ESSENTIAL OIL YIELD AND COMPOSITION REFLECT BROWSING DAMAGE OF JUNIPERS

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Abstract – The impact of browsing on the vegetation is dependent on the relative density and species composition of browsers. Both herbivore density and plant damage can be either site specific or change seasonally and spatially. For juniper (*Juniperus communis*) forests of a sand dune region of Hungary it was therefore assumed that plant damage investigated at different temporal and spatial scales would reflect selective herbivory. The level of juniper damage was tested for a possible correlation with the concentration of plant secondary metabolites (PSMs) in the plants, and its seasonal changes in browsing pressure. Heavily browsed and non-browsed junipers were also assumed to differ in their chemical composition and the spatial distribution of browsing damage within each forest was also analyzed to reveal the main browser. It was also expected that long-term differences in local browsing pressure would be reflected in site-specific age distributions of distant juniper populations. The results showed that concentrations of PSMs (essential oils) varied significantly among junipers and seasons. Heavily browsed shrubs contained the lowest oil yield and it was highest in shrubs bearing no damage, indicating that PSMs might have contributed to the reduced browsing in the undamaged shrubs. Yield of essential oil showed a seasonal fluctuation that was lower in the summer period than in other seasons. An additional gas chromatography (GC) assay showed differences in certain components of essential oil, indicating that certain chemicals could have contributed to the reduced consumption. The consequential long-term changes were reflected in the differences in age distribution between distant juniper forests. The results confirm that both the concentration of PSMs and specific compounds of the essential oil could play an important role in the selective browsing damage by local herbivores.

Key Words – *Juniperus communis*, Essential oil, Age distribution, Herbivory, Rabbit, Sheep

This is a post-peer-review, pre-copyedit version of an article published in Journal of Chemical Ecology.
The final authenticated version is available online at: https://doi.org/10.1007/s10886-008-9557-5

INTRODUCTION

Many plants contain secondary metabolites (Karaman et al., 2001) that can be harmful for plant pathogens and bacteria (Dorman and Deans, 2000) and also for herbivores (Freeland and Janzen, 1974; Miller et al., 2005). The effect of plant secondary metabolites (PSMs) depends on the sensitivity of the herbivore but also on its capacity of detoxification (Freeland and Janzen, 1974; Iason and Villalba, 2006; Miller et al., 2005) that is influenced both by the quantity and composition of PSMs (Villalba and Provenza, 2005). These can vary with habitat and season (Gomez et al., 2003; Vourc'h et al., 2001)), as well as among individuals (Miller et al., 2005). In a study on the impact of snowshoe hare (*Lepus americanus*) on birch (*Betula pendula*), the concentration of tannins in the plants was found to increase during growth and also to be induced by the browsing of herbivores (Haukioja and Neuvonen, 1985).

The impact of herbivory is certainly dependent on the relative density and species composition of browsers (Mysterud, 2006). Both herbivore density and plant damage can be either site specific or change seasonally, and even spatially. Larger bodied mammals mostly affect the vegetation around farms and corrals, where overgrazing is common (Wezel and Bender, 2004). Large scale variation in plant damage was correlated with herbivore density (Tsujino and Yumoto, 2004). Herbivores living in certain patch types may cause extensive damage in nearby plant associations as rabbits do in grasslands surrounding junipers (Villafuerte and Moreno, 1997) resulting in a short scale patchiness.

In previous studies, we focused on the juniper-poplar forests of a sandy region of Hungary. The differences in the vegetation could be attributed partly to local and seasonal changes in browsing pressure (Kertesz et al., 1993) by either the European rabbit (*Oryctolagus cuniculus*) or domestic sheep (*Ovis aries*) as the density of other browsers was negligible in the juniper forests (Katona and Altbacker, 2002). Earlier studies showed that browsing habits of the main herbivores differ by sites, as junipers (*Juniperus communis*) were browsed by rabbits at the Bugac site during the winter period exclusively (Matrai et al., 1998), by sheep but not the rabbits at the Orgovány site. Junipers were seemingly intact at Bócsa site, even though both rabbits and sheep were present in the area. Traditional sheep grazing, mostly occurring from pens surrounding the forest was restricted to the spring and autumn period, and sheep grazing was discontinued by the authorities of Kiskunság National Park in 2001. This change in the management and recent collapse of the rabbit population (Katona and Altbacker, 2002) resulted in a reduced browsing pressure. As the regeneration of juniper shrubs is relatively slow, comparing signs of herbivory in local juniper stands enabled us to evaluate the distribution of PSMs without current confounding impacts such as induced responses in damaged branches (Miller et al., 2005; Nagy et al., 2000). Assuming that present plant damage still reflects previous browsing pressure, we described spatial and temporal pattern of the morphological and chemical constituents. The following hypotheses were tested: (1.) the level of juniper damage should correlate negatively with the essential oil yield; (2.) oil yield should change by season in accordance with browsing pressure; (3.) heavily browsed and non-browsed junipers should differ in their chemical composition; (4.) browsing damage reveals the main browser: it should be lowest in the middle of the forest if junipers were eaten by sheep, or highest if rabbits were the main agents shaping the shrubs; (5.) long term differences in local browsing pressure will be reflected in site-specific age distributions of juniper populations.

METHODS AND MATERIALS

Study Area The study was conducted in the sand dunes the Kiskunság National Park, central Hungary (Figure 1). Mosaics of juniper patches and intermittent sandy grasslands form the semidesert vegetation categorized as Juniper-poplar forests (Kertesz et al., 1993). The focus area of Orgovány (46°47’30”N; 19°27’34”E) was chosen for analyzing herbivore-juniper interactions. For a broader scale analysis, we selected an additional two shrubland areas: Bugac (46°38'58"N; 19°36'38"E), and Bócsa (46°38'56"N; 19°27'31"E), where the age distribution of juniper stands was analyzed for a comparison (see below).

Classification of browsing damage level The shape of damaged junipers was studied at the area of Orgovány of Kiskunság National Park (see Figure 1), Browsing damage was classified from the ground to a height 100 cm on a scale of 1-5 from non-browsed to heavily browsed (Figure 2). Junipers were labeled as non-browsed when damage (shape distortion) was between 0-20 %; mildly browsed when damaged between 21-40 %; medium-browsed when damaged between 41-60 %; well-browsed when damaged between 61-80 %; and heavily browsed if damage was between 81-100 % of the bottom part of the shrub.

Seasonal Pattern of Essential Oil Yield 10-10 randomly chosen independent juniper shrubs were chosen in July and October in 2003, as well as February and May in 2004, two nonadjacent shrubs per damage level category. The height of these shrubs (2-2.5m) and their gender (female) were similar. The samples, 100-120 g of foliage after removal of berries, were 10 cm long tips of new branches collected from the same height (about 1 m) of each shrub. Plant material was gathered in plastic bags that were immediately placed in a cooler (5 °C), until they were oven dried at 37±3˚C (Memmert U-type oven). For the extraction of oil yield, the method described by Pharmacopoea Hungarica (Vegh, 1986) was used with the modification of shortening the time of steam distillation to 1.5 hours because of the negligible (less than 5 % of the total) additional yield afterwards (Bernath, unpublished data). For this, 40-50 g of dried samples chopped into 2-3 cm pieces were distilled with a Clevenger-apparatus constructed according to Vegh (1986). Statistical analysis was made by two way repeated measures ANOVA using damage level as a fixed factor and seasons as a repeated factor.

Determination of Essential Oil Composition by Gas Chromatography Plant material was collected during February 2004 from the Orgovány area of KNP. Heavily browsed and non-browsed shrubs (N=10-10) were selected randomly from the most browsed part of the forest. The terminal 10-15 cm of foliage was clipped from several branches from ground level to a height of 2 m. The preparation of the samples and the extraction of essential oil was the same as described above. The chemical composition of essential oils extracted from the above 10-10 shrubs was analyzed using a Shimadzu 14B gas chromatograph equipped with a FID detector and SE-30 capillary column (30 m × 0.25 mm, and 0.25 mm film thickness). The carrier gas was nitrogen (1 ml min^{-1}), and the split ratio was 75/1. The temperature of the oven was programmed as follows: 110 ˚C for 3 min, up to 220 ˚C at 8 ˚C min^{-1} (5 min), 21.75 min analysis time, IB 220 ˚C and detection 250˚C. Data were analyzed using Shimadzu Class VP Chromatography Data System (Shimadzu, Japan) software. Relative percentage of the oil constituent was calculated from the GC (FID) peak areas in percent of the total area by using the area normalization method (Novak et al., 2001, 2003). The identification of compounds was performed by peak enrichment with GC-standards by Carl Roth (Karlsruhe, Germany). Statistical difference of essential oil components of heavily browsed and non-browsed samples was tested by *Student (t) tests*.

Effect of Grazing Pressure on Juniper The spatial distribution of grazing damage was analyzed at the Orgovány Juniper Forest of Kiskunság National Park (see Figure 1). Here traditional sheep grazing was discontinued in 2001, and rabbits also disappeared from the area in 1995. From the spatial distribution of signs of browsing indicated by the shape of shrubs, we investigated if browsing was more pronounced at the periphery or in the middle of the forest. For this, three plots were defined approximately 100 m in diameter. They were established along a transect between the last active sheep pen found at the border of juniper forest and the middle of the juniper forest. Within each plot the shape of junipers (N=100-100) was classified by using a spiral sampling from the centre point. We expected a decreasing trend if sheep were the most important agents shaping the shrubs, an opposite trend if rabbits inhabiting the central part of the forest browsed most intensively, or an inverse U-shaped spatial pattern if both species contributed to browsing damage. For the analysis of data, the Mantel-Haenszel χ^2 test was used for linear trend.

Long-term Consequences of Juniper Browsing The age distribution of juniper shrubs was evaluated in three separate forests (Bugac, Bócsa, Orgovány) of the Kiskunság National Park in May 2003. At each site, the basal diameter of the stems of 100-100 plants was measured around 5-5 randomly chosen points using spiral sampling (altogether 500 individuals per area). The diameter of the stems was measured with a slide caliper (MEBA, Croatia) at a height of 10 cm. Polished cross-sections of dead junipers (N=13) of different diameter were prepared for counting annual rings to calculate age. Data were compared by sites as a fixed factor and age categories as a repeated factor using two-way repeated measures ANOVA followed by *Duncan* post-hoc tests.

RESULTS

Seasonal Pattern of Essential Oil Yield of Differently Browsed Junipers

The relationship between the oil yield and the damage level and its seasonal pattern is shown in Figure 3. The damage level was inversely proportional to the essential oil yield. The analysis of oil yield, done with a two-way repeated measures ANOVA with *Duncan* post-hoc tests, indicated a significant effect of browsing level ($F(4,5)=5.636$, $P=0.027$) and season ($F(3,15)=48.242$, $P<0.001$), without a significant interaction ($F(12,15)=1.701$, $P=0.164$), suggesting a similar seasonal change in the different browsing level categories. Oil yield was lower in the summer period than in the other seasons.

Essential Oil Components of Heavily Browsed and Non-browsed Juniper Shrubs

Essential oil components of heavily browsed and non-browsed juniper shrubs (Figure 4) were tested with *Student t-tests* using *the Welch-correction*). Significant differences were found between the following chemical constituents of the essential oil extracted by GC: α-pinene ($t(10)=2.187$, $P=0.05$); δ-3-carene ($t(9)=3.061$, $P=0.01$); β-myrcene ($t(17)=2.111$, $P=0.04$); and other unidentified components designated by their retention time 11.14 ($t(12)=2.488$, $P=0.02$); and 12.22 ($t(12)=2.405$, $P=0.03$). In some cases the concentration of the chemical was lower (δ-3-carene and β-myrcene), and sometimes higher (α-pinene) in the browsed shrubs than in the undamaged junipers.

Browsed Shrubs and Herbivore Pressure

The frequency of browsed shrubs was compared on three plots (Figure 5). There was a significant decrease in the ratio of heavily browsed shrubs toward the centre of the shrubland (Mantel-Haenszel χ^2 test for linear trend: $\chi^2(2)=46.143$; $P<0.001$). The majority of shrubs were browsed on the plot closest to the formerly active sheep pen indicating a high browsing pressure by sheep. There was much less damage evident deep in the forest, indicating that it was mainly browsing by sheep rather than by rabbits which contributed to the shaping of junipers at Orgovány.

Long-term Consequences of Juniper Browsing

We determined the age of junipers measuring their stem diameter. The linear regression equation of $y=0.386x+4.97$ described a significant stem-age function ($R^2=0.87$, $P<0.001$) and was used to calculate age depicted in Figure 6. We compared the frequency distributions using a two-way repeated measures ANOVA followed by *Duncan* post-hoc tests for all pairwise comparisons, and found that the age distributions were significantly different among the areas ($F(2,12)=0.417$, $P<0.0001$), age classes ($F(7,84)=0.279$, $P<0.0001$) and the interaction between areas and age classes ($F(14,84)=0.112$, $P<0.0001$). Further analyses of site differences within age classes showed that the juniper stand at Bugac is different from the other two populations. The juniper population at Bugac lacks young individuals, as there is a decreased proportion for age classes less than 20 years, and is older than the other two stands. Junipers at Bócsa and Orgovány seem to form stable populations with a linear decrease by age.

DISCUSSION

For juniper forests in Hungary it was assumed that plant damage investigated at different temporal and spatial scales would reflect selective herbivory. The level of juniper damage was tested for a possible correlation with the concentration of plant secondary metabolites (PSMs) in the plants, and its seasonal changes in browsing pressure, as plant damage can reflect this latter according to herbivore density (Tsujino and Yumoto, 2004). Assuming that both the appearance and chemical composition of shrubs reflect grazing pressure, we found support for several predictions of plant animal interaction. A diminishing impact toward the middle of the forests was expected in areas of sheep browsing damage. In contrast, an opposite spatial distribution of grazing damage should be found in rabbit-dense areas, where damage is concentrated deep in the forest, as rabbits feed around their warrens and avoid open areas (Moreno et al., 1996). It was found that the level of damage was highest close to a sheep pen (the activity centre for the pastoral grazing system) whereas no evidence of rabbit browsing was present (Figure 5).

Variation in the shape of juniper shrubs corresponded to their chemical composition (Figure 4), with truncated shrubs containing the lowest oil yield. This indicates that plant secondary compounds might have lead to avoidance of the undamaged shrubs (Provenza et al., 2003). Concentrations of volatile oils varied significantly among junipers and seasons (Figure 3). Even though our simplified method of essential oil extraction could underestimate the total oil yield (Milojevic et al., 2008; Venskutonis, 1997), differences among shrubs showing high or low damage levels are still meaningful. Certain chemicals might have contributed to the reduced consumption as browsed junipers contained essential oil with elevated levels of terpenoids (Figure 4) similarly to conifers (Vourc'h et al., 2002; Vourc'h et al., 2001). In our case, the most frequent constituents, α-pinene and sabinene, were even more concentrated in the damaged plants than in the avoided ones. It should be noted that those plant individuals poor in α-pinene were rich in δ-3-carene, β-myrcene, and borneol, whose concentration was high in damaged shrubs. This suggests that complex features of oil composition and not the concentration of single components could explain variation in browse damage. Sheep avoided food when exposed to the odor of α-pinene or a mixture of monoterpenes (Narjisse et al., 1996), and lambs exposed to odors of two monoterpenes (camphor and α-pinene) reduced food intake (Estell et al., 2005). The strong selective pressure by black-tailed deer (*Odocoileus hemionus sitkensis*) contributed to the persistence of individual seedlings with high concentrations of chemical defenses (Vourc'h et al., 2001). In a semiarid environment like our study site, a considerable portion of aromatic plants might contain high levels of monoterpenoids that interfere with digestion (Carpenter et al., 1979). A ten-fold increase of δ-3-carene was found in the non-browsed junipers of Orgovány. In other studies, monoterpenes were either positively (like cymene) or negatively (including α-pinene, sabinene and β-pinene, myrcene, terpineol) correlated to the juniper intake of goats (Riddle et al., 1996). High avoidance was detected for α-pinene, myrcene and sabinene in black-tailed deer (Vourc'h et al., 2002). The anti-herbivore defense of red cedars (*Thuja plicata*) was directly proportional to the level of herbivory, as the essential oil yield and quality were adapted to the feeding pressure (Vourc'h et al., 2001).

The production of PSMs could be coupled seasonally with that of herbivore browsing. Seasonal changes in essential oils have been well documented for many coniferous species

(Nerg et al., 1994; Riddle et al., 1996). There was a strong seasonal effect on volatile oil concentration, where concentrations were greatest during the winter and spring months (January through May) and lowest during the summer and fall in leaves of ashe juniper (*J. ashei*) and redberry juniper (*J. pinchottii*) (Owens et al., 1998). As reported for thyme (*Thymus vulgaris*), its essential oil yield was highest in the middle of the summer (Badi et al., 2004), when browsing by goats is probably most intensive (Riddle et al., 1996). Production of volatile terpenoids such as monoterpenes, sesquiterpenes and homoterpenes can be induced by herbivore feeding (Arimura et al., 2005), a mechanism well studied in conifers (Martin et al., 2003; McKay et al., 2003; Miller et al., 2005; Trapp and Croteau, 2001). In the present study, juniper oil content was lowest in the summer period. The high concentration of essential oils in the other seasons is remarkable, as sheep were present in the spring and autumn period due to management rules, and based from feces analysis, rabbits most likely browsed junipers in winter (Matrai et al., 1998). As browsing by the main browsers were no longer present in the study area in the present sampling period, the induced plant protection system known for conifers was unlikely to be responsible for the seasonal changes observed. It is more likely that pre-existing individual differences in PSMs production resulted in various levels of browsing damage and that was still obvious at the time of sampling due to slow growth of junipers.

Differences in age distribution of junipers were expected among the areas due to local herbivores affecting survival of shrubs. The difference in the age distributions of compared stands might stem from herbivore species composition and foraging behavior of local herbivores. The foraging habits of European rabbits resulted in a high mortality of young shrubs at Bugac (Matrai et al., 1998), and age distribution of juniper stands reflects that impact (see Figure 6). In spite of the recent browsing by sheep, the age distribution of the juniper population in Orgovány has remained the same as in Bócsa, where neither sheep nor rabbits consumed juniper. Sheep forage mainly in the grassy patches and only start eating juniper when grass becomes scarce. The occasional browsing of tall junipers from the side did not affect the survival of junipers and only modified their morphology. Rabbits, on the contrary, forage in well-hidden places close to shrubs and not only browse the older plants' bottom twigs, but can destroy young junipers as well as our former field experiment proved (Matrai et al., 1998). For the renewal of the juniper stands it is essential that seedlings survive, especially as *J. communis* becomes fertile only after several years and ageing results in reduced fertility (Ward, 2007). The long-term accumulation of such impacts could have resulted in the distorted age distribution of junipers at Bugac.

Herbivores may circumvent negative effects of plants by physiological (Dearing et al., 2000) or behavioral means (Provenza, 1995). The effect of plant metabolites can be reduced by several strategies, for example certain herbivores can avoid tannin-containing plants by learned avoidance (Provenza, 1995), and selective browsing minimizes the intake of harmful plants (Launchbaugh et al., 2001). Therefore, within the same plant population, individuals with a higher concentration of PSMs enjoy a relatively better protection (Bryant et al., 1985). Juniper browsing by mule deer (*Odocoileus hemionus*) was found to be inversely correlated to the essential oil yield (Schwartz et al., 1980a). This could be attributed to the oxygenated monoterpenes and sesquiterpenes in the oil that inhibit the microbial activity in the deer's rumen after the ingestion of a certain number of branches (Schwartz et al., 1980b). As Welch et al. (1983) showed the PSMs concentration was not the only determinant of food intake, more detailed studies are necessary.

Signs of a varying amount of juniper consumption were found by site and seasons. Such differences might stem either from plant toxicity, feeding responses of animals, or an interaction of both (Iason and Villalba, 2006). Thyme was one of the main components of the summer diet of the rabbits in Bugac (Matrai et al., 1998) and naive animals in captivity consumed small amounts if their mother was forced to eat thyme despite its toxicity (Altbacker et al., 1995). Rabbits could adjust their diet both by individual and social learning

(Bilko et al., 1994). With a mixed diet the accumulation of certain PSMs can be avoided and consumption increased (Marsh et al., 2006), and thereby the energy and toxin intake optimized (Bryant et al., 1985; Bryant et al., 1992; Provenza et al., 2003) as can be observed in several generalist vertebrates. Nevertheless, some specialists are physiologically better adapted to ecological constraints and have a more efficient detoxification system than generalists (Dearing et al., 2000). Experimental manipulation of chemical composition of junipers in food choice tests are currently undertaken in order to reveal how the oil content and carene concentration reduces juniper consumption. Further studies using different herbivore species and manipulating experience levels when alternative food sources are present would be valuable (Dearing et al., 2000; Villalba and Provenza, 2005). Substantial changes in the grazing regime are considered as the major force of succession in semidesert shrublands (Evans et al., 2006). Understanding the mechanisms contributing to the interaction of plant defenses and browsing behavior of local herbivores is increasingly important.

Acknowledgments – We are grateful to István Németh for his contribution to the fieldwork. We thank the Kiskunság National Park for the support of our field surveys. This manuscript was greatly improved due to valuable suggestions of two anonymous reviewers. This study was supported by the Hungarian Scientific Research Foundation (OTKA T 29703) and by the National Research and Development Program (NKFP 3B-0008/2002).

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This is a post-peer-review, pre-copyedit version of an article published in Journal of Chemical Ecology. The final authenticated version is available online at: https://doi.org/10.1007/s10886-008-9557-5

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FIGURES

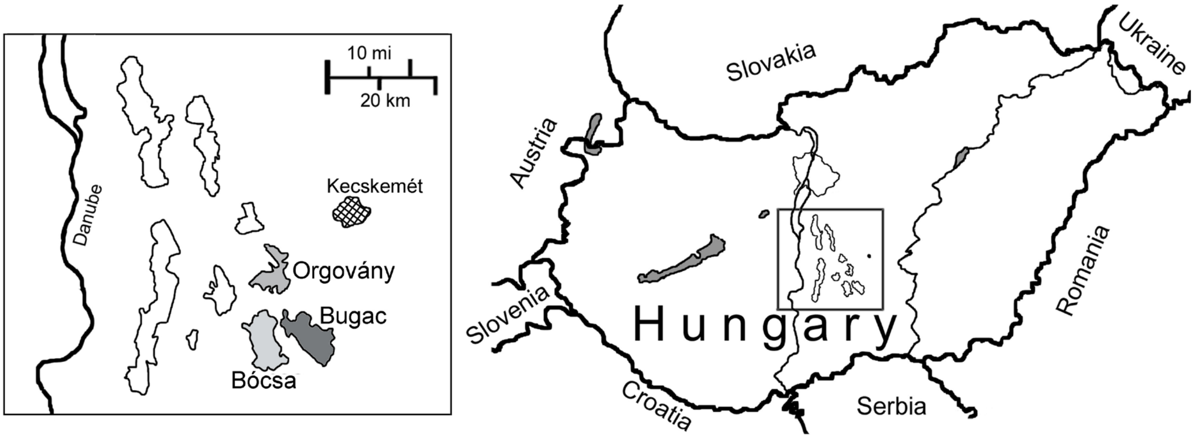

Figure 1. Location of our study areas, Orgovány, Bugac and Bócsa belonging to the Kiskunság National Park, Hungary.

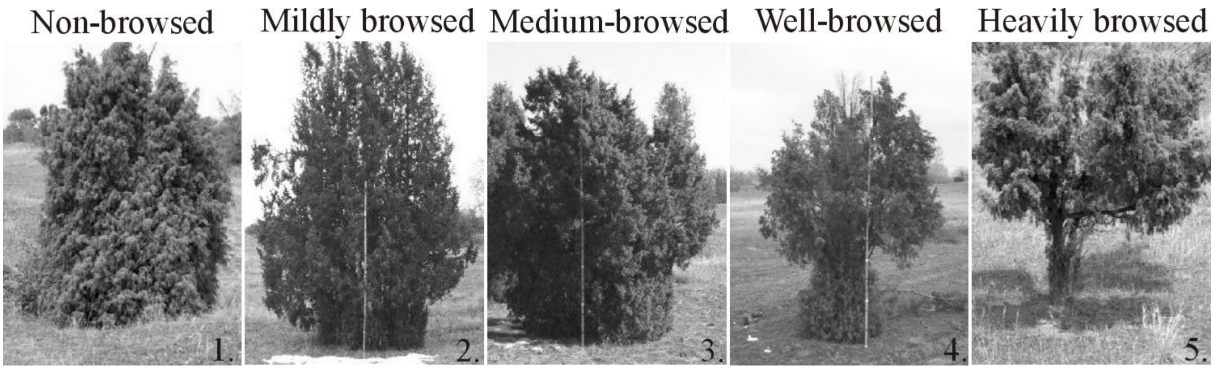

Figure 2. Classification of browsing damage level of junipers. Pictures show 1. non-browsed (damage between 0-20 %); 2. mildly browsed (damage between 21-40 %); 3. medium-browsed (damage between 41-60 %); 4. well-browsed (damage between 61-80 %); 5. heavily browsed (damage between 81-100 %) shrubs.

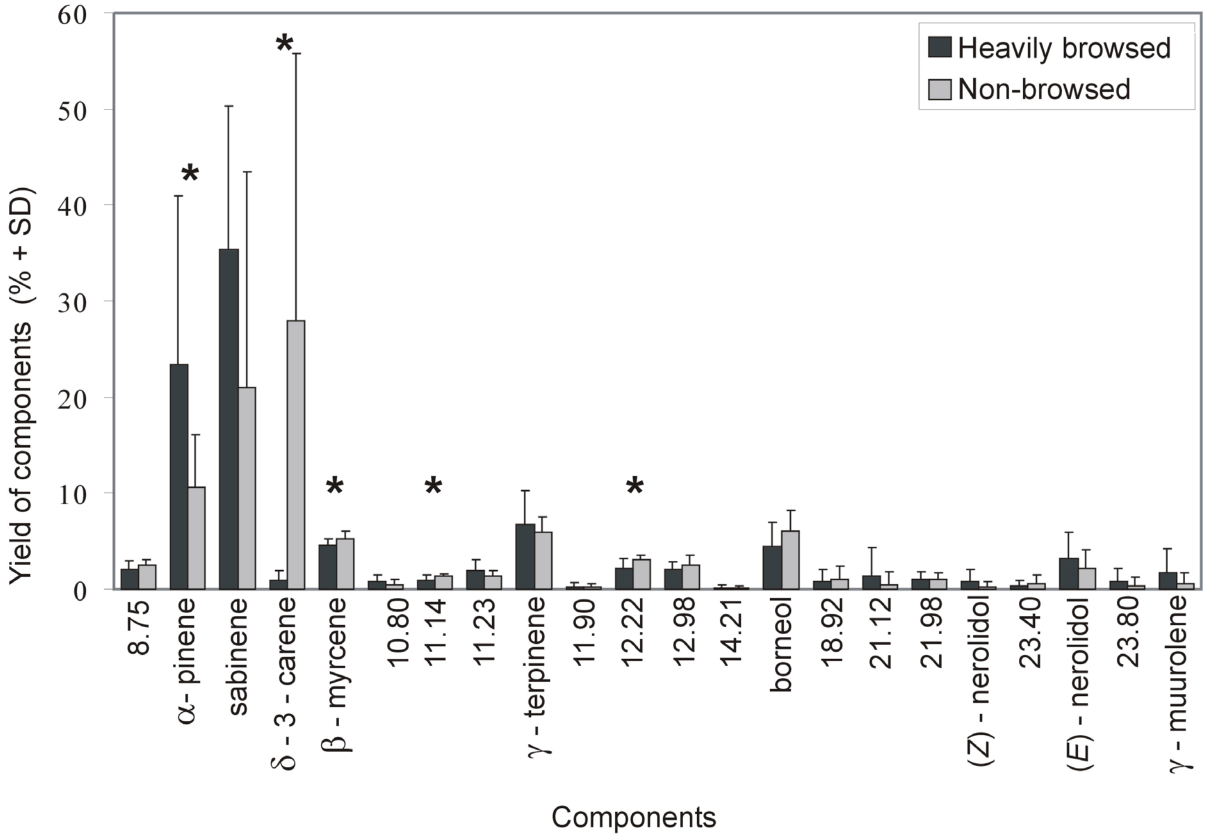

Figure 3. Seasonal pattern of essential oil yield of junipers (N =2–2) belonging to different browsing damage levels. Mean values were tested for significant differences (P < 0.05) by two-way repeated measures ANOVA followed by *Duncan* post-hoc tests for all pairwise comparisons. Significant differences among damage level categories are indicated by different letters, while differences among seasons are labeled by asterisks (* - *P*<0.05; ** - *P*<0.01; *** - *P*<0.001).

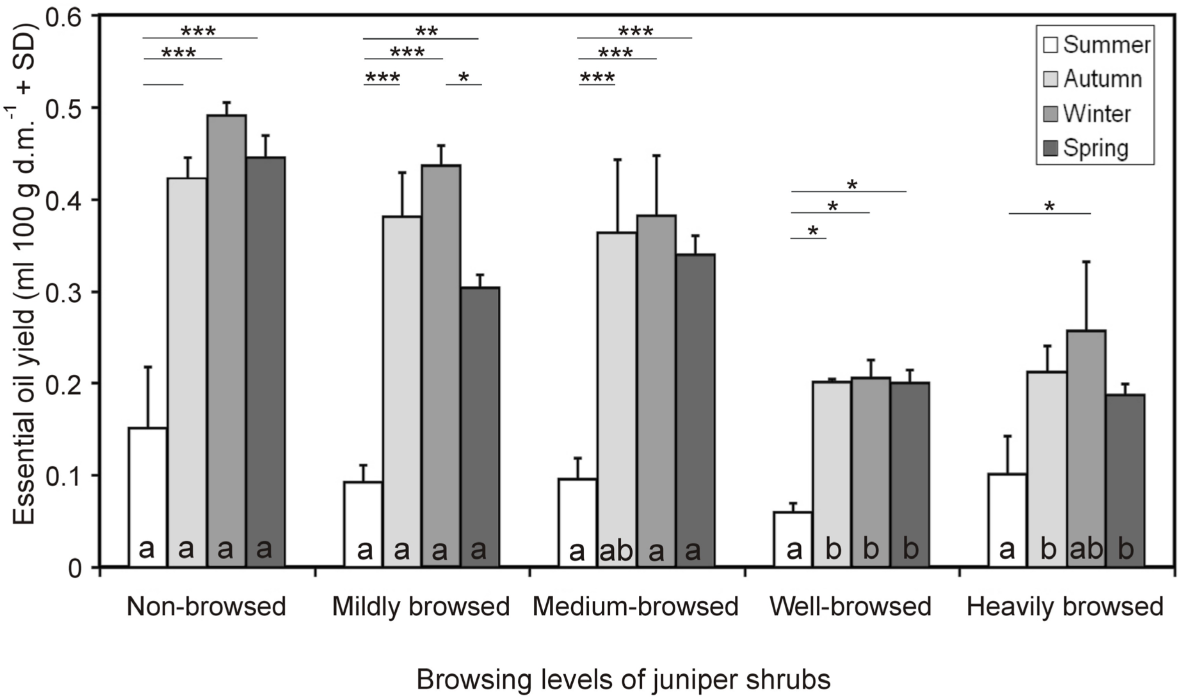

Figure 4. Quantification of essential oil components of heavily browsed and non-browsed juniper shrubs (*N*=10-10) from the Orgovány area as determined by gas chromatography. The identification of the oil components was based on a comparison of GC-standards by Carl Roth (Karlsruhe, Germany). The percentage of components was computed from GC (FID) peak areas by using the area normalization method. Bars represent mean values + SD. Statistical difference of essential oil components of heavily browsed and non-browsed samples was verified by *Student (t) tests with Welch-correction*. Asterisks (*) label significant differences (P<0.05).

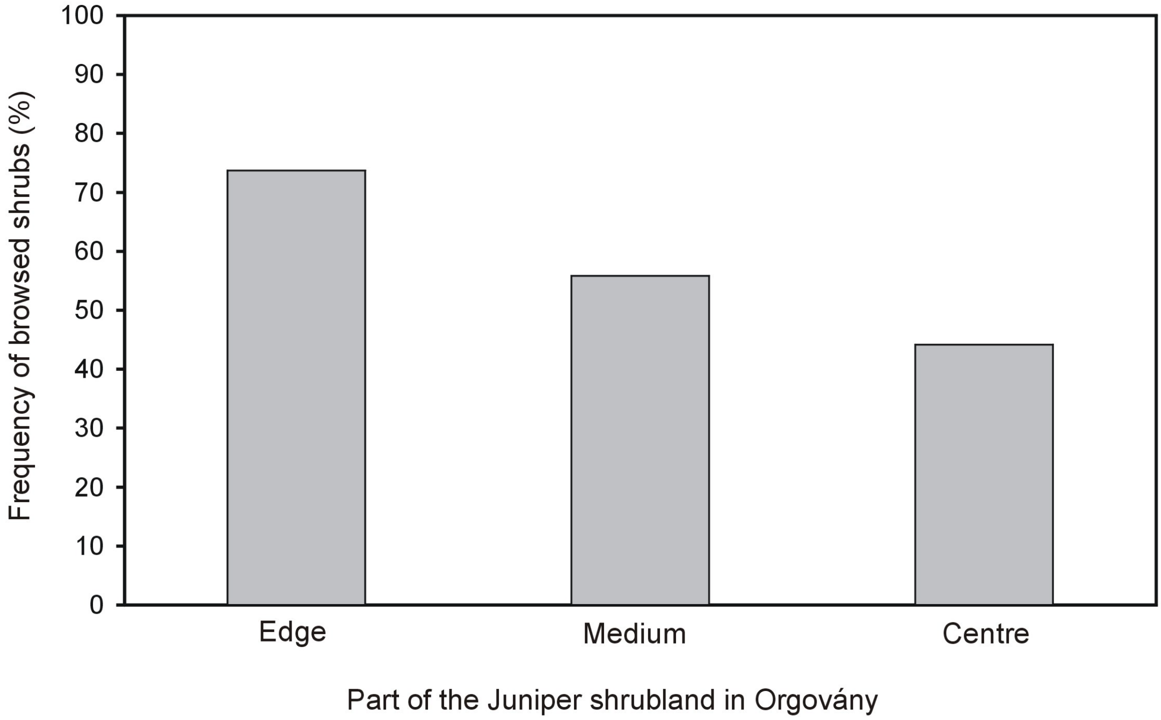

Figure 5. Spatial distribution of damaged junipers along an axis from edge to centre of the juniper shrubland at Orgovány. Bars represent frequency of juniper shrubs with any signs of browsing determined in one hectare plots. In each plot, 100 individuals were classified. Mantel-Haenszel χ^2 test for linear trends showed damage frequency to decrease from edge to centre, indicating that distance from the sheep fold was more important than presence of rabbits in determining browsing damage.

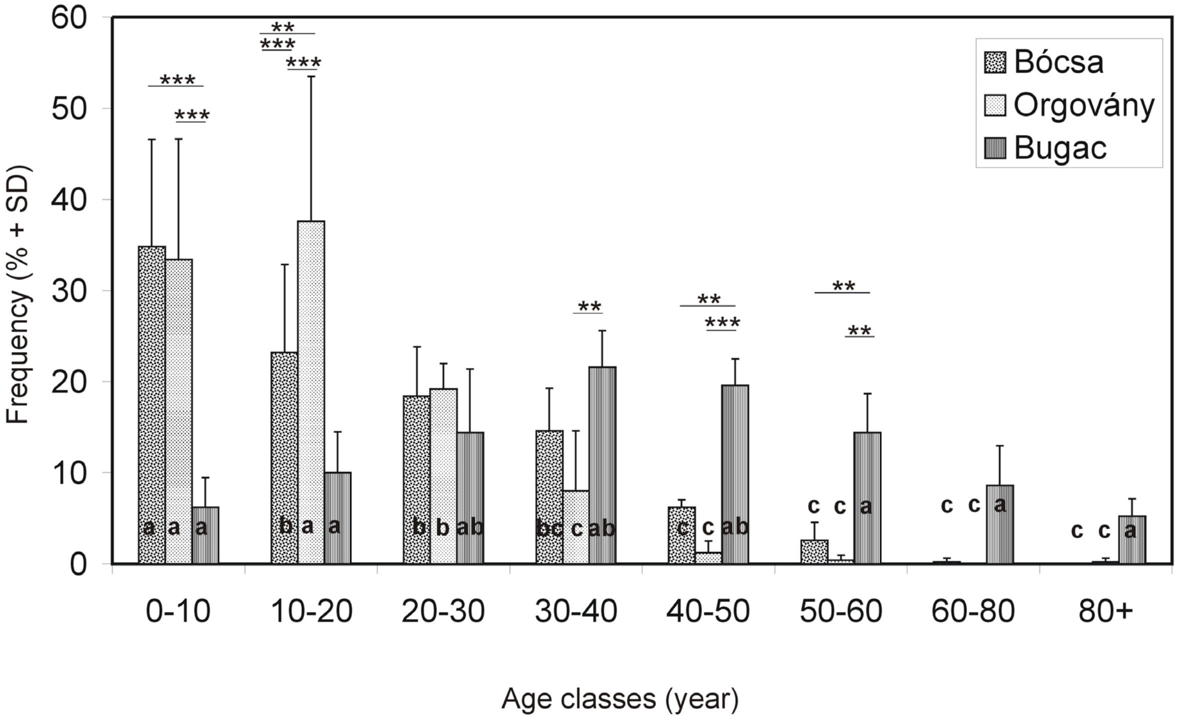

Figure 6. Age structure of *J. communis* populations in the three study areas (Bócsa, Orgovány and Bugac) of the Kiskunság National Park, represented as the percentage of individuals belonging to the corresponding age class. In each area, the age distribution was determined from 5 different plots where stem diameter of 100-100 individuals was measured. Age was determined using the regression equation y=0.386x+4.97 based on counting age rings for 13 reference stems collected in the same locality. Mean values were tested for significant differences (P < 0.05) by two-way repeated measures ANOVA followed by *Duncan* post-hoc tests for all pairwise comparisons. Significant differences among age classes are indicated by different letters, while differences among areas are labeled by asterisks (* - *P*<0.05; ** - *P*<0.01; *** - *P*<0.001).