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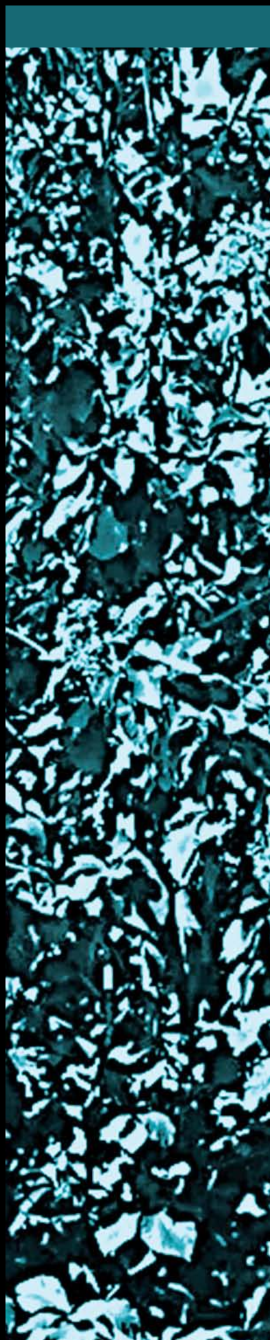

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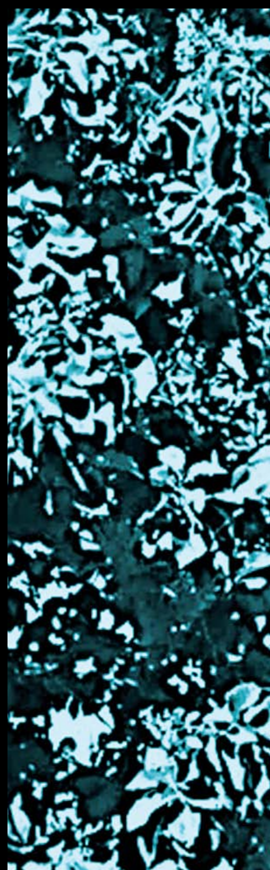

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Effect of ultrasonic algae control devices on non-target organisms: a review

Vpliv ultrazvoka za zaviranje rasti alg na netarčne organizme – pregled literature

Pija Klemenčič[a], Aleksandra Krivograd Klemenčič[b*]

[a]Čušperk 51, SI-1290 Grosuplje, Slovenija
[b]Faculty of Civil and Geodetic Engineering, University of Ljubljana, Hajdrihova 28, SI-1000 Ljubljana, Slovenia

* Correspondence: aleksandra.krivograd-klemencic@fgg.uni-lj.si

Abstract: There is an increasing interest in using ultrasonication in controlling algal (cyanobacterial) blooms and biofouling, a physical method with presumably no adverse effects on non-target organisms, such as fish and zooplankton. At the beginning the use of ultrasound (US) to control algae and biofouling has focused on high-power US causing cavitation; however, due to the potential damage to non-target organisms including marine mammals and human divers, high-power US causing cavitation are not used anymore for algae control in natural environment. Current ultrasonic algae control devices use low-power and thus control algae and biofouling by utilising resonance frequencies and the sound pressure caused by a sound wave propagating through a water column. There are only few studies existing on the effect of US on non-target organisms with incomplete information on wavelengths and intensities of US devices. However, we can conclude that non-cavitation US devices used to control algae and reduce biofouling had no adverse health effects on studied fish species with no feeding and behaviour changes noticed. Caution should be taken when installing US devices in marine locations since they may interfere with communication between sea mammals or may cause adverse effects on fish from subfamily Alosinae, the only known fish able to detect US. The studies dealing with non-cavitation US used to control algae and biofouling on non-target zooplankton have conflicting results from high mortality to no evident effects. Therefore, caution should be taken when using US for counteract algal growth in ponds or lakes, especially in terms of zooplankton and natural balance maintenance.

Key words: algal blooms, algal control, fish, ultrasound, zooplankton

Izvleček: V zadnjih letih narašča zanimanje za uporabo ultrazvoka (UZ) za nadzor prekomerne razrasti alg (cianobakterij) v vodnih telesih in tvorbe biofilma na plovilih, hladilnih napravah in drugih industrijskih objektih. UZ deluje po fizikalnih načelih in po trditvah proizvajalcev nima negativnih vplivov na netarčne organizme, kot so ribe in zooplankton. Prvi poskusi uporabe UZ za zaviranje rasti alg in tvorbe biofilma so potekali z uporabo visoko energijskih UZ, ki povzročajo kavitacijo, vendar se je uporaba le-teh kmalu opustila zaradi morebitnih negativnih vplivov na netarčne organizme, kamor sodijo tudi morski sesalci in potapljači. Danes se za zaviranje rasti alg uporabljajo nizko energijski UZ, ki za kontrolo rasti alg in biofilma uporabljajo resonančne frekvence in zvočni tlak, ki ga povzroča zvočni val s širjenjem skozi vodni stolpec. O vplivu UZ na netarčne organizme obstaja le malo raziskav in še to z nepopolnimi informacijami o karakteristikah testiranih UZ. Kljub temu lahko povzamemo, da nizko energijski UZ, ki se uporabljajo za zatiranje alg in zmanjšanje tvorbe biofilma, nimajo škodljivih vplivov na preučevane vrste rib. Kljub temu priporočamo previdnost pri uporabi UZ v morskem okolju, saj lahko ultrazvočni valovi ovirajo komunikacijo med morskimi sesalci ali škodijo ribam iz poddružine Alosinae - edine znane ribe s sposobnostjo zaznavanja UZ. Raziskave o vplivu nizko energijskih UZ na netarčne zooplanktonske organizme kažejo nasprotujoče si rezultate, od akutnih letalnih učinkov do odsotnosti vpliva. Zato je potrebna previdnost pri uporabi UZ za zmanjševanje rasti alg v ribnikih ali jezerih, zlasti z vidika varovanja zooplanktona in s tem ohranjanja naravnega ravnovesja.

Ključne besede: kontrola alg, prekomerna namnožitev alg, ribe, ultrazvok, zooplankton

Introduction

Current methods to control algal (cyanobacterial) blooms and to reduce their (toxic) by-products include chemical or biological additives, ozonation, activated carbon filters and ultrasonic products (Jarni et al. 2017). A major concern with chemical or biological additives is their environmentally hostile impact and the possible release of toxins when cyanobacterial cells are broken which exacerbate the problem. Therefore, there is an increasing interest in using ultrasonication, a physical method, in controlling algal blooms, which is effective also in degrading the cyanotoxins (Song et al. 2005) when released into water. Although, the effect of the ultrasound (US) on algae is well studied, the knowledge on the safety of the US on non-target organisms, such as fish and zooplankton, is limited. Therefore, this paper focuses on the literature review on the effects of ultrasonication used for counteract algal growth in water bodies on non-target organisms, namely fish and zooplankton.

US, a sound with a frequency of 20 kHz or above, is beyond the limits of human hearing. According to frequency, US is divided into three categories including low frequency US with a frequency of 20–100 kHz, high frequency US with a frequency of 0.1–1 MHz and diagnostic US with a frequency of 1–500 MHz. Low frequency US is commonly used in chemically important systems in which chemical and physical changes are desired, as it has the ability to cause acoustic cavitation, a phenomenon, where US causes the formation of bubbles that implode upon themselves, causing intense heat (4,500–7,500 °C) and pressure (approximately 10,000 Bar) (Askokkumar et al. 2007). In acoustic cavitation microscopic gas bubbles that are generally present in a liquid will be forced to oscillate due to an applied acoustic field. If the oscillation amplitude is large enough cavitation bubbles may appear within the liquid bulk (Dular et al. 2016). The degree of cavitation, and thus the effect on the cell, is regulated by the frequency, intensity, and duration of the sound waves (Rajasekhar et al. 2012). Whenever low frequency and high intensity US is applied to liquids, for example in applications as sonar, industrial processing, and bio-medical research, cavitation may occur (Neppiras 1984). Lower sound pressure limit for appearance of acoustic cavitation is at sound pressure amplitude of about 2 kPa or 186 dB re 1 μPa (Margulis 1995).

US technologies are effective means of minimizing algal (cyanobacterial) blooms in freshwater bodies, minimizing biofilm formation in industrial applications, and preventing biofouling on ships (Lee et al. 2001, Zhang et al. 2006, Krivograd Klemenčič and Griessler Bulc 2010). At first the use of US to control algae has focused on high-power US causing cavitation (Lee et al. 2001, Zhang et al. 2006, Joyce et al. 2010). However, the potential damage to non-target organisms, including marine mammals and human divers, has been one of the reasons why high-power US causing cavitation is not an ideal solution for algae control in natural environment. This is why current ultrasonic algae control devices use low-power and thus do not control algae through the high pressures and temperatures associated with cavitation; but instead by utilising resonance frequencies and the sound pressure caused by a sound wave propagating through a water column (Lowe 2011). It was discovered that certain ultrasonic sound vibrations in the water produce critical resonance frequencies of algae gas vesicles, vacuoles, and plasmalemma cell lining. Exposure to these sounds cause the cell membranes to break or tear (Rajasekhar et al. 2012). Although low-power US is believed to be safer to non-target organisms the knowledge about the effects of low-power US on non-target organisms when implemented on a large scale in complex natural systems is limited.

The US algae control devices currently available on the market are operating in the range from 20–200 kHz with low-power and in contrast to US used in industry and medicine applications do not induce cavitation effect.

Effect of ultrasound on fish

Sensory systems in fish

Fishes, like other vertebrates, have a variety of different sensory systems that enable them to glean information from the world around them. Vision is often most useful when a fish is close to the source of the signal, in daylight, and when the water is clear. However, vision does not work well at night or in deep waters. Chemical signals can be highly specific (e.g., a particular pheromone used to indicate danger). However, chemical signals travel slowly in still water and diffusion of the chemicals depends upon currents. Therefore, chemical signals are not directional and, in many cases, they may diffuse quickly to a non-detectable level. As a consequence, chemical signals may not be effective over long distances (Popper 2008). In contrast, acoustic signals in water travel very rapidly, travel great distances without substantially attenuating (declining in level) in open water, and they are highly directional. Thus, acoustic signals provide the potential for two animals to communicate quickly (Zelick et al. 1999, Popper et al. 2003). Since sound is potentially a good source of information, fishes have evolved two sensory systems to detect acoustic signals, and therefore many species use sound for communication (e.g., mating, territorial behaviour) (Zelick et al. 1999). The two systems are the ear, for detection of a sound from 20 Hz to 1 kHz or more, and the lateral line for detection of hydrodynamic signals (water motion) from less than 1 Hz to 100 or 200 Hz (Zelick et al. 1999).

If a fish can hear a biologically irrelevant human-generated sound (e.g., sonar, ship noise, US for algae control) it might interfere with the ability of fish to detect other biologically relevant signals. In effect, anthropogenic sounds and explosions (e.g., detonations) may affect behaviour, and result in short and long-term tissue damage, but only at significantly high levels. Fish hearing can be affected by the presence of a background noise that is in the same general frequency band as the biologically relevant signal (Fay and Megela-Simmons 1999, Popper et al. 2003). In the case the background noise is increased due to human-generated sources it may be harder for a fish to detect the biologically relevant sounds needed for its survival (Popper 2008). According to Popper (2008) the effects of military mid- and high-frequency active sonars on fishes has not been studied yet.

Detection of ultrasound by fish

It is known that fish species are not producing communication sounds within the ultrasonic frequency range (Bass and Ladich 2008). Fish, with few exceptions from family Clupeidae described below, cannot hear sounds above 3–4 kHz, and the majority of fish species are only able to detect sounds to 1 kHz (Hawkins 1981, Fay 1988, Popper 2008). In contrast, a healthy young human can detect sounds to about 20 kHz, while dolphins and bats can detect sounds to well over 100 kHz (Popper 2008).

In 1982 it was discovered that ultrasonic sonar (about 160 kHz) caused behavioural responses in migrating *Alosa sapidissima* (Kynard and O'Leary 1990). Ten years later Nestler et al. (1992) and

Dunning et al. (1992) reported that high frequency sounds at 110–140 kHz and with high intensities (180 dB re 1 µPa) were effective in deterring two fish species belonging to the family Clupeide (subfamily of Alosine (shads)) from power plant intakes: blueback herring (*Alosa aestivalis*) and alewife (*Alosa pseudoharengus*). Mann et al. (1997) measured the audiogram of *Alosa sapidissima* which confirmed that the species could detect sound in the ultrasonic frequency range up to 180 kHz. Later behavioural and physiological studies showed that additional species belonging to the subfamily Alosine can detect and respond to US. These include gulf menhaden (*Brevoortia patronus*) (Mann et al. 2001) and two species of European shad, *Alosa fallax fallax* (Gregory et al. 2007) and *Alosa alosa* (Wilson et al. 2008). Wilson et al. (2011) found out that the response of Alosine to US is an antipredatory response against echolocating toothed whales and this is why the fish always turn away from the sound source. According to Plachta et al. (2004) the ultrasonic pathway in Alosine appears to be a feature-rich US detector that is likely to be adapted (e.g., frequency, intensity) to odontocete echolocation signals.

According to known data the ability to detect US is limited to the subfamily Alosinae and has not been found in other fish species from the family Clupeidae, for e.g., from the subfamily Clupeinae (Mann et al. 2001, 2005) or the subfamily Dorosomatinae (Narins et al. 2013). It also does not appear that fishes from other families are able to detect US, although very few hearing studies have tested this ability (Narins et al. 2013). One study conditioned *Gadus morhua* to ultrasonic pulses at 38 kHz with a threshold for detection of 204 dB re 1 µPa (Astrup and Møhl 1993). A follow-up study by Schack et al. (2008) found that unconditioned *Gadus morhua* did not show any behavioural or physiological response when exposed to the same type of stimulus generated with the same equipment as used in the study performed by Astrup and Møhl (1993).

Effects of ultrasound used for counteract algal growth and biofouling on fish

Summary of the effects caused by the US devices used to control algae and reduce biofouling on fish is shown in Table 1. De Lange (2007) conducted a research on the effects of US used for cyanobacteria control in surface waters on different fish: bream, silver bream, bass, common roach (*Rutilus rutilus*), ruffle (*Gymnocephalus cernua*), common rudd (*Scardinius erythrophthalmus*), tenches (*Tinca tinca*), and pikes (*Esox lucius*). Bream, bass, and silver bream are names for several species or higher taxonomic groups of fish. However, in De Lange (2007) Latin names or more exact names for fish species are not reported; therefore, we cannot know which species of fish from the groups bream, bass, and silver bream were used for the research. Results showed that the fish population was evenly distributed across two basins, with and without the US, even after 4 months of US operation revealing that US had no effect on fish migration between the two basins. Also, the length of fish in both basins did not differ significantly. Given that the fish in the basin with US did not massively flee, it can be concluded that the US with the applied load (no data is provided about the type, frequencies or intensities of the US used) is not noticeable or considered unsafe for tested fish species. Furthermore, no excessive mortality of fish has been observed in the basin where the US was used.

Oyib (2009) reported on reduction of cage net fouling at a salmon-farming facility by US (LG Sonic, 12 W, dual core multi frequency technology, 20–200 kHZ) installed in a salmon cage with nets covered with marine fouling organisms. As reported by Oyib (2009) there were no detectable changes in salmon behaviour during the US exposure of 28 days.

Table 1: The list of effects caused by the ultrasound (US) devices used to control algae (cyanobacteria) and to reduce biofouling on non-target fish and zooplankton.

Tabela 1: Seznam učinkov, ki jih povzročajo ultrazvočne (US) naprave za zaviranje rasti alg in biofilma na netarčne ribe in zooplankton.

US effect	Test organisms	US characteristics	Literature
No long-term effect on fish migration or mortality	Bream, silver bream, bass, *Rutilus rutilus, Gymnocephalus cernua, Scardinius erythrophthalmus, Tinca tinca, Esox lucius*	No data on the type, power and frequency of the US used	De Lange 2007
No changes in salmon behaviour during the US exposure of 28 days	Salmon	LG Sonic, 12 W, 20–200 kHZ	Oyib 2009
No long-term effect noticed on the body weight increase, fish productivity, feeding, and behaviour during the US exposure of 1 year	*Cyprinus carpio*	LG Sonic Tank, range 50 m, 12 W, 20–200 kHZ	Griessler Bulc et al. 2011
Continuous US exposure deterred fish from feeding	*Ictalurus punctatus*	No data on the type, power and frequency of the US used	Zimba and Grimm 2008
No effect noticed on the body weight increase, fish productivity, feeding, and behaviour	Juvenile *Cyprinus carpio*	LG Sonic SSS, range 10 m, 11 W, 20–200 kHZ	Krivograd Klemenčič and Griessler Bulc 2013
No effect noticed on the fish productivity, feeding, and behaviour	*Cyprinus carpio*	LG Sonic Tank, range 70 m, 13 W, 20–200 kHz	Krivograd Klemenčič and Griessler Bulc 2015
No long-term effect noticed on fish physiology during the US exposure of 30 days	*Cyprinus carpio*	SOFCHEM TWIN-f system, 15 W, 23–46 kHZ	Techer et al. 2017
Acute lethal effect	*Daphnia magna*	Flexidal AL-10, acoustic power 0.7 W, $8.5x10^{-4}$ W mL^{-1}, 12–200 kHz	Lürling and Tolman 2014a
Acute lethal effect	*Daphnia magna*	Flexidal AL-05, acoustic power 0.63 W, 20–44 kHz	Lürling and Tolman 2014b
Acute lethal effect	*Daphnia magna*	Flexidal AL-50, US characteristics not reported	Govaert et al. 2007
No acute effect noticed	*Daphnia* ssp.	Pool Tec 10”, Huges Sonic Systems, power not reported, 110-240 V, 45–60 kHz	Hedge 2013
No acute nor long-term effect noticed	*Daphnia* ssp.	producer and power not reported, ~580 kHz	Hedge 2013
No acute effect noticed	Juvenile and adult *Daphnia magna*	LG Sonic e-line, 25 W, 20–100 kHz	Klemenčič and Krivograd Klemenčič 2021

Griessler Bulc et al. (2011) studied a water treatment system for common carp (*Cyprinus carpio*) which included among other treatment devices also US. The research was conducted in two fish ponds, namely an experimental pond with treatment, including a roughing filter, a glass fibre filter, a UV-C unit, and a US device and a control pond without any treatment. The US device was a low-power commercially available US transducer (LG Sonic® Tank, range 50 m, 12 W, with dual core multi frequency technology, 20–200 kHZ) floatingly installed in the corner of the experimental pond. The research was performed continuously for more than one year and during the whole experiment the US device was switched on. The results showed that in the experimental pond with US rearing conditions for common carps were better with higher body weight increase and higher fish productivity than in the pond without treatment. It can be concluded that the US device showed no negative effect on fish regarding body weight increase and fish productivity. Moreover, fish mortality was the same in both ponds correlated with transportation stress at the beginning of the experiment. The authors of the research did not report any feeding problems or behaviour changes in fish in the pond with US. On the contrary, in the research performed by Zimba and Grimm (2008) in tank trials with channel catfish fingerlings (*Ictalurus punctatus*), continuous operation of the US devices deterred fish from feeding. Their trials were therefore modified to allow a four hour period without US treatment around the feeding time. Turning off the US signal during the feeding resulted in the fish feeding and no further adverse effects. However, in the research performed by Zimba and Grimm (2008) no information is available on the main characteristics of the US used for trials, namely intensity, power or frequencies. Therefore, it is very hard to compare both studies.

Krivograd Klemenčič and Griessler Bulc (2013) compared at a lab-scale two treatment systems for fish farms (a) a system with a constructed wetland and a US device, and (b) a system with a constructed wetland in order to find out the effect of US on fish productivity and behaviour. Model fish was juvenile common carp. The US device used was a commercially available US transducer (LG Sonic® SSS, range 10 m, 11 W, with dual core multi-frequency technology). The research showed no negative effect of the US device on fish, resulting in higher body weight increase and higher fish productivity in the system with the US unit compared to the control system without the US. There was no fish mortality observed during the experiment nor authors stated any difference in feeding habits or behaviour of fish in the tank with the US. Another research was performed in 2015 by the same authors (Krivograd Klemenčič and Griessler Bulc 2015) with the similar type of low-power non-cavitation US device (LG Sonic® Tank, range 70 m, 13 W, 20–200 kHz) and the same test organism common carp. Again, in the pond with treatment system, including the US device, no negative effects of the US on fish were noticed, resulting in higher fish productivity compared to the fish pond without treatment. In addition, no adverse effects were noticed on fish.

Techer et al. (2017) studied the effect on the fish physiology of a long-term exposure to anti-cyanobacterial US on the common carp (*Cyprinus carpio*). Two-years-old carps were chronically (for 30 days) exposed to a low-power US. The used US system consisted of a dual-frequency US device emitting continuous signals and powered by a solar floating platform with sound pressure level (SPL) up to 187 dB re 1μPa. There were two transducers operating independently. They were immersed at a depth of around 0.5 m with one emitting at an average frequency of 23 kHz and the other at 46 kHz. The two frequencies are simultaneously transmitted using the two transducers mounted adjacent one another, with a distance of 10 cm between them. The supplied power to the transducers was initially fixed at 15 W. According to the manufacturer Sofchem (France) the TWIN-f® ultrasonic system, which was used for the experiments, has been especially developed for algae growth inhibition. After seven and 30 days of exposure to ultrasonication, fish were sacrificed, condition factor indices were determined, and a panel of biochemical markers linked to fish physiological homeostasis was assessed, encompassing (i) hepatic antioxidant enzyme biomarkers, i.e., total superoxide dismutase (total SOD), catalase (CAT), total glutathione peroxidase (total GPx), and glutathione S-transferase (GST) activities, (ii) lactate dehydrogenase activity related to cellular energetic metabolism, and (iii) circulating cortisol levels subsequent to stress. Results showed that carps were not affected by US exposure when exposed in floating cages in fish ponds over a 30-day period. Cortisol levels slightly increased over the duration of the experiment, but its variation did not show US exposure related stress. Moreover, an overall diminution of the expression levels of different biomarkers was reported during the experiment including cellular antioxidant enzyme activities such as superoxide dismutase, glutathione peroxidase, catalase and glutathione S-transferase, and lactate dehydrogenase. Subtle changes in these biomarkers were dependent on the type of enzyme activity and especially of the origin of fish (i.e., sampled pond) regardless of the presence of US equipment, reflecting thereby fish adaptation to local environmental conditions in each pond. In conclusion, this study does not provide indication that ultrasonication in the aforementioned conditions affects the welfare and physiological homeostasis of carps.

Effects of ultrasound used for other applications on fish

Summary of the effects caused by the US devices used to control algae and reduce biofouling on zooplankton is shown in Table 1. Duchene (2016) reported that US deployed underwater directly into fish pens has a lethal effect on juvenile stages of the Chilean sea lice (*Caligus rogercresseyi*). The application as reported by Duchene (2016) is not harmful to the fish (salmon) or marine mammals due to the low-power (20 W) and low frequencies (20 KHz) used per transmitter. However, no tests were performed to confirm this statement.

According to Frenkel et al. (2000a) US at therapeutic intensity levels enhances uptake of particles into fish cells by widening intercellular spaces, thus increasing permeability of the skin (e.g., sonication at 3 MHz, intensity 2.2 W cm^{-2}, power 11 W). The first signs of biological effects in the sonicated tissues were observed at 1.7 W cm^{-2} or 8.5 W and at sonication time of 90 s with the ultrasonic beam perpendicular to the skin surface and the distance of 15 cm between the fish and the transducer. This effect of US has been used for a variety of applications in aquaculture, including transport of silver chloride nanoparticles (Frenkel et al. 2000b) and vaccination. Fernandez-Alonso et al. (2001) used US (24 s at 40 kHz and 40 W in a small bath sonicator) to transfer viral hemorrhagic septicemia plasmids into trout fingerlings as a form of immersion vaccination. Zohar et al. (1991) noted that for fish, crustaceans, and molluscs, compounds which can be administered by their US-enhanced method include proteins, nucleic acid sequences, antibiotics, antifungals, steroids, vitamins, nutrients, minerals, hormones, and vaccines. Zohar et al. (2001) stated that the frequencies and intensities used to implement the molecule transfer range from 20 kHz to 10 MHz, below 3 W cm^{-2} with exposure time of a few minutes. According to LaLiberte and Haber (2014) it is possible that fish in natural systems could be at risk for disease or possibly environmental contaminant uptake if US exposure is great enough to induce epidermal permeability (e.g., 20 kHz-10 MHz; 8.5-40 W). Although, US used for algae control could be in the same frequency and power range as the US which is reported to increase fish skin permeability (Lowe 2011), the volumes of water in which fish are exposed to sonication are much different. In the experiments where fish were treated or vaccinated with US the sonication took place in small volumes of water in order to apply US directly on fish skin (the distance between the fish and the transducer was 15 cm or less), which contributed to the increased impact of US on fish. On the other hand, in nature systems treated by US, fish are exposed to long-term sonication (days or even months). Therefore, research on skin damage and thus the possible environmental contaminant uptake in fish exposed to algae control US devices is needed in order to evaluate possible long-term effects of algae control US devices on fish.

Effect of ultrasound on zooplankton

Effects of ultrasound used for counteract algal growth on zooplankton

Very few studies are available on the effect of commercially available US systems for algae-control on non-target aquatic organisms; moreover, we could identify only five studies with focus on zooplankton, namely Govaert et al. (2007), Hedge (2013), and Lürling and Tolman (2014a,b) together with our recent study Klemenčič and Krivograd Klemenčič (2021). Lürling and Toldman (2014a) tested commercially available US (Flexidal AL-10, Belgium) which is according to the manufacturer used to reduce algae growth in small ponds, aquaria and small water reservoirs (~12 kHz to ~200 kHz, acoustic power 0.7 (±0.2) W, 8.5×10^{-4} W mL^{-1}) on non-target zooplankton species *Daphnia magna* in 1 L jars (actual volume of 800 mL). After 15 minutes of sonication all *D. magna* organisms exposed to sonication died (acute lethal effect), while all *D. magna* in control groups survived. Effect of temperature (possible overheating due to sonication) was excluded. The same year Lürling and Tolman (2014b) published a similar research in which they exposed *D. magna* (~2 mm body size) in 1 L jars (actual volume of 800 mL) to the US device of the same producer (Flexidal AL-05, Belgium) supplied at 20 kHz, 28 kHz, 36 kHz or 44 kHz with acoustic power of 0.63 (±0,05) W. All animals were killed between 10 min (44 kHz) and 135 min (20 kHz) (acute lethal effect). An experiment with differently sized *Daphnia* (0.7–3.2 mm) testing the hypothesis that juveniles are more susceptible than adults showed that all animals were killed between 4 and 30 min when exposed to 44 kHz. The survival time in organisms of different body-size were lowest in animals between 1.1 and 1.7 mm and larger in the smallest and largest animals tested. Increasing water volumes up to 3.2 L and thus lowering the US intensity did not markedly increase survival of *Daphnia* exposed to 44 kHz US. A tank experiment with six 85 L tanks containing a mixture of green algae, cyanobacteria and *D. magna* was performed to study the effect of US over a longer period of time (25 days). The results showed animal densities were extremely low in the treatments compared to the controls. Higher frequencies exerted a stronger effect on *D. magna* than lower frequencies. Animals between 1 and 2 mm seemed strongly affected by 44 kHz US than smaller and larger specimens, but again survival times were very short (about 2 to 17 min only). Increasing water volume and thereby lowering the intensity of ultrasonication did not elevate the survival time significantly. Hence, even in small ponds and aquaria the tested transducers are expected to exert an effect on non-target organisms such as *Daphnia*. This finding is supported by a field study conducted by Govaert et al. (2007) (the original report is not available, data are cited from Lürling and Tolman (2014b)) in two identical ponds that were interconnected and received the same water. One of the ponds was treated with Flexidal AL-50 (Belgium) transducer (the same manufacturer as in Lürling and Tolman, 2014a,b), while the other served as a control. The authors reported an almost complete disappearance of *Daphnia* from the US treated pond, while *Daphnia* remained abundant in the non-treated control pond. Since the original report (Govaert et al. 2007) is not available, the volume of the ponds, the frequency and the output power of the US are not known.

Hedge (2013) performed a lab-scale experiment (5 days) in 65 L tanks with low- frequency US (Pool Tec 10", Hughes Sonic Systems, 45-60 kHz, 110-240 V, output power not reported), while long-term (2.5 months) field-scale experiment was performed with high-frequency US (~580 kHz, producer and output power not reported) in an artificial lake with the approximate area of 2.4 km^2 being partitioned into four sections. Separation allowed for two sections of the lake to be subjected to the US while the other two were controls. As model organisms different species from genera *Daphnia* were used. According to the results there was no negative effect of ultrasonication found on *Daphnia* in lab and field-scale experiments. High frequency ultrasonication did not reduce reproduction, increase mortality rates or negatively alter the environment in a way that decreases its suitability for zooplankton. Moreover, ultrasonication did not influence the dispersion of *Daphnia* within the tank in lab-scale conditions. Similarly, Klemenčič and Krivograd Klemenčič (2021) reported that commercially available US for algae control of the Dutch producer LG Sonic (LG Sonic e-line, 25 W, 20–100 kHz) had no acute effect on the mobility of juvenile and adult *D. magna* specimens in lab-scale experiment with up to 48 h exposure to ultrasonication. They concluded that US devices from different manufacturers can have different effects on target- and non-target organisms.

Effect of ultrasound used for ballast water treatment on zooplankton

The US has been investigated not only for algae control but also as a control for zooplankton in ballast waters (e.g., Sassi et al. 2005, Laliberte and Haber 2014). However, usage of acoustic cavitation in ballast water treatment is relatively new and remains insufficiently researched (Gregg et al. 2009, Lloyd's Register 2014). Sassi et al. (2005) performed laboratory and onshore test trials for ballast water treatment with US unit specially designed for disintegration (e.g., cell disruption, emulsifying, homogenising), thermoplastic molding, coating-lacquer removal, intensive surface cleaning, wire cleaning, cutting, drilling, lapping and compressing, used in industry or sonochemistry laboratories. The operating frequency of US unit used was 20 kHz with output power of 2000 W. Laboratory tests showed total reduction rates of 84-100% for Artemia salina with the best results obtained with a flow rate of 200 L h^{-1} and a maximum transducer amplitude of 50%. For test organisms Nereis virens, Acartia tonsa, Tisbe battagliai and Alexandrium tamarense the mortality attained was always below 40% for all tests. In the onshore trials, the mortality rates achieved were 94-99% for copepods, 86-99% for copepod nauplii, 95-98% for cladocerans, 80% for rotifers and 97% for barnacle nauplii. Holm et al. (2008) tested the effect of high-power US (19 kHz) on a cladoceran (*Ceriodaphnia dubia*), rotifers (*Brachionus plicatilis, B. calyciflorus*, and *Philodina sp*.), and brine shrimp (*Artemia* sp.) in a flow-through system. Ultrasonic intensities were 13.5-25.5 W cm^{-2}. The results showed that most effective treatment against zooplankton larger than 100 μm were exposure times below 10 seconds and energy densities less than 20 J mL^{-1}resulted in 90% mortality. Microjets within the zooplankton caused by the collapse of cavitation bubbles were the hypothesized cause of zooplankton mortality in the experiments.

Effects of ultrasound used for other applications on zooplankton

Wells (1968) studied the effect of ultrasonication on *Daphnia magna* (~0.2 cm length). He used the US transducer with diagnostic frequency (~3 MHz) and diameter 0.95 cm specially constructed to permit the irradiation of single specimens of *Daphnia*. The length of the water jacket in front of the transducer was 4–7 cm. The animal was placed in a small irradiation chamber filled with water. The results showed that exposure of *D. magna* to the US with power 10 W corresponding to 29 W cm^{-2} and frequency of 3 MHz caused the death of all exposed animals in 2 minutes (acute lethality). However, at lower power (below 8 W corresponding to 23 W cm^{-2}) and the same frequency all animals survived. There was a threshold level at about 5 W at the experimental conditions below which ultrasonic irradiation had no significant effect on *D. magna* survival. Kamenskii (1970) studied the influence of the US on eggs and larvae of some fish trematodes together with their intermediate hosts *Cyclops*, *Daphnia* and *Lymnaea stagnalis* (shell height 0.8–1.2 cm, width 0.3–0.6 cm). The animals were treated with ultrasonic waves 50, 500 or 1,000 kHz in standing water for 3 seconds or in flowing water for 10 seconds. In still water, all organisms except *Lymnaea* were killed, even at the lowest rate of frequency. *Lymnaea* was not killed at any frequency. Analogous results were obtained in flowing water. Unfortunately, only frequency and no output power of the US unit used is reported. However, from acute lethal effect in very short contact time (up to 10 seconds) also at low frequencies we can assume that author tested high-power US.

Conclusions

According to known data, fish species are not producing communication sounds within the ultrasonic frequency range and also cannot hear sounds above 3–4 kHz, although very few hearing studies have tested this ability. The only known exception are marine fish species from family Clupeidae (subfamily Alosinae) which are able to detect sound up to 180 kHz as an anti-predatory response against echolocating toothed whales.

Therefore, caution should be taken when installing ultrasonic devices in marine locations since they may interfere with communication between sea mammals or may cause adverse effects on fish from subfamily Alosinae. There are only few publically-available studies on the effects of ultrasound (US) on fish. Furthermore, not all of them report information on wavelengths and intensities of the used US devices, because that usually remains proprietary information, making the comparison between the studies very difficult. Nevertheless, based on the available per-reviewed studies, we can conclude that non-cavitation US devices used to control algae (cyanobacteria) and to reduce biofouling had no known adverse health effects on the studied fish species with no feeding and behaviour changes noticed. US is used in aquaculture for immersion vaccination or antibiotic treatment because it can make fish skin permeable. Although, research on long-term exposure of fish to low-power algae control US devices show no adverse effects on fish, it is possible that fish exposed to US in natural systems could be at risk for diseases or contaminant uptake because of increased skin permeability. We should also be aware that the studies reviewed in this paper are not considering the possible effects of the ultrasonication on molecular or genetic levels in fish.

We identified identify only four studies dealing with the effects of US used for algae control on non-target zooplankton with conflicting results. According to some authors low-frequency and low-power US (Flexidal) have acute lethal effect on *Daphnia* in lab and field conditions. On the other hand, low-frequency US of different producer had no negative effect on *Daphnia* productivity and mortality. Therefore, caution should be taken when using US for counteract algal growth in ponds or lakes especially in terms of zooplankton and natural balance maintenance. In the last decades US has been investigated together with ultraviolet irradiation, ozone and hydrodynamic cavitation as a control for zooplankton in ballast waters. The research of sonication as ballast water treatment is promising with mortality rates for zooplankton up to 100%. In contrast with algae-control systems for ballast water treatment high-power US units are used in order to achieve high mortality rates. Acute lethal effect on zooplankton can be achieved also by the use of low-power and diagnostic frequency US.

Povzetek

Obstajajo različne metode za zaviranje rasti alg in cianobakterij v vodnih telesih, kot so dodajanje kemijsko in biološko aktivnih snovi, ozonacija, različni tipi filtracije in uporaba ultrazvoka (UZ). Glavni problem dodajanja kemijsko in biološko aktivnih snovi je tvorba različnih stranskih produktov in povečano sproščanje cianotoksinov iz cianobakterijskih celic ob njihovi poškodbi oz. odmrtju, kar običajno problem še poslabša. UZ naprave, ki so prosto dostopne na trgu za namen nadzora rasti alg, delujejo v razponu od 20 do 200 kHz in so nizko energijske, kar pomeni, da ne povzročajo učinka kavitacije. Te UZ napave po trditvah proizvajalcev nimajo negativnih vplivov na netarčne organizme, kot so ribe in zooplankton. V naravnih vodnih ekosistemih, kot so na primer jezera in ribniki, je bistvenega pomena, da z uporabo UZ ne poškodujemo ostalih, t. i., netarčnih organizmov, ter s tem ne porušimo naravnega ravnovesja v vodnem telesu.

O vplivu UZ na netarčne organizme obstaja le malo raziskav in še to z nepopolnimi informacijami o karakteristikah testiranih UZ. Kljub temu raziskave kažejo, da nizko energijski UZ, ki se uporabljajo za zatiranje alg nimajo škodljivih vplivov na preučevane vrste rib. Kljub temu se priporoča previdnost pri uporabi UZ v morskem okolju, saj lahko ultrazvočni valovi ovirajo komunikacijo med morskimi sesalci ali škodijo ribam iz poddružine Alosinae - edine znane ribe s sposobnostjo zaznavanja UZ. Raziskave o vplivu nizko energijskih UZ na netarčne zooplanktonske organizme pa kažejo nasprotujoče si rezultate, od akutnih letalnih učinkov do odsotnosti vpliva. Zato je potrebna previdnost pri uporabi UZ za zmanjševanje rasti alg v ribnikih ali jezerih, zlasti z vidika varovanja zooplanktona in s tem ohranjanja naravnega ravnovesja.

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Imunski sistem pri rakih: predstavitev raziskav s kopenskim rakom *Porcellio scaber*

Immune system in crustaceans: a presentation of research with terrestrial crustacean *Porcellio scaber*

Andraž Dolar*, Damjana Drobne, Rok Kostanjšek, Anita Jemec Kokalj

Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za biologijo, Večna pot 111, SI-1000 Ljubljana, Slovenija

*Korespondenca: andraz.dolar@bf.uni-lj.si

Izvleček: Zaradi stalne interakcije organizmov z okoljem so le ti tekom evolucije razvili zmožnost prepoznavanja in razlikovanja med lastnimi in tujimi molekulami in oblikovanja ustreznega odziva na tujke, kar je temeljna funkcija imunskega sistema. Enakonožci *Porcellio scaber* so predstavniki kopenskih rakov, podobno kot tudi druge skupine rakov pa imajo razvito naravno oziroma prirojeno imunost, ki jo tvorijo imunske celice ali hemociti ter humoralne komponente. Opisali smo tri različne tipe hemocitov, ki se nahajajo v hemolimfi: semigranularne (povprečno 65 %), granularne (17 %) in hialine (18 %). Podobno veliki semigranulociti (premer 11,2 ± 0,4 µm) in granulociti (premer 12,0 ± 0,3 µm) so ovalno do okrogle oblike, razlikujejo pa se v gostoti citoplazemskih granul, ki pri granulocitih prevladujejo. Okrogli hialinociti so manjši (8,1 ± 0,3 µm) in brez granul. Pri slednjih smo identificirali dva podtipa, pri čemer domnevamo, da podtip 1 z velikim in okroglim jedrom ter prevladujočim evkromatinom ustreza lastnostim zarodnih celic. Povprečna gostota prostih hemocitov v hemolimfi *P. scaber* je 3,50 ± 0.19 x 10^6 celic/mL. Poleg metod za merjenje celičnih komponent smo uspešno vpeljali metode za merjenje humoralnih komponent, kot so: fenol oksidazi podobna aktivnost, koncentracija dušikovega oksida ter aktivnosti antioksidativnih encimov in alfa-2 makroglobulina. V primeru okužbe *P. scaber* z bakterijo (*Rhabdochlamydia porcellionis*) smo dokazali aktivacijo celičnega imunskega odziva z izrazito tvorbo nodulov s prevladujočim deležem semigranulocitov. S temi bazičnimi raziskavami smo si začrtali pot za nadaljnje raziskave na področju ekotoksikologije, metodologijo spremljanja imunskih procesov pa bi lahko v prihodnosti prenesli tudi na druge vrste rakov.

Ključne besede: enkapsulacija, fagocitoza, hemocit, melanizacija, nodulacija, signalna pot

Abstract: The immune system is crucial to recognise and distinguish between self and nonself, and react to challenges posed by the environment. We present a review on the innate immunity of crustaceans, in particular terrestrial crustacean woodlice *Porcellio scaber*. The main effectors of immunity in crustaceans are haemocytes which carry out the cellular immune processes and synthesise humoral defence components. We described three types of haemocytes in *P. scaber*: semigranulocytes (65% of freely circulating haemocytes), granulocytes (17%) and hyalinocytes (18%). The average density of free circulating haemocytes is 3.50 ± 0.19 x 10^6 cells/mL. Semigranulocytes and granulocytes are both oval shaped with a similar diameter (11.2 ± 0.4 µm and 12.0 ± 0.3 µm, respectively), but granulocytes have a higher density of granules which are also larger than in the case of semigranulocytes. Hyalinocytes are round, agranular and smaller (8.1 ± 0.3 µm). Two types of hyalinocytes were discovered. One subtype is of particular interest, as it has a very large nucleus with dominating euchromatine resembling properties of the stem progenitor cells. We have implemented new methods to measure humoral components in the haemolymph of *P. scaber*. These are: phenoloxidase like activity, nitric oxide levels, antioxidant enzyme activity and alpha-2-macroglobulin. The formation of nodules was noted in the case of *P. scaber* infection with bacteria *Rhabdochlamydia porcellionis*. Our future research will be focused to investigate the immune response of *P. scaber* at the proteome and transcriptome level.

Keywords: encapsulation, haemocyte, melanization, nodulation, phagocytosis, signal pathway

Uvod

Organizmi zaznavajo zunanje in notranje okolje, se nanj odzivajo in s tem ohranjajo notranje ravnovesje, kar omogoča njihov razvoj in preživetje. Ob soočenju organizma z neznanimi dejavniki v okolju, kot so npr. okužbe s potencialno patogenimi mikroorganizmi in paraziti, abiotskimi spremembami in onesnaženostjo okolja, se sproži obrambni odziv ali pa se le-ta prilagodi na nove razmere (Boraschi in sod. 2020). Zaradi stalne interakcije z omenjenimi biotskimi oziroma abiotskimi dejavniki v okolju so organizmi tekom evolucije razvili zmožnost prepoznave in razlikovanja med lastnimi in tujimi molekulami ter nabor imunskih odzivov z raznolikimi efektorskimi mehanizmi (Jiravanichpaisal in sod. 2006, Milutinović in sod. 2016, Ihan 2020).

V znanstveni literaturi je na voljo veliko študij o imunskem odzivu nevretenčarjev na različne vrste stresa (Hauton 2012, Labaude in sod. 2017, Boraschi in sod. 2020), medtem ko je proučevanje odziva imunskega sistema nevretenčarjev v slovenskem raziskovalnem prostoru dokaj novo raziskovalno področje (Kostanjšek in Marolt 2015, Tesovnik in sod. 2017, Dolar in sod. 2020). Na Katedri za zoologijo, Oddelka za biologijo, Biotehniške fakultete, Univerze v Ljubljani, smo v zadnjih letih opisali imunski odziv kopenskega enakonožnega raka vrste *Porcellio scaber* (navadni prašiček), kot odziv na različne vrste stresa (Kostanjšek in Marolt 2015, Dolar in sod. 2020, 2021, Mayall in sod. 2021). Vpeljali smo tehnike za analizo posameznih komponent imunskega sistema enakonožnih rakov ter opisali ultrastrukturo in morfologijo krvnih celic oziroma hemocitov. V tem prispevku želimo širši publiki podrobneje predstaviti imunski sistem rakov in raziskave, ki so bile opravljene pri kopenskih rakih s poudarkom na vrsti *P. scaber*.

Imunski sistem rakov

Temeljno obrambo rakov pred zunanjimi dejavniki predstavljajo vedenjske prilagoditve (izogibanje) ter fizikalna in kemijska pregrada, ki jo tvorijo zunajcelični matriksi in izločki žlez integumenta (Jiravanichpaisal in sod. 2006, Rowley in Powell 2007) in črevesnega epitela (npr. kutikula, peritrofna membrana). Struktura kutikule telesne površine in prebavne cevi ter prisotnost peritrofne membrane se pri različnih skupinah rakov zelo razlikujeta (Van Der Zande in sod. 2020). Debelina kutikule, prisotnost in velikost por ter peritrofna membrana pomembno vplivajo na potencialen prehod mikroorganizmov v telesno votlino. Poleg omenjenega pa k obrambi pred patogenimi mikroorganizmi oziroma tujimi molekulami, ki lahko resno ogrozijo organizem, dodatno prispevajo še encimi, antimikrobni peptidi, reaktivne kisikove zvrsti, surfaktanti in nizek pH v prebavilu (Jiravanichpaisal in sod. 2006, Buchon in sod. 2014, Van Der Zande in sod. 2020).

Vsi nevretenčarji, vključno z raki, imajo razvite mehanizme prirojene imunosti, ki zagotavljajo hitro in nespecifično obrambo (Boraschi in sod. 2020). Poleg teh pa so pri nevretenčarjih potrdili tudi obstoj imunskega spomina, ki omogoča hitrejši in učinkovitejši odziv organizma ob ponovnem stiku s patogenom ali drugim dražljajem (Melillo in sod. 2018), kar je sicer osnovna lastnost pridobljene imunosti pri vretenčarjih. Eden izmed argumentov o obstoju specifične imunosti in imunskega spomina pri dekapodnih rakih je izrazita variabilnost vezavne domene celične adhezijske molekule, ki so jo prvič opisali pri ljudeh z Downovim sindromom (DSCAM, angl. down syndrome cell adhesion molecule) (Hauton 2012). Kljub temu pri nevretenčarjih redko govorimo o pridobljeni imunosti, saj so pojav imunskega spomina doslej opisali predvsem na nivoju celotnega organizma, (npr. kot povečana imunokompetenca), molekulski mehanizmi procesa pa še niso pojasnjeni.

Prirojeno imunost tvorijo celične komponente (celice z različnimi obrambnimi mehanizmi) in humoralne komponente (proteinski kompleksi v tkivih in telesnih tekočinah). Obe komponenti sta soodvisni in delujta usklajeno (Hauton 2012). Glavni nosilci celične komponente prirojenega imunskega odziva rakov so celice hemolimfe oz. hemociti, ki poleg vrste obrambnih procesov na nivoju celic sintetizirajo tudi večino molekul humoralne obrambe. Hkrati pa je znano, da pri rakih molekule humoralne telesne obrambe izločajo tudi celice drugih tkiv, npr. prebavne žleze poznane kot hepatopankreas (Xu in sod. 2020).

Hemociti

Hemociti so celice krvožilnega sistema pri rakih, ki se pretežno nahajajo prosto v hemolimfi, lahko pa tudi migrirajo v druga tkiva (Chevalier in sod. 2011, Söderhäll 2016, Tassanakajon in sod. 2018). V splošnem pri rakih ločimo tri tipe hemocitov, ki se razlikujejo po velikosti, razmerju med citoplazmo in jedrom in v prisotnosti ter gostoti citoplazemskih granul (Bauchau 1981). Semigranularne (t. i. semigranulociti) in granularne celice (t. i. granulociti) imajo podobno velikost, manjše okroglo ali ledvičasto jedro in citoplazemske granule različnih velikosti. Ta dva celična tipa se ločita v gostoti citoplazemskih granul, ki je pri granulocitih občutno večja. Hialine celice (t. i. hialinociti) so najmanjše celice z relativno velikim jedrom in brez granul v citoplazmi (Jiravanichpaisal in sod. 2006, Chevalier in sod. 2011, Söderhäll 2016). Izraz hialin pomeni »steklast, prosojen«, in izhaja iz odsotnosti citoplazemskih granul teh celic (Mikrobiološki slovar 2013). Na primeru hemocitov pri ostrigah (*Crassostrea rhizophorae*) so pokazali, da različni tipi hemocitov predstavljajo različne razvojne stopnje, torej, da se hialinociti pretvorijo v semigranulocite ti pa v granulocite. V primeru stresa, npr. zaradi okoljskih sprememb ali onesnažila, pa granulociti lahko degranulirajo in postanejo semigranulociti (Rebelo in sod. 2013). Slednje se kaže kot trend povečevanja števila granulocitov ter manjšanja števila semigranulocitov ob naraščanju koncentracije onesnažila, kar smo pokazali na primeru *P. scaber* izpostavljenemu pesticidu v zemlji (Dolar in sod. 2021).

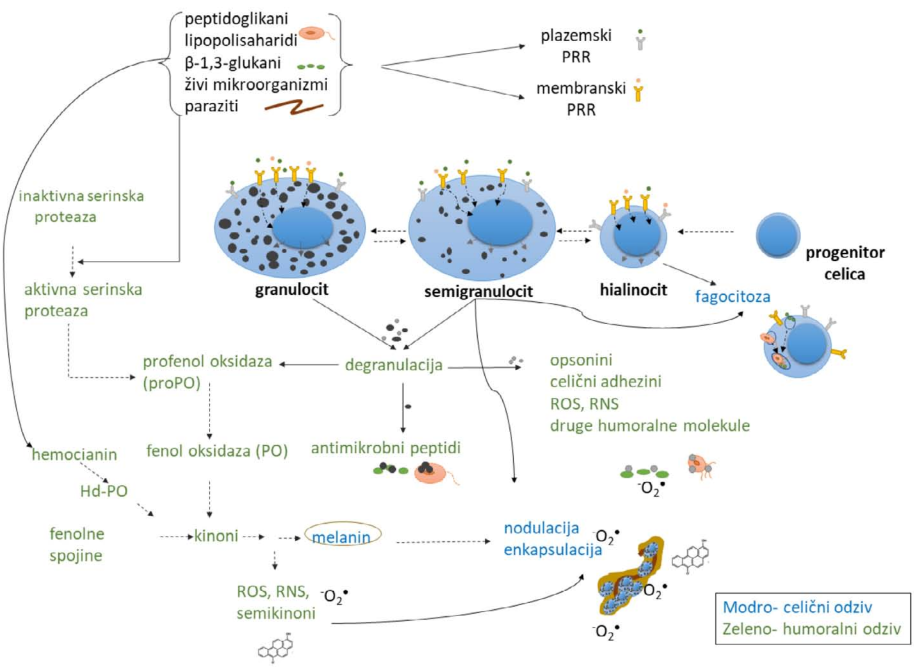
Vsak tip hemocitov opravlja določeno funkcijo, ki pa se med različnimi skupinami rakov lahko razlikujejo (Söderhäll 2016). Kljub temu so hialinociti večine rakov udeleženi zlasti v proces fagocitoze, semigranulociti pri nodulaciji in enkapsulaciji ter v manjšem obsegu fagocitozi ter sintezi humoralnih molekul, granulociti pa so pretežno vključeni v sintezo in izločanje molekul humoralne obrambe (Chevalier in sod. 2011, Mangkalanan in sod. 2014, Kostanjšek in Pirc Marolt, 2015, Jia in sod. 2017, Zhou in sod. 2018) (Sl. 1). Poleg osrednje vloge v procesih celične imunosti, hemociti sodelujejo tudi pri regeneraciji poškodovane kutikule (Halcrow in Smith 1986) in mišičnega tkiva (Uhrík in sod. 1989) ter nevrogenezi (Da Silva in sod. 2015).

Hematopoetski organ

Podobno kot pri ostalih nevretenčarjih, hemociti pri rakih nastajajo in zorijo v specializiranem tkivu, t. i. hematopoetskem organu. Le-ta je sestavljen iz lobulov, ki vsebujejo hemocite različnih razvojnih stopenj: zarodne celice, diferencirajoče in zrele celice. Zorenje celic poteka od centra nodula navzven, od koder se nato sproščajo v hemolimfo, ki organ obliva, kot diferencirani hemociti (Chevalier in sod. 2011, Lin in Söderhäll 2011, Söderhäll 2016). Nekatere raziskave poročajo, da so hemociti lahko sproščeni v hemolimfo tudi kot nediferencirane celice (Söderhäll in sod. 2003). Hemociti, ki krožijo v hemolimfi se ne delijo, zato se odmrli hemociti v obtoku neprestano nadomeščajo s sproščanjem novih celic iz hematopoetskega organa (Söderhäll in Cerenius 1992). Morfologija lobulov se pri različnih vrstah rakov lahko zelo razlikuje (van de Braak in sod. 2002).

Prepoznavanje tujih in lastnih molekul ter aktivacija humoralnega in celičnega imunskega odziva

Imunski sistem deluje v treh ključnih zaporednih korakih: (i) vezavi molekularnih vzorcev mikroorganizmov oziroma patogenov (M/PAMP, angl. microbial/pathogen-associated molecular patterns) in molekularnih vzorcev povezanih s poškodbo (DAMP, angl. damage-associated molecular patterns) na receptorje za prepoznavo molekularnih vzorcev M/PAMP in DAMP (PRR, angl. pattern recognition receptors) (Wang in Wang 2013, Tran in sod. 2020), (ii) aktivaciji



Slika 1: Poenostavljen shematski model celičnih in humoralnih mehanizmov prirojene imunosti rakov. Shema prirejena po Smith in sod. (2003) in Dolar (2021). Hd-PO; molekula hemocianina s fenol oksidazno aktivnostjo, PRR; receptorji za prepoznavo molekularnih vzorcev, ROS; reaktivne kisikove zvrsti, RNS; reaktivne dušikove zvrsti. Puščica s polno črto prikazuje povezavo različnih komponent in odzivov prirojene imunosti, puščica s prekinjeno črto pa prikazuje zaporedje aktivacije komponent telesne obrambe.

Figure 1: Simplified schematic model of the cellular and humoral mechanisms of innate immunity in crustaceans. Scheme adapted from Smith et al. (2003) and Dolar (2021). Hd- PO; molecule of hemocyanin with phenoloxidase activity, PRR; pattern recognition receptors, ROS; reactive oxygen species, RNS; reactive nitrogen species. Arrows with solid line indicate the connection of the different components and reactions of innate immunity, arrows with dashed line represent the activation sequence of body defense.

signalnih poti in (iii) proženju ustreznih celičnih in humoralnih efektorskih mehanizmov (Loker in sod. 2004, Tassanakajon in sod. 2018). Pri uporabi slovenske terminologije smo se deloma oprli na strokovna dela avtorjev Kotnik in sod. (2010) in Ihan (2020), v priloženem slovarju (Tab. 1) pa smo ključne imunološke pojme in kratice tudi razložili.

Tabela 1: Slovar imunoloških pojmov in kratic.
Table 1: Glossary of immunological terms and abbreviations.

Pojem	Opis
Imunski sistem	Organski sistem večceličnih organizmov s sposobnostjo razlikovanja med telesu lastnimi in telesu tujimi molekulami ter odstranjevanja tujkov
Naravna ali prirojena imunost	Evolucijsko starejši mehanizmi imunskega odziva, ki zagotavljajo hiter in nespecifičen odziv na prisotnost tujka in poškodovanih lastnih celic oziroma molekul (angl. innate immunity)
Celični imunski mehanizmi	Mehanizmi, ki vključujejo fagocitiranje, noduliranje in enkapsuliranje tujkov v telesu gostitelja
Humoralni imunski mehanizmi	Odstranjevanje in uničevanje tujkov in lastnih poškodovanih celic oziroma molekul s komponentami humoralnega odziva, kot npr. antimikrobni peptidi, melanizacija (profenol oksidazni sistem), antioksidativni encimi, citokini, opsonini
Nodulacija	Tvorba skupkov hemocitov okoli večjega tujka v telesu gostitelja, ki ga celice ne morejo fagocitirati
Enkapsulacija	Večje nodulom podobne strukture, ki nastanejo v primeru večjih tujkov v telesu gostitelja (npr. gliste)
Fagocitoza	Endocitotski proces požiranja tujkov
Hemociti	Celice hemolimfe oziroma krvožilnega sistema pri rakih
Semigranulociti	Semigranularni hemociti - večje celice hemolimfe z zmerno gostoto citoplazemskih granul
Granulociti	Granularni hemociti - večje celice hemolimfe podobne velikosti kot semigranulociti, z visoko gostoto granul v citoplazmi
Hialinociti	Hialini hemociti - manjše in agranularne celice hemolimfe z velikim jedrom glede na citoplazmo
M/PAMP	Molekularni vzorci, povezani z mikroorganizmi oziroma patogeni (angl. microbial/pathogen-associated molecular patterns)
DAMP	Molekularni vzorci, povezani s poškodbo (angl. damage-associated molecular patterns)
PRR	Receptorji za prepoznavo molekularnih vzorcev tujkov (angl. pattern recognition receptors)
Profenol oksidazni sistem	Kompleksen sistem, ključen pri tvorbi rdeče-rjavega pigmenta melanina, ki je reguliran s številnimi encimi (npr. fenol oksidaza)
Antimikrobni peptidi	Obrambni peptidi gostitelja so evolucijsko ohranjene komponente prirojene imunosti, ki sodelujejo pri humoralnem odzivu
NO	Dušikov oksid (angl. nitric oxide) je reaktivna dušikova zvrst (RNS, angl. reactive nitrogen species), ki ima v celicah vlogo sekundarnega sporočevalca. Kot komponenta humoralne telesne obrambe deluje citotoksično in je udeležena pri razgradnji fagocitiranih tujkov
ROS	Reaktivne kisikove zvrsti (angl. reactive oxygen species) nastajajo tekom aerobnega metabolizma in so udeležene v številne procese, med drugim imajo vlogo sekundarnih sporočevalcev, delujejo citotoksično in sodelujejo v razgradnji fagocitiranih tujkov

Receptorji za prepoznavo molekularnih vzorcev so netopni proteini na površini hemocitov in celic drugih tkiv (Wang in Wang 2013) oziroma topni proteini v hemolimfi (Coates 2012, Jin in sod. 2013). Pri rakih je poznana vrsta genov za različne skupine PRR, kot na primer: Toll-u podobni receptorji (TLRs, angl. Toll-like receptors), vezavni protein za lipopolisaharide (LPS) in β-1,3-glukane (LGBPs, angl. lipopolysaccharide and β-1,3-glucan-binding proteins), lektin tipa C, galektin, odstranjevalni receptor (SR, angl. scavenger receptor), s fibrinogenom povezani proteini (FREPs, angl. fibrinogen-related proteins) in receptorji DSCAM (Wang in Wang 2013, Tassanakajon in sod. 2018, Tran in sod. 2020).

Vezava molekularnih vzorcev M/PAMP ali DAMP na PRR sproži vnetni odziv s sproščanjem vnetnih mediatorjev (citokinov), hkrati pa se aktivira odstranjevanje tujkov s fagocitozo in eksocitoza humoralnih molekul (Ihan 2020). Z vezavo M/PAMP ali DAMP povzročena aktivacija receptorjev PRR inducira celične signalne poti, ki v jedru inducirajo izražanje genov imunskega odziva (Huang in Ren 2020, Tran in sod. 2020). Pri rakih poznamo tri glavne tovrstne signale poti: pot Toll, pot Imd in pot Jak/Stat. Signalno pot Toll aktivirajo zlasti po Gramu pozitivne bakterije, glive (oz. peptidoglikani in β-1,3-glukani) ter virusni proteini, signalno pot Imd aktivirajo po Gramu negativne bakterije in RNA virusi, signalna pot Jak/Stat pa kontrolira številne biološke procese, med drugim proliferacijo in diferenciacijo hemocitov, embrionalni razvoj, regeneracijo poškodovanih tkiv, tvorbo antimikrobnih proteinov in protivirusni odziv z RNA interferenco (Li in Xiang 2013, Morin-Poulard in sod. 2013, Du in sod. 2016, Huang in Ren 2020). Poznan je tudi širok nabor signalni proteinov omenjenih poti. Ta pri signalni poti Toll na primer vključuje proteine Spätzle, MyD88, Pelle, Dorsal, Cactus, Traf6 (Tassanakajon in sod. 2018). Aktivacija signalne poti Toll se odraža v regulaciji izražanja velikega števila z imunskim sistemom povezanih genov, ki kodirajo antimikrobne peptide, manjše peptide z neznano funkcijo, proteine, ki so udeleženi v melanizacijo in sistem strjevanja hemolimfe (De Gregorio in sod. 2002). Aktivacija signalne poti Imd vodi v aktivacijo transkripcijskega faktorja Relish, ki regulira izražanje genov z zapisom za antimikrobne peptide, kot so npr. krustin, lizocim in armadilidin (Becking in sod. 2020, Huang in Ren 2020).

Celični imunski mehanizmi

Med glavne efektorske mehanizme prirojene celične imunosti uvrščamo fagocitozo, nodulacijo ter enkapsulacijo tujkov. Fagocitoza je evolucijsko ohranjen mehanizem prirojene imunosti. Izraz fagocitirati označuje endocitotski proces požiranja delcev večjih od 0,5 µm, kot so npr. mikroorganizmi in fragmenti odmrlih celic (Jiravanichpaisal in sod. 2006, Liu in sod. 2020). Fagocitoza poteka po naslednjem sosledju: i) prepoznavanje molekularnih vzorcev M/PAMP, DAMP ali opsoninov vezanih na površino mikroorganizmov s PRR na površini hemocitov, ii) pripenjanje fagocitirajočih celic na delce, (iii) privzem delcev v notranjost celic z reorganizacijo citoskeleta in (iv) znotrajcelični vezikularni transport do fagosomov, v katerih se delci razgradijo (Jiravanichpaisal in sod. 2006, Liu in sod. 2020). Fagocitno aktivnost regulirajo komponente melanizacijske kaskade, peroksinektin, reaktivne kisikove zvrsti in antimikrobni peptidi (Cerenius in sod. 2008, Boraschi in sod. 2020, Liu in sod. 2020).

Obrambna mehanizma nodulacije in enkapsulacije nastopita v primeru, ko hemociti tujkov ne uspejo fagocitirati zaradi njihove velikosti in številčnosti. Pri nodulaciji gre za agregacijo hemocitov na tujek, pri čemer nastajajo t. i. noduli. Pri procesu enkapsulacije pa nastanejo večje, nodulom podobne strukture, ko se organizem sooči z večjimi tujki v telesu, kot so npr. gliste. V obeh primerih je končni rezultat večslojna kapsula iz sploščenih hemocitov na površini tujka, ki je pogosto tudi melanizirana. Ujeti mikroorganizmi in paraziti znotraj nodula navadno odmrejo zaradi odsotnosti kisika ali delovanja strupenih reaktivnih kisikovih in dušikovih zvrsti, oziroma drugih toksičnih molekul, ki nastanejo med melanizacijo. Poleg tega fizična izolacija ujetim mikroorganizmom preprečuje nadaljnjo rast in širjenje po telesu gostitelja (Jiravanichpaisal in sod. 2006).

Humoralni imunski mehanizmi

Ključne komponente humoralne telesne obrambe so profenol oksidazni (proPO) sistem, reaktivne kisikove (ROS, angl. reactive oxygen species) in dušikove zvrsti (RNS, angl. reactive nitrogen species), antimikrobni peptidi ter drugi encimi in molekule (Tassanakajon in sod. 2018).

Profenol oksidazni sistem je odgovoren za sintezo melanina, ki se nalaga okoli poškodovanih tkiv in tujkov v telesu gostitelja (Cerenius in Söderhäll 2004, Amparyup in sod. 2013), hkrati pa je soudeležen tudi v drugih imunskih procesih kot sta že omenjeni enkapsulacija in nodulacija, ter sintezi citotoksičnih antimikrobnih peptidov. Poleg imunske vloge proPO sistem pomembno sodeluje tudi pri pigmentaciji in sklerotizaciji tkiv (Cerenius in Söderhäll 2004, Lee in sod. 2004, Amparyup in sod. 2013). Ključna komponenta proPO sistema je aktiven encim fenol oksidaza (PO), ki je rezultat aktivacijske kaskade, odgovorne za pretvorbo in aktivacijo neaktivne oblike proPO v PO. Aktivacijo proPO sproži vezava M/PAMP in DAMP na PRR ter aktivnost proteolitičnih encimov mikroorganizmov (Cerenius in sod. 2008). ProPO kaskado regulirajo številne serinske proteaze, med katerimi sta pomembnejša terminalni proPO-aktivacijski encim (Cerenius in Söderhäll 2004) in proteazni inhibitor alfa-2 makroglobulin (Jiravanichpaisal in sod. 2006, Ponprateep in sod. 2017).

Aktivna oblika PO deluje kot tirozinaza oziroma katehol oksidaza in katalizira pretvorbo monofenolov in *o*-difenolov v *o*-kinone, iz slednjih se nato spontano tvori osrednji končni produkt, rdeče-rjavi pigment melanin, ter stranski produkti, kot so ROS, RNS in semikinoni (Cerenius in Söderhäll 2004, Lee in sod. 2004, Amparyup in sod. 2013). Zaradi potencialnega kvarnega delovanja glavnih in stranskih produktov melanizacije za gostiteljske celice, je proPO kaskada negativno regulirana (inhibirana) na več ravneh preko (i) inhibicije serinskih proteaz, (ii) inhibicije aktivnosti PO in (iii) sinteze ROS in RNS (Amparyup in sod. 2013). Izkazalo se je, da pri nekaterih nevretenčarskih skupinah, med drugim tudi pipalkarjih in rakih enakonožcih, PO podobno aktivnost posedujejo tudi drugi proteini hemolimfe, med njimi hemocianin, ki ima podobno kot encim PO v aktivnem centru bakrov atom (Fan in sod. 2009, Jaenicke in sod. 2009, Arockiaraj in sod. 2013, Pan in sod. 2019, Ramasamy in sod. 2017). Za PO podobno aktivnost hemocianina je ključna konformacijska pretvorba molekule hemocianina v aktivno katehol oksidazo, inducirana pa je z enakimi komponentami kot aktivacija kaskade proPO (Coates 2012).

Pomemben del humoralne komponente prirojenega imunskega sistema predstavljajo ROS in RNS, ki sodelujejo pri uničenju fagocitiranih tujih delcev znotraj fagosomov hemocitov (Raman in sod. 2008, Jia in sod. 2017). Nastanejo tudi kot stranski produkti melanizacije in drugih celičnih procesov. Njihovo koncentracijo regulirajo antioksidativni encimi in drugi proteini, ki omejujejo oksidativni stres induciran s prekomerno sintezo reaktivnih spojin (Tassanakajon in sod. 2013). Med encimi v tovrstnem omejevanju velja omeniti superoksid dismutazo, katalazo in glutation reduktazo.

Antimikrobni peptidi ali obrambni peptidi gostitelja so evolucijsko ohranjene komponente prirojene imunosti. Zanje so značilni: (i) kratka aminokislinska zaporedja (7–100), (ii) hidrofobne in kationske lastnosti ter (iii) amfipatska struktura. Zaradi učinkovite in hitre protimikrobne aktivnosti proti najpogostejšim mikroorganizmom so ti peptidi poznani tudi kot »naravni antibiotiki«, na mikroorganizme pa delujejo preko tvorbe por v njihovi membrani ali vplivajo na različne celične procese mikrobov (Rosa in Barracco 2010, Ageitos in sod. 2017). Pri rakih poznamo kar 15 različnih družin antimikrobnih peptidov, med katerimi so najbolje poznani: krustin, penaedin, anti-lipopolisaharidni faktor, lizocim, defenzin in armadilidin (Smith in Dyrynda 2015).

Raziskave kopenskih rakov vrste *Porcellio scaber*

Celični imunski mehanizmi

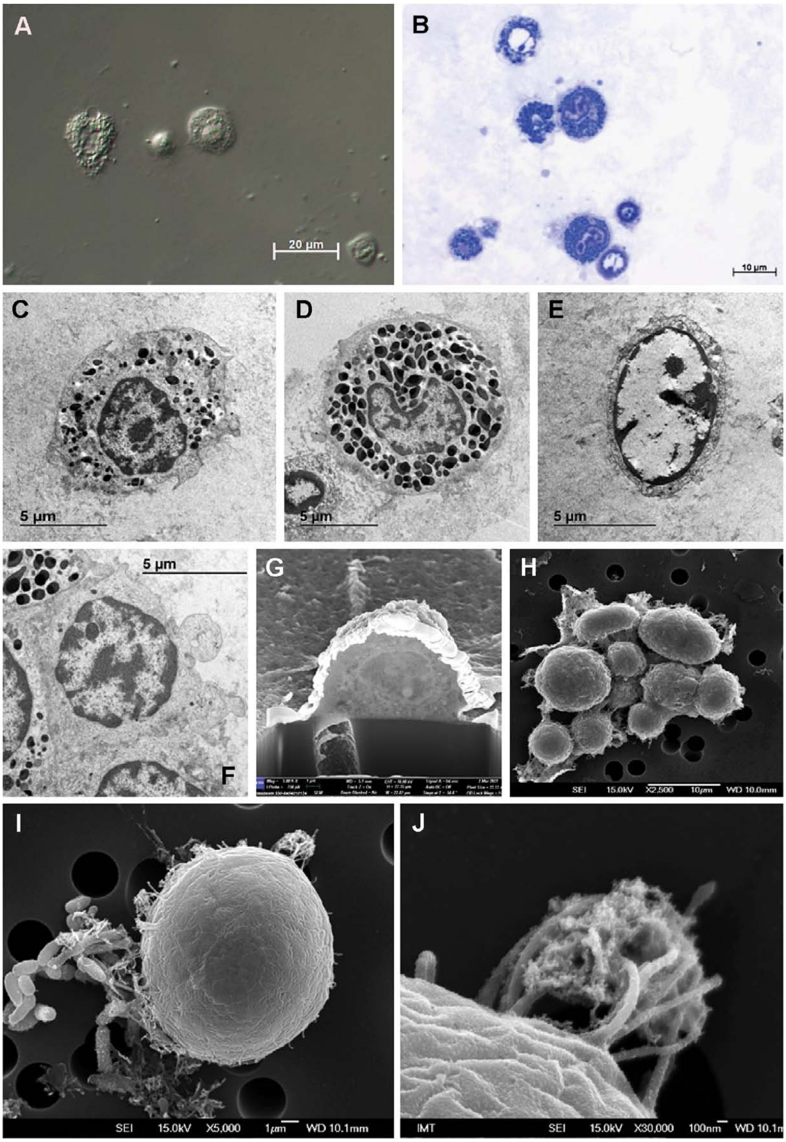
Večina raziskav imunskega sistema rakov je bila opravljena na morskih in sladkovodnih predstavnikih, tovrstni mehanizmi kopenskih enakonožcev pa so slabo raziskani (Cole in Morris 1980, Hess in Poinar 1985, Herbinière in sod. 2005, Irmak in sod. 2005, Jaenicke in sod. 2009, Sicard in sod. 2010, Chevalier in sod.

2011, Lupetti in sod. 2013, Kostanjšek in Pirc Marolt 2015, Becking in sod. 2020, Dolar in sod. 2020). V nedavnih študijah (Dolar in sod. 2020, 2021) smo pri kopenskem enakonožnem raku *P. scaber* opisali tri različne tipe hemocitov. Ti ustrezajo predhodnim opisom semigranularnih, granularnih in hialinih hemocitov (Sl. 2) drugih rakov (Ding in sod. 2012, Lv in sod. 2014, Zhou in sod. 2018), zlasti hemocitom sorodnega kopenskega enakonožca *Armadillidium vulgare* (Chevalier in sod. 2011). Gostota hemocitov v hemolimfi *P. scaber* je podobna vrednostim za vrsto *Porcellio spinicornis* (Alikhan in Naich 1986) in približno 10-krat nižja kot pri *A. vulgare* (Sicard in sod. 2010, Chevalier in sod. 2011). Viabilnost hemocitov *P. scaber* je zelo podobna vrednostim drugih kopenskih rakov (Tab. 2). Semigranulociti so najpogostejši tip celic, saj predstavljajo kar 65 % vseh prostih hemocitov v hemolimfi *P. scaber* (Sl. 2c). Gre za celice ovalne do okrogle oblike, ki v premeru merijo 11,2 ± 0,4 µm, v citoplazmi pa imajo majhno gostoto citoplazemskih granul manjše velikosti (premer 0,3 ± 0,1 µm). Granulociti predstavljajo 17 % vseh prostih celic hemolimfe in so po velikosti in obliki zelo podobni semigranulocitom (12,0 ± 0,3 µm), medtem ko je gostota citoplazemskih granul pri njih večja (Sl. 2d). Povprečna velikost citoplazemskih granul pa je 0,8 ± 0,1 µm. Hialinociti v premeru merijo le 8,1 ± 0,3 µm in predstavljajo v povprečju 18 % vseh prostih hemocitov hemolimfe (Sl. 2e in f). So okrogli in agranularni.

Razmerja med posameznimi hemociti pri *P. scaber* s prevladujočim deležem semigranulocitov ustrezajo podatkom za vrsto *A. vulgare* (Chevalier in sod. 2011), medtem, ko so pri *P. spinicornis* poročali o precej manjšem deležu semigranulocitov (12 %) (Alikhan in Naich 1986), kar pa ni v skladu s podatki za nekatere morske rake deseteronožce (Martin in Graves 1985, Jussila in sod. 1997).

S transmisijsko elektronsko mikroskopijo smo identificirali dva podtipa agranularnih hialinocitov (Dolar in sod. 2020). Podtip 1 hialinocita ima veliko in okroglo jedro s prevladujočim evkromatinom in tanko plastjo heterokromatina tik pod notranjo membrano jedrnega ovoja (Sl. 2e). Podtip 2 hialinocita pa ima manjše celično jedro s prevladujočim heterokromatinom (Sl. 2f). Nekateri avtorji predvidevajo, da bi lahko celice s podobnimi morfološkimi znaki (majhne celice z velikim razmerjem med jedrom in citoplazmo) kot smo jih opisali pri podtipu 1 hialinocita, predstavljale nezrele hemocite oziroma zarodne celice (Roulston in Smith 2011, Rebelo in sod. 2013, Ramlingam in sod. 2015). Slednje bi lahko potrdili z nadaljnjimi analizami, kot so npr. genska ekspresija transkripcijskih faktorjev udeleženih v proliferaciji in diferenciaciji zarodnih celic ali pa z imunolokalizacijo omenjenih molekul (Matatall in sod. 2018). Podobne razlike med podtipi hemocitov so bile sicer opažene tudi pri drugih rakih (Battison in sod. 2003, Lv in sod. 2014, Mangkalanan in sod. 2014).

Z vrstično elektronsko mikroskopijo smo glede na velikost in prisotnost citoplazemskih granul prepoznali dve populaciji celic (Sl. 2h-j). Pri populaciji večjih celic smo na površini oziroma v liziranih celicah prepoznali citoplazemske granule (Dolar in sod. 2020), podobno kot Zhou in sod. (2018), ki so jih na površini hemocitov in v liziranih hemocitih rakovice *Scylla paramamosain* opisali kot citoplazemskim granulam podobne izbokline. Prisotnost citoplazemskih granul v citoplazmi večjih celic smo potrdili tudi s kombinacijo tehnik fokusiranega ionskega žarka in vrstične elektronske mikroskopije (FIB-SEM) (Sl. 2g). Za razliko od večjih celic, pa je površina manjših celic (Sl. 2h in i) gladka, brez granulam podobnih izboklin, hkrati pa so na površini bolj pogosto opazne filopodijem podobne strukture (Sl. 2i in j). Domnevamo, da populacija manjših celic predstavlja hialinocite, katerih glavna funkcija je fagocitoza (Sl. 2j).



Slika 2: Mikrografije hemocitov kopenskega enakonožca *Porcellio scaber*. **A** - DIC mikroskopska tehnika hemocitov; **B** - presevna svetlobna mikroskopija poltankih rezin hemocitov barvanih z barvilom Azur II-metilen modro. Transmisijska elektronska mikroskopija **C**- semigranulocita, **D** - granulocita, **E** - hialinocita podtipa 1 in **F** - hialinocita podtipa 2. **G** - FIB-SEM analiza granularnega hemocita. **H**, **I**, **J** - vrstična elektronska mikroskopija hemocitov.

Figure 2: Micrographs of haemocytes of the terrestrial isopod *Porcellio scaber*. **A** - DIC microscopic technique of haemocytes and **B** - transmitted light microscopy of semi-thin slices of haemocytes stained with Azure II -methylene blue dye. Transmission electron microscopy of **C** - semigranulocyte, **D** - granulocyte, **E** - subtype 1 hyalinocyte and **F** - subtype 2 hyalinocyte. **G** - FIB-SEM analysis of granular haemocyte. **H**, **I**, **J** - scanning electron microscopy of haemocytes.

Pri kopenskih rakih enakonožcih so za fagocitiranje odgovorne hialine celice, v manjšem obsegu pa tudi semigranularne celice (Chevalier in sod. 2011). Znano je, da so pri navadnem pasavčku *Armadillidium vulgare* v tvorbo nodulov in enkapsulacijo udeleženi predvsem semigranulociti in v manjšem obsegu tudi granulociti, medtem ko so pri *P. scaber* za to odgovorni predvsem semigranulociti (Kostanjšek in Pirc Marolt 2015). Pri *P. scaber* sta Kostanjšek in Pirc Marolt (2015) podrobno opisala tvorbo nodulov v primeru okužbe z bakterijo *Rhabdochlamydia porcellionis*. Opisala sta različne oblike nodulov na organih z znotrajcelično okužbo z omenjeno bakterijo. Avtorja sta sklepala, da je tvorba nodulov velikosti med 20 in 200 µm na površini organov posledica odstranjevanja teh bakterij v telesni votlini oziroma hemocelu. Na podlagi *R. porcellionis* v hemocitih hemolimfe in semigranulocitih hematopoetskega tkiva sta potrdila njihovo fagocitno aktivnost.

Pri rakih enakonožcih je hematopoetski organ sestavljen iz treh parnih nodulov, ki se nahajajo v 6. in 7. segmentu pereona ter v 1. segmentu pleona (Chevalier in sod. 2011). Procese enkapsulacije in melanizacije so ob vstavitvi sintetičnih filamentov v telesno votlino *P. scaber* opisali tudi Irmak in sod. (2005). Kot primer celičnega odziva *P. scaber* lahko pokažemo primer okužbe z glisto, pri kateri smo v hemolimfi opazili aglutinacijo hemocitov na njeni površini. V skupkih smo opazili predvsem granulocite, vendar pa so za potrditev tega potrebne nadaljnje raziskave z večjim številom vzorcev (Sl. 3).

Humoralni obrambni mehanizmi

V hemolimfi *P. scaber* smo analizirali fenol oksidazno (PO) oziroma PO podobno aktivnost (Dolar in sod. 2020). Slednjo, pa tako kot pri drugih rakih, poleg fenol oksidaze posedujejo tudi drugi proteini hemolimfe, med drugim hemocianin. Kljub številnim raziskavam še vedno ni povsem jasno ali je dokazana PO aktivnost pri rakih enakonožcih posledica plazemske PO, PO podobne aktivnost hemocianina ali kar obeh (Sicard in sod. 2010). Pri *P. scaber* (Jaenicke in sod. 2009) in dveh morskih enakonožcih (Smith in Söderhäll 1991, Pless in sod. 2003) je najvišja PO aktivnost hemocianina v *in vitro* pogojih dosežena z dodatkom detergentov (npr. natrijevega dodecilsulfata; SDS) v reakcijsko mešanico, ki posnemajo naravne aktivatorje in povzročijo konformacijsko pretvorbo molekule hemocianina in s tem sprostitev aktivnega mesta za fenolne spojine (Baird in sod. 2007). Slednje smo potrdili

Slika 3: Aglutinacija hemocitov na površini gliste v hemolimfi *Porcellio scaber*.
Figure 3: Agglutination of haemocytes on the surface of nematode in the *Porcellio scaber* haemolymph.

tudi v naših raziskavah, v katerih brez dodatka SDS hemolimfi aktivnosti nismo zasledili, ta pa se je povečevala z večanjem koncentracije SDS v hemolimfi. Ker ne moremo z gotovostjo trditi, da je bila z meritvami potrjena encimska aktivnost zgolj posledica aktivnost encima PO, smo jo opredelili kot PO podobno encimsko aktivnost (analogno z ustaljenim angleškim izrazom »PO--like«). Aktivnosti smo merili po optimiziranem protokolu Jaenicke in sod. (2009), kot je opisano v Dolar in sod. (2020). Vrednosti PO podobne aktivnosti za *P. scaber* ustrezajo vrednostim, ki so jih podali Jaenicke in sod. (2009), primerjava z literaturo pa je zahtevna, saj avtorji za izražanje aktivnosti uporabljajo različne enote (Irmak in sod. 2005, Sicard in sod. 2010).

Dušikov oksid (NO) v hemolimfi enakonožca smo merili z uporabo Griessovega reagenta, po prilagojeni metodi Faraldo in sod. (2005). Zaznane vrednosti NO pri *P. scaber* ustrezajo vrednostim, ki so jih pri tem organizmu pomerili tudi Mayall in sod. (2021) (Tab. 2). Poleg meritev reaktivnih dušikovih spojin smo spremljali tudi aktivnost antioksidativnih encimov katalaze in superoksid dismutaze, ki posredno nakazujeta prisotnost ROS. Ustaljene protokole za merjenje aktivnosti na nivoju celotnega organizma (Beauchamp in Frodovich 1971, Jemec in sod. 2008) smo prilagodili za merjenje na majhnih vzorcih hemolimfe (5 µl), kar je omogočilo merjenje na vzorcih posameznih živali (Dolar 2021). Z uporabo specifičnega inhibitorja katalaze, natrijevega azida, smo dodatno dokazali, da v hemolimfi merimo katalazno aktivnost. V literaturi podatkov o katalazni aktivnosti v hemolimfi drugih kopenskih rakov sicer nismo našli. Poleg reaktivnih zvrsti smo v naših raziskavah kot prvi vpeljali tudi meritve aktivnosti alfa-2 makroglobulina (Dolar 2021).

Tabela 2: Vrednosti imunskih komponent merjenih v vzorcih hemolimfe osebkov *Porcellio scaber,* iz kontrolne laboratorijske populacije brez simptomov okužbe ali stresa (povzeto po Dolar in sod. 2020, 2021).

Table 2: Values of immune components measured in samples of haemolymph from *Porcellio scaber* individuals from the control laboratory population without symptoms of infection or stress (summarized after Dolar et al. 2020, 2021).

Imunska komponenta	**Povprečna vrednost ± standardna napaka**	**Podatki iz literature**
Gostota hemocitov v hemolimfi (št. celic/mL hemolimfe)	$3{,}50 \pm 0{,}19 \times 10^6$	$25\text{-}41 \times 10^6$ [a] $32 \pm 4 \times 10^6$ [b] $4{,}93 \times 10^6$ [c] $4{,}3 \times 10^6$ [d]
Viabilnost hemocitov (%)	93,77 ± 0,42	91 [b] 92,16 ± 0,66 [c] 78 [e]
Delež semigranulocitov (odstotek vseh hemocitov, %)	65,01 ± 1,89	72 [b] 12 [d]
Delež granulocitovov (odstotek vseh hemocitov, %)	17,25 ± 1,55	21 [b] 28 [d]
Delež hialinocitov (odstotek vseh hemocitov, %)	17,74 ± 1,30	7 [b] 60 [d]
Fenol oksidazi podobna aktivnost (ΔA/min mL hemolimfe)	10,23 ± 0,22	15 [f]
Koncentracija dušikovega oksida (µM)	14,16 ± 1,24	10,5 [c]
Aktivnost superoksid dismutaze (U/mg proteinov)	4,58 ± 0,66	n.p.
Aktivnost katalaze (ΔA/min mg proteinov)	0,55 ± 0,07	n.p.
Aktivnost alfa-2 makroglobulina (ΔA/min mg proteinov)	1,06 ± 0,12	n.p.

[a] *Armadillidium vulgare* (Sicard in sod. 2010); [b] *A.vulgare* (Chevalier in sod. 2011); [c] *P. scaber (*Mayall in sod. 2021); [d] *P. spinicornis* (Alikhan in Naich 1986); [e] *P. laevis* (Snyman in Odendaal 2009); [f] *P. scaber* (Jaenicke in sod. 2009); n.p., ni podatka

Med pomembnejše komponente humoralnega imunskega odziva sodijo tudi antimikrobni peptidi, ki pri *P. scaber* do sedaj še niso bili opisani, medtem ko so Herbinière in sod. (2005) iz hemocitov in plazme hemolimfe *A. vulgare* izolirali in opisali nov antimikrobni peptid imenovan armadillidin. V nedavni raziskavi so Becking in sod. (2020) z *in silico* analizami dokazali, da so sekvence armadillidinov pogosto zastopane v transkriptomih kopenskih rakov enakonožcev, vključno s *P. scaber*.

Transkriptomske analize in dostopnost nukleotidnih zaporedij je spodbudilo uporabo omskih pristopov tudi v okoljskih študijah. Chevalier in sod. (2012) so tako analizirali izražanje genov povezanih z imunskim sistemom v hemocitih *A. vulgare*, okuženih z bakterijo iz rodu *Wolbachia*. Podobno so izražanje tarčnih genov preverjali tudi Ferreira in sod. (2019), pri kopenskem enakonožcu *Porcellionides pruinosus*. Podatki o tovrstnih študijah pri *P. scaber* zaenkrat še niso poznani, smo pa v našem laboratoriju že optimizirali metodo in naredili prve poskuse izražanja genov povezanih z imunskim sistemom.

Zaključek in pogled v prihodnost

V prispevku smo predstavili pregled temeljnih komponent in procesov prirojene imunosti pri rakih. Podrobneje smo orisali raziskave s kopenskimi raki vrste *Porcellio scaber,* ki jih na tem področju izvajamo na Katedri za zoologijo, Oddelka za Biologijo. Trenutno se intenzivno ukvarjamo s proteomom hemolimfe *P. scaber*, v teku pa so tudi raziskave, v sklopu katerih bomo v celicah hemolimfe (hemocitih) analizirali izražanje genov, ki so povezani z imunostjo. V ta namen smo že optimizirali protokole za izolacijo zadostne količine RNA ustrezne kvalitete. Transkriptom *P. scaber*, ki smo ga pridobili iz Univerze v Cardiffu (Velika Britanija, Wales) in iz podatkovne baze NCBI (SRA), smo z orodjem BLAST primerjali proti znanim zaporedjem iz podatkovne baze (NCBI) in tako identificirali tarčne sekvence ter pripravili oligonukleotidne začetnike. Za slednje smo tudi preverili učinkovitost naleganja na cDNA prevedene iz RNA zaporedja hemocitov *P. scaber*.

Z opisanimi raziskavami smo začrtali pot za nadaljnje raziskave na področju fiziologije rakov, biologije stresa in ekotoksikologije, kjer izbrane imunske komponente merimo pri organizmih izpostavljenih različnim pogojem in vrstam onesnažil (Dolar in sod. 2021, Mayall in sod. 2021). Pričakujemo, da bo na ta način omogočen celostni vpogled v delovanje prirojenega imunskega sistema pri kopenskih rakih, hkrati pa tudi možnost presoje vplivov okoljskih sprememb in onesnažil na več nivojih biološke organizacije organizma. Vpeljano metodologijo spremljanja imunskih procesov, na podlagi merjenja imunskih komponent, bi v prihodnosti lahko prenesli tudi na druge vrste rakov, ki jih proučujemo v okviru programske skupine P1-0184.

Summary

Organisms interact with their environment in an attempt to maintain a dynamic equilibrium that allows for the survival and development of the organism. The immune system is crucial to recognise and distinguish between self and nonself, and react to challenges posed by the environment. The purpose of this review is to present the basic features of innate immunity of crustaceans and in particular of terrestrial crustacean woodlice *Porcellio scaber*. This organism has been an established test model at the Chair of Zoology, Department of Biology (Biotechnical Faculty, University of Ljubljana) for more than 3 decades. In the last decade we have implemented the methods for tracking the cellular and humoral immune response to infections and other types of interactions, such as pollutants. We have performed basic research on the ultrastructure and morphology of haemocytes in *P. scaber*. Three types were identified: semigranulocytes (65% of freely circulating haemocytes), granulocytes (17%), and hyalinocytes (18%). The average density of free circulating haemocytes is $3.50 \pm 0.19 \times 10^6$ cells/mL. Semigranulocytes and granulocytes are both oval shaped and similar in diameter (11.2 ± 0.4 µm and 12.0 ± 0.3 µm, respectively), but granulocytes have a higher density of granules, which are also larger than in semigranulocytes. Hyalinocytes are round, agranular and smaller (8.1 ± 0.3 µm). Two types of hyalinocytes have been discovered: subtype 1 has a very large nucleus with dominant euchromatin, while subtype 2 has a

smaller nucleus with predominant heterochromatin. Subtype 1 is of particular interest because it resembles the properties of stem progenitor cells. We have implemented new methods to measure humoral components in *P. scaber* haemolymph, including: Phenoloxidase-like activity, nitric oxide levels, antioxidant enzyme activity, and alpha-2 macroglobulin. Similarly, as for other crustaceans, the activity of phenoloxidase was not limited only to this enzyme, but also included other proteins in the haemolymph, most likely hemocyanin. As for the cellular immune response in *P. scaber*, when infected with the bacterium *Rhabdochlamydia porcellionis*, the formation of nodules by mostly semigranulocytes was noted, and agglutination of haemocytes on the parasite (nematode) was observed. Our future goal is to study the immune response of *P. scaber* at the proteome and transcriptome levels. We intend to use these immune components in other research areas, for example in ecotoxicology, to study the effects of pollutants and other types of biotic and abiotic stress on organisms. Finally, newly developed methods can be implemented to crustaceans from other phylogenetic groups which are also studied in the framework of our Research programme P1-0184.

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Polhova mast in protimikrobno učinkovanje

Dormouse fat and antimicrobial activity

Leon Drame[a], Sara Skok[a], Janez Mulec[a,b*]

[a] Znanstvenoraziskovalni center Slovenske akademije znanosti in umetnost, Inštitut za raziskovanje krasa, Titov trg 2, 6230 Postojna
[b] Univerza v Novi Gorici, Krasoslovno študijsko središče Unesco, Glavni trg 8, 5271 Vipava

*Korespondenca: janez.mulec@zrc-sazu.si

Izvleček: Polhovo mast se še vedno v ljudskem zdravilstvu uporablja za lajšanje številnih zdravstvenih težav, vendar zaenkrat ne obstajajo sistematične raziskave o njeni vlogi kot protimikrobne učinkovine. V sveži pohovi masti, pridobljeni na tradicionalen način ter tudi v tisti, ki je že bila uporabljena, so bili prisotni mikroorganizmi. Polhova mast v poskusu *in vitro* ni izkazala inhibitornega učinka na rast indikatorskih bakterij *Escherichia coli* in *Staphylococcus epidermidis*. Protimikrobno učinkovitost, o kateri priča ljudsko zdravilstvo, in jo potrjuje njena stalna uporaba, gre najverjetneje pripisati njeni stimulaciji imunskega sistema gostitelja. Potrebne so nadaljnje raziskave, ki bodo potrdile uporabnost polhove masti oziroma njenih aktivnih sestavnih delov pri zdravljenju mikrobnih okužb z večkratno odpornimi sevi.

Ključne besede: mikroorganizmi, polhova mast, protimikrobna učinkovitost

Abstract: Dormouse fat continues to be used in traditional medicine to alleviate various health problems but, to date, there has been no systematic research into its role as an antimicrobial agent. Microorganisms have been confirmed as being present both in fresh dormouse fat, that has been produced in a traditional way and in previously applied fat. When studied *in vitro,* dormouse fat showed no inhibitory effect on the growth of the indicator bacteria *Escherichia coli* and *Staphylococcus epidermidis*. The apparent antimicrobial efficacy attested by its continuing and seemingly successful use in traditional medicine is most likely related to stimulation of the host immune system. Further research is needed to investigate and confirm the usefulness of dormouse fat, or its active components, in the treatment of infections related to multiple resistant microbial strains.

Keywords: antimicrobial activity, dormouse fat, microorganisms

Uvod

Za lajšanje zdravstvenih tegob so si ljudje v preteklosti pomagali na različne načine. Eden izmed tradicionalnih zdravilnih pripravkov, značilen za slovenski prostor, je tudi polhova mast (Peršič 1998). Polhova mast se v ljudskem zdravilstvu uporablja vsestransko in se jo na kožo nanaša pri težavah z bradavicami, brazgotinami, izpuščaji, ranami, urezninami, odrgninami, zlomi, zvini, ozeblinami in oteklinami, glivičnimi ter virusnimi (herpes) infekcijami. Poročajo tudi o uspehih pri odpravljanju težav s hemeroidi, prhljajem, koprivnico, luskavico, artritisom, išiasom in revmatizmom. O uspehih pri oralni uporabi poročajo pri angini, gripi, kašlju, vneti sluznici prebavil, zaprtju, težavah z jetri in dvanajstnikom. Polhova mast naj bi bila učinkovita tudi proti raku na želodcu in debelem črevesu.

Ker vsebuje polhova mast visok delež nenasičenih maščobnih kislin (~90%), je tudi pri nižjih temperaturah, npr. -20 °C, v tekočem stanju. Raziskave kažejo, da imata polhova mast in žirovo olje, podobno maščobnokislinsko sestavo, posebej glede vsebnosti metil palmitata, metil oleata, metil linoleata in metil eikozenoata (Štos 2019). Kakovost in kemijska sestava polhove masti je odvisna od prehrane polhov in življenjskega cikla – hibernacije (Fietz in sod. 2005, Šiftar 2020).

Pridobivanje polhove masti se razlikuje pri posameznih pridelovalcih in vključuje počasno taljenje in razpuščanje maščobnega tkiva navadnih polhov *Glis glis* v posodi nad paro, z izpostavljanjem močnim sončnim žarkom, ali pa s cvrtjem v ponvi (Vesel 2016). Zaradi tradicionalnega postopka pridobivanja polhova mast ni sterilna. Zaenkrat ne obstajajo sistematične raziskave o njeni vlogi kot protimikrobne učinkovine oziroma mehanizmih delovanja na imunski sistem. V tej predhodni raziskavi smo želeli preveriti protimikrobno delovanje polhove masti na dveh indikatorskih bakterijskih vrstah, *Escherichia coli* in *Staphylococcus epidermidis*.

Material in metode

V raziskavi smo uporabili vzorce polhove masti različnih starosti, pridobljenih na tradicionalen način, iz treh lovskih območij (Tab. 1). V vzorcih smo preverili prisotnosti kultivabilnih mikroorganizmov na šestih mikrobioloških gojiščih (CNA: Colistin Nalidixic Acid, kolistin nalidiksična kislina, za selektivno izolacijo po Gramu pozitivnih bakterij, Služba za pripravo gojišč in reagentov IMI MF UL; CHROMID®ESBL: Extended-Spectrum β-Lactamase, široko spektralna β-laktamaza, za izolacijo enterobakterij, ki sintenizirajo β-laktamaze, Biomérieux; KA: krvni agar za gojenje prehransko nezahtevnih bakterij, Služba za pripravo gojišč in reagentov IMI MF UL; MACC: MacConkey agar za selektivno izolacijo po Gramu negativnih enterobakterij glede na sposobnost fermentacije laktoze, Služba za pripravo gojišč in reagentov IMI MF UL; NA: Nutrient agar za neselektivno gojenje različnih mikroorganizmov, Sigma-Aldrich; CHROMID®VRE: Vancomycin-Resistant Enterococci, vankomicin odporni enterokoki, za izolacijo enterokokov, odpornih proti vankomicinu, Biomérieux). Vsako gojišče je bilo enakomerno inokulirano s 100 µl polhove masti, vendar so se na površini gojišč zaradi hidrofobnosti vzorca ohranile vidne kapljice masti ves čas inkubacije (Sl. 1A), ki je potekala aerobno 48 ur pri temperaturi 37 °C (CNA, CHROMID®ESBL, KA, MACC, NA in CHROMID®VRE) oziroma sedem dni pri temperaturi 20 °C (NA). Po preteku inkubacije smo številčno ovrednotili koncentracijo mikroorganizmov (kolonijske enote v CFU na mililiter) ter precepili zrasle kolonije. Nadaljnja subkultivacija kolonij iz primarnih plošč v enakih razmerah in na enakih gojiščih ni bila uspešna, kakor tudi ne gojenje v tekočem gojišču LB (Luria broth base, Miller, Sigma).

Za testiranje protimikrobne učinkovitosti polhove masti, ki se kaže v inhibiciji rasti indikatorskega mikroba, smo uporabili dve bakteriji in sicer vrsto *Escherichia coli*, sev DH5α [*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δlac*(lacZYA-argF) (φ80 lacZ ΔM15)*] ter sev ST100 bakterije *Staphylococcus epidermidis* (interna zbirka Inštituta za raziskovanje krasa ZRC SAZU). *E. coli* je značilna predstavnica človekovega prebavnega trakta (Eckburg in sod. 2005), *S. epidermidis* pa človekovega kožnega mikrobioma (Otto 2009). Prekonočno kulturo indikatorske bakterije smo postrgali s trdnega gojišča ter jo resuspendirali v 500 µl sterilne fiziološke raztopine in jo z vatenko (FLOQSwab, Copan) razmazali na gojišče NA, da smo zagotovili dovoljšnjo gostoto inokuluma za izvedbo testa, približno 10^8 CFU/ml. Na inokulirano gojišče smo nato položili sterilne diske iz filter papirja (Filtrak 388, Spezialpapierfabrik Niederschlag, Nemčija) na katere smo nanesli 5 µl vzorca polhove masti. Vzporedno smo na inokulirano gojišče nanesli še dve kapljici vzorca masti (5 µl) ter eno od njiju s sterilno plastično mikrobiološko zanko razpotegnili v ravni liniji (Sl. 1B). Pojav cone inhibicije rasti indikatorskega organizma smo preverili po 24 in 48 urah inkubacije pri 37 °C.

Rezultati in razprava

Rast mikroorganizmov na primarnih selektivnih in neselektivnih gojiščih se je pojavila tako v svežem (polhova mast, pridobljena leta 2020) kot v starejših vzorcih polhove masti (Tab. 1). Barvne reakcije pri kolonijah, zraslih na primarnih ploščah CHROMID®VRE in CHROMID®ESBL, niso bile značilne za odporne seve, glede na posamezno gojišče in navodila proizvajalca. Čiščenje kolonij iz primarnih plošč in njihovo gojenje za nadaljnjo identifikacijo v enakih laboratorijskih razmerah in na enakih gojiščih ni bilo uspešno. V tem in podobnih primerih je zato potrebna previdnost pri interpretaciji rezultatov gojenja. Predvidevamo, da so primarno zrasle bakterije mikroaerofilne, ki za preživetje potrebujejo kisik, za uspešno rast pa nižje ravni kisika od tistih, ki so prisotne v ozračju in so se vzpostavile v kapljicah masti (Sl. 1A). V nadaljnjih raziskavah polhove masti bo treba posebno pozornost posvetiti tej skupini mikroorganizmov.

Polhova mast je vsebovala spremljajoče mikroorganizme, na katere je treba biti pozoren v procesu zdravljenja in jih bo treba v nadaljnjih raziskavah identificirati. Zaradi prisotnosti mikroorganizmov, morebiti tudi anaerobnih, polhova mast ni primerna za neposreden nanos na odprte rane. Smiselno bo v prihodnje tudi testiranje, ali v polhovi masti preživijo ključni oportunistični in patogeni mikrobi. Polhova mast se v steklenički, ko si jo uporabnik nanese na prste za nanos na obolelo mesto, lahko tudi dodatno kontaminira s kožnim mikrobiomom uporabnika.

Polhova mast *in vitro* ni inhibirala rasti indikatorskih bakterij *E. coli* (Sl. 1B) in *S. epidermidis.* Inhibitorna oziroma protimikrobna učinkovitost polhove masti o kateri govori ljudsko zdravilstvo, se lahko izkazuje posredno preko stimulacije imunskega odgovora gostitelja. Polhova mast oziroma njeni prečiščeni sestavni deli bi zato lahko predstavljali alternativno možnost pri zdravljenju okužb z večkratno odpornimi sevi. Preučiti bo torej treba tudi morebiten vpliv metabolnih produktov mikrobiote polhove masti na hitrejši potek zdravljenja. Vsekakor bo treba ponovno poskusiti z izolacijo in identifikacijo mikroor-

Tabela 1: Kolonijsko število mikroorganizmov v polhovi masti na primarnih ploščah.
Table 1: Colony-forming units of microorganisms in dormouse fat inoculated onto primary plates.

	Kolonijsko število mikroorganizmov (CFU/ml) v polhovi masti iz treh lovskih področij (leto produkcije)		
	LD Babno Polje (2011)	**LD Rakek (2020)**	**LD Senožeče (2013)**
NA[1]	460	2370	4320
NA[2]	1990	3570	0
CHROMID®ESBL[2]	1970	17320	710
CHROMID®VRE [2]	6350	1060	0
CNA[2]	10	+	+
MACC[2]	260	2190	4170
KA[2]	30	+	130

Oznake: [1], 20 °C, 7 dni; [2], 37 °C, 48 ur; +, prisotnost rasti; NA, nutrient agar; CHROMID®ESBL, Extended--Spectrum β-Lactamase, široko spektralna β-laktamaza; CHROMID®VRE, Vanconycin-Resistant Enterococci, vamkomicin odporni enterokoki; CNA, Colistin Nalidixic Acid, kolistin nalidiksična kislina; MACC, MacConkey agar; KA, krvni agar ganizmov v polhovi masti ter ugotoviti stalnost vrstne sestave ter njeno delovanje na izbrane indikatorske, tudi večkratno odporne, potencialno patogene bakterijske vrste. Čeprav nismo dokazali neposredne protimikrobne učinkovitosti polhove masti na testiranih bakterijah, s pričujočim zapisom želimo spodbuditi nadaljnje proučevanje obstoječih tradicionalnih receptur ljudskega zdravilstva ter njihovo znanstveno ovrednotenje v luči razširjenja mikroorganizmov, odpornih proti protimikrobnim učinkovinam.

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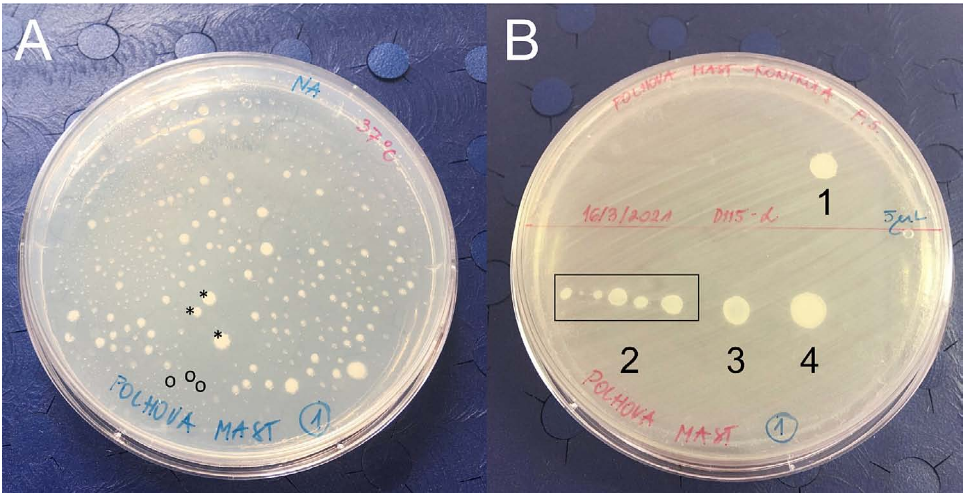

Slika 1: Mikrobiološko testiranje, primer vzorca polhove masti iz lovskega področja LD Rakek. **A** - rast bakterijskih kolonij (obarvane pike, *) na primarni plošči NA in kapljice masti (prosojne pike, o) brez opazne mikrobne rasti; **B** - testiranje protimikrobne učinkovitosti polhove masti z indikatorskim sistemom *E. coli*: petrijevka nad rdečo horizontalno črto (kontrola), (1) disk namočen v sterilni fiziološki raztopini ter mikrobna prerast; petrijevka pod rdečo črto, (2) odsotnost inhibicije rasti *E. coli* vzdolž linearnega nanosa polhove masti, kjer je prišlo do nastajanja kapljic zaradi hidrofobnosti vzorca, (3) okoli kapljice masti ter (4) okoli diska, namočenega v polhovi masti.

Figure 1: Microbiological examination; example of a sample of dormouse fat from the hunting area of LD Rakek. **A** - growth of bacterial colonies (opaque dots, *) on the primary plate of nutrient agar (NA) and fat droplets (transparent dots, o) without any apparent microbial growth; **B** - testing of antimicrobial activity of dormouse fat using an *E. coli* indicator strain: petri dish above the red horizontal line (control), (1) disc soaked with sterile saline and microbial overgrowth; petri dish below the red line, (2) no inhibition of *E. coli* growth along a linear application of dormouse fat, where droplets formed due to the hydrophobicity of the sample, (3) around a fat droplet and (4) around the disc soaked with dormouse fat.

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UV radiation and temperature effects on functional traits in *Helianthemum nummularium* subsp. *grandiflorum* at the alpine and montane site in the Slovenian Alps

Učinki UV sevanja in temperature na funkcionalne značilnosti *Helianthemum nummularium* subsp. *grandiflorum* na alpinskem in montanskem rastišču v Julijskih Alpah

Tadeja Trošt Sedej*, Rok Damjanič

Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, SI-1000 Ljubljana, Slovenia

*Correspondence: tadeja.trostsedej@bf.uni-lj.si

Abstract: Alpine plants have evolved strategies to survive harsh conditions, which include high UV and visible radiation, extreme temperatures, dryness and lack of nutrients. Survival strategies include biochemical, physiological and morphological responses, which are scarcely studied because of the time-demanding and complex experimental conditions. We researched functional traits in the alpine plant common rockrose *Helianthemum nummularium* subsp. *grandiflorum* growing under ambient UV-B and reduced UV-B radiation at different altitudes (1600 and 2000 m a.s.l.) of mount Vogel in the Slovenian Alps. Leaf anatomy, pigments and optical properties were investigated at the beginning and at the end of the growing season. Plants showed high constitutive UV-absorbing compounds content (UV-AC) throughout the season. Most leaf thickness parameters were not altered according to UV and altitude conditions. Leaves did not transmit any UV spectrum, in agreement with high UV-AC. High photosynthetic spectrum transmittance at alpine altitudes was due to complex biochemical and anatomical responses to these conditions, rather than to UV radiation. Unchanged chlorophyll content of *H. nummularium* could be related to shrub life form, where leaves shade out high UV and PAR irradiance as well as contribute to lower leaf temperature. This study shows the complexity of alpine plant response, where specific characteristics of plant species should not be overlooked.

Keywords: *Helianthemum nummularium*, leaf optical properties, pigments, UV radiation

Izvleček: Alpinske in gorske rastline so razvile strategije za preživetje v skrajnih razmerah, ki vključujejo visoko jakost UV in vidnega sevanja, skrajne temperature, sušo in pomanjkanje hranil. Strategije preživetja alpinskih rastlin vključujejo biokemične, fiziološke in morfološke odzive, ki jih raziskovalci zaradi zahtevne izvedbe redko proučujejo. V naši raziskavi smo proučevali funkcionalne značilnosti alpske rastline velecvetni popon *Helianthemum nummularium* subsp. *grandiflorum*, in sicer pod naravnim in zmanjšanim sevanjem UV-B na dveh različnih nadmorskih višinah (1600 in 2000 m n.m.) gore Zadnji Vogel v Julijskih Alpah.

Anatomijo listov, pigmente in optične lastnosti smo raziskovali na začetku in na koncu rastne sezone. Rastline so ob obeh merjenjih vsebovale veliko UV-absorbirajočih snovi (UV-AC). Večina parametrov, s katerimi smo ocenjevali debeline listnih tkiv, se ni odzivala na različne sevalne in temperaturne razmere. Listi niso prepuščali UV spektra sevanja, kar se je ujemalo z veliko vsebnostjo UV-AC. Velika prepustnost fotosinteznega spektra sevanja na alpinski nadmorski višini je bila povezana z biokemičnimi in anatomskimi značilnostmi rastline in ne z jakostjo UV sevanja. Nespremenjena vsebnost klorofila pri *H. nummularium* bi lahko bila povezana z grmičasto rastno obliko rastline, kjer zgornji listi zasenčijo spodnje liste ter tako prispevajo k manjšim prejetim odmerkom UV in fotosintezno aktivnega sevanja ter k nižji temperaturi listov. Študija kaže na kompleksen odziv alpinskih rastlin na UV sevanje in temperaturne razmere v gorah, pri čemer je pomembna tudi rastna oblika rastline.

Ključne besede: *Helianthemum nummularium,* optične značilnosti listov, pigmenti, UV sevanje

Introduction

Alpine plants come in a variety of life forms and have evolved various strategies to allow their survival under harsh mountain conditions (Körner 2003). Alpine plants adapt and acclimate to high ultraviolet (UV) and photosynthetically active radiation (PAR), low temperatures and frost, rapid temperature changes, soil dryness, lack of nutrients, low air humidity, strong winds and short growing season (Nicotra et al. 2010). Alpine environmental conditions can be stressful for plant life, where topography, exposure, diseases and human activities can also contribute to the stressful environment (Turunen and Latola 2005).

Ultraviolet-B radiation has been reported to cause changes in leaf morphology that lead to smaller and thicker leaves, with increased thickness of the epidermis or cuticle. These appear to be adaptive responses to stressful alpine environments (Jansen 2002; Robson et al. 2015). High UV-B radiation can negatively affect growth and productivity (Wargent et al. 2009; Gruber et al. 2010), carbon assimilation (Guidi et al. 2011; Hideg et al. 2013) and stomatal function (Nogues et al. 1999). Photosynthetic rates can be sustained under ambient doses of UV-B radiation through enhanced photoprotection, which depends on the induced synthesis of UV-B–absorbing compounds (UV-B–AC) and activation of the plant antioxidant defence system (Wargent et al. 2011; Kataria et al. 2014) or induction of photo-repair, which lead to the recovery of photochemical efficiency (Xu and Gao 2010).

Damaging UV-B effects at high altitudes and low latitudes are an exception rather than norm (Searles et al. 2001). The responses of high-altitude plants to UV-B radiation are often less pronounced compared to those of low-altitude plants, which suggests that alpine plants are acclimated and well-adapted to greater intensities of UV-B radiation. Indeed, these plants might have simultaneous co-tolerance to several stress factors, as acclimation or adaptation to harsh climates can also increase tolerance to UV-B radiation, and *vice versa.* Such interactions might aggravate or mitigate plant responses. Plant can increase their tolerance to UV-B, low temperatures or drought through increased acclimation to a second stressor (Chalker-Scott and Scott 2004; Trošt Sedej and Gaberščik 2008; Terfa et al. 2014).

The present study investigated adaptations and acclimatisation to increased UV-B radiation at two different alpine altitudes in the alpine plant common rockrose *Helianthemum nummularium* subsp. *grandiflorum* (Scop.) Schinz & Thell.). Common rockrose is widespread from upper montane (1200-1600 m a.s.l.) to alpine (1600-2000 m a.s.l.) altitudes in Central and Southern European mountains. This indicates its successful adaptation and acclimation to the alpine environment. Current research of *Helianthemum* has been limited to phylogenetics (Volkova et al. 2016).

The objective of the present study was to investigate *H. nummularium* responses to different UV radiation doses at different altitudes in its natural habitat. We aimed to study the interactive effects of diverse environmental conditions on the responses of *H. nummularium* in its natural habitat.

Materials and methods

Plant species

Helianthemum grandiflorum subsp. *grandiflorum* is an alpine herbaceous perennial that sometimes has a woody base. It grows 10 to 30 cm high, and flowers from June to September. *H. nummularium* is commonly distributed over warm, dry, rocky limestone meadows from montane to alpine altitudes throughout the Alps and other high mountains of Central Europe (Martinčič et al. 2010).

Experimental site

The experimental site was located at Mount Zadnji Vogel in the Julian Alps, Slovenia, and comprised a montane and an alpine altitude plot (1600 m a.s.l.: 46°19'04.4"N, 13°48'33.9"E; 2000 m a.s.l.: 46°19'46.7"N, 13°48'07.3"E) (Fig. 1). Both plots were above the timberline, on southerly exposed slopes. Slope steepness was 70% to 100%. The vegetation consisted of meadows on calcareous ground that lacked grazing and tourism. Their mean annual precipitation from 1981-2010 was 2600 to 3200 mm (Slovenian Environment Agency).

Two different UV exposures were provided to the plants using two types of filters: a Quinn XT UV filter (UV-), which absorbed UV-B and UV-A; and a Quinn cast UVT filter (UV), which was transparent to UV-B and UV-A (Quinn-Plastics, UK). Five UV and five UV- filters (each 20 × 20 cm) were positioned 15 cm above a group of three to six plants at each altitude. Similar soil water contents near and under the filters were maintained due to slope steepness, heavy rainfall and small size of the filters.

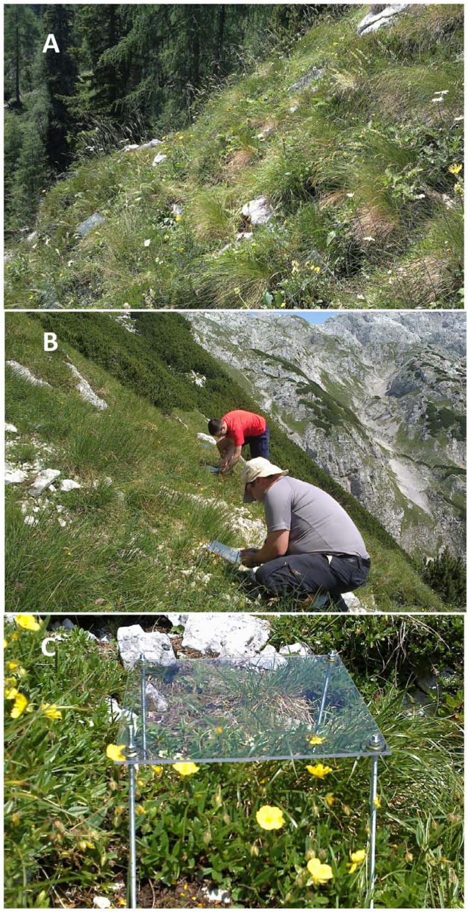

Figure 1: Experimental plot at Zadnji Vogel. **A** - at 1600 m a.s.l. **B** - at 2000 m a.s.l. **C** - *Helianthemum nummularium* subsp. *grandiflorum.*

Slika 1: Poskusna ploskev na Zadnjem Voglu. **A** - na 1600 m n.m. **B** - na 2000 m n.m. **C** - *Helianthemum nummularium* subsp. *grandiflorum.*

UV-B radiation was monitored over four days with a clear sky for all four different treatments (two altitudes × two UV exposures), using a radiometer with a UV-B radiometric sensor (RM-22; Opsytec Dr. Gröbel, Germany). The data collected were used to determine the proportion of the UV-B radiation that reached the plants under the different UV filters. The standard daily UV-B doses (UV) and the reduced daily UV-B doses (UV-) were calculated over three months (July, August, September) and for the four treatments using a model (Björn and Murphy 1993) that included Caldwell's generalised plant action spectra (Caldwell et al. 1986) for daily clear-sky measures (Tab. 1).

Table 1. Total and biologically active UV-B doses calculated for daily clear-sky (UV) and reduced daily clear-sky (UV-) UV-B according to month and altitude (Björn and Murphy 1993).
Tabela 1. Celokupni in biološko aktivni odmerki UV-B, izračunani za sončen dan (UV) in sončen dan pri zmanjšani jakosti (UV-) UV-B glede na mesec in nadmorsko višino (Björn in Murphy 1993).

		UV-B dose (kJ m^{-2} day^{-1})					
		July		August		September	
Filter	UV-B	1600 m	2000 m	1600 m	2000 m	1600 m	2000 m
UV	Total	55.91	57.58	47.75	49.27	34.52	35.73
	Biologically active	7.13	7.34	5.86	6.05	3.9	4.04
UV-	Total	16.77	17.28	14.32	14.78	10.36	10.38
	Biologically active	2.14	2.2	1.76	1.82	1.17	1.18

For both research plots, temperatures were monitored 10 cm underground once per hour during the experimental period, using temperature data loggers (HOBO TidbiTv2; Onset Computer Corporation, USA) (Fig. 2).

Measurements

Plants were analysed in July and September. The UV filters were placed above the plants at the end of June. The first sampling and measurements were conducted three weeks after the UV filters were placed. Measurements were performed on 10 randomly selected fully developed upper leaves per treatment at two altitudes. All the analysed leaves developed under the filters.

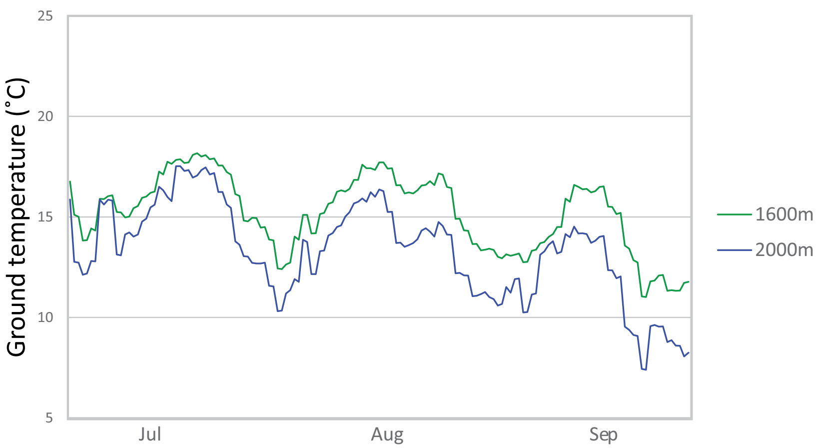

Figure 2. Mean daily ground temperatures at 1600 m and 2000 m a.s.l. at Zadnji Vogel, monitored once per hour in July, August and September, using temperature data loggers (HOBO TidbiTv2).
Slika 2. Srednje dnevne temperature tal na 1600 m in 2000 m n.m. na Zadnjem Voglu, merjene vsako uro julija, avgusta in septembra s pomočjo temperaturnih senzorjev (HOBO TidbiTv2).

Anatomical analysis

For the anatomical studies, 10 fully developed leaves were sampled from 10 plants per treatment at each altitude. Anatomical observations of the leaf cross-sections, which included palisade and spongy parenchyma thickness, and epidermis and cuticle thickness measurements, were performed under an optical binocular microscope (CX41; Olympus, Japan), with the measurements recorded and photographs taken with a digital camera (XC30; Olympus, Japan), using CellSens software (Olympus, Japan). The nail polish peel method was used to measure the stomatal features of the upper and lower leaf surfaces, under the same optical binocular microscope.

Pigment analyses

Photosynthetic pigments

Fully developed leaves from 10 plants per treatment at each altitude were collected at noon and were kept on moist paper in a refrigerated box. Fresh mass (FM) of the leaf tissue (10 mg) was extracted in 80% (v/v) acetone in buffered distilled water (pH 7.8). Absorbances were measured using a UV/VIS spectrophotometer (Lambda 25; Perkin Elmer, USA). Chlorophyll a (Chl a) and chlorophyll b (Chl b) contents were calculated from the absorbances measured at 663.6 nm and 646.6 nm (Porra et al. 1989). Pigment contents were expressed per leaf dry weight (mg g^{-1}).

UV-B- and UV-A-absorbing compounds

The leaf tissue that was collected as described above (10 mg FM) from 10 fully developed leaves from 10 plants per treatment at each altitude were extracted in 5 mL of acidified MeOH (MeOH/H_2O/HCl (37%), 79:20:1, v/v) (Caldwell 1968). Absorbance was measured over the spectral ranges for UV-B (280-315 nm) and UV-A (316-400 nm) using a UV/VIS spectrometer (Lambda 25; Perkin Elmer, USA), and were calculated per dry weight (g^{-1}) and integrated to estimate the total content of UV-B- (UV-B-AC) and UV-A-absorbing compounds (UV-A-AC) (a.u. g^{-1}).

Leaf optical properties

Fully developed leaves from 10 plants per treatment at each altitude were collected at noon and were kept on moist paper in a refrigerated box. The leaf transmittance and reflectance spectra were measured in the laboratory on fresh leaves, using the Jaz Modular Optical Sensing Suite (Ocean Optics, USA) with a measurement sphere (ISP-30-6-R; Ocean Optics, USA), applying UV/VIS/near infrared light from a deuterium and halogen light source (DH-2000; Ocean Optics, USA). The leaf reflectance spectra were measured for the upper leaf surface. The spectrometer was calibrated to 100% reflectance using a white reference panel with >99% diffuse reflectance (Spectralon; Labsphere, North Sutton, NH, USA). The leaf transmittance spectra were measured for the lower leaf surface by illumination of the upper surface. The spectrometer was calibrated to 100% transmittance with a light beam that passed directly into the interior of the integrating sphere. The transmittance and reflectance measurements were processed using SpectraSuite software (Ocean Optics, USA).

Data analysis

The plant responses and characteristic data were analysed using the SPSS Statistics 22.0 software (IBM, USA). Statistical tests were performed on 10 samples. The normal distributions of the data were evaluated using Shapiro-Wilk tests. Homogeneity of variance was analysed using Levene's tests. One-way ANOVA with multiple comparison tests and Tukey's *post-hoc* tests or Kruskal-Wallis tests and Bonferroni *post-hoc* tests were used to compare differences between the four treatments at each experimental site. Factorial ANOVA was performed to investigate the effects of UV radiation and temperature, and their interaction on the measured parameters. Redundancy analysis (RDA, CANOCO 5) was used to determine whether variations in the measured variables were related to the differences in temperature and UV radiation between experimental plots.

Results

Leaf anatomical characteristics

Most of the *H. nummularium* leaf thickness parameters were significantly higher at the montane altitude compared to the alpine altitude in July, which might be due to the delayed phenological events at the alpine altitude. The upper and lower cuticle thicknesses significantly increased under reduced UV radiation (UV-) at the montane and alpine altitudes in September, while spongy mesophyll thickness decreased. Total leaf thickness and specific leaf area did not change according to the different UV radiation and altitude conditions in September. The measured stomatal characteristics showed no changes at all (Tab. 2).

Table 2: Leaf anatomical characteristics for *Helianthemum nummularium* according to month and altitude for near-ambient (UV) and reduced (UV-) UV radiation.

Tabela 2: Anatomske značilnosti listov pri *Helianthemum nummularium* julija in septembra v montanskem in alpinskem pasu pri naravnem (UV) in zmanjšanem (UV-) UV sevanju.

Measure	Detail	July		September						
		1600 m	2000 m	1600 m		2000 m		Factorial ANOVA		
		UV	UV-	UV	UV-	UV	UV-	T	UV	T*UV
Stomatal density (mm^{-2})	Upper	87.7 ±5.1^{a}	90.3 ±3.4^{a}	96.6 ±4.8^{a}	92.5 ±7.7^{a}	95.5 ±3.6^{a}	92.7 ±2.0^{a}	ns	ns	ns
	Lower	157.8 ±5.3^{a}	154.5 ±3.9^{a}	164.6 ±12.5^{a}	161.1 ±9.3^{a}	160.2 ±3.9^{a}	152.1 ±3.5^{a}	ns	ns	ns
Stomatal length (µm)	Upper	32.9 ±0.5^{a}	32.3 ±0.5^{a}	34.7 ±0.4^{a}	34.8 ±0.8^{a}	33.9 ±0.3^{a}	34.2 ±0.5^{a}	ns	ns	ns
	Lower	33.7 ±0.2^{a}	34.7 ±0.5^{a}	34.1 ±0.4^{a}	34.3 ±0.2^{a}	33.7 ±0.3^{a}	34.5 ±0.7^{a}	ns	ns	ns
Cuticle thickness (µm)	Upper	3.2 ±0.1^{a}	2.2 ±0.1^{b}	2.9 ±0.1^{a}	3.6 ±0.2^{b}	3.5 ±0.2^{b}	4.0 ±0.2^{c}	ns	*	*
	Lower	2.2 ±0.2^{a}	1.5 ±0.1^{b}	2.5 ±0.2^{a}	2.9 ±0.2^{a}	3.0 ±0.2^{b}	3.1 ±0.2^{b}	ns	**	*
Epidermis thickness (µm)	Upper	22.5 ±0.9^{a}	21.7 ±1.0^{a}	22.8 ±0.8^{a}	25.2 ±0.8^{a}	24.3 ±0.6^{a}	22.6 ±0.7^{a}	ns	ns	ns
	Lower	17.9 ±0.9^{a}	18.0 ±0.7^{a}	19.9 ±1.0^{a}	23.6 ±1.0^{a}	22.4 ±0.7^{a}	19.2 ±0.7^{a}	ns	ns	ns
Leaf thickness (µm)	-	302.8 ±6.3^{a}	259.9 ±7.9^{b}	320.6 ±12.5^{a}	316.2 ±9.3^{a}	309.2 ±3.9^{a}	288.5 ±6.5^{a}	ns	ns	ns
Mesophyll thickness (µm)	Palisade	133.0 ±3.7^{a}	112.4 ±3.9^{b}	140.7 ±7.7^{a}	140.2 ±5.3^{a}	127.8 ±2.0^{ab}	124.6 ±4.0^{b}	**	ns	*
	Spongy	132.7 ±3.9^{a}	103.5 ±4.4^{b}	131.6 ±5.1^{a}	120.4 ±4.9^{a}	128.0 ±3.0^{ab}	114.4 ±3.8^{b}	ns	*	ns
Specific leaf area (cm^{2} g^{-1})	-	0.2 ±0.0^{a}	0.2 ±0.1^{a}	0.1 ±0.0^{a}	0.1 ±0.0^{a}	0.1 ±0.0^{a}	0.1 ±0.0^{a}	ns	ns	ns

Data are means ± standard error (n = 10 plants). Different letters indicate significant differences between the treatments ($p \leq 0.05$; one-way ANOVA); *, $p \leq 0.05$; **, $p \leq 0.01$; ns, $p > 0.05$, as significant responses to environmental factors temperature (T) and UV radiation, and their interaction (Factorial ANOVA).

Pigment contents

Pigment contents of *H. nummularium* showed the least changes for UV radiation and altitude for these three experimental plant species. The UV-B–AC contents in these leaves were lower at the montane than the alpine altitude in July and September (Tab. 3).

Leaf optical properties

Leaf reflectance for *H. nummularium* showed minor changes according to UV radiation and altitude, while leaf transmittance of the total photosynthetic spectrum (except for violet) was significantlly higher at the alpine than the montane altitude. Reduced UV radiation (UV-) increased the transmittance of the green and yellow spectra of all the three plant species. (Fig. 3, Tab. 4).

Table 3: Pigment contents of *Helianthemum nummularium* according to month and altitude for near-ambient (UV) and reduced (UV-) UV radiation.

Tabela 3: Vsebnost barvil v listih pri *Helianthemum nummularium* glede na mesec in nadmorsko višino pri naravnem (UV) in zmanjšanem (UV-) UV sevanju.

Measure	**July**		**September**						
	1600 m	**2000 m**	**1600 m**		**2000 m**		**Factorial ANOVA**		
	UV	**UV-**	**UV**	**UV-**	**UV**	**UV-**	**T**	**UV**	**T*UV**
Chlorophyll a (mg g $^{-1}$ DW)	1.60 ±0.20^{a}	1.93 ±0.16^{a}	1.65 ±0.16^{a}	1.61 ±0.15^{a}	1.44 ±0.09^{a}	1.40 ±0.13^{a}	ns	ns	ns
Chlorophyll b (mg g $^{-1}$ DW)	0.83 ±0.18^{a}	1.36 ±0.19^{a}	1.44 ±0.42^{a}	1.37 ±0.26^{a}	1.06 ±0.16^{a}	0.99 ±0.12^{a}	ns	ns	ns
UV-A–AC (a.u. g^{-1})	5.80 ±0.20^{a}	6.14 ±0.70^{a}	5.00 ±0.21^{a}	4.46 ±0.30^{a}	4.89 ±0.22^{a}	4.65 ±0.43^{a}	ns	ns	ns
UV-B–AC (a.u. g^{-1})	5.08 ±0.40^{b}	7.30 ±0.23^{a}	4.80 ±0.28^{b}	6.42 ±0.68^{ab}	7.02 ±0.25^{a}	6.37 ±0.54^{ab}	*	ns	ns

Data are means ±standard error (n = 10 plants). Different letters indicate significant differences between the treatments (one-way ANOVA); *, p ≤0.05; ns, p >0.05, as significant responses to environmental factors temperature (T) and UV radiation, and their interaction (Factorial ANOVA).

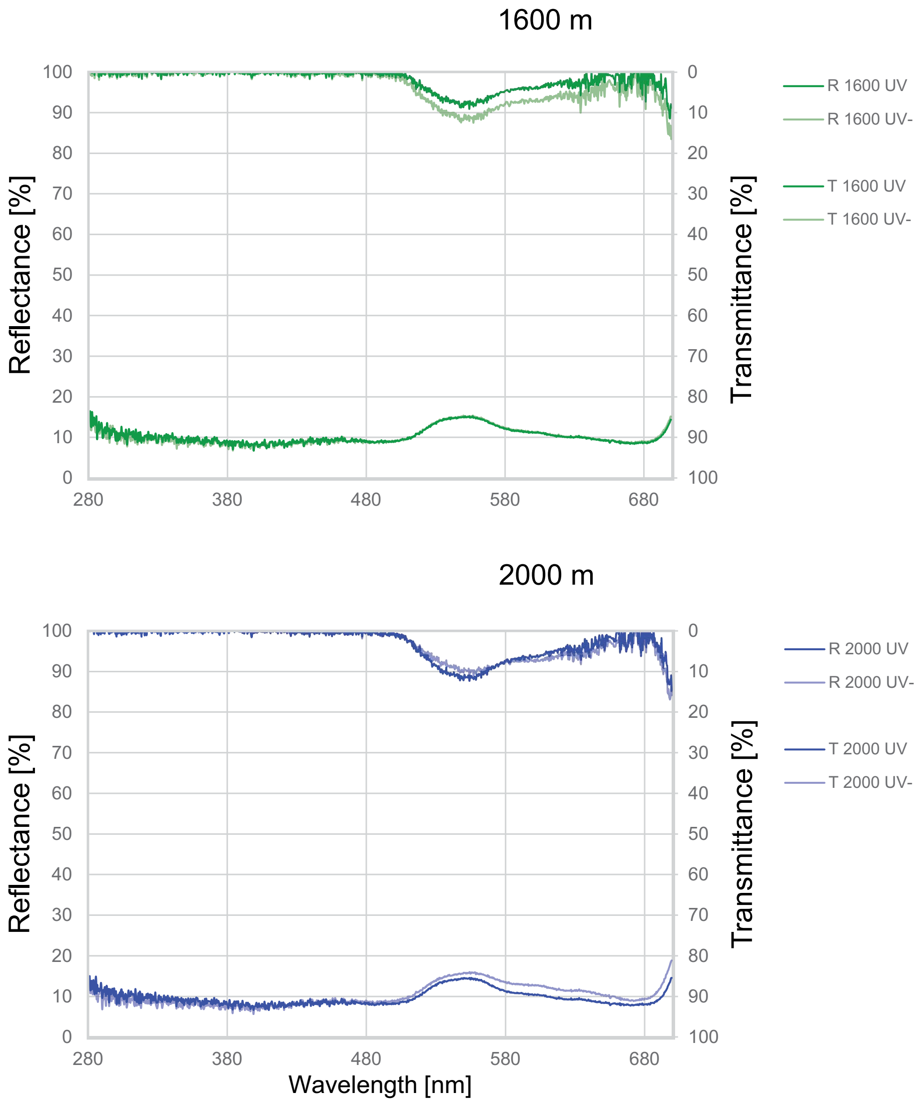

Figure 3: Leaf optical properties in September, as leaf transmittance and reflectance of *Helianthemum nummularium* at the indicated altitudes under near-ambient (UV) and reduced (UV-) UV radiation. Data are means for every 5-nm interval (n = 10 plants).

Slika 3: Optične lastnosti listov v septembru pri *Helianthemum nummularium* na označenih nadmorskih višinah pod naravnim (UV) in zmanjšanim (UV) UV sevanjem. Podatki so aritmetične sredine 5-nm intervalov (n = 10 rastlin).

Table 4: Significance of the effects of temperature and UV radiation on leaf reflectance and transmittance for *Helianthemum nummularium* over the light spectral ranges in September.
Tabela 4: Učinek temperature in UV sevanja na odbojnost in prepustnost listov *Helianthemum nummularium* v različnih spektralnih območjih v septembru.

Light spectrum	T	UV	T*UV
Reflectance			
UV-B	ns	ns	ns
UV-A	ns	ns	ns
Violet	ns	ns	ns
Blue	ns	ns	ns
Green	ns	ns	ns
Yellow	ns	ns	ns
Red	*	ns	ns
Near-infrared	*	ns	ns
Transmittance			
UV-B	ns	ns	ns
UV-A	ns	ns	ns
Violet	ns	ns	ns
Blue	*	ns	ns
Green	**	*	ns
Yellow	**	*	ns
Red	**	ns	ns
Near-infrared	*	ns	ns

*, p ≤0.05; **, p ≤0.01; ***, p ≤0.001; ns, p >0.05, as (light colour coded) significant effects of environmental factors temperature (T) and UV radiation, and their interaction (Factorial ANOVA).

In July, the diversity among the treated plants was low; the morphological variables and UV-absorbing compounds content accounted for 66% of the total variation between the two altitudes and different UV-B radiation treatments according to RDA analyses. In September, the diversity between the treated plants was high; the morphological variables and UV-absorbing compounds content accounted for 19% of the total variation between the different UV-B radiation and altitudinal treatments according to RDA analyses. Explanatory variables accounted for 19.9% of the total variation, where T explained 14.7% and UV-B 5.1%. (Fig. 4).

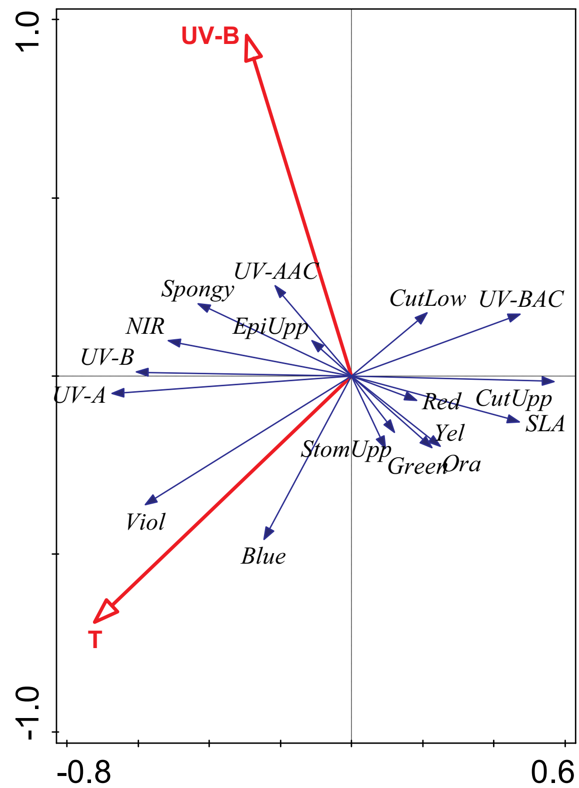

Figure 4: Redundancy analysis ordination diagram showing the strengths of the associations between the environmental factors (T, UV-B) and functional traits of *Helianthemum nummularium.*

Slika 4: Diagram redundančne ordinacijske analize prikazuje moč povezav med okoljskimi dejavniki (T, UV-B) in funkcionalnimi značilnostmi *Helianthemum nummularium.*

Discussion

Unaffected Chl a and Chl b contents of a shrub life form

Chl a and Chl b contents did not change in *H. nummularium* according to UV radiation and altitude throughout the season. Previous studies have reported that UV radiation can lead to reductions, no changes, and increases in chlorophyll contents (Smith et al. 2000; Bassman et al. 2003). It has been shown that UV radiation generates free radicals and leads to degradation of chlorophylls and carotenoids (Middleton and Teramura 1993), and also to increased biosynthesis of photosynthetic pigments under adequate intensities of PAR and UV-A (Verdaguera et al. 2017). Furthermore, heterogeneous responses can reflect differences in species and/or cultivars (Teramura 1983). Decreases in chlorophyll contents under strongly reduced solar UV-B radiation have been shown for *Zea mays* and *Citrus aurantifolia* (Barsig and Malz 2000; Ibanez et al. 2008). John et al. (2001) demonstrated that exposure to UV radiation can lead to the induction of genes associated with senescence, which can result in loss of plant chlorophyll content. Trošt Sedej et al. (2020) showed a significant decrease in chlorophyll contents in an alpine plant *Saxifraga hostii* under the ambient UV radiation at alpine altitudes at the end of the growing season, which might suggest earlier senescence. The different response of *H. nummularium* and *S. hostii* could be due of different life forms, where the leaves of *S. hostii* rosettes are more exposed to irradiance than the leaves of *H. nummularium* shrub growth form.

High levels of constitutive UV-absorbing compound contents

H. nummularium showed high UV-AC contents throughout the season. In agreement with this, other studies have demonstrated that species from locations with naturally high UV radiation at either high altitude or low latitude are less sensitive to UV radiation than species from low UV radiation locations (Biswas and Jansen 2012). Flavonoids have key roles in plant UV protection due to their antioxidant and UV-screening properties (Smith et al. 2000). Flavonoid formation is not only induced by UV radiation, but is also affected by other environmental variables, such as photosynthetic irradiance, temperature and nutrient supply (Bassman et al. 2003).

In *H. nummularium*, UV-AC contents were generally not affected by the different environmental factors, which demonstrated high constitutive UV-AC contents. Constitutive levels of UV-AC under increased UV radiation (flavonoids and other phenylpropanoid derivatives) have been correlated with plant tolerance to UV radiation (Qaderi et al. 2008). High constitutive and inducible UV-AC explain the good protection of the photosystem from UV radiation penetration of the plants at the alpine altitude. Constitutive and inducible UV radiation tolerances reflect phenotypic plasticity in response to the different UV environments; lowland *Arabidopsis* ecotypes show more inducible UV radiation tolerance, whereas highland *Arabidopsis* ecotypes show constitutive UV radiation tolerance (Jansen et al. 2010). These studies suggest that for alpine plants, constitutive defence is a more advantageous strategy than inducible defence, where UV stress is constantly high.

Most leaf thickness parameters showed no response to the UV and altitude conditions

Most of the *H. nummularium* leaf thickness parameters and all of the stomatal characteristics showed no changes according to the UV radiation and altitude in September. The exceptions were for spongy mesophyll thickness, upper and lower cuticle thickness, which increased and decreased, respectively, under the near-ambient UV radiation at the montane and alpine altitudes in September. UV radiation can induce several photomorphogenic responses, which include decreased leaf area and increased leaf thickness. Such modifications represent effective mechanisms that reduce the transmittance of UV radiation to the inner leaf tissue (Czégény et al. 2016). Increases in leaf thickness were attributed to increases in the number of spongy parenchyma cells in *Brassica carinata* and *Medicago sativa* (Bornman and Vogelmann 1991). It has been reported that cuticle thickness of the upper needle surface of *Abies balsamea* decreases at high altitudes (DeLucia and Berlyn 1984). These studies indicate that plants that are less adapted to UV radiation show more changes to their leaf parameters than those that are better adapted to UV radiation. This suggests that *H. nummularium* plants are well-adapted to UV radiation and high altitude.

Leaves did not transmit any UV spectrum

The leaf reflectance of *H. nummularium* showed small changes according to UV radiation and altitude. Another study on *S. hostii* showed that alpine plants, but not montane plants, can be grouped according to their different UV radiation exposures in September, meaning that UV radiation had a distinct influence on leaf optical properties (Trošt Sedej et al. 2020).

The leaves of *H. nummularium* did not transmit any UV spectrum, which corresponded to their high constitutive and inducible UV-AC contents. Similar responses were shown for *Arnica montana,* where enhanced UV radiation was not the key factor that triggered changes in the flavonoid composition. The key factor was temperature, which decreased with altitude (Albert et al. 2009). A tropical alpine study indicated that high levels of UV radiation screening are common to native and non-native plant species, and to different growth forms in an alpine environment. The plasticity of the epidermal UV radiation transmittance is a mechanism that is used by some, but not all, species to cope with varying solar UV radiation exposure. In the non-native *Verbascum thapsus*, leaf transmittance of UV-A was shown to be variable along an alpine altitude gradient, and to be strongly correlated with UV-B radiation and altitude. However, in the native *Vaccinium reticulatum*, leaf transmittance of UV-A was consistently low and did not change with altitude (Barnes et al. 2017).

The leaf transmittance of the photosynthetic spectrum increased at the alpine altitude, while the transmittance of the green and yellow spectra increased under the reduced UV radiation in September. The leaf optical properties are dependent on the anatomical and biochemical characteristics of the leaf, such as the structure of the leaf, the concentration and type of flavonoids it contains, chlorophylls and other pigments, and the thickness of the epidermis, cuticle, waxes and hairs (Ziska et al. 1992), and they are correlated with plant life form (Day et al. 1992). The optical properties can be species-specific, but can also vary within a species, due to ontogenetic development of plants (Liew et al. 2008). The environmental conditions, to which a plant is exposed to, can alter leaf optical properties, including for UV radiation and PAR, temperature, and water and soil properties (Ustin and Jacquemoud 2020). Therefore, the high photosynthetic spectrum transmittance of *H. nummularium* at the alpine altitude is more due to complex biochemical and anatomical responses to the alpine environmental conditions than to UV radiation itself.

Conclusions

H. nummularium showed high UV-AC contents throughout the season. The UV-AC contents were generally not affected by the different environmental factors, which indicated high constitutive UV-AC contents. Constitutive and inducible UV radiation tolerances reflect phenotypic plasticity in response to different UV environments. Most leaf thickness parameters showed no response to the different UV radiation and altitude conditions. Plants, less adapted to UV radiation, show more changes in leaf parameters than plants, well-adapted to UV radiation, which suggests that *H. nummularium* is well-adapted to UV radiation and high altitude. The leaf reflectance of *H. nummularium* showed small changes according to UV radiation and altitude. The leaves of *H. nummularium* did not transmit any UV spectrum, which corresponded to their high constitutive and inducible UV-AC contents. Leaf transmittance of the photosynthetic spectrum increased for the alpine altitude, while the transmittance of the green and yellow spectra increased under the reduced UV radiation in September. The high photosynthetic spectrum transmittance of *H. nummularium* at the alpine altitude was due to complex biochemical and anatomical responses to the alpine environmental conditions, rather than to UV radiation. Unchanged chlorophyll content of *H. nummularium* could be related to shrub life form, where the leaves shade out the high UV and PAR irradiance as well as contribute to lower leaf temperature. The study shows complexity of alpine plant response, where specific characteristics of plant species should not be overlooked.

Povzetek

Alpinske in gorske rastline so razvile strategije za preživetje v skrajnih razmerah, ki vključujejo visoko jakost UV in vidnega sevanja, skrajne temperature, sušo in pomanjkanje hranil. Strategije preživetja alpinskih rastlin vključujejo biokemične, fiziološke in morfološke odzive, ki jih raziskovalci zaradi zahtevne izvedbe redko proučujejo. V naši raziskavi smo proučevali funkcionalne značilnosti alpske rastline velecvetni popon *Helianthemum nummularium* subsp. *grandiflorum* pod naravnim in zmanjšanim sevanjem UV-B na dveh različnih nadmorskih višinah (1600 in 2000 m n.m.) gore Zadnji Vogel v Julijskih Alpah. Anatomijo listov, pigmente in optične lastnosti smo raziskovali na začetku in na koncu rastne sezone. *H. nummularium* je ob obeh merjenjih vseboval veliko UV-absorbirajočih snovi (UV-AC) v listih. V splošnem različni okoljski dejavniki na vsebnost UV-AC niso vplivali, kar kaže na visoko vsebnost konstitutivnih UV-AC. Konstitutivne in inducibilne UV-AC odražajo fenotipsko plastičnost rastline v

odzivu na različna UV okolja. Večina parametrov, s katerimi smo ocenjevali debeline listnih tkiv, ni pokazala odziva na različno UV sevanje in temperaturne razmere. UV sevanje pri manj prilagojenih rastlinah izzove večje spremembe anatomije listov kot pri rastlinah, prilagojenih na veliko UV sevanje, kar kaže, da je *H. nummularium* dobro prilagojena alpinska rastlina. Listi niso prepuščali UV sevanja, kar ustreza njihovi visoki konstitutivni in inducibilni vsebnosti UV-AC. Prepustnost fotosinteznega spektra sevanja skozi liste se je povečala na alpinski nadmorski višini, medtem ko se je prepustnost zelenega in rumenega spektra povečala pod zmanjšanim UV sevanjem septembra. Velika prepustnost fotosinteznega spektra sevanja na alpinski nadmorski višini je bila povezana z biokemičnimi in anatomskimi značilnostmi rastline in ne z jakostjo UV sevanja. Nespremenjena vsebnost klorofila pri *H. nummularium* bi lahko bila povezana z grmičasto rastno obliko rastline, kjer zgornji listi zasenčijo spodnje liste ter tako prispevajo k manjšim prejetim odmerkom UV in fotosintezno aktivne svetlobe ter k nižji temperaturi listov. Študija kaže na kompleksen odziv alpinskih rastlin na UV sevanje in temperaturne razmere v gorah, pri čemer je pomembna tudi rastna oblika rastline.

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Bark spectral signatures of one-year-old twigs of different shrubs mainly depend on their biochemical traits

Spektralni podpisi skorje enoletnih vejic različnih grmovnih vrst so večinoma odvisni od biokemijskih lastnosti skorje

Mateja Grašič*, Bojana Ropret, David Gradinjan, Alenka Gaberščik

Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia

*Correspondence: mateja.grasic@bf.uni-lj.si

Abstract: The interaction of bark with light depends on the optical properties of the bark, which are important for plant energy balance especially out of the vegetation season. Light reflected from bark also represents a kind of "bark spectral signature" that may be species-specific. This study examines some morphological, biochemical, and physiological traits that may affect the reflectance of the bark of 1-year-old twigs of different shrubs: *Corylus avellana*, *Rosa canina*, *Ligustrum vulgare*, *Sambucus nigra*, *Cornus sericea* var. *flaviramea*, and *Viburnum lantana*. High variability was seen across these species for morphological, biochemical, and physiological traits, except for photochemical efficiency of photosystem II. The bark spectral signatures differed significantly across these species. The reflectance peak for *C. sericea* var. *flaviramea* was in red, for *C. avellana* in green, and the other species showed a wide peak from green to red light. Redundancy analysis revealed that UV-B–absorbing substances and anthocyanin content, along with outer and inner bark thickness, together explained 61% of the reflectance spectra variability. Outer bark thickness was important for reflectance in UV, violet, and blue, while anthocyanins were important for reflectance in green and yellow. Chlorophyll *b* was negatively related to the reflectance of visible light. This study revealed great importance of biochemical traits for explaining bark reflectance. Differences in "bark spectral signatures" enable differentiation across species out of the vegetation season.

Keywords: bark, pigments, reflectance, UV-B–absorbing substances, woody plants

Izvleček: Interakcija skorje s svetlobo je odvisna od optičnih lastnosti skorje, ki so pomembne za energijsko ravnovesje rastlin zlasti izven vegetacijske sezone. Svetloba, ki se odbije od skorje, predstavlja tudi nekakšen "spektralni podpis skorje", ki je lahko vrstno specifičen. V tej raziskavi smo proučili nekatere morfološke, biokemijske in fiziološke lastnosti, ki lahko vplivajo na odbojnost skorje enoletnih vejic različnih grmovnih vrst: *Corylus avellana*, *Rosa canina*, *Ligustrum vulgare*, *Sambucus nigra*, *Cornus sericea* var. *flaviramea* in *Viburnum lantana*. Opazili smo veliko variabilnost med proučevanimi vrstami glede njihovih morfoloških, biokemijskih in fizioloških lastnosti, razen fotokemične učinkovitosti fotosistema II. Med izbranimi vrstami so se značilno razlikovali tudi spektralni podpisi skorje. Vrh odbojnosti pri vrsti *C. sericea* var. *flaviramea* je bil v rdečem, pri *C. avellana* v zelenem, druge vrste pa so pokazale širok vrh, ki se je raztezal od zelenega do rdečega spektralnega območja. Redundančna analiza je pokazala, da vsebnost UV-B–absorbirajočih snovi in antocianinov skupaj z debelino zunanje in notranje skorje skupaj pojasnjujejo 61 % variabilnosti odbojnih spektrov. Debelina zunanje skorje je bila pomembna za odbojnost v UV, vijoličnem in modrem spektralnem območju, vsebnost antocianinov pa za odbojnost v zelenem in rumenem spektralnem območju. Klorofil *b* je bil negativno povezan z odbojnostjo vidnega dela spektra. Ta raziskava je pokazala velik pomen biokemijskih lastnosti za razlago odbojnosti skorje. Razlike v "spektralnih podpisih skorje" omogočajo razlikovanje med vrstami izven vegetacijske sezone.

Ključne besede: lesnate rastline, odbojnost, pigmenti, skorja, UV-B–absorbirajoče snovi

Introduction

Bark comprises tissues outside the vascular cambium, and consists of the inner bark, which contains secondary phloem, and the outer bark or periderm, produced by the cork cambium (Romero 2014). Bark protects stems against physical disturbances (e.g., fire) and environmental factors (e.g., extreme temperatures, drought), and it has high repair potential (Romero et al. 2009). In addition, the outer bark is an effective barrier against pollutants (Pfanz 1999), and enables transport of assimilates and water (Poorter et al. 2014). Bark physiological and biochemical traits vary significantly across species, and these specific bark traits define the bark interactions with the abiotic and biotic environments (Larcher 2003).

The surface structure of bark determines the composition and structure of epiphytic communities (Tewari et al. 2009). In various forest types, the suitability of bark for epiphytic lichen and bryophyte colonisation increases with increasing bark roughness (Black and Harper 1979). Ferrenberg and Mitton (2014) demonstrated that smooth bark limits the attacks of insects specialised in attacking tree stems. Bark may also contain high concentrations of flavonoids, which are known to have multiple functions (Carrillo-Parra et al. 2012). These substances protect plants from different biotic and abiotic stresses and act as filters for UV radiation, signal molecules, detoxifying agents, and antimicrobial compounds (Samanta et al. 2011).

The bark chlorenchyma is located under the rhytidomal or outer peridermal layers; it can harvest transmitted light and use the stem internal CO_2 for photosynthesis (Pfanz et al. 2002, Wittmann and Pfanz 2008, 2014). The transmission of light through the periderm is low and depends on the species and age of the branches or stems (Aschan et al. 2001). A study by Wittmann and Pfanz (2016) revealed that transmittance of photosynthetically active radiation (PAR) through the periderm of young stems varied between 8.5% and 42.0%, while PAR transmittance through the total bark ranged from 2.2% to 6.2%.

The fixation of CO_2 via photosynthesis reduces internal carbon dioxide losses and thus contributes to a favourable carbon balance (Filippou et al. 2007, Eyles et al. 2009). Wittmann and Pfanz (2008) showed that photosynthesis of the bark of young stems can replace 60% to 90% of respiratory carbon losses. Assimilation of CO_2 in stems has several advantages over photosynthesis in leaves, as stems lose less water and contain high concentrations of CO_2, which reduce the level of photorespiration in stems (Damesin 2003). In young stems, respiration and the photosynthetic rate are generally higher than in older stems (Wittmann and Pfanz 2008). The amount of light that can reach different stem tissues affects plastid differentiation, and thus also their photosynthetic properties (Wittmann and Pfanz 2016). As branches grow older, the bark and the peridermal layer become thicker, and consequently the amount of chlorophyll and photosynthetic activity decrease (Pilarski 1999, Wittmann et al. 2001). Bark photosynthesis does not only have an important role in maintaining the carbon balance, but it also improves the oxygen content and enables undisturbed respiration of metabolically active tissues (Wittmann and Pfanz 2014). Green stems with poorly developed periderm have functional stomata and can assimilate large amounts of carbon, especially during the period when the plants are leafless (Filippou et al. 2007). Also, in spite of the low light levels in winter, bark photosynthetic activity and respiration contribute to a more favourable carbon balance from late autumn to early spring, when photosynthetic activity in evergreen organs is reduced (Wittmann and Pfanz 2007) or is absent in deciduous species.

The light regime within the stem affects the differentiation of plastids and thus affects the photosynthetic properties of individual tissues in the stem (Wittmann and Pfanz 2016). Light penetration depends on the optical properties of the bark. Light reflected from bark represents a kind of "bark spectral signature" that is species-specific and can reveal the physiological and biochemical properties of the bark (Tokarz and Pilarski 2005, Levizou and Manetas 2007). In deciduous species, the bark spectral signatures enable differentiation among woody species out of the vegetation season using remote sensing (Atkilt Girma et al. 2013), and host discrimination by bark and timber beetles (Campbell and Borden 2005). Bark optical properties also contribute to the bark energy balance and prevent overheating (Henrion and Tributsch 2009).

In the present study, we examined different plant traits that affect bark reflectance of 1-year-old twigs of different woody species: *Corylus avellana*, *Rosa canina*, *Ligustrum vulgare*, *Sambucus nigra*, *Cornus sericea* var. *flaviramea*, and *Viburnum lantana*. We hypothesised that the bark biochemical traits and the bark thickness have important roles in shaping the bark reflectance. To show this, we analysed different pigments and UV-absorbing substances, and measured the inner and outer bark thicknesses, and the bark reflectance spectra.

Materials and methods

Plant species

The species studied were the woody plants *Corylus avellana* L. (Betulaceae), *Rosa canina* L. (Rosaceae), *Ligustrum vulgare* L. (Oleaceae), *Sambucus nigra* L. (Adoxaceae), *Cornus sericea* var. *flaviramea* L. (Cornaceae), and *Viburnum lantana* L. (Caprifoliaceae). All of these species are shrubs that are native to Europe, except for *C. sericea*, which is native to North America. However, the cultivar *C. sericea* var. *flaviramea* was developed in Europe. The studied species are all deciduous, with the exception of *L. vulgare*, which is a semi-evergreen or deciduous shrub that is frequently grown as a hedge. *C. avellana* is an important component of hedgerows. *R. canina* is a climbing shrub that has stems, which are covered with small, sharp, hooked prickles. *S. nigra* is also very common in hedgerows and scrubland, and it is also widely grown as an ornamental shrub or small tree. *C. sericea* var. *flaviramea* is widely grown as an ornamental plant that grows best in moderate warmth in sunny places, although it can also tolerate shade. *V. lantana* is a tree-like shrub that is common along waysides and is also grown as an ornamental plant.

One-year-old and about 0.5 cm thick twigs from 10 plants of each species were sampled from open places in Ljubljana (46°3'N; 14°28'E) and Golnik (46°19'N; 14°19'E), and were processed on the day of sampling. The sampling and analysis took place before the development of leaves, from February to the end of March.

Morphological properties

Analysis of bark thickness was carried out on transverse sections of vital 1-year-old twigs. The measurements included the thickness of the inner and outer bark (periderm). The measurements were performed using a light microscope (CX41; Olympus, Tokyo, Japan) equipped with a digital camera (XC30, Olympus) and the CellSens software (Olympus).

Biochemical properties

The chlorophyll *a*, chlorophyll *b*, and carotenoid contents were determined for bark extracts according to Lichtenthaler and Buschmann (2001a, b), with absorbance measured at 470 nm, 645 nm, and 662 nm using a UV/VIS spectrometer (Lambda 25; Perkin-Elmer, Norwalk, CT, USA). Anthocyanin content was determined on bark extracts as described by Drumm and Mohr (1978), with absorbance measured at 530 nm. Total methanol-soluble UV-B–absorbing and UV-A–absorbing substances (as a measure of total phenolics) were also extracted from fresh twigs, according to Caldwell (1968), with absorbance measured from 280 nm to 319 nm, and from 320 nm to 400 nm, respectively. The extinction values were integrated for each UV region. The biochemical parameters are expressed per bark area.

Physiological properties

Chlorophyll fluorescence was measured using a portable chlorophyll fluorometer (PAM-2100; Heinz Walz GmbH, Effeltrich, Bavaria, Germany). The potential and effective photochemical efficiency of photosystem (PS) II (Fv/Fm and Fq'/Fm', respectively) were evaluated according to Schreiber et al. (1996), with the measurements performed on fresh twigs. Prior to the Fv/Fm measurements, twigs were kept in the dark for 20 minutes. Fq'/Fm' measurements were performed at daylight at >1000 µmol m^{-2} s^{-1}, with temperatures ranging from 10 to 15 °C. Fq'/Fm' was calculated using equation 1,

$$\mathrm{Fq'/Fm'} = \frac{Fm' - F'}{Fm'} \quad (1)$$

where Fm' is maximum fluorescence from dark- or light-adapted leaf, respectively (PS II centers closed), and F' is fluorescence emission from dark- or light-adapted leaf, respectively (Brestic and Zivcak 2013).

Potential photochemical efficiency (Fv/Fm) was calculated using the equation 2,

$$Fv/Fm = 1 - \frac{Fo}{Fm} \quad (2)$$

where Fo is minimal fluorescence from dark-adapted leaf (PS II centres open), and Fm is maximum fluorescence from dark- or light-adapted leaf, respectively (PS II centres closed) (Brestic and Zivcak 2013).

Optical properties

Bark reflectance was determined in the laboratory on the day of sampling. The bark was carefully removed from the twigs. The reflectance spectrum was measured from 290 nm to 800 nm, at a resolution of ~1.3 nm, using a portable spectrometer (Jaz Modular Optical Sensing Suite; Ocean Optics, Inc., Dunedin, FL, USA; grating, #2; slit size, 25 µm) with an optical fibre (QP600-1-SR-BX, Ocean Optics, Inc.) and an integrating sphere (ISP-30-6-R, Ocean Optics, Inc.). The reflectance spectrum was measured for the bark surface by illumination with a UV/VIS-near infrared (NIR) light source (DH-2000; Ocean Optics, Inc.). The spectrometer was calibrated to 100% reflectance using a white reference panel with >99% diffuse reflectance (Spectralon, Labsphere, North Sutton, NH, USA). More details on the procedure for measurement of optical properties can be found in Klančnik et al. (2015).

Statistical analysis

Statistical analysis was carried out in IBM SPSS statistics 22.0 using one-way multivariate 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 in Past 3.14. We performed redundancy analysis (RDA) using CANOCO for Windows 4.5 programme package to determine whether variations in the reflectance spectra were related to morphological and biochemical parameters (ter Braak and Šmilauer 2002). The spectra were divided into spectral bands (i.e., UV-B, UV-A, violet, blue, green, yellow, red, and NIR) to obtain more detailed information about the correlations between explanatory and response variables. Forward selection of the explanatory variables was used to avoid co-linearity between the variables (Lepš and Šmilauer 2003). The level of significance was accepted at $p \leq 0.05$. Non-significant explanatory variables were excluded from further analysis. All of the variables in the RDA were standardised to eliminate the influence of magnitude differences between scales and units.

Results

In the comparison of the biochemical traits, the greatest variability was seen for anthocyanin content (Tab. 1). The chlorophyll *a* to *b* ratio varied a lot across the species. The bark of *C. sericea* var. *flaviramea* differed the most from the other species, with the highest total chlorophylls, UV-absorbing substances, and anthocyanins, but not carotenoids. The peridermal layers had different thicknesses in the species studied. *V. lantana* had the thickest outer bark, while for *S. nigra* the inner bark was the thickest. *R. canina* and *C. sericea* var. *flaviramea* still had the epidermal layer and a very thin layer of periderm. Fv/Fm was high in the majority of species, however, Fq'/Fm' was low and did not differ across the species.

The bark spectral signatures were very variable. The bark reflectance differed the most in the visible part of the spectrum. *C. sericea* var. *flaviramea* had high reflectance in the UV-B and NIR regions, and low reflectance in the visible region (Fig. 1). Its reflectance spectra had distinct peaks in the red region. However, the reflectance peak of other species was wide, and ranged from green to red, with the exception of *C. avellana*, which had a peak in the green region. The reflectance of *V. lantana* gradually increased along the spectra, with a slight depression in the red edge.

Table 1: Selected bark biochemical and physiological traits, along with bark reflectance and thicknesses for the different woody species examined in this study.

Tabela 1: Izbrane biokemijske in fiziološke lastnosti ter odbojnost in debelina skorje različnih lesnatih vrst, obravnavanih v tej raziskavi.

Traits	**Units**	**Data measures according to species**					
		Corylus avellana	***Rosa canina***	***Ligustrum vulgare***	***Sambucus nigra***	***Cornus sericea***	***Viburnum lantana***
Biochemical							
Chlorophyll *a*	mg cm^{-2}	0.012 ±0.001^{bc}	0.022 ±0.002^{a}	0.010 ±0.002^{b}	0.016 ±0.002^{bc}	0.019 ±0.012^{abc}	0.014 ±0.009^{bc}
Chlorophyll *b*	mg cm^{-2}	0.006 ±0.001^{a}	0.013 ±0.001^{bc}	0.007 ±0.003^{ab}	0.011 ±0.003^{b}	0.029 ±0.023^{c}	0.017 ±0.014^{bc}
Carotenoids	mg cm^{-2}	0.005 ±0.000^{ac}	0.008 ±0.001^{b}	0.003 ±0.001^{a}	0.005 ±0.001^{c}	0.006 ±0.003^{abc}	0.006 ±0.001^{bc}
Anthocyanins	au cm^{-2}	0.44 ±0.03^{a}	0.84 ±0.17^{bc}	0.23 ±0.07^{a}	0.63 ±0.05^{b}	1.48 ±0.53^{c}	1.12 ±0.28^{c}
UV-B–AS	au cm^{-2}	4.90 ±0.45^{a}	9.64 ±0.93^{bc}	7.69 ±1.11^{ab}	10.64 ±1.43^{c}	23.10 ±4.32^{d}	12.27 ±1.42^{c}
UV-A–AS	au cm^{-2}	4.09 ±0.37^{a}	9.11 ±0.89^{bc}	7.45 ±1.13^{ab}	8.94 ±1.01^{bc}	7.69 ±1.41^{b}	10.89 ±1.76^{c}
Bark thickness							
Inner bark	µm	84 ±7^{a}	163 ±20^{c}	173 ±18^{ac}	210 ±17^{b}	137 ±25^{c}	169 ±40^{bc}
Outer bark$^{\#}$	µm	64 ±8^{a}	33 ±1^{b}	92 ±5^{a}	165 ±10^{c}	37 ±3^{b}	284 ±32^{c}
Physiological							
Photochemical efficiency of PS II							
Fq'/Fm'	au	0.12 ±0.05^{a}	0.12 ±0.07^{a}	0.14 ±0.11^{a}	0.15 ±0.04^{a}	0.11 ±0.03^{a}	0.14 ±0.03^{a}
Fv/Fm	au	0.36 ±0.07^{ac}	0.62 ±0.03^{b}	0.65 ±0.06^{abd}	0.71 ±0.03^{d}	0.48 ±0.05^{c}	0.69 ±0.05^{d}
Reflectance	%						
UV-B		4.3 ±3.4^{abd}	8.8 ±1.1^{b}	4.9 ±2.2^{a}	10.0 ±1.7^{bc}	11.7 ±0.6^{c}	0.3 ±1.8^{d}
UV-A		6.6 ±2.2^{ab}	9.3 ±1.0^{ab}	7.7 ±1.2^{a}	13.6 ±1.8^{c}	9.2 ±0.5^{b}	0.1 ±2.0^{c}
Violet		14.1 ±8.7^{ab}	12.6 ±7.3^{a}	11.2 ±2.8^{a}	13.8 ±2.3^{a}	8.1 ±0.4^{b}	3.6 ±2.0^{c}
Blue		16.8 ±8.9^{ab}	14.3 ±7.9^{a}	14.2 ±3.0^{a}	15.0 ±2.3^{a}	7.6 ±0.4^{c}	7.2 ±3.6^{bc}
Green		23.5 ±7.4^{a}	20.2 ±7.4^{a}	23.5 ±3.6^{a}	25.5 ±3.7^{a}	7.3 ±0.5^{b}	11.1 ±4.3^{b}
Yellow		26.5 ±6.7^{ab}	21.8 ±6.7^{ab}	24.5 ±3.4^{a}	23.3 ±3.2^{a}	10.9 ±1.2^{c}	15.5 ±4.9^{bc}
Red		25.9 ±6.7^{a}	20.3 ±6.6^{a}	21.8 ±3.0^{a}	19.2 ±2.8^{a}	13.0 ±1.4^{b}	19.1 ±5.4^{ab}
NIR		49.0 ±7.5^{ab}	56.4 ±4.9^{a}	56.8 ±1.9^{a}	55.7 ±6.2^{a}	67.4 ±1.7^{c}	43.8 ±7.5^{b}

Data are means ±SD (n = 5-10 for each species); different superscript letters within each row indicate significant differences ($p \leq 0.05$; Tukey tests); reflectance spectra represent means within 5-nm intervals ($p \leq 0.05$, Tukey tests). Abbreviations: au, arbitrary unit; Fq'/Fm', effective photochemical efficiency; Fv/Fm, potential photochemical efficiency; UV-B–AS, UV-B–absorbing substances; UV-A–AS, UV-A–absorbing substances; $^{\#}$epidermal layer with periderm.

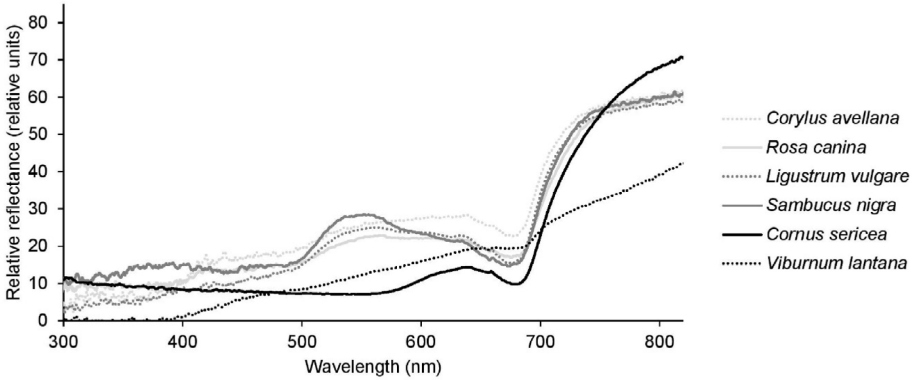

Figure 1: Relative reflectance spectra of bark of the different shrub species.
Slika 1: Relativni odbojni spektri skorje različnih grmovnih vrst.

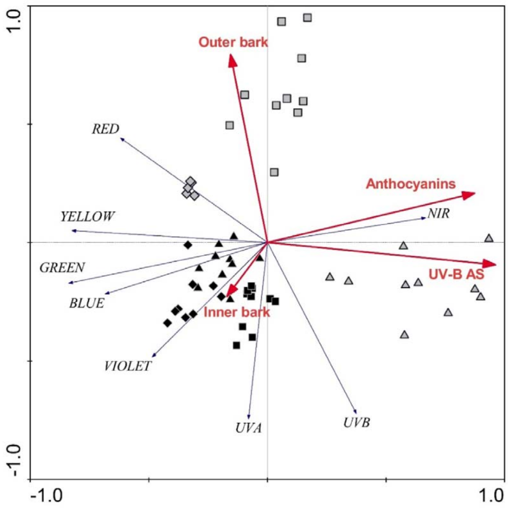

Figure 2: The strength of the associations between the bark properties, in terms of inner and outer bark thicknesses, anthocyanins, UV-B–absorbing substances (UV-B–AS), and different regions of the reflectance spectra. Grey diamonds, *Corylus avellana*; black squares, *Rosa canina*; black diamonds, *Ligustrum vulgare*; black triangles, *Sambucus nigra*; grey triangles, *Cornus sericea* var. *flaviramea*; grey squares, *Viburnum lantana*. The plot was generated using redundancy analysis.
Slika 2: Moč povezav med lastnostmi skorje, in sicer med debelino notranje in zunanje skorje, antocianini, UV-B–absorbirajočimi snovmi (UV-B–AS) ter različnimi območji odbojnih spektrov. Sivi diamanti, *Corylus avellana*; črni kvadrati, *Rosa canina*; črni diamanti, *Ligustrum vulgare*; črni trikotniki, *Sambucus nigra*; sivi trikotniki, *Cornus sericea* var. *flaviramea*; sivi kvadrati, *Viburnum lantana*. Graf je bil narejen v okviru redundančne analize.

RDA revealed negative relationships between the biochemistry and the reflectance in the visible region of the spectrum (Fig. 2). Here, UV-B–absorbing substances alone explained 36% of the variability of the bark reflectance spectra, anthocyanins alone explained 31%, while periderm and chlorophyll *b* alone explained 15% each. When considered together, UV-B–absorbing substances explained 38% of the variability ($p = 0.001$), and the outer bark thickness, inner bark thickness, and anthocyanin content explained an additional 12% ($p = 0.001$), 7% ($p = 0.001$), and 4% ($p = 0.009$) of the variability, respectively. With the measured parameters, as much as 61% of the reflectance spectra variability was explained.

In the explanation of the different parts of the reflectance spectra redundancy analyses, outer bark thickness was seen to have a crucial role in explaining the reflectance in the UV, violet, and blue regions. Furthermore, anthocyanins were important to explaining the reflectance in the green and yellow regions, while the UV-B–absorbing substances explained most of the reflectance in the red and NIR regions (Tab. 2).

Discussion

In general, high variability was seen across the species for the morphological and biochemical traits, with less variability for the physiological traits. One of the important traits was the amount of photosynthetic pigments, which are responsible for bark photosynthesis (Pfanz 1999). The stems of woody plants usually contain much lower amounts of photosynthetic pigments than the leaves. However, in young stems with a thin layer of cork, the pigment content can be similar to that in leaves (Kocurek and Pilarski 2012). This was also the case in the present study, as we measured

Table 2: Explained variance of the different reflectance spectra regions for the woody species according to the morphological and biochemical parameters, as obtained by redundancy analysis.
Tabela 2: Pojasnjena varianca različnih spektralnih območij odbojnih spektrov za lesnate vrste glede na morfološke in biokemijske parametre, ugotovljena s pomočjo redundančne analize.

Spectral region	Bark trait	Explained variance (%)	p
UV	Outer bark thickness	35	0.001
	Inner bark	9	0.002
	UV-A–AS	4	0.040
	Anthocyanins	4	0.031
Violet, blue	Outer bark thickness	39	0.001
	UV-A–AS	6	0.030
Green, yellow	Anthocyanins	48	0.001
	Inner bark thickness	13	0.001
	Carotenoids	8	0.002
	Outer bark thickness	5	0.010
	Chlorophyll *b*	4	0.032
	Chlorophyll *a*	3	0.020
Red, NIR	UV-B–AS	40	0.002
	Outer bark thickness	7	0.010
	Inner bark thickness	7	0.012

Abbreviations: p, p-value; UV-B–AS, UV-B–absorbing substances; UV-A–AS, UV-A–absorbing substances.

comparable amounts of photosynthetic and other pigments per area, as seen for the leaves of different herbaceous species (Grašič et al. 2019a, b, c). The bark usually contains 90% of the total photosynthetic pigments, while the remaining 10% is located in the deeper layers of the woody stem (Tokarz and Pilarski 2005). The main reason for this distribution is that the formation of bark reduces the intensity of radiation that reaches the chlorophyll layer and affects the content of photosynthetic pigments in the inner tissues (Pilarski et al. 2008). In the species studied here, there were differences across the levels of chlorophyll *a*, and even more pronounced differences for chlorophyll *b*, which resulted in variable chlorophyll *a*/*b* ratios that ranged from 2.00 in *C. avellana* to 0.65 in *C. sericea* var. *flaviramea*. According to these ratios, we cannot generally speak about a shady character of the bark inner environment. Shady character can only be confirmed for two species in this study, namely *C. sericea* var. *flaviramea* and *V. lantana*, with chlorophyll *a*/*b* ratios below 1. Such ratios reveal higher contents of the accessory pigment chlorophyll *b* (in comparison to the main pigment chlorophyll *a*), which is needed to increase light-use efficiency in shady environments (Larcher 2003).

The bark of *C. sericea* var. *flaviramea* was the thinnest, but it had the highest total chlorophylls, UV-absorbing substances, and anthocyanins, as was also evident from the noticeably red colour of the bark. In *V. lantana*, anthocyanin content was almost the same as in *C. sericea* var. *flaviramea*, even though its bark colour was completely different, as also revealed from the reflectance spectra. The colour of anthocyanins varies according to the vacuolar pH, and therefore it cannot be implicitly assumed that non-red tissues lack anthocyanins (Gould et al. 2000). Anthocyanins have multiple functions in plants. They can protect shade-adapted chloroplasts from exposure to high light intensity (Gould et al. 2000), and act as chemical repellents and visual signals. Therefore, they are also important in plant and animal interactions (Carrillo-Parra et al. 2012). Some anthocyanins also have significant antiviral, antibacterial, and fungicidal activities (Lev-Yadun and Gould 2008), which provides an advantage for plants with high anthocyanin levels. Their levels can also increase due to low temperatures, which was the case for a container-grown red maple in Georgia (Sibley et al. 1999). The anthocyanin production is related to the redox potential of the plastoquinone pool in the photosynthetic electron transport chain that regulates the anthocyanin biosynthesis genes (Kumar Das et al. 2011). In the study with green and red stems of *C. stolonifera*, Gould et al. (2010) showed high attenuation of PAR by anthocyanins, especially of green and yellow light, and pronounced differences in photoprotective potential and anthocyanin concentrations in the red stems compared to the green stems. *C. sericea* var. *flaviramea* showed the highest reflectance in the UV and NIR regions, and the lowest reflectance in the visible regions. The reflectance peak of other species was wide, and ranged from green to red, except for *C. avellana*, which had a peak in the green region. The reflectance of *V. lantana* gradually increased along the spectra, with a slight depression in the red edge. These reflectances were related to bark morphology and biochemistry, as shown by RDA. Anthocyanins act as effective light screening substances (Kumar Das et al. 2011), as also seen in the present study. Negative relationship was seen between the anthocyanins and the reflected light especially in the green, yellow, and blue regions. Their importance was also shown by RDA performed with specific regions of the spectrum, where anthocyanins explained 48% of the green and yellow reflectance spectra variability. In addition to anthocyanins, stems can contain high levels of other products of the phenylpropanoid pathway. The accumulation of UV-absorbing substances represents a primary mechanism by which plants can acclimatise to changing UV environments, including altered UV-B (Bornman et al. 2015). These phenolic substances strongly absorb in the UV region, while they reflect light of longer wavelengths. However, when a variety of vegetable crop plants were examined for tolerance to UV, it was shown that the levels of chlorophyll and UV-absorbing substances did not correlate directly with their sensitivity (Smith et al. 2000). The reflectance in the NIR region was explained by the UV-absorbing substances. The study of Kokaly and Skidmore (2015) showed that reflectance of different phenolic compounds in plants increases with increasing wavelength and reaches its peak at around 1600 nm. We obtained positive relationship between the amount of UV-absorbing substances and the reflectance spectrum in the NIR region.

The presence of photosynthesis in green stems and in the bark of woody stems has been reported for different tree species (Pfanz 1999). Green stems without a well-developed periderm and with abundant, functional stomata can carry out photosynthesis (Nilsen 1995). The lack of stomata on woody stems results in the accumulation of respiratory CO_2, which supports CO_2-rich and O_2-poor environments (Mancuso and Marras 2003). CO_2 might also diffuse into the chlorenchyma through the lenticels (Eyles et al. 2009). The highest photosynthetic activity takes place in the outer bark, which contains higher amounts of photosynthetic pigments compared to the inner bark, which has less chlorophyll and carotenoids, and mainly serves for storage of assimilates that are produced in the outer bark or in the leaves (Pilarski 1999). The photochemical efficiency of PS II in the bark has been reported to be much lower than in the leaves (Manetas and Yiotis 2009). The Fv/Fm measured in the present study ranged from relatively low in *C. avellana* (0.36) to relatively high in *S. nigra* (0.71). These efficiencies reveal the presence of strong and mild permanent stress, respectively. The mean for the photosynthetic apparatus for undisturbed functioning is close to 0.83 (Björkman and Demmig 1987). Fq'/Fm' was low and did not differ across these species (means from 0.11 to 0.15), which showed strong transient stress that was possibly the consequence of the low temperatures during the sampling period. Solhaug and Haugen (1998) reported that the lowest photochemical efficiency of PS II in bark was detected during the winter period, due to the low temperatures. The chlorophyll fluorescence might also reflect the wood structural changes, such as changes in the bark chlorenchyma (Johnstone et al. 2014). High Fv/Fm along with efficient uptake of internal CO_2 might support the maintenance of a favourable carbon balance in the plant, especially during winter (Ivanov et al. 2006).

Conclusions

The parameters that affected bark reflectance of 1-year-old twigs varied across the studied species. The present results revealed high importance of twig biochemistry for light reflectance. However, these relations may not be directly transferable to the plant level, since they depend on twig age and also on environmental conditions that may significantly alter plant traits. Therefore, additional research is needed to study this relation in twigs of different age, and in different times of the season, which is especially important in the case of deciduous species. Nevertheless, bark of the species examined here shows different spectral signatures, which enables differentiation across species out of the vegetation season.

Summary

Bark comprises tissues outside the vascular cambium, and consists of the inner bark, which contains secondary phloem, and the outer bark or periderm. Bark protects the stem from physical disturbances, environmental factors and pollutants, and enables transport of assimilates and water. The physiological and biochemical traits of bark vary greatly between species. This study examined factors that affect bark reflectance of 1-year-old twigs of different shrub species: *Corylus avellana*, *Rosa canina*, *Ligustrum vulgare*, *Sambucus nigra*, *Cornus sericea* var. *flaviramea*, and *Viburnum lantana*. We hypothesised that bark biochemical properties and bark thickness play an important role in shaping bark reflectance and thus also the so-called "bark spectral signatures". We analysed contents of various pigments and UV-absorbing substances in the bark and measured bark thickness and bark reflectance spectra. Bark morphological and biochemical properties showed great variability between the species. The bark of *C. sericea* var. *flaviramea* differed the most from the other species, showing the highest amounts of total chlorophyll, UV-absorbing substances, and anthocyanins. The peridermal layer was developed differently in the studied species. *V. lantana* had the thickest outer bark, while the inner bark was thickest for *S. nigra*. *R. canina* and *C. sericea* var. *flaviramea* had an epidermis and a very thin peridermal layer. Photosynthetic pigments in the bark allow the process of photosynthesis, which mostly takes place in the outer bark. Outer bark contains higher amounts of pigments compared to the inner bark, which serves primarily as an assimilate storage. Fv/Fm was relatively high in most of the studied species, while Fq'/Fm' was relatively low and did not differ between the species. Redundancy analysis revealed associations between bark biochemical properties and reflectance in the visible part of the spectrum. UV-B–absorbing substances explained 38% of the variability of the reflectance spectra ($p = 0.001$), outer bark thickness explained 12% ($p = 0.001$), inner bark thickness 7% ($p = 0.001$), and anthocyanins explained an additional 4% ($p = 0.009$) of the variability. Therefore, these parameters together explained as much as 61% of the variability of the reflectance spectra. Bark of the studied species showed different spectral signatures resulting from different bark physiological and biochemical properties. This allows us to differentiate between species out of the vegetation season.

Povzetek

Skorja zajema tkiva zunaj vaskularnega kambija in je sestavljena iz notranje skorje, ki vsebuje sekundarni floem, ter zunanje skorje ali periderma. Skorja ščiti steblo pred fizičnimi motnjami, okoljskimi dejavniki in onesnaževali ter omogoča transport asimilatov in vode. Fiziološke in biokemijske lastnosti skorje se med vrstami močno razlikujejo. V raziskavi smo proučili dejavnike, ki vplivajo na odbojnost skorje enoletnih vejic različnih grmovnih vrst: *Corylus avellana*, *Rosa canina*, *Ligustrum vulgare*, *Sambucus nigra*, *Cornus sericea* var. *flaviramea* in *Viburnum lantana*. Predvidevali smo, da imajo biokemijske lastnosti in debelina skorje pomembno vlogo pri oblikovanju odbojnosti oziroma tako imenovanih "spektralnih podpisov" skorje. Analizirali smo vsebnost različnih pigmentov in UV-absorbirajočih snovi v skorji ter izmerili debelino skorje in njene odbojne spektre. Morfološke in biokemijske lastnosti skorje so pokazale veliko variabilnost med vrstami. Najbolj se je od ostalih vrst razlikovala skorja vrste *C. sericea* var. *flaviramea*, ki je vsebovala največ skupnih klorofilov, UV-absorbirajočih snovi in antocianinov. Proučevane vrste so imele različno razvito peridermalno plast. Najdebelejšo zunanjo skorjo je imela vrsta *V. lantana*, medtem ko je bila notranja skorja najdebelejša pri vrsti *S. nigra*. Vrsti *R. canina* in *C. sericea* var. *flaviramea* sta imeli prisotno povrhnjico in zelo tanko plast periderma. Fotosintezni pigmenti v skorji omogočajo proces fotosinteze, ki večinoma poteka v zunanji skorji. Ta vsebuje več pigmentov v primerjavi z notranjo skorjo, ki je namenjena predvsem shranjevanju asimilatov. Potencialna fotokemična učinkovitost FS II je bila pri večini proučevanih vrst razmeroma visoka, dejanska fotokemična učinkovitost FS II pa relativno nizka in se ni razlikovala med vrstami. Redundančna analiza je razkrila povezave med biokemijskimi lastnostmi in odbojnostjo skorje v vidnem delu spektra. UV-B–absorbirajoče snovi so razložile 38 % variabilnosti odbojnih spektrov ($p = 0{,}001$), debelina zunanje skorje 12 % ($p = 0{,}001$), debelina notranje skorje 7 % ($p = 0{,}001$), vsebnost antocianinov pa še dodatne 4 % ($p = 0{,}009$) variabilnosti. Omenjeni parametri so skupaj torej pojasnili kar 61 % variabilnosti odbojnih spektrov. Skorja proučevanih vrst je pokazala različne spektralne podpise, ki so posledica različnih fizioloških in biokemijskih lastnosti skorje. To nam omogoča razlikovanje med vrstami izven vegetacijske sezone.

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Crown stratification ratio models for *Tectona grandis* L. f in Oluwa Forest reserve, Nigeria

Modeli razmerja stratifikacije krošnje za vrsto *Tectona grandis* L. fv gozdnem rezervatu Oluwa, Nigerija

Omobolanle Temitope Faleye[a]*, Lucas Aderemi Akomolede[b], Olalekan Kehinde Ajayi[b], Opeyemi Philips Akinsulire[c]

[a]Departmentof Forest Resources Management, University of Ibadan, Ibadan, Nigeria
[b]Forestry Research Institute of Nigeria, Jericho Hill, Ibadan, Nigeria
[c]Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria

*Correspondence: temitopefaleye1@gmail.com

Abstract: This research investigated crown ratio models for *Tectona grandis* plantation in Oluwa Forest reserve (Ondo State, Nigeria) using variables of slenderness coefficient and merchantable height. With three non-linear regression functions - logistic, Chapman-Richard and exponential we showed that basal area, tree stem volume and mean tree height per hectare were of high significance. In the same vein, there were fewer tree species in the class of higher diameter and height than there were in lower class. There were also more trees in the co-dominant and intermediate classes than in the dominant and suppressed layers. The lack of emergence in the plantation reflected the past disturbance of the ecosystem. Most of the tree growth variables were significantly different in different canopy layers in the study area. Based on the evaluation models, the three functions investigated for tree crown ratio modeling gave constant and reliable results in all canopy layers considering their indices. Especially, Chapman-Richard and exponential functions gave consistent trends and good fits for crown ratio models. It is recommended to put strict measures in place to avert any level of illegalities that may likely disrupt the delicate equilibrium of the ecosystem. It is also recommended that complexity revealed in this study is sustained in the region, and encouraged in other parts of Nigeria.

Keywords: crown ratio, modeling, Oluwa forest reserve, *Tectona grandis,* tree growth

Izvleček: Raziskava preizkuša tri različne modele razmerja krošnje v nasadih vrste *Tectona grandis* v gozdnem rezervatu Oluwa (država Ondo, Nigerija) z uporabo spremenljivk koeficienta vitkosti in tržne višine. Modeli, ki temeljijo na nelinearnih regresijskih funkcijah (logistični, Chapman-Richard in eksponentni) so pokazali, da so parametri kot so bazalna površina, prostornina debel in povprečna višina drevesa na hektar zelo pomembni. V razredu višjega premera in višine je bilo manj drevesnih vrst kot v nižjem razredu. V kodominantnem in srednjem razredu je bilo tudi več dreves kot v dominantni in zavrti plasti. Omejeno pojavljanje mladih rastlin odraža pretekle motnje ekosistema. Poleg tega se je večina rastnih spremenljivk dreves v različnih plasteh krošenj na preučevanem območju bistveno razlikovala. Vsi trije modeli so glede na indekse, ki smo jih uporabili, dali zanesljive in ponovljive rezultate v vseh plasteh krošenj. Chapman-Richardova in eksponentna funkcija sta pokazali skladne trende in ujemanje. Priporočamo stroge ukrepe za kakršnekoli nezakonite posege, ki bi lahko porušili občutljivo ravnovesje ekosistema ter da se celovitost sestojev ohrani v regiji in spodbuja v drugih delih Nigerije.

Ključne besede: gozdni rezervat Oluwa modeliranje, rast drevesa, razmerje krošnje, *Tectona grandis*

Introduction

Forests are viewed, defined, assessed and valued through different points of view and from different vantage points. It can be seen as a source of timber products, an ecosystem composed of trees along with myriad forms of biological diversity, a home for indigenous people, a repository for carbon storage, a source of multiple ecosystem services, and a social-ecological system or as all of the above (Chazdon et al. 2016). Within the forest, complex layers of vegetation are common from ground cover of mosses and low flowering plants through middle layers of bushes and secondary trees (Adekunle and Ige 2006**).** According to Hardiman et al. (2011), tree crown enables net primary production. It is also known that crown ratio is the ratio of live crown length to aboveground tree height (Schomaker et al. 2007). It is often used as an important predictor variable for tree growth equation. It indicates tree vigor and is a useful parameter in forest health assessment. According to Kozlowski et al. (1991), dense and large crowns are associated with potential growth rates while sparse and small crowns may reflect unfavorable site conditions (competition, moisture, diseases).

Hasenauer and Monserud (1996) suggested that crown ratio is a useful indicator of tree vigor, wood quality (Kershaw et al. 1990), resistance against wind (Navaratil 1997), stand density (Clutter et al. 1983) and it is important in management of many non-timber resources including wildlife habitat and recreation (Mcgraughey 1997). Crown dimensions can be important components of forest growth and yield models, and are used in many tree and crown level growth–modeling systems (Cole and Lorimer 1994). For instance, tree crown parameters can be considered when simple competition indices are not able to adequately predict recovery from competition when a competitor is removed (e.g. by thinning) (Vanclay 1994). Tree crown parameters were used as predictor variables in diameter and height growth equations (Monserud and Sterba 1996). Similarly, stand crown parameters were used to distinguish different stages of stand development (Soares 1999).

Within a forest stand, there are different forms of canopy layers. These canopy layers provide protection from strong winds and storms, while also intercepting sunlight and precipitation, leading to a relatively sparse vegetated understory layer (Lowman and Moffett 1993). The canopy of a rainforest is typically about 10m thick, and intercepts around 95% of sunlight. The canopy is below the emergent layer - a sparse layer of very tall trees that are typically very rare, one or two per hectare. With high availability of water and a near ideal temperature in rainforests, light and nutrients are two factors that limit tree growth from the understory to the canopy.

A canopy layer presents the horizontal and vertical distribution of tree crowns in a forest stand. Vertical canopy arrangement is often simplified by dividing canopy cover into height layers while horizontal canopy is commonly quantified as the vertically-projected percentage of cover of plant canopies, and the abundance and size of canopy gaps. According to Fiala (2003), additional attributes that are used to describe tree crown, include the number and height of vertical canopy layers and the proportions of cover contributed by different species groups.

Forest canopy is strongly influenced by stand density due to changing competitive interactions among the individual trees and in turn directly influences stem-wood volume production. Stand density also influenced the amount and distribution of leaf area in the forest stands. It is a measure of the stocking of a stand of trees based on the number of trees per unit area and diameter at breast height of the tree of average basal area. Stand density index is usually well correlated with stand volume and growth, and several variable-density yield tables have been created using this index (Chazdon et al. 2016).

The most commonly cited stand development model is Oliver's (1981) four-stage model, which comprises stand-initiation, stem-exclusion, understory re-initiation, and old-growth phases, all of which include canopy cover criteria. Franklin et al. (2002) proposed an alternative stand development model for natural stands. Their model highlights eight commonly encountered development stages: disturbance and legacy creation, cohort establishment, canopy closure, biomass accumulation/competitive exclusion, maturation, vertical diversification, horizontal diversification, and pioneer cohort loss, with canopy attributes described for each of these stages. Quantifying canopy structure attributes across forest stands of different ages can aid in evaluating these stand development models. The development of understory plant communities is usually related to changes in the over-story (Henderson 1981, Oliver 1981, Zamora 1981, Stewart 1988, Franklin et al. 2002, Naesset and Okland 2002). According to Connell and Slatyer (1977) "tolerance" model of succession, shade-tolerant species are generally present in all stages of succession, but invade the understory and increase in their abundance across the gradient of development stages.

Waide (2008) noted that very little of the World's tropical rainforest area can be considered to be under effective management including Nigerian forests. Clear-cut logging, tree planting, and short stand rotation lengths have greatly reduced the structural variability of forests (Smith 1988). Moreover, it is generally understood that tree canopy changes as forests develop with age (Oliver 1981, Van Pelt and Franklin 2000, Bond and Franklin 2002, Franklin et al. 2002), but these changes have rarely been quantified. The lack of data and the difficulty of accurately measuring the height to the live crown base which is even more pronounced in species with asymmetric crowns may however justify the relatively little research done on modeling crown parameters (Soares and Tome 2001).

Though much is known about how forests change in the number and size and identity of their stems (e.g. Oliver and Larson 1990), scanty information is available on the forest canopy (Aber 1979, Brown and Parker 1994) and almost nothing is known of the development of canopy layers in a forest stand (Parker 1995). This presents a problem since numerous functional characteristics of forests are linked to developmental stages (Waring and Schlesinger 1985). Thus, lack of information on the development of the outer canopy precludes the prediction of some stand functional characteristics from remote information. The shape of the outer canopy as well as the different levels below is important for several reasons. This part of a forest is the interface of atmospheric interactions. The extent, shape and disposition of this surface have implications for the penetration of light and heat, and for the extent of turbulent mixing, among other properties. Furthermore, the shape of the outer canopy necessarily constrains some aspects of the internal structure included below that surface. In addition, changes in the canopy reflect the development of the forest.

Tree crown research contributes to several key forest ecosystem attributes: biodiversity, productivity, forest management, forest environment, and wildlife. Crown ratio is used as an input variable to estimate growth and mortality of individual trees and also to display changes in the appearances of stands over time for habitat suitability and visual changes (Avery and Burkhart 2001). The crown leaves capture radiant energy for photosynthesis. The size of a tree crown has a marked effect and strong correlation with the growth of the tree and its various parts (Temesgen et al. 2005). Thus, measurement of a tree crown is often used to estimate the tree growth (Kozlowski et al. 1991).

No single model can be expected to be best for all purposes. It is therefore important to consider forest crown under different canopy layers to provide useful alternative model for management decisions. Many authors have based crown ratio equation on logistic functions (Hasenauer and Monserud 1996, Temesgen et al. 2005), exponential function (Holdaway 1986, Dyer and Burhart 1987) or Chapman Richard function (Soares and Tome 2001, Adesoye and Oluwadare 2008). However, most of these models were formulated for either even aged single species stands, or multi-species stands comprising of different ages or mixed stand with two or more species. These statistical functions have been tested on even aged stand consisting of different canopy layers (Popoola and Adesoye 2012), but none or rare in Oluwa Forest reserve.

The purpose of this study was to enhance understanding of canopy structure at different strata across different ages of forest stands using inventory data. Specifically, horizontal and vertical canopies were compared under different layers and the impact on the growth at different ages were quantified and assessed in Oluwa Forest reserve in Ondo State of Nigeria, as well as the determination of stand density indices.

Materials and methods

Study area

This study was carried out in Oluwa Forest reserve located in Odigbo Local government area, the southern part of Ondo State, Nigeria (Figs. 1 and 2). Four stands of different ages (39, 34, 30 and 26 years) which covered a total area of 2000 ha were investigated for the study. The forest is under the management of the Department of Forestry of the Ondo State Government of Nigeria. Oluwa Forest reserve covers 828 km^2 in area. It is located along the Lagos-Benin expressway and it is the largest forest reserve in the state. Most

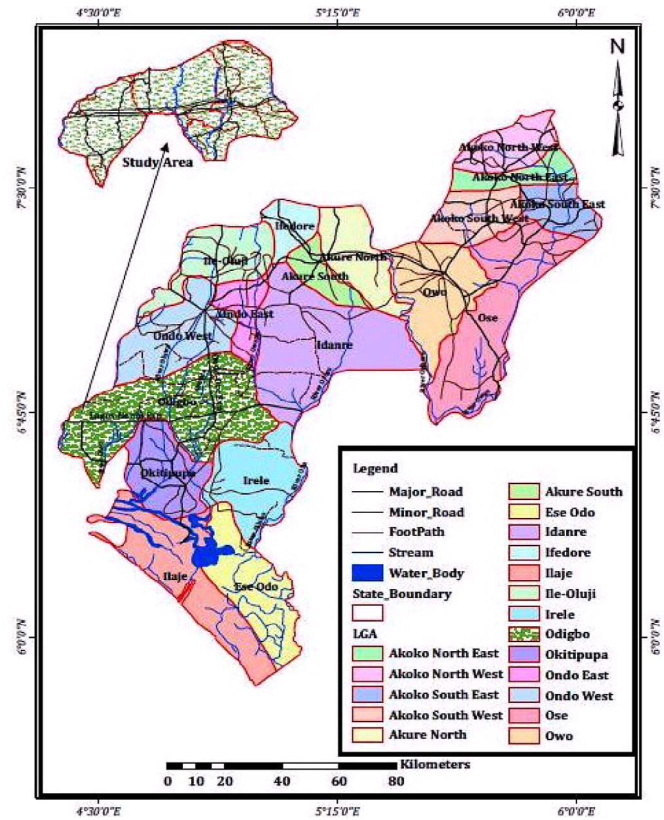

Figure 1: Administrative map of Ondo State showing the study area (Oluwa Forest reserve). Source**:** Ministry of Land and Housing, Akure, Ondo State, Nigeria.

Slika 1: Zemljevid pokrajine Ondo s prikazom območja proučevanja (gozdni rezervat Oluwa). Vir: Ministrstvo za okolje in stanovanjske zadeve, Akure, pokrajina Ondo, Nigerija.

part of the reserve lies north of the road while a minor part (about one-sixth of the total area) lies south of the road. Its eastern border is very close to the Ondo road, a major road that leads to both Ondo and Akure towns (Figs. 1 and 2). The area is part of the western plains of Nigeria. According to Iloeje (1981), the experimental site lies approximately between latitudes 635' N and 720' N, and longitudes 345' E and 432' E with much of it lying approximately between 300 and 600 m above the sea level. Most rivers and streams draining this area originated at the southern part of the study area. Important rivers are Oni, Oluwa, Ominla and Owena rivers. The study area is under the influence of Koppens humid tropical rain forest climate. Mean annual rainfall according to the observations of the Nigerian Meteorological Society (2007) ranges from 1,200 mm to 1,450 mm and temperatures are high throughout the year with a mean of about 27 °C with annual range of 3 °C. Natural vegetation of the area is tropical rainforest, characterized by multiple canopies and lianas. Some of the most commonly found trees in the area are: *Melicia excels* C.C Berg (Welw.), *Afzelia bipindensis* Harms, *Antiaris africana* Lesch, *Brachystegia nigerica* Hoyle & A.P.D Jones, *Lophira alata* Banks ex C.F. Gaertn, *Lovoa trichilioides* Harms, *Terminalia ivorensis* A. Chev, *T. superba* Engl. & Diels and *Triplochiton scleroxylon* K. Schum. However, the natural vegetation of the area, with the exception of the areas within the forest reserve had been reduced to secondary re-growth forest thickets and fallow re-growth in various stages of development, or was replaced by perennial and annual crops (Osunade 1991). These perennial crops include cocoa, kola and citrus. Most of the rural settlements in the study area had been established between 1920 and 1950 and by 1970 human colonization of the area was completed (Adejuwon 1971). Oluwa Forest reserve used to be contiguous with Omo Forest reserve in Ogun State of Nigeria. However, along the western border, the area is now densely populated.

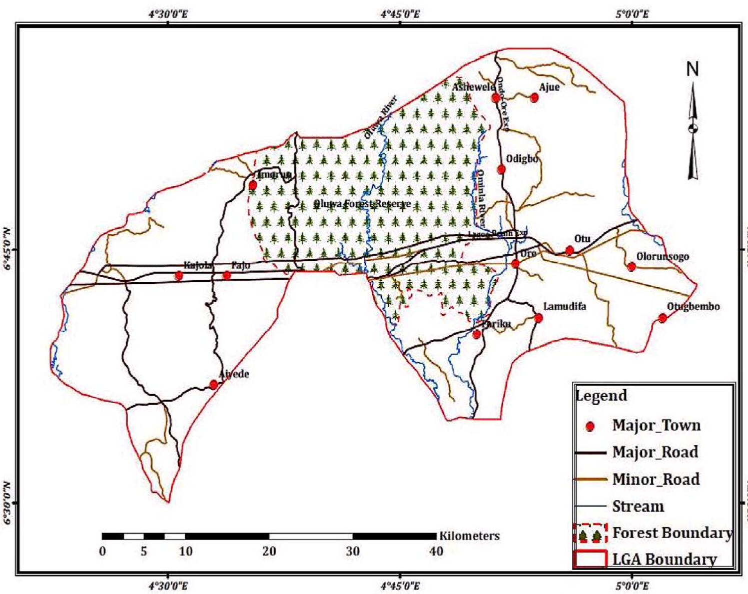

Figure 2: Odigbo Local government area showing Oluwa Forest reserve and road network pattern.
Slika 2: Zemljevid območja Odigbo z gozdnim rezervatom Oluwa in cestnim omrežjem.

Data collection

Four stands of different ages which were planted in 1982, 1987, 1991, and 1995 (ages 39, 34, 30 and 26 years, respectively) were studied in this investigation. Within each stand, five sample plots of 25 m x 25 m (0.0625 ha) were determined and surveyed for the purpose of this study. This was in a total 20 sample plots.

Within each sample plot, tree growth variables such as total height of trees (THT), merchantable height (MHT), crown length (CL) and crown diameter (CD) were measured. Similarly, diameters at breast height (DBH) were measured at 1.3 m above the ground level while diameters at the base, middle, and top of the tree were also measured with Spiegel Relascope (Relaskop). In addition, trees within each plot were classified into four canopy layers namely; dominant (height above 25 m), co-dominant (height range of 20 m to 24.9 m), intermediate (height range of 14 m to 19.9 m), and suppressed (height lower than 13.9 m) layers.

Data analysis

Data collected from tree measurements were used to estimate basal area, crown projection area at different layers, slenderness coefficient, crown ratio of individual tree, stand volume per plot, mean crown ratio and stand density index of trees at different layers. The following formulae (eq. 1-14) were used for calculations.

Basal area (eq. 1)

$$BA = \frac{\pi D^2}{4} \qquad (1)$$

where: BA, basal area (m^2); D, diameter at breast height (m).

Tree slenderness coefficient (eq. 2)

$$TSC = \frac{THT}{D} \qquad (2)$$

where: TSC, tree slenderness coefficient; THT, tree total height (m); D, diameter at breast height (m).

Crown projection area (eq. 3)

$$CPA = \pi \frac{CD^2}{4} \qquad (3)$$

where: CPA, crown projection area; CD, crown diameter (m).

Tree volume per plot (eq. 4)

$$v = h \left[\frac{A_b + 4A_m + A_t}{6}\right] \qquad (4)$$

where: v, tree volume (m^3); h, tree height (m); A_b, cross-sectional area at the base of a tree (m^3); A_m, cross-sectional area at the middle of a tree (m^3); A_t, cross-sectional area at the top of a tree (m^3).

Stand density index

The maximum density line was expressed by the equation 5 (Reineke 1933),

$$log_{10}N = -1.605\ (log_{10}D) + k \qquad (5)$$

where: N, number of trees per acre; D, diameter at breast height (DBH) of the tree of average basal area; k, a constant varying with the species (a specific species constant).

When the quadratic mean diameter equals 10 inches (250 mm), the log of N equals the stand density index (SDI) in equation 6,

$$log_{10}SDI = -1.605(1) + k \qquad (6)$$

which means that: k = log_{10}SDI + 1.605

Substituting the value of k above into the reference-curve formula gives the equation 7,

$$log_{10}N = log_{10}SDI + 1.605 - 1.605\ log_{10}D \qquad (7)$$

This equation can be rewritten as equation 8:

$$log_{10}SDI = log_{10}N + 1.605\ log_{10}D - 1.605 \qquad (8)$$

The above equation is an expression for computing the stand density index from the number of trees per acre and the diameter of the tree of average basal area. Estimates of stand density express the degree to which the growing space available for tree growth is utilized. Thus, stand density is a function of three elements:

i. Number of trees - which is readily determined by counting.
ii. Tree size - which involves a number of factors, e.g.:
    - Stem - characterized by diameter, height and taper
    - Crown - characterized by spread and height
    - Root - characterized by spread and depth (both difficult to measure).
iii. Spatial distribution on the ground - which is not readily determined. Generally, a square or triangular spacing is assumed.

In even-aged stands, crown closure may be proportional to basal area per ha. This relationship has led to the development of indices between estimates of crown closure obtained from aerial photographs and basal area. The value of crown closure as a variable depends on how well variation in stand volume is correlated with it.

The functional form of this relationship is (eq. 9),

$$Log_{10} N = k - 1.605 (Log_{10} QMD) \quad (9)$$

where: N, number of trees per hectare; k, a species-specific constant; QMD, quadratic mean diameter.

Crown ratio model

The tree crown ratio models employed for this study are: Logistic function, Chapman-Richard and Exponential models since a crown ratio value ranges from 0 (i.e. no crown, dead or defoliated tree) to 1 (i.e. crown extends over the entire tree bole). The tree crown ratio model formulated to express crown ratio as a function of tree size (e.g. basal area, merchantable height). The original forms of the models are (eqs. 10-12):

$$CR = (1 - e^{-\beta X}) \quad (10)$$

$$CR = ((1 + e^{-\beta X})^{0.5})^{-1} \quad (11)$$

$$CR = b_o + e^{-\beta X} \quad (12)$$

where: CR, estimated crown ratio; βx, linear equation with parameter β and independent variable x (which includes individual tree characteristics such as merchantable height, diameter at breast height, crown length and total height); e, Naperian constant (2.72).

The multiple linear regression equation 13 for the independent variables is given as follows:

$$\beta_x = \beta_o + b_{x1} + c_{x2} + d_{x3} \quad (13)$$

where: β_x, linear equation with parameter β and independent variable x; β_o, the intercept; b, c, d are the parameters; x_1, x_2, x_3 are the independent variables (eq. 14).

$$CR = (1 - e^{-\beta_0 + bx_1 + cx_2 + dx_3})^{-1} \quad (14)$$

where: CR, crown ratio; e, Naperian constant (2.72); β_o, the intercept; b, c, d are the parameters; x_1, x_2, x_3 are the independent variables.

Model evaluation

The models were evaluated with a view of selecting the best estimator for tree crown ratio. In this study, the evaluation was based on coefficient of determination (R^2) and standard error of estimate (SEE), computed in order to evaluate the fitted models. The significance of the parameter estimates was observed. In addition, residual values were plotted against the predicted crown ratio values to check constant error assumptions. The selected versions of the models are presented in Supplement 1.

The level of statistical significance was set at p-value > 0.05.

Results and discussion

A total of 1042 individual trees were identified and recorded from the sampling plot which covers an area of 2000 ha. In this sampling, 95 trees were recorded in the dominant canopy layer, covering about 9.1% of the total population of the forest plantation. 267 trees were recorded in the co-dominant layer and this accounted for about 25.6% while 403 trees were recorded for intermediate height layer with 38.7% of the total population. Meanwhile, the suppressed layer accounted for 26.6 % with 277 trees (Supplement 3).

This research revealed that the number of individual trees per hectare in the forest plantation was higher, compared to those reported by previous researchers for other tropical rainforests in Nigeria (Adekunle et al. 2004, Ojo 2004, Adekunle and Olagoke 2008). This is an indication that the protection of the Oluwa Forest reserve is very effective. Another scientific statement is that the mean basal area per hectare obtained for the forest plantation (92.65m^2/ha) was comparable with that reported in Onigambari Forest Reserve (Akinyemi et. al. 2002) and also higher than in Omo Forest reserve (Adekunle 2007).

It was also discovered that the values of the mean basal area were comparatively higher to those reported by Temesgen et al. (2005) who conducted a research on multispecies and multilayered stands of southeastern British Columbia. The mean values of stem volume values obtained for the forest plantation (7184.46m^2/ha) were as well far higher than those reported by Adekunle et al. (2004), Adekunle and Olagoke (2008) and Alder and Abayomi (1994) for tropical rainforest ecosystem in Nigeria.

Correlation among tree growth variables for individual dominant trees

The Pearson's correlation coefficients for the growth variables are presented (Tabs. 1-4). The correlation values obtained for individual dominant trees varied considerably between -0.01 and 0.68. Most of the tree growth variables have significant positive correlation with one another. The highest correlation was between merchantable height and total height (r = 0.68), followed by correlation between diameter breast height and total height (r = 0.62) and between diameter breast height and merchantable height (r = 0.53). Negative correlation was obtained between diameter breast height and crown length (r = -0.01), merchantable height and slender coefficient (r = -0.31), diameter breast height and crown ratio (r = -0.30), and total height and crown ratio (r = -0.26).

Meanwhile the correlation values obtained for individual co-dominant trees varied between -0.72 and 0.78 (Tab. 2). Most of the tree growth variables have significant positive correlation with one another. The highest correlation was between merchantable height and total height (r = 0.78), followed by crown length and crown ratio (r = 0.78) and diameter breast height and merchantable height (r = 0.66). Negative correlation was obtained between merchantable height and crown length, total height and crown ratio (r = -0.09), diameter breast height and crown ratio (r = -0.15) and total height and slender coefficient (r = -0.22).

The correlation values recorded for individual trees in the intermediate layer varied between -0.34 and 0.92 (Tab. 3). Most of the tree growth variables have significant positive correlation with one another. The correlation was between crown length and crown ratio (r = 0.92), followed by diameter breast height and slender coefficient (r = 0.57), total height and slender coefficient (r = 0.52) and diameter breast height and merchantable height (r = 0.51). Negative correlation was obtained between diameter breast height and crown ratio (r = -0.34); total height and crown ratio (r = -0.61), crown ratio and slender coefficient and merchantable height and crown length (r = 0.28).

The values obtained for suppressed trees varied between -0.24 and 0.77 (Tab. 4). Most of the tree growth variables have significant positive correlation with one another. The highest correlation was between crown length and crown ratio (r = 0.77), followed by diameter breast height and slender coefficient (r = 0.54), merchantable height and slender coefficient (r = 0.40), and diameter breast height and crown length (r = 0.38). Negative correlation was obtained between total height and crown ratio, (r = -0.24), crown length and slender coefficient (r = -0.24), and crown ratio and slender coefficient (r = -0.26).

Generally, most of the tree growth variables were significantly and positively correlated with one another, which imply that an increase in one tends to be associated with an increase in the other variables. However, the correlation between the tree slenderness coefficient and the tree crown ratio, and the crown length and slenderness coefficients were low (r = 0.02 and 0.03 respectively). The result revealed negative correlations between crown ratio and diameter at breast height, crown length and diameter at breast height, crown ratio and total height. These negative correlation trends are expected and suggested that tall and slender tree with small diameter has lower crown ratio values. Similar pattern was observed by previous researchers (Chukwu et al. 2018)

Table 1: Correlation matrix for individual dominant tree growth variables.
Tabela 1: Matrika korelacij za rastne spremenljivke pri dominantih drevesih.

	DBH	MHT	THT	CL	SC	CR
DBH (m)	1.00					
MHT (m)	0.53**	1.00				
THT (m)	0.62**	0.68**	1.00			
CL (m)	-0.01	-0.56**	0.22**	1.00		
SC	-0.85**	-0.31**	-0.27**	0.10	1.00	
CR	-0.30**	-0.88**	-0.26**	0.88**	0.22**	1.00

Abbreviations: THT, total height; MHT, merchantable height; DBH, diameter at breast height; SC, slenderness coefficient; CL, crown length; CR, crown ratio; **, statistically significant correlation

Table 2: Correlation matrix for individual co-dominant tree growth variables.
Tabela 2: Matrika korelacij za rastne spremenljivke pri kodominantnih drevesih.

	DBH	MHT	THT	CL	SC	CR
DBH (m)	1.00					
MHT (m)	0.57**	1.00				
THT (m)	0.66**	0.78**	1.00			
CL (m)	0.28**	-0.09	0.54**	1.00		
SC	-0.72**	-0.27**	-0.21**	0.02	1.00	
CR	-0.15**	-0.66**	-0.09**	0.78**	0.21**	1.00

Abbreviations: THT, total height; MHT, merchantable height; DBH, diameter at breast height; SC, slenderness coefficient; CL, crown length; CR, crown ratio; **, statistically significant correlation.

Table 3: Correlation matrix for individual intermediate tree growth variables.
Tabela 3: Matrika korelacij za rastne spremenljivke pri srednjih drevesih.

	DBH	MHT	THT	CL	SC	CR
DBH (m)	1.00					
MHT (m)	0.51**	1.00				
THT (m)	0.28**	0.30**	1.00			
CL (m)	0.12**	0.03	0.18**	1.00		
SC	-0.57**	-0.17**	0.52**	0.03	1.00	
CR	-0.03	-0.30**	-0.06	0.92**	0.03	1.00

Abbreviations: THT, total height; MHT, merchantable height; DBH, diameter at breast height; SC, slenderness coefficient; CL, crown length; CR, crown ratio; **, statistically significant correlation.

Table 4: Correlation matrix for individual suppressed tree growth variables
Tabela 4: Matrika korelacij za rastne spremenljivke pri zavrtih drevesih.

	DBH	MHT	THT	CL	SC	CR
DBH (m)	1.00					
MHT (m)	0.19**	1.00				
THT (m)	0.39**	-0.70**	1.00			
CL (m)	0.38**	0.54**	0.29**	1.00		
SC	0.64**	0.40**	0.16	-0.24**	1.00	
CR	0.35**	0.38**	-0.24**	0.77**	-0.26**	1.00

Abbreviations: THT, total height; MHT, merchantable height; DBH, diameter at breast height; SC, slenderness coefficient; CL, crown length; CR, crown ratio; **, statistically significant correlation.

Basal area and stem volume estimation

The forest had an average tree basal area of 92.65 m^2/ha while the average tree stem volume was 7184.46 m^2/ha. Information on tress basal area and stem volume per hectare for the forest plantation is presented in Supplement 2.

Height distribution of tree per plot in canopy layers in the study area

The distribution in height classes corresponding to each of the strata (canopy layers) in the study area is shown in Table 5. Four layers existed in the all the plots sampled in the plantation. Trees belonging to the dominant height class accounted for about 9.1% of the individuals sampled in the plantation. About 25.6% of the forest plantation belonged to the co-dominant layer while the intermediate layer accounted for about 38.7%. The suppressed layer accounted for about 26.6%. There was a major decrease in the number of trees reaching the heights above 25 m, compared to the significant increase in the number of trees reaching the heights of 14 m – 19.9 m. Also, there was lower number of individuals in the dominant canopy layer than in the suppressed and co-dominant height classes. The intermediate class recorded the highest number of trees in the overall canopy layer.

Crown ratio model

Three non-linear regression models (Logistics, Chapman-Richard, and Exponential) were used in the study. All the tree growth variables apart from the crown ratio (the dependent variable), were tested during model fitting processes. The selected version of the Logistics, Chapman-Richard and Exponential models, their parameters estimation and fit statistics for the canopy layers are represented in Table 6. Merchantable height and slenderness coefficient were found to consistently predict crown ratio in all the functions.

Table 5: Distribution of tree height per hectare in canopy layers in the study area.
Tabela 5: Razporeditev višine dreves v slojih krošenj na proučevanem območju.

Canopy layer	**Height (m)**	**Number of trees / ha**
Dominant	≥ 25	95
Co-dominant	20 - 24.9	267
Intermediate	14 – 19.9	403
Suppressed	≤ 13.9	277
Total		1042

The R^2 values for the three functions were fairly high for the dominant and co-dominant and intermediate layer with low values of standard errors of estimates (SEE). The suppressed layer which gave a lower fit to the data set in the functions gave significant result for the estimated parameters for all applied functions.

However, there were significant differences among growth variables under different canopy layers; hence the three models were fitted to the data set on the layer basis. The merchantable height and slenderness coefficient gave better fit to the data set and were found to be important in defining the tree crown ratio for *Tectona grandis* stand in Oluwa Forest reserve. The suitability of the other tree growth variable was investigated and failed to explain the tree crown ratio in the entire canopy layer, and were therefore not included in the model presentation result. The R^2 values for the three functions were consistently high under dominant, co-dominant, and intermediate layers with low SEE. The suppressed layers, which gave much lower fit to the data set in all the functions, however produced significant result for all the estimated parameters in all the functions. The R^2 value obtained in this study were generally higher in comparison with those reported previously for less diverse ecosystem (Temesgen et.al. 2005, Adesoye and Oluwadare 2008) with lower SEE value. This indicates better fit of the three functions to the data set than those fitted by previous researchers.

Table 6: Crown ratio models selected with parameter estimate and fit statistic for dominant, co-dominant, intermediate and suppressed layers in Oluwa Forest reserve using Logistic, Chapman-Richard and Exponential functions. Mean ± standard error is presented.

Tabela 6: Modeli razmerja krošenj, izbrani z oceno spremenljivk in statistiko primernosti za dominantne, kodominantne, srednje in zavrte plasti v gozdnem rezervatu Oluwa z uporabo logističnee, Chapman-Richard-ove in eksponentne funkcije. Predstavljena je srednja vrednost ± standardna napaka.

Function	Parameter	Dominant layer	Co-dominant layer	Intermediate layer	Suppressed layer
Logistic	a_o	1.21±0.14	0.27±0.09	0.25±0.03	-1.27±0.09
	a_1	-0.11±0.01	-0.11±0.01	0.03±0.00	0.02±0.01
	a_2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Chapman	a_o	0.09±0.22	0.59±0.02	-0.61±0.06	-0.56±0.07
-Richard	a_1	0.17±0.01	0.27±0.03	0.17±0.07	14.16±1.49
	a_2	-0.00±0.00	0.02±0.00	-0.02±0.00	0.00±141.22
Exponential	a_o	0.19±0.05	-0.40±0.03	-0.07±0.07	-0.47±0.02
	a_1	-0.13±0.03	0.03±0.00	-0.05±0.01	-32.28±6.87
	a_2	0.00±0.00	0.00±0.00	-5.74±3.82	0.23±49387.19

Logistic: R^2 (Dominant layer) = 0.657; R^2 (Co-dominant layer) = 0.643, R^2 (Intermediate layer) = 0.647; SEE (for all) = 0.2.

Chapman-Richard: R^2 (Dominant layer) = 0.704; R^2 (Co-dominant layer) = 0.763; R^2 (Intermediate layer) = 0.747; R^2 (Suppressed layer) = 0.430, SEE (for all) = 0.2.

Exponential: R^2 (Dominant layer) = 0.711; R^2 (Co-dominant layer) = 0.738; R^2 (Intermediate layer) = 0.720; R^2 (Suppressed layer) = 0.427, SEE (for all) = 0.2.

Table 7: Growth variables for dominant, co-dominant, intermediate and suppressed layers in Oluwa Forest reserve. Mean ± standard deviation and minimum to maximum range are presented.
Tabela 7: Spremenljivke rasti za prevladujoče, kodominantne, srednje in zavrte plasti v gozdnem rezervatu Oluwa. Predstavljene so srednje vrednosti ± standardni odklon in razpon vrednosti.

Statistical value	Canopy layer	Growth variable				
		BA	DBH	MHT	SC	CR
Mean ± SD	Dominant	0.06±0.04	27.19±0.73	14.43±3.32	97.58±31.42	0.41±0.10
	Co-dominant	0.05±0.03	23.68±0.01	12.60±3.09	94.06±33.45	0.38±0.00
	Intermediate	0.06±0.06	19.08±8.00	9.75±2.48	92.62±39.98	0.36±0.01
	Suppressed	0.16±0.67	10.48±0.01	4.88±0.21	90.40±36.38	0.37±0.01
Minimum – maximum	Dominant	0.01–0.18	9.55–47.43	6.60– 1.20	209.42-533.48	0.17–0.72
	Co-dominant	0.00–0.20	4.45–56.93	4.50–19.80	49.09–330.34	0.42–0.70
	Intermediate	0.00–0.52	4.46– 2.20	3.80–17.40	29.69–583.88	0.04–0.82
	Suppressed	0.00–0.16	0.03–0.92	1.70–14.90	6.28–202.80	0.78–0.98

Abbreviations: BA, basal area; MHT, merchantable height; DBH, diameter at breast height; SC, slenderness coefficient; CR, crown ratio; SD, standard deviation.

Mean comparison of the growth variables for the canopy layers

The mean slenderness coefficient decreases with decreasing crown ratio at different stages of the canopy layer (Tab. 7). Dominant class had the highest value and suppressed individual class recorded the lowest value. This indicates that trees that are tall and slender had lower crown ratio values. However, the average diameter at breast height followed the same trend as the dominant class recorded the highest value (27.19 m) and the suppressed class had the lowest (10.48 m).

Relationship between residual and estimated Crown ratio

The evaluation of the residual plots (Figs. 3-5) and its error analysis revealed that error variance is constant across the predicted crown ratio. Logistics, Chapman-Richard and Exponential functions were observed to have constant error variances.

In the graphical relationship between the residuals and estimated crown ratio obtained with the three functions, all the residual values are in the positive and negative region which implied that crown ratio values were consistently predicted. Exponential and Chapman-Richard functions judging from their error analyses appeared well in the three functions owing to the fact that constant error variances distributed well both in the positive and negative region of the x-axis (i.e. the estimated crown ratio values). This is desirable for a good model. This trend was similar to the findings of Soares and Tome (2001) and Adesoye and Oluwadare (2008). Therefore, based on the evaluation of the error analyses, Chapman-Richard and Exponential functions are recommended for predicting crown ratio in the stand because they were more precise in their predictive abilities.

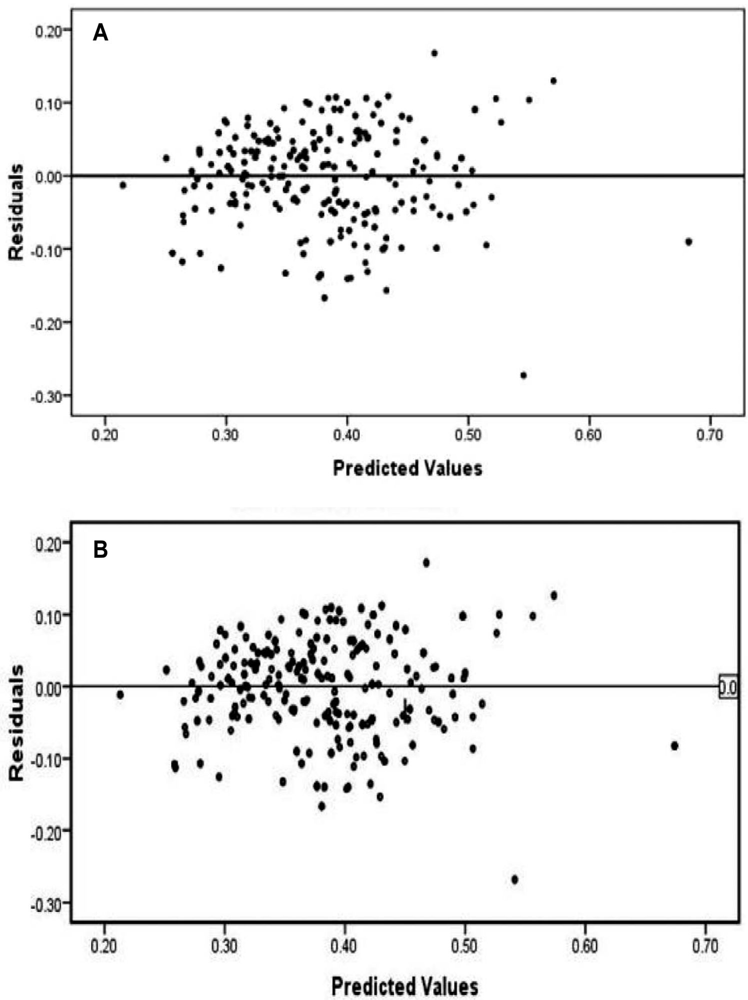

Figure 3: Relationship between residual and predicted values using **A** - Logistic and **B** - Exponential models in dominant layer.

Slika 3: Razmerje med rezidualno in predvideno vrednostjo pri **A** - logističnem in **B** - eksponentnem modelu za dominantni sloj.

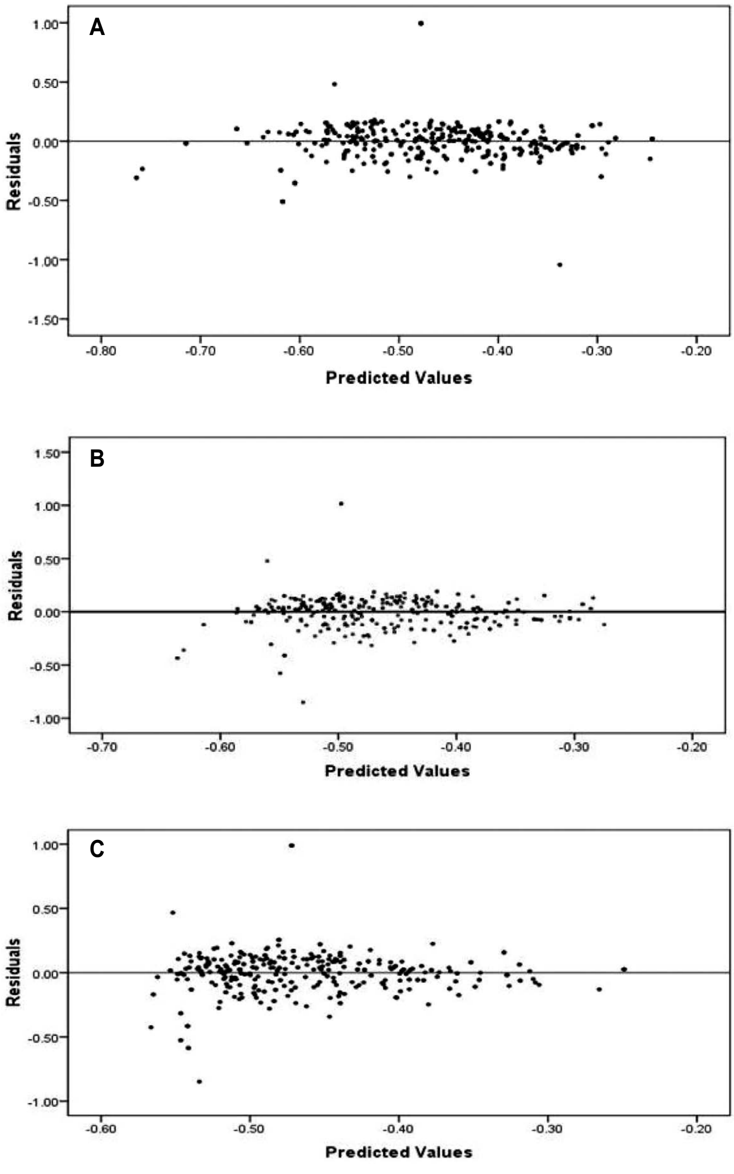

Figure 4: Relationship between residual and predicted values using **A** - Logistic, **B** - Exponential and **C** - Chapman-Richard model in intermediate layer.

Slika 4: Razmerje med rezidualno in predvideno vrednostjo pri **A** - logističnem, **B** - eksponentnem in **C** - Chapman-Richardovem modelu za srednji sloj.

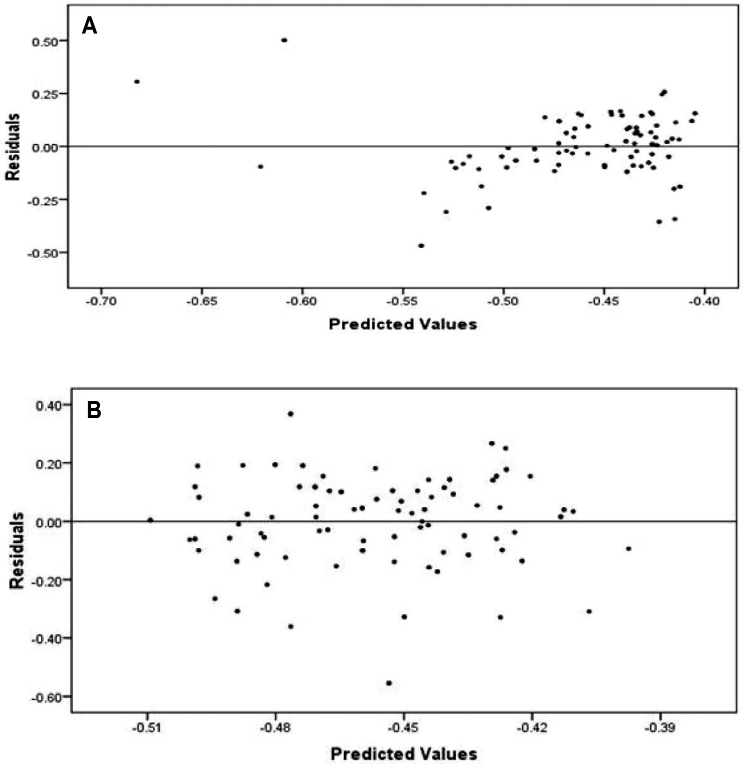

Figure 5: Relationship between residual and predicted values using **A** - Logistic and **B** - Chapman-Richard model in suppressed layer.

Slika 5: Razmerje med rezidualno in predvideno vrednostjo pri **A** - logističnem in **A** - Chapman-Richardovem modelu za zavrti sloj.

Model fitting and evaluation

Model fitting and evaluation are important parts of model building. Fitting of crown ratio models was based on the total data set. A number of different models were examined for predicting crown ratio using Logistic, Chapman-Richard and Exponential functions and the selected versions of the models are presented in Supplement 1.

One unique independent variable that features in all the models was tree slenderness coefficient. This proves that tree slenderness coefficient is one of the factors contributing to the size of tree crown ratio. Similar pattern was observed in the studies conducted by Hanus et al. (2000), Hasenauer and Monserud (1996) and Hann (1997). Merchantable height was another important variable used in modeling. Adesoye and Oluwadare (2008) and Marshall et al. (2003) also found merchantable height as an important variable for modeling crown.

The other variables failed to adequately explain crown ratio variation and were therefore not included in the models. The SEE differences were small since crown ratio is constrained to the interval of 0 and 1. This was also noticed in the work carried out by Temesgen et al. (2005) and Adesoye and Oluwadare (2008). Generally, the models consistently gave good fit to the *Tectona grandis* plantation data in Oluwa Forest reserve.

The evaluation of the residual plots (Figs. 3-5) revealed that error variance was constant across the predicted crown ratio. In the graphical relationship between the residuals and estimated crown ratio obtained with the logistic function, all the residual values were in both negative and positive regions but were farther from one another which implied that crown ratio values were slightly consistent. Exponential and Chapman-Richard functions judging from their error analysis appeared 'constant' error variance distributed both in the positive and negative regions of the x-axis (i.e. the estimated crown ratio values), which is highly desirable. Similar trend was observed by Soares and Tome (2001) and Adesoye and Oluwadare (2008). Based on the evaluation of the error analysis, Chapman-Richard and exponential functions are highly recommended for predicting crown ratio in the stand. Although the R^2 values for suppressed canopy layer were lower compared to other layers. They were more precise in their predictive abilities.

Conclusions

Based on the examined result in the study, the basal area and tree stem volume per hectare in forest plantation were higher than the values suggested for a well-stocked tropical rainforest in Nigeria. There were fewer tree species in the class with higher diameter and height than in the classes with smaller diameter and lower height. These trees of higher diameter classes formed the dominant layer which could be the result of their early and fast growth as there were more trees in the co-dominant and intermediate layer than in the dominant and suppressed layer. Meanwhile, the lack of emergence reflected the past disturbance of the ecosystem. The higher mean basal area, stem volume, and tree height per hectare recorded in this study compared to other tropical rainforest in other part of Nigeria are also very significant. Most of the tree growth variables were significantly different in different canopy layers of the study area.

The forest canopy was very diverse and the tree growth variables related considerably well with each other. It is recommended on this note that strict measure should be put in place to prevent any illegal action that may disrupt the delicate equilibrium of the ecosystem. The mean basal area obtained was greater than $90m^2$ that is suggested for a well-stocked forest plantation and a robust tropical forest plantation in Nigeria. It is comparable with data obtained in other parts of West Africa in similar ecosystems. It is highly recommended that such structure should be sustained in the region and by extension, encouraged in other parts of the World. However, based on the evaluation models, the three functions investigated in this study for crown ratio modeling (Chapman-Richard, Logistic and Exponential functions) gave constant and accurate result for all the canopy layers. Chapman-Richard and Exponential functions are recommended as crown ratio models for *Tectona grandis* plantation in Oluwa Forest reserve.

Povzetek

Raziskave drevesnih krošenj prispevajo k poznavanju več ključnih atributov gozdnih ekosistemov: biotske raznovrstnosti, produktivnosti, gospodarjenja z gozdovi, gozdnega okolja in prisotnosti prostoživečih živali. Spremenljivka razmerje krošenj se uporablja za oceno rasti in umrljivosti posameznih dreves in za oceno primernosti habitatov. Velikost drevesne krošnje je v povezavi z rastjo drevesa in njegovih različnih delov. Raziskava preizkuša tri različne nelinearne modele (logistični, Chapman-Richardov in eksponentni) za nasad vrste *Tectona grandis* v gozdnem rezervatu Oluwa (država Ondo, Nigerija). Modeli so pokazali, da so parametri bazalna površina, prostornina debel in povprečna višina drevesa na hektar zelo pomembni. Ker noben model ni uporaben za vse namene, je pri testiranju pomembno upoštevati različne plasti krošenj. Namen te študije je bil z uporabo empiričnih podatkov izboljšati razumevanje strukture krošenj v različnih plasteh gozdnih sestojev različne starosti ter določili indekse gostote sestojev.

Rezultati so pokazali, da je bila bazalna površina in obseg drevesnega debla na hektar v gozdnih nasadih višja od vrednosti, izmerjenih v visoko produktivnem tropskem deževnem gozdu v Nigeriji. V razredu z višjim premerom in višino je bilo manj drevesnih vrst kot v razredih z manjšim premerom in manjšo višino. Drevesa višjih razredov premera so tvorila prevladujočo plast, kar bi lahko bil rezultat njihove zgodnje hitre rasti, saj je bilo v kodominantni in srednji plasti plasti več dreves kot v dominantni in zavrti plasti. Omejeno pojavljanje mladih rastlin odraža pretekle motnje ekosistema. V tej študiji smo zabeležili tudi višjo povprečno osnovno površino, prostornino stebla in višino dreves na hektar v primerjavi z drugimi tropskimi deževnimi gozdovi v Nigeriji. Večina spremenljivk rasti dreves se je v različnih krošnjah na območju proučevanja bistveno razlikovala.

Plast krošenj je bila zelo raznolika in spremenljivke rasti dreves so se med seboj precej dobro povezovale. Zato priporočamo sprejetje strogih ukrepov za preprečevanje kakršnihkoli nezakonitih ukrepov, ki bi lahko porušili občutljivo ravnovesje ekosistema. Povprečna bazalna površina je bila večja od 90 m^2, kar je značilno za produktiven gozdni nasad oziroma za vitalne tropske gozdove v Nigeriji. Ta podatek je primerljiv tudi s podatki, pridobljenimi v drugih delih Zahodne Afrike v podobnih ekosistemih. Zelo priporočljivo je, da se takšna struktura ohranja v regiji in spodbuja tudi v drugih delih sveta. Vse tri funkcije, uporabljene v tej študiji za modeliranje razmerja krošenj (Chapman-Richardova, logistična in eksponentna), so dale zanesljive rezultate za vse plasti krošenj. Uporabo Chapman-Richardove in eksponentne funkcije pa priporočamo za modeliranje razmerja krošnje za nasad *Tectona grandis* v gozdnem rezervatu Oluwa.

Acknowledgements

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List of supplements

Supplement 1: Fitting of experimental data with selected tree crown ratio models for dominant, co-dominant, intermediate and suppressed layers.
Dodatek 1: Ujemanje podatkov z izbranimi modeli krošnje za dominantni, kodominantni, srednji in zavrti sloj.

Function	Dominant layer	Co-dominant layer	Intermediate layer	Suppressed layer
Logistic	0.66±0.20	0.64±0.20	0.65±0.16	0.41±0.15
Chapman-Richard	0.70±0.17	0.76±0.20	0.75±0.17	0.43±0.16
Exponential	0.71±0.17	0.74±0.20	0.72±0.16	0.43±0.16

Values are expressed as $R^2 \pm$ SEE

Supplement 2: Tree basal area and stem volume per plot in forest reserve.
Dodatek 2: Površina drevesne baze in prostornina debla na vzorčnem mestu v gozdnem rezervatu.

Plot	Basal area (m^2)	Stem volume (m^3)
1	43.29	3, 716.89
2	49.56	4, 204.16
3	53.02	4, 816.72
4	53.26	4, 941.89
5	38.77	3, 476.27
6	133.91	10, 259.63
7	160.17	10, 477.57
8	101.55	3, 064.33
9	106.03	7, 892.71
10	151.61	11, 081.72
11	132.94	9, 215.09
12	136.93	9, 368.11
13	78.87	5, 544.59
14	112.22	8, 478.86
15	147.01	9, 598.89
16	82.61	7, 182.57
17	103.26	8, 616.16
18	64.33	6, 460.89
19	76.53	7, 214.86
20	87.09	8, 077.26
Total	1, 912.96	143, 686.17
Mean	95.65	7, 184.31

Supplement 3: Tree canopy and percentage of individual layer.
Dodatek 3: Delež dreves po slojih drevesnih krošenj.

Canopy layer	Number of trees	Percentage (%)
Dominant	95	9.12
Co-dominant	267	25.62
Intermediate	403	38.68
Suppressed	277	26.58
Total	1042	100.00

INSTRUCTIONS FOR AUTHORS

1. Types of Articles

SCIENTIFIC ARTICLES are comprehensive descriptions of original research and include a theoretical survey of the topic, a detailed presentation of results with discussion and conclusion, and a bibliography according to the IMRAD outline (Introduction, Methods, Results, and Discussion). In this category ABS also publishes methodological articles, in so far as they present an original method, which was not previously published elsewhere, or they present a new and original usage of an established method. The originality is judged by the editorial board if necessary after a consultation with the referees. The recommended length of an article including tables, graphs, and illustrations is up to fifteen (15) pages; lines must be double-spaced. Scientific articles shall be subject to peer review by two experts in the field.

REVIEW ARTICLES will be published in the journal after consultation between the editorial board and the author. Review articles may be longer than fifteen (15) pages.

BRIEF NOTES are original articles from various biological fields (systematics, biochemistry, genetics, physiology, microbiology, ecology, etc.) that do not include a detailed theoretical discussion. Their aim is to acquaint readers with preliminary or partial results of research. They should not be longer than five (5) pages. Brief note articles shall be subject to peer review by one expert in the field.

CONGRESS NEWS acquaints readers with the content and conclusions of important congresses and seminars at home and abroad.

ASSOCIATION NEWS reports on the work of Slovene biology associations.

2. Originality of Articles

Manuscripts submitted for publication in *Acta Biologica Slovenica* should not contain previously published material and should not be under consideration for publication elsewhere.

3. Language

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.

4. Titles of Articles

Title must be short, informative, and understandable. It must be written in English and in Slovene language. The title should be followed by the name and full address of the authors (and if possible, fax number and/or e-mail address). The affiliation and address of each author should be clearly marked as well as who is the corresponding author.

5. Abstract

The abstract must give concise information about the objective, the methods used, the results obtained, and the conclusions. The suitable length for scientific articles is up to 250 words, and for brief note articles, 100 words. Article must have an abstract in both English and Slovene.

6. Keywords

There should be no more than ten (10) keywords; they must reflect the field of research covered in the article. Authors must add keywords in English to articles written in Slovene.

7. Running title

This is a shorter version of the title that should contain no more than 60 characters with spaces.

8. Introduction
The introduction must refer only to topics presented in the article or brief note.

9. Illustrations and Tables
Articles should not contain more than ten (10) illustrations (graphs, dendrograms, pictures, photos etc.) and tables, and their positions in the article should be clearly indicated. All illustrative material should be provided in electronic form. Tables should be submitted on separate pages (only horizontal lines should be used in tables). Titles of tables and illustrations and their legends should be in both Slovene and English. Tables and illustrations should be cited shortly in the text (Tab. 1 or Tabs. 1-2, Fig. 1 or Figs. 1-2; Tab. 1 and Sl. 1). A full name is used in the legend title (e.g. Figure 1, Table 2 etc.), written bold, followed by a short title of the figure or table, also in bold. Subpanels of a figure have to be unambiguously indicated with capital letters (A, B, …). Explanations associated with subpanels are given alphabetically, each starting with bold capital letter, a hyphen and followed by the text (A - text...).

10. The quality of graphic material
All the figures have to be submitted in the electronic form. The ABS publishes figures either in pure black and white or in halftones. Authors are kindly asked to prepare their figures in the correct form to avoid unnecessary delays in preparation for print, especially due to problems with insufficient contrast and resolution. Clarity and resolution of the information presented in graphical form is the responsibility of the author. Editors reserve the right to reject unclear and poorly readable pictures and graphical depictions. The resolution should be 300 d.p.i. minimum for halftones and 600 d.p.i. for pure black and white. The smallest numbers and lettering on the figure should not be smaller than 8 points (2 mm height). The thickness of lines should not be smaller than 0.5 points. The permitted font families are Times, Times New Roman, Helvetica and Arial, whereby all figures in the same article should have the same font type. The figures should be prepared in TIFF, EPS or PDF format, whereby TIFF (ending *.tif) is the preferred type. When saving figures in TIFF format we recommend the use of LZW or ZIP compression in order to reduce the file sizes. The photographs can be submitted in JPEG format (ending *.jpg) with low compression ratio. Editors reserve the right to reject the photos of poor quality. Before submitting a figure in EPS format make sure first, that all the characters are rendered correctly (e.g. by opening the file first in the programs Ghostview or GSview – depending on the operation system or in Adobe Photoshop). With PDF format make sure that lossless compression (LZW or ZIP) was used in the creation of the *.pdf file (JPEG, the default setting, is not suitable). Figures created in Microsoft Word, Excel, PowerPoint etc. will not be accepted without the conversion into one of the before mentioned formats. The same goes for graphics from other graphical programs (CorelDraw, Adobe Illustrator, etc.). The figures should be prepared in final size, published in the magazine. The dimensions are 12.5 cm maximum width and 19 cm maximum height (width and height of the text on a page).

11. Conclusions
Articles shall end with a summary of the main findings which may be written in point form.

12. Summary
Articles written in Slovene must contain a more extensive English summary. The reverse also applies.

13. Literature
References shall be cited in the text. If a reference work by one author is cited, we write Allan (1995) or (Allan 1995); if a work by two authors is cited, (Trinajstić and Franjić 1994); if a work by three or more authors is cited, (Pullin et al. 1995); and if the reference appears in several works, (Honsig-Erlenburg et al. 1992, Ward 1994a, Allan 1995, Pullin et al. 1995). If several works by the same author published in the same year are cited, the individual works are indicated with the added letters a, b, c,

etc.: (Ward 1994a,b). If direct quotations are used, the page numbers should be included: Toman (1992: 5) or (Toman 1992: 5–6).The bibliography shall be arranged in alphabetical order beginning with the surname of the first author, comma, the initials of the name(s) and continued in the same way with the rest of the authors, separated by commas. The names are followed by the year of publication, the title of the article, the full name of the journal (periodical), the volume, the number in parenthesis (optional), and the pages. Example:

Mielke, M.S., Almeida, A.A.F., Gomes, F.P., Aguilar, M.A.G., Mangabeira, P.A.O., 2003. Leaf gas exchange, chlorophyll fluorescence and growth responses of Genipa americana seedlings to soil flooding. Experimental Botany, 50(1), 221–231.

Books, chapters from books, reports, and congress anthologies use the following forms:

Allan, J.D., 1995. Stream Ecology. Structure and Function of Running Waters, 1st ed. Chapman & Hall, London, 388 pp.

Pullin, A.S., McLean, I.F.G., Webb, M.R., 1995. Ecology and Conservation of *Lycaena dispar*: British and European Perspectives. In: Pullin A. S. (ed.): Ecology and Conservation of Butterflies, 1st ed. Chapman & Hall, London, pp. 150-164.

Toman, M.J., 1992. Mikrobiološke značilnosti bioloških čistilnih naprav. Zbornik referatov s posvetovanja DZVS, Gozd Martuljek, pp. 1-7.

14. Format and Form of Articles

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.

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PREGLEDNA ČLANKA – REVIEW PAPERS:

KRATKA NOTICA – BRIEF NOTE:

ZNANSTVENI ČLANKI – SCIENTIFIC ARTICLES: