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Corylus avellana bark optical properties differ during and out of the
vegetation season

Optične lastnosti skorje navadne leske se razlikujejo med vegetacijsko sezono
in izven nje

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Abstract: We compared different bark traits of the common hazel (*Corylus avellana* L.) in four different months during the vegetation season (September, November) and out of the vegetation season (January, February) to get an insight into the changes in bark morphological, biochemical, and optical properties. Since bark of woody plants contains a layer of chlorenchyma, which can harvest transmitted light and perform photosynthesis, we also measured bark potential photochemical efficiency of photosystem II. The values of the latter parameter decreased during the research period, ranging from 0.77 in September to 0.22 in February. This could be attributed to low temperatures. The shapes of the bark reflectance curves were similar between the four samplings, with a peak in red and pronounced reflectance in the near-infrared spectrum. However, the level of reflectance differed between the four samplings. Regarding the time of the season, we obtained the most pronounced changes in the green, yellow, and red reflectance spectra. Light reflectance in these regions was positively related with chlorophyll *b* and carotenoid contents, while it was negatively related with anthocyanins and UV-B-absorbing substances. Transmittance spectra showed less variability between the four samplings. Regarding the studied pigments, the most pronounced changes were obtained for anthocyanin and UV-B-absorbing substances contents, which decreased in accordance with decreasing environmental temperatures. On the contrary, the level of photosynthetic pigments remained high, thus enabling undisturbed primary metabolism.

Keywords: bark, *Corylus avellana*, optical properties, pigments, potential photochemical efficiency of photosystem II (Fv/Fm)

Izveček: Primerjali smo lastnosti skorje navadne leske (*Corylus avellana* L.) v štirih različnih mesecih med vegetacijskim obdobjem in izven vegetacijskega obdobja. Predvidevali smo, da se bodo biokemijske in posledično optične lastnosti skorje po odpadanju listov spremenile. Ker skorja lesnatih rastlin vsebuje tudi plast klorenhima, ki prestreza presevano svetlobo in vrši fotosintezo, smo na skorji merili tudi potencialno fotokemično učinkovitost fotosistema II. Izmerjene vrednosti slednjega parametra so se tekom raziskave zniževale, in sicer od 0,77 pri septembrskih vzorcih do 0,22 pri februarskih vzorcih, kar bi lahko pripisali nizkim temperaturam. Oblika

krivulj odbojnosti sevanja skorje je bila med štirimi vzorčenji zelo podobna, z viškom v rdečem območju in veliko odbojnostjo v bližnjem infrardečem območju, medtem ko je bila raven odbojnosti med temi vzorčenji različna. Največje razlike v odbojnosti sevanja med vegetacijskim obdobjem in izven vegetacijskega obdobja smo zaznali v zelenem, rumenem in rdečem spektralnem območju. Odbojnost svetlobe v teh območjih je bila pozitivno povezana z vsebnostjo klorofila *b* in karotenoidov, negativno pa z antocianini in UV-B–absorbirajočimi snovmi. Presevni spektri so pokazali manjšo variabilnost. Primerjava različnih pigmentov je pokazala najbolj izrazite spremembe v vsebnosti antocianinov in UV-B–absorbirajočih snovi, ki so se manjšale skladno z zniževanjem okoljskih temperatur. Raven fotosinteznih pigmentov je skozi celotno obdobje ostala na visoki ravni in s tem omogočila nemoten primarni metabolizem.

Ključne besede: barvila, *Corylus avellana*, optične lastnosti, potencialna fotokemična učinkovitost fotosistema II (Fv/Fm), skorja

Introduction

Bark is a plant tissue outside the cambium layer, which can be found in stems, branches, and roots of woody plants, and has an important physiological and protective function (Martin and Crist 1970). It consists of the inner bark with secondary phloem and the outer bark or periderm, which is produced by the cork cambium (Romero 2014). Under the outer peridermal layers (rhytidomal), bark contains a layer of chlorenchyma that can harvest transmitted light and perform photosynthesis (Filippou et al. 2007, Wittmann and Pfanz 2008, 2014). The study of 24 different species revealed lower chlorophyll contents in twigs in comparison to leaves, but a higher chlorophyll/carotenoid ratio, indicating improved light harvesting (Levizou and Manetas 2007). Ultrastructural studies of bark and stem chloroplasts revealed that their features are typical for shade plants (Leong and Anderson 1984). However, in the case of *Eucalyptus nitens* bark exposed to high light conditions, the level of photosynthetic pigments was comparable to that of sun leaves (Tausz et al. 2005). Besides chlorophyll content, the level of bark photosynthesis also depends on other bark traits that shape bark optical properties. Light that reaches the plant tissue is a result of bark optical properties that comprise light reflectance, absorptance, and transmittance (Pilarski et al. 2008, Klančnik et al. 2015). It was shown that bark reflects light in the wavelength range between 700 and 2,000 nm that presents an optical window, in which light is reflected and transmitted in green plants (Henrion and Tributsch

2009). Transmittance of visible light through the periderm is low and depends on species and age of branches or stems (Aschan et al. 2001). Periderm absorbs a significant share of visible light, thus transmitting only 10–50% of ambient light (Manetas and Pfanz 2005). In the case of *Fagus sylvatica*, absorption of the bark decreased with increasing wavelength from about 92% at 400 nm to about 15% at 700 nm (Pilarski et al. 2008). Optical properties of a certain plant tissue are strongly affected by its overall biochemical properties (Klančnik et al., 2014a,b, 2016), primarily by the level of photosynthetic pigments. The level of photosynthetic pigments is changing due to varying environmental conditions and throughout the season (Larcher 2003). For example, in *Populus tremuloides* chlorophylls and carotenoids in bark showed the highest levels in late summer and the lowest levels in winter (Barr and Potter 1974). The variability in bark optical and biochemical properties also affects bark photosynthesis, which is enabled by CO₂ from the respiring living cells of xylem parenchyma, cambium, and phloem (Aschan and Pfanz 2003). Bark photosynthesis is especially important in deciduous trees out of the vegetation season, when plants are leafless. However, in the evergreen shrub *Myrica cerifera*, it was reported that bark photosynthesis increased carbon- and water-use efficiency, which contributed to the expansion of this species in the coastal environments (Vick and Young 2009). The level of photosynthesis in four-year-old stems of *Pinus monticola* and in young stems of *Betula pendula* was about 1 mmol m⁻² s⁻¹ (Cernusak and Marshall

2000, Wittmann et al. 2006), which is rather low in comparison to the level of photosynthesis, typical of leaves (Larcher 2003). Light-use efficiency of chlorenchyma may be estimated by measuring potential photochemical efficiency (F_v/F_m), which can also be used for monitoring changes in the physiological condition of bark in various trees (Alekseev et al. 2007). In *Pinus sylvestris* needles, Ivanov et al. (2006) detected a 65% reduction in F_v/F_m in winter, while in bark chlorenchyma, F_v/F_m remained relatively high.

In this study, we aimed to examine changes in the functional traits of bark in one-year-old twigs of the deciduous shrub *Corylus avellana* during and out of the vegetation season. We hypothesised that bark biochemical properties and consequently optical properties will change after the leaves will fall off. In addition, we also expected changes in F_v/F_m due to unfavourable temperatures in winter.

Materials and methods

Species and site description

The common hazel (*Corylus avellana* L.) is a 3–8 m high deciduous shrub, assigned to the vascular plant family Corylaceae, that thrives in open spaces at forest edges but is also used for hedgerows. It is native to Europe and western Asia. Monoecious flowers occur on bare branches in late winter to early spring before leaf development (Martinčič et al. 2007).

Plant samples were collected in open spaces in the area of Otočec (194 m a.s.l.; 45.8339 °N, 15.2202 °E). At each of the four samplings, one-year-old twigs were collected from ten randomly selected 1.5–2 m high plants. The twigs were processed on the day of sampling. The sampling and the analysis took place in September and November 2019, before the leaves fell off, and afterwards in January and February 2020, before the development of new leaves. Climate records on minimum daily air temperatures in the period from 1 September 2019 to 17 February 2020, obtained from the nearest meteorological station Novo mesto, are shown in Fig. 1.

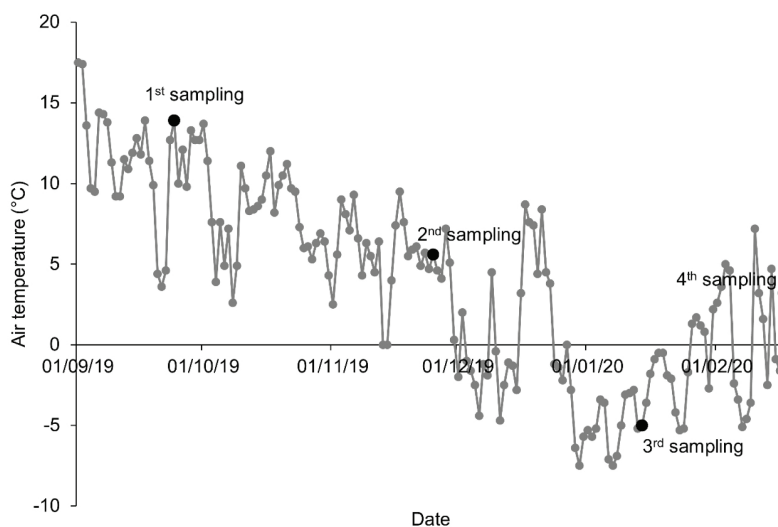


Figure 1: Dynamics of minimum daily air temperatures in the period 1 September 2019 – 17 February 2020 at the meteorological station Novo mesto, nearest to the sampling site.

Slika 1: Minimalne dnevne temperature zraka od 1. septembra 2019 do 17. februarja 2020, izmerjene na najbližji meteorološki postaji (Novo mesto).

Morphological properties

The analysis of bark thickness was carried out on transverse sections of vital one-year-old twigs. The thicknesses of secondary phloem and periderm were measured using a light microscope (CX41; Olympus, Tokyo, Japan) equipped with a digital camera (XC30; Olympus) and the CellSens software (Olympus).

Biochemical and physiological properties

Chlorophyll *a*, chlorophyll *b*, and carotenoid contents were determined for bark extracts according to the methodology described by Lichtenthaler and Buschmann (2001a,b). Acetone extracts' absorbances of ground bark samples were measured at three different wavelengths, namely 470 nm, 645 nm, and 662 nm, using a UV/VIS spectrometer (Lambda 25; Perkin-Elmer, Norwalk, CT, USA). Anthocyanin content was determined following the methodology of Drumm and Mohr (1978). Bark samples were ground, extracted in methanol:37% HCl = (99:1 (v/v)), centrifuged (4,000 rpm, 4 °C, 4 min) and then stored at 3–5 °C in the dark for 24 hours. Absorbances of the extracts were measured with a UV/VIS spectrometer at a wavelength of 530 nm. Total methanol-soluble UV-B-absorbing and UV-A-absorbing substances, which are a measure of total phenolics in ground bark samples, were extracted in a mixture of methanol, distilled water, and 37% HCl (79:20:1 (v/v)), according to the method described by Caldwell (1968). The samples were centrifuged (4,000 rpm, 10 °C, 10 min) and then scanned in the range from 280 nm to 319 nm for UV-B-absorbing substances, and in the range from 320 nm to 400 nm for UV-A-absorbing substances. The absorbance values were integrated for each UV region. All biochemical parameters are expressed per bark area. Fv/Fm was measured on fresh twigs as described by Schreiber et al. (1996), using a portable chlorophyll fluorometer (PAM-2100; Heinz Walz GmbH, Effeltrich, Bavaria, Germany). Before the measurement, twigs were kept in dark for 20 minutes.

Bark and leaf reflectance and transmittance

The optical properties of bark and leaves (when these were present) were determined in the laboratory on the day of sampling. Bark was carefully removed from the twigs with a razor blade. The measurements were performed in the range from 290 nm to 800 nm for reflectance and in the range from 290 nm to 800 nm for transmittance, using a portable spectrometer (Jaz Modular Optical Sensing Suite; Ocean Optics Inc., Dunedin, FL, USA; grating, #2; slit size, 25 µm) that was connected with an optical fibre (QP600-1-SR-BX; Ocean Optics Inc.) and an integrating sphere (ISP-30-6-R; Ocean Optics Inc.). The resolution of the measurements was ~1.3 nm. The reflectance spectrum was measured for the bark/leaf surface by illumination with a UV/VIS-near-infrared (NIR) light source (DH-2000; Ocean Optics, Inc.). We calibrated the spectrometer to 100% reflectance using a white reference panel with > 99% diffuse reflectance (Spectralon; Labsphere, North Sutton, NH, USA). In the case of the transmittance spectra, periderm surface was illuminated with a light source, while the integrating sphere that captured the transmitted light was positioned below the bark (or leaf). The incident angle of bark/leaf illumination was 90°. Prior to the measurement, we calibrated the spectrometer to 100% transmittance by light beam passing directly into the interior of the integrating sphere.

Statistical analysis

The significance of the differences between the four different samplings for each of the measured bark parameter was tested using one-way analysis of variance followed by Duncan's post-hoc multiple comparison tests. Prior to this analysis, normal distributions of the data were tested using Shapiro-Wilk tests, and homogeneity of variance from the means was analysed using Levene's tests. The relationships between the physiological, biochemical, and morphological bark properties, and bark reflectance and transmittance were evaluated using Pearson's correlation analysis. IBM SPSS statistics 22.0 (IBM, Armonk, NY, United States) was used for these statistical analyses. The graphs were drawn in Microsoft Excel 2016 (Microsoft,

Redmond, WA, USA). In addition, in the case of leaf reflectance and transmittance spectra, we performed Student's t-tests in Microsoft Excel 2016 to examine the differences between the first two samplings for each region of the leaf reflectance and leaf transmittance spectra separately, with significance accepted at $P \leq 0.05$.

Results

Bark and leaf optical properties

Bark reflectance spectra measurements revealed similar shapes, but different levels of light reflectance (Fig. 2). For all these measurements, we detected a peak in red and a pronounced increase in reflectance in NIR. We observed marked differences from violet to red between the autumn (September and November) and winter samples (January and February) (Tab. 1).

Bark transmittance spectra measurements revealed different shapes. The first peak was wider, ranging from green to red (Fig. 3). The autumn samples differed more than winter samples (Tab. 1).

There was practically no transmittance at short wavelengths.

Leaf optical properties were measured in September, while the leaves were still green, and in November, when they turned yellow. This is clearly seen in the reflectance curves, which display lower values and a distinct peak in green in September, and much higher values with a very wide peak ranging from green to orange in November (Fig. 4). In September, the second peak only starts in NIR, while in November it already starts in red. The reflectance in short wavelengths did not differ significantly between the two samplings (Tab. 2).

In the case of leaf transmittance spectra, the differences between the two samplings were even more pronounced. When the leaves turned yellow, they became more transparent throughout the whole spectrum (Fig. 5). However, the differences in short wavelengths were not significant (Tab. 2). The shape of the leaf transmittance curves was very similar to those of the leaf reflectance spectra. The curve from September shows a distinct peak in green, while the one from November has a very wide peak, ranging from green to orange, as was the case in leaf reflectance spectra.

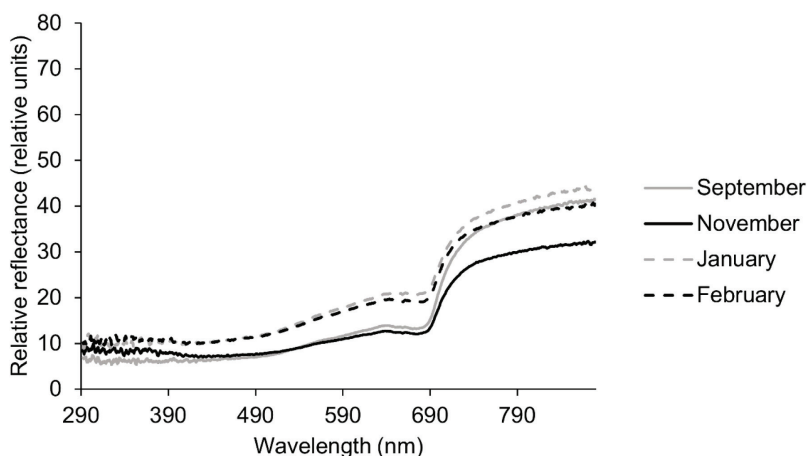


Figure 2: Mean relative bark reflectance (290 nm – 880 nm) for *Corylus avellana*, sampled in September, November, January, and February; the data were smoothed using moving averages with a period of five consecutive measurements ($N = 10$).

Slika 2: Povprečna relativna odbojnost skorje navadne leske med 290 in 880 nm ob vzorčenju v septembru, novembru, januarju in februarju; podatki so bili zglajeni z drsečimi povprečji iz vsakih sledečih petih zaporednih meritev ($N = 10$).

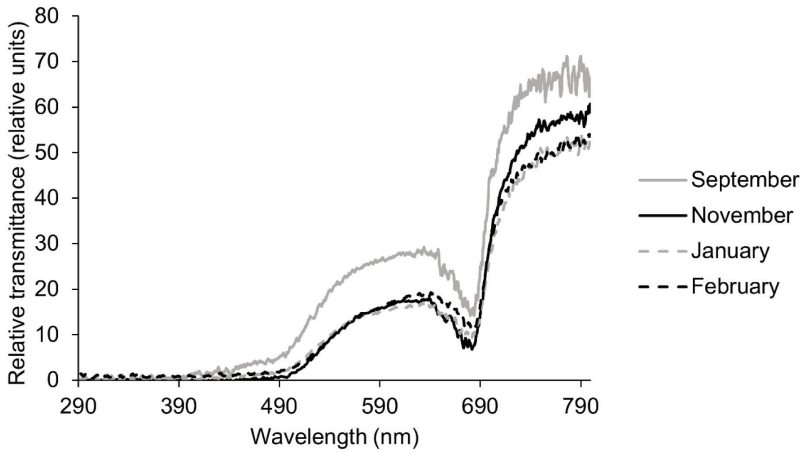


Figure 3: Mean relative bark transmittance (290 nm – 800 nm) for *Corylus avellana*, sampled in September, November, January, and February; the data were smoothed using moving averages with a period of five consecutive measurements (N = 10).

Slika 3: Povprečna relativna presevnost skorje navadne leske med 290 in 800 nm ob vzorčenju v septembru, novembru, januarju in februarju; podatki so bili zglajeni z drsečimi povprečji iz vsakih sledečih petih zaporednih meritev (N = 10).

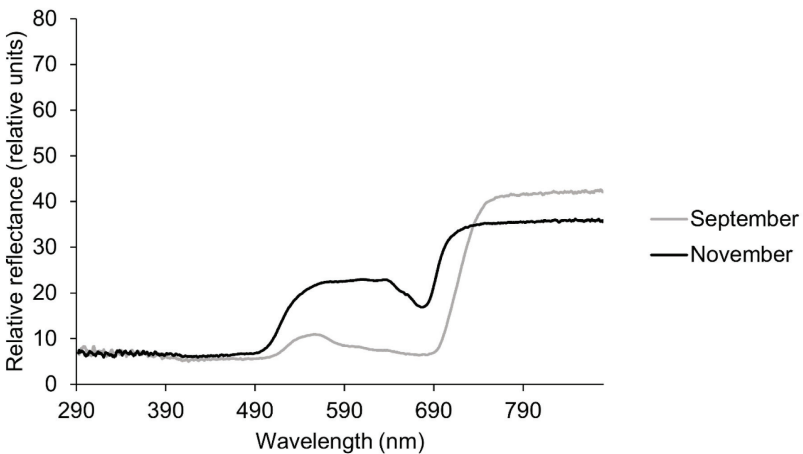


Figure 4: Mean relative leaf reflectance (290 nm – 880 nm) for *Corylus avellana*, sampled in September and November; the data were smoothed using moving averages with a period of five consecutive measurements (N = 10).

Slika 4: Povprečna relativna odbojnost listov navadne leske med 290 in 880 nm ob vzorčenju v septembru in novembru; podatki so bili zglajeni z drsečimi povprečji iz vsakih sledečih petih zaporednih meritev (N = 10).

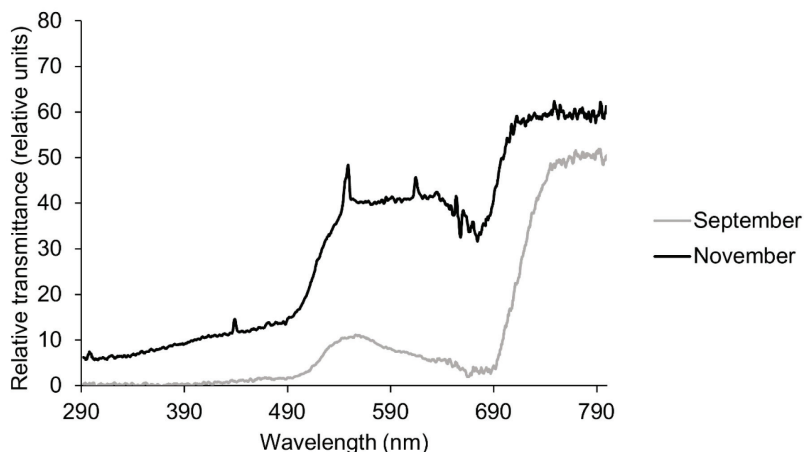


Figure 5: Mean relative leaf transmittance (290 nm – 800 nm) for *Corylus avellana*, sampled in September and November; the data were smoothed using moving averages with a period of five consecutive measurements ($N = 10$).

Slika 5: Povprečna relativna presevnost listov navadne leske med 290 in 800 nm ob vzorčenju v septembru in novembru; podatki so bili zglajeni z drsečimi povprečji iz vsakih sledečih petih zaporednih meritev ($N = 10$).

Besides optical properties, Tab. 1 also summarises physiological, biochemical, and morphological bark properties of *Corylus avellana* at different times of the year. There are some hints of changes in bark biochemical properties, however, most of them are not significant. There was an evident reduction in anthocyanins from September to February, and a decrease in photosynthetic pigments in November, while these showed an increase again towards spring. The

level of UV-B-absorbing substances was somewhat higher in September and was later reduced. Small changes were also seen for bark morphological properties, which was expected. More pronounced changes were obtained when comparing F_v/F_m at different times of the year, which was decreasing along with decreasing temperatures. The values were optimal in September, while later on they revealed the presence of stress.

Table 1: Optical, physiological, biochemical, and morphological bark properties of *Corylus avellana*, sampled in September, November, January, and February.**Tabela 1:** Optične, fiziološke, biokemijske in morfološke lastnosti skorje navadne leske ob vzorčenju v septembru, novembru, januarju in februarju.

| Bark traits | Units | September | November | January | February |
|-------------------------------|---------------------|-----------------------------|-----------------------------|-----------------------------|----------------------------|
| Optical: reflectance | | | | | |
| | au | | | | |
| UV-B | | 6.33 ± 1.57 ^a | 8.55 ± 1.96 ^b | 10.54 ± 2.00 ^c | 10.38 ± 1.97 ^c |
| UV-A | | 6.17 ± 1.18 ^a | 8.21 ± 1.47 ^b | 10.25 ± 1.74 ^c | 10.80 ± 1.58 ^c |
| Violet | | 6.32 ± 1.43 ^a | 7.25 ± 1.24 ^a | 10.26 ± 2.08 ^b | 10.20 ± 1.46 ^b |
| Blue | | 6.90 ± 1.91 ^a | 7.53 ± 1.41 ^a | 11.27 ± 2.53 ^b | 11.15 ± 1.88 ^b |
| Green | | 9.22 ± 3.15 ^a | 9.17 ± 2.18 ^a | 14.65 ± 3.94 ^b | 14.12 ± 2.74 ^b |
| Yellow | | 12.17 ± 3.65 ^a | 11.37 ± 2.47 ^a | 18.55 ± 4.22 ^b | 17.62 ± 3.12 ^b |
| Red | | 14.24 ± 4.41 ^a | 12.86 ± 3.18 ^a | 21.31 ± 4.37 ^b | 19.82 ± 4.13 ^b |
| NIR | | 36.74 ± 7.46 ^b | 29.04 ± 7.06 ^a | 39.80 ± 8.35 ^b | 36.88 ± 6.96 ^b |
| Optical: transmittance | | | | | |
| | au | | | | |
| UV-B | | -0.13 ± 1.54 ^a | -0.18 ± 0.63 ^a | 0.06 ± 0.68 ^{ab} | 1.00 ± 2.29 ^b |
| UV-A | | 0.27 ± 1.20 ^a | -0.16 ± 0.50 ^a | 0.08 ± 0.58 ^a | 0.92 ± 2.31 ^a |
| Violet | | 1.83 ± 2.19 ^b | -0.02 ± 0.44 ^a | 0.58 ± 0.94 ^{ab} | 1.11 ± 2.47 ^{ab} |
| Blue | | 4.44 ± 3.81 ^b | 0.48 ± 0.70 ^a | 1.54 ± 1.63 ^a | 1.75 ± 2.63 ^a |
| Green | | 18.30 ± 9.81 ^b | 8.77 ± 6.04 ^a | 9.46 ± 6.79 ^a | 9.07 ± 5.87 ^a |
| Yellow | | 26.99 ± 7.48 ^b | 16.50 ± 5.55 ^a | 15.51 ± 7.06 ^a | 17.03 ± 5.86 ^a |
| Red | | 24.83 ± 8.97 ^b | 14.83 ± 7.99 ^a | 14.94 ± 7.74 ^a | 17.35 ± 7.46 ^a |
| NIR | | 62.71 ± 8.52 ^b | 52.61 ± 12.11 ^a | 46.48 ± 14.81 ^a | 47.48 ± 10.31 ^a |
| Physiological | | | | | |
| Fv/Fm | au | 0.768 ± 0.038 ^d | 0.604 ± 0.077 ^c | 0.316 ± 0.072 ^b | 0.226 ± 0.050 ^a |
| Biochemical | | | | | |
| Chlorophyll <i>a</i> | mg cm ⁻² | 0.012 ± 0.006 ^b | 0.006 ± 0.002 ^a | 0.012 ± 0.006 ^b | 0.016 ± 0.003 ^b |
| Chlorophyll <i>b</i> | mg cm ⁻² | 0.016 ± 0.005 ^b | 0.009 ± 0.003 ^a | 0.019 ± 0.009 ^b | 0.020 ± 0.005 ^b |
| Carotenoids | mg cm ⁻² | 0.004 ± 0.001 ^{ab} | 0.002 ± 0.001 ^a | 0.004 ± 0.001 ^{ab} | 0.008 ± 0.009 ^b |
| Anthocyanins | au cm ⁻² | 0.884 ± 0.378 ^b | 0.672 ± 0.288 ^{ab} | 0.649 ± 0.249 ^{ab} | 0.480 ± 0.170 ^a |
| UV-B-AS | au cm ⁻² | 6.076 ± 2.178 ^b | 4.144 ± 1.108 ^a | 4.587 ± 1.114 ^a | 4.335 ± 0.588 ^a |
| UV-A-AS | au cm ⁻² | 4.566 ± 1.590 ^a | 3.495 ± 1.063 ^a | 4.224 ± 1.516 ^a | 4.131 ± 0.418 ^a |
| Morphological | | | | | |
| Periderm thickness | µm | 68.56 ± 8.19 ^{ab} | 63.14 ± 10.54 ^{ab} | 72.28 ± 8.46 ^b | 61.49 ± 7.99 ^a |
| Secondary phloem thickness | µm | 103.85 ± 13.52 ^b | 88.09 ± 11.95 ^{ab} | 91.06 ± 12.53 ^{ab} | 78.29 ± 17.40 ^a |

Data are means ± SD (N = 10 for each column); different superscript letters within each row indicate significant differences ($P \leq 0.05$; Duncan tests); reflectance and transmittance spectra represent means within 5-nm intervals ($P \leq 0.05$, Duncan tests); au, arbitrary units; NIR, near-infrared; Fv/Fm, potential photochemical efficiency; UV-B-AS, UV-B-absorbing substances; UV-A-AS, UV-A-absorbing substances.

Table 2: Leaf reflectance and transmittance of *Corylus avellana* leaves, sampled in September and November.**Tabela 2:** Odbojnost in presevnost listov navadne leske ob vzorčenju v septembru in novembru.

| Colour region | Reflectance (au) | | Transmittance (au) | |
|---------------|---------------------------|---------------------------|----------------------------|----------------------------|
| | September | November | September | November |
| UV-B | 7.30 ± 1.62 ^a | 6.75 ± 1.53 ^b | 0.28 ± 0.66 ^a | 6.19 ± 17.16 ^a |
| UV-A | 6.60 ± 1.04 ^a | 6.75 ± 1.52 ^a | 0.24 ± 0.50 ^a | 7.90 ± 17.05 ^a |
| Violet | 5.45 ± 0.41 ^a | 6.24 ± 1.26 ^a | 0.80 ± 0.89 ^a | 11.36 ± 16.97 ^a |
| Blue | 5.64 ± 0.30 ^a | 6.76 ± 1.31 ^b | 1.61 ± 1.31 ^a | 13.80 ± 16.79 ^b |
| Green | 9.10 ± 2.09 ^a | 17.93 ± 6.67 ^b | 8.12 ± 5.47 ^a | 34.34 ± 18.42 ^b |
| Yellow | 8.28 ± 0.98 ^a | 22.75 ± 6.45 ^b | 7.43 ± 4.25 ^a | 41.34 ± 14.09 ^b |
| Red | 7.17 ± 0.93 ^a | 21.06 ± 8.06 ^b | 4.74 ± 3.53 ^a | 39.27 ± 15.35 ^b |
| NIR | 38.03 ± 8.10 ^a | 35.17 ± 9.01 ^a | 42.02 ± 13.59 ^a | 58.90 ± 10.48 ^b |

Data are means ± SD (N = 10 for each column); different superscript letters within each row indicate significant differences ($P \leq 0.05$; Student's t-tests); reflectance and transmittance spectra represent means within 5-nm intervals ($P \leq 0.05$; Student's t-tests); au, arbitrary units; NIR, near-infrared.

Pearson's correlation analysis between bark optical properties (reflectance and transmittance), and bark physiological, biochemical, and morphological properties revealed some strong relations (Tab. 3). The strongest negative correlation was obtained between bark reflectance in all regions of the spectrum except NIR, and Fv/Fm. Moderate to strong negative correlation was also obtained between bark reflectance and anthocyanins as well as UV-B-absorbing substances. As in the case of Fv/Fm, this relationship was significant across all

regions of the spectrum, with the exception of the NIR region. There was also positive correlation between bark reflectance in green, yellow, and red, and carotenoids, and negative correlation between bark reflectance in short wavelengths (UV-B, UV-A, violet) and secondary phloem thickness.

We obtained less significant relations between bark transmittance spectra and bark physiological, biochemical, and morphological properties. All of these relations were positive.

Table 3: Pearson correlation coefficients between bark optical properties (reflectance and transmittance) and bark physiological, biochemical, and morphological properties for *Corylus avellana*; significant correlations are indicated in bold.

Tabela 3: Pearsonovi korelacijski koeficienti med optičnimi lastnostmi skorje (odbojnost in presevnost) ter fiziološkimi, biokemijskimi in morfološkimi lastnostmi skorje navadne leske; statistično značilne korelacije so označene s krepko pisavo.

| Bark trait | Fv/Fm | Chl <i>a</i> | Chl <i>b</i> | Carotenoids | Anthocyanins | UV-B-AS | UV-A-AS | Periderm | Sec. phloem |
|----------------------|----------------|--------------|--------------|--------------|---------------|----------------|---------|----------|---------------|
| Reflectance | | | | | | | | | |
| UV-B | -0.80** | 0.16 | 0.22 | 0.15 | -0.38* | -0.41** | -0.19 | 0.14 | -0.34* |
| UV-A | -0.81** | 0.21 | 0.26 | 0.23 | -0.38* | -0.43** | -0.21 | 0.07 | -0.40* |
| Violet | -0.70** | 0.26 | 0.29 | 0.28 | -0.34* | -0.41** | -0.24 | 0.12 | -0.32* |
| Blue | -0.65** | 0.26 | 0.28 | 0.30 | -0.34* | -0.41** | -0.26 | 0.12 | -0.29 |
| Green | -0.59** | 0.31 | 0.33* | 0.32* | -0.35* | -0.39* | -0.25 | 0.11 | -0.26 |
| Yellow | -0.57** | 0.31 | 0.34* | 0.35* | -0.36* | -0.36* | -0.23 | 0.15 | -0.22 |
| Red | -0.56** | 0.25 | 0.28 | 0.34* | -0.36* | -0.35* | -0.24 | 0.20 | -0.20 |
| NIR | -0.23 | 0.34* | 0.34* | 0.23 | -0.07 | -0.13 | -0.10 | 0.24 | 0.13 |
| Transmittance | | | | | | | | | |
| UV-B | -0.27 | 0.34* | 0.39* | 0.15 | -0.14 | 0.05 | 0.05 | -0.06 | 0.04 |
| UV-A | -0.14 | 0.34* | 0.38* | 0.12 | -0.07 | 0.13 | 0.07 | -0.03 | 0.12 |
| Violet | 0.11 | 0.30 | 0.30 | 0.11 | 0.10 | 0.36* | 0.18 | 0.09 | 0.21 |
| Blue | 0.27 | 0.25 | 0.22 | 0.09 | 0.20 | 0.39* | 0.17 | 0.13 | 0.21 |
| Green | 0.41** | 0.16 | 0.14 | 0.07 | 0.29 | 0.28 | 0.10 | 0.08 | 0.13 |
| Yellow | 0.37* | 0.13 | 0.10 | 0.10 | 0.30 | 0.33* | 0.17 | 0.05 | 0.11 |
| Red | 0.25 | 0.18 | 0.13 | 0.15 | 0.30 | 0.42** | 0.27 | 0.10 | 0.11 |
| NIR | 0.43** | 0.07 | 0.06 | 0.05 | 0.36* | 0.27 | 0.14 | 0.03 | 0.19 |

*, $P \leq 0.05$; **, $P \leq 0.01$; NIR, near-infrared; Fv/Fm, potential photochemical efficiency; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; UV-B-AS, UV-B-absorbing substances; UV-A-AS, UV-A-absorbing substances; Sec. phloem, secondary phloem.

Discussion

We compared different traits of *Corylus avellana* bark at different times of the year, during and out of the vegetation season. Bark of woody plants contains a layer of chlorenchyma that can harvest transmitted light and perform photosynthesis. During the vegetation season, the presence of leaves alters the environmental conditions affecting bark out of two reasons. One is changing of the quality and quantity of solar radiation reaching the bark due to filtering by leaves, which absorbs a significant share of radiation, as shown in the present study. The other reason is related with changes in temperature, which are more

extreme out of the vegetation season (Středa et al. 2015). The period out of the vegetation season in temperate climates is related with low winter temperatures that may affect bark structure and function, as shown by the Fv/Fm measurements in our study. These were decreasing in accordance with decreasing minimum daily temperatures. Alekseev et al. (2007) also reported about the winter decrease of Fv/Fm activity and spring recovery, depending on climatic conditions of a particular year. In the case of bark of *Populus tremula*, Fv/Fm recovered only in late April, right before the start of the vegetation season (Solhaug and Haugen 1998).

Bark optical properties changed significantly during the studied period. Besides the time of the season, they also depend on twig age, which both determine their physical structure (Pilarski 1989, Kharouk et al. 1995, Pilarski et al. 2008) and their biochemical properties (Tokarz and Pilarski 2005), as also shown for different leaf types (Klančnik et al. 2014a, 2014b, 2016, Grašič et al. 2020). In the present study, bark spectral signatures (reflectance) revealed significant differences between the vegetation season and the period out of the vegetation season. The most pronounced differences were seen in the green, yellow, and red spectra. Light reflectance in these regions was positively related with chlorophyll *b* and carotenoid contents, while it was negatively related with anthocyanins and UV-B-absorbing substances. This is supported by previous studies, which showed that increased anthocyanin production in leaves enhanced the absorbance in the green and yellow regions (Neill and Gould 1999). Bark has optimised its reflection of incoming radiation in the range from 700 nm to 2,000 nm, which enables its temperature control (Henrion and Tributsch 2009). This was confirmed by high reflectance and transmittance in NIR in the present study with *C. avellana*. However, the relation between light transmittance and the measured parameters was less consistent. Thus, some additional parameters that were not included in the study might be involved. More pronounced changes were obtained in leaf optical properties in autumn. The leaf reflectance curves showed lower reflectance and a distinct green peak in September, and much higher reflectance and a very wide peak ranging from green to orange in November. The differences between the September and November samples were significant across the whole spectrum except for the UV-A, violet, and NIR regions. Leaves become much more transparent in November, especially in the range from 400 nm to 600 nm. These changes occur due to degradation of photosynthetic pigments and the persistence of other pigments, especially anthocyanins (Junker and Ensminger 2016).

Bark biochemistry changed only slightly during the studied period. The most pronounced changes were obtained for anthocyanins, which have multiple functions in bark. Besides affecting optical properties, anthocyanins exert beneficial effects on plant physiological processes, play a

role in plant interactions with other organisms, for instance in repellence of herbivores and parasites, and provide the camouflage of plants against their background (Lev-Yadun and Gould 2008). The experiment with red maple revealed that higher anthocyanin levels in bark were induced by lower temperatures in winter (Sibley et al. 1999). This is contrary to our results, as we observed a decrease in anthocyanin levels with decreasing environmental temperatures (Fig. 1). This decrease was possibly due to lower photosynthetic activity, which was limited by low Fv/Fm under low temperatures. In white pine bark, the level of photosynthesis increased with increasing light intensity and bark temperature, maximum net photosynthesis being approximately 76% of dark respiration (Cernusak and Marshall 2000). We also observed a decrease in the production of UV-absorbing substances, which are various phenolic substances that present an efficient UV radiation filter (Rozema et al. 2002) and have a strong antioxidant and antibacterial effect, acting as radical scavengers and biocides (Pietarinen et al. 2006). Their production is an energetically demanding process (Germ et al. 2006). Therefore, the synthesis of these substances is the result of a trade-off between their production and investment of plants in primary metabolism. Conversely, the level of chlorophylls remained at a high level, which enabled undisturbed primary metabolism. These levels of chlorophylls corresponded to more than a half of the levels of these pigments in leaves of green plants (Grašič et al. 2019a, 2019b, 2020). In the study with aspen, bark was shown to contain 17–40% of the whole tree chlorophyll (Kharouk et al. 1995). The share of different photosynthetic pigments reflects the character of bark regarding light. A relatively low chlorophyll *a* to *b* ratio obtained in this study indicates a shady environment within the bark (Dale and Causton 1992). The contents of carotenoids in the bark of *C. avellana* were comparable with those in green leaves of some species (Grašič et al. 2019a, 2019b, 2020). In the study of Levizou et al. (2004), it was shown that carotenoid composition of the periderm in twigs does not fully reveal acclimation to shade. The physical environment within the twigs, especially hypoxia, seems to be more important than shade.

Summary

The common hazel (*Corylus avellana* L.) is a deciduous shrub thriving in open places at forest edges. One-year-old twigs from ten randomly selected plants in the area of Otočec (194 m a.s.l.; 45.8339 °N, 15.2202 °E) were sampled four times, in September and November 2019, and in January and February 2020. We compared different traits of *C. avellana* bark during and out of the vegetation season. We hypothesised that bark biochemical and consequently optical properties will change after the leaves will fall off, due to altered radiation environment. In addition, we also expected changes in potential photochemical efficiency of PS II (Fv/Fm) due to unfavourable temperature conditions in winter. Indeed, the Fv/Fm values were decreasing in accordance with decreasing minimum daily temperatures. Bark optical properties changed during the studied period. Spectral curves from different samplings revealed similar shapes, but different levels of light reflectance. For all the measurements, we detected a peak in red and pronounced reflectance in NIR. Regarding the time of the season, the most pronounced changes in reflectance were obtained in the green, yellow, and red spectra. Light reflectance in these regions was positively related with chlorophyll *b* and carotenoid contents, while it was negatively related with anthocyanins and UV-B-absorbing substances. Bark biochemistry showed only slight changes during the studied period. The most pronounced changes were obtained for anthocyanin contents, which have multiple functions in bark. We observed a decrease in anthocyanin contents with lowering environmental temperatures. The decrease in anthocyanin contents was possibly due to low photosynthetic activity on account of low Fv/Fm under low temperatures. In the period out of the vegetation season, we also observed a decrease in the production of UV-absorbing substances, which are basically various phenolic substances. This decrease is possibly the result of a trade-off between the production of these substances and investment of plants in primary metabolism. On the contrary, the level of photosynthetic pigments remained at a high level, which enabled undisturbed primary metabolism. The levels of chlorophylls in the bark measured in this study corresponded to more than a half of the levels of

these pigments in leaves of green plants. Relatively low chlorophyll *a* to *b* ratio obtained in this study indicates a shady environment within the bark. The contents of carotenoids in *C. avellana* bark were relatively high and comparable to the contents of these pigments in green leaves.

Povzetek

Navadna leska (*Corylus avellana* L.) je listopadni grm, ki uspeva na odprtih rastiščih ob robu gozda. Med septembrom in februarjem smo štirikrat vzorčili enoletne vejice desetih naključno izbranih rastlin na območju Otočca (194 m n. m.; 45,8339 °N, 15,2202 °E). Primerjali smo različne lastnosti skorje navadne leske med vegetacijskim obdobjem in izven vegetacijskega obdobja. Predvidevali smo, da se bodo po odpadanju listov zaradi spremenjenega sevalnega okolja spremenile biokemijske in posledično optične lastnosti skorje. Poleg tega smo pričakovali tudi spremembe v potencialni fotokemični učinkovitosti fotosistema II (Fv/Fm) zaradi neugodnih temperaturnih razmer pozimi. Vrednosti Fv/Fm so se zmanjševale z zniževanjem minimalne dnevne temperature. Optične lastnosti skorje so se v proučevanem obdobju spreminjale. Spektralne krivulje različnih vzorcev so imele podobne oblike, vendar različne stopnje odbojnosti svetlobe. Pri vseh meritvah smo zaznali vrh v rdečem območju in veliko odbojnost v bližnjem infrardečem območju. Primerjava vzorcev iz vegetacijskega obdobja in obdobja izven vegetacijske sezone je najbolj izrazite spremembe odbojnosti pokazala v zelenem, rumenem in rdečem delu spektra. Odbojnost svetlobe je bila v teh regijah pozitivno povezana z vsebnostjo klorofila *b* in karotenoidov, negativno pa z vsebnostjo antocianinov in UV-B-absorbirajočih snovi. Biokemijske lastnosti skorje so se v proučevanem obdobju le nekoliko spreminjale. Najbolj izrazite spremembe smo opazili v vsebnosti antocianinov, ki imajo v skorji več pomembnih funkcij. Zaznali smo skladno zmanjševanje vsebnosti antocianinov z zniževanjem okoljskih temperatur. To zmanjšanje je bilo verjetno posledica majhne fotosintezne aktivnosti, ki je bila omejena z majhno Fv/Fm pri nizkih temperaturah. V obdobju izven vegetacijske sezone smo opazili tudi zmanjšanje proizvodnje UV-absorbirajočih snovi, ki so v osnovi različne

fenolne snovi. To zmanjšanje je bilo verjetno posledica kompromisa med proizvodnjo teh snovi in vlaganjem asimilatov v primarni metabolizem. Nasprotno pa je raven fotosinteznih pigmentov ostala na visoki ravni, kar je omogočilo nemoten primarni metabolizem. Raven vsebnosti klorofilov v skorji je dosegla več kot polovico vsebnosti teh barvil v listih zelenih rastlin. Razmeroma nizko razmerje klorofil *a/b* kaže na senčno okolje znotraj skorje. Razmeroma velika vsebnost karotenoidov v skorji navadne leske je bila primerljiva z vsebnostjo teh barvil v zelenih listih.

References

- Alekseev, A. A., Matorin, D. N., Osipov, V. A., Venediktov, P. S., 2007. Investigation of the photosynthetic activity of bark phelloderm of arboreous plants using the fluorescent method. *Moscow University Biological Sciences Bulletin*, 62 (4), 164-170.
- Aschan, G., Wittmann, C., Pfanz, H., 2001. Age-dependent bark photosynthesis of aspen twigs. *Trees*, 15, 431-437.
- Aschan, G., Pfanz, H., 2003. Non-foliar photosynthesis – a strategy of additional carbon acquisition. *Flora*, 198 (2), 81-97.
- Barr, M. L., Potter, L. D., 1974. Chlorophylls and carotenoids in aspen bark (*Populus tremuloides*). *The Southwestern Naturalist*, 19 (2), 147-154.
- Caldwell, M. M., 1968. Solar ultraviolet radiation as an ecological factor for alpine plants. *Ecological Monographs*, 38 (3), 243-268.
- Cernusak, L. A., Marshall, J. D., 2000. Photosynthetic refixation in branches of western white pine. *Functional Ecology*, 14 (3), 300-311.
- Dale, M. P., Causton, D. R., 1992. Use of the chlorophyll *a/b* ratio as a bioassay for the light environment of a plant. *Functional Ecology*, 6 (2), 190-196.
- Drumm, H., Mohr, H., 1978. The mode of interaction between blue (UV) light photoreceptor and phytochrome in anthocyanin formation of the *Sorghum* seedling. *Photochemistry and Photobiology*, 27 (2), 241-248.
- Filippou, M., Fasseas, C., Karabourniotis, G., 2007. Photosynthetic characteristics of olive tree (*Olea europaea*) bark. *Tree Physiology*, 27 (7), 977-984.
- Germ, M., Mazej, Z., Gaberščik, A., Trošt Sedej, T., 2006. The response of *Ceratophyllum demersum* L. and *Myriophyllum spicatum* L. to reduced, ambient, and enhanced ultraviolet-B radiation. *Hydrobiologia*, 170 (1), 47-51.
- Grašič, M., Škoda, B., Golob, A., Vogel-Mikuš, K., Gaberščik, A., 2019a. Barley and spelt differ in leaf silicon content and other leaf traits. *Biologia*, 74, 929-939.
- Grašič, M., Piberčnik, M., Zelnik, I., Abram, D., Gaberščik, A., 2019b. Invasive alien vines affect leaf traits of riparian woody vegetation. *Water*, 11, 2395.
- Grašič, M., Sakovič, T., Abram, D., Vogel-Mikuš, K., Gaberščik, A., 2020. Do soil and leaf silicon content affect leaf functional traits in *Deschampsia caespitosa* from different habitats? *Biologia Plantarum*, 64, 234-243.
- Henrion, W., Tributsch, H., 2009. Optical solar energy adaptations and radiative temperature control of green leaves and tree barks. *Solar Energy Materials and Solar Cells*, 93 (1), 98-107.
- Ivanov, A. G., Krol, M., Svshnikov, D., Malmberg, G., Gardeström, P., Hurry, V., Oquist, G., Huner, N. P., 2006. Characterization of the photosynthetic apparatus in cortical bark chlorenchyma of Scots pine. *Planta*, 223, 1165-1177.

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- Junker, L. V., Ensminger, I., 2016. Relationship between leaf optical properties, chlorophyll fluorescence and pigment changes in senescing *Acer saccharum* leaves. *Tree Physiology*, 36 (6), 694-711.
- Kharouk, V. I., Middleton, E. M., Spencer, S. L., Rock, B. N., Williams, D. L. 1995. Aspen bark photosynthesis and its significance to remote sensing and carbon budget estimate in the boreal ecosystem. *Water, Air, and Soil Pollution*, 82, 483-497.
- Klančnik, K., Pančić, M., Gaberščik, A., 2014a. Leaf optical properties in amphibious plant species are affected by multiple leaf traits. *Hydrobiologia*, 737, 121-130.
- Klančnik, K., Vogel-Mikuš, K., Gaberščik, A., 2014b. Silicified structures affect leaf optical properties in grasses and sedge. *Journal of Photochemistry and Photobiology B: Biology*, 130, 1-10.
- Klančnik, K., Zelnik, I., Gnezda, P., Gaberščik, A., 2015. Do reflectance spectra of different plant stands in wetland indicate species properties? In: Vymazal, J. (ed.): *The Role of Natural and Constructed Wetlands in Nutrient Cycling and Retention on the Landscape*. Springer, Cham, pp. 73-86.
- Klančnik, K., Levpušček, M., Gaberščik, A., 2016. Variegation and red abaxial epidermis define the leaf optical properties of *Cyclamen purpurascens*. *Flora*, 224, 87-95.
- Larcher, W., 2003. *Physiological Plant Ecology: Ecophysiology and Stress Physiology of Functional Groups*, 4th ed. Springer-Verlag, Berlin, 514 pp.
- Leong, T.-Y., Anderson, J. M., 1984. Adaptation of the thylakoid membranes of pea chloroplasts to light intensities. I. Study on the distribution of chlorophyll-protein complexes. *Photosynthesis Research*, 5, 101-115.
- Levizou, E., Petropoulou, Y., Manetas, Y., 2004. Carotenoid composition of peridermal twigs does not fully conform to a shade acclimation hypothesis. *Photosynthetica*, 42 (4), 591-596.
- Levizou, E., Manetas, Y., 2007. Photosynthetic pigment contents in twigs of 24 woody species assessed by in-vivo reflectance spectroscopy indicate low chlorophyll levels but high carotenoid/ chlorophyll ratios. *Environmental and Experimental Botany*, 59 (3), 293-298.
- Lev-Yadun, S., Gould, K., 2008. Role of anthocyanins in plant defence. In: Winefield, C., Davies, K., Gould, K. (eds.): *Anthocyanins*. Springer, New York, pp. 22-28.
- Lichtenthaler, H. K., Buschmann, C., 2001a. Extraction of photosynthetic tissues: chlorophylls and carotenoids. *Current Protocols in Food Analytical Chemistry*, 1 (1), 165-170.
- Lichtenthaler, H. K., Buschmann, C., 2001b. Chlorophylls and carotenoids: measurement and characterization by UV-VIS spectroscopy. *Current Protocols in Food Analytical Chemistry*, 1 (1), 171-178.
- Manetas, Y., Pfanz, H., 2005. Spatial heterogeneity of light penetration through periderm and lenticels and concomitant patchy acclimation of corticular photosynthesis. *Trees*, 19, 409-414.
- Martin, R. E., Crist, J. B., 1970. Elements of bark structure and terminology. *Wood and Fiber Science*, 3, 269-279.
- Martinčič, A., Wraber, T., Jogan, N., Podobnik, A., Turk, B., Vreš, B., Ravnik, V., Frajman, B., Strgulc Krajšek, S., Trčak, B., Bačič, T., Fischer, M. A., Eler, K., Surina, B., 2007. *Mala flora Slovenije. Ključ za določanje praprotnic in semenk*, 4th ed. Tehniška založba Slovenije, Ljubljana, 967 pp.
- Neill, S., Gould, K., 1999. Optical properties of leaves in relation to anthocyanin concentration and distribution. *Canadian Journal of Botany*, 77 (12), 1777-1782.
- Pietarinen, S., Willför, S., Ahotupa, M., Hemming, J., Holmbom, B., 2006. Knotwood and bark extracts: strong antioxidants from waste materials. *Journal of Wood Science*, 2 (5), 436-444.
- Pilarski, J., 1989. Optical properties of bark and leaves of *Syringa vulgaris* L.. *Bulletin of the Polish Academy of Sciences: Biological sciences*, 37, 253-269.
- Pilarski, P., Tokarz, K., Kocurek, M., 2008. Optical properties of the cork of stems and trunks of beech (*Fagus sylvatica* L.). *Polish Journal of Environmental Studies*, 17 (5), 773-779.
- Romero, C., 2014. Bark Structure and Functional Ecology. In: Cunningham, A. B., Campbell, B. M., Luckert, M. K. (eds.): *Bark: Use, Management, and Commerce in Africa*. *Advances in Economic Botany*, Vol. 17. New York Botanical Garden Press, New York, pp. 5-25.

- Rozema, J., Björn, L. O., Bornman, J. F., Gaberščik, A., Hader, D. P., Trošt, T., Germ, M., Klisch, M., Gröniger, A., Sinha, R. P., Lebert, M., He, Y. Y., Buffoni-Hall, R., de Bakker, N. V. J., van de Staaij, J., Meijkamp, B. B., 2002. The role of UV-B radiation in aquatic and terrestrial ecosystems—an experimental and functional analysis of the evolution of UV-absorbing compounds. *Journal of Photochemistry and Photobiology B: Biology*, 66, 2-12.
- Schreiber, U., Kühl, M., Klimant, I., Reising, H., 1996. Measurement of chlorophyll fluorescence within leaves using a modified PAM fluorometer with a fiber-optic microprobe. *Photosynthesis Research*, 47, 103-109.
- Sibley, J. L., Ruter, J., Eakes, D. J., 1999. Bark anthocyanin levels differ with location in cultivars of red maple. *HortScience*, 34 (1), 137-139.
- Solhaug, K., Haugen, J., 1998. Seasonal variation of photoinhibition of photosynthesis in bark from *Populus tremula* L.. *Photosynthetica*, 35, 411-417.
- Středa, T., Litschmann, T., Středová, H., 2015. Relationship between tree bark surface temperature and selected meteorological elements. *Contributions to Geophysics and Geodesy*, 45 (4), 299-311.
- Tausz, M., Warren, C. R., Adams, M. A., 2005. Is the bark of shining gum (*Eucalyptus nitens*) a sun or a shade leaf? *Trees*, 19, 415-421.
- Tokarz, K., Pilarski, J., 2005. Optical properties and the content of photosynthetic pigments in the stems and leaves of the apple tree. *Acta Physiologiae Plantarum*, 27, 183-191.
- Vick, J. K., Young, D. R., 2009. Corticular photosynthesis: a mechanism to enhance shrub expansion in coastal environments. *Photosynthetica*, 47 (1), 26-32.
- Wittmann, C., Pfan, H., 2008. General trait relationships in stems: a study on the performance and interrelationships of several functional and structural parameters involved in corticular photosynthesis. *Physiologia Plantarum*, 134 (4), 636-648.
- Wittmann, C., Pfan, H., Loreto, F., Centritto, M., Pietrini, F., Alessio, G., 2006. Stem CO₂ release under illumination: corticular photosynthesis, photorespiration or inhibition of mitochondrial respiration? *Plant, Cell and Environment*, 29 (6), 1149-1158.
- Wittmann, C., Pfan, H., 2014. Bark and woody tissue photosynthesis a means to avoid hypoxia or anoxia in developing stem tissues. *Functional Plant Biology*, 41 (9), 940-953.

Stinkwort (*Dittrichia graveolens*) organic extracts as potential biofungicides for *Fusarium poae*

Organski izvlečki smrdljive ditrihovke (*Dittrichia graveolens*) kot možni biofungicidi za *Fusarium poae*

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Abstract: In the present study, we tested the antifungal activity of ethanol, methanol and acetone extracts of stinkwort against pathogenic fungus grown from ecological wheat grain, molecularly identified as *Fusarium poae*. Its susceptibility to the stinkwort extracts was tested *in vitro* with agar dilution method. The results of antifungal effect of the organic stinkwort extracts showed that the growth of *F. poae* was significantly reduced by these extracts compared to the control and that the antifungal activity is dose-dependent. The methanol extract showed stronger inhibition than the ethanol and acetone ones at all three concentrations. All organic extracts showed a similar antifungal activity against *F. poae* as a broad-spectrum fungicide azoxystrobin. We tested the effects of stinkwort extracts on the germination of radish seeds. The methanol extract delayed the germination of the radish seeds during the first 24 hours, but thereafter all three organic extracts had a comparable germination rate as the control seeds. Despite the delayed germination of the radish seeds, the methanol extract did not reduce the final germination rate and at the same time reduced the fungal infection by almost 50%. These results indicate that the methanol stinkwort extract has the potential to be used as a biofungicide in organic farming.

Keywords: *Dittrichia graveolens*, *Fusarium poae*, antifungal activity, seed germination

Izvleček: V raziskavi smo testirali protiglavno aktivnost etanolnega, metanolnega in acetonskega izvlečka smrdljive ditrihovke proti patogeni glivi, ki smo jo izolirali iz ekoloških semen pšenice in molekularno določili kot *Fusarium poae*. Njeno občutljivost za izvlečke smrdljive ditrihovke smo določali *in vitro* z dilucijsko metodo na agarju. Vsi organski izvlečki smrdljive ditrihovke so glede na kontrolo značilno zmanjšali rast glivnega micelija. Protiglavno delovanje je bilo koncentracijsko odvisno. Rast je najbolj zavrl metanolni izvleček. Vsi organski izvlečki so imeli primerljivo protiglavno delovanje kot azoksistrobin, ki velja za fungicid s širokim spektrom delovanja. Testirali smo tudi vpliv izvlečkov na kalitev semen redkvice. Metanolni izvleček je v prvih 24 urah kalitev zakasnil, pozneje pa je bila kaljivost vseh treh izvlečkov primerljiva s kontrolo. Kljub zakasneni kalitvi je metanolni izvleček inhibiral rast glivnega micelija za okoli 50 %. Rezultati kažejo na možnost uporabe metanolnega izvlečka smrdljive ditrihovke kot biofungicida v ekološkem kmetijstvu.

Ključne besede: *Dittrichia graveolens*, *Fusarium poae*, protiglavna aktivnost, kalitev semen

Introduction

Stinkwort (*Dittrichia graveolens* (L.) Greuter, family Asteraceae) is an annual aromatic plant that is distributed mainly in disturbed areas e.g. along roadsides, on fallow land and pastures (Sellem et al. 2020). It reaches a height of about 80 cm, has sticky leaves and bright yellow flowers and is characterized by a very strong aromatic odor (Frajman and Kaligarič 2009). The stinkwort is originally a Mediterranean species that has been introduced in several other European countries and worldwide where it is considered invasive alien plant. In recent decades, the species has spread very rapidly along motorways in Central Europe and has also been discovered in Slovenia. The mapping has shown that it grows along most parts of Slovenian motorways (Frajman and Kaligarič 2009), and along regional roads (Grašič et al. 2016). Invasive alien plant species have negative impacts on the environment and associated economic losses (e.g. infrastructure damage), and regular annual management of these habitats is necessary to reduce their populations (Richardson et al. 2000). Organized mowing and removal of invasive plants produces large amounts of biomass that can be used as a source for searching for natural products from plants. In the past, aromatic herbs have been used as a valuable source of many biologically active compounds because they contain many secondary metabolites. Stinkwort is an aromatic plant and is therefore a potentially good source of biologically active compounds. It has been used in Asian and European traditional medicine and it possess potential antioxidant and antibacterial activity (Mazandarani et al. 2014). There have also been reports about its allelopathic effects on the germination of selected crops and weeds (Omezzine et al. 2011) and as a potential bio-herbicide for control of invasive common ragweed (Grašič et al. 2016).

On the other hand, antifungal activity of stinkwort against *Fusarium poae* has not been studied yet. *Fusarium poae* is a harmful fungus, mainly because it produces mycotoxins. *Fusarium poae* causes Fusarium head blight (FHB) disease which is an important and insidious disease affecting mainly wheat, barley and other cereals worldwide (Nogueira et al. 2018). FHB is a pre-harvest disease; however, *Fusarium* species can grow in

the post-harvest period if grains are not properly dried or stored in moist and semi-moist conditions (Stenglein 2009). FHB reduces the germination rate and seedling vigour, resulting in a loss of grain yield. FHB can also cause indirect losses, as *Fusarium* species produce mycotoxins (Nogueira et al. 2018). Among the *Fusarium* species causing this disease, *F. poae* is relatively weakly pathogenic compared to the others but produces a large number of mycotoxins (Stenglein 2009). The presence of mycotoxins in cereal grains, in particular in wheat, is a major problem worldwide due to the toxicological risks for human and animal health. The use of fungicides is therefore crucial to protect consumers and modern agricultural pest control practices rely heavily on the use of synthetic fungicides. However, the use of synthetic fungicides is not permitted in organic farming. There are also reports of less fungal disease in organic production, but the mechanisms behind this are poorly understood (Karlsson et al. 2017). Yogeve et al. (2011) reported on the suppressive properties of organic compost against the development of *Fusarium oxysporum* in soils with a history of organic farming compared to conventional cultivation. Although organic farming increases beneficial endophytic fungal communities that lead to healthier plants (Karlsson et al. 2017, Xia et al. 2019), it is also confronted with pathogenic fungi that can have negative effects on cereals.

In our previous study we isolated several fungal endophytes from organically produced wheat grain, including *Alternaria alternata*, *Alternaria infectoria*, *Aspergillus flavus*, *Epicoccum nigrum* and *Fusarium poae* (Anžlovar et al. 2017). Among these fungi, *Aspergillus* and *Fusarium* spp. are known to produce harmful mycotoxins (Nogueira et al. 2018, Ponzilacqua et al. 2018). Treatment with fungicides is therefore necessary to protect plants and/or crops from spoilage and to reduce damage. However, the use of synthetic fungicides has led to the development of fungal resistance to antifungal agents, not to mention that fungicide residues may also be harmful to other organisms (Chen et al. 2008). Furthermore, the organic farming standards are designed to allow the use of naturally occurring substances, while synthetic substances are banned or strictly limited. For these reasons, interest in new and “greener” bioprotection products has increased. Efforts to

discover new active compounds, that are more environmentally friendly have kept scientists looking for natural products from plant. Invasive alien plant species are a rich source of bioactive compounds (Ajao and Moteetee 2015, Bezuneh 2015, Shuping and Eloff 2017) and have a large biomass, that is usually destroyed. Therefore, invasive plants, despite their harmful effects, are also a potential source of useful and beneficial compounds.

The aim of this research was to investigate a possible antifungal effect of extracts from stinkwort. This aromatic plant is an invasive plant in Slovenia and could be a source of natural compounds that could possibly be used as biofungicides for organic farming. We tested the inhibitory effect of organic extracts on the growth of the phytopathogenic fungus *F. poae* isolated from organically grown wheat seeds.

Materials and methods

Plant material

Fresh shoots of stinkwort (*Dittrichia graveolens* (L.) Greuter) were collected during the flowering period in September 2018 (leg. and det. S. Strgulc Krajšek) in Ljubljana, Slovenia (Roje, Obvozna cesta), on gravel road bank (N 46°6'17.17», E 14°28'54.36»). The shoots were air dried at room temperature in the dark.

Certified organic seeds of *Raphanus sativus* L. cv. Cherry Belle (Bio radish cherry belle) were obtained from Natur aktiv (Austria).

Preparation of organic extracts

Dry stinkwort shoots were ground to a powder by a mill (M20; IKA-Werke, Germany), and 30 g of this ground material was dissolved in 100 ml 96% ethanol, methanol or acetone. The mixtures were left shaking for 24 h at room temperature on an orbital shaker (Laboshake 500; Gerhardt, Germany) at 130 rpm. After this extraction, the mixtures were vacuum filtered (520A filter paper; Whatman, GE Healthcare Life Sciences, UK) and extracts were used for antifungal tests. The yield of extract (extractable component) expressed on

dry weight basis of pulp was calculated according to the following equation:

$$\text{Yield (\%)} = (W1 \times 100) / W2$$

where W1 is the weight of the extract residue obtained after solvent removal and W2 is the weight of the dry plant material before the extraction.

Fungal growth inhibition assay

The antifungal activities of the stinkwort extracts were tested against *F. poae*, which was previously isolated from wheat grain and molecularly identified by PCR (Anžlovar et al. 2017).

The inhibitory effects of the stinkwort extracts on radial growth of the fungal mycelia were tested following the method in our previous study (Anžlovar and Dolenc Koce 2014). One hundred microliters of organic extract (30%, 15% or 5% concentration) was spread over each Petri dish (diameter, 90 mm) containing 2% potato dextrose agar (Biolife, Italy) using a Drigalski spatula. Disks of fungal mycelia (diameter, 5 mm) were cut from the margins of 7-day-old *Fusarium* cultures and aseptically inoculated by placing them in the center of a fresh plate with the extract. Control samples with ethanol, methanol or acetone and without extracts were prepared at the same time. The fungicide azoxystrobin (10 mg/ml) was used as the positive control. The fungal colonies were incubated at room temperature (23±2 °C) in the dark for 7 days.

Mycelial growth was assessed on the 4th and 7th day after inoculation. All plates were photographed with a digital camera (EOS 1000D, Canon, Tokyo, Japan) and the growth area (cm²) was calculated with image processing software (ImageJ).

Inhibition of the fungal growth was expressed as percentage of growth reduction and calculated according to modified Lira-De León et al. (2014):

$$\text{Inhibition (\%)} = (AC - AT) / AC \times 100$$

where AC is the area of mycelian growth of the control colonies, and AT is the area of mycelian growth of the treated colonies. Three replicates were carried out for control and each treatment (N = 3).

Germination test

To set the germination test, we used sterile Petri dishes (diameter, 90 mm) with one layer of autoclaved filter paper soaked with 1 ml of the extract or distilled water as a control. After the paper was dried overnight, 3 ml of distilled water were added.

For each treatment we used 3 replicates each with 20 seeds in a 1.5 x 1.5 cm array (N = 3). Germination test took place in a growth chamber at 22±2 °C, 60% humidity and photoperiod of 16 h light / 8 h dark. The experiment lasted for four days. If necessary, a few drops of water were added to ensure sufficient humidity. Seeds were examined every day at roughly 24-hour intervals. A seed was considered germinated on the day of root emergence.

Statistical analysis

For the antifungal activities, three fungal colonies per treatment were measured and mean values were calculated. From these data, inhibition of fungal growth was calculated. The differences between treated and positive control samples were tested using one-way ANOVA and Holm-Sidak *post-hoc* test. The level of significance was set at $P < 0.05$.

Results and discussion

Since bioactive compounds belong to different groups of plant metabolites, the choice of extraction solvent is important and specific to each plant material. Methanol and ethanol are both polar solvents and are able to extract polar and non-polar secondary metabolites. The polarity of methanol is lower than that of ethanol. Methanol has a polarity index of 5.1 and is usually used in the extraction of polar bioactive components

Table 1: Yield of stinkwort extracts. The yield was calculated as percentage of extract dry mass according to the starting material. Data are means ± standard error (N = 3).

Tabela 1: Izkoristek izvlečkov smrdljive ditrihovke. Izkoristek je delež suhe snovi glede na maso začetnega materiala, izražen v odstotkih. Podatki so povprečja ± standardne napake (N = 3).

| Extract | Yield (%) |
|----------|-----------|
| Ethanol | 9.3±0.2 |
| Methanol | 10.8±0.4 |
| Acetone | 7.8±0.2 |

Table 2: Mycelium growth of *Fusarium poae* at 4 days and 7 days after treatment with stinkwort ethanol, methanol and acetone extracts. Data are means ± standard error (N = 3). Different letters indicate statistically significant differences ($P < 0.05$) according to One-way ANOVA and Holm-Sidak *post-hoc* test between stinkwort organic extracts and control on the same day.

Tabela 2: Rast micelija glive *Fusarium poae* 4. in 7. dan po tretmaju z etanolnim, metanolnim in acetonskim izvlečkom smrdljive ditrihovke. Podatki so povprečja ± standardne napake (N = 3). Različne črke v stolpcu prikazujejo statistično značilne razlike ($P < 0,05$) med organskimi izvlečki smrdljive ditrihovke in kontrolo na isti dan, glede na enosmerno ANOVA in Holm-Sidakov *post-hoc* test.

| Treatment | Fungal growth area (cm ²) | |
|-----------|---------------------------------------|------------------------|
| | Days after treatment | |
| | 4 | 7 |
| Control | 26.9±0.9 ^a | 58.4±0.9 ^a |
| Ethanol | 8.3±0.5 ^b | 16.2±1.0 ^b |
| Methanol | 2.0±0.1 ^c | 4.7±0.4 ^c |
| Acetone | 2.7±0.1 ^d | 13.4±2.0 ^{bc} |

of plant extracts (Zaidel et al. 2019). However, ethanol is better than methanol in the extraction of polyphenols (Do et al. 2014). Acetone was chosen as the extraction agent because it dissolves many hydrophilic and lipophilic components, is miscible with water and has low toxicity to fungi and is therefore very useful in bioassays (Eloff 1998). In our study of stinkwort extracts that could potentially be used as biofungicides, we found that methanol is the most efficient of the organic solvents we used (Tabs. 1, 3, 4). Methanol extract also contained more dry mass and had a higher yield than ethanol, while acetone extract had the lowest yield (Tab. 1).

The results of antifungal effect of the organic stinkwort extracts showed that the growth of *F. poae* was significantly reduced by these extracts when compared to the control on the 4th and 7th day after the treatment (Tab. 2). The antifungal activity also significantly differed among different organic extracts on the 4th day after the treatment (Tab. 3). The reduction of mycelium growth was the most affected by methanol extracts, following acetone and ethanol (Tab. 2). After 7 days, the antifungal activity of methanol extract remained above 90%, while the activity of acetone extract decreased to 77% and showed similar antifungal activity as ethanol extract (Tab. 3). The growth areas of fungi after acetone treatment were more variable; therefore, the results of growth reduction and antifungal activity did not differ significantly from those of

methanol and ethanol. Moderate antifungal activity has also been reported for an aqueous-methanol extract from stinkwort, which inhibited growth of several soil-borne fungi (Abu Irmaileh et al. 2017). The prolific nature and successful invasion of new habitats suggests that invasive species are likely to have a novel biochemistry that repels native species (Cappuccino and Arnason 2006). In this aspect, it is not surprising that many invasive plants have antimicrobial activity, including antifungal ones (Rashmi and Rajkumar 2011, Aghel et al. 2011, Bajpai et al. 2012, Jankovec 2016). The antifungal activity of invasive plants against the genus *Fusarium* is not well studied. Acetone extracts from seven common invasive alien plant species in South Africa had moderate activity on *Fusarium oxysporum* (Mdee et al. 2009). The invasive species *Ageratina adenophora*, also from the *Asteraceae* family like stinkwort, has antifungal properties that can be used as alternative fungicides. The antifungal activity was performed by poisoned food technique and the methanol leaf extract showed a positive effect against *Fusarium oxysporum* by completely inhibiting its growth at concentration 250 mg/ml (Das and Devkota 2018). The invasive goldenrod ethanol extracts show moderate activity against *F. poae* (Hladnik 2017). Methanol and ethanol extracts from rhizomes of invasive *F. japonica* inhibited the growth of *F. poae*, while leaf extracts even stimulated the growth of *F. poae* (Gioahin 2016).

Table 3: Antifungal activity of stinkwort ethanol, methanol and acetone extracts 4 and 7 days after treatment. Data are means \pm standard error (N = 3). Different letters indicate statistically significant differences (P < 0.05) according to One-way ANOVA and Holm-Sidak post-hoc test between stinkwort organic extracts and azoxystrobin on the same day.

Tabela 3: Protiglivna aktivnost 4. in 7. dan po tretmaju z etanolnim, metanolnim in acetonskim izvlečkom smrdljive ditrihovke. Podatki so povprečja \pm standardne napake (N = 3). Črke v stolpcu prikazujejo statistično značilne razlike (P < 0,05) med organskimi izvlečki smrdljive ditrihovke in azoksistrobinom na isti dan, glede na enosmerno ANOVA in Holm-Sidakov post-hoc test.

| Treatment | Growth inhibition (%) | |
|--------------|------------------------------|------------------------------|
| | Days after treatment | |
| | 4 | 7 |
| Ethanol | 69.2 \pm 1.8 ^a | 72.3 \pm 1.7 ^a |
| Methanol | 92.6 \pm 0.1 ^b | 91.9 \pm 0.7 ^b |
| Acetone | 90.1 \pm 0.3 ^c | 77.0 \pm 3.5 ^{ab} |
| Azoxystrobin | 91.2 \pm 1.8 ^{bc} | 81.2 \pm 1.5 ^a |

In the present study, all organic extracts of *Dittrichia graveolens* showed similar antifungal activity against *F. poae* as azoxystrobin, a fungicide with a broad spectrum of activity (Tab. 3). In particular, the antifungal activity of methanol extract was even higher than of azoxystrobin on 7th day after treatment, whereas on 4th day was comparable to azoxystrobin. The efficiency of acetone extract was slightly lower than of azoxystrobin, but not significantly. On the other hand, ethanol extracts were on 4th day significantly lower comparable to azoxystrobin, but on 7th day antifungal efficiency was in the same range. The long-term antifungal activity of all organic extracts was comparable or higher than that of azoxystrobin. Elshafie et al. (2019) reported that the antifungal activity of essential oil from the invasive plant *Solidago canadensis* was higher than that of azoxystrobin against *M. fructicola*, while the activity against *P. expansum* and *A. niger* was lower than that of azoxystrobin. The methanolic extract of *Ageratina adenophora* also inhibited *Fusarium oxysporum* more strongly than the synthetic fungicides Bavistin (carbendazim 50% WP) and Mancozeb (ethylene-bis-dithiocarbamate) (Das and Devkota, 2018).

The antifungal activity of organic extracts of *D. graveolens* against *F. poae* is dose-dependent (Tab. 4). The methanol extract showed stronger inhibition than the ethanol and acetone ones at all three concentrations. The 5% methanol extract showed almost 50% inhibition, while ethanol and acetone ones were less active at the same concentration (about 14%). The differences between all three concentrations within the same

extract were statistically significant (Tab. 4). In the case of methanol extract, it would be useful to prepare even more concentrated extracts to obtain 100% inhibition. Similarly, the essential oils of *Solidago* inhibited mycelium growth of *F. poae* in a dose-dependent manner (Jankovec 2017). On the other hand, ferulic acid, which is considered the strongest phenolic acid with antifungal activity against *Fusarium* species, significantly reduced the growth of *F. poae* mycelium with increasing concentration, while the lowest concentration of ferulic acid stimulated the growth of mycelium (Schöneberg et al. 2018). In this context it would also be good to check the stinkwort extract of concentrations lower than 5%.

Beside growth inhibition of fungal mycelia, we observed the morphological anomalies after the treatment with stinkwort methanol extract. The control colonies were woolly to cotton-like with white, fluffy aerial mycelia. The treated mycelium, on the other hand, was denser and grew up instead of growing on the surface of the PDA plate (Fig. 1). This result may be explained by the fact that stinkwort methanolic extract not only inhibits the growth of *F. poae* mycelium, but also alters hyphal development. Similar observations were reported for ferulic acid affecting growth of *F. poae*, in which 0.5% ferulic acid significantly reduced growth and caused colonies to grow in very dense aerial mycelium. The morphology of the colonies was visibly affected, showing irregular growth and low to no colony elevation compared to control (Schöneberg et al. 2018). Similarly, Alwahshi et al. (2019) found that the fungicide Cidely Top (mixture of cyflufenamid and difenoconazole)

Table 4: Dose-dependent activity of stinkwort ethanol, methanol and acetone extracts 7 days after treatment. Data are means \pm standard error (N = 3). Different letters indicate statistically significant differences ($P < 0.05$) according to One-way ANOVA and Holm-Sidak post-hoc test among three concentrations of the each extract.

Tabela 4: Koncentracijsko odvisna protiglivna aktivnost izvlečkov smrdljive ditrihovke 4. in 7. dan po tretmaju z etanolnim, metanolnim in acetoniskim izvlečkom smrdljivke. Podatki so povprečja \pm standardne napake (N = 3). Črke v vrstici prikazujejo statistično značilne razlike ($P < 0,05$) med tremi različnimi koncentracijami istega organskega izvlečka smrdljive ditrihovke, glede na enosmerno ANOVA in Holm-Sidakov post-hoc test.

| Extract | Growth inhibition (%) | | |
|----------|-----------------------------|-----------------------------|-----------------------------|
| | 5% | 15% | 30% |
| Ethanol | 13.7 \pm 1.7 ^a | 22.4 \pm 0.4 ^b | 72.3 \pm 1.7 ^c |
| Methanol | 47.1 \pm 2.6 ^a | 66.0 \pm 2.7 ^b | 91.9 \pm 0.7 ^c |
| Acetone | 14.0 \pm 0.7 ^a | 38.4 \pm 5.5 ^b | 77.0 \pm 3.5 ^c |

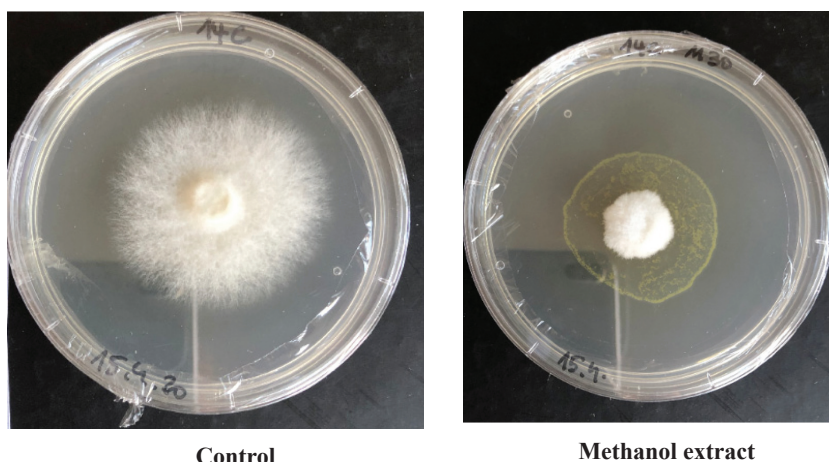


Figure 1: Mycelium growth of *Fusarium poae* on potato dextrose agar without (control) and with stinkwort methanol extract on day 4 after inoculation.

Slika 1: Rast micelija glive *Fusarium poae* na krompirjevem agarju brez metanolnega izvlečka smrdljive ditrihovke (kontrola) in z metanolnim izvlečkom smrdljive ditrihovke 4. dan po inokulaciji.

inhibited *F. solani* by affecting hyphal development, septum formation, cytoplasmic integrity and altered conidial formation. An altered hyphal morphology, e.g. shrunken and thinner hyphae of *Botrytis cinerea*, was observed after treatment with the essential oil of *Solidago canadensis* (Liu et al. 2016). A change in mycelium morphology was also observed during treatment with essential oil from *Solidago canadensis* flowers, where the treatments caused fading of the mycelium of *Aspergillus flavus* (Jankovec 2016), which could correspond to reduced virulence (Liu et al. 2010).

The seed germination and seedling growth is generally considered as the critical process of the

plant development. High yield losses are caused by fungi of *Fusarium* genus, which through infected grains are transferred to seedlings and damage them (Knudsen et al. 1995). The main source of *Fusarium* infection is the soil. Therefore, the treatment of soil or seeds before sowing is very important and is currently among the biggest problems in organic farming, as the use of synthetic preparations is strictly forbidden. They must be replaced by biopreparations, which must be as efficient and reliable as chemical protection, but their choice is rather limited (Pekarskas and Sinkevicienė 2015). Bioactive compounds from stinkwort methanol extract could be a good treatment before sowing,

Table 5: Germination rate of radish seeds treated with stinkwort ethanol, methanol and acetone extracts. Data are means \pm standard error (N=60). Different letters indicate statistically significant differences ($P < 0.05$) among different extracts on the same day.

Tabela 5: Delež kaljivosti semen redkvice, tretiranih z etanolnim, metanolnim in acetonskim izvlečkom smrdljive ditrihovke. Podatki so povprečja \pm standardne napake (N = 60). Črke v vrstici prikazujejo statistično značilne razlike ($P < 0,05$) med tretmaji na isti dan.

| | Germination rate (%) | | | |
|-------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| | Control | Ethanol | Methanol | Acetone |
| Day 1 | 95.0 \pm 2.9 ^a | 72.7 \pm 4.4 ^a | 63.3 \pm 4.4 ^b | 80.0 \pm 2.9 ^a |
| Day 2 | 97.7 \pm 3.3 ^a | 80.0 \pm 5.8 ^a | 87.7 \pm 6.0 ^a | 92.7 \pm 1.7 ^a |
| Day 3 | 97.7 \pm 3.3 ^a | 82.7 \pm 7.3 ^a | 87.7 \pm 6.0 ^a | 93.3 \pm 1.7 ^a |

as it is more fungicidal than the chemical agent azoxystrobin. A good pre-sowing treatment must therefore have an antifungal effect and at the same time mustn't impact the germination of the seeds. To determine the phytotoxic effect of *Dittrichia* organic extracts, we observed the germination of organically produced radish seeds, which is frequently used in ecotoxicological studies.

In the preliminary experiments, we tested three different concentrations of stinkwort ethanol extract: 15%, 5% and 2.5%. The 15% concentration inhibited germination almost completely (75%), whereas 5% and 2.5% concentrations were comparable with germination of control seeds (data not shown). Therefore, we used 5% extracts to observe the germination rate and seedlings development (Tab. 5). The organic stinkwort extracts influenced the germination of radish seeds in different ways. After 24 h, 95% of the control seeds germinated, while the germination rate of the treated seeds was lower as follows: acetone extract (80%), ethanol extract (72%) and methanol extract (63%). The inhibition of germination was significant only for methanol extract. After 48 h and 72 h, the differences in germination rate between treated and control seeds were not significant anymore. The methanol extract delayed germination of the radish seeds for the first 24 h, but after that all three organic extracts had a comparable germination rate to the control seeds. The development of the treated radish seedlings was slightly slower than that of the seedlings growing under control conditions (data not shown). On the contrary, 5% aqueous extract of stinkwort had a negative effect on the germination of weed common ragweed (*Ambrosia artemisiifolia*), while the germination rate of wheat seeds treated with 2.5% aqueous extract of stinkwort was comparable to that of the control (Grašič et al. 2016). Furthermore, the root and coleoptile growth of treated wheat seedlings were also comparable to the control (Grašič et al. 2016). There are also reports on the negative effects of aqueous and organic extracts of *D. graveolens* on weed germination and growth (Omezzine et al. 2011). The bioherbicidal potential of aqueous extracts of *Dittrichia* on weeds could be the result of their epicuticular exudate, which is water-soluble and also has acaricidal (Sofou et al. 2017) and insecticidal activity (Lampiri et al. 2020).

The 5% methanol extract despite the delayed germination of the radish seeds in the first 24 hours did not reduce the germination rate of the radish seeds (Tab. 5) but at the same time did reduce the fungal infection by almost 50% (Tab. 4). These results suggest that methanol stinkwort extract has a potential to be used as biofungicide for organic farming.

Conclusion

Fungal diseases cause considerable morbidity and mortality worldwide and increase healthcare costs. In the light of increasing fungicide resistance and the general objective of reducing the environmental impact of agriculture, biocontrol strategies are likely to become more important in the future. The present study confirmed the strong antifungal activity of stinkwort organic extracts against *F. poae*. The exact antifungal ingredients and mechanisms of action are under investigation in our laboratories. As an invasive plant, the stinkwort represent valuable source in research for novel natural antifungal products.

Povzetek

Smrdljiva ditrihovka je aromatična enoletnica in je v Sloveniji invazivna tujerodna vrsta. Na otip je lepljiva, saj je porasla z žleznimi laski, ima tudi močen vonj, po katerem je dobila ime. Kot zdravilna rastlina se uporablja tako v azijskem kot evropskem tradicionalnem zdravilstvu. Številne aromatične rastline so pomemben vir biološko aktivnih snovi, saj vsebujejo veliko sekundarnih metabolitov. Smrdljiva ditrihovka kot aromatična in zdravilna rastlina tako predstavlja možen vir biološko aktivnih snovi, hkrati pa bi njena uporabnost pospešila njeno odstranjevanje, kar je pomemben ukrep pri omejevanju invazivnih vrst.

V predhodnih raziskavah smo iz semen pšenice izolirali glivo *Fusarium poae*, patogeno glivo, ki povzroča veliko ekonomsko škodo. Povzročča bolezen bledenja klaskov (fusarium head blight, FHB), ki prizadene pšenico, ječmen in ostala žita. Glede na ostale vrste iz tega rodu je relativno šibko patogena, nevarna pa je, ker izloča veliko mikotoksinov, ki so nevarni za zdravje ljudi. Pro-

tiglivna aktivnost izvlečkov smrdljive ditrihovke proti glivi *F. poae* je slabo raziskana.

V raziskavi smo testirali protigitlivno aktivnost organskih izvlečkov smrdljive ditrihovke proti patogeni glivi *F. poae*. Iz nadzemnih delov smrdljive ditrihovke smo pripravili 30-odstotne etanolne, metanolne in acetonske izvlečke. Protigitlivno aktivnost smo določali z dilucijsko metodo na agarju. Vsi organski izvlečki smrdljive ditrihovke so glede na kontrolo značilno zmanjšali rast glivnega micelija. Protigitlivno delovanje je bilo koncentracijsko odvisno. Pri vseh treh koncentracijah (5 %, 10 %, 30 %) je rast najbolj zavrl metanolni izvleček. Vsi organski izvlečki so imeli primerljivo protigitlivno

delovanje kot azoksistrobin, ki velja za fungicid s širokim spektrom delovanja. Metanolni izvleček je imel celo večjo končno protigitlivno učinkovitost kot azoksistrobin. Za možnost uporabe izvlečkov kot biofungicidov smo poleg protigitlivne aktivnosti testirali tudi njihov vpliv na kalitev semen redkvice. Metanolni izvleček je v prvih 24 urah kalitev semen redkvice zakasnil, pozneje pa je bila kaljivost vseh treh izvlečkov primerljiva s kontrolo. Kljub zakasneni kalitvi je 5-odstotni metanolni izvleček inhibiral rast glivnega micelija za okoli 50 %. Rezultati kažejo na možnost uporabe metanolnega izvlečka smrdljive ditrihovke kot biofungicida v ekološkem kmetijstvu.

References

- Abu Irmaileh, B.E., Salem, N.M., AlAboudi, A.M.F., Abu Zarqa, M.H., Abdeen, A.O., 2017. Antifungal activity of the stinkwort (*Inula graveolens*) extracts. *Journal of Plant Pathology & Microbiology*, 8, 8.
- Aghel, N., Mahmoudabadi, A. Y., Darvishi, L., 2011. Volatile constituents and anti-candida activity of the aerial parts essential oil of *Ditrichia graveolens* (L.) Greuter grown in Iran. *African Journal of Pharmacy and Pharmacology*, 5(6), 772-775.
- Ajao, A.A., Moteetee, A.N., 2015. *Tithonia diversifolia* (Hemsl) A. Gray. (Asteraceae: Heliantheae), an invasive plant of significant ethnopharmacological importance: A review. *South African Journal of Botany*, 113, 396-403.
- Alwahshi, K.J., Saeed, E.E., Sham, A., Alblooshi, A.A., Alblooshi, M.M., El-Tarabily, K.A., AbuQamar, S.F., 2019. Molecular identification and disease management of date palm sudden decline syndrome in the United Arab Emirates. *International Journal of Molecular Sciences*, 20, 923.
- Anžlovar, S., Dolenc Koce, J., 2014. Antibacterial and antifungal activity of aqueous and organic extracts from indigenous and invasive species of Goldenrod (*Solidago* spp.) grown in Slovenia. *Phyton*, 54, 135-147.
- Anžlovar, S., Likar, M., Dolenc Koce, J., 2017. Antifungal potential of thyme essential oil as a preservative for storage of wheat seeds. *Acta Botanica Croatica*, 76, 64-71.
- Bajpai, V.K., Baek, K.H., Kim, E.S., Han, J.E., Kwak, M., Oh, K., Kim, J.C., Kim, S., Choi, G.J., 2012. In vivo antifungal activities of the methanol extracts of invasive plant species against plant pathogenic fungi. *The Plant Pathology Journal*, 28 (3), 317-321.
- Bezunch, T.T., 2015. Phytochemistry and antimicrobial activity of *Parthenium hysterophorus* L.: A Review. *Science Journal of Analytical Chemistry*, 3 (3), 30-38.
- Cappuccino, N., Arnason, J.T., 2006. Novel chemistry of invasive exotic plants. *Biology Letters*, 2, 189-193.
- Chen, P.J., Moore, T., Nesnow, S., 2008. Cytotoxic effects of propiconazole and its metabolites in mouse and human hepatoma cells and primary mouse hepatocytes. *Toxicology in Vitro*, 22, 1476-1483.
- Das, R.K., Devkota, A., 2018. Antifungal activities and phytochemical screening of two invasive alien species of Nepal. *Studies in Fungi*, 3(1), 293-301.
- Do, Q.D., Angkawijaya, A.E., Tran-Nguyen, P.L., Huynh, L.H., Soetaredjo, F.E., Ismadji, S., Ju, Y.H., 2014. Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. *Journal of Food and Drug Analysis*, 22 (3), 296-302.
- Eloff, J.N., 1998. Which extractant should be used for the screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology*, 60, 1-8.

- Elshafie, H. S., Gruľová, D., Baranová, B., Caputo, L., De Martino, L., Sedlák, V., Camele, I., De Feo, V., 2019. Antimicrobial activity and chemical composition of essential oil extracted from *Solidago canadensis* L. growing wild in Slovakia. *Molecules*, 24, 1206.
- Frajman, B., Kaligarič, M., 2009. *Dittrichia graveolens*, a new alien species of the Slovenian flora (*Dittrichia graveolens*, nova tujerodna vrsta slovenske flore). *Hladnikia*, 24, 35-43.
- Grašič, M., Anžlovar, S., Strgulc Krajšek, S., 2016. The impact of aqueous extracts of stinkwort (*Dittrichia graveolens*) and false yellowhead (*D. viscosa*) on germination of selected plant species. *Phyton*, 56 (2), 293-301.
- Gioahin, E., 2016. Antimicrobial activity of Japanese knotweed (*Fallopia japonica*) essential oils and extracts. MSc Thesis, University of Ljubljana, Biotechnical faculty, Department of food science and technology, 63 pp.
- Hladnik, S., 2017. Antifungal activity of aqueous and organic extracts from Goldenrod. MSc Thesis, University of Ljubljana, Faculty of education, Department of biology, 46 pp.
- Jankovec, M., 2016. Biological activity of goldenrod (*Solidago spp.*) essential oil. MSc Thesis, University of Ljubljana, Biotechnical faculty, Department of biology, 60 pp.
- Karlsson, I., Friberg, H., Kolseth, A.K., Steinberg, C., Persson, P., 2017. Organic farming increases richness of fungal taxa in the wheat phyllosphere. *Molecular Ecology*, 26: 3424-3436.
- Knudsen, M.B., Hockenhull, J., Jensen, D.F., 1995. Biocontrol of seedling diseases of barley and wheat caused by *Fusarium culmorum* and *Bipolaris sorokiniana*: Effects of selected fungal antagonists on growth and yield components. *Plant Pathology*, 44, 467-77.
- Lampiri, E., Agrafioti, P., Levizou, E., Athanassiou, C.G., 2020. Insecticidal effect of *Dittrichia viscosa* lyophilized epicuticular material against four major stored-product beetle species on wheat. *Crop Protection*, 132, 105095.
- Lira-De León, K.I., Ramírez-Mares, M.V., Sánchez-López, V., Ramírez-Lepe, M., Salas-Coronado, R., Santos-Sánchez, N.F. et al., 2014. Effect of crude plant extracts from some Oaxacan flora on two deleterious fungal phytopathogens and extract compatibility with a biofertilizer strain. *Frontiers in Microbiology*, 5, 383.
- Liu, G.Y., Nizet, V., 2010. Color me bad: microbial pigments as virulence factors. *Trends in Microbiology*, 17, 406-413.
- Liu, S., Shao, X., Wei, Y., Li, Y., Xu, F., and Wang, H., 2016. *Solidago canadensis* L. essential oil vapor effectively inhibits *Botrytis cinerea* growth and preserves postharvest quality of strawberry as a food model system. *Frontiers in Microbiology*, 7, 1179.
- Mazandarani, M., Ghafourian, M., Khormali, A., 2014. Ethnopharmacology, antibacterial and antioxidant activity of *Dittrichia graveolens* (L.) W. Greuter. which has been used as remedies antirheumatic, anti-inflammation and antiinfection against leishmaniasis in the traditional medicine of Gorgan, Iran. *Crescent Journal of Medical and Biological Sciences*, 1, 125-129.
- Mdee, L.K., Masoko, P., Eloff, J.N., 2009. The activity of extracts of seven common invasive plant species on fungal phytopathogens. *South African Journal of Botany*, 75, 375-379.
- Nogueira, M. S., Decundo, J., Martinez, M., Dieguez, S. N., Moreyra, F., Moreno, M.V., Stenglein, S.A., 2018. Natural contamination with mycotoxins produced by *Fusarium graminearum* and *Fusarium poae* in malting barley in Argentina. *Toxins*, 10, 78.
- Omezzine, F., Ladhari, A., Rinez, A., Haouala, R., 2011. Allelopathic potential of *Inula graveolens* on crops and weeds. *Allelopathy Journal*, 28, 63-76.
- Pekarskas, J., Sinkevičienė, J., 2015. Effect of biopreparations on seed germination and fungal contamination of winter wheat. *Biologija*, 61 (1), 25-33.
- Ponzilacqua, B., Corassin, C. H., Fernandes Oliveira, C. A., 2018. Antifungal activity and detoxification of aflatoxins by plant extracts: Potential for food applications. *The Open Food Science Journal*, 10, 24-32.

- Rashmi, S., Rajkumar, H.G., 2011. Preliminary phytochemical analysis and *in vitro* evaluation of antifungal activity of five invasive plant species against *Macrophomina Phaseolina* (Tassi) Goid. *International Journal of Plant Research*, 1 (1), 11-15.
- Richardson, D.M., Pyšek, P., Rejmanek, M., Barbour, M.G., Panetta, F.D., West, C.J., 2000. Naturalization and invasion of alien plants: concepts and definition. *Diversity and Distributions*, 6, 93–107.
- Schöneberg, T., Kibler, K., Sulyok, M., Musa, T., Bucheli, T.D., Mascher, F., Bertossa, M., Voegelé, R.T., Vogelgsang, S., 2018. Can plant phenolic compounds reduce *Fusarium* growth and mycotoxin production in cereals? *Food Additive and Contaminants, part A* 35 (12), 2455–2470.
- Sellem, I., Chakchouk-Mtibaaa, A., Zaghdenb, H., Smaouia, S., Ennouria, K., Mellouli, L., 2020. Harvesting season dependent variation in chemical composition and biological activities of the essential oil obtained from *Inula graveolens* (L.) grown in Chebba (Tunisia) salt marsh. *Arabian Journal of Chemistry*, 13 (3), 4835-4845.
- Sofou, K., Isaakidis, D., Spyros, A., Büttner, A., Giannis, A. and Katerinopoulos, H.E., 2017. Use of cistic acid, a natural extract from *Dittrichia viscosa*, for the control of *Varroa destructor*, a parasite of the European honey bee. *Beilstein Journal of Organic Chemistry*, 13, 952-959.
- Stenglein, S. A., 2009. *Fusarium poae*: a pathogen that needs more attention. *Journal of Plant Pathology*, 91 (1), 25-36.
- Shuping, D.S.S., Elof, J.N., 2017. The use of plants to protect plants and food against fungal pathogens. *African Journal of Traditional, Complementary and Alternative Medicines*, 14 (4), 120-127.
- Yogev, A., Laor, Y., Katan, J., Hadar, Y., Cohen, R., Medina, S., Raviv, M., 2011. Does organic farming increase soil suppression against *Fusarium* wilt of melon? *Organic agriculture*, 1, 203-216.
- Xia, Y., Sahib, M.R., Amna, A., Opiyo, S.O., Zhao, Z., Gao, Y.G., 2019. Culturable endophytic fungal communities associated with plants in organic and conventional farming systems and their effects on plant growth. *Nature Scientific Reports*, 9, 1669.
- Zaidel, D.N.A., Muhamad, I.I., Daud, N.S.M., Muttalib, M.A.A., Khairudd, N., Lazim, N.A.M., 2019. Production of biodiesel from rice bran oil. In: Verma, D., Fortunati, E., Jain, S., Zhang, X.: Biomass, biopolymer-based materials, and bioenergy: construction, biomedical, and other industrial applications. Woodhead Publishing Series in Composites Science and Engineering, 409-447.

The quality of Slovenian chestnut honey and its specific properties relevant for medical application and functional nutrition

Kakovost slovenskega kostanjevega medu in njegove poznane lastnosti v prid medicinski uporabi in za funkcionalno prehrano

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Abstract: Chestnut honey is well-described in terms of sensory properties, pollen and chemical composition. Specific bitter taste is accompanied with other typical sensory properties derived from its chemical composition, especially in the nectar of sweet chestnut. Compounds from other sources of nectar and honeydew, especially linden, fir and spruce, with smaller amounts from meadow plants, create the specific sensory and chemical properties of Slovene chestnut honey. Based on the chemical composition of the honey, especially the content and proportions of different inorganic ions, it is possible to track the geographical origin of the pasture. Bees contribute significantly to recognized antimicrobial properties of honey by secretion of enzymes and antimicrobial peptides via the food processing glands. When the honey is used for medical purposes, we have to take precautions to avoid microbial and chemical contamination. For the planning of specific use of honey as a medical application we need to explore in detail specific pharmacological properties of single compounds from the chestnut honey and its contribution to the whole activity during wound treatment. In this paper we present a review of most distinct properties of chestnut honey important for its medical application.

Keywords: antimicrobial activity, antioxidant activity, kynurenic acid, honey contamination, melissopalinalogy, organic honey

Izvleček: Kostanjev med je dobro opisan tako z osnovnimi senzoričnimi lastnostmi, z melissopalinaloško analizo in s kemijsko sestavo. Specifičen grenek okus spremljajo še druge tipične senzorične lastnosti, ki pa imajo osnovo v kemijski sestavi, predvsem

izvirne medicīne na pravem kostanju. Temu se lahko pridruŕujejo tudi snovi iz drugih virov medenja, predvsem medenja lipe, jelke in smreke, v manjši meri pa travniških rastlin. Geografskega porekla znotraj Slovenije ne moremo zanesljivo opredeliti na osnovi melisopanlinološke analize, lahko pa na specifični kemijski sestavi, predvsem vsebnosti in razmerji med posamezni anorganskimi ioni. K zaznanemu protimikrobnemu delovanju kostanjevega medu pa bistveno prispevajo same čebele z dodajanje encimov in protimikrobnih peptidov. Za uspešno uporabo kateregakoli medu, tudi kostanjevega, v medicinske namene je nujno zagotavljati visoko kvaliteto pridelave brez mikrobnega in kemičnega onesnaŕenja. Z natančnejšim poznavanjem farmacevtskih učinkov posameznih snovi iz kostanjevega medu k celoviti negi ran lahko načrtujemo specifične pogoje pridelave in uporabe kostanjevega medu za pripravo ustreznih medicinskih pripomočkov.

Ključne besede: antioksidativna aktivnost, ekološki med, kinurenska kislina, pelodna analiza, onesnaŕenost medu, protimikrobna aktivnost

Introduction

Chestnut honey was always recognized as something special, especially in Europe. Even nonexperts can recognize some of the key properties of the chestnut honey, such as its specific amber color and bitter taste. In folk medicine it is believed that chestnut honey's specific sensory properties might contribute to its healing strength. However, it is even more likely that this belief comes from the healing experience through decades or even centuries. In the recent decades there has been a substantial increase in new scientific data regarding different properties of chestnut honey, especially its chemical composition that supports the healing effects proposed by folk medicine. Chestnut honey has been added to the list of specific honeys that have been registered for medical use in the last twenty years.

General properties of chestnut honey

Chestnut honey is characterized by a reddish-brown, dark amber color, and is usually clear. The taste of chestnut honey is medium sweet, medium to very bitter, and sometimes a slightly acidic or metallic taste may also be present. The odor and aroma are characteristic and intense, reminiscent of chestnut leaves, caramel, burnt sugar, and it can sometimes give off a slight animal note (Bertoncelj et al. 2011a). This type of honey is characterized by a long-lasting aroma, so its sensory accept-

ability, especially among younger consumers, is poorer. The specific sensory properties of chestnut honey are mainly influenced by compounds present in small quantities, like organic acids, other aromatic compounds, pigments and phenolic compounds.

The main constituents of honey are sugars, mainly fructose and glucose, with small amounts of other sugars (Bogdanov et al. 2004, Korošec et al. 2016b). Within the project of Slovenian honey characterization in the years 2014-2016, fructose, glucose, sucrose, raffinose, turanose, melezitose and maltose were determined in Slovenian chestnut honey samples (Tab. 1). Chestnut honey is characterized by a high fructose to glucose ratio ($F/G = 1.5 - 1.6$), suggesting that typical chestnut honey usually remains liquid and is not prone to crystallization. Some physicochemical properties of chestnut honey are presented in Tab. 2. Compared to other types of Slovenian honey, chestnut honey is characterized by a high electrical conductivity and pH value, high content of amino acid proline and high activity of enzyme diastase.

The complex composition of honey derives from components present in small amounts, such as elements, enzymes, organic acids, phenolic compounds, proteins, and vitamins, which also contribute to antimicrobial and antioxidant activity of honey. Compared to other types of Slovenian honey (acacia, linden, fir, spruce, multifloral and forest honey), chestnut honey contains more potassium, calcium and manganese (Kropf et al. 2010). The potassium content is very high

Table 1: Sugar content in chestnut honey (N = 15) (Korošec et al. 2016a). Abbreviations: SD, standard deviation; Min, minimum; Max, maximum.

Tabela 1: Vsebnost sladkorjev v kostanjevem medu (N = 15) (Korošec s sod. 2016a). Okrajšave: SD, standardni odklon; Min, minimum; Max, maksimum.

| Statistics | Sugar content (g/kg honey) | | | | | | |
|------------|----------------------------|---------|---------|-----------|----------|------------|---------|
| | Fructose | Glucose | Sucrose | Raffinose | Turanose | Melezitose | Maltose |
| Mean | 413.3 | 261.3 | 1.94 | 7.64 | 27.7 | 75.9 | 44.0 |
| SD | 49.8 | 29.8 | 1.55 | 14.57 | 5.0 | 34.0 | 9.1 |
| Min. | 336.7 | 207.7 | 0.73 | 0.00 | 21.2 | 46.3 | 30.6 |
| Max. | 479.6 | 303.5 | 5.00 | 43.17 | 37.0 | 173.3 | 54.2 |

Table 2: Results of physicochemical analysis of chestnut honey samples (N = 29) (Bertoncelj et al. 2011a). Abbreviations:SD, standard deviation; Min, minimum; Max, maximum; DN, diastase number.

Tabela 2: Rezultati fizikalno-kemijskih analiz kostanjevega medu (N = 29) (Bertoncelj s sod. 2011a). Okrajšave: SD, standardni odklon; Min, minimum; Max, maksimum; DN, diastazno število.

| Statistics | Water content | Electrical conductivity | | Free acids (meq/kg) | Lactones (meq/kg) | Total acids (meq/kg) | Proline content (mg/kg) | Diastase (DN) |
|------------|---------------|-------------------------|------|---------------------|-------------------|----------------------|-------------------------|---------------|
| | (g/100 g) | (mS/cm) | pH | | | | | |
| Mean | 15.9 | 1.61 | 5.51 | 13.3 | 2.5 | 15.8 | 558 | 18.6 |
| SD | 0.9 | 0.24 | 0.42 | 4.7 | 1.6 | 5.7 | 108 | 4.5 |
| Min | 13.7 | 1.05 | 4.75 | 7.3 | 0.0 | 8.9 | 390 | 13.9 |
| Max. | 17.7 | 2.25 | 6.18 | 26.0 | 6.5 | 32.5 | 776 | 31.9 |

with an average 3590 mg/kg. The ash content in Slovenian chestnut honey is between 0.55 to 1.04 g/100 g, which is on average 25% higher than in honeydew types of honey and almost 20 times higher than in acacia honey. Chestnut honey also contains various phenolic compounds (phenolic acids and flavonoids). Of the phenolic acids, *p*-coumaric, caffeic and cinnamic acid predominate. The main flavonoids detected in chestnut honey are propolis-derived flavonoids: pinocembrin, chrysin, galangin and pinobanksin, as well as kaempferol and apigenin (Bertoncelj et al. 2011b). Phenolic compounds have been proven to act as antioxidants. Moreover, samples of Slovenian chestnut honey exhibit antioxidant activity; the results are comparable to the antioxidant activity of honeydew honey and are related to the color; darker honeys have higher antioxidant activity (Korošec et al. 2016b).

Geographical variability of chestnut honey in Slovenia

Kropf with colleagues (Kropf et al. 2010) analyzed the composition of chestnut honey from different geographical regions of Slovenia (Perko 1998) and evaluated different physicochemical parameters: electrical conductivity, ash content, pH value, contents of total and free acids, lactones, proline, proteins, color parameters L^* , a^* and b^* , specific rotation, elemental content (S, Cl, K, Ca, Mn, Rb), stable carbon and nitrogen isotope ratios ($\delta^{13}C_{\text{honeys}}$, $\delta^{13}C_{\text{proteins}}$ and $\delta^{15}N$). Samples of chestnut honey originate from all four Slovenian natural geographical macroregions. In the Mediterranean region, chestnut honey is rarely represented, so chestnut honey samples from Alpine (n=17), Dinaric (n=12) and Pannonian macroregion (n=8) were included in the comparison of geographical variability. Chestnut honey from the three different Slovenian regions differed in the content of proline, potassium, rubidium and

in the color parameters. Chestnut honey from the Pannonian macroregion contained more rubidium, while honey from the Dinaric macroregion was darker in color and contained less proline. Using the method of linear discriminant analysis (LDA) Kropf with colleagues (Kropf et al. 2010) successfully proved the discrimination of chestnut honey samples according to the geographical origin based on the treated parameters, as the first two axes explained the overall variability of the data. Parameters with the major factors in discriminating Slovenian chestnut honeys were the contents of sulfur, ash and potassium, the color parameter L^* (lightness) and the ratio of sulfur to calcium (S/Ca).

Melissopalynology of the chestnut honey

In one of our previous studies we attempted to identify the geographical origin of chestnut honey based on pollen analysis of 28 samples (Golob et al. 2008). We were not able to match it. Partial matching was observed only in acacia honey (honey of the black locust, *Robinia pseudacacia*), most likely because of more evident differences in the phenology of melliferous plants and bigger differences in diversity during blooming of black locust in different phytogeographical regions of Slovenia. Regardless of the extraction of a honey by a beekeeper before chestnut blooming, there was still pollen of the black locust present in more than half of the samples. We found following types of pollen: *Trifolium repens*, *Tilia* sp., *Plantago* sp., *Asteracea* type J and *Filipendula* sp. in more than half samples and their phenophases match with the blooming of chestnut. Large-leaved linden (*Tilia palthyphyllus*) blooms regularly just before chestnut, but small-leaved linden (*Tilia cordata*) finishes flowering usually at the beginning of the chestnut bloom. Presence in honey samples of the pollen from plants that bloom before chestnut is usually confirmation of partial mix of honey from previous nectar source. Maple (*Acer* sp.) and flowering ash (*Fraxinus ornus*) are also present in more than half the samples (Golob et al. 2008). Pollen analysis of the chestnut honey is mainly useful for checking the conditions of beekeeping but not for estimation of geographical origin on the territory of Slovenia, although, this is pos-

sible by analyzing different chemical parameters (see previous chapter). Later analysis done on 82 samples of chestnut honey (Kandolf 2011) showed some possibility to identify some specific melissopalynological characteristics related to phytogeographical regions when compared sets of chestnut honey samples between the regions. Although, we don't have a single pollen type that would be always present or at list at specific relative abundance for the specific phytogeographical region, it is possible to compare current sample with existing already analyzed and get the likelihood of the best fit.

Antimicrobial activity of honey and its possible mechanisms in chestnut honey

The main determinants of honey's antimicrobial activity are hydrogen peroxide formation, antimicrobial peptides (AMP), high osmolarity and low pH (Szveda 2017). The antimicrobial activity in all types of honey is significantly influenced by the action of the enzyme glucose oxidase (GOX). It is secreted from the honeybee's feeding glands (hypopharyngeal glands) into the honey. The level of expression of this enzyme in the bee and thus its presence in honey is genetically determined (Bucekova et al. 2014). GOX is an oxidoreductase that catalyzes the oxidation of glucose to gluconic acid during the dilution of honey with water in the presence of oxygen, thereby lowering the pH of honey and producing one molecule of hydrogen peroxide. Both low pH and hydrogen peroxide have antimicrobial activity (Kwakman and Zaat 2012). The general mechanism for inhibiting bacterial growth in honey is its high osmolarity - honey consists of about 80% (w/v) sugar. Other antimicrobial factors are more specific and can vary considerably between different types of honey. For example, an important antimicrobial agent in Manuka honey is methylglyoxal (MGO), which is of plant origin (Mavric et al. 2008; Kwakman and Zaat 2012). By reducing the ability of bacterial adherence and movement, it inhibits both Gram-positive and Gram-negative bacteria. It has been found that polyphenols entering honey from plant nectar greatly increase the antibacterial activity of hydrogen peroxide in honey (Kwakman and Zaat 2012, Bucekova et al. 2018). An example of

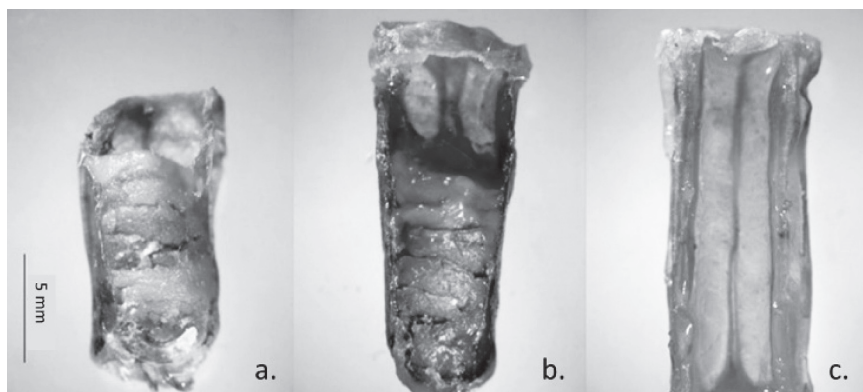


Figure 1: We can find different cell types at the same bee comb with stored food a) comb cell with bee bread, b) comb cell with bee bread covered by honey and closed by wax cap and c) closed comb cell with honey content. In the same comb frame at the same source of nectar, honey above bee bread had significantly higher antimicrobial activity than honey in comb cells without bee bread (Podrižnik and Božič 2015). Photo: B. Podrižnik)

Slika 1: V istem satu lahko najdemo različne tipe satnih celic s skladiščeno hrano, a) satna celica s čebeljim kruhkom, b) satna celica s čebeljim kruhkom pokritim z medom, ki je zaprta z voščenim poklopcem in c) zaprta satna celica, ki vsebuje samo med. V istem satu in pri istem viru medicinskega meda, skladiščen nad čebeljim kruhkom, znatno večjo protimikrobno aktivnost, kot med v satnih celicah brez čebeljega kruhka (Podrižnik and Božič 2015) (Slika: B. Podrižnik).

such honey is melon honeydew (Bucekova et al. 2018). Antimicrobial peptides may also be a very important factor in the antimicrobial activity of honey (Kwakman et al. 2010). Their content and type can vary greatly between different types of honey (Bucekova et al. 2018, Erban et al. 2019). Some types of honey, e.g. Manuka and Kanuka, practically do not contain them, while they are abundant in others, e.g. sunflower or linden.

Chestnut honey has the strongest antimicrobial activity among Slovenian honeys, as evidenced by growth inhibition tests of selected bacterial and fungal species (Kunčič et al. 2012). However, it is not yet known what kind of AMP and GOX activity chestnut honey has. Additional antimicrobial activity was observed in chestnut honey stored over bee bread (pollen stored in honeycomb cells, Fig. 1) (Podrižnik and Božič 2015). The additional processing of honey by bees and the effect of stored pollen probably contributed to the fact that under laboratory conditions with the agar diffusion method a 30% larger zone of growth inhibition in *Staphylococcus aureus* was observed. It is difficult to explain which of the factors described above are decisive.

Influence of food processing glands on honey quality

Food processing glands have a key role in final quality of the honey (Winston 1991). Excretion of the gland secretions of the foraging bee starts already during collection of the nectar. Among glands, the subesophageal glands, also known as feeding glands, are the most important and intensively studied contributor to honey processing. Foraging bees secrete mainly two enzymes from these glands. The first is α -glucosidase, which separates glucose from compound sugars attached by an α bound, and it is most effective when splitting sucrose into simple sugars, for this reason it is also called sucrose (Kubo et al. 1996). The second enzyme is glucose oxidase (GOX) (Takenaka et al. 1990), which contributes to antimicrobial activity of the honey by producing hydrogen peroxide through oxidation of glucose into gluconic acid. Peroxidase activity is the major antimicrobial activity in honeys from melliferous plants (see discussion before). Secretion of glucose oxidase is also present in younger nurse bees which are feeding larvae with royal jelly. During this

period, nurse bees are secreting proteins of royal jelly, among them antimicrobial peptides (Kubo et al. 1996, Fujita et al. 2010). The dynamic of well-known antimicrobial peptide defensin-1 secretion is unclear, especially regarding age of the bee and her role in the colony. Young bees are contributing to royal jelly also with the secretions from the mandibular glands. This gland contributes the fatty part of the royal jelly, with 10-hydroxy-decanoic acid the best known (Huo et al. 2016). Some components of the royal jelly also end up in honey due to intensive food exchange between hive and foraging bees (Crailsheim 1991). The involvement of salivary glands in these processes is unclear. It is known that these glands are more involved in other bees in production of pheromones (Feng et al. 2013; Martin et al. 2018).

Excretion of AMP from food processing glands in honey bees

AMP can be an important factor in the antimicrobial activity of a particular type of honey. They are evolutionarily very old defense molecules and represent an important part of the innate immune system. Therefore, they are found in very different organisms as well as in insects. In general, they are amphipathic, cationic peptides, which kill the bacterium by permeabilization of its cell (cytoplasmic) membrane. Several types of AMP are known in bees. While proline-rich apidaecins (Casteels et al. 1993) and abaecin (Casteels et al. 1990), the former 18 to 20 and the latter 34 amino acid residues long, have not yet been detected in honey, defensins, hymenoptaecine and jelleins (Kwakman and Zaat 2012) have all been found.

The most known and researched bee AMP are defensins (Bilikova et al. 2015). Genes for two structural forms of defensin, defensin-1 and defensin-2 (Klaudiny et al. 2005), have been discovered in the genome of the honey bee (*Apis mellifera*). The better understood defensin-1 is constitutively expressed in pharyngeal, hypopharyngeal, and mandibular glands of the bee. Defensin-2, which is expressed in fat and hemolymph, is 55.8% structurally identical to defensin-1 and appears to be expressed, *i.e.* inducible, only in the case of infection. Defensin-1 consists of 51 amino acid residues (Fujiwara et al. 1990) and defensin-2

consists of 43 (Klaudiny et al. 2005), both of them containing 3 disulphide bridges. Bee defensins show a high degree of polymorphism. This can significantly affect the level of their expression and their antimicrobial activity. While defensin-1 is responsible for the collective immunity of bees, defensin-2 is responsible for their individual immunity (Ilyasov et al. 2012). Defensin-1 is a common component of royal jelly (Ramanathan et al. 2018) and honey (Erban et al. 2019). Because it was first discovered in royal jelly, it is also known by an alternative name - royalisin. Defensin-2 has not yet been detected in honey. Defensin-1 is primarily directed against Gram-positive bacteria, but is also toxic to some Gram-negative bacteria (Bilikova et al. 2015). Importantly, it is quite effective in destroying multivariate bacterial biofilms (Sojka et al. 2016). It is interesting to note that although defensin-1 has an antibacterial effect, it also promotes the healing of open wounds. By stimulating the secretion of MMP-9 metalloproteinase from keratinocytes, it stimulates their migration and thus wound closure and re-epithelialisation (Bucekova et al. 2017). Hymenoptaecin (Casteels et al. 1993) is a 93 amino acid residue long AMP, which is inducibly expressed in the case of infection. This is probably the reason why it is only occasionally found in honey (Erban et al. 2019). Its bactericidal activity is broad and directed against both Gram-positive and Gram-negative bacteria. Honey also contains the protein MRJP1 (Major Royal Jelly Protein 1) (Erban et al. 2019). This 61 kDa protein (Tian et al. 2018) is a precursor of three antibacterial peptides, jellein-1, 2, and 3 (Fontana et al. 2004). Jelleins, peptides with a length of 8 to 9 amino acid residues, are probably formed *in situ* after proteolytic processing of the C-terminal part of MRJP1 (Buttstedt et al. 2014) by serine proteases that are also present in honey. The content of MRJP1 was high in all honeys tested so far, so the contribution of jelleins to the antimicrobial activity of honey is probably important. They are toxic to bacteria, both G⁺ and G⁻, and to yeast (Fontana et al. 2004).

Microorganisms in honey

Honey itself is not a sterile food; it contains both bacteria and fungi (yeasts and molds). However, due to its high osmolarity and low pH, only certain groups of microorganisms can thrive in it, and this community is usually stable. They even found that some bacteria in this community produce bacteriocins that prevent the growth of other bacteria (Szweda 2017). Thus, among the Gram-positive bacteria we can find representatives of the genera *Bacillus*, *Micrococcus*, *Streptococcus* and *Clostridium*, while among the Gram-negative bacteria species of the genera *Achromobacter*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia*, *Flavobacterium*, *Klebsiella*, *Proteus* and *Pseudomonas* are present. Fungal contaminants are often species of the genera *Penicillium*, *Aspergillus*, *Saccharomyces*, *Zygosaccharomyces* and sometimes representatives of the former genus *Torulopsis*. Secondary sources of microbial contamination in honey may be humans, equipment, utensils, wind, dust, etc. Most bacteria and other microorganisms cannot grow or multiply in honey; they are dormant due to the antibacterial activity of honey. Thus spore-forming microorganisms such as *Bacillus cereus*, *Clostridium perfringens* and *Clostridium botulinum*, or more precisely their spores, can survive in honey at low temperatures for several months or even a year (Olaitan et al. 2007). The worst possible contamination route is contamination due to poor beekeeping practices, which can be avoided by appropriate beekeeping measures during beekeeping and honey delivery. Disease caused by honey consumption has rarely been suspected, but it has never been directly demonstrated that honey is one of the main sources of infection (Grabowski and Klein 2017). Unfortunately, possible contamination from environmental sources cannot be completely avoided. In such a case, honey can be treated with γ radiation (Jo et al. 2005), and some new research also suggests the use of high pressure and ultrasound (Leyva-Daniel et al. 2017, Janghu et al. 2017). Together with the improvement of honey quality, we must ensure that during the production of the honey product there is no contamination with microorganisms that could cause health problems (Snowdon and Cliver 1996, Olaitan et al. 2007, Silva et al. 2017).

Chemical contamination of chestnut honey and legislation in this field

Environment pollution is reflected also in the contamination of honey. Amounts of pollutants like metals, organic pollutants and biocides rarely exceed regulated minimal residual levels for humans (Al-Waili et al. 2012). Older EU legislation required honey to be completely pollutant-free (Directive 1974). Honey is now included in newer general directive about food products that regulates labeling, residues of pollutants and analytical methods (European Commission 2018). In recent research of Slovene honey samples, Česnik et al. (2019) did not find excessive amounts of pollutants according to current legislation. Nevertheless, remedies of miticides used to fight Varroa mites are often detected, in a smaller extent even in the samples from organic beekeeping (Česnik et al. 2019). French researchers (Wiest et al. 2011) as well Italian (Saitta et al. 2017) also reported on residues of miticides and fungicides. In Italian research (Saitta et al. 2017) low contamination of chestnut honey with pollutants was found. Chestnut nectar contains a lot of minerals (see chapter “Geographical variability...”), so it is not a surprise that chestnut honey was rich with radioactive ^{137}Cs (Panatto et al. 2007). For medical use, honey must be produced under organic standards (Hermanns et al. 2020) and it is necessary to be cautious about all possible sources of contamination, especially about the use of illegal chemicals to treat bee diseases (e.g. acaricides and antibiotics). Even though that bees forage on chestnut in the forest, the chestnut honey could be contaminated with nectar from melliferous plants in intensive agriculture area, as well as the treatments against Varroa mites.

Additional interesting medical properties of honey

In addition to the antimicrobial activity, honey has many other biological activities that are therapeutically interesting, especially in wound healing process (Oryan et al. 2016). Honey has proven antioxidant and anti-inflammatory activity and stimulates immune system cells involved in wound healing and tissue regeneration. Antioxidant

activity is driven by a whole range of substances, such as flavonoids, phenolic acids, ascorbic acid, tocopherols, antioxidant enzymes, specific amino acids, and selenium. Among them, phenolic compounds have the greatest antioxidant role, e.g. gallic acid, which together with antimicrobial activity contribute to wound healing. Their role is primarily the reduction of reactive oxygen and nitrogen species and thus the reduction of oxidative reactions in inflammation.

The specificity of chestnut honey is that it has a sufficiently high content of kynurenic acid (Turski et al. 2016). Kynurenic acid is a metabolic product of the amino acid tryptophan. It is also a natural metabolite in human body, where it plays an important role in regulation of metabolism (Milart et al. 2019), immune response (Małaczewska et al. 2014) and neurotransmission. Alteration in homeostasis of kynurenic acid and its metabolites has been implicated in different neurological and psychological disorders (Meier 2019). It is a natural component of human breast milk and regulates weight gain in breast-fed infants. Insufficient levels of kynurenic acid in artificial milk formulas contribute to overweight in infants, which is a risk factor for obesity in later stages of child development (Milart et al. 2019). It has also been proven that kynurenic acid and other tryptophan derivatives are involved in the healing of superficial wounds (Poormasjedi-Meibod et al. 2014, Matysik-Woźniak et al. 2017). High proline content and biochemical conditions in honey also allow the synthesis of additional kynurenic acid derivatives as 3-pyrrolidinyl quinurenic acid and its gamma lactone (Beretta et al. 2009). Their phar-

macological activity, as well as the possible role of other specific chemical substances from chestnut honey, is unknown (Truchado et al. 2009a,b,c).

Examples of medical use and opportunity of the chestnut honey

Complications due to the bacterial resistance to classical antibiotics triggered biomedical research on the potential use of honey for wound care (Molan 1992). Researchers tested different types of honey, among them only few were recognized as medical. The first recognized medical honey was monofloral Manuka from New Zealand, and another early example was Revamil® Neatherland, but they both differ in their mode of action (Kwakman et al. 2011). Recently, new products for wound care from different producers have emerged (Hermanns et al. 2020).

Substantial antibacterial and antifungal activity was found in Slovene chestnut honey (Kunčič et al. 2012) and therefore it was recommended for medical use. This knowledge was used in the commercial product line Vivamel® (Fig. 2). We can expect more specific solutions on the market based on specific properties of individual type of honey along with clearer definition of medical honey quality (Hermanns et al. 2020). Chestnut honey has an excellent opportunity for product development, especially because of pharmacological relevant concentrations of kynurenic acid and other derivatives of the kynurenic pathway.

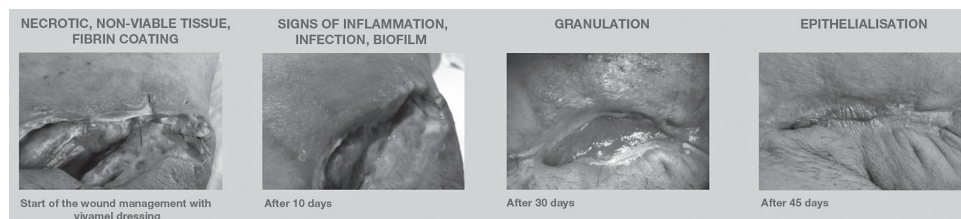


Figure 2: Demonstration of wound healing with use of chestnut honey. (Figure is from directions for use of wound dressing with medical honey by Tosama d.o.o.)

Slika 2: Prikaz procesa celjenja ran z uporabo kostanjevega medu. (Slika je izrezana iz "Smernice za uporabo oblog z medicinskim kostanjevim medom", Tosama d.o.o.)

Conclusions

1. Slovene chestnut honey is relatively well characterized from all quality aspects. Differences in its physical and chemical characteristics are due to its geographical origin.
2. Melissopatology is a good tool to verify beekeeping practice regarding specific monofloral production of the chestnut honey, also partially for geographical origin.
3. The antimicrobial activity of chestnut honey is considerable but also varies and requires further study. Antimicrobial peptides probably contribute strongly to the overall antimicrobial activity of the chestnut honey.
4. Activity of the hypopharyngeal glands during honey processing is the major source of enzymes and antimicrobial peptides in the honey.
5. The production of honey for medical purposes requires special attention to avoid microbial contamination. Organic beekeeping is the safest way for medicinal honey production, but even in this case, one must be aware of potential contamination by pollutants.
6. Chestnut honey has unique medical properties with pharmacologically relevant concentrations of kynurenic acid and other compounds derived from the kynurenic metabolic pathway.
7. Chestnut honey is well recognized in the field of wound care. New findings may lead to novel specific applications as medicinal honey.

Povzetek

Kostanjev med je bil od nekdaj prepoznan kot nekaj posebnega, predvsem v evropskem prostoru. Tudi laiki lahko hitro prepoznajo nekaj ključnih lastnosti kostanjevega medu – specifično temno jantarno barvo in grenek okus. Za to vrsto medu je značilna dolgo obstojna aroma (Bertoncelj s sod. 2011a), zato je senzorična sprejemljivost te vrste medu, predvsem med mlajšimi potrošniki, slabša. V zadnjih desetletjih se tudi za med pravega kostanja kopičijo znanstveni podatki o različnih lastnostih, predvsem kemijski sestavi medu, pridružil pa se je tudi naboru vrst medu, ki so bile prepoznane kot primerne za medicinsko uporabo. Značilno

za kostanjev med je visoko razmerje med fruktozo in glukozo, ki znaša okoli 1,5, zato tipičen kostanjev med običajno ne kristalizira (Tab. 1). V primerjavi z ostalimi vrstami slovenskega medu je za kostanjev med značilna visoka električna prevodnost, visoka vrednost pH, velika vsebnost aminokislinske prolin in visoka aktivnost encima diastaze (Tab. 2). Kostanjev med v primerjavi z ostalimi vrstami slovenskega medu (akacijevim, lipovim, hojevim, smrekovim, cvetličnim in gozdnim medom) vsebuje največ K^+ , Ca^{2+} in Mn^{2+} ionov (Kropf s sod. 2010). Kostanjev med je tudi relativno bogat s fenolnimi spojinami (Bertoncelj s sod. 2011b), kar se odraža tudi o relativno visokih antioksidativnih učinkih, primerljivo z antioksidativno učinkovitostjo maninih medov (Korošec s sod. 2016b). Kropf in sod. (2010) so analizirali sestavo medu iz različnih geografskih regij Slovenije (Perko 1998) in vrednotili različne parametre. Kostanjev med iz panonske makroregije je vseboval več rubidija, med iz dinarske makroregije pa je bil statistično značilno temnejše barve in je vseboval manj prolina. Z metodo linearne diskriminantne analize (LDA) so dokazali, da ima najpomembnejši prispevek k razlikovanju slovenskega kostanjevega medu glede na geografski izvor vsebnost žvepla, pepela in kalija, parameter barve L^* (svetlost) ter razmerje S/Ca (Kropf s sod. 2010). Fenološko v več kot polovici vzorcev kostanjevega medu najdemo poleg močno prevladujočega pravega kostanja tipe cvetnih prahov kot so *Trifolium repens*, *Tilia* sp. *Plantago* sp. *Asteracea* tip J in *Filipendula* sp., ki cvetijo sočasno s pravim kostanjem (Golob s sod. 2008, Kandolf 2011). V več kot polovici vzorcev so bili prisotni tudi tipi cvetnih prahov sadnega drevja in javorjev (*Acer* sp.), ki cvetijo pomladi pred pravim kostanjem, kar nakazuje na težavo pridelave čistega monoflornega medu, lahko pa tudi zaradi primesi zaradi skladiščenja kostanjevega medu nad čebeljim kruhkom starejšega izvora.

Protimikrobna aktivnost pri vseh vrstah medu je pomembno pogojena z delovanjem encima glukoza oksidaze (GOX) in z njim povezanim vodikovim peroksidom (Szweda 2017). GOX se v med izloča iz čebeljih krmilnih žlez, poleg encimov pa te žleze izločajo tudi protimikrobne peptide (AMP) (Bucekova s sod. 2014). Polifenoli, ki v med pridejo iz rastlinskega nektarja, močno ojačajo protibakterijsko delovanje vodikovega peroksida

v medu (Kwakman and Zaat 2012, Bucekova s sod. 2018). Ostali protimikrobni dejavniki so bolj specifični in se lahko med različnimi vrstami medu precej razlikujejo. Tako je ključni protimikrobni dejavnik medu manuka metilglioksal (MGO), ki je rastlinskega izvora (Mavric s sod. 2008, Kwakman in Zaat 2012). Kostonj med ima od slovenskih vrst medov najmočnejšo protimikrobno aktivnost, kot je bilo dokazano s testi inhibicije rasti izbranih bakterijskih in glivnih vrst (Kunčič s sod. 2012). Vendar pa še ni poznano, kakšne vsebnosti AMP in aktivnosti GOX ima kostonj med. Dodatna protimikrobna aktivnost je bila opažena v kostonj medu, skladiščenim nad čebeljim kruhkom (cvetni prah, skladiščen v satnih celicah, sl. 1) (Podrižnik in Božič 2015). Encime, AMP in še druge sestavine se izločajo v med iz čeljustnih žlez čebel (Winston 1991), vanj pa zaidejo tako med nabiranjem kot predelavo v panju (Crailsheim 1991). Najbolj znani in raziskani čebelji AMP so defenzini (Bilikova s sod. 2015), med njimi se defenzin-1 izraža v podžrelni žlezi, ki poleg proti mikrobnega delovanja pospešuje tudi celjenje odprtih ran. V medu se nahaja tudi protein MRJP1 (od angl. »Major Royal Jelly Protein 1«) (Erban s sod. 2019). Ta 61 kDa protein je prekurzor treh protibakterijskih peptidov, jelleina-1, 2 in 3, ki so toksični za bakterije, tako G^+ kot G^- , kot tudi za kvasovke (Fontana s sod. 2004).

Sam med ni sterilno živilo, v njem najdemo tako bakterije kot glive (kvasovke in plesni). Vendar lahko zaradi visoke osmolarnosti in nizkega pH uspevajo le določene skupine mikroorganizmov, ta združba je večinoma stabilna. Ugotovili so celo, da nekatere bakterije v tej združbi producirajo bakteriocine, ki preprečujejo razrast drugih bakterij (Szweida 2017). Sekundarni viri mikrobnega kontaminacije v medu so lahko ljudje, oprema, posoda, veter, prah itd. Na žalost se možni kontaminaciji iz okoljskih virov ne moremo popolnoma izogniti. V takšnem primeru lahko med obdelamo z γ žarčenjem (Jo s sod. 2005), nekatere nove raziskave pa predlagajo tudi uporabo visokega pritiska in ultrazvoka (Leyva-Daniel s sod. 2017, Janghu s sod. 2017). Onesnaženje okolja se odraža tudi v onesnaženosti medu. Vrednosti onesnažil, kot so kovine, organska onesnažila in biocidi redko

presežejo predpisane mejne vrednosti za človeka (Al-Waili s sod. 2012, European Commission 2018, Česnik s sod. 2019). Za medicinske namene naj bi se primarno uporabljal med pridobljen po ekoloških standardih (Hermanns s sod. 2020), vseeno pa je potrebno biti pozoren na vse možne vire onesnaženja, zlasti morebitno zlorabo nedovoljenih sredstev za zatiranje bolezni (n.p. akaracidi in antibiotiki).

Posebnost kostonjevega medu je, da vsebuje kinurensko kislino (Turski s sod. 2016). Kinurenska kislina je presnovni produkt aminokislina triptofan. Je tudi naravni metabolit v človeškem telesu, kjer ima pomembno vlogo pri uravnavanju metabolizma (Milart s sod. 2019), imunskega odziva (Małaczewska s sod. 2014) in prenašanju živčnih signalov. Dokazano pa je tudi, da so kinurenska kislina in drugi derivati triptofana udeleženi v procesih celjenja površinskih ran (Poonmasjedi-Meibod s sod. 2014, Matysik-Woźniak s sod. 2017). Zapleti pri zdravljenju ran zaradi odpornosti bakterij na klasične antibiotike je stimulirala biomedicinske raziskave potencialne uporabe medu za pripravke za nego ran (Molan 1992). Znatno protibakterijsko in protiglivično delovanje je bilo potrjeno za slovenski kostonj med (Kunčič s sod. 2012) (Sl. 2). Z jasnejšo opredelitvijo kvalitete za deklaracijo medicinskega medu (Hermanns s sod. 2020) bodo na trgu ponujene nove rešitve na osnovi specifičnih lastnosti posameznih vrst medu. Tu ima pravi kostonj posebno priložnost, predvsem zaradi farmakološko relevantnih koncentracij kinurenske kisline in drugih derivatov kinurenske poti.

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References

- Al-Waili, N., Salom, K., Al-Ghamdi, A., Ansari, M.J., 2012. Antibiotic, Pesticide, and Microbial Contaminants of Honey: Human Health Hazards. *The Scientific World Journal* 2012, 1–9.
- Beretta, G., Artali, R., Caneva, E., Orlandini, S., Centini, M., Facino, R.M., 2009. Quinoline alkaloids in honey: Further analytical (HPLC-DAD-ESI-MS, multidimensional diffusion-ordered NMR spectroscopy), theoretical and chemometric studies. *Journal of Pharmaceutical and Biomedical Analysis*, 50, 432–439.
- Bertoncelj, J., Golob, T., Kropf, U., Korošec, M., 2011a. Characterisation of Slovenian honeys on the basis of sensory and physicochemical analysis with a chemometric approach. *International Journal of Food Science & Technology*, 46, 1661–1671.
- Bertoncelj, J., Polak, T., Kropf, U., Korošec, M., Golob, T., 2011b. LC-DAD-ESI/MS analysis of flavonoids and abscisic acid with chemometric approach for the classification of Slovenian honey. *Food Chemistry*, 127, 296–302.
- Bilikova, K., Krakova, T.K., Yamaguchi, K., Yamaguchi, Y., 2015. Major royal jelly proteins as markers of authenticity and quality of honey. *Archives of Industrial Hygiene and Toxicology*, 66, 259–267.
- Bogdanov, S., Ruoff, K., Oddo, L.P., 2004. Physico-chemical methods for the characterisation of unifloral honeys: a review. *Apidologie*, 35, S4–S17.
- Bucekova, M., Buriova, M., Pekarik, L., Majtan, V., Majtan, J., 2018. Phytochemicals-mediated production of hydrogen peroxide is crucial for high antibacterial activity of honeydew honey. *Scientific Reports*, 8, 9061.
- Bucekova, M., Sojka, M., Valachova, I., Martinotti, S., Ranzato, E., Szep, Z., Majtan, V., Klaudiny, J., Majtan, J., 2017. Bee-derived antibacterial peptide, defensin-1, promotes wound re-epithelialisation in vitro and in vivo. *Scientific Reports*, 7, 7340.
- Bucekova, M., Valachova, I., Kohutova, L., Prochazka, E., Klaudiny, J., Majtan, J., 2014. Honeybee glucose oxidase—its expression in honeybee workers and comparative analyses of its content and H₂O₂-mediated antibacterial activity in natural honeys. *Naturwissenschaften*, 101, 661–670.
- Buttstedt, A., Moritz, R.F.A., Erler, S., 2014. Origin and function of the major royal jelly proteins of the honeybee (*Apis mellifera*) as members of the yellow gene family. *Biological Reviews*, 89, 255–269.
- Casteels, P., Ampe, C., Jacobs, F., Tempst P., 1993. Functional and chemical characterization of Hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honeybee (*Apis mellifera*). *The Journal of Biological Chemistry*, 268, 7044–7054.
- Casteels, P., Ampe, C., Rivière, L., Van Damme, J., Elicone, C., Fleming, M., Jacobs, F., Tempst P., 1990. Isolation and characterization of abaecin, a major antibacterial response peptide in the honeybee (*Apis mellifera*). *European Journal of Biochemistry*, 187, 381–386.
- Česnik, H.B., Kmecl, V., Bolta, Š.V., 2019. Pesticide and veterinary drug residues in honey - validation of methods and a survey of organic and conventional honeys from Slovenia. *Food Additives & Contaminants: Part A*, 36, 1358–1375.
- Council Directive, 1974. 74/409/EEC of 22 July. On harmonization of the laws of the member states relating to honey. *Official Journal of the European Communities* 17, 10–14.
- Crailsheim, K., 1991. Interadult feeding of jelly in honeybee (*Apis mellifera* L.) colonies. *Journal of Comparative Physiology B*, 161, 55–60.
- Erban, T., Shcherbachenko, E., Talacko, P., Harant, K., 2019. The unique protein composition of honey revealed by comprehensive proteomic analysis: Allergens, venom-like proteins, antibacterial properties, royal jelly proteins, serine proteases, and their inhibitors. *Journal of Natural Products*, 82, 1217–1226.
- European Commission, 2018. Technical guidelines for determining the magnitude of pesticide residues in honey and setting maximum residue levels in honey. SANTE/11956/2016 rev.9, pp. 41.

- Feng, M., Fang, Y., Han, B., Zhang, L., Lu, X., Li, J., 2013. Novel aspects of understanding molecular working mechanisms of salivary glands of worker honeybees (*Apis mellifera*) investigated by proteomics and phosphoproteomics. *Journal of Proteomics*, 87, 1–15.
- Fontana, R., Mendes, M.A., Souza, B.M., Konno, K., César, L.M.M., Malaspina, O., Palma M.S., 2004. Jelleines: a family of antimicrobial peptides from the royal jelly of honeybees (*Apis mellifera*). *Peptides*, 25, 919–928.
- Fujita, T., Kozuka-Hata, H., Uno, Y., Nishikori, K., Morioka, M., Oyama, M., Kubo, T., 2010. Functional analysis of the honeybee (*Apis mellifera* L.) salivary system using proteomics. *Biochemical and Biophysical Research Communications*, 397, 740–744.
- Fujiwara, S., Imai, J., Fujiwara, M., Yaeshima, T., Kawashima, T., Kobayashi, K., 1990. A potent antibacterial protein in royal jelly. Purification and determination of the primary structure of royalisin. *Journal of Biological Chemistry*, 265, 11333–11337.
- Golob, T., Korošec, M., Bertoneclj, J., Kropf, U., Kandolf Borovšak, A., Božič, J., Zdešar, P., Meglič, M., Goljat, A., Šivic, F., Borovšak U., Veljanovski-Geremia, V., Sonc, C., Kandolf Borovšak, A., 2008. Med: značilnosti slovenskega medu. Čebelarstva zveza Slovenije, Javna svetovalna služba v čebelarstvu, Lukovica.
- Grabowski, N.T., Klein, G., 2017. Microbiology and foodborne pathogens in honey. *Critical Reviews in Food Science and Nutrition*, 57, 1852–1862.
- Hermanns, R., Mateescu, C., Thrasyvoulou, A., Tananaki, C., Wagener, F.A.D.T.G., Cremers, N.A.J., 2020. Defining the standards for medical grade honey. *Journal of Apicultural Research*, 59, 125–135.
- Huo, X., Wu, B., Feng, M., Han, B., Fang, Y., Hao, Y., Meng, L., Wubie, A.J., Fan, P., Hu, H., Qi, Y., Li, J., 2016. Proteomic analysis reveals the molecular underpinnings of mandibular gland development and lipid metabolism in two lines of honeybees (*Apis mellifera ligustica*). *Journal of Proteome Research*, 15, 3342–3357.
- Ilyasov, R., Gaifullina, L., Saltykova, E., Poskryakov, A., Nikolenko, A., 2012. Review of the expression of antimicrobial peptide defensin in honey bees *Apis mellifera* L. *Journal of Apicultural Science*, 56, 115–124.
- Janghu, S., Bera, M.B., Nanda, V., Rawson, A., 2017. Study on power ultrasound optimization and its comparison with conventional thermal processing for treatment of raw honey. *Food Technology and Biotechnology*, 55, 570–579.
- Jo, C., Kim, J.K., Kang, H.J., Young, L.E., Byun, M.W., 2005. Irradiation effects on the decontamination of microorganisms in honey. *International Symposium »New frontiers of irradiated food and non-food products«*, September 22-23, 2005, Bangkok, Thailand. pp. 8.
- Kandolf, A., 2011. Pelodna analiza medu iz različnih fitogeografskih območij Slovenije = Pollen spectrum of honey from different phytogeographical areas of Slovenia. M. Sc. Thesis, University of Ljubljana, Biotechnical faculty.
- Klaudiny, J., Albert, Š., Bachanová, K., Kopernický, J., Šimúth, J., 2005. Two structurally different defensin genes, one of them encoding a novel defensin isoform, are expressed in honeybee *Apis mellifera*. *Insect Biochemistry and Molecular Biology*, 35, 11–22.
- Korošec, M., Kandolf Borovšak, A., Božič, J., Bertoneclj, J., Justinek, J., Lilek, N., Kozmus, P., 2016a. Končno poročilo projekta Karakterizacija slovenskega medu / Final report of the project 'Characterisation of Slovene honey'. Čebelarstva zveza Slovenije, Biotehniška fakulteta, Lukovica, Ljubljana.
- Korošec, M., Kropf, U., Golob, T., Bertoneclj, J., 2016b. Functional and nutritional properties of different types of slovenian honey. In: Kristbergsson, K., Ötles, S. (eds.): *Functional Properties of Traditional Foods*. Springer US, Boston, MA, pp. 323–338.
- Kropf, U., Korošec, M., Bertoneclj, J., Ogrinc, N., Nečemer, M., Kump, P., Golob, T., 2010. Determination of the geographical origin of Slovenian black locust, lime and chestnut honey. *Food Chemistry*, 121, 839–846.

- Kubo, T., Sasaki, M., Nakamura, J., Sasagawa, H., Ohashi, K., Takeuchi, H., Natori, S., 1996. Change in the expression of hypopharyngeal-gland proteins of the worker honeybees (*Apis mellifera* L.) with age and/or role. *The Journal of Biochemistry*, 119, 291–295.
- Kunčič, M.K., Jaklič, D., Lapanje, A., Gunde-Cimerman, N., 2012. Antibacterial and antimycotic activities of Slovenian honeys. *British Journal of Biomedical Science*, 69, 154–158.
- Kwakman, P.H.S., Velde, A.A. te, Boer, L., de, Speijer, D., Vandenbroucke-Grauls, C.M.J.E., Zaat, S.A.J., 2010. How honey kills bacteria. *The FASEB Journal*, 24, 2576–2582.
- Kwakman, P.H.S., Velde, A.A. te, Boer, L., de, Vandenbroucke-Grauls, C.M.J.E., Zaat, S.A.J., 2011. Two major medicinal honeys have different mechanisms of bactericidal activity. *PLoS ONE* 6, e17709.
- Kwakman, P.H.S., Zaat, S.A.J., 2012. Antibacterial components of honey. *IUBMB Life*, 64, 48–55.
- Leyva-Daniel, D.E., Escobedo-Avellaneda, Z., Villalobos-Castillejos, F., Alamilla-Beltrán, L., Welti-Chanes, J., 2017. Effect of high hydrostatic pressure applied to a Mexican honey to increase its microbiological and functional quality. *Food and Bioprocesses*, 102, 299–306.
- Małaczewska, J., Siwicki, A.K., Wójcik, R.M., Kaczorek, E., Turski, W.A., 2014. Effect of oral administration of kynurenic acid on the activity of the peripheral blood leukocytes in mice. *Central-European Journal of Immunology*, 39, 6–13.
- Martin, S.J., Correia-Oliveira, M.E., Shemilt, S., Drijfhout, F.P., 2018. Is the salivary gland associated with honey bee recognition compounds in worker honey bees (*Apis mellifera*)? *Journal of Chemical Ecology*, 44, 650–657.
- Matysik-Woźniak, A., Paduch, R., Turski, W.A., Maciejewski, R., Jünemann, A.G., Rejda, R., 2017. Effects of tryptophan, kynurenine and kynurenic acid exerted on human reconstructed corneal epithelium in vitro. *Pharmacological Reports*, 69, 722–729.
- Mavric, E., Wittmann, S., Barth, G., Henle, T., 2008. Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (*Leptospermum scoparium*) honeys from New Zealand. *Molecular Nutrition & Food Research*, 52, 483–489.
- Milart, P., Paluszkiwicz, P., Dobrowolski, P., Tomaszewska, E., Smolinska, K., Debinska, I., Gawel, K., Walczak, K., Bednarski, J., Turska, M., Raba, n M., Kocki, T., Turski, W.A., 2019. Kynurenic acid as the neglected ingredient of commercial baby formulas. *Scientific Reports*, 9, 1–8.
- Molan, P.C., 1992. The antibacterial activity of honey. *Bee World*, 73, 59–76.
- Olaitan, P.B., Adeleke, O.E., Iyabo, O.O., 2007. Honey: a reservoir for microorganisms and an inhibitory agent for microbes. *African Health Sciences*, 7, 159–165.
- Oryan, A., Alemzadeh, E., Moshiri, A. 2016. Biological properties and therapeutic activities of honey in wound healing: A narrative review and meta-analysis. *Journal of Tissue Viability*, 25, 98–118.
- Panatto, D., Gasparini, R., Lai, P., Rovatti, P., Gallelli, G., 2007. Long-term decline of ¹³⁷Cs concentration in honey in the second decade after the Chernobyl accident. *Science of the Total Environment*, 382, 147–152.
- Perko, D., 1998. The regionalization of Slovenia. *Acta Geographica*, 38, 11–57.
- Podrižnik, B., Božič, J., 2015. Maturation and stratification of antibacterial activity and total phenolic content of bee bread in honey comb cells. *Journal of Apicultural Research*, 54, 81–92.
- Poormasjedi-Meibod, M.-S., Hartwell, R., Kilani, R.T., Ghahary, A., 2014. Anti-scarring properties of different tryptophan derivatives. *PLOS ONE* 9, e91955.
- Ramanathan, A.N.K.G., Nair, A.J., Sugunan, V.S., 2018. A review on royal jelly proteins and peptides. *Journal of Functional Foods*, 44, 255–264.
- Saitta, M., Bella, G.D., Fede, M.R., Turco, V.L., Potorti, A.G., Rando, R., Russo, M.T., Dugo, G., 2017. Gas chromatography-tandem mass spectrometry multi-residual analysis of contaminants in Italian honey samples. *Food Additives & Contaminants: Part A*, 34, 800–808.
- Silva, M.S., Rabadzhiev, Y., Eller, M.R., Iliev, I., Ivanova, I., Santana, W.C., 2017. Microorganisms in honey. *Honey Analysis*.
- Snowdon, J.A., Cliver, D.O., 1996. Microorganisms in honey. *International Journal of Food Microbiology*, 31, 1–26.

- Sojka, M., Valachova, I., Bucekova, M., Majtan, J., 2016. Antibiofilm efficacy of honey and bee-derived defensin-1 on multispecies wound biofilm. *Journal of Medical Microbiology*, 65, 337–344.
- Szweda, P., 2017. Antimicrobial activity of honey. *Honey Analysis*, 1, 215–232.
- Takenaka, T., Ito, H., Yatsunami, K., Echigo, T., 1990. Changes of glucose oxidase activity and amount of gluconic acid formation in the hypopharyngeal glands during the lifespan of honey bee workers (*Apis mellifera* L.). *Agricultural and Biological Chemistry*, 54, 2133–2134.
- Tian, W., Li, M., Guo, H., Peng, W., Xue, X., Hu, Y., Liu, Y., Zhao, Y., Fang, X., Wang, K., Li, X., Tong, Y., Conlon, M.A., Wu, W., Ren, F., Chen, Z., 2018. Architecture of the native major royal jelly protein 1 oligomer. *Nature Communications*, 9, 3373.
- Truchado, P., Gil-Izquierdo, A., Tomás-Barberán, F., Allende, A., 2009a. Inhibition by chestnut honey of N-Acyl-l-homoserine lactones and biofilm formation in *Erwinia carotovora*, *Yersinia enterocolitica* and *Aeromonas hydrophila*. *Journal of Agricultural and Food Chemistry*, 57, 11186–11193.
- Truchado, P., López-Gálvez, F., Gil, M.I., Tomás-Barberán, F.A., Allende, A., 2009b. Quorum sensing inhibitory and antimicrobial activities of honeys and the relationship with individual phenolics. *Food Chemistry*, 115, 1337–1344.
- Truchado, P., Martos, I., Bortolotti, L., Sabatini, A.G., Ferreres, F., Tomas-Barberan, F.A., 2009c. Use of quinoline alkaloids as markers of the floral origin of chestnut honey. *Journal of Agricultural and Food Chemistry*, 57, 5680–5686.
- Turski, M.P., Chwil, S., Turska, M., Chwil, M., Kocki, T., Rajtar, G., Parada-Turska, J., 2016. An exceptionally high content of kynurenic acid in chestnut honey and flowers of chestnut tree. *Journal of Food Composition and Analysis*, 48, 67–72.
- Wiest, L., Buleté, A., Giroud, B., Fratta, C., Amic, S., Lambert, O., Pouliquen, H., Arnaudguilhem, C., 2011. Multi-residue analysis of 80 environmental contaminants in honeys, honeybees and pollens by one extraction procedure followed by liquid and gas chromatography coupled with mass spectrometric detection. *Journal of Chromatography A*, 1218, 5743–5756.
- Winston, M.L., 1991. *The biology of the honey bee*. Harvard University Press.

Vpliv vojaških aktivnosti na ohranjanje vrstne pestrosti dnevnih metuljev na osrednjem vadišču slovenske vojske – poligon Poček

The impact of military activities on preserving butterfly diversity in the central Slovenian military area – Poček

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Izvleček: V letih 2007 in 2019 je bil opravljen kvantitativni popis favne dnevnih metuljev v osrednjem delu vojaškega poligona Poček s ciljem primerjave stanja obeh vzorčenj ter ovrednotenja vpliva vojaških aktivnosti na pestrost favne dnevnih metuljev. S standardizirano metodo transektnega popisa na linijah, ki so vključevale vse za območje poligona reprezentativne habitatne tipe, smo v obeh letih skupaj opazili 73 vrst (2007: 62, 2019: 61), med njimi 11 ogroženih (2007: 9, 2019: 8). Favni obeh vzorčenj sta se značilno razlikovali v sestavi vrst, ne pa v vrstni pestrosti, povprečni populacijski gostoti vrst na posameznem transektu in povprečni populacijski gostoti vrst na poligonu. Različnost v sestavi favne je posledica vrstnega obrata ter sprememb v velikosti in prostorski razporeditvi populacijskih gostot vrst po transektih. Med vrstami, ki se jim je populacijska gostota v 2019 zmanjšala v primerjavi z 2007 so prevladovala take, ki so imele v 2007 največje gostote v gozdnih vegetacijskih tipih; med vrstami, ki so v 2019 dosegale višje populacijske gostote kot v 2007 pa tiste, ki so bile najštevilčnejše na traviščih. To pomeni, da redne, vendar časovno in prostorsko neenakomerne vojaške aktivnosti predstavljajo heterogene motnje, ki vzdržujejo izjemno pester mozaik habitatnih tipov, v katerem imajo največjo naravovarstveno vrednost obsežni ekstenzivno vzdrževani suhi kamniti kraški travniki in suhi travniki v zgodnjih fazah zaraščanja. Vojaške aktivnosti tako pomembno prispevajo k ohranjanju biotske in krajinske pestrosti območja.

Ključne besede: dnevni metulji, transektni monitoring, vrstna pestrost, sestava favne, Lepidoptera

Abstract: In 2007 and 2019, a quantitative inventory of the butterfly fauna was conducted in the central part of the Poček military training area, with the aim of comparing the fauna from both periods and evaluating the impact of military activities on the butterfly diversity. Using a standardized linear transect method and covering all habitat types represented in the military area, a total of 73 species were observed in both years (2007: 62, 2019: 61), including 11 threatened species (2007: 9, 2019: 8). The fauna of both sampling periods differed significantly in composition but not in species diversity, average population density of species on single transects, and in average population density of species in the area. The difference in the faunal composition is the result

of species turnover and changes in the size and spatial distribution of the population densities of the species at an individual transects. Among species whose population density decreased in 2019 compared to 2007, those with the highest densities in forest vegetation types in 2007 prevailed. On the other hand, the predominantly grassland species became more abundant in 2019. This means that regular, but temporally and spatially uneven military activities, represent heterogeneous disturbances that maintain an extremely diverse mosaic of habitat types, in which large scale extensively maintained dry calcareous grasslands and dry grasslands in the early stages of succession have the greatest conservation value. Military activities thus make an important contribution to the conservation of the biodiversity and landscape diversity of the area.

Key words: butterflies, transect monitoring, species richness, species composition, Lepidoptera

Uvod

Dnevni metulji (Papilionoidea) so ena najbolj proučenih skupin žuželk in pomembni modelni organizmi na raziskovalnih področjih kot so sinekologija, biogeografija, klimatske spremembe, interakcije žuželke-rastline in v raziskavah speciacije (Wiemers in sod. 2018). S približno 19.000 opisanimi vrstami predstavljajo 12 % vseh znanih vrst metuljev – Lepidoptera (Van Nieukerken in sod. 2011). V Sloveniji je bilo doslej zanesljivo opaženih 181 vrst dnevnih metuljev, kar Slovenijo uvršča med vrstno najbolj bogate evropske države (Maes in sod. 2019). Med njimi je 6 vrst, ki v Sloveniji v zadnjih desetletjih niso bile več opažene in so opredeljene kot izumrle (Wiemers in sod. 2018).

Dnevni metulji s kratkimi generacijskimi časi, številčno majhnimi populacijami in kompleksnimi ekološkimi potrebami, specifičnimi za posamezno razvojno fazo, hitreje sledijo majhnim spremembam v okolju kot dolgo živeči organizmi z daljšimi razmnoževalnimi cikli. Zato so dnevni metulji prepoznani kot ena najpomembnejših nevretenčarskih bioindikatorskih skupin (npr. Kudrna 1986, Settele in sod. 2009) in so reprezentativni indikatorji populacijskih trendov opaženih pri večini kopenskih žuželk, ki predstavljajo približno dve tretjini vseh svetovnih vrst (Thomas 2005). Na podlagi tega je Evropska okoljska agencija, z namenom zmanjšanja upadanje biodiverzitete do leta 2010, v okviru projekta »Streamlining European 2010 Biodiversity Indicators« že leta 2007 dnevne metulje uvrstila med evropske biodiverzitetne indikatorje (SEBI 2010 Indicators),

s katerimi se vrednoti splošne trende v stanju biodiverzitete v Evropi (Van Sway in Van Strien 2008, Van Sway 2010). Dnevni metulji so bili izbrani kot indikatorji za oceno stanja in trendov evropskih travnišč (European Grassland Butterfly Indicator), ki so najpomembnejši habitati evropskih vrst dnevnih metuljev, saj jih poseljuje več kot polovica evropskih vrst (Van Sway in sod. 2006).

Vojaška vadišča so zaradi heterogenosti habitatov kot posledica stalne, vendar neenakomerne rabe in odsotnosti intenzivnega kmetijstva znana kot pomembna območja ohranjanja biodiverzitete, vključno z metulji (Smith in sod. 2002, Warren in sod. 2007, Warren in Büttner 2008). Eno največjih vadišč v Sloveniji je območje Počka pri Postojni, kjer je bil prvi popis favne dnevnih metuljev (ti. inventarizacija) opravljen leta 1997, najdenih pa je bilo 59 vrst (Polak 1997). Druga inventarizacija je bila izvedena leta 2007, ugotovljenih je bilo 96 vrst. V okviru te raziskave je bil opravljen tudi kvantitativni transektni popis favne dnevnih metuljev (Verovnik 2008), ki smo ga v letu 2019 ponovili (Čelik 2019). Cilj te raziskave je primerjati sedanje stanje s stanjem pred dvanajstimi leti ter ovrednotiti vpliv rednih vojaških aktivnosti na pestrost favne dnevnih metuljev na poligonu Poček.

Metode

Opis raziskovanega območja

Vojaški poligon Poček je del Osrednjega vadišča slovenske vojske (OSVAD Postojna), ki se razprostira JV od Postojne, na južnih obron-

kih Javornikov. Za območje je značilen izredno razgiban kraški relief. Poligon leži večinoma v submontanskem pasu (500–800 m n.m.v.), le vzhodni del poligona sega v montanski pas z vrhovi Veliki vrh (826 m), Gadovec (972 m), Kamena gora (1040 m) in Baba (1085 m).

Poligon leži v pasu bukovih in hrastovih conalnih gozdov (Čarni in sod. 2002), vendar gozd površinsko ne prevladuje, ker se predvsem zaradi rednih vojaških aktivnosti (uporaba vozil, streljanja) v zadnjih petih letih, vzpostavljajo in ohranjajo obsežne odprte, negozdne površine (travišča, kamnišča, ruderalne površine) in površine v različnih stopnjah zaraščanja (zaraščajoča travišča, grmišča). Negozdne, ekstenzivno vzdrževane površine pa so najpomembnejši habitati dnevnih metuljev zmernega pasu (Settele in sod. 2009).

Raziskovano območje je del Posebnega ohranitvenega območja Javorniki–Snežnik (SI3000231), ki vključuje visoki kraški planoti Javorniki in Snežnik ter Pivško podolje. V tem območju se varuje 2 rastlinski in 15 živalskih kvalifikacijskih vrst. Med njimi so 3 vrste dnevnih metuljev: močvirski cekinček (*Lycaena dispar*), strašničin mravljiščar (*Phengaris teleius*) in travniški postavnež (*Euphydryas aurinia*). Prvi dve se pojavljata le na vlažnih traviščih Pivškega podolja, slednja pa poseljuje suha travišča podolja in kraških planot (Čelik in sod. 2005).

Metoda transektnega popisa

Standardizirana metoda transektnega popisa (Pollard in Yates 1993, Thomas 2005) se izvaja s počasno hojo po transektni liniji, med katero štejemo vse osebkne dnevnih metuljev, ki jih opazimo pred seboj v navidezni kocki z dolžino stranic 5 metrov. Na terenu je transekt navidezna linija v krajini, njen položaj mora biti enak pri vsaki ponovitvi transektnega popisa.

Metoda transektnega popisa je vremensko in časovno definirana: geografski položaj Slovenije določa, da se popis izvaja pri temperaturi zraka vsaj 15 °C, v brezvetrju ali ob rahlem vetru, v sončnem ali vsaj pretežno jasnem vremenu, med 10.00 in 18.00 uro.

Izbor transektnih linij

Število in lokacijo transektnih linij smo izbrali tako, da smo v popis vključili vse habitatne tipe, ki so reprezentativni za območje vojaškega poligona Poček. V letu 2007 smo popise izvedli na petih transektnih linijah skupne dolžine 1845 metrov, v letu 2019 na šestih linijah s celotno dolžino 1870 m (Sl. 1, Tab. 1). Zaradi rednih, vsakodnevnih vojaških aktivnosti na poligonu Poček v zadnjih petih letih ter posledično časovno in prostorsko zelo strogih omejitev našega gibanja v vojaškem območju, smo v letu 2019 transektne popise lahko ponovili le na treh transektih iz leta 2007

Tabela 1: Značilnosti transektnih linij za popis pestrosti favne dnevnih metuljev na vojaškem poligonu Poček v letih 2007 in 2019.

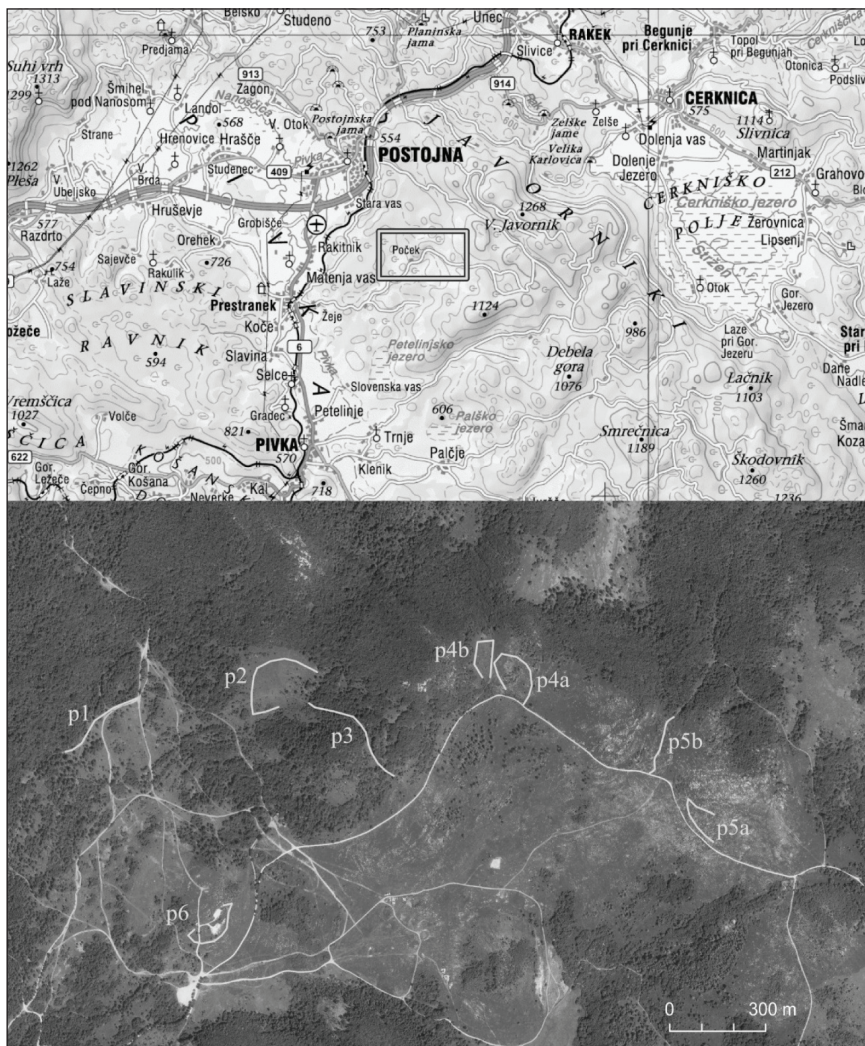
Table 1: The characteristics of the transect lines for butterfly counts at Poček military area in 2007 and 2019.

| Transekt | Dolžina transektu (m) | | | Tip vegetacije na transektu | |
|----------|-----------------------|------|--|--|--|
| | 2007 | 2019 | 2007 | 2019 | |
| p1 | 360 | 290 | Gozdni rob (gozdni sestoj le na eni strani transektne linije, na drugi suho zaraščajoče travišče) | Gozdni rob (gozdni sestoj le na eni strani transektne linije, na drugi suho zaraščajoče travišče) | |
| p2 | 470 | 434 | Zaraščajoč suh kraški travnik | Zaraščajoč suh kraški travnik | |
| p3 | 395 | 378 | Gozdna pot (pretežno rahli sklep drevesnih krošenj in manjše gozdne jase) | Gozdna pot (pretežno rahli sklep drevesnih krošenj in manjše gozdne jase) | |
| p4 | 320 | 284 | Zaraščajoč suh kraški travnik (v času popisov sveže pogorel) | Zaraščajoč suh kraški travnik (vključuje manjši mezofilen sestoj s trstikasto stožko – <i>Molinia arundinacea</i>) | |
| p5 | 300 | 201 | Suh kamnit kraški travnik | Suh kamnit kraški travnik | |
| p6 | – | 283 | – | Suh kamnit kraški travnik | |
| Skupaj | 1845 | 1870 | | | |

(Sl. 1: p1, p2, p3). Zaradi prepovedi vstopa na določena območja smo morali transektne linije p4 in p5 spremeniti. Ohranili smo ju v bližini linij iz leta 2007 tako, da smo vključili čim več površine primerljivega habitatnega tipa (skaloviti kraški travnik) iz leta 2007 (Sl. 1). Ker zgolj s premaknitvijo linij nismo popolnoma zadostili temu pogoju (transekt p4 v 2019 je zaraščajoč suh kraški travnik), smo dodali novo transektno linijo – p6 (Tab. 1).

Izvedba popisov

Transektne popise smo izvajali od aprila do septembra, v vsakem letu v šestih terenskih dnevih (2007: 10. 5., 25. 5., 10. 6., 6. 7., 27. 7., 15. 8.; 2019: 22. 4., 1. 6., 25. 6., 15. 7., 30. 7., 13. 9.). V letu 2007 so bili prvi popisi izvedeni v mesecu maju, saj so bili vezani na dovoljenja za terensko delo v okviru raziskave favne vojaških vadbišč (Verovnik 2008). V letu 2019 popisa v mesecu maju nismo mogli izvesti zaradi slabega vremena oziroma prepovedi vstopa na poligon zaradi nevarnih vojaških aktivnosti.



Slika 1: Transektne linije za popis stanja dnevnih metuljev na vojaškem poligonu Poček v letih 2007 (p1, p2, p3, p4a, p5a) in 2019 (p1, p2, p3, p4b, p5b, p6).

Figure 1: Position of transect lines for butterfly counts at Poček military area in 2007 (p1, p2, p3, p4a, p5a) and 2019 (p1, p2, p3, p4b, p5b, p6).

V obeh letih smo beležili tudi prisotnost vrst, ki smo jih opazili izven transektnih linij, bodisi v neposredni bližini linije, ali pa v času hoje med posameznimi transektnimi linijami.

Taksonomijo in poimenovanje dnevnih metuljev smo povzeli po Wiemers s sod. 2018.

Analiza podatkov

Populacijsko gostoto vsake vrste smo izračunali za vsak transekt in je izražena v številu osebkov na mesec na 100 m transekta in izračunali smo jo po Kitahara in sod. (2008) na sledeč način: število osebkov vrste v mesecu je izraženo kot število opaženih osebkov v tem mesecu, če je bil transektni popis izveden le enkrat v mesecu oz. kot povprečje obeh mesečnih vrednosti, če sta bila izvedena dva popisa. Za izračun povprečnega števila osebkov na mesec preko celotne sezone smo uporabili le tiste mesece, v katerih je bila vrsta opazovana in tako izključili vpliv različnega števila generacij v eni sezoni med vrstami. Populacijska gostota vrste je nato izračunana kot kvocient med povprečnim številom osebkov na mesec preko celotne sezone in dolžino transekta (tj. populacijska gostota vrste na transektu) oz. skupno dolžino vseh transektov (tj. populacijska gostota vrste na poligonu Poček).

Razliko v vrstni pestrosti transektov med letoma 2007 in 2019 smo testirali z neparametričnim Mann-Whitneyevim testom. Povezanost med vrstno pestrostjo in vegetacijskimi tipi v letih 2007 in 2019 smo preverjali s Hi-kvadrat testom. Za ugotavljanje razlik v povprečni populacijski gostoti vrst na transektu (med primerjanimi pari transektov: p1_2007 vs p1_2019, p2_2007 vs p2_2019, p3_2007 vs p3_2019, p4a_2007 vs p4b_2019, p5a_2007 vs p5b_2019) oz. na poligonu Poček med letoma smo uporabili Wilcoxonov test predznačenih rangov (Wilcoxon signed-rank test). Spremembe v sestavi favne dnevnih metuljev med letoma na podlagi na razporeditve vrst in njihovih populacijskih gostot na transektih, smo ocenjevali s permutacijsko multivariatno analizo variance (PERMANOVA – PERmutational Multivariate Analysis of VAriance), v kateri smo uporabili Bray-Curtisov koeficient podobnosti in 9999 permutacij. Rezultat PERMANOVA smo grafično prikazali z diagramom nemetričnega

večrazsežnostnega lestvičenja (NM-MDS – Non-Metric MultiDimensional Scaling). Z analizo SIMPER (SIMilarity PERcentage species contributions) smo ugotavljali, katere vrste največ prispevajo k razlikam v sestavi favne med letoma. Statistične analize smo izvedli s programskima paketoma IBM SPSS STATISTICS Version 22 in PAST (Hammer 1999–2018).

Skupne vrste so tiste, ki so se pojavljale na obeh primerjanih transektih. Unikatne vrste za transekt so tiste, ki so bile v posameznem letu opažene le na tem transektu. Odstotek skupnih vrst smo izračunali kot kvocient med številom skupnih vrst in vrstno pestrostjo obeh primerjanih transektov.

Za prikaz stopnje ogroženosti vrst na nacionalnem in evropskem nivoju smo uporabili naslednje kazalnike:

- Priloga k Pravilnik o uvrstitvi ogroženih rastlinskih in živalskih vrst v rdeči seznam (Uradni list RS 82, 2002), (v nadaljevanju: RS–SLO);
- European Red List of Butterflies (van Sway s sod. 2010), (v nadaljevanju: RS–Evropa);
- Uredba o zavarovanih prosto živečih živalskih vrstah (Uradni list RS 46, 2004), (v nadaljevanju: UZŽV);
- Direktiva o ohranjanju naravnih habitatov ter prostoživečih živalskih in rastlinskih vrst (Direktiva Sveta 92/43/EGS), (v nadaljevanju: FFH).

Rezultati

Vrstna pestrost in populacijske gostote na transektih

Na transektnih linijah smo skupno registrirali 73 vrst dnevnih metuljev; 62 vrst (1519 osebkov) v letu 2007 in 61 vrst (1597 osebkov) v letu 2019 (Tab. 2). Petdeset vrst je bilo takih, ki so se na transektih pojavljale v obeh letih, 12 vrst, ki so se na transektih pojavljale le v letu 2007 in 11 vrst, ki so bile na transektih registrirane le v letu 2019 (Tab. 2).

Tabela 2: Seznam in populacijska gostota vrst, ki so bile opažene na transektih vojaškega poligona Poček v letih 2007 in 2019. S krepkim tiskom je označena maksimalna populacijska gostota vrste; + vrsta opažena v osrednjem delu poligona, vendar izven transektnih linij; – vrsta na transektu ni opažena; svetlo siva – vrste, ki so bile na transektih opažene le v letu 2007; temno siva – vrste, ki so bile na transektih opažene le v letu 2019.

Table 2: List and population density of species observed on transects at Poček military area in 2007 and 2019. Bold indicates the maximum population density of a species; + indicates species observed in the central part of the military area, but outside the transects; – denotes species not observed on a transect; light gray - species observed on transects only in 2007; dark gray - species observed on transects only in 2019.

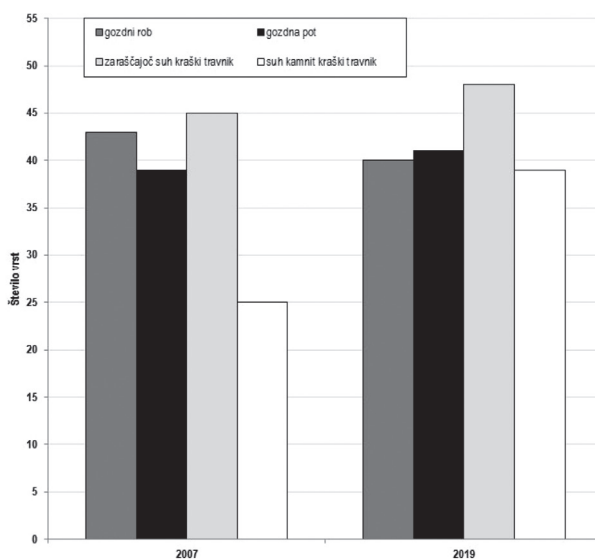
| Takson | Populacijska gostota | | | | | | | | | | | Izven transek. linij |
|-------------------------------------|----------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|----------------------|
| | p1 | | p2 | | p3 | | p4 | | p5 | | p6 | |
| | 2007 | 2019 | 2007 | 2019 | 2007 | 2019 | 2007 | 2019 | 2007 | 2019 | 2019 | |
| Papilionidae | | | | | | | | | | | | |
| <i>Iphiclides podalirius</i> | 0.56 | 0.52 | – | 0.46 | – | – | 0.31 | 0.35 | 1.00 | – | – | |
| <i>Papilio machaon</i> | 0.46 | 0.34 | 0.21 | 0.23 | 0.87 | – | 0.73 | 0.35 | – | 0.50 | 0.35 | |
| <i>Zerynthia polyxena</i> | – | – | – | 0.23 | – | 0.53 | – | – | – | – | – | |
| Hesperiidae | | | | | | | | | | | | |
| <i>Heteropterus morpheus</i> | 0.28 | 0.69 | – | – | – | 0.79 | 0.31 | 0.35 | – | – | – | |
| <i>Carterocephalus palaemon</i> | – | 0.69 | – | 0.69 | – | 0.53 | – | – | – | – | – | + (2007) |
| <i>Ochlodes sylvanus</i> | 0.28 | 1.72 | – | 0.35 | 0.25 | 0.66 | 0.31 | 0.70 | – | 0.62 | 1.06 | |
| <i>Hesperia comma</i> | 1.11 | – | 1.38 | 0.23 | 1.27 | – | 0.63 | – | – | – | – | |
| <i>Thymelicus lineola</i> | 0.28 | 0.34 | 0.21 | 0.46 | 1.01 | 0.26 | 0.31 | 0.35 | – | – | – | |
| <i>Carcharodus floccifera</i> | 0.28 | – | – | – | – | – | – | – | – | – | – | + (2019) |
| <i>Erynnis tages</i> | – | 0.34 | 0.64 | – | 0.42 | – | – | 0.70 | – | 0.50 | 0.35 | |
| <i>Pyrgus malvae</i> | – | 0.52 | 0.21 | 0.92 | 0.25 | 0.26 | – | 0.35 | – | 1.24 | 0.35 | |
| <i>Pyrgus armoricanus</i> | – | – | – | – | – | – | 0.31 | – | – | – | 0.71 | |
| <i>Pyrgus alveus</i> | – | – | – | – | – | – | – | – | – | – | – | + (2007) |
| <i>Pyrgus serratulae</i> | – | – | – | – | – | – | 0.31 | – | – | – | – | |
| <i>Spialia sertorius</i> | 0.28 | – | – | – | – | – | – | – | – | – | – | |
| Pieridae | | | | | | | | | | | | |
| <i>Leptidea sinapis/ juvernica*</i> | 0.65 | 0.34 | – | 0.23 | 1.01 | 0.26 | 0.47 | 0.35 | 0.33 | 1.00 | 0.35 | |
| <i>Gonepteryx rhamni</i> | 0.28 | 0.69 | – | 0.23 | 0.51 | 0.40 | – | 0.35 | – | 0.50 | 0.35 | |
| <i>Colias alfaciensis/ hyale*</i> | – | 0.69 | – | – | – | – | – | – | – | – | – | + (2007) |
| <i>Colias crocea</i> | – | 0.34 | – | 0.23 | – | 0.26 | – | – | – | – | – | + (2007) |

| | | | | | | | | | | | |
|------------------------------|-------------|-------------|------|--------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| <i>Fabriciana adippe</i> | - | - | - | - | - | - | 0.31 | - | 0.33 | - | 0.26 |
| <i>Boloria dia</i> | - | 0.34 | - | 0.54 | - | 0.53 | - | 0.70 | - | - | 0.71 |
| <i>Boloria euphrosyne</i> | - | - | - | - | 0.76 | - | - | - | - | - | - |
| <i>Vanessa cardui</i> | - | 0.52 | 0.21 | 0.46 | - | 0.53 | 0.63 | 0.53 | 0.67 | 1.00 | 1.41 |
| <i>Vanessa atalanta</i> | 0.28 | 0.34 | - | - | 0.25 | - | - | - | - | - | - |
| <i>Aglais io</i> | - | - | - | - | - | - | - | 0.35 | - | - | - |
| <i>Aglais urticae</i> | - | - | - | - | - | - | 0.63 | - | - | - | - |
| <i>Polygonia c-album</i> | 0.28 | - | - | - | - | 0.26 | - | - | - | - | - |
| <i>Nymphalis polychloros</i> | 0.28 | - | - | - | - | - | - | - | - | - | - |
| <i>Euphydryas aurinia</i> | 0.28 | - | - | - | - | - | - | 0.35 | - | 1.49 | 0.35 |
| <i>Euphydryas maturna</i> | - | - | - | - | - | - | - | - | - | - | + (2007) |
| <i>Melitaea trivia</i> | - | - | - | 0.23 | - | - | - | - | 0.67 | - | 0.35 |
| <i>Melitaea didyma</i> | - | 1.55 | 0.21 | 1.15 | - | 0.53 | - | 0.70 | - | 0.50 | 0.88 |
| <i>Melitaea cinxia</i> | 0.28 | 0.69 | - | 0.23 | - | - | - | 0.35 | - | 0.75 | - |
| <i>Melitaea phoebe</i> | - | - | - | - | - | - | - | - | - | - | + (2007) |
| <i>Melitaea athalia</i> | 0.97 | 2.76 | - | 3.00 | 1.01 | 2.12 | 0.31 | 1.41 | - | 1.49 | 1.77 |
| <i>Melitaea britomartis</i> | - | - | - | - | - | - | - | - | - | - | + (2007) |
| <i>Melitaea aurelia</i> | - | - | - | - | - | - | - | - | - | - | + (2007) |
| <i>Coenonympha pamphilus</i> | 0.28 | 0.46 | 0.32 | 0.23 | 0.38 | 0.53 | 0.31 | 1.41 | - | 1.08 | 0.71 |
| <i>Coenonympha glycerion</i> | 3.06 | 2.07 | 4.75 | 8.41 | 1.27 | 2.12 | - | 5.63 | 4.00 | 2.99 | 5.65 |
| <i>Coenonympha arcania</i> | 7.22 | 2.41 | 3.40 | 0.63 | 9.87 | 1.72 | 4.06 | 1.41 | 8.44 | 1.24 | 2.12 |
| <i>Lopinga achine</i> | 0.83 | 0.34 | - | - | 0.76 | 1.32 | - | 0.35 | - | - | - |
| <i>Pararge aegeria</i> | - | - | - | - | 0.38 | 0.26 | - | - | 0.33 | - | - |
| <i>Lasiommata maera</i> | 0.28 | - | 0.21 | - | 0.76 | - | 0.31 | 0.35 | 1.00 | - | - |
| <i>Melanargia galathea</i> | 13.75 | 10.78 | 7.77 | 16.36 | 9.62 | 10.05 | 1.78 | 8.27 | 3.17 | 2.86 | 8.39 |
| <i>Hipparchia fagi</i> | - | - | - | - | - | 0.34 | - | 0.35 | - | 0.50 | 1.06 |
| <i>Minois dryas</i> | 1.67 | 2.24 | 2.98 | 3.46 | 1.65 | 0.53 | - | 0.35 | 0.33 | - | 4.95 |
| <i>Brintesia circe</i> | 0.46 | 0.34 | 0.28 | 0.23 | 1.52 | 0.26 | 0.78 | - | 0.67 | - | 0.35 |
| <i>Arethusa arethusa</i> | 0.56 | - | - | - | - | - | - | - | - | - | 0.35 |
| <i>Aphantopus hyperantus</i> | - | 0.34 | - | - | - | 0.79 | - | - | - | - | + (2007) |
| <i>Maniola jurtina</i> | - | 0.80 | - | 0.23 | 1.14 | 0.79 | 0.63 | 1.06 | - | 1.00 | 1.06 |
| <i>Erebia medusa</i> | 1.94 | 1.03 | 2.02 | 4.84 | 1.60 | 2.65 | 0.63 | 2.82 | 1.00 | 1.00 | 1.06 |
| Število vrst | 43 | 40 | 30 | 40 | 39 | 41 | 34 | 37 | 25 | 31 | 33 |

* Obravnavano kot kompleks dveh vrst, ker vrsti znotraj kompleksa z metodologijo transektnega popisa na terenu ni mogoče razlikovati.

Število vrst po transektih se ni razlikovalo med letoma (Mann-Whitney $Z = 1,12$; $P = 0,31$). V obeh letih je bila vrstna pestrost največja na zaraščajočih suhih travnikih, najmanjša pa na suhih kamnitih travnikih (Sl. 2), razlike v številu vrst po vegetacijskih tipih med letoma niso bile statistično značilne ($X^2 = 2,52$; $P = 0,47$).

Najvišjo populacijsko gostoto na transektu (16,36 osebkov/mesec/100 m) je imela vrsta *M. galathea* (2019: p2). Populacijska gostota vrst na posameznem transektu se je med letoma značilno razlikovala le na transektu p2 (2007: $Me = 0,00$; 2019: $Me = 0,23$; $Z = 2,434$, $P = 0,015$), na ostalih primerjanih transektih (p1, p3, p4, p5) razlike niso bile statistično značilne.

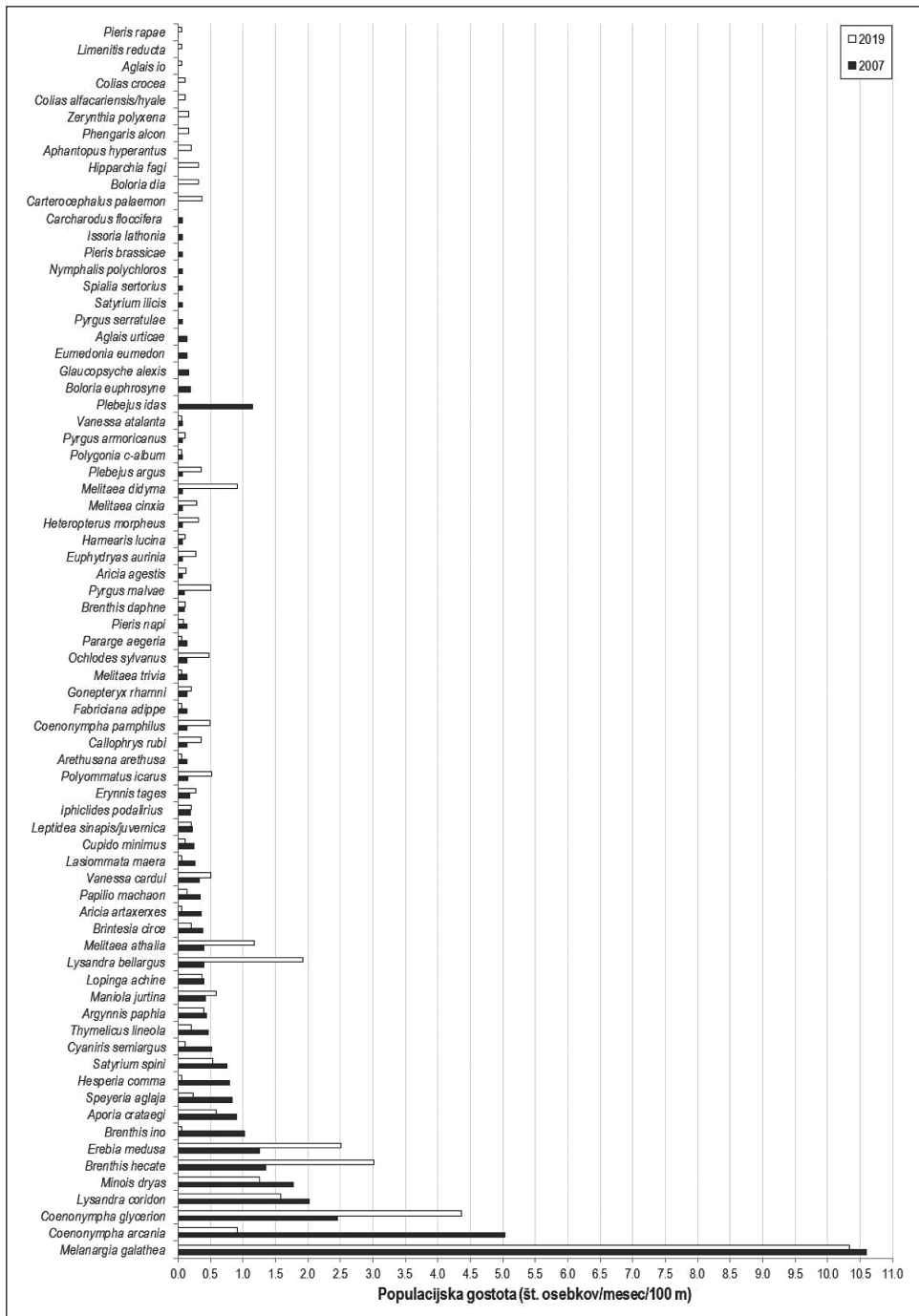


Slika 2: Vrstna pestrost dnevni metuljev po vegetacijskih tipih na transektih vojaškega poligona Poček v letih 2007 in 2019.

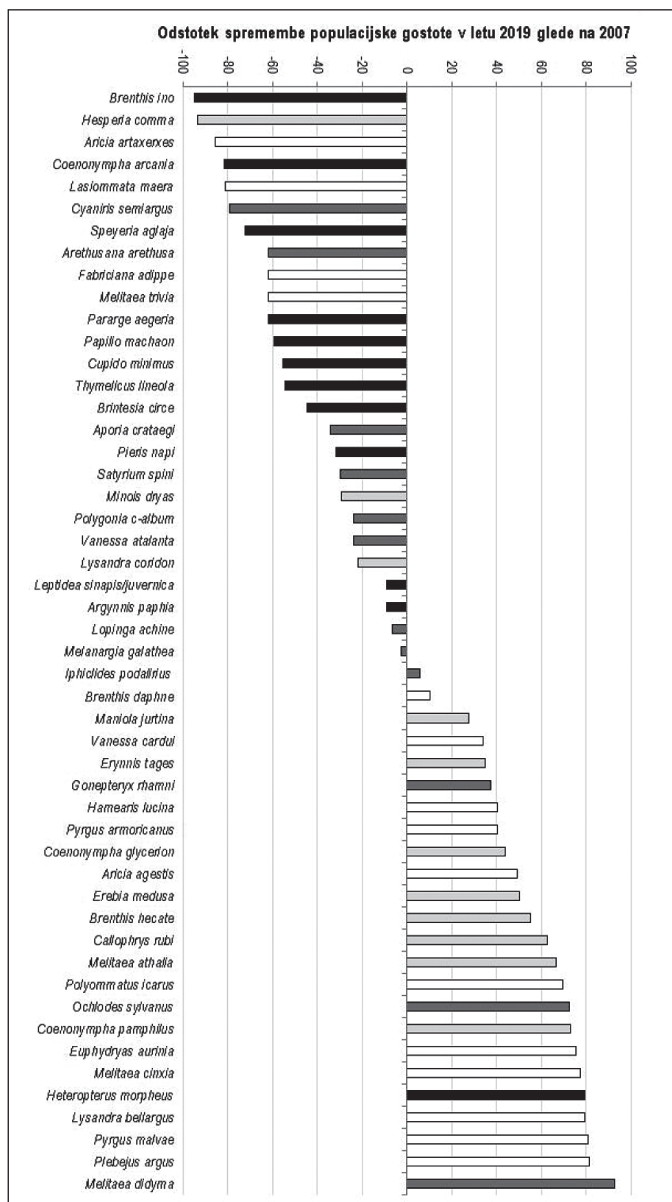
Figure 2: Species richness in different vegetation types on transects at Poček military area in 2007 and 2019.

Populacijske gostote vrst na poligonu se v povprečju niso značilno razlikovale med letoma (2007: $Ma = 0,13$; 2009: $Me = 0,16$; $Z = 0,228$, $P = 0,820$). V letu 2007 smo zabeležili 9 vrst, ki so imele populacijsko gostoto vrste na poligonu 1 ali več kot 1 osebkov/mesec/100 m, v letu 2019 je bilo takih vrst 8 (Sl. 3). Vrsta z največjo številčnostjo je bila v obeh letih *M. galathea*. V obeh vzorčenjih so bile vrste z najvišjimi populacijskimi gostotami tiste, ki so ekološko vezane na suha zaraščajoča travišča (npr. *M. dryas*, *C. arcania*, *C. glycerion*, *E. medusa*, *B. hecate*) ali pa na kamnite kraške travnike (npr. *L. coridon*, *P. idas*).

Med 23 vrstami, ki so bile na transektih opažene zgolj v enem od obeh vzorčenj, so imele vse vrste, z izjemo *P. idas*, populacijsko gostoto vrste na poligonu nižjo od 0,5 osebkov/mesec/100 m (Sl. 3). Med petdesetimi vrstami, ki so se na transektih pojavljale v obeh letih, smo nižjo populacijsko gostoto vrste na poligonu v 2019 glede na 2007 opazili pri 26 vrstah (52 %; Sl. 4). Med njimi je bilo največ vrst (18) takih, ki so v 2007 maksimalno populacijsko gostoto dosegale na gozdni poti ali gozdnem robu. Med vrstami, ki so v 2019 imele na poligonu višjo populacijsko gostoto kot v 2007, je največ vrst (19) takih, ki so v 2019 imele najvišjo populacijsko gostoto na enem od obeh tipov travnikov (Sl. 4).



Slika 3: Primerjava populacijskih gostot vrst dnevnih metuljev na poligonu Poček med letoma 2007 in 2019.
 Figure 3: Comparison of population densities of butterflies at Poček military area in 2007 and 2019.



Slika 4: Odstotek spremembe populacijske gostote vrste na poligonu Poček med letoma 2007 in 2019, za vrste, ki so se na transektih pojavljale v obeh letih. Vegetacijski tipi, v katerih so vrste imele najvišjo populacijsko gostoto v 2007 (vrste v upadu) in 2019 (vrste v porastu): temno siva – gozdni rob, črna – gozdna pot, svetlo siva – zaraščajoč suhi kraški travnik, bela – suh kamnit kraški travnik.

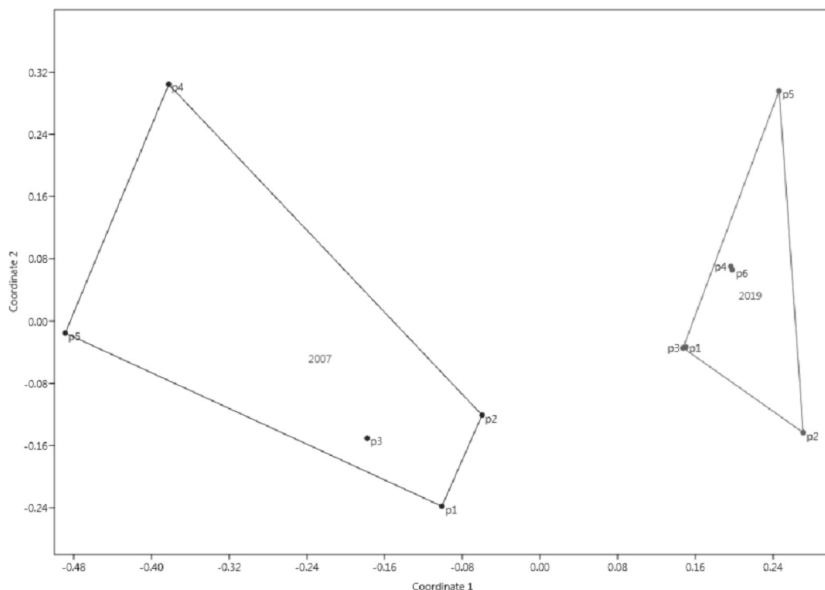
Figure 4: Proportion of change in the population density of the species at the Poček military area between 2007 and 2019, for species occurring on the transects in both years. Vegetation types in which the species had the highest population density in 2007 (declining species) and 2019 (increasing species): dark grey - forest edge, black - forest path, light grey - overgrown dry calcareous meadows, white - dry rocky calcareous meadows.

Sestava favne dnevnih metuljev na transektih v letih 2007 in 2019

Razporeditev vrst in njihovih populacijskih gostot na transektih se je med letoma statistično značilno razlikovala (PERMANOVA: pseudo-F = 4,14; df = 5; P = 0,0027; Sl. 5). Različnost v sestavi favne dnevnih metuljev med letoma je bila 53,18 % (SIMPER test). K razliki največ prispevajo vrste z visokimi populacijskimi gostotami oz. velikimi spremembami tega parametra v vrednosti in prostorski razporeditvi na transektih med vzorčenjema (Tab. 3). Vrste, ki so k različnosti obeh favn prispevale vsaj 1 % je bilo 29, do 50 % različnosti je skupaj pojasnilo 8 vrst. Kumulativni prispevek vrst, ki so se pojavljale le v enem od vzorčenj (23 vrst; Tab. 2) je 11,4 % (ni prikazano v Tab. 3), med njimi sta le 2 vrsti (*P. idas*, *B. dia*), ki sta k različnosti posamič prispevali več kot 1 % (Tab. 3).

Med vrstami, ki so k različnosti favn obeh let posamično prispevale vsaj 1 %, je bil v 2019 upad v populacijski gostoti zabeležen pri vrstah, ki so v 2007 imele največje gostote v obeh gozdnih vegetacijskih tipih (7 vrst) ali na obeh tipih travnikov (4 vrste); med vrstami, pri katerih smo v 2019 opazili povečanje populacijske gostote so prevladoval tiste (14 vrst; Tab. 3), ki so največje gostote dosegale na travnikih.

Primerjava med letoma kaže (Tab. 4), da sta si bili po sestavi najbolj podobni (Bray-Curtis = 0,611) favni dnevnih metuljev na transektih p2_2007 (zaraščajoč suh kraški travnik) in p6_2019 (suh kamnit kraški travnik), ki sta imeli 20 (46 %) skupnih vrst, najbolj različni (Bray-Curtis = 0,309) sta si bili favni na transektih p4_2007 (sveže pogorel zaraščajoč suh kraški travnik) in p2_2019 (zaraščajoč suh kraški travnik) s 24 (48 %) skupnimi vrstami. Med tremi transekti, na katerih je monitoring v obeh letih potekal na istih linijah (p1, p2, p3), je največjo podobnost v sestavi favne imel transekt p3 (gozdna pot), medtem ko je transekt p2 (zaraščajoč suh kraški travnik) imel



Slika 5: Razporeditev transektov na podlagi podobnosti v sestavi favne med letoma 2007 in 2019 z uporabo nemetričnega večrazsežnostnega lestvičenja (NM-MDS). Letnici sta centroida posamezne skupine. Stres = 0,13.

Figure 5: Non-metric multidimensional scaling ordination (NM-MDS) of butterfly assemblages on transects on years 2007 and 2019. Years are the centroids of each group. Stress = 0.13.

Tabela 3: Prispevek (%) vrst k razlikam v sestavi favne dnevnih metuljev na transektih poligona Poček med letoma 2007 in 2019 (SIMPER test). Vključene so le vrste, ki so k različnosti prispevale vsaj 1%.

Table 3: Contribution (%) of individual species to the differentiation in the composition of the fauna of butterflies on the transects of the Poček military area between 2007 and 2019 (SIMPER test). Only species that have contributed at least 1% to the differentiation are included.

| Takson | Povprečna različnost | Prispevek (%) | Kumulativni prispevek (%) | Povprečna populacijska gostota | |
|------------------------------|----------------------|---------------|---------------------------|--------------------------------|-------|
| | | | | 2007 | 2019 |
| <i>Melanargia galathea</i> | 6.08 | 11.44 | 11.44 | 7.220 | 9.450 |
| <i>Coenonympha arcania</i> | 5.68 | 10.68 | 22.12 | 6.600 | 1.590 |
| <i>Coenonympha glycerion</i> | 3.28 | 6.17 | 28.28 | 2.620 | 4.480 |
| <i>Brenthis hecate</i> | 2.50 | 4.70 | 32.98 | 1.600 | 3.730 |
| <i>Plebejus idas</i> | 2.34 | 4.39 | 37.37 | 1.880 | 0.000 |
| <i>Lysandra bellargus</i> | 2.32 | 4.36 | 41.73 | 0.548 | 2.320 |
| <i>Minois dryas</i> | 2.06 | 3.88 | 45.61 | 1.330 | 1.920 |
| <i>Melitaea athalia</i> | 1.96 | 3.68 | 49.29 | 0.458 | 2.090 |
| <i>Lysandra coridon</i> | 1.78 | 3.34 | 52.63 | 2.220 | 1.850 |
| <i>Erebia medusa</i> | 1.48 | 2.79 | 55.42 | 1.440 | 2.230 |
| <i>Hesperia comma</i> | 0.99 | 1.86 | 57.28 | 0.878 | 0.038 |
| <i>Melitaea didyma</i> | 0.99 | 1.85 | 59.13 | 0.042 | 0.885 |
| <i>Aporia crataegi</i> | 0.90 | 1.68 | 60.82 | 1.080 | 0.510 |
| <i>Satyrrium spini</i> | 0.84 | 1.57 | 62.39 | 0.976 | 0.478 |
| <i>Brenthis ino</i> | 0.83 | 1.56 | 63.94 | 0.780 | 0.057 |
| <i>Ochlodes sylvanus</i> | 0.82 | 1.55 | 65.49 | 0.168 | 0.852 |
| <i>Speyeria aglaja</i> | 0.82 | 1.54 | 67.03 | 0.858 | 0.410 |
| <i>Cyaniris semiargus</i> | 0.79 | 1.48 | 68.51 | 0.776 | 0.082 |
| <i>Maniola jurtina</i> | 0.74 | 1.39 | 69.90 | 0.354 | 0.823 |
| <i>Callophrys rubi</i> | 0.72 | 1.35 | 71.25 | 0.112 | 0.722 |
| <i>Brintesia circe</i> | 0.65 | 1.21 | 72.46 | 0.742 | 0.197 |
| <i>Polyommatus icarus</i> | 0.62 | 1.17 | 73.62 | 0.370 | 0.870 |
| <i>Coenonympha pamphilus</i> | 0.61 | 1.15 | 74.77 | 0.258 | 0.737 |
| <i>Plebejus argus</i> | 0.61 | 1.15 | 75.92 | 0.098 | 0.575 |
| <i>Pyrgus malvae</i> | 0.61 | 1.14 | 77.06 | 0.092 | 0.607 |
| <i>Vanessa cardui</i> | 0.59 | 1.10 | 78.16 | 0.302 | 0.742 |
| <i>Boloria dia</i> | 0.55 | 1.04 | 79.20 | 0.000 | 0.470 |
| <i>Lasiommata maera</i> | 0.55 | 1.04 | 80.24 | 0.512 | 0.058 |
| <i>Lopinga achine</i> | 0.54 | 1.01 | 81.25 | 0.318 | 0.335 |

največji odstotek skupnih vrst. Razlike v prostorski umestitvi in vegetacijskih značilnostih transektov p4 in p5 med obema letoma (Sl. 1, Tab. 1) so se odražale v znatno manjši podobnosti v sestavi favne in številu, ne pa v odstotkih (transekt p4) skupnih vrst glede na primerjave med ostalimi tremi transekti (Tab. 4: krepki tisk).

Parametra število in odstotek skupnih vrst torej ne kažeta enakega vzorca (tj. v isti primerjavi enako število skupnih vrst ne pomeni nujno tudi enakega odstotka le teh), prav tako oba omenjena parametra ne odražata enakega vzorca kot koeficient podobnosti (tj. v isti primerjavi višje oz. nižje število ali odstotek skupnih vrst ne pomeni nujno tudi višje oz. nižje vrednosti koeficienta podobnosti). To velja za primerjave med letoma in znotraj posameznega leta (Tab. 4), kar pomeni, da parametra število in odstotek skupnih vrst nista ustrezen pokazatelj podobnosti v sestavi favne.

V obeh letih je bilo največje število unikatnih vrst opazovano na transektu p1 (gozdni rob), najnižje na transektih p2 (zaraščajoč suh kraški travnik) in p5 (suh kamnit kraški travnik) (Tab. 4: diagonalna).

Ogrožene vrste

V osrednjem delu vojaškega poligona Poček smo v letu 2007 našli 9, v letu 2019 pa 7 ogroženih vrst in eno na evropskem nivoju potencialno ogroženo vrsto – *H. fagi* (Tab. 5). V Sloveniji zavarovane (UZŽV) in evropsko pomembne vrste (FFH) imajo največjo številčnost na zaraščajočih suhih travnikih (*C. floccifera*, *P. alcon*), kamnitih kraških travnikih (*E. aurinia*) in presvetljenih gozdnih poteh (*Z. polyxena*, *L. achine*). V letu 2019 se je glede na leto 2007 populacijska gostota zmanjšala štirim ogroženim vrstam (*S. sertorius*, *P. idas*, *M. trivialis*, *L. achine*), štirim (*Z. polyxena*, *P. armoricanus*, *E. aurinia*, *H. fagi*) se je povečala, za dve ogroženi vrsti (*C. floccifera*, *P. alcon*) pa spremembe ni mogoče ovrednotiti. Domnevamo, da odsotnost vrste *E. eumedon* v letu 2019 ni posledica upada populacijske gostote, temveč je razlog v drugačni poziciji transektne linije p4, ki ni vključevala za vrsto ustreznega življenjskega okolja.

Tabela 4: Bray-Curtis koeficient podobnosti v sestavi favne dnevnih metuljev na transektih v letih 2007 in 2019 (zgornja desna polovica), število/odstotek skupnih vrst (spodnja leva polovica) in število unikatnih vrst (diagonalna). Svetlo siva – podobnost med transekti v letu 2007, temno siva – podobnost med transekti v letu 2019, črna – podobnost med transekti obeh let.

Table 4: Bray-Curtis coefficient of similarity in the composition of the butterfly fauna of the 2007 and 2019 transects (upper right half), number/percentage of common species (lower left half), and number of unique species (diagonal). Light gray - similarity between transects in 2007, dark gray - similarity between transects in 2019, black - similarity between transects of both years.

| | p1_2007 | p2_2007 | p3_2007 | p4_2007 | p5_2007 | p1_2019 | p2_2019 | p3_2019 | p4_2019 | p5_2019 | p6_2019 |
|---------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|---------|
| p1_2007 | 8 | 0.666 | 0.686 | 0.415 | 0.485 | 0.574 | 0.561 | 0.574 | 0.507 | 0.399 | 0.472 |
| p2_2007 | 24/49 | 1 | 0.592 | 0.435 | 0.492 | 0.580 | 0.550 | 0.551 | 0.605 | 0.429 | 0.611 |
| p3_2007 | 29/55 | 25/57 | 2 | 0.495 | 0.537 | 0.589 | 0.464 | 0.584 | 0.499 | 0.393 | 0.498 |
| p4_2007 | 24/45 | 19/42 | 26/55 | 6 | 0.521 | 0.426 | 0.309 | 0.409 | 0.384 | 0.411 | 0.393 |
| p5_2007 | 19/39 | 17/45 | 20/46 | 18/44 | 1 | 0.369 | 0.347 | 0.399 | 0.399 | 0.354 | 0.406 |
| p1_2019 | 26/45 | 21/43 | 28/55 | 22/42 | 15/30 | 5 | 0.630 | 0.709 | 0.639 | 0.514 | 0.666 |
| p2_2019 | 28/51 | 25/56 | 29/58 | 24/48 | 19/41 | 31/63 | 1 | 0.615 | 0.609 | 0.485 | 0.643 |
| p3_2019 | 27/47 | 23/48 | 28/54 | 22/42 | 17/35 | 29/58 | 34/72 | 2 | 0.679 | 0.502 | 0.633 |
| p4_2019 | 26/48 | 21/46 | 26/52 | 22/45 | 14/29 | 28/57 | 28/57 | 29/59 | 3 | 0.662 | 0.737 |
| p5_2019 | 23/45 | 20/49 | 23/49 | 19/41 | 12/27 | 24/51 | 26/58 | 26/57 | 25/58 | 1 | 0.602 |
| p6_2019 | 21/38 | 20/46 | 23/47 | 20/36 | 15/35 | 24/49 | 27/59 | 26/54 | 26/59 | 25/64 | 3 |

Tabela 5: Ogrožene vrste v območju transektnih linij vojaškega poligona Poček v letih 2007 in 2019. Za razlago kratic ogroženosti glej poglavje 2.3.**Table 5:** Endangered species on the transects at Poček military area in 2007 and 2019. For the explanation of the acronyms see section 2.3.

| Vrsta | RS-SLO | RS-Evropa | UZŽV (prilogi 1, 2) | FFH (prilogi II, IV) |
|-------------------------------|--------|-----------|------------------------|-------------------------|
| <i>Zerynthia polyxena</i> | V | LC | 1, 2 | IV |
| <i>Carcharodus floccifera</i> | E | NT | 1, 2 | |
| <i>Pyrgus armoricanus</i> | V | LC | | |
| <i>Spialia sertorius</i> | V | LC | | |
| <i>Phengaris alcon</i> | E | LC | 1, 2 | |
| <i>Eumedonia eumedon</i> | V | LC | | |
| <i>Plebejus idas</i> | V | LC | | |
| <i>Euphydryas aurinia</i> | V | LC | 1, 2 | II, IV |
| <i>Melitaea trivia</i> | V | LC | | |
| <i>Lopinga achine</i> | | VU | 1 | IV |
| <i>Hipparchia fagi</i> | | NT | | |

Razprava

Rezultati transektnih popisov kažejo na veliko vrstno pestrost vojaškega poligona Poček, saj 73 ugotovljenih vrst v obeh letih predstavlja kar 42 % vseh vrst dnevnih metuljev, ki se pojavljajo v Sloveniji (Verovnik 2019). Tako visoko število vrst registriranih na manj kot 2 kilometra dolžine transektnih linij potrjuje tudi to, da je bila izbira transektnih linij z vidika reprezentativnosti življenjskih okolij (vegetacijskih tipov) ustrežna.

Vrstno najbolj pestri so zaraščajoči suhi travniki. Ti so v zgodnjih fazah zaraščanja primerno življenjsko okolje tako za nekatere vrste, ki imajo sicer večje populacijske gostote na suhih kamnitih travnikih, npr. *Lysandra bellargus*, *Lysandra coridon*, *Plebejus argus*, *Aricia agestis*, *Melitaea trivia*, kot tudi za vrste, ki so sicer ekološko bolj vezane na presvetljene grmiščne in/ali gozdne habitate, npr. *Iphiclides podalirius*, *Zerynthia polyxena*, *Carterocephalus palaemon*, *Ochlodes sylvanus*, *Gonepteryx rhamni*, *Coenonympha arcania*. V obeh vzorčenjih je bilo najmanjše število vrst opaženo na suhih kamnitih travnikih, kar je presenetljivo, saj so načeloma gozdovi v Evropi oz. zmernem pasu življenjsko okolje z

najmanj vrstami dnevnih metuljev (npr. Settele in sod. 2009). Razlog za visoko vrstno pestrost dnevnih metuljev v obeh gozdnih vegetacijskih tipih je v razgibani strukturiranosti lesne vegetacije z nesklenjenim sklepom krošenj, ki dopušča veliko svetlobe pri tleh ter posledično pestro zeliščno vegetacijo s primerno mikroklimo za metulje. Poleg tega je ena stran transektne linije na gozdnem robu vključevala zaraščajoče suho travišče, transektna linija na gozdni poti pa je ponekod mejila na travnate jase.

Med obema vzorčenjema nismo ugotovili razlik v vrstni pestrosti dnevnih metuljev (število vrst na transektih), povprečni populacijski gostoti vrst na posameznem transektu in povprečni populacijski gostoti vrst na poligonu. Favni sta se med letoma značilno razlikovali v sestavi; razlika pa je posledica vrstnega obrata (species turnover) ter sprememb v velikosti in prostorski razporeditvi populacijskih gostot vrst po transektih. Znano je, da vrstna pestrost ni zadostno merilo za oceno biodiverzitete, ker ne pokaže časovnih in prostorskih sprememb v vrstnem obratu (Asiss in sod. 2018, Hillebrand in sod. 2018). V naši raziskavi smo pokazali, da tudi parametra število in odstotek skupnih vrst nista ustrezna pokazatelja podobnosti v sestavi favn, ker ne odražata

enakega vzorca kot koeficient podobnosti, ki upošteva prostorske in časovne spremembe v populacijskih gostotah vrst. Časovni vrstni obrat na poligonu Poček je med letoma 2007 in 2019 vključeval 23 vrst (12 vrst opaženih izključno v 2007 in 11 vrst opaženih izključno v 2019) in je deloma posledica razlik v poziciji transektov p4 in p5, ter v dodani transektni liniji p6 v letu 2019. Namreč, štiri od vrst, ki jih nismo našli v letu 2019, so bile v letu 2007 najdene izključno na transektih p4 in p5: *A. urticae*, *E. eumedon*, *P. brassicae* in *P. serratule*. V letu 2019 smo izključno na transektih p4, p5, p6 našli dve vrsti, ki zato verjetno nista bili zabeleženi v 2007: *P. alcon* in *A. io*. Tovrstno metodološko spremembo odražata tudi koeficient podobnosti v sestavi favne in število skupnih vrst, ki sta za transekt p4 in p5 znatno nižja kot pri treh transektih, na katerih je monitoring v obeh letih potekal na istih linijah (p1, p2, p3). Drugi vzrok, da določene vrste v posameznem letu niso bile zabeležene, je lahko tudi različna fenologija in posledično številčnost posamezne vrste v obeh primerjanih sezonah zaradi različnih klimatskih razmer in zaradi drugačne časovne razporeditve vzorčenj v posameznem letu.

Sprememba v velikosti in prostorski razporeditvi populacijskih gostot vrst med letoma je pomembnejši razlog za razliko v sestavi obeh favn kot časovni vrstni obrat, saj je bil prispevek posamezne vrste, ki se je pojavljala le v enem od vzorčenj, pri 21 vrstah (izjemi *P. idas* in *B. dia*) manjši od 1%, kumulativni prispevek vseh 23 vrst pa le 11,4 %, kar je enako kot je k različnosti med favnoma obeh let prispevala najštevilčnejša vrsta, *M. galathea*. K različnosti sestave obeh favn torej kumulativno največ (88,6 %) prispevajo vrste, ki so se na transektih pojavljale v obeh vzorčenjih (50 vrst). Glede na to, da (i) so med vrstami, ki se jim je populacijska gostota v 2019 zmanjšala v primerjavi z 2007 prevladovale take, ki so imele v 2007 največje gostote v obeh gozdnih vegetacijskih tipih (tj. 64 % upošteva 29 vrst z največjim prispevkom k različnosti) in (ii) da so med vrstami, ki so v 2019 dosegale višje populacijske gostote kot v 2007 prevladovale tiste, ki so bile najštevilčnejše na obeh tipih travnikov (78 % upošteva 29 vrst z največjim prispevkom k različnosti) sklepamo, da se s travniškimi površinami v osrednjem delu poligona Poček

ustrezno upravlja in da imajo velik naravovarstveni pomen.

V obeh letih je bilo v osrednjem delu poligona opaženih 11 ogroženih vrst, vrstni obrat med vzorčenjema je vključeval 5 vrst (*Z. polyxena*, *S. sertorius*, *E. eumedon*, *P. idas*, *H. fagi*). Vzroka razlik v prisotnosti ogroženih vrst med obema letoma sta v večini primerov sprememba v poziciji transektih p4, p5, p6) in različna časovna razporeditev vzorčenj v posameznem letu. Razširjenost in številčnost registriranih ogroženih vrst kaže na veliko naravovarstveno vrednost poligona Poček, saj visoke populacijske gostote nekaterih ogroženih vrst (npr. *L. achine*, *P. idas*, *E. aurinia*) predstavljajo pomemben delež celotne populacije posamezne vrste v Sloveniji.

Vrsta olivni slezovček (*P. serratule*) se v Sloveniji pojavlja le na dveh lokalitetah, v vojaškem poligonu Poček in na Volovji rebri nad Ilirsko Bistrico (Verovnik in sod. 2012). V letu 2007 sta bila na Počku opažena le dva osebkna na transektu p4 in v njegovi neposredni bližini, medtem ko v 2019 vrste nismo zabeležili. Kljub starejšim navedbam o prisotnosti vrste v Sloveniji, je bila ta zagotovo potrjena šele leta 2007. Zato vrsta še ni uvrščena v rdeči seznam slovenskih metuljev, ki je nastal leta 2002 (UL RS, 2002). Glede na razširjenost in ekološke potrebe vrste v Sloveniji, jo je v prihodnje smiselno uvrstiti na rdeči seznam kot prizadeto vrsto (Verovnik in sod. 2012).

S povečevanjem heterogenosti krajine se povečuje vrstna pestrost favne dnevnihih metuljev (Slancarova in sod. 2014, Loos in sod. 2014, van der Merwe in sod. 2019). Veliko število različnih habitatnih tipov v krajini (ti. *compositional heterogeneity*) ima pozitiven učinek na vrstno pestrost favne dnevnihih metuljev, s povečevanjem geometrijske heterogenosti (ti. *configurational heterogeneity*) – raznolikost v številu, velikosti in prostorski razporeditvi habitatnih krp – pa se povečuje funkcionalna pestrost v sestavi favne dnevnihih metuljev, ki zato vključuje tudi več ekološko specializiranih vrst (Perović in sod. 2015). Motnje (kot npr. vojaške aktivnosti) povečujejo heterogenost krajine in s tem pestrost združb, pri čemer je vrstna pestrost največja v krajini z optimalnim številom motenj; ta ugotovitev je osnova hipoteze o uravnovešeni frekvenci pojavljanja motenj (*intermediate disturbance hypothesis*,

Conell 1978). Z raziskavami biotske raznolikosti vojaških vadišč, kjer so motnje stalne, so Warren in sodelavci (2007) predlagali hipotezo o heterogeni frekvenci pojavljanja motenj (*heterogeneous disturbance hypothesis*), ki predpostavlja, da je biotska pestrost največja, kjer je frekvenca pojavljanja motenj zelo heterogena. To pomeni, da se različno v prostoru in času pojavljajo motnje, ki se razlikujejo po tipu, frekvenci, intenziteti, periodičnosti, obsegu in obliki ter trajanju. Glede na to, da se v zadnjih letih na vojaškem poligonu Poček vojaške aktivnosti (npr. streljanja s požiganjem, vožnja vojaških vozil) izvajajo redno, vendar časovno in prostorsko neenakomerno, ter da znotraj poligona ni kmetijskih površin (le na zelo majhnem deležu travnikov na obrobju poligona se izvaja ekstenzivna košnja), ugotavljamo, da vojaške aktivnosti predstavljajo heterogene motnje, ki vzdržujejo izjemno pester mozaik habitatnih tipov (suha travišča, strukturirani gozdni robovi, presvetljene gozdne poti, erodirane in ruderalne površine, omrežje kamnitih in peščenih cest), v katerem imajo največjo naravovarstveno vrednost obsežni ekstenzivno vzdrževani suhi kamniti kraški travniki in suhi travniki v zgodnjih fazah zaraščanja. Vojaške aktivnosti tako pomembno prispevajo k ohranjanju biotske in krajinske pestrosti območja.

Zaključek

S primerjavo pestrosti favne dnevnih metuljev med obema vzorčenjema nismo ugotovili razlik v vrstni pestrosti, povprečni populacijski gostoti vrst na posameznem transektu in povprečni populacijski gostoti vrst na poligonu. Favni sta se med letoma značilno razlikovali v sestavi; razlika je posledica vrstnega obrata (species turnover) ter sprememb v velikosti in prostorski razporeditvi populacijskih gostot vrst na transektih. R. različnosti sestave obeh favn kumulativno največ prispevajo pogoste vrste, ki so se na transektih pojavljale v obeh vzorčenjih. V obeh letih skupaj je bilo v osrednjem delu poligona opaženih 73 vrst (42 % vrst dnevnih metuljev, ki se pojavljajo v Sloveniji), med njimi 11 ogroženih vrst, med katerimi nekatere (npr. *L. achine*, *E. aurinia*) dosegajo populacijske gostote, ki predstavljajo pomemben delež celotne populacije posamezne vrste v Sloveniji. Med

vrstami, ki se jim je populacijska gostota v 2019 zmanjšala v primerjavi z 2007 so prevladovale take, ki so imele v 2007 največje gostote v obeh gozdnih vegetacijskih tipih; med vrstami, ki so v 2019 dosegale višje populacijske gostote kot v 2007 so prevladovale tiste, ki so bile najštevilčnejše na obeh tipih travnikov. To pomeni, da se s traviščnimi površinami v osrednjem delu poligona Poček ustrezno upravlja in da imajo velik naravovarstveni pomen. Redne, vendar časovno in prostorsko neenakomerne vojaške aktivnosti predstavljajo heterogene motnje, ki vzdržujejo izjemno pester mozaik habitatnih tipov in tako pomembno prispevajo k ohranjanju biotske in krajinske pestrosti območja.

Summary

By comparing the butterfly fauna diversity between the two sampling years, we found no differences in species richness, average population density of species at each transect, and average population density of species in the Poček military area. The fauna varied significantly in composition over the years; the difference is due to species turnover and changes in the size and spatial distribution of the population densities of species at a given transect. The cumulative difference in the faunas is mostly due to the changes in common species that were present at the transects in both sampling years. In both years together, 73 species (42 % of the species of butterflies occurring in Slovenia) were observed in the central part of the military area, including 11 endangered species, some of which (eg. *L. achine*, *E. aurinia*) reach population densities representing a significant proportion of the total population of each species in Slovenia. Among the species whose population density decreased in 2019 compared to 2007, those with the highest densities in both forest vegetation types in 2007 were predominant. Among the species that reached higher population densities in 2019 than in 2007, those that were most abundant in both types of meadows in 2019 prevailed. This means that the grasslands in the central part of the Poček military area are managed properly and are of high conservation importance. Regular, but temporally and spatially uneven military activities represent heterogeneous disturbances that maintain

an extremely diverse mosaic of habitat types and thus significantly contribute to the conservation of the biodiversity and landscape diversity of the area.

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Literatura

- Asiss, D.S., Santos, I.A.D., Ramos, F.N., Barrios-Rojas, K.E., Majer, J.D., Vilela, E.F., 2018. Agricultural matrices affect ground ant assemblage composition inside forest fragments. *PLoS ONE*, 13(5), e0197697.
- Braü, M., Dolek, M., 2013. *Phengaris alcon* (Dennis & Schiffermüller 1775). V: Braü M., Bolz R., Kolbeck H., Nunner A., Voith J., Wolf W. (ur.): Tagfalter in Bayern. Verlag Eugen Ulmer, Stuttgart, pp. 266–272.
- Conell, J.H. 1978. Diversity in tropical forest and coral reef. *Science*, 24, 1302–1310.
- Čelik, T., Verovnik, R., Gomboc, S., Lasan, M., 2005. Natura 2000 v Sloveniji: Metulji (Lepidoptera). Založba ZRC, ZRC SAZU, Ljubljana, 288 pp.
- Direktiva Sveta 92/43/EGS. Direktiva o ohranjanju naravnih habitatov ter prostoživečih živalskih in rastlinskih vrst. <https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:1992L0043:20070101:SL:PDF>
- Čarni, A., Marinček, L., Seliškar, A., Zupančič, M., Puncer, I., Marinček, L., Wraber, M., Prešeren, M., Žagar, V., Accetto, M., Tregubov, V., Dakskobler, I., Mršič, N., Jarnjak, M., 2002. Vegetacijska karta gozdnih združb Slovenije (s komentarjem), merilo 1:400.000. Biološki inštitut Jovana Hadžija ZRC SAZU, Ljubljana.
- Čelik, T., 2019. Monitoring dnevnih metuljev na osrednjem vadišču slovenske vojske – poligona Poček in Bač. Končno poročilo. Biološki inštitut Jovana Hadžija ZRC SAZU, Ljubljana, 23 pp.
- Hillebrand, H., Blasius, B., Borer E.T., Chase, J.M., Downing, J.A., Eriksson, B.K., Filstrup, C.T., Harpole, W.S., Hodapp, D., Larsen, S., Lewandowska, A.M., Seabloom, E.W., Van de Waal, D.B., Ryabov, A.B., 2018. Biodiversity change is uncoupled from species richness trends: Consequences for conservation and monitoring. *Journal of Applied Ecology*, 55, 169–184.
- Kitahara, M., Yumoto, M., Kobayashi, T., 2008. Relationship of butterfly diversity with nectar plant species richness in and around the Aokigahara primary woodland of Mount Fuji, central Japan. *Biodiversity and Conservation*, 17, 2713–2734.
- Kudrna, O., 1986. Butterflies of Europe. Vol. 8, Aspects of the conservation of butterflies in Europe. Aula Verlag, Wiesbaden, 323 pp.
- Loos, J., Dorresteyn, I., Hanspach, J., Fust, P., Rakosy, L., Fischer, J., 2014. Low-intensity agricultural landscapes in Transylvania support high butterfly diversity: Implications for conservation. *PLoS ONE*, 9(7), e103256.
- Maes, D., Verovnik, R., Wiemers, M., Brosens, D., Beškov, S., Bonelli, S., Buszko, J., Cantú-Salazar, L., Cassar, L.F., Collins, S., in sod. (69 avtorjev), 2019. Integrating national Red Lists for prioritising conservation actions for European butterflies. *Journal of Insect Conservation*, 23, 301–330.

- Perović, D., Gámez-Virués, S., Börschig, C., Klein, A.M., Krauss, J., Steckel, J., Rothenwöhrer, C., Erasmi, S., Tscharncke, T., Westphal, C., 2015. Configurational landscape heterogeneity shapes functional community composition of grassland butterflies. *Journal of Applied Ecology*, 52, 505–513.
- Polak, S., 1997. Okoljevarstvene ocene za vadbišče Bile – živalstvo. Elaborat. Notranjski muzej Postojna, 21 pp.
- Rebeušek, F., 2006. Mravljiščarji Slovenije – razširjenost, ekologija, varstvo. Center za kartografijo favne in flore Slovenije, Miklavž na Dravskem polju, 14 pp.
- Settele, J., Shreeve, T., Konvička, M., Van Dyck, H., 2009. *Ecology of Butterflies in Europe*. Cambridge University Press, 513 pp.
- Slancarova, J., Benes, J., Kristynek, M., Kepka, P., Konvicka, M., 2014. Does the surrounding landscape heterogeneity affect the butterflies of insular grassland reserves? A contrast between composition and configuration. *Journal of Insect Conservation*, 18, 1–12.
- Smith, M.A., Turner, M.G., Rusch, D.H., 2002. The effect of military training activity on eastern lupine and the Karner blue butterfly at Fort McCoy, Wisconsin, USA. *Environmental Management*, 29, 102–115.
- Thomas, J.A., 2005. Monitoring change in the abundance and distribution of insects using butterflies and other indicator groups. *Philosophical Transactions of the Royal Society B*, 360, 339–357.
- Uradni list RS, 82, 2002. Pravilnik o uvrstitvi ogroženih rastlinskih in živalskih vrst v rdeči seznam. Uradni list Republike Slovenije (24. 9. 2002). Uredbe, 82, 8893–8975.
- Uradni list RS, 46, 2004. Uredba o zavarovanih prosto živečih živalskih vrstah. Uradni list Republike Slovenije (30.4.2004), 5933–6016.
- Van der Merwe, L.J., Pryke, J.S., Samways, M.J., 2019. Well-managed grassland heterogeneity promotes butterfly conservation in a corridor network. *Journal of Environmental Management*, 238, 382–395.
- Van Nieuwerkerken, E.J., Kaila, L., Kitching, I.J., Kristensen, N.P., Lees, D.C.,...in sod., 2011. Order Lepidoptera Linnaeus, 1758. V: Zhang Z.-Q. (ed.): *Animal biodiversity. An outline of higher-level classification and survey of taxonomic richness*. *Zootaxa*, 3148: 212–221.
- Van Sway, C.A.M., Warren, M.S., Loïs, G., 2006. Biotope use and trends of European butterflies. *Journal of Insect Conservation*, 10 (2), 189–209.
- Van Sway, C.A.M., Van Strien, A.J., 2008. The European butterfly indicator for grassland species 1990–2007. Report VS2008.022. De Vlinderstichting, Wageningen.
- Van Swaay, C.A.M., Cuttelod, A., Collins, S., Maes, D., López Munguira, M., Šašić, M., Settele, J., Verovnik, R., Verstrael, T., Warren, M., Wiemers, M., Wynhof, I., 2010. *European Red list of butterflies*. Publications Office of the European Union, Luxembourg, 47 pp.
- Van Sway, C.A.M., 2010. The European grassland butterfly indicator. Newsletter No 1 (September 2010), Butterfly Conservation Europe.
- Verovnik, R., 2008. Dnevni metulji. V: Tome D. (ur.): *Naravovarstveno ovrednotenje izbranih vojaških območij v Sloveniji: primerjalna študija z referenčnimi območji*, CRP Znanje za varnost in mir 2006–2010. Nacionalni inštitut za biologijo, Ljubljana, pp. 150–178.
- Verovnik, R., 2019. Prenovljeni seznam dnevnih metuljev (Lepidoptera: Papilionidea) Slovenije. *Acta Entomologica Slovenica*, 27, 5–15.
- Verovnik, R., Rebeušek, F., Jež, M., 2012. Atlas dnevnih metuljev (Lepidoptera: Rhopalocera) Slovenije. Center za kartografijo favne in flore, Miklavž na Dravskem polju, 456 pp.
- Warren, S.D., Büttner, R., 2008. Active military training areas as refugia for disturbance-dependent endangered insects. *Journal of Insect Conservation*, 12, 671–676.
- Warren, S.D., Holbrook, S.W., Dale, D.A., Whelan, N.L., Elyn, M., Grimm, W., Jentsch, A., 2007. Biodiversity and the heterogeneous disturbance regime on military training lands. *Restoration Ecology*, 15, 606–612.
- Wiemers, M.W., Balleto, E., Dinca, V., Fric, Z.F., Lamas, G., Lukhtanov, V., Munguira, M.L., van Sway, C.A.M., Vila, R., Vliegenthart, A., Wahlberg, N., Verovnik, R., 2018. An updated checklist of the European Butterflies (Lepidoptera, Papilionoidea). *ZooKeys*, 811, 9–45.

Characteristics of spatial use and importance of landscape features for recovering populations of Eurasian lynx (*Lynx lynx*)

Značilnosti rabe prostora in pomen krajinskih značilnosti za ponovno naseljene populacije Evrazijskega risa (*Lynx lynx*)

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Abstract: Following the extirpation from Central and Southeastern Europe by the end of the 19th century, Eurasian lynx (*Lynx lynx*) was reintroduced in the Alps and Dinarics. The recovering population sizes fluctuated over the years; however, they remained relatively small and isolated since their distribution after the establishment has not significantly expanded by natural colonization. One of the most radical changes to the landscape of Europe over the past centuries has been the creation of vast urban and agricultural areas and subsequent extension of infrastructure, causing increasingly fragmented landscape, especially for weak dispersers like lynx. There is a pressing need to establish greater connectivity between the genetically isolated populations, particularly throughout the Alps and Dinarics, to achieve a viable meta-population structure. In the paper, we review scientific knowledge on Eurasian lynx spatial requirements and behaviour, and critically evaluate the methods used. Apart from habitat suitability and connectivity studies, we also provide a review of lynx home range sizes and movement activity, including dispersion. We present habitat suitability model constructed to examine suitable habitat for recovering Dinaric - SE Alpine population and compare home range sizes of lynx from Dinarics in Slovenia with other lynx populations in Europe. Future considerations for lynx conservation should include the impact of climate change, protection of corridor areas, reducing impact of barriers and, if needed, “complement” dispersal via translocations to achieve viable pan-European lynx metapopulation in the future.

Keywords: connectivity, conservation management, dispersal, Eurasian lynx, fragmentation, habitat suitability, home range, *Lynx lynx*

Izvleček: Po izumrtju evrazijskega risa (*Lynx lynx*) v srednji in jugovzhodni Evropi ob koncu 19. stoletja, so ris v 70. in 80. letih prejšnjega stoletja ponovno naselili v Alpe in Dinaride, čemur je v zadnjih dveh desetletjih sledilo še nekaj nadaljnjih naselitev. Vzpostavile so se razmeroma majhne in izolirane populacije, saj se njihova prostorska razširjenost z naravno kolonizacijo ni bistveno povečala. V preteklih stoletjih se je pokrajina v Evropi najbolj temeljito spremenila zaradi širjenja obsežnih urbanih in kmetijskih površin ter posledično širjenja prometne infrastrukture. To je povzročilo vse večjo razdrobljenost življenjskega prostora, zlasti za šibke dispergerje, kot je ris. Zato se vse bolj povečuje potreba po večji povezljivosti genetsko izoliranih populacij,

zlasti v Alpah in Dinaridih, s čimer bi vzpostavili viabilno metapopulacijsko strukturo. V prispevku predstavljamo pregled obstoječega poznavanja prostorskih zahtev in vedenja evrazijskega risa ter izpostavljamo prednosti in slabosti uporabljenih metod proučevanja. Poleg študij primernosti in povezljivosti habitata za risa je v prispevku predstavljen tudi pregled značilnosti domačih okolišev in gibanja risov, vključno z disperzijo. V prispevku predstavljamo model primernosti prostora za risa, ki je bil izdelan za širše območje dinarske - jugovzhodno alpske populacije in primerjavo velikosti domačih okolišev risov iz Slovenskega dela Dinaridov z drugimi populacijami risov v Evropi. Prihodnji vidiki ohranjanja evrazijskega risa v Evropi bi morali vključevati vpliv podnebnih sprememb, varovanje območij koridorjev med habitatnimi krpami, zmanjševanje vpliva (zlasti) linijskih ovir (npr. avtoceste) in po potrebi »dopolnjevanje« disperzijo s premeščitvami oziroma translokacijami osebkov, s čimer bi lahko v prihodnosti vzpostavili viabilno panevropsko metapopulacijo.

Ključne besede: disperzija, domači okoliš, Evrazijski ris, fragmentacija, *Lynx lynx*, povezljivost prostora, primernost prostora, varstveno upravljanje

Introduction

One of the most radical changes to the landscape of Europe over the past centuries has been the creation of vast urban and agricultural areas and subsequent extension of infrastructure networks, and after more than 5000 years of intense human activities only 2% of original prime forest remains (Iuell et al. 2003).

The Eurasian lynx, once widespread throughout Europe, disappeared from Central and Southern Europe and many other parts of the continent during the 18th and 19th centuries, as a consequence of direct persecution, habitat loss through forest destruction, expansion of cultivated land, and the excessive reduction of wild ungulates (Breitenmoser 1998, Schadt et al. 2002, Zimmermann 2003, Potočnik et al. 2009). Since the end of the nineteenth century, forests have regenerated in many mountainous regions of Europe (Breitenmoser 1998, Zimmermann 2004), and the wild ungulate populations have recovered quickly (Apollonio et al. 2010). The improvement of the ecological conditions as well as protective legislation was favourable for the return of large carnivores as lynx populations reintroduced in the Central Europe in the 1970s and 1980s still persist in the Swiss Jura Mountains, Northwest Swiss Alps, Dinarics, French Vosges and Chartreuse Alps mountains (Breitenmoser 1998, Chapron et al. 2014).

The population sizes have fluctuated over the years, but distribution has not significantly expanded by natural colonization. Following the first reintroductions, lynx were translocated to Northeastern Switzerland in 2001 (Ryser et al. 2004), and to the Kalkalpen (Austria) in 2011-2013. Lynx' current distribution in Central and Southeastern Europe seems to be mainly limited to sites that were used for reintroductions and translocations where they were successful.

One of these reintroduced populations is Dinaric - SE Alpine lynx population, which formally/administratively belong to the Alpine and Dinaric populations (Kaczensky et al. 2013), however they are both demographically and genetically the same population since the SE Alps have been colonized by lynx from Slovenia. As such, the lynx in Dinaric - SE Alpine population represent a genetically continuous unit, which is however presently divided in two distinct subpopulations: the larger Dinaric and the smaller SE Alpine subpopulation. This population plays an important role as the "stepping stone" that connects lynx populations in the southeastern Europe (Albania/Kosovo/North Macedonia) with the reintroduced populations in the northwestern and eastern Alps in Switzerland, France and Austria (Kalkalpen).

The reintroduced/translocated populations in Europe are relatively isolated, and only limited movement occurs between some of them (Zimmermann and Breitenmoser 2007). In the human dominated and highly fragmented landscape

of Europe, dispersal is constrained by natural (e.g. rivers, deep valleys) and anthropogenic barriers such as vast urban and agricultural areas and subsequent extension of traffic infrastructure networks (Potočník et al. 2019a). Furthermore, dispersing sub-adult lynx show a strong tendency to establish home ranges in territories adjacent to conspecifics (Zimmermann et al. 2005). Thus, the combination of environmental and ecological factors make it unlikely that lynx will spontaneously colonize new areas in the Alps. It is therefore a conservation priority to link the existing lynx populations in the Alps to the Jura and Dinaric Mountains (Molinari-Jobin et al. 2003), potentially to the Vosges, Bohemian-Black Forest and Balkan populations, and in long-term possibly even to Carpathian populations (European Commission 2013). Natural dispersal alone likely would be insufficient to establish this interconnectivity, making translocations and reintroductions necessary (e.g. Zimmermann and Breitenmoser 2007, Molinari-Jobin et al. 2010).

Here we review scientific knowledge on Eurasian lynx spatial requirements and behavior as well as to examine the advantages and weaknesses of the methods used. It is crucial to assess and mitigate the negative effect of habitat fragmentation on lynx populations and facilitate genetic exchange among isolated (sub)-populations or demes in Central and Southeastern Europe. Knowledge on amount and distribution of suitable habitat available to particular lynx population and to the obstacles it is exposed to is important for improvement of our understanding of lynx population connectivity within each population and across habitat patches at the metapopulation level. Apart from habitat suitability and connectivity studies, we provide also information on lynx home range size and movement activity, including dispersion, as a critical part of its ability to occupy sufficient interconnected areas to compensate for demographic variations and subsequently support genetic exchange between (sub)-populations, ensuring viability of the metapopulation. We also present lynx habitat suitability model we constructed for Dinaric Mountains - SE Alps and adjacent regions as well as preliminary data on home range sizes of GPS-collared lynx in Dinaric Mountains in Slovenia between 2003 and 2020.

Habitat suitability

Top predators are generally not very sensitive to a particular habitat structure, vegetation or ecosystem type (Mladenoff et al. 1995). But among the European large carnivores, Eurasian lynx is certainly the one with the most specific demands regarding habitat and prey (Breitenmoser 1998). However, lynx can adapt to semi-natural landscape and their permanent disturbances (Breitenmoser-Würsten et al. 2001). The Eurasian lynx is present in large continuous lowland forest areas with more than 50% of forest cover. It is linked to forest areas with high amount of forest fringe (Breitenmoser et al. 2000). The Eurasian lynx can also tolerate interruptions by open land habitat patches and land use types such as pastures or agriculture. Telemetry studies in 1990s in the Swiss Alps (Breitenmoser-Würsten et al. 2001) showed that re-introduced lynx originating from highly forested Carpathian Mountains, already adapted to open areas, when compared to the first telemetry studies in the early 1970s (Haller and Breitenmoser 1986). Intensive land use is tolerated as long as there is enough connected forest area for retreat (Breitenmoser 1998, Schadt et al. 2002).

Presence and availability of food/prey sources is important parameter determining habitat suitability for animal species. Lynx diet varies greatly depending on prey availability and accessibility. Although other species within *Lynx* genus developed specializations for hunting lagomorphs, Eurasian lynx staple prey in Central Europe are roe deer (*Capreolus capreolus*) and Alpine chamois (*Rupicapra rupicapra*) as well as other ungulate species like red deer (*Cervus elaphus*) and European mouflon (*Ovis aries musimon*). In other parts of its distribution, it can also prey on semi-domestic reindeer (*Rangifer tarandus*) and white-tailed deer (*Odocoileus virginianus*). In areas with low roe deer density Eurasian lynx diet can seasonally shift to other types of prey like rodents and birds (Krofel et al. 2012). In a research of lynx diet in Dinaric forests with low density of ungulates, rodents represented considerable part of the diet (7.7%) at peaks of their population dynamics. This proves that Eurasian lynx is able to adapt to various food sources. Given present high population densities of ungulate species across Europe (e.g. Apollonio et al. 2010) it is

assumed that prey availability is not a limiting factor for its habitat suitability in Central and Southern Europe.

Habitat suitability modeling has become a common conservation management tool to analyze and predict suitable habitat for a range of species including mammals, other vertebrates and invertebrates as well as plants and other organisms (Guisan et al. 2013, Morrison et al. 2012). Various modeling methods exist (e.g. Maxent, ENFA, GARP etc.), but in general, they all involve evaluating the potential habitat of one or more species based on known occurrences (presence-only), sometimes absence data (presence-absence), and environmental conditions at those locations, to identify additional areas of suitable habitat with similar environmental conditions (Elith and Leathwick 2009).

One of the first habitat suitability models for lynx has been developed by Zimmermann and Breitenmoser (2002) using probability model for distribution in Swiss Jura Mountains. Later they adapted it and extended it over entire Jura Mountains (Zimmermann and Breitenmoser 2007). Schadt et al. (2002) used a new approach in habitat suitability modelling. They prepared habitat model using presence-absence data and logistic regression to formalize the relationship between environmental conditions and species habitat requirements in quantifying the amount of potential habitat. They generated a home range suitability model based on local radio-tracking data obtained from lynx in the French and Swiss Jura Mountains (a landscape similar to the Central Europe low mountain ranges in fragmentation and population density) and extrapolated the model to Germany. Zimmermann (2004) used Ecological Niche Factor Analysis (ENFA) modelling to predict the potential distribution of lynx in the Alps. The first factor, called marginality, accounted for 29 % of the total specialization and showed that lynx preferred forest, shrubs and herbaceous vegetation, but avoided areas of heterogeneous agriculture. The other three factors accounted for more specialization, mostly regarding distance to towns, heterogeneous agriculture, forest and open space frequencies.

Signer (2010) used an adapted habitat suitability model by Zimmermann and Breitenmoser (2007) for the Jura Mountains and extrapolated it

over the Alps, which have a different relief and other environmental characteristics. Hence, the validity of the model for the Alps is questionable (Graf et al. 2006).

Skrbinšek (2004) made a habitat suitability model for Slovenian Alps and Dinaric mountains which we extrapolated (this paper) over entire range of Slovenia, Croatia, Bosnia and Hercegovina, NE Italy and border area with Austria (Fig. 1). The area encompasses the entire areal of re-introduced Dinaric population that is now recognized as Dinaric - SE Alpine population. We generated a home range suitability model based on methodology used by Schadt et al. (2002). They used telemetry data obtained from lynx in the French and Swiss Jura Mountains and evaluated with independent radio-tracking data from the low mountain range along the German-Czech border (Bufka et al. 2000) and from the Dinaric mountain range of southern Slovenia (Staniša and Huber 1997). We adapted the model to the updated environmental data and validated it with GPS and VHF telemetry data from Dinarics, as well as C1 and C2 data from lynx monitoring in Slovenia using Boyce index method (Boyce et al. 2002, Hirzel 2001, Hirzel et al. 2001, Hirzel et al. 2006) modified by (Skrbinšek, unpublished data). Our results indicate 16.300 km² of study area as high-quality habitat and additional 20.900 km² as still suitable habitat. Dinaric Mountains and SE Alps represent 11.400 km² and 9.500 km² of suitable habitat, respectively.

Becker (2013) constructed lynx habitat suitability model using Maximum Entropy Species Distribution Modeling software (Maxent), a popular modeling software using a machine-learning algorithm to determine suitable habitat based on species occurrence and environmental data. This was first habitat suitability study that combined lynx presence data from the Jura Mountains and six locations in the Alps to assess suitable habitat for lynx throughout the entire Alpine region (Fig. 2). She used data of 102 different lynx from seven different study areas. She found that approximately 103.600 km² of suitable lynx habitat exists in the Alps, covering approximately 54% of the total Alpine Convention area and identified 22 patches of suitable habitat ranging from 400 to over 17.000 km², representing potential individual lynx subpopulations.

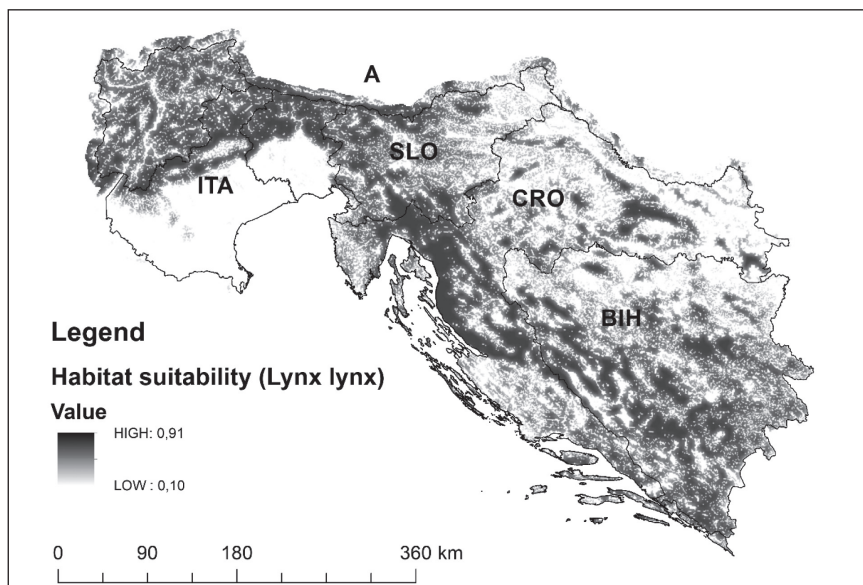


Figure 1: Habitat (home range) suitability map based on logistic regression model (after Schadt et al. 2002) and validated with GPS and VHF telemetry data from Dinarics in Slovenia, along with C1 and C2 presence data from lynx monitoring in Slovenia (SCALP methodology).

Slika 1: Karta primernosti habitata izračunana s pomočjo modela logistične regresije (po Schadt in sod. 2002), validiranega s podatki GPS in VHF telemetrično spremljanih risov iz Dinaridov v Sloveniji ter C1 in C2 podatki monitoringa risa v Sloveniji (po metodologiji SCALP).

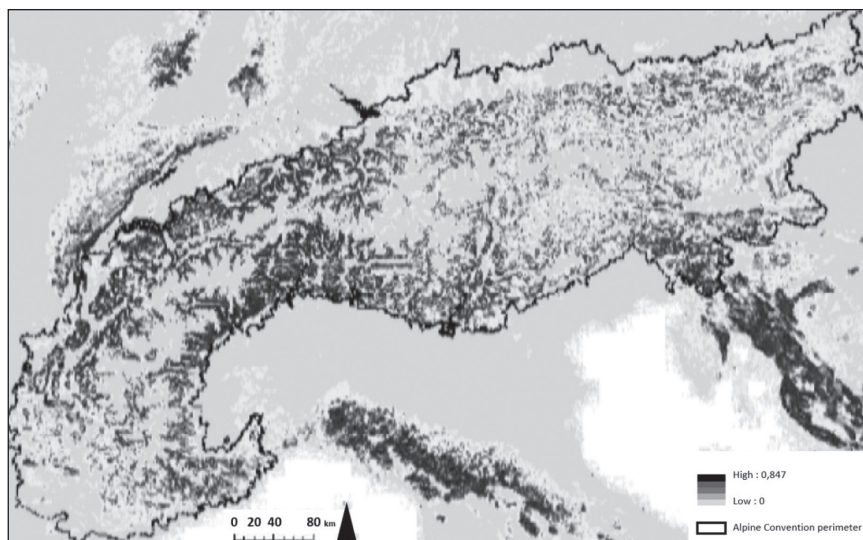


Figure 2: Lynx habitat suitability map for the Alps and adjacent regions based on Maxent model by Becker (2013). Dark colour, highly suitable habitat; light colour, unsuitable habitat.

Slika 2: Model primernosti habitata za risa na območju Alp in okoliških regij izdelan po metodi Maxent (Becker 2013). Temna barva, zelo primeren habitat; svetla barva, neprimeren habitat.

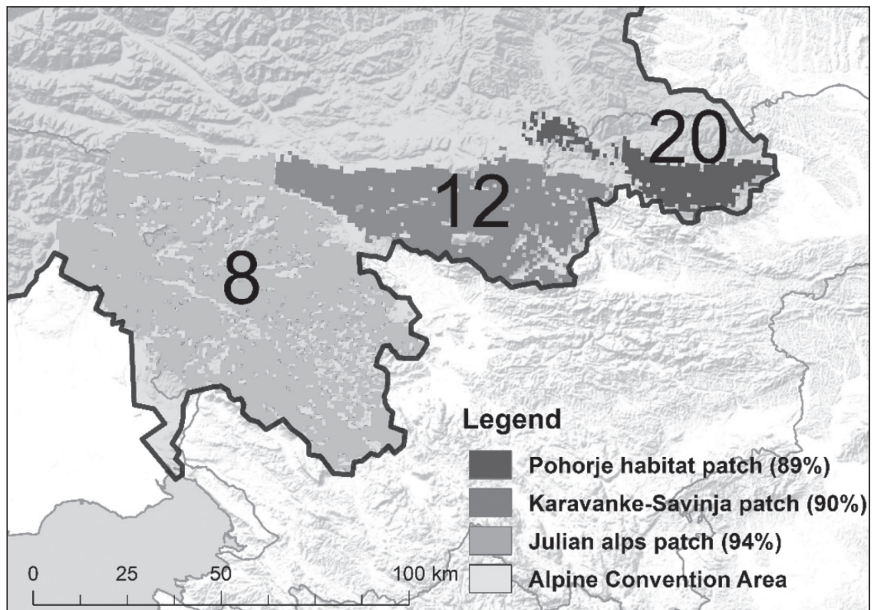


Figure 3: Relative size of three habitat patches of suitable lynx habitat from Slovenian Alpine area according to our model compared to the sizes from MaxEnt model (Becker 2013) for the same habitat patches numbered 8, 12 and 20. The patches cover regions of Julian Alps (Nr. 8), Karavanke - Savinja Alps (Nr. 12) and Pohorje (Nr. 20). The sizes of suitable habitat in our model corresponds to 94%, 90% and 89% of the Becker's (2013) habitat patch sizes.

Slika 3: Relativna velikost območij, kjer je primeren habitat za risa v slovenskem alpskem prostoru po našem modelu v primerjavi z modelom MaxEnt (Becker 2013) za iste habitatne krpe označene s števkami 8, 12 in 20. Habitatne krpe pokrivajo območja Julijskih Alp (št. 8), Karavank - Savinjskih Alp (št. 12) in Pohorja (št. 20). Velikosti primernehabitata v našem modelu ustrezajo 94 %, 90 % in 89 % velikosti habitatnih krp habitatnega modela, ki ga je izdelala Becker (2013).

The model shows very similar pattern of lynx suitable habitat areas with hotspots in Dinaric Mountains and in the Alpine area (Becker 2013) to our habitat suitability model. We also compared the sizes of three suitable lynx habitat patches stretching into the Slovenian Alps with sizes of corresponding areas in our model (Fig. 3). The patches cover regions of Julian Alps (Nr. 8), Karavanke - Savinja Alps (Nr. 12) and Pohorje (Nr. 20). The sizes of suitable habitat in our model corresponds to 94%, 90 and 89% of the Becker's habitat patch sizes, which indicates similar results of both models, however, slightly smaller size of suitable habitat seems our model a bit more conservative.

Habitat connectivity

Habitat loss and excessive fragmentation is a well-documented threat to wildlife (e.g. Andrén 1994, Hagan et al. 1996). As habitat is reduced, wildlife populations decrease in size and become more isolated. The extinction risks may be reduced by rescue effect due to dispersal between local populations (Hanski et al. 1996). Connectivity between suitable habitat patches depends on the number of dispersers available in the population, the distance between the source and the target populations, and the dispersal ability of the species under consideration (Wiens 1997).

The Central and South-Eastern European lynx populations are relatively isolated, and only limited movement occurs between some populations (Zimmermann and Breitenmoser 2007, Potočnik et

al. 2009). In the fragmented mountainous regions of the Alps and Dinarics dispersal is constrained by barriers including high mountain peaks, deep valleys, canyons and glaciers, fenced highways, large rivers as well as settlements, agricultural, industrial and other urban areas. The ongoing refugee crisis in Europe has seen many countries rush to construct border security fencing to divert or control the flow of people (Linnel et al. 2016). The process of border fencing can represent an important additional threat to wildlife because it can cause additional fragmentation of habitat, reducing its connectivity and lower effective population size.

Several studies have modeled lynx habitat connectivity in central Europe, using different methodologies (e.g. Zimmermann 2003, Schadt et al. 2004, Zimmermann 2004, Becker 2013, Magg et al. 2016). Zimmermann (2003, 2004) evaluated the habitat connectivity for the lynx in the Alps in order to reveal the suitable habitat patches (subpopulations), their potential size and connections between them. Connectivity was calculated in the GIS using a friction grid and a cost distance function. The results of the cost distance analysis showed that all 37 patches were within the range of dispersal cost of subadult lynx moving through unfavourable habitat. However, experience suggests that only few dispersers will cross unsuitable areas and anthropogenic linear barriers such as fenced highways.

Becker (2013) also evaluated habitat connectivity for lynx in the Alps. Firstly, she created a map of suitable habitat in Maxent and used ArcGIS RegionGroup to assign each cell to a connected region (see Becker (2013) for more details). Secondly, based on assumed barriers including major highways, rivers, and areas of high elevation she divided these regions into 32 habitat patches. Lastly, patches smaller than 400 km² were discarded, resulting in 22 patches considered large enough to support lynx subpopulations.

Similar to Zimmermann (2004), Magg et al. (2016) calculated least-cost paths based on a resistance (friction) grid to assess the connectivity between suitable habitat patches in Bohemian - Bavarian lynx population. They assigned resistance grid values within the range of the inverse habitat suitability. Generally, the biggest anthropogenic barriers between different lynx habitat patches

were highways followed by other large urban settlements, main roads, and municipal roads. They pointed out that not habitat suitability, but habitat connectivity is limiting distribution of the Bohemian-Bavarian lynx population.

Kramer-Schadt et al. (2004) introduced an individual-based spatially explicit dispersal model to assess the probability of a dispersing lynx reaching another suitable patch in the complex heterogeneous German landscape. Results of the modeling revealed that most of the suitable patches could be interconnected by movements of dispersing lynx within 10 years of reintroduction or recolonization. However, when realistic levels of mortality risks on roads were applied, most patches became isolated except ones along the German-Czech border. Consequently, patch connectivity was limited not so much by the distribution of dispersal habitat but by the high mortality of dispersing lynx. Accordingly, they suggest that rather than solely investing in habitat restoration, management efforts should try to reduce road mortality, too.

Movement

Movement is one of the most studied yet least understood concepts in ecology and evolutionary biology. It has been considered as a glue cementing subpopulations and allowing connections between usually isolated populations (Waser et al. 2001, Wiens 2001). Movements have consequences for individuals as well as for populations and communities, and their effects on inclusive fitness are ultimately the selecting forces for dispersal, migration, exploration, and other types of movement that affect the distribution, abundance, and dispersion of individuals (Clobert and Wolff 2001).

Understanding animal movement is fundamental to interpret spatial-temporal patterns of habitat selection, foraging behavior, and the interactions between predator and prey (Bell 1990). Animal movements are influenced by intrinsic physiological factors (e.g., hunger and reproduction) and the sensory capabilities of organisms. Spatial structure also influences movement as long as there is a perceived difference in quality of the varying cover types as individuals search for resources such as food, mates, or den sites or use different

cover types to avoid intraspecific and interspecific agonistic encounters (Zollner and Lima 1997).

Eurasian lynx movements are related to the needs of foraging, mating and rearing of young. Despite its relatively small size, this species uses large home ranges, therefore their moving paths are longer, too (Schmidt et al. 1997). The movement paths of an individual arise from sequential decisions regarding their needs and perceptions of the surrounding habitat, and it is these decisions that ultimately give rise to the functional connectivity of the landscape (Tracey et al. 2013). Eurasian lynx is highly territorial species and if individuals are to maintain their rights to a territory, they need to move fast and widely enough to advertise their presence over as much area and in as short intervals as possible.

Jędrzejewski et al. (2002) made a research on Eurasian lynx movement patterns in Białowieża Primeval Forest (Poland). A total of 18 lynx (6 adult males, 5 adult females, 2 subadult males 1 subadult female and 4 kittens) were captured and radio collared.

Adult males moved the longest distances, whereas non-reproductive females moved the shortest distances (Tab. 1). Females with kittens moved significantly longer distances than single females. The longest uninterrupted movement of lynx (male) covered 31 km during which it covered up approximately 18% of its home range only (Jędrzejewski et al. 2002). The ratio between straight-line distances (SLD) and daily

median distances (DMD) was significantly higher in males than in females with kittens, which indicates that males moved in a different manner than females. Males moved more directly as they covered longer routes than females, which moved more intensively, but their movements were more concentrated and they stayed relatively close to the place visited on a previous day (Jędrzejewski et al. 2002). Compared to solitary male lynx, females must encounter more prey to meet their energy demands and those of their kittens, which accompanied them on 100% of movement paths (Fuller et al. 2010). Okarma et al. (1997) reported that males were on average killing 1,5 times fewer deer per time unit than females with kittens.

There is much evidence that predators adjust their circadian rhythm of activity to that of their main prey (Curio 1976, Ferguson, et al. 1988). The synchronization of predator and prey activity would occur more closely in species that rely on small or medium-sized prey which rest hidden in burrows. This prey is detectable mainly when moving outside the burrows. Lynx main prey are ungulates, which do not use burrows to rest. In Białowieża Primeval Forest, snow-tracking revealed lynx's successful attacks on both feeding or lying roe deer (Jędrzejewski et al. 1993). Thus, it is not the pattern of prey activity but the conditions that increase its vulnerability (Schmidt 1999).

GPS tracking in Dinaric mountains revealed movements of the lynx were primarily affected by daytime period, time since the last kill/den

Table 1: Daily movement distances (DMD) and straight-line distances (SLD) for radio-tracked *Lynx lynx* in Białowieża Primeval Forest. N, number of individuals; SD, standard deviation. (Jędrzejewski et al. 2002).

Tabela 1: Skupna dnevna prepotovana razdalja (DMD) in dnevni premik (SLD) evrazijskih risov v gozdu Białowieża. N, število osebkov; SD, standardna deviacija. (Jędrzejewski in sod. 2002).

| Sex/age group of lynx | DMD (km) | | SLD (km) | |
|------------------------------|-----------|------------|-----------|-----------|
| | Mean ± SD | (min-max) | Mean ± SD | (min-max) |
| Adult males (N = 5) | 9.0 ± 7.0 | 0 – 24.8 | 3.3 ± 3.2 | 0.7 – 2.5 |
| Females with kittens (N = 4) | 6.8 ± 4.3 | 0 – 18.6 | 1.5 ± 1.8 | 0.4 – 1.7 |
| Females (N = 3) | 3.7 ± 4.1 | 0 – 11.8 | 1.4 ± 1.6 | 0.8 – 1.9 |
| Subadult males (N = 2) | 7.4 ± 4.6 | 0 – 18.4 | 2.0 ± 2.1 | 0.5 – 2.0 |
| Kittens (N = 3) | 5.8 ± 2.9 | 0.9 – 10.3 | 1.9 ± 1.8 | 0.7 – 1.4 |
| All lynx (N = 17) | 7.2 ± 5.6 | 0 – 24.8 | 2.3 ± 2.7 | 0.4 – 2.5 |

translocation, lynx demographic category, and their interactions (Krofel et al. 2013). The lynx tended to stay closer to the prey immediately after the kill, but were found increasingly further away, especially during the day, as the time progressed. This effect was especially pronounced in the females with immobile kittens, but was practically nonexistent in the subadult male. There was a notable difference in movement pattern of female lynx during consumption process in period of denning (Krofel et al. 2013). While their kittens were immobile, the females were frequently found further away from the kill compared to when they were alone or had mobile kittens, as they kept regularly returning to the den site. This was particularly the case during daytime, when the females spent a lot of time at the den site.

Jędrzejewski et al. (1993) recorded longest lynx distances (mean 14 km, SD = 3,4 km) during the days when they made no kill. The distances in all consecutive days after making a kill were significantly shorter. Straight-line distances between consecutively killed prey varied from 2.9

km in reproducing female lynx to 6.4 km in adult males. In male lynx these distances were longer in mating season, whereas in females they were longer during the period of kitten's high mobility.

The influence of snow on Eurasian lynx movements is not clear, although there is information that snow conditions could affect their movements and subsequent predation success and distribution, especially for adult females with kittens (Pullianen 1995, Pulliainen and Hyypä 1975, Haglund 1966), as it shown in Canada lynx (Murray and Boutin 1991, Stenseth et al. 2004).

Home range

A home range is the area in which an animal lives and moves on a periodic basis. It is among the most basic of ecological parameters that is regularly described for a given species. An understanding of the requirements for use of space is fundamental for species management and conservation (Schwartz 1999). Furthermore, home

Table 2: Minimum Convex Polygon (100%) home range estimates for adult Eurasian lynx in different sites in Europe. Some authors also provided 95% Kernel method home range estimates.

Tabela 2: Velikost domačih okolišev evrazijskega risa različnih območij v Evropi ocenjena z metodo minimalnega konveksnega poligona z uporabo vseh lokacij (100% MCP) in kernelsko metodo (95% Kernel).

| Study site | No. of animals | | 100% MCP home range size [km ²] | | 95% Kernel home range size [km ²] | | Reference |
|------------------------------------|----------------|--------|---|--------|---|--------|--|
| | Male | Female | Male | Female | Male | Female | |
| Sarek, Sweden | 8 | 21 | 709 | 407 | 431 | 251 | Linnell et al. 2001 |
| Northwestern Alps, Switzerland | 11 | 12 | 159 | 106 | / | / | Breitenmoser-Wursten et al. 2001 |
| Hedmark, Norway | 7 | 10 | 1456 | 832 | 886 | 535 | Herfindal et al. 2004 |
| Białowieża Primeval forest, Poland | 5 | 3 | 248 | 133 | 235 | 152 | Schmidt et al. 1997 |
| Swiss Jura, Switzerland | 3 | 5 | 264 | 168 | / | / | Breitenmoser et al. 1993 |
| French Jura, France | 3 | 5 | 258 | 150 | / | / | Stahl et al. 2002 |
| Nord-Trøndelag, Norway | 3 | 2 | 1515 | 561 | 1719 | 235 | Linnell et al. 2001, Sunde et al. 2000 |
| Akershus, Norway | 2 | 2 | 812 | 350 | / | / | Herfindal et al. 2004 |
| Bergslagen, Sweden | 4 | 1 | 632 | 307 | 305 | 97 | Linnell et al. 2001 |
| Vosges mountains, France | 3 | 1 | 235 | 516 | / | / | Schmidt et al. 1997 |
| Kočevje, Slovenia | 2 | 2 | 200 | 177 | / | / | Huber et al. 1995 |
| Slovenian Dinarics | 3 | 7 | 222 | 178 | 306 | 217 | This study |
| Average | | | 562 | 313 | | | |

range size is one of the most important parameters in producing population estimates. It is important to know how much space individuals need when estimating potential carrying capacities to plan conservation or recovery programs (Schmidt et al. 1997). Home range size is not easy to determine. One big problem is that home ranges vary greatly between interspecific and intraspecific samples. While some interspecific variations in home range can be explained in body mass and feeding styles (Guarino, 2002) in many cases patterns of space-use within species vary by factors of 10 to 1000 (Gompper and Gittleman 1991). Another problem is variety of concepts, methodologies and estimators used to determine home ranges within and between species. The simplest estimator of a home range from a set of location data is the minimum convex polygon (MCP) (Mohr 1947) that has been widely used in Eurasian lynx studies, although it has many drawbacks including often overestimating the size of home ranges (Burgman and Fox 2003). The other estimators, especially in more recent studies, that have been frequently employed for constructing utilization distribution home ranges in lynx are the so-called (fixed or adaptive) kernel density estimators (Worton 1989, Burgman and Fox 2003). We collected these estimators for lynx home range data from different studies across Europe and added lynx home range size estimates obtained in our previous studies and conservation projects in Dinarics in Slovenia (Tab. 2). In 2003 we equipped first lynx with GPS collar in the Dinaric region. Since then we collared 8 more lynx (3 males and 5 females) in Slovenian Dinarics.

Schmidt et al. (1997) obtained data on home range sizes of different demographic categories of Eurasian lynx in Białowieża Primeval forest, Poland. They studied the effects of different parameters like age, sex, season and sociality on home range variation (Tab. 3). They therefore divided monitored individuals into four groups: (1) adult males (body mass > 19 kg), (2) adult females (only females with kittens), (3) subadult males of known age < 2.5 years (Kvam 1991) or body mass < 15 kg, and (4) subadult females less than 2 years old (Kvam 1991) or weighing up to 15 kg and not accompanied by kittens.

The results showed that sex of lynx was most important variable affecting home range size, following by time of monitoring, and number of localizations. In adult males, a notable growth in the utilized area was found in December-January, i.e. the pre-mating season (increase by 94% compared to October-November range) and February-March, i.e. the mating season (increase by 36%). There were no data for subadults in December-March, when the increase of the adult's home ranges was largest. In reproducing adult females, the most conspicuous change occurred in May -June, when the area utilized decreased by 81% in comparison to November-December. Female home ranges were largest in January-February (39% increase) (Schmidt et al. 1997). Lynx is territorial species, so one of important parameters defining home range size is the social status of individuals. Schmid et al. (1997) studied overlap of home ranges and identifying parameters that influence it. The results showed that home range overlap among lynx depended mainly on

Table 3: Minimum Convex Polygon (100%) average yearly and seasonal home range estimates for different Eurasian lynx age groups in Białowieża Primeval forest, Poland.

Tabela 3: Povprečna velikost celoletnih in sezonskih domačih okolišev evrazijskega risa v pragozdu Białowieża na Poljskem. Velikost je ocenjena z metodo MCP (100 %).

| | Home range [km ²] | | |
|-----------------|-------------------------------|---------------|---------------|
| | Average | Autumn/Winter | Spring/Summer |
| Adult male | 248 | 165 | 143 |
| Adult female | 133 | 94 | 56 |
| Subadult male | 199 | 65 | 67 |
| Subadult female | 85 | 65 | 71 |

the age and sex of the individuals. The average overlap of home range among males was 30%. The most extensive overlap (75%) was between the home ranges of adult and subadult males. The average home range overlap between adult females was 29%. Except for females with kittens, the lynx usually avoided contact with conspecifics. Adult males were always found > 1 km away from each other. The average distance between simultaneously radio-tracked males was 11.6 km, however the distance between females was shorter (8.1 km). The nearest females were 0.5 km apart (Schmidt et al. 1997).

Many authors suggest that intraspecific variation in home range size is highly correlated with food/prey availability (Sandell 1989, Powel et al. 1997, Girgione et al. 2002). However, estimating the availability of food and prey can be both difficult and expensive in the field and it is desirable to employ indirect methods that could explain some of the existing variation in home range size (Herfindal et al. 2005). Throughout their range in Europe home range sizes of lynx vary by a factor of 10 (Linnell et al. 2001, Jedrzejewski et al. 2002). In initial attempts to compare home range size between populations, researchers generally used latitude as a simple measure of variation in environmental productivity (Buskirk and McDonald 1989, Gompper and Gittleman 1991). However, Herfindal et al. (2005) stressed that this is not the best method, as it does not take the effect of altitude and oceanic influence into account. They tried to explain variations in home range using FPAR index. It reflects the fraction of incident photosynthetically active radiation absorbed by the green leaves and has been used as a measure of net primary production (Sellers et al. 1997). Herfindal et al. (2005) study was trying to explain this variation in terms of available indices of prey density and environmental productivity. On the individual level they explored Eurasian lynx home range variations compared to roe deer density in south-eastern Norway, which is rarely demonstrated through quantification of prey density within home ranges for carnivore species. They used hunting statistics as an index of variation in prey density assuming that roe deer harvest reflected population density. Results showed significant decrease in home range size of lynx with increasing roe deer density. On a

European level, they related home range sizes of 111 lynx from 10 study sites to estimates derived from remote sensing of environmental productivity and seasonality. Results of multivariate regression models indicated a clear relationship between lynx home range size and study area productivity. Although there is no independent data that relates productivity directly to prey density one can reasonably assume that such relationship exists.

The analyses also revealed that for a given prey density/productivity, males had larger home ranges than females (Herfindal et al. 2005). It probably reflects both the larger body size of the male lynx and the predicted sex differences in reproductive strategies of the sexes (Sandell 1989). Sandell (1989) also predicted that female home range size should closely follow prey density, but that at some point male home range size should increase more rapidly due to a change in mating tactics and abandon territoriality. However, lynx males remained territorial at any point of density of prey or female lynx (Herfindal et al. 2005).

Dispersal

Dispersal is any movement of individual organisms in which they leave their home area, sometimes establishing a new home area. It is a crucial parameter in population dynamics, especially in threatened subpopulations within a meta-population (Levins 1970, Hanski 1999). Dispersal alone can help a population recover, if the reasons for the decline were demographic or genetic. Ultimate mechanisms that most likely affect dispersal are environmental variation and demographic structure. The proximate mechanisms include genetics, competition, individual fitness, and (breeding) habitat selection. These act via the fitness traits of survival and reproduction. If dispersal enhances these functions, it will be selected for independently of whatever proximate factors may serve to trigger it (Shields 1982). Another evolutionary issue is the fitness that often follows successful colonization of empty habitat or the discovery of new habitat beyond the species' current range. Possibility of inbreeding or outbreeding depression are also potential concerns (Shields 1982). A final evolutionary issue concerns the maintenance of an appropriate level

of genetic variability in a population (e.g. Cooper and Kaplan 1982). This is often considered a population level process involving the long-term probability of demic survival and reproduction. We can distinguish breeding dispersal i.e. the subsequent movement between sites or groups and natal dispersal i.e. the movement an individual makes from its birth site or previous breeding site to the site where it potentially reproduces (Zimmermann 2004). Ultimate factors are the selective forces that shape the evolution of the behavior. Main factors that drive individual into a dispersal are: genetic predisposition to disperse, local population density, habitat change, age of individual, reproductive status and perturbation (Zimmermann 2004). The decision to stop dispersal may involve various elements of habitat selection or patch choice, such as conspecific attraction, habitat quality or physiological factors (Wiens 2001).

The chances for successful dispersal depend on the connectivity of the landscape and is consequently decreased in intensively used landscapes i.e. matrix by barriers mostly imposed by humans, such as transportation infrastructure and the loss of suitable habitat (Schadt et al. 2004). Dispersal allows a species to recolonize former habitats after severe range depression. Natal dispersal rate and dispersal distances are generally male biased in mammals and female biased in birds (Greenwood 1980, Dobson 1982, Clarke et al. 1997). However, the significance of dispersal for the spread of a population is less obvious in felids. Natal dispersal patterns are generally male biased for large solitary felids (Smith 1993, Beier 1995, Machr et al. 2002), whereas the patterns are less clear among the four species of the *Lynx* genus, with no clear patterns within species and findings ranging from male biased dispersal (Mowat and Slough 1998, Mowat et al. 2000, Schmidt 1998, Janečka et al. 2007) to male and female lynx dispersing equally far and with equal frequency (O'Donoghue et al. 1997, O'Donoghue et al. 1998, Ferreras et al. 2004, Zimmermann et al. 2005, Campbell and Strobeck 2006).

A study comparing dispersing lynx from populations in the Nordics, Baltics, and Dinaric Mountains as well as Central Europe found that the mean dispersal distance was 39 kilometers, and 68% of dispersing lynx settled within

50 kilometers (Molinari-Jobin et al. 2010). Lynx tend to establish home ranges adjacent to those of other lynx (Zimmermann et al. 2005), which affects their likelihood of establishing new colonies. Thus, while a lynx population may expand in spatial size, solitary lynx are unlikely to disperse and establish entirely new, separate populations (Zimmermann et al. 2007).

A comprehensive research on the spatio-temporal behaviour of subadult lynx in two re-introduced populations was carried out in Switzerland between 1988 and 2001 (Zimmermann 2004, Zimmermann et al. 2005, Zimmermann et al. 2007). The study was based on telemetry and other data for 39 juvenile lynx; 22 in the north-west Swiss Alps and 17 lynx in Jura Mountains. The lynx became independent at the age range from 9.3 – 10.6 months (there was no significant difference between males and females). Subadult lynx separated from their mother 91 - 100 days after the beginning of the year (first decade in April). Mothers mostly left their kittens at the edge of their territory by undertaking excursions on the other side of their territories or even out of their home range. In most cases the mother seemed to have left the juveniles. The cause of separation may be the sense of the female that she is not able to kill enough prey for her kittens (Molinari and Molinari-Jobin 2001). Different aspects of the spatio-temporal behavior suggest that disintegration of litters of the free-ranging lynx is not caused by female parent aggression as pointed out by Stroganov (1962) and later by Jonsson (1984). After the separation, the subadults usually stayed a few days close to the place where the separation occurred and then moved on (Zimmermann 2004). The mean distance made by lynx that completed dispersion was 68.8 km (N = 7) in the one study area and 21.4 (N = 12) in the other. In both areas no difference between sexes was observed for centroid, total or maximum dispersal distances whichever dispersal category was considered (Zimmermann et al. 2007).

Dispersing lynx were recovered mean = 41.2 km (N = 14) (in Jura Mountains) and mean = 24.3 km (N = 13) (in North Western Swiss Alps) away from their point of origin. However, expressed in relative units as the mean circular resident female's and male's home range diameters, the median recovery distance in the North-West Swiss Alps was

2.0 (range 0.4-5.6) times the mean circular resident female's home range diameter and 1.4 (range 0.3-3.8) times the mean circular resident male's home range diameter. In the Jura Mountains, it was 2.0- (range 0.1-6.6) and 1.5- (range 0.1-5.1) times the respective means (Zimmermann et al. 2007). For some subadults (N = 5) the researchers were able to document a transient home range but most subadults established a definitive home range directly after their dispersal. Subadults from the north-west Swiss Alps and the Jura Mountains appeared to have the same dispersal potential as there were no observed differences between the two areas in the total and maximum distances dispersed. However, a larger proportion of individuals in the north-west Swiss Alps, all males, moved through unfavourable habitat but all stopped at fenced highways and turned back, except one male, which left the area. The apparent

reduced ability of subadults to cross barriers led to circular dispersal (Zimmermann et al. 2007). Within the study, they did not detect any positive density dependent effects in lynx dispersal and hence could not confirm the hypothesis that high population density encourages the expansion of the population.

Similar study of various aspects of lynx natal dispersal was carried out in Scandinavia by comparing dispersal patterns of 120 radio-marked lynx in two study areas in Sweden (Sarek and Bergslagen areas) and two study areas in Norway (Hedmark and Akershus areas, Samelius et al. 2012). They found, contrary to the Swiss study, that male lynx dispersed farther than female lynx with mean dispersal distances of 148 and 47 km for male and female lynx that were followed to the age of 18 months or older. In fact, female lynx often established home ranges that overlapped or

Table 4: Mean age at separation from mothers and mean dispersal distance for lynx in Jura Mountains, North-western Alps (CH), Sarek and Bergslagen (SWE), Hedmark and Akershus (NOR). Date of the birth in Jura Mountain and Northwestern Alps was estimated as the mean birth date of all known births, while 1st of June was assumed as date of the birth for other study areas. Researchers used the arithmetic center of the area for starting and finishing point to calculate the distance of dispersal.

Tabela 4: Povprečna starost mladičev ob ločitvi od samice in povprečna dolžina disperzijske poti risov v Jurskem pogorju, Severozahodnih Alpah (CH), Sareku in Bergslagnu (SWE), ter Hedmarku in Akershusu (NOR). Datum kotitve risov v Jurskem Pogorju in Severnozahodnih Alpah je podan kot povprečje vseh znanih datumov iz predhodnjih raziskav, za ostala območja pa je datum kotitve 1. junij. Raziskovalci so za lokacijo začetka in konca disperzije določili središče domačega območja.

| Lynx pop. | Gender | Age at separation from mother [days] | Dispersal distance [km] | Reference |
|-------------------------|--------|--------------------------------------|-------------------------|------------------------|
| Jura Mountain (CHE) | M + F | 330.9 (N = 14) | 41.2 (N = 14) | Zimmermann et al. 2005 |
| Northwestern Alps (CHE) | M + F | 413.6 (N = 13) | 24.3 (N = 14) | Zimmermann et al. 2005 |
| Sarek, Sweden | M | 303 (N = 14) | 130 ± 82 (N=11) | Samelius et al. 2012 |
| Sarek, Sweden | F | 276 (N = 10) | 47 ± 19 (N=21) | Samelius et al. 2012 |
| Hedmark, Norway | M | 288 (N = 7) | 136 ± 72 (N = 5) | Samelius et al. 2012 |
| Hedmark, Norway | F | 297 (N = 8) | 69 ± 107 (N = 5) | Samelius et al. 2012 |
| Akershus, Norway | M | 285 (N = 2) | 83 ± 34 (N = 4) | Samelius et al. 2012 |
| Akershus, Norway | F | 300 (N = 1) | 15 ± 23 (N = 4) | Samelius et al. 2012 |
| Bergslagen, Sweden | M | 255 (N = 4) | 205 ± 69 (N = 9) | Samelius et al. 2012 |
| Bergslagen, Sweden | F | 249 (N = 11) | 47 ± 19 (N = 19) | Samelius et al. 2012 |
| Mean | M | 282.75 (N = 27) | 138.5 (N = 29) | |
| Mean | F | 280.5 (N = 30) | 44.5 (N = 49) | |
| Mean | | 299.75 | 79.75 | |

partly overlapped that of their mothers. Similarly, the dispersal rate was greater among male lynx than among female lynx, with 100% of the males dispersing compared with 65% of the females dispersing.

The central and south-eastern European lynx populations are relatively isolated, and only limited movement occurs between some populations (Zimmermann and Breitenmoser 2007, Potočnik et al. 2009). In the fragmented mountainous regions of the Alps and Dinarics dispersal is constrained by barriers including high mountain peaks, deep valleys, canyons and glaciers, fenced highways, large rivers as well as settlements, agricultural, industrial and other urban areas. The ongoing refugee crisis in Europe has seen many countries rush to construct border security fencing to divert or control the flow of people (Linnel et al. 2016). The process of border fencing can represent an

important additional threat to wildlife because it can cause additional fragmentation of habitat, thus reduce its connectivity and lower effective population size.

Among Eurasian lynx re-introduction programs in Europe in the 1970s, three male and three female lynx were translocated from Slovakia and released in the northwestern Dinarics in Slovenia in 1973. Although little experience and no guidelines were available for carnivore recovery programs (Breitenmoser et al. 2001), data on signs of presence like sightings, reproduction events, scats, prey kills, attacks on domestic animals and mortalities were collected opportunistically but recorded systematically, both in Slovenia and Croatia (Čop and Frković 1988). Thus, the monitoring was mainly oriented in collection of spatial distribution of lynx signs of presence and on recording of dead (culled) lynxes. The founder

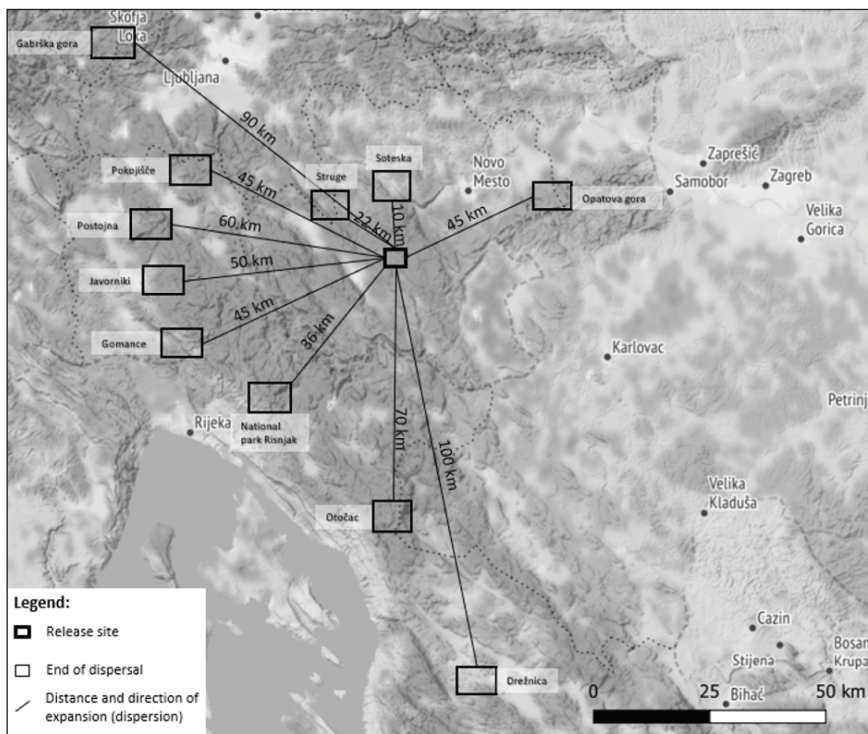


Figure 4: Dispersal of lynx released in Kočevski rog between 1973 and 1981 (modified after Čop and Frković 1988) (map source: © Stamen Design).

Slika 4: Disperzija risov od mesta izpusta v Kočevskem Rogu med leti 1973 in 1981 (prirejeno po Čop in Frković 1988) (vir kartografskih podatkov: © Stamen Design).

individuals established territories near the release site. Second year after the release, young lynx started to disperse into neighboring areas (Čop and Frković 1988). The monitoring data made it possible to follow the forefront of the expansion of the growing population in subsequent years. Eight years after the reintroduction young dispersing lynx or adult territorial lynx were recorded in all directions (but mainly along the Dinaric Mountains, at distances from 36 to 100 km from the release site (Čop and Frković 1988) (Fig. 4). Four decades after the reintroduction, it seems that the lynx dispersal was more successful towards southeast, along the Dinaric Mountains in Croatia and Bosnia and Hercegovina (BiH), than toward northwest, into the southeastern Alps in Slovenia, Austria and Italy. The maximum distance of recorded area of presence of lynx in BiH from the release site is around 390 km, while from the northwest (NE Italy) is around 140 km. The proximate cause for that is not clear, yet; however, it is obvious that fenced highway Ljubljana - Trieste represents an extremely strong barrier for dispersing lynx (Skrbinšek 2004). Given the apparent reduced ability of subadults, especially females, to cross the highway in almost a half of century after the reintroduction, it is unlikely that lynx will be able to spontaneously establish new reproductive area towards the SE Alps.

Conclusions

Conservation biology and conservation management rarely get a straightforward and clear answers for all the problems we may want to address. That is also the case for the habitat suitability modelling. Regardless of which methods and concepts are used creating habitat suitability models, they often reveal more questions to ask than to answer them. Different habitat suitability modelling approaches have proved to be useful according to the evaluation criteria established, but there may be no single “best” model or modelling concept.

We should highlight the need to carefully consider models’ parameters and data input, which could be even more important in the future. Especially considering the fact that the use of GPS telemetry data (that is often clustered) for study-

ing animals’ movements and use of resources is expected to increase (Cagnacci et al. 2010). For future models, it would be useful to include lynx presence data from different populations/areas, which might expand the range of environmental conditions that lynx occupy, and thus contribute to a more comprehensive model (Becker 2013).

Another option could be to assess suitable lynx habitat using other modelling methods, e.g. an ensemble approach combining multiple methods or a spatially explicit individual based movement model. As a subsequent step, we could combine the GIS models (habitat and land tenure system of the lynx) with population viability considerations into meta-population models, and hence bring such theoretical approaches closer to the real world (Zimmermann 2004).

Habitat restoration is of little concern for the conservation of the Eurasian lynx as forest and shrubs have continuously increased since the eradication of the species at the beginning of the 20th century and seem to further increase as a consequence of the economic changes. Prey base is not a limiting factor either since roe deer, red deer or chamois, lynx’s main prey, have recolonized almost all suitable habitat in the Alps and Dinarics and are more abundant than ever (Apolonio et al. 2010).

Due to contradicting results, available field studies (Zimmermann 2004, Zimmerman et al. 2007, Samelius et al. 2012) haven’t provided clear answers about possible male biased dispersal rate and distance in lynx as predicted in mammals (Greenwood 1980) although the study from Swiss Jura and the Alps did not reveal any sex bias. Dispersal directions and distances were shaped by the surrounding habitat, topography, as well as linear barriers (e.g. highways) (Zimmermann 2004). These outcomes have consequences for the calibration of individual based dispersal models: not only road mortality should be considered (see Kramer-Schadt et al. 2004), but habitat-type specific mortalities as well as the effect of linear barriers should furthermore be included. Such models have several clear advantages: among others, they force one to develop explicit hypothesis, to organize existing knowledge, and to estimate values for unknown parameters.

For reintroduced, small or recovering populations like Dinaric - SE Alpine population a metapopulation management in the form of translocating lynx from one habitat patch to another may be an alternative conservation tool. Such a management, reproducing dispersal, may be necessary if proved natural colonisation is hindered in increasingly fragmented landscapes of Europe.

Future considerations about suitable habitat for lynx should also include the impact of climate change. Mountainous environments are considered particularly at high risk, with significantly reduced snow depth and duration of snow-cover (Beniston et al. 2003) upward shifts of flora (Gehrig-Fasel et al. 2007) and risks of shifts of areals of new/alien predator/competitor species like golden jackals (*Canis aureus*) (Potočnik et al. 2019b) could impact lynx's predation and the amount of available habitat. Influence of human activities, like forest exploitation, game management and direct disturbance through recreation in forest ecosystems, lynx main habitat, should be taken into account while setting nature conservation strategies. Conservation efforts to protect corridor areas or reduce the impact of barriers remain important for the establishment of a viable Pan-European lynx metapopulation.

Povzetek

Evrazijski ris (*Lynx lynx*), nekoč razširjen po vsej Evropi, je v 18. in 19. stoletju izginil iz srednje in južne Evrope ter mnogih drugih delov celine kot posledica neposrednega preganjanja, izgube življenjskega prostora ob izsekavanju gozdov, širjenja obdelovalne zemlje in izrazitega zmanjšanja številčnosti prostoživečih parkljarjev (Breitenmoser 1998, Schadt in sod. 2002, Zimmermann 2003, Potočnik in sod. 2009). Izboljšanje ekoloških razmer v začetku 20. stoletja je bilo ugodno za vrnitev velikih zveri, zato so v 70. letih prejšnjega stoletja izpeljali nekaj ponovnih naselitev na območju Alp, eno pa so leta 1973 izvedli tudi v Dinaridih v Sloveniji. Do danes so se od naselitev ohranile risje populacije Jurskem pogorju v Švici, severozahodnih švicarskih Alpah, Dinaridih in v francoskih Alpah na območju Vogezov in Chartreuse (Breitenmoser 1998).

Velikosti teh populacij so se skozi leta spreminjale, vendar se po vzpostavitvi populacij njihova razširjenost z naravno kolonizacijo ni bistveno povečevala. Po teh naselitvah so rise leta 2001 preselili še v severovzhodno Švico (Ryser et al. 2004) in v Apneniške Alpe v Avstriji (2011-2013). Zdi se, da je sedanja razširjenost risov v srednji in jugovzhodni Evropi večinoma posledica vzpostavitve populacij omejenih na območja uspešnih ponovnih naselitev oziroma translokacij risov v preteklih desetletjih. Zato je prednostna naloga njihovega ohranjanja povezati obstoječe populacije risov v Alpah s populacijami v Juri in Dinaridih (Molinari-Jobin et al. 2003), potencialno pa tudi s populacijami v Vogezih, ob češko-nemški meji in na Balkanu ter dolgoročno, morda celo s Karpatsko populacijo (Evropska komisija 2013).

V prispevku predstavljamo pregled obstoječega poznavanja prostorskih zahtev in vedenja evrazijskega risa ter izpostavljamo prednosti in slabosti uporabljenih metod proučevanja. Poleg študij primernosti in povezljivosti habitata za risa je v prispevku tudi pregled značilnosti domačih okolišev in gibanja risov, vključno z disperzijo. Predstavljamo model primernosti prostora za risa, ki je bil izdelan za širše območje dinarske - jugovzhodne alpske populacije in primerjavo velikosti domačih okolišev risov iz Dinaridov v Sloveniji, ki so bili GPS-telemetrično spremljani med leti 2003 in 2020 z drugimi populacijami risov v Evropi.

Modeliranje primernosti prostora za posamezno vrsto je postalo običajno orodje za varstveno upravljanje, za analizo in napovedovanje primerne habitata za številne vrste, vključno s sesalci, drugimi vretenčarji in nevretenčarji ter rastlinskimi vrstami in združbami. Enega prvih modelov primernosti habitata za risa sta razvila Zimmermann in Breitenmoser (2002) z uporabo verjetnostnega modela za območje Jurskega pogorja v Švici. Kasneje sta ga dopolnila in razširila na celotno območje Jurskih Alp (Zimmermann in Breitenmoser 2007).

Skrbinšek (2004) je izdelal model primernosti habitata za območje Slovenije, ki smo ga za namen te študije ekstrapolirali na območje celotne Slovenije, Hrvaške, Bosne in Hercegovine, SV Italije ter obmejno območje z Avstrijo. Območje zajema celotno širše območje ponovno naseljene

dinarske populacije, ki jo danes prepoznavamo kot dinarsko - jugovzhodno alpsko populacijo. Model smo razvili na osnovi metodologije, ki so jo uporabili Schadt in sodelavci (2002). Uporabili so telemetrične podatke risov spremljanih v francoski in švicarski Juri, model pa nato ovrednotili z neodvisnimi telemetričnimi podatki risov iz območja vzdolž nemško-češke meje (Bufka in sod. 2000) in iz dinarskega območja južne Slovenije (Staniša 1998). Njihov model smo prilagodili novim okoljskim podatkom in ga evalvirali z GPS in VHF telemetričnimi podatki, kot tudi podatki C1 in C2 monitoringa risov v Sloveniji (po Molinari in sod. 2003).

Becker (2013) je z uporabo programskega paketa »Maximum Entropy Species Distribution Modelling Model« (Maxent) izdelala model primernosti habitata risa za celotno območje Alp, ter del Dinaridov in Apeninov. Ugotovila je, da v Alpah približno 103.600 km² primerne habitata za risa, ki pokriva približno 54 % celotnega območja Alpske konvencije, znotraj katerega je opredelila 22 krp primerne habitata v velikosti od 400 do več kot 17.000 km², ki predstavljajo območja potencialnih sub-populacij risov. Model kaže zelo podoben vzorec primerne habitata na območju Dinaridov kot habitatni model, ki smo ga izdelali mi za območje dinarske-jugovzhodno alpske populacije. Primerjali smo tudi velikosti treh habitatnih krp (Becker 2013), ki se raztezajo v slovenske Alpe, in velikosti ustreznih površin v našem modelu in sicer za območja Julijskih Alp, Karavank - Savinjskih Alp in Pohorja. Velikosti primerne habitata v našem modelu ustrezajo 94 %, 90 % in 89 % velikosti habitatnih krp Beckerjevega modela. To kaže na podobne rezultate obeh modelov, vendar nekoliko manjša velikost krp primerne habitata v našem modelu nakazuje na njegovo nekoliko bolj konzervativno opredeljevanje prostora primerne za risa.

Povezljivost prostora za risa v srednji Evropi so modelirali v različnih študijah, v katerih so uporabljali različne metodološke pristope (npr. Zimmermann 2003, Schadt in sod. 2004, Zimmermann 2004, Becker 2013, Magg in sod. 2016). Zimmermann (2004) je povezljivost prostora za alpski prostor modeliral v GIS programskem orodju z uporabo frikcijskih rastrov (angl. friction grid) in funkcije stroškovnih razdalj (angl. Cost Distance Function). Rezultati analize

stroškovnih razdalj so pokazali, da je bilo vseh 37 evidentiranih habitatnih krp znotraj disperzijske razdalje odraščajočih risov, ki se gibljejo skozi neugoden življenjski prostor. Kljub temu pa so rezultati terenskih študij pokazali, da je v resnici povezljivost prostora veliko manjša, predvsem na račun linijskih barrier (ograjenih avtocest), ki jih večina risov v disperziji ni uspela prečkati (Zimmermann in sod. 2007).

Prav tako je Becker (2013) je ocenila povezljivost prostora za risa v Alpah in opredelila 22 habitatnih krp. Povezljivost prostora je najprej izračunala s programskim orodjem ArcGIS RegionGroup, nato pa je večje habitatne krpe še subjektivno razdelila na več podregij na podlagi upoštevanja avtocest, velikih rek ali visokih gorskih grebenov kot razmejitvenih območij, ob predpostavki da bi risi tako velike ovire le redko prečkali.

Schadt in sod. (2004) so za preučevanje povezljivosti prostora in simulacijo uspešnosti disperzije risov uporabili individualni prostorsko eksplicitni model disperzije (IBSEDM), ki temelji na simulaciji verjetnosti posameznih osebkov v disperziji, da bodo dosegli sosednje primerne habitatne krpe. Model so aplicirali na kompleksno heterogeno nemško pokrajino. Ugotovili so, da bi risi, glede na svoje disperzijske sposobnosti večino primerne prostora kolonizirali v približno desetih letih. Ko pa so v modelu upoštevali tudi pričakovane stopnje smrtnosti risov ob prečkanju cest, je večina habitatnih krp ostala izoliranih, razen ob nemško-češki meji. Posledično se je izkazalo, da je bila povezljivost habitatnih krp bolj omejena z visoko smrtnostjo na cestah, kot z razdrobljenostjo in razpršenostjo primerne habitata. V skladu s tem so predlagali, naj se prizadevanja za varstvo upravljanje namesto zgolj v vlaganja v obnovo habitatov usmerijo tudi v ukrepe za zmanjšanje smrtnosti na cestah.

Gibanje risov v prostoru je povezano s potrebami po iskanju hrane, parjenju in vzreji mladičev. Kljub razmeroma majhni telesni velikosti pa ta vrsta uporablja velike domače okoliše, zato je tudi njihovo gibanje v prostoru zelo intenzivno (Schmidt in sod. 1997). Jędrzejewski in sod. (2002) so opravili raziskavo o vzorcih gibanja evrazijskih risov v pragozdnem območju Białowieża na Poljskem. Skupno so telemetrično spremljali 18 risov (6 odraslih samcev, 5 odraslih samic, 2 subadultna samca 1 subadultna samica

in 4 mladiči). Odrasli samci so se v povprečju premikali na največje razdalje, medtem ko so se samice, ki niso bile reproduktivne, premikale na najkrajših razdaljah. Samice z mladiči so se premakale na bistveno daljših razdaljah kot samice brez mladičev. Najdaljše neprekinjeno gibanje risjega samca je merilo 31 km, pri čemer je obhodil približno 18 % njegovega domačega okoliša (Jędrzejewski in sod. 2002).

Vpliv snega na gibanje evrazijskih risov ni jasen, čeprav obstajajo študije, ki nakazujejo, da bi snežne razmere lahko vplivale na njihovo gibanje ter na nadaljnji uspeh in razporeditev njihovega plenjenja, zlasti pri odraslih samicah z mladiči (Pullianen in sod. 1995, Pulliainen in Hyypiä 1975, Haglund 1966) Takšen vpliv snega je bil jasnejše opažen pri kanadškem risu (*Lynx canadensis*) (Murray in Boutin 1991, Stenseth in sod. 2004).

Schmidt in sod. (1997) so pridobili podatke o velikosti domačih okolišev risov iz različnih demografskih kategorij (Białowieża, Poljska). Poskušali so prikazati učinke različnih parametrov, kot so starost, spol, sezona in socialnost na velikost in spreminjanje domačih okolišev. Izkazalo se je, da je spol risa najpomembnejša spremenljivka, ki vpliva na velikost domačih okolišev, temu je sledil čas spremljanja osebkov in število zbranih lokalizacij, ki so jih uporabili za izračun. V celotnem območju njihove razširjenosti v Evropi se velikosti domačih okolišev risov razlikujejo za faktor 10 (Linnell in sod. 2001; Jędrzejewski in sod. 2002) V začetnih poskusih primerjanja velikosti domačih okolišev med populacijami so raziskovalci na splošno uporabili zemljepisno širino kot preprost indikator gradienta okoljske produktivnosti (Buskirk in McDonald 1989, Gompper in Gittleman 1991). Kasneje so Herfindal in sod. (2004) pokazali, da ta spremenljivka ni ustrezna, saj ne upošteva vpliva nadmorske višine in regionalnih klimatskih pogojev. Njihovi rezultati multivariatnih regresijskih modelov so pokazali jasno povezavo med velikostjo domačega območja risa in produktivnostjo proučevanega območja. Čeprav ni neodvisnih podatkov, ki bi produktivnost okolja neposredno povezovali z gostoto plena, utemeljeno domnevajo, da takšna povezava obstaja.

Disperzija je vsako gibanje posameznega osebk, pri katerem zapusti domače območje, včasih

pa ob tem vzpostavi novo domače območje (Levins 1970). Je ključni parameter v populacijski dinamiki, zlasti pri ogroženih subpopulacijah znotraj metapopulacije (Levins 1970, Hanski 1999).

Študija v kateri so primerjali disperzijske vzorce risov iz populacij v Nordijskih, Baltskih in Dinarskih gozdnih območjih ter iz srednje Evrope, je pokazala, da je bila povprečna disperzijska razdalja risov 39 kilometrov, 68 % risov, ki so dispergirali pa se je naselilo na območju znotraj 50 kilometrov (Molinari-Jobin in sod. 2010). Mladi risi tako pogosto vzpostavljajo domače okoliše na mestih, ki mejijo na območja drugih risov (Zimmermann in sod. 2005), kar pomembno vpliva na verjetnost vzpostavljanja populacij na novih območjih. Medtem ko se ob večanju populacija risov sicer lahko prostorsko širi, je verjetnost, da bodo posamezni risi dispergirali daleč stran in ustanovili povsem nove, ločene populacije zelo majhna (Zimmermann in sod. 2007).

Štiri desetletja po ponovni naselitvi risov v Dinaride v Sloveniji se zdi, da je bilo širjenje risov uspešnejše proti jugovzhodu, vzdolž Dinarska območja na Hrvaškem in v Bosni in Hercegovini (BiH), kot proti severozahodu, v jugovzhodne Alpe v Sloveniji, Avstriji in Italiji. Največja razdalja zabeleženega območja prisotnosti risa v BiH od mesta izpustov je približno 390 km, medtem ko je od severozahoda (SV Italija) približno 140 km. Zanesljivega odgovora za to sicer še nimamo; očitno pa je, da ograjena avtocesta Ljubljana - Trst predstavlja izjemno močno oviro za disperzijo risov iz dinarskega območja proti Alpam (Skrbinšek 2004).

Sodeč po dosedanjem poznavanju in izkušnjah bi morali v prihodnje vidike ohranjanja primernega življenjskega okolja risa vključevati tudi vpliv podnebnih sprememb. Gorska okolja veljajo za posebej ogrožena, pri čemer se globina in trajanje snežne odeje v zadnjih desetletjih močno zmanjšujeta (Beniston in sod. 2003), spreminja se naravna vegetacija (Gehrig-Fasel in sod. 2007) povečuje pa se tudi tveganje za spreminjanje in širjenje novih/tujerodnih plenilcev/kompetitorjev, kot je na primer evrazijski šakal (*Canis aureus*) (Potočnik in sod. 2019b). Vsi omenjeni dejavniki bi lahko pomembno vplivali tako na razpoložljivost prehranskih virov za rise, kot tudi na količino razpoložljivega ustreznega prostora zanje. Ne glede na to pa prizadevanja za ohranjanje območij in

koridorjev med habitatnimi krpami, zmanjševanje vpliva (zlasti) linijskih ovir (avtoceste) in, po potrebi, »imitacija« disperzije s premestitvami oziroma translokacijami osebkov ostajajo pomembni deli varstvenih ukrepov za vzpostavljajanje viabilne pan-evropske metapopulacije risa v Evropi.

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References

- Andren, H., 1994. Effects of habitat fragmentation on birds and mammals in landscapes with different proportions of suitable habitat: a review. *Oikos*, 71 (3), 355-366.
- Apollonio, M., Andersen, R., Putman, R., (Eds.), 2010. European ungulates and their management in the 21st century. Cambridge University Press, Cambridge, UK, pp. 604.
- Becker, T., 2013. Modelling Eurasian lynx distribution and estimation of patch and population size in the Alps. Master thesis. University of London, London, United Kingdom.
- Beier, P., 1995. Dispersal of juvenile cougars in fragmented habitat. *The Journal of Wildlife Management*, 59 (2), 228-237.
- Bell, W.J., 1990. Searching behavior patterns in insects. *Annual Review of Entomology* 35, 447-67.
- Beniston, M., Keller, F., Koffi, B., Goyette S., 2003. Estimates of snow accumulation and volume in the Swiss Alps under changing climate conditions. *Theoretical and Applied Climatology*, 76, 125- 140.
- Bernhart, F., 1990. Untersuchungen des Aktivitätsmusters des Luchses (*Lynx lynx*) in der Schweiz: Experimente und Felduntersuchungen. Doctoral dissertation. University of Bern, Bern, Switzerland.
- Boyce, M.S., Vernier, P.R., Nielsen, S.E., Schmiegelow, F.K., 2002. Evaluating resource selection functions. *Ecological Modelling*, 157 (2-3), 281-300.
- Breitenmoser, U., 1998. Large predators in the Alps: the fall and rise of man's competitors. *Biological Conservation*, 83 (3), 279-289.
- Breitenmoser, U., Breitenmoser-Würsten, C., Okarma, H., Kaphegyi, T., Kaphegyi-Wallmann, U., Müller, U.M., 2000. Action plan for the conservation of the Eurasian lynx (*Lynx lynx*) in Europe. Strasbourg, Council of Europe. Convention on the conservation of European wildlife and natural habitats, 22, 1-83.
- Breitenmoser, U., Breitenmoser-Würsten, C. 2001. Die ökologischen und anthropogenen Voraussetzungen für die Existenz grosser Beutegreifer in der Kulturlandschaft. *Forest Snow and Landscape Research*, 76 (1/2), 23-39.
- Breitenmoser, U., Breitenmoser-Würsten, C., Capt, S., Ryser, A., Zimmermann, F., Angst, C., Laass, J., 1999. Lynx management problems in the Swiss Alps. *Cat News*, 30, 16-18.
- Breitenmoser-Würsten, C., Obexer-Ruff, G., 2003. Population and conservation genetics of two reintroduced lynx (*Lynx lynx*) populations in Switzerland—a molecular evaluation 30 years after translocation. In: *Proceedings of the 2nd Conference on the Status and Conservation of the Alpine Lynx Population (SCALP)*, Strasbourg, France, 7-9.
- Breitenmoser-Würsten, C., Zimmermann, F., Molinari-Jobin, A., Molinari, P., Capt, S., Vandel, J. M., Breitenmoser, U., 2007. Spatial and social stability of a Eurasian lynx (*Lynx lynx*) population: an assessment of 10 years of observation in the Jura Mountains. *Wildlife Biology*, 13 (4), 365-380.
- Breitenmoser-Würsten, C., Zimmermann, F., Ryser, A., Capt, S., Laass, J., Siegenthaler, A., Breitenmoser, U., 2001. Untersuchungen zur Luchspopulation in den Nordwestalpen der Schweiz 1997-2000. *Kora Bericht*, 9, 92.
- Brotons, L., Thuiller, W., Araújo, M.B., Hirzel, A.H., 2004. Presence-absence versus presence-only modelling methods for predicting bird habitat suitability. *Ecography*, 27 (4), 437-448.
- Bufka, L., Cervený, J., Koubek, P., Horn, P. 2000. Radiotelemetry research of the lynx (*Lynx lynx*) in Šumava: preliminary results. In: *Proceedings Predatori v Myslivosti*. Czech Forestry Society, Praga, Czech Republic, 143-153.

- Burgman, M.A., Fox, J.C., 2003. Bias in species range estimates from minimum convex polygons: implications for conservation and options for improved planning. *Animal Conservation*, 6 (1), 19-28.
- Buskirk, S.W., McDonald, L.L., 1989. Analysis of variability in home range size of the American marten. *The Journal of Wildlife Management*, 53 (4), 997-1004.
- Cagnacci, F., Boitani, L., Powell, R.A., Boyce, M.S., 2010. Animal ecology meets GPS-based radiotelemetry: a perfect storm of opportunities and challenges. *Philosophical Transactions of the Royal Society B Biological Sciences*, 365 (1550), 2157-2162.
- Campbell, V., Strobeck, C., 2006. Fine-scale genetic structure and dispersal in Canada lynx (*Lynx canadensis*) within Alberta, Canada. *Canadian Journal of Zoology*, 84 (8), 1112-1119.
- Chapron, G., Kaczensky, P., Linnell, J.D.C., von Arx, M., Huber, D., Andr n, H., Jerina, K., Kos, I., Krofel, M., Maji  Skrbin ek, A., Poto nik, H., Skrbin ek, T., ... Anders, O., 2014. Recovery of large carnivores in Europe's modern human-dominated landscapes. *Science*, 346 (6216), 1517-1519.
- Clarke, A.L., S ether, B.E., R skaft, E., 1997. Sex biases in avian dispersal: a reappraisal. *Oikos*, 79 (3), 429-438.
- Clobert, J., Wolff, J.O., 2001. Introduction. In: Clobert, J., Danchin, E., Dhondt, A. A., Nichols, J. D. (eds.): *Dispersal*. Oxford University Press, New York, pp. 17 - 21.
-  op, J., Frkovic, A., 1998. The re-introduction of the lynx in Slovenia and its present status in Slovenia and Croatia. *Hystrix*, 10 (1), 65-76.
- Cooper, W.S., Kaplan, R.H., 1982. Adaptive "coin-flipping": a decision-theoretic examination of natural selection for random individual variation. *Journal of Theoretical Biology*, 94 (1), 135-151.
- Curio, E., 1976. The ethology of predation. *Zoophysiology and ecology (Vol.7)*. Springer-Verlag, Berlin - New York, pp. 250.
- Danchin, E., Heg, D., Doligez, B., 2001. Public information and breeding habitat selection. In: Clobert, J., Danchin, E., Dhondt, A. A., Nichols, J. D. (eds): *Dispersal*. Oxford University Press, New York, pp. 243-258.
- Dobson, F.S., 1982. Competition for mates and predominant juvenile male dispersal in mammals. *Animal Behaviour*, 30 (4), 1183-1192.
- Elith, J., Leathwick, J.R., 2009. Species distribution models: ecological explanation and prediction across space and time. *Annual Review of Ecology, Evolution and Systematics*, 40, 677-697.
- Ferguson, J.W.H., Galpin, J.S., Wet, M.D., 1988. Factors affecting the activity patterns of black-backed jackals *Canis mesomelas*. *Journal of Zoology*, 214 (1), 55-69.
- Ferreras, P., Delibes, M., Palomares, F., Fedriani, J.M., Calzada, J., Revilla, E., 2004. Dispersal in the Iberian lynx: factors affecting the start, duration, distance and dispersal success. *Behavioural Ecology*, 15, 31-40.
- Fuller, A.K., Harrison, D.J., 2010. Movement paths reveal scale-dependent habitat decisions by Canada lynx. *Journal of Mammalogy*, 91 (5), 1269-1279.
- Gehrig-Fasel, J., Guisan, A., Zimmermann, N.E., 2007. Tree line shifts in the Swiss Alps: Climate change or land abandonment? *Journal of Vegetation Science*, 18, 571-582.
- Graf, R.F., Bollmann, K., Sachot, S., Suter, W. Bugmann, H., 2006. On the generality of habitat distribution models: a case study of capercaillie in three Swiss regions. *Ecography*, 29, 319-328.
- Greenwood, P.J., 1980. Mating systems, philopatry and dispersal in birds and mammals. *Animal Behaviour*, 28 (4), 1140-1162.
- Gompper, M. E., Gittleman, J.L., 1991. Home range scaling: intraspecific and comparative trends. *Oecologia*, 87, 343-348.
- Guarino, F., 2002. Spatial ecology of a large carnivorous lizard, *Varanus varius* (Squamata: Varanidae). *Journal of Zoology (London)*, 258, 449-457.
- Guisan, A., Zimmermann, N.E., 2000. Predictive habitat distribution models in ecology. *Ecological Modelling*, 135 (2-3), 147-186.

- Guisan, A., Tingley, R., Baumgartner, J.B., Naujokaitis-Lewis, I., Sutcliffe, P.R., Tulloch, A.I.T., Regan, T.J., Brotons, J., McDonald-Madden, E., Mantyka-Pringle, C., Martin, T.G., Rhodes, J.R., Maggini, R., Setterfield, S.A., Elith, J., Schwartz, M.W., Wintle, B.A., Broennimann, O., Austin, M., Ferrier, S., Kearney, M.R., Possingham, H.P., Buckley, Y.M., 2013. Predicting species distributions for conservation decisions. *Ecology Letters*, 16 (12), 1424-1435.
- Hagan, J.M., Vander Haegen, W.M., McKinley, P.S., 1996. The early development of forest fragmentation effects on birds. *Conservation Biology*, 10 (1), 188-202.
- Haglund, B., 1966. Winter habits of the lynx (*Lynx lynx* L.) and wolverine (*Gulo gulo* L.) as revealed by tracking in the snow. *Viltrevy*, 4, 81-229.
- Haller, H Breitenmoser, U., 1986. Zur Raumorganisation der in den Schweizer Alpen wiederangesiedelten Population des Luchses *Lynx lynx*. *Zeitschrift fuer Saeugetierkunde*, 51 (5), 289-311.
- Hanski, I., 1999. *Metapopulation Ecology*. Oxford University Press. New York, USA, pp. 324.
- Hanski, I., Moilanen, A., Gyllenberg, M., 1996. Minimum viable metapopulation size. *The American Naturalist*, 147 (4), 527-541.
- Herfindal, I., Linnell, J. D., Odden, J., Nilsen, E. B., Andersen, R., 2005. Prey density, environmental productivity and home range size in the Eurasian lynx (*Lynx lynx*). *Journal of Zoology*, 265 (1), 63-71.
- Hirzel, A., 2001. Linking landscape-and population ecology for large population management modelling: the case of Ibex (*Capra ibex*) in Switzerland. Doctoral dissertation. Université de Lausanne, Faculté des sciences, Switzerland.
- Hirzel, A.H., Helfer, V., Metral, F., 2001. Assessing habitat-suitability models with a virtual species. *Ecological Modelling*, 145 (2-3), 111-121.
- Hirzel, A.H., Le Lay, G., Helfer, V., Randin, C., Guisan, A., 2006. Evaluating the ability of habitat suitability models to predict species presences. *Ecological Modelling*, 199 (2), 142-152.
- Iuell, B., Bekker, G.J., Cuperus, R., Dufek, J., Fry, G., Hicks, C., Hlavác, V., Keller, V.B., Rosell, C., Sangwine, T., Tørsløv, N., Wandall, Maire, B. (Eds.), 2003. *Wildlife and Traffic: A European Handbook for Identifying Conflicts and Designing Solutions. Habitat Fragmentation due to Transportation Infrastructure*. COST 341.
- Janečka, J.E., Blankenship, T.L., Hirth, D.H., William Kilpatrick, C., Tewes, M.E., Grassman, L.I., 2007. Evidence for male-biased dispersal in bobcats *Lynx rufus* using relatedness analysis. *Wildlife Biology*, 13 (1), 38-47.
- Jędrzejewski, W., Nowak, S., Schmidt, K., Jędrzejewska, B., 2002. The wolf and the lynx in Poland—results of a census conducted in 2001. *Kosmos*, 51 (4), 491-499.
- Jędrzejewski, W., Schmidt, K., Miłkowski, L., Jędrzejewska, B., Okarma, H., 1993. Foraging by lynx and its role in ungulate mortality: the local (Białowieża Forest) and the Palaearctic viewpoints. *Acta Theriologica*, 38 (4), 385-403.
- Jonsson, S., 1984. Lodjurets jakt och sociala liv. *Zool. Ti.*, 1, 28-34. [In Swedish]
- Kaczensky, P., Chapron, G., von Arx, M., Huber, H., Andrén, H., Linnell, J., 2003. Status, management and distribution of large carnivores - bear, lynx, wolf and wolverine in Europe. Report. European Commission, pp. 72.
- Kramer-Schadt, S., Revilla, E., Wiegand, T., Breitenmoser, U., 2004. Fragmented landscapes, road mortality and patch connectivity: modelling influences on the dispersal of Eurasian lynx. *Journal of Applied Ecology*, 41 (4), 711-723.
- Krofel, M., Huber, D., Kos, I., 2011. Diet of Eurasian lynx *Lynx lynx* in the northern Dinaric Mountains (Slovenia and Croatia). *Acta Theriologica*, 56 (4), 315-322.
- Krofel, M., Skrbinšek, T., Kos, I., 2013. Use of GPS location clusters analysis to study predation, feeding, and maternal behavior of the Eurasian lynx. *Ecological Research*, 28 (1), 103-116.
- Kvam, T., 1991. Reproduction in the European lynx, *Lynx lynx*. *Zeitschrift fur Saeugetierkunde*, 56, 146-158.
- Levins, R., 1970. Extinction. In: Gerstenhaber, M. (ed.): *Some Mathematical Problems in Biology*. American Mathematical Society, Providence, Rhode Island, USA, pp. 75-107.

- Linnell, J.D.C., Andersen, R., Kvam, T., Andren, H., Liberg, O., Odden, J., Moa, P.F., 2001. Home range size and choice of management strategy for lynx in Scandinavia. *Environmental Management* 27 (6), 869-879.
- Linnell, J.D., Trouwborst, A., Boitani, L., Kaczensky, P., Huber, D., Reljic, S., Hayward, M.W., 2016. Border security fencing and wildlife: the end of the transboundary paradigm in Eurasia? *PLoS Biology*, 14 (6), e1002483.
- Macdonald, D.W., Johnson, D.D.P., 2001. Dispersal in theory and practice: consequences for conservation biology. In: Clobert, J., Danchin, E., Dhondt, A.A., Nichols, J.D. (eds.): *Dispersal*. Oxford University Press, New York, 358-372.
- Maehr, D.S., Land, E.D., Shindle, D.B., Bass, O.L., Hoctor, T.S., 2002. Florida panther dispersal and conservation. *Biological Conservation*, 106 (2), 187-197.
- Magg, N., Müller, J., Heibl, C., Hackländer, K., Wölfl, S., Wölfl, M., Heurich, M., 2016. Habitat availability is not limiting the distribution of the Bohemian-Bavarian lynx *Lynx lynx* population. *Oryx*, 50 (4), 742-752.
- Mladenoff, D.J., Sickley, T.A., Haight, R.G., Wydeven, A.P., 1995. A regional landscape analysis and prediction of favorable gray wolf habitat in the northern Great Lakes region. *Conservation Biology*, 9 (2), 279-294.
- Mohr, C.O., 1947. Table of equivalent populations of North American small mammals. *The American Midland Naturalist*, 37 (1), 223-249.
- Molinari, P., Molinari-Jobin, A., 2001. Behavioural observations of interactions in a free-ranging lynx *Lynx lynx* family at kills. *Acta Theriologica*, 46, 441-445.
- Molinari-Jobin, A., Marboutin, E., Wölfl, S., Wölfl, M., Molinari, P., Fasel, M., Huber, T., 2010. Recovery of the Alpine lynx *Lynx lynx* metapopulation. *Oryx*, 44 (2), 267-275.
- Molinari-Jobin, A., Molinari, P., Breitenmoser-Würsten, C.H., Woelel, M., Staniša, C., Fasel, M., Stahl, P., Vandel, J.M., Rotelli, L., Kaczensky, P., Huber, T., Adamič, M., Koren, I., Breitenmoser, U., 2001. Pan-Alpine Conservation Strategy for the lynx. SCALP, Council of Europe, pp. 19.
- Molinari-Jobin, A., Molinari, P., Breitenmoser-Würsten, C., Wölfl, M., Staniša, C., Fasel, M., Stahl, P., Vandel, J.M., Rotelli, L., Kaczensky, P., Huber, T., Adamič, M., Koren, M., Breitenmoser, U., 2003. The pan-alpine conservation strategy for the lynx. *Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention) Nature and environment*, No. 130. Council of Europe Publishing, pp. 24.
- Morrison, M.L., Marcot, B., Mannan, W., 2012. *Wildlife-habitat relationships: concepts and applications*. Island Press, Washington, DC., USA, pp. 54.
- Mowat, G.K., Poole, E., O'Donoghue, M., 2000. Ecology of lynx in northern Canada and Alaska. In: Ruggiero, L.F. et al. (eds.): *Ecology and conservation of lynx in the United States*. University Press of Colorado, Boulder, USA, 265-306.
- Mowat, G., Slough, B.G., 1998. Some observations on the natural history and behaviour of the Canada lynx, *Lynx canadensis*. *Canadian Field-Naturalist*, 112 (1), 32-36.
- Murray, D.L., Boutin, S., 1991. The influence of snow on lynx and coyote movements: does morphology affect behavior? *Oecologia*, 88 (4), 463-469.
- O'Donoghue, M., Boutin, S., Krebs, C.J., Hofer, E.J., 1997. Numerical responses of coyotes and lynx to the snowshoe hare cycle. *Oikos*, 80, 150-162.
- O'Donoghue, M., Boutin, S., Krebs, C.J., Zuleta, G., Murray, D.L., Hofer, E.J., 1998. Functional responses of coyotes and lynx to the snowshoe hare cycle. *Ecology*, 79 (4), 1193-1208.
- Okarma, H., Jedrzejewski, W., Schmidt, K., Kowalczyk, R., Jedrzejewska, B., 1997. Predation of Eurasian lynx on roe deer and red deer in Białowieża Primal Forest, Poland. *Acta Theriologica*, 42 (2), 203-224.
- Potočnik, H., Skrbinšek, T., Kos, I., 2009. The reintroduced Dinaric lynx population dynamics in PVA simulation: the 30 years retrospective and the future viability. *Acta Biologica Slovenica*, 52 (1), 3-18.

- Potočnik, H., Al Sayegh-Petkovšek, S., De Angelis, D., Huber, Đ., Jerina, K., Kusak, J., Mavec, M., Pokorny, B., Reljić, S., Rodriguez R., M., Skrbinšek, T., Vivoda, B., 2019a. Handbook for integrating the bear habitat suitability and connectivity to spatial planning: prepared within the framework of the Life Dinalp Bear project. University of Ljubljana, Ljubljana, Slovenia, pp. 66.
- Potočnik, H., Pokorny, B., Flajšman, K., Kos, I., 2019b. Evrazijski šakal. Zlatorogova knjižnica 42. Lovska zveza Slovenije, Ljubljana, Slovenia, pp. 248.
- Pulliaainen E., Hyypiä V., 1975. Winter food and feeding habits of lynx (*Lynx lynx*) in south-eastern Finland. Suomen Riista, 26, 60-63.
- Pulliaainen E., Lindgren E., Tunkkari P.S., 1995. Influence of food availability and reproductive status on the diet and body condition of the European lynx in Finland. Acta Theriologica, 40 (2), 181-196.
- Ripple, W.J., Estes, J.A., Beschta, R.L., Wilmers, C.C., Ritchie, E.G., Hebblewhite, M., Schmitz, O.J., 2014. Status and ecological effects of the world's largest carnivores. Science, 343 (6167), 1241-1248.
- Ryser, A., Von Wattenwyl, K., Ryser-Degiorgis, M.P., Willisch, C., Zimmermann, F., Breitenmoser, U. 2004. Luchsumsiedlung Nordostschweiz 2001-2003, Schlussbericht Modul Luchs des Projektes LUNO. Muri bei Bern. KORA Bericht. 22, 1-60.
- Samelius, G., Andrén, H., Liberg, O., Linnell, J.D.C., Odden, J., Ahlqvist, P., Sköld, K., 2012. Spatial and temporal variation in natal dispersal by Eurasian lynx in Scandinavia. Journal of Zoology, 286 (2), 120-130.
- Sandell, M., 1989. The mating tactics and spacing behaviour of solitary carnivores. In: Gittleman, J.L. (ed.): Carnivore behavior, ecology and evolution. Cornell University Press, New York, USA, 164-182.
- Schadt, S.A., 2002. Scenarios assessing the viability of a lynx population in Germany. Szenarien für eine lebensfähige Luchspopulation in Deutschland. Doctoral dissertation. Technisches Univeritat München, Germany.
- Schadt, S., Revilla, E., Wiegand, T., Knauer, F., Kaczensky, P., Breitenmoser, U., 2002. Assessing the suitability of central European landscapes for the reintroduction of Eurasian lynx. Journal of Applied Ecology, 39 (2), 189-203.
- Schmidt, K., 1998. Maternal behaviour and juvenile dispersal in the Eurasian lynx. Acta Theriologica, 43, 391-408.
- Schmidt, K., Jedrzejewski, W., Okarma, H., 1997. Spatial organization and social relations in the Eurasian lynx population in Białowieża Primeval Forest, Poland. Acta Theriologica, 42 (3), 289-312.
- Schmidt, K., 1999. Variation in daily activity of the free-living Eurasian lynx (*Lynx lynx*) in Białowieża Primeval Forest, Poland. Journal of Zoology, 249 (4), 417-425.
- Schwartz, M.W., 1999. Choosing the appropriate scale of reserves for conservation. Annual Review of Ecology and Systematics, 30, 83-108.
- Sellers, P.J., Dickinson, R.E., Randall, D.A., Betts, A.K., Hall, F.G., Berry, J.A., Collatz, G.J., Denning, A.S., Mooney, H.A., Nobre, C.A., Sato, N., Field, C.B., Henderson-Sellers, A., 1997. Modeling the exchanges of energy, water, and carbon between continents and the atmosphere. Science, 275, 502-509.
- Shields, W.M., 1982. Philopatry, inbreeding, and the evolution of sex. SUNY press. New York, USA, pp. 245.
- Signer, J., 2010. Distribution and Connectivity of Eurasian Lynx (*Lynx lynx*) in the Alps. ECONNECT, Austrian Environmental Agency, Vienna, Austria, pp 14.
- Skrbinšek, T., 2004. Model primernega prostora za risa v Sloveniji. eng. Lynx in Slovenia, background documents for conservation and management. University of Ljubljana, Biotechnical Faculty, Ljubljana, Slovenia, 122-147.
- Smith, J.L.D., 1993. The role of dispersal in structuring the Chitwan tiger population. Behaviour, 124, 165-195.
- Staniša, C., Huber, T., 1997. Kolikšen je življenjski prostor največje evropske mačke? LZS, Ljubljana, Lovec, 80, 59-61.

- Stahl, P., Vandell, J.M., Ruetter, S., Coat, L., Coat, Y., Balestra, L., 2002. Factors affecting lynx predation on sheep in the French Jura. *Journal of Applied Ecology*, 39 (2), 204-216.
- Stenseth, N.C., Shabbar, A., Chan, K.S., Boutin, S., Rueness, E.K., Ehrlich, D., Jakobsen, K.S., 2004. Snow conditions may create an invisible barrier for lynx. *Proceedings of the National Academy of Sciences*, 101 (29), 10632-10634.
- Stroganov, S.U., 1962. *Mammals of Siberia. Carnivore*. Izdatelstvo AN USSR, Moscow, Russia [In Russian].
- Tracey, J.A., Zhu, J., Boydston, E., Lyren, L., Fisher, R.N., Crooks, K.R., 2013. Mapping behavioral landscapes for animal movement: a finite mixture modeling approach. *Ecological Applications*, 23 (3), 654-669.
- Zimmermann, F., 2003. Lynx habitat fragmentation of the Alps-a preliminary model. In: Molinari-Jobin, A. (ed.): 2nd SCALP Conference, Amden, 7-9 May 2003.
- Zimmermann, F., 2004. Conservation of the Eurasian Lynx (*Lynx lynx*) in a fragmented landscape-habitat models, dispersal and potential distribution. Doctoral dissertation. Université de Lausanne, Faculté de biologie et médecine, Switzerland.
- Zimmermann, F., Breitenmoser, U., 2007. Potential distribution and population size of the Eurasian lynx *Lynx lynx* in the Jura Mountains and possible corridors to adjacent ranges. *Wildlife Biology*, 13 (4), 406-416.
- Zimmermann, F., Breitenmoser-Würsten, C., Breitenmoser, U., 2005. Natal dispersal of Eurasian lynx (*Lynx lynx*) in Switzerland. *Journal of Zoology*, 267 (4), 381-395.
- Zimmermann, F., Breitenmoser-Würsten, C., Breitenmoser, U., 2007. Importance of dispersal for the expansion of a Eurasian lynx *Lynx lynx* population in a fragmented landscape. *Oryx*, 41 (3), 358-368.
- Zollner, P.A., Lima, S.L., 1997. Landscape-level perceptual abilities in white-footed mice: perceptual range and the detection of forested habitat. *Oikos*, 80, 51-60.
- Wandeler, P., 2004. Spatial and temporal population genetics of Swiss red foxes (*Vulpes vulpes*) following a rabies epizootic. Doctoral dissertation. Cardiff University, Wales, UK.
- Waser, P.M., Strobeck, C., Paetkau, D., 2001. Estimating interpopulation dispersal rates. In: Gittleman, J.L., Funk, S.M., Macdonald, D.W., Wayne K. (eds.): *Carnivore conservation*. Cambridge University Press, Cambridge, UK, 484-497.
- White, S., Briers, R.A., Bouyer, Y., Odden, J., Linnell, J.D.C., 2015. Eurasian lynx natal den site and maternal home-range selection in multi-use landscapes of Norway. *Journal of Zoology*, 297 (2), 87-98.
- Wiens, J.A. 1997. The emerging role of patchiness in conservation biology. In: Pickett, S.T.A., Ostfeld, R.S., Shachak, M., Likens, G.E. (eds.): *The ecological basis for conservation: heterogeneity, ecosystems, and biodiversity*. Chapman and Hall, New York, USA, pp. 93-107.
- Wiens, J.A., 2001. The landscape context of dispersal. In: Clobert, J., Danchin, E., Dhondt, A.A., Nichols, J.D. (eds.): *Dispersal*. Oxford University Press, New York, USA, 96-109.
- Worton, B.J., 1989. Kernel methods for estimating the utilization distribution in home-range studies. *Ecology*, 70 (1), 164-168.

Effects of different environmental and sampling variables on the genotyping success in field-collected scat samples: a brown bear case study

Vpliv različnih dejavnikov okolja in vzorčenja na uspešnost genotipizacije vzorcev iztrebkov, zbranih na terenu: primer pri rjavem medvedu

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Abstract: The paper investigates how different field conditions and sample characteristics influence genotyping success in field-collected brown bear scat samples. Genotyping performance of 413 samples collected in a pilot study in southern Slovenia was evaluated, and statistical modelling was used to control confounding between predictor variables and to quantify their specific effects on genotyping success. The best predictors of genotyping success were subjectively estimated scat age, sampling month, and contents of a scat. Even when the other confounded variables were controlled for, genotyping success dropped rapidly with the age estimate, from 89% (82-94%) for 0-day scats to 33% (19-52%) for scats estimated to be 5 days old. Sampling month was also an important predictor, and samples collected during the bear hyperphagia period in late summer / autumn performed considerably better (90%, 78-96%) than the samples collected in spring / early summer (66%, 57-74%). This effect was stronger for fresh than for older samples. Effects of different food types were also considerable, but less important for practical use. Since noninvasive genetic sampling already became the key method for surveying wild populations of many species, efficiency of studies is becoming increasingly important. Understanding the effect of the month of sampling allows the field season to be timed for maximum genotyping success, while subjective scat age provides a useful metric that indicates a sample's viability for genotyping, allowing for prioritization of samples and culling of non-viable samples before resources are wasted for their analysis. This provides higher useful data yields per invested resources and may ultimately lead to better study results.

Keywords: genetics, genotyping success, molecular ecology, noninvasive sampling, scat sampling, *Ursus arctos*

Izvlček: V članku je predstavljen učinek različnih terenskih pogojev in lastnosti vzorca na uspešnost genotipizacije iztrebkov rjavega medveda, nabranih na terenu. Ocenil sem uspešnost genotipizacije 413 vzorcev, zbranih v pilotni študiji v južni Sloveniji ter uporabil statistično modeliranje za popravek motenja med spremenljivkami in kvantifikacijo njihovih učinkov na uspeh genotipizacije. Uspeh genotipizacije so najbolje pojasnili subjektivno ocenjena starost vzorca, mesec vzorčenja in vsebina iztrebka. Tudi ko sem kontroliral moteče spremenljivke, je uspešnost z višjo oceno starosti hitro padala, od 89 % (82 – 94 %) pri iztrebkih starih 0 dni na 33 % (19 – 52 %)

za iztrebke ocenjene kot stare 5 dni. Pomembna pojasnjevalna spremenljivka je tudi mesec vzorčenja, saj so imeli iztrebki, zbrani v obdobju hiperfagije medvedov pozno poleti in jeseni znatno višjo uspešnost (90 %, 78 – 96 %) kot iztrebki zbrani pomladi in zgodaj poleti (66 %, 78 – 96 %). Ta učinek je bil izrazitejši za sveže kot za starejše vzorce. Učinki različne prehrane so bili prav tako precejšnji, kar pa je za praktično uporabo manjšega pomena. Neinvazivno genetsko vzorčenje je že postalo ključna metoda za preučevanje prostoživečih populacij številnih živalskih vrst, zato postaja učinkovitost takšnih študij vse bolj pomembna. Razumevanje učinka meseca vzorčenja nam omogoča načrtovanje terenskega dela tako, da bo uspešnost genotipizacije kar najvišja. Po drugi strani nam subjektivna ocena starosti iztrebka podaja dobro merilo uporabnosti vzorca in nam omogoča prioritizacijo vzorcev ter odstranitev slabih vzorcev, preden porabimo sredstva za njihovo analizo. To omogoča višji izplen uporabnih podatkov glede na porabljena sredstva in delo ter lahko prispeva k boljšim rezultatom študije.

Ključne besede: genetika, molekularna ekologija, neinvazivno vzorčenje, *Ursus arctos*, uspešnost genotipizacije, vzorčenje iztrebkov

Introduction

Noninvasive (or minimally invasive) genetic sampling is increasingly becoming the key method for surveying wild populations of many species (DeYoung and Honeycutt 2005, Carroll et al. 2018). It allows us to collect large numbers of genetic samples in a cost-effective manner without disturbing the animals or affecting their behavior. Consequently, it is being used for a wide range of research and management questions such as population genetics, breeding behavior, population abundance, and breeding behavior (DeWoody 2005, Waits and Paetkau 2005, Mumma et al. 2016, Carroll et al. 2018). New applications and new analytical approaches emerge regularly as researchers develop new ideas on how to use these powerful tools (De Barba et al. 2017, Andrews et al. 2018, Carroll et al. 2018).

There is also a downside to using noninvasive genetic samples. Exposed to harsh field conditions, DNA in such samples rapidly deteriorates, decreasing genotyping success and generating errors that must be correctly handled in laboratory analysis and downstream analytical procedures (Taberlet et al. 1996, 1999). Consequently, development of noninvasive genetic sampling has not been without its growing pains, and serious errors were made by researchers that were not sufficiently aware of the specifics of data obtained from noninvasive genetic samples (e.g. Gagneux et al. 1997, retracted 2001). Nevertheless, the methods matured over the last

two decades into routinely applicable tools which can be, with some care, applied by any competent researcher for a wide range of research questions.

Researchers have dedicated considerable effort to improve the laboratory procedures and maximize genotyping success (e.g. De Barba and Waits 2010, Skrbinšek et al. 2010). Regardless of the well understood analytical approaches for noninvasive genetic samples, one critical issue remains: DNA quality. As a rule, not all noninvasive genetic samples collected in the field will provide useful data, and in some cases the success rate can be quite low (Waits and Paetkau 2005). A non-amplifying sample still requires effort and generates costs. A large proportion of failed samples increases the cost and effort, even in well-planned studies that prepare for that contingency, and can even cause studies to fail in meeting their goals if such contingency planning is insufficient. A firm understanding of when and how to plan the field season to maximize genotyping success rate, which field conditions can influence that parameter and how to decide whether or not a sample is viable for analysis are critical factors that can help a researcher design a more efficient study that can provide more useful data per given field effort and costs. While there are several published experimental studies where some specific field factors affecting genotyping success in noninvasive samples have been looked at (e.g. Murphy et al. 2007, Santini et al. 2007), there are not many studies that would specifically

try to examine field-collected samples post-hoc and thoroughly evaluate how they performed with regard to the sampling conditions, appearance and content (but see Kopatz et al. 2020).

In this paper, I explore how different field conditions and sample characteristics affect genotyping success, the proportion of samples that can be successfully genotyped, in field-collected brown bear scat samples. I use statistical modelling to correct for confounding between environmental variables and scat appearance to evaluate specific effects of individual variables on genotyping success. Finally, I summarize the findings into recommendations that can be used to increase fieldwork efficiency in studies utilizing scat samples and provide suggestions for further research of the topic.

Materials and methods

Data collection and laboratory analysis protocols are described in details in Skrbinšek et al. (2010). Here I use 413 samples collected in a pilot study in two predefined sampling areas (170 and 240 km², respectively) between May and November of 2004 and 2005. The samples were collected by volunteer samplers and by professional hunters of Slovenia Forest Service. Both areas are in Dinaric Mountains of southern Slovenia, (area 1: 45.68328N, 14.67084E; area 2: 45.61652N, 14.43271E).

Each person involved in sampling (sampler) received an oral presentation about the sampling procedures and precise written instructions for sample collection, evaluation of its age, and recording of other environmental parameters. Samplers were instructed to collect the sample from the outer layer of the scat, possibly a part not in contact with the floor, and not from the top of the scat where the DNA is most exposed to being washed away by rain. Samples were collected in 50 ml screw-cap tubes in non-denatured 96% ethanol. Upon delivery to the lab the samples were stored at -20°C until analysis.

The length of the period that the scat has been exposed to the elements affects the quality of the target DNA (Murphy et al. 2007, Panasci et al. 2011), so we provided the samplers with guidelines how to estimate the scat's age. These guidelines

were not precise, but rather helpful pointers to distinguish old scats from fresh ones (contents specific smell, visual appearance, presence of mucous and insect larvae). We also instructed the samplers not to collect samples they judge to be older than 5 days, thus providing an upper subjective 'limit' on what they considered to be a fresh enough scat for analysis. The samplers also collected additional data about the sample – date and location where it was found, and how protected it was by foliage (exposure). During DNA extraction the main contents of each scat were recorded, and later organized into five broad categories: green vegetation, material of animal origin, beech nuts, corn, and fruits.

Laboratory analysis is explained in detail in Skrbinšek et al. (2010). Briefly, DNA from the samples was extracted in a dedicated laboratory for noninvasive genetic samples with very strict contamination prevention protocols. DNA template was amplified using 12 microsatellite markers and a sex marker in a single multiplex PCR following the modified multiple-tube approach (Taberlet et al. 1996, Skrbinšek et al. 2010). Fragment analysis was performed on an Applied Biosystems ABI 3130xl automatic sequencer, and data analyzed using GeneMapper 4.0 software (Applied Biosystems) to provide single-PCR genotypes. The genotype data from multiple amplifications for each sample were pooled to obtain consensus genotypes and calculate the quality index (Miquel et al. 2006b), the average proportion of genotyping analyses that provided the correct (consensus) genotype.

We were able to successfully genotype many poor-quality samples because they matched a good-quality sample, but which we would not be able to reliably genotype on their own. To get a sample-level objective criterion, I used the quality index (QI) as a measure of DNA quality rather than the actual information if we were able to successfully genotype the sample or not. Since this metric was distributing bimodally (Fig. 1), I recoded it into a binary variable 'genotyping success', with samples that had QI < 0.4 considered as 'failed'. This threshold was obtained through experience and was used also in the original study as the 'fail' threshold for samples that didn't match any other sample of the same animal (Skrbinšek et al. 2010).

I obtained meteorological data (temperature, precipitation) from automatic meteorological stations in the area. For each sample I extracted the data from the closest meteorological station for the time period it was expected to be exposed in the environment – since it was presumably deposited by the animal (estimated using scat age estimate) until it was collected (recorded date). Total precipitation and average temperature during that period were calculated for each sample. Temperature was corrected for difference in elevation between the meteorological station and the location where the sample was collected.

I explored the effects of the sample and environmental parameters on genotyping success using Generalized Linear Models (GLM) and information-theoretic analysis approach (Burnham and Anderson 2002). All analyses were done in R language for statistical computing (R Core Team 2020) using RStudio IDE version 1.3.1073 (R Studio Team 2020). I used ‘genotyping success’ as the response variable with the binomial link function, and constructed an a-priori model set that reflected the following hypotheses: a) the DNA in scat degrades with its age - the time it is exposed in the environment, b) an increase in environment temperature can affect the DNA quality in the scat either by conservation through dehydration, or increased decomposition due to higher enzymatic and bacterial activity, c) rain can wash target DNA from the scat sample, d) scat contents directly affects the quantity and quality of target DNA through “scraping” of intestinal epithelium by rough food particles and conservation of DNA in the environment but can also negatively affect genotyping success because of PCR inhibiting substances, e) food and climate conditions change through the cycle of seasons and can have complex effects on analytical viability of target DNA, f) content of the scat possibly exacerbates or ameliorates the effect of age, temperature, sampling month and rain, and g) age of the scat can have different effects in different months because of different climatic and food conditions.

I first fitted the full model of all variables that I hypothesized could affect the genotyping success, without interactions, and explored Variance Inflation Factors (VIF) to check for multicollinearity. I fitted the models that reflected the hypotheses stated above, with the limitation that the maximum

number of estimated parameters in each included model didn’t exceed 12 to avoid overparameterization and used AICc as the model selection criterion (Akaike 1973). Since there is an obvious relation between variables ‘month of sampling’ and ‘temperature’, I didn’t use both variables in the same models. I used the most parsimonious model (with the lowest AICc) as the best model for inference, but I also performed averaging of models within $\Delta\text{AICc} \leq 5$ using Akaike’s weights (Burnham and Anderson 2002) to explore model selection uncertainty and the effects of parameters and interactions that were not included in the best model. I used R packages *ggeffects* (Lüdtke 2018) and *ggplot2* (Wickham 2016) to explore and visualize the effects of different explanatory variables on the response variable and package *MuMIn* (Bartoň 2020) for calculating AICc and model averaging.

Results

Analysis of Variance Inflation Factors (VIFs) didn’t indicate any problematic multicollinearity between variables (generalized VIFs between 1.02 and 1.67).

Models that included the ‘month of sampling’ variable were considerably better than the models that included ‘temperature’ ($\Delta\text{AICc} = 5.43$ between best models), and the ‘temperature’ variable was not considered any longer. All models within $\Delta\text{AICc} < 3$ included scat age, month of sampling and scat content variables. Interaction between age and month of sampling was also represented in many high-ranking models (Akaike weight sum = 0.69). These variables were included in the highest-ranking (best) model, which was used for inference, and their distribution is shown in Fig. 1. Use of the other variables had much less support in the data and were not considered anymore.

Observed effects of the variables predicted using the best model were compared with model predictions obtained with the model-averaged model. The differences were small, and the averaged model was not considered anymore.

Estimated age of the scat had the most prominent effect on genotyping success (Fig. 2A). When controlled for the other parameters (consecutive day in a year (sampling month variable) = 222 (average),

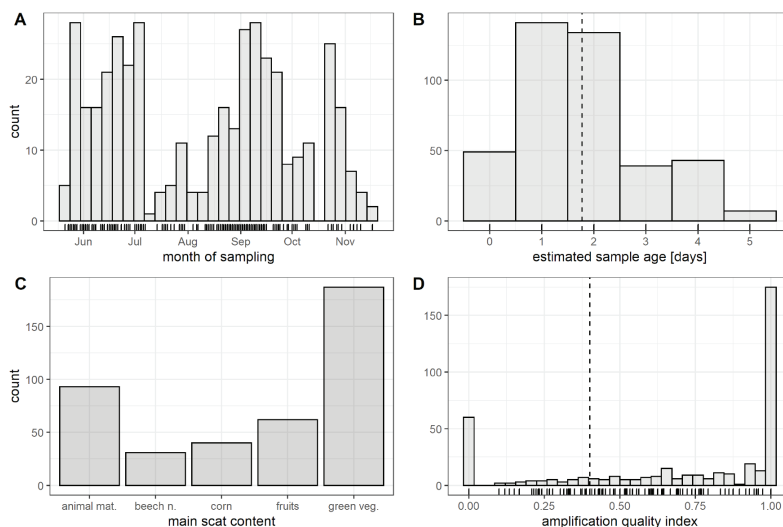


Figure 1: Distribution of samples by month of sampling (A), subjectively estimated sample age (B), the main visually estimated scat content (C), and amplification quality index (Miquel et al. 2006b) (D). The dashed vertical line in (B) indicates the average estimated age. The dashed vertical line in (D) indicates the 0.4 quality index threshold above which the sample was considered suitable for genotyping.

Slika 1: Razporeditev vzorcev po mesecu vzorčenja (A), subjektivni oceni starosti vzorca (B), glavni vizualno ocenjeni vsebini iztrebka (C) in indeksu kvalitete (Miquel et al. 2006a) (D). Črčkana navpična črta v (B) prikazuje povprečno ocenjeno starost vzorca. Črčkana navpična črta v (D) prikazuje prag indeksa kvalitete 0.4, nad katerim se vzorec smatra kot ustrezen za genotipizacijo.

content = 'green vegetation' (most frequent), the expected success rate for samples estimated to be 0 days old was 89% (82-94%). This expectation drops to 33% (19-52%) for the samples estimated to be 5 days old.

The sampling month was another important predictor of genotyping success (Fig. 2B), with one and two days old samples (scat age = 1.5, content = 'green vegetation') collected in spring and early summer having a considerably lower expected success rate (66%; 57-74% CI) than similar samples collected in late autumn (90%; 78-96% CI). The interaction between age of the scat and the sampling month indicates that this effect is stronger for fresher samples and disappears for samples estimated to be 4 and 5 days old (Fig. 2D). However, the confidence intervals, which are relatively narrow for fresh samples, are much wider for older samples (Fig. 2D) since there were considerably more fresh samples collected (Fig. 1B), so the actual effect for older samples is less clear.

Scat content was also an important predictor of genotyping success (Fig. 2C), with beech nuts having considerably higher expected success rate, followed by corn. However, it must be noted that beechnuts are available only in autumn and early winter when success rate is generally higher. Most of the samples with fruit content were also sampled in autumn.

Discussion

In this study, I explored a number of field-collected variables about environmental conditions, time of the year and visual appearance of scat samples collected for genotyping. Using statistical modelling, I was able to disentangle the effects of otherwise confounded variables and found some of them to be useful predictors of genotyping success. Understanding these effects can help researchers in both fieldwork planning

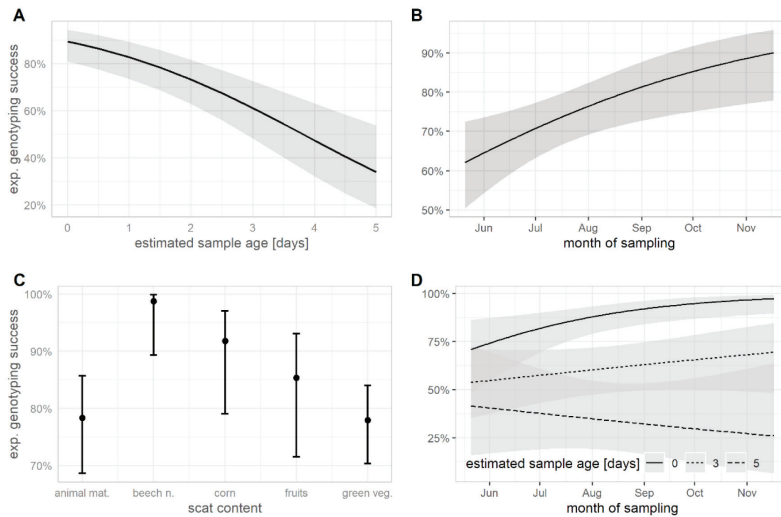


Figure 2: Model-predicted expected genotyping success by estimated sample age (**A**), month of sampling (**B**), main content of scat (**C**) and month of sampling by estimated sample age (**D**). For calculations of predictions, all other parameters were set to the average value (for continuous variables) or set to the most frequent value (for categorical variables). Gray ribbons in A, B and D and bars in graph C indicate 95% confidence intervals of predictions. Graph D doesn't show all values for sample age to be able to show the confidence intervals. None of the graphs were extrapolated outside of the collected data.

Slika 2: Modelne napovedi pričakovane uspešnosti genotipizacije glede na ocenjeno starost vzorca (**A**), mesec vzorčenja (**B**), glavno vsebino v iztrebku (**C**) in mesecu vzorčenje glede na ocenjeno starost vzorca (**D**). V vseh primerih so bile za izračun napovedi vse ostale spremenljivke nastavljene na aritmetično sredino (pri zveznih spremenljivkah) oziroma na najbolj pogosto vrednost (pri kategoričnih spremenljivkah). Siv pas v A, B in D ter navpične črte v C kažejo 95% intervale zaupanja. Graf D ne prikazuje vseh vrednosti ocenjene starosti vzorca, da se lahko prikažejo intervale zaupanja. Grafov nisem ekstrapoliral izven okvirjev zbranih podatkov.

and laboratory analysis, potentially increasing the amount of useful data collected in a study and reducing costs.

A failed analysis of a noninvasive genetic sample means that all (usually considerable) resources that went into its collection and laboratory analysis have been wasted. Consequently, one of the major targets during implementation of any study utilizing such material is to optimize both the sample collection and the laboratory analysis in a manner that maximizes the genotyping success, providing the maximum yield of useful data for the invested effort and funds. An understanding of the field-observable variables that influence the genotyping success can help in fieldwork planning and provide field personnel with useful information on which samples to collect and what data to record. A good understanding of the expected

genotyping success rate also helps in scaling of the field effort for maximum efficiency – to avoid wasting resources through oversampling, but to still provide enough sample coverage to obtain the desired result. When these samples are being analyzed in a laboratory, we can use the success rate predictions to prioritize samples, which is particularly useful if more samples have been collected than there are resources available to analyze them.

One of the major observations is that a subjective estimate of scat age is an excellent predictor of the scat's amplification success. The fact that target DNA degrades with time in a noninvasive genetic sample is intuitive, and has been demonstrated experimentally by several authors (Murphy et al. 2007, Santini et al. 2007, Panasci et al. 2011, Demay et al. 2013), with most authors

describing rapid degradation of DNA in the first 5-7 days (but see King et al. 2018). The interesting observation of my work is that a person in the field, equipped just with basic instructions, their senses, and common sense, does an excellent job in estimating freshness of a scat. Scats will deteriorate at a different pace depending on many factors, environmental and intrinsic, most of them difficult or impossible to evaluate in the field. This means that a subjective estimate of a scat's "age" will not necessarily reflect the exact period it spent being exposed in the environment, but rather the sum effects of both the time, the scat's composition, and the environmental factors that impacted its subjective appearance of "freshness". However, the high predictive power of this subjective estimate suggests that it is possibly more useful than knowing the objective age of a scat would be.

Another interesting finding is the considerable effect of the month of sampling. A similar effect of season on genotyping success from feces has been described before (Piggott and Taylor 2003, De Barba et al. 2010, Kopatz et al. 2020). Interestingly, the Kopatz et al. (2020) study done recently on brown bear scats collected in northern Norway as well as De Barba et al. (2010) study done in Italian Alps both found almost the same effect, in the same species, but in very different landscapes. It is difficult to determine what is causing this effect, but we can speculate on some interactions of climate and food conditions. An interesting speculation is also that this higher success may be caused by the brown bear seasonal physiological cycle since the higher genotyping success coincided with the late summer / autumn hyperphagia period (López-Alfaro et al. 2013), both in our study and in the study done in northern Norway. The interaction between the month of sampling and the estimated age of a sample also indicates that the month of sampling had a stronger effect on fresh samples. While there is a decent sample coverage for most of the period when brown bear scat sampling is typically feasible in our climate, the coverage is lower from mid-July until mid-August, making predictions for that period less reliable.

The effect of the scat content (diet) has been observed to influence genotyping success of scat samples (Murphy et al. 2003, Broquet et al. 2007, Panasci et al. 2011), but it is more dif-

icult to interpret than other variables, and of less practical use. Beech nuts, one of the main bear foods in autumn during high beech mast years, has extremely high success rate, but this finding may be biased by scat age estimates. Scats with high beech nut content may look old very quickly (personal observation), and there is a fair chance that the samplers were overestimating the age of the scat, or even skipped collecting some relatively fresh scats they deemed too old (i.e. subjectively estimated to be older than five days).

Even if I have not found support for the effects of the other parameters I considered, this does not mean that they do not exist. Rainfall and exposure were recognized as a considerable factor of DNA degradation by other authors (Murphy et al. 2007, Brinkman et al. 2010), but didn't show in any of the high-support models in this study. The reason for this may be that we didn't collect scats that were subjectively considered older than 5 days, and high rainfall on an exposed scat could have considerably altered a scat's appearance, making it look older than it was and hence not considered for collection. For environmental temperature I did observe an effect, but as this variable is highly correlated with the sampling month, I didn't use both variables in the same model, and use of the sampling month was much better supported.

Conclusions

While not all the results presented here will be applicable to other geographic locations and/or species, there are concrete practical applications of the findings described in this paper.

A surprisingly good predictor of genotyping success, the subjectively estimated age of the scat should be recorded in any noninvasive genetic study. While it is safe to assume that its predictive power would be considerable in any study of brown bears, it should also be a reasonably good predictor in other species where scat samples are used to obtain genotypes. In the latter case, caution should be exercised since it does seem that in certain species and environments the DNA degradation process in scat can be much slower (King et al. 2018). But with some preliminary research into the effect of this variable on genotyping success, it can be effectively used

both during the field collection as well as in the lab to select and prioritize samples for analysis, increasing effectiveness of the study and decreasing costs.

The effect of the month of sampling seems like another very important predictor of genotyping success, and one that can have a considerable impact on study design since a researcher can considerably increase the effectiveness of a study just by correctly planning the sampling season. The fact that we can see nearly identical effects of the month of sampling in southern and northern Europe indicates that at least for brown bears in Europe, we may be able to generalize this observation. The data I show here come from a pilot study, and when the effect of the sampling month became evident in our preliminary analyses, we timed our country-wide sampling of brown bears from September until December, and achieved 88% genotyping success (Skrbinšek et al. 2019). While seasonal effects will almost certainly be different in other species and landscapes (e.g. Piggott 2004), a thought should be given to this issue during the planning phase of any study utilizing noninvasive genetic samples. A well-conceived pilot study that investigates this may end up saving a lot of effort and costs in the long run.

As noninvasive genetic sampling slips from the domain of cutting-edge science into a more mundane domain of routine everyday use, optimization and cost-effectiveness increasingly become the critical issues. For many studies, understanding the factors that improve or deteriorate genotyping success may just mean the difference between a study succeeding, or failing miserably.

Povzetek

Nein vazivno genetsko vzorčenje je postalo ključna metoda za preučevanje prostoživečih populacij različnih živalskih vrst, saj nam omogoča cenovno učinkovito zbiranje velikega števila genetskih vzorcev, ne da bi s tem živali motili ali vplivali na njihovo vedenje. So pa takšni vzorci z analitičnega vidika zahtevni, uspešnost analize (genotipizacije) pa je lahko v posameznih študijah zelo nizka. Zaradi tega je pomembno poznati dejavnike, ki vplivajo na uspešnost analiz, saj nam lahko to pomaga pri načrtovanju in laboratorijskih

analizah, kar lahko prispeva k učinkovitejši in uspešnejši študiji.

V članku sem ocenil vplive različnih okoljskih dejavnikov in zabeleženih lastnosti vzorcev na uspešnost genotipizacije 413 vzorcev, zbranih v pilotni študiji v južni Sloveniji. Ker je spremenljivk veliko in ker je med njimi precej motenja, sem uporabil statistično modeliranje z generaliziranimi linearnimi modeli in informacijsko-teoretični pristop izbire modelov za prepoznavo najpomembnejših pojasnjevalnih spremenljivk, popravek motenja med njimi in kvantifikacijo njihovih učinkov.

Uspeh genotipizacije so najboljše pojasnili subjektivno ocenjena starost vzorca, mesec vzorčenja in vsebina iztrebka. Tudi ko sem kontroliral moteče spremenljivke, je uspešnost z višjo oceno starosti hitro padala, od 89 % (82 – 94 %) pri iztrebkih starih 0 dni na 33 % (19 – 52 %) za iztrebke ocenjene kot stare 5 dni. Pomembna pojasnjevalna spremenljivka je bil tudi mesec vzorčenja, saj so imeli iztrebki, zbrani v obdobju hiperfagije medvedov pozno poleti in jeseni znatno višjo uspešnost (90 %, 78 – 96 %) kot iztrebki zbrani pomladi in zgodaj poleti (66 %, 78 – 96 %). Ta učinek je bil izrazitejši za sveže kot za starejše vzorce. Učinki različne prehrane so se zdeli prav tako precejšnji, vzorci z visoko vsebnostjo žira ali koruze pa so se izkazali kot najboljši. Za praktično uporabo je sicer ta spremenljivka manjšega pomena, zaradi sezonske dostopnosti posamezne hrane pa tudi motenja nisem mogel v celoti kontrolirati.

Nein vazivno genetsko vzorčenje vse bolj prehaja iz domene vrhunske znanosti v domeno rutinske vsakodnevne uporabe, kar še bolj poveča potrebo po čim višji učinkovitosti študij. Razumevanje učinka meseca vzorčenja nam omogoča načrtovanje terenskega dela tako, da bo uspešnost genotipizacije kar najvišja. Po drugi strani nam subjektivna ocena starosti iztrebka podaja dobro merilo uporabnosti vzorca in nam omogoča prioritizacijo vzorcev ter odstranitev slabih vzorcev, preden porabimo sredstva za njihovo analizo. To omogoča višji izplen uporabnih podatkov glede na porabljena sredstva in delo ter izboljša uspešnost študije, pri nekaterih študijah pa lahko celo pretehta, ali bo študija uspela ali ne.

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References

- Akaike, H., 1973. Information theory and an extension of the maximum likelihood principle. Pages 267–281 in B. N. Petrov and F. Caski, editors. *Proceedings of the Second International Symposium on Information Theory*. Akademiai Kiado, Budapest, Hungary.
- Andrews, K.R., De Barba, M., Russello, M.A., Waits, L.P., 2018. Advances in using non-invasive, archival, and environmental samples for population genomic studies. *Springer Link*, 1–37. doi: 10.1007/13836_2018_45
- Bartoń, K., 2020. MuMIn: Multi-model inference. Available from <https://cran.r-project.org/package=MuMIn>.
- Brinkman, T.J., Schwartz, M.K., Person, D.K., Pilgrim, K.L., Hundertmark, K.J., 2010. Effects of time and rainfall on PCR success using DNA extracted from deer fecal pellets. *Conservation Genetics*, 11, 1547–1552.
- Broquet, T., Ménard, N., Petit, E., 2007. Noninvasive population genetics: A review of sample source, diet, fragment length and microsatellite motif effects on amplification success and genotyping error rates. *Conservation Genetics*, 8, 249–260.
- Burnham, K.P., Anderson, D.R., 2002. *Model selection and multimodel inference*. New York.
- Carroll, E.L., Bruford, M.W., De Woody, J.A., Leroy, G., Strand, A., Waits, L., Wang, J., 2018. Genetic and genomic monitoring with minimally invasive sampling methods. *Evolutionary Applications*, 11, 1094–1119.
- De Barba, M., Miquel, C., Lobréaux, S., Quenette, P.Y., Swenson, J.E., Taberlet, P., 2017. High-throughput microsatellite genotyping in ecology: improved accuracy, efficiency, standardization and success with low-quantity and degraded DNA. *Molecular Ecology Resources*, 17, 492–507.
- De Barba, M., Waits, L.P., 2010. Multiplex pre-amplification for noninvasive genetic sampling: Is the extra effort worth it? *Molecular Ecology Resources*, 10, 659–665.
- Demay, S.M., Becker, P.A., Eidson, C.A., Rachlow, J.L., Johnson, T.R., Waits, L.P., 2013. Evaluating DNA degradation rates in faecal pellets of the endangered pygmy rabbit. *Molecular Ecology Resources*, 13, 654–662.
- De Barba, M., Waits, P.L., Genovesi, P., Randi, E., Chirichella, R., Cetto, E., 2010. Comparing opportunistic and systematic sampling methods for non-invasive genetic monitoring of a small translocated brown bear population. *Journal of Applied Ecology*, 47, 172–181.
- DeWoody, A.J., 2005. Molecular approaches to the study of parentage, relatedness, and fitness: practical applications for wild animals. *Journal of Wildlife Management*, 69, 1400–1418.
- DeYoung, R.W., Honeycutt, R.L., 2005. The molecular toolbox: genetic techniques in wildlife ecology and management. *Journal of Wildlife Management*, 69, 1362–1384.
- Gagneux, P., Woodruff, D.S., Boesch, C., 1997. Furtive mating in female chimpanzees. *Nature*, 387, 358–359.

- King, S.R.B., Schoenecker, K.A., Fike, J.A., Oyler-McCance, S.J., 2018. Long-term persistence of horse fecal DNA in the environment makes equids particularly good candidates for noninvasive sampling. *Ecology and Evolution*, 8, 4053–4064.
- Kopatz, A., Kleven, O., Flagstad, Ø., 2020. Seasonal variation of success in DNA- extraction from brown bear fecal samples. Trondheim. Available from <https://brage.nina.no/nina-xmlui/bitstream/handle/11250/2640529/1775.pdf?sequence=1>.
- López-Alfaro, C., Robbins, C.T., Zedrosser, A., Nielsen, S.E., 2013. Energetics of hibernation and reproductive trade-offs in brown bears. *Ecological Modelling*, 270, 1–10.
- Lüdtke, D., 2018. ggeffects: Tidy data frames of marginal effects from regression models. Available from <https://doi.org/10.21105/joss.00772> (accessed November 19, 2020).
- Miquel, C., Bellemain, E., Poillot, C., Bessière, J., Durand, A., Taberlet P., 2006a. Quality indexes to assess the reliability of genotypes in studies using noninvasive sampling and multiple-tube approach. *Molecular Ecology Notes*, 6, 985–988.
- Miquel C., Bellemain E., Poillot J., Bessiere J., Durand A., Taberlet P., 2006b. Quality indexes to assess the reliability of genotypes in studies using noninvasive sampling and multiple-tube approach. *Molecular Ecology Notes*, 6, 985–988.
- Mumma, M.A., Adams, J.R., Zieminski, C., Fuller, T.K., Mahoney, S.P., Waits, L.P., 2016. A comparison of morphological and molecular diet analyses of predator scats. *Journal of Mammalogy*, 97, 112–120.
- Murphy, M.A., Kendall, K.C., Robinson, A., Waits, L.P., 2007. The impact of time and field conditions on brown bear (*Ursus arctos*) faecal DNA amplification. *Conservation Genetics*, 8, 1219–1224.
- Murphy, M.A., Waits, L.P., Kendall, K.C., 2003. The influence of diet on faecal DNA amplification and sex identification in brown bears (*Ursus arctos*). *Molecular Ecology*, 12, 2261–2265.
- Panasci, M., Ballard, W.B., Breck, S., Rodriguez, D., Densmore, L.D., Wester, D.B., Baker, R.J., 2011. Evaluation of fecal DNA preservation techniques and effects of sample age and diet on genotyping success. *Journal of Wildlife Management*, 75, 1616–1624.
- Piggott, M.P., 2004. Effect of sample age and season of collection on the reliability of microsatellite genotyping of faecal DNA. *Wildlife Research*, 31, 485–493.
- Piggott, M.P., Taylor, A.C., 2003. Extensive evaluation of faecal preservation and DNA extraction methods in Australian native and introduced species. *Australian Journal of Zoology*, 51, 341–355.
- R Core Team., 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
- RStudio Team, 2020. RStudio: Integrated Development for R. RStudio, PBC, Boston, MA. url: <http://www.rstudio.com/>.
- Santini, A., Lucchini, V., Fabbri, E., Randi, E., 2007. Ageing and environmental factors affect PCR success in wolf (*Canis lupus*) excremental DNA samples. *Molecular Ecology Notes*, 7, 955–961.
- Skrbinšek, T., Jelenčič, M., Waits, L., Kos, I., Trontelj, P., 2010. Highly efficient multiplex PCR of noninvasive DNA does not require pre-amplification. *Molecular Ecology Resources*, 10, 495–501.
- Skrbinšek, T., Luštrik, R., Majjić-Skrbinšek, A., Potočnik, H., Kljun, F., Jelenčič, M., Kos, I., Trontelj P., 2019. From science to practice : genetic estimate of brown bear population size in Slovenia and how it influenced bear management. *European Journal of Wildlife Research*, 65, 1–15.
- Taberlet, P., Griffin, S., Goossens, B., Questiau, S., Manceau, V., Escaravage, N., Waits, L. P., Bouvet, J., 1996. Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research*, 24, 3189–3194.
- Taberlet, P., Waits, L. P., Luikart, G., 1999. Noninvasive genetic sampling: look before you leap. *Trends in Ecology & Evolution*, 14, 323–327.
- Waits, L.P., Paetkau, D.W., 2005. Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection. *Journal of Wildlife Management*, 69, 1419–1433.
- Wickham, H., 2016. *ggplot2: elegant graphics for data analysis*. Springer.

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Articles and notes should be submitted in English, or as an exception in Slovene if the topic is very local. As a rule, congress and association news will appear in Slovene.

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The manuscripts should be sent exclusively in electronic form. The format should be Microsoft Word (*.doc) or Rich text format (*.rtf) using Times New Roman 12 font with double spacing, align left only and margins of 3 cm on all sides on A4 pages. Paragraphs should be separated by an empty line. The title and chapters should be written bold in font size 14, also Times New Roman. Possible sub-chapter titles should be written in italic. All scientific names must be properly italicized. Used nomenclature source should be cited in the Methods section. The text and graphic material should be sent to the editor-in-chief as an e-mail attachment. For the purpose of review the main *.doc or *.rtf file should contain figures and tables included (each on its own page). However, when submitting the manuscript the figures also have to be sent as separate attached files in the form described under paragraph 10. All the pages (including tables and figures) have to be numbered. All articles must be proofread for professional and language errors before submission.

A manuscript element checklist (For a manuscript in Slovene language the same checklist is appropriately applied with a mirroring sequence of Slovene and English parts):

English title – (Times New Roman 14, bold)

Slovene title – (Times New Roman 14, bold)

Names of authors with clearly indicated addresses, affiliations and the name of the corresponding author – (Times New Roman 12)

Author(s) address(es) / institutional addresses – (Times New Roman 12)

Fax and/or e-mail of the corresponding author – (Times New Roman 12)

Keywords in English – (Times New Roman 12)

Keywords in Slovene – (Times New Roman 12)

Running title – (Times New Roman 12)

Abstract in English (Times New Roman 12, title – Times New Roman 14 bold)

Abstract in Slovene – (Times New Roman 12, title – Times New Roman 14 bold)

Introduction – (Times New Roman 12, title – Times New Roman 14 bold)
Material and methods – (Times New Roman 12, title – Times New Roman 14 bold)
Results – (Times New Roman 12, title – Times New Roman 14 bold)
Discussion – (Times New Roman 12, title – Times New Roman 14 bold)
Summary in Slovene – (Times New Roman 12, title – Times New Roman 14 bold)
Figure legends; each in English and in Slovene – (Times New Roman 12, title – Times New Roman 14 bold, figure designation and figure title – Times New Roman 12 bold)
Table legends; each in English and in Slovene – (Times New Roman 12, title – Times New Roman 14 bold, table designation and table title – Times New Roman 12 bold)
Acknowledgements – (Times New Roman 12, title – Times New Roman 14 bold)
Literature – (Times New Roman 12, title – Times New Roman 14 bold)
Figures, one per page; figure designation indicated top left – (Times New Roman 12 bold)
Tables, one per page; table designation indicated top left – (Times New Roman 12 bold)
Page numbering – bottom right – (Times New Roman 12)

15. Peer Review

All Scientific Articles shall be subject to peer review by two experts in the field (one Slovene and one foreign) and Brief Note articles by one Slovene expert in the field. With articles written in Slovene and dealing with a very local topic, both reviewers will be Slovene. In the compulsory accompanying letter to the editor the authors must nominate one foreign and one Slovene reviewer. However, the final choice of referees is at the discretion of the Editorial Board. The referees will remain anonymous to the author. The possible outcomes of the review are: 1. Fully acceptable in its present form, 2. Basically acceptable, but requires minor revision, 3. Basically acceptable, but requires important revision, 4. May be acceptable, but only after major revision, 5. Unacceptable in anything like its present form. In the case of marks 3 and 4 the reviewers that have requested revisions have to accept the suitability of the corrections made. In case of rejection the corresponding author will receive a written negative decision of the editor-in-chief. The original material will be erased from the ABS archives and can be returned to the submitting author on special request. After publication the corresponding author will receive the *.pdf version of the paper.

