is generally a widely used antibiotic for the treatment of gastrointestinal infections caused by non-invasive *Escherichia coli* strains. Resistance to colistin is usually mediated by chromosomally encoded mechanisms, however, mobile colistin resistance encoded by *mcr* genes have been recently described. So far, a total of 10 variants of *mcr* genes (*mcr-1* to *mcr-10*) carried by various plasmid families have been identified in Enterobacterales (most commonly in *E. coli*) from patients, food, food animals and the environment, including wildlife. Current evidence suggests that due to the historically high consumption of colistin in livestock and the increased incidence of plasmid-encoded colistin resistance in animal isolates, the veterinary sector is a likely source of *mcr* genes and a driving force for the spread of plasmid-borne colistin resistance to humans. Genes encoding resistance to other classes of antibiotics, such as $bla_{\text{CTX-M-1}}$ for extended-spectrum beta-lactams have also been detected on some groups of plasmids carrying the *mcr* genes (particularly IncHI2 plasmids). The co-transfer of ESBL and *mcr* genes by plasmids poses a significant risk in the spread of multidrug-resistant strains.

Material and methods

Within the project a set of samples from patients and food-producing animals at slaughter between 2017 and 2020 were obtained within a national surveillance program. A total of 1440 human clinical isolates of Gram-negative bacteria resistant to colistin (MIC >2 mg/l)) were collected by Public Health Institute. Other 400 samples of fresh meat and caecum originating from slaughtered poultry (n=366), pigs (n=17) and cattle (n=17) were collected by State Veterinary Institutes. These samples were enriched in buffered peptone water (PPV) and used for selective cultivation.

Selection of colistin-resistant isolates and *mcr* detection

Primary samples from slaughtered animals were incubated overnight into 3 ml of PPV and then subjected to selective cultivation on media (Eosin methylene blue and MacConkey agar) with colistin (3.5 mg/l) to obtain resistant isolates. Species identification was performed by MALDI-TOF and the susceptibility to 21 antibiotics and production of beta-lactamases was determined by disk diffusion method (CLSI, 2018). In human isolates the minimum inhibition concentration (MIC) by broth microdilution method was determinated (EUCAST). Detection of plasmid-encoded colistin resistance genes (*mcr-1* to *mcr-9*) was performed by PCR.

Whole genome sequencing, bioinformatics data analysis and conjugation experiments

All isolates positive for *mcr* genes were subjected to whole genome sequencing (WGS). Genomic DNA was obtained using the NucleoSpin Tissue kit (Macherey-Nagel) and DNA libraries were prepared with the Nextera XT DNA Library Preparation Kit (Illumina). The obtained sequencing data were qualitatively processed by the program Trimmomatic v0.36. and then assembled into fast format using SPAdes v3.13.1 software. Bacterial sequence types, antibiotic resistance genes, virulence factors and plasmid replicons were determined using CGE tools available at http://www.genomicepidemiology.org. Transferability of plasmids to recipient *E. coli* were tested by conjugative experiment in 3 different cultivate temperature (IncX4 in 42°C, IncI2 in 37°C and IncHI2 in 28°C).

Results

By selective cultivation on agar medium with colistin a total of 94 (23,5 %, n=400) resistant isolates were obtained from samples of veterinary origin (poultry 91,5 %, pigs 4 % and cattle 4 %). Resistant isolates were identified as *E. coli* (n=79/94), *Klebsiella spp.* (n=11/94) and *Moellerella wisconsensis* (n=4/94). The presence of *mcr-1* genes was confirmed in 54 isolates from which 51 were from poultry, 2 from cattle and 1 from a pig. Other variants of *mcr* genes have not been found. In terms of sample type, isolates with *mcr* genes were primarily detected in samples from meat (n=51/53, n=94 %) compared to those from caecum. Susceptibility testing revealed resistance to more than 7 antibiotics (ampicillin, streptomycin, sulphonamides, tetracycline, trimethoprim-sulfamethoxazole, nalidixic acid and ciprofloxacin) in 44 % of veterinary isolates (24/54) while ESBL production was detected only in 4 isolates. All *mcr-1* positive isolates were sensitive to carbapenems and fosfomycin.

From 1440 pure bacterial cultures resistant to colistin, 69 *mcr*-positive isolates were obtained by PCR testing. The *mcr-1* gene was detected in *E. coli* (n= 44/69) and *Klebsiella pneumoniae* (n=4/69), the second most frequent variant was *mcr-9* found in *Enterobacter cloaceae complex* (n=19/69), *Citrobacter freundii* (n=1/69) and *Serratia* spp. (n=1/69). In two isolates *mcr-4.3* variant was detected. Twenty-nine isolates (42 %) were resistant to more than 5 antibiotics and production of ESBL/AmpC has been demonstrated in 11 isolates.

A total of 121 *mcr*-positive isolates (52 from slaughtered animals and 69 of human origin) were subjected to WGS. The genetic diversity of sequence type (ST) was overall high. The most common ST among *E. coli* veterinary isolates were ST1011 and ST162 (both n=9).

Genes for resistance to aminoglycosides (*aph(6)-Id, aph(3'')-Ib*), sulphonamides (*sul1, sul2, sul*3), quinolones (*qnrS1, qnrB19*) and narrow-spectrum beta-lactams (bla_{TEM-1B}) have been demonstrated in these isolates. Genes encoding ESBL (bla_{CTX-M}, bla_{CMY}) were detected in 4 isolates. WGS revealed a chromosomal mutations of *gyrA p.S83L*, *gyrA p.D87N* and *parC p.S80I* conferring quinolone resistance in 60% of *E. coli*. In human clinical isolates, most *E. coli* strains belonged to ST744 (10/69) while the majority of *Enterobacter cloaceae complex* isolates were identified as ST484 (8/69). Different variants of *bla* genes encoding resistance to beta-lactams (bla_{TEM}, $bla_{CTX-M,}$ bla_{CMY}, bla_{ACT}), sulphonamides (*sul1, sul2*) and aminoglycosides (*aac(3)-IId, aadA1, aadA5, aph(3'')-Ib, aph(6)-Id*) dominated from the detected antibiotic resistome.

Plasmid replicons IncFIB (n=44/52; 84 %) and IncX4 (n=42/52; 80%) dominated in veterinary isolates. However, the transferability of *mcr*-carrying plasmids have not yet been tested. In case of clinical isolates, the most frequent plasmid replicons carrying *mcr-1* were IncX4 (n=34/69; 49%), IncI2 (n=8/69; 11,5%) and IncHI2 (n=6/69; 9%). The rest of the isolates (n=19/69) carried *mcr-9* gene located on IncHI2 plasmid or incorporated on chromosome. Conjugation experiment confirmed the transfer of *mcr* to the recipient *E. coli* in 45 out of 50 isolates.

Discussion and conclusion

Plasmid-mediated colistin resistance in Enterobacterales was firstly detected in 2015 in China. Over time, colistin resistance was detected in all continents. According to the European Centre for Disease Prevention and Control (ECDC; 2017), human isolates resistant to colistin represented 8.5% [2]. In human isolates we observed low prevalence of mcr genes (opposite to veterinary sector), yet the occurrence of colistin-resistant bacteria should be considered as a possible risk for health. Our study showed that prevalence of *mcr* genes (primarily *mcr-1*) in the meat samples from poultry was higher than from other animal samples as was found in other studies [3]. Nevertheless, colistin consumption and the occurrence of *mcr* genes is still lower in Europe compared to other continents, especially Asia. The most frequent type of plasmids was IncX4 and IncI2 (both carrying *mcr-1* gene) which are often connected with plasmid-mediated colistin resistance [3]. Rational using of colistin and obtaining information of mechanism its resistance will help to limit the spread of these resistant bacteria and thus reduce the risk of the development of life-threatening infections

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