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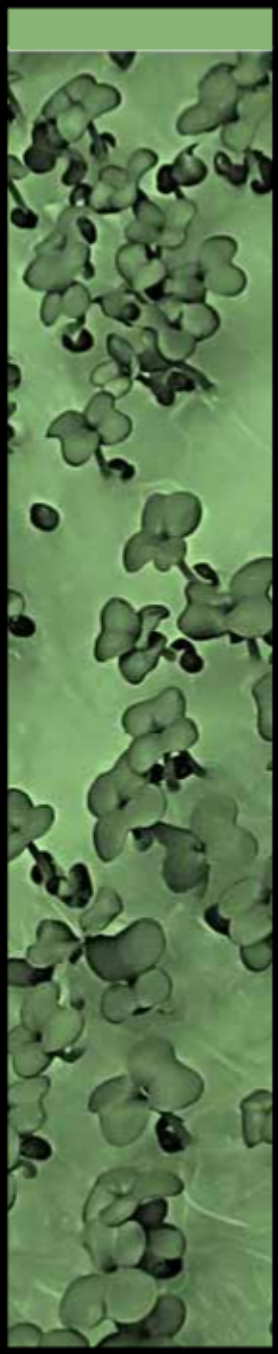

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Occurrence, toxins and possibilities of control of bloom-forming cyanobacteria of European freshwaters: a review

Pojavljanje, toksičnost in kontrola cvetenja cianobakterij v evropskih celinskih vodah: pregled

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Abstract: Blooming of cyanobacteria is a common problem of eutrophic water bodies in Europe and worldwide and can cause severe problems with toxicity, taste and odour of the water. Toxins produced by cyanobacteria (cyanotoxins) are structurally diverse and their effects range from liver damage, including liver cancer, to neurotoxicity and thus they may present a serious threat for drinking water safety. Cyanobacterial blooms present major challenges for the management of rivers, lakes and reservoirs and are predicted to cause even worse problems in the future due to the climate change associated with global warming, increased availability of light to phytoplankton and rising levels of atmospheric CO_2. This paper presents the literature review of occurrence, toxins (along with their effects on human health) and possibilities of control of bloom-forming cyanobacteria.

Keywords: algal blooms, cyanobacteria, cyanobacterial control, cyanotoxins, Europe, freshwaters

Izvleček: Cvetenje cianobakterij je pogost problem v evtrofnih vodnih telesih v Evropi in po svetu. Povzroča lahko resne težave zaradi toksičnosti, spremenjenega okusa in vonja vode. Toksini, ki jih izločajo cianobakterije (cianotoksini), so po zgradbi različni, njihovi učinki pa zajemajo vse od poškodb jeter, vključno z rakom na jetrih, do nevrotoksičnosti in lahko predstavljajo resno nevarnost pri zagotavljanju varne pitne vode. Cvetenje cianobakterij predstavlja velik izziv za upravljalce rek, jezer in zbiralnikov, predvideva pa se, da bo v prihodnosti ta problematika še naraščala zaradi klimatskih sprememb in z njimi povezanih učinkov globalnega segrevanja, povečane dostopnosti svetlobe za fitoplankton in naraščajočih koncentracij atmosferskega CO_2. Članek predstavlja pregled literature o pojavljanju, toksinih (vključno z njihovimi učinki na zdravje ljudi) in kontroli cianobakterijskih vrst, ki cvetijo v evropskih celinskih vodah.

Ključne besede: cvetenje alg, cianobakterije, kontrola cianobakterij, cianotoksini, Evropa, celinske vode

Introduction

When environmental conditions such as temperature, light and nutrient status are conducive, surface waters (both freshwater and marine) may host increased growth of algae and/or cyanobacteria. If and when such proliferation is dominated by a single (or a few) species, the phenomenon is referred to as an algal or cyanobacterial bloom (CB) (Chorus and Bartram 1999). CBs are a common problem of stagnant water bodies in Europe (Eiler and Bertilson 2004, Jacquet et al. 2005) and worldwide (Paerl and Huisman 2009, Kosten et al. 2012, Michalak et al. 2013). They present major challenges for the management of rivers, lakes and reservoirs (Carey et al. 2012) and are predicted to cause even worse problems in the future due to the climate change associated with global warming, increased availability of light to phytoplankton and rising levels of atmospheric CO_2 (Jöhnk et al. 2008, Kosten et al. 2012, O'Neil et al. 2012, Paerl and Huisman 2009, Paerl and Paul 2012, Zhang et al. 2012). Some lakes, rivers and estuaries have seasonal blooms that start in summer and last into autumn, some have persistent blooms that encompass all seasons, and some have blooms that occur as extreme peaks and crashes lasting just a few days or weeks (Havens 2008). In temperate regions, CBs generally occur during the late summer and early autumn and may last two to four months (Cook et al. 2004). This is also the time when demand for recreational water is the highest (Chorus et al. 2000). In regions with Mediterranean (mild, wet winter and warm, dry summer) or subtropical climates, the bloom season may start earlier and persist longer (Cook et al. 2004).

The CBs increase the turbidity of eutrophied lakes and in turn supress growth of aquatic macrophytes affecting invertebrates and fish species in addition to oxygen depletion and odour problems (Paerl and Huisman 2009). Lastly, some cyanobacterial species produce toxic peptides and alkaloids, which are a major threat to the use of freshwater ecosystems, and reservoirs for drinking water, irrigation, fishing and recreation (Carmichael 2001). If cyanobacteria are present or even dominant for most of the year, the problems associated with high cyanobacterial biomass and the potential health threats from their toxins increase. Proliferation of toxic cyanobacteria often causes a reduction in biodiversity, leading to disruption of the trophic chain and to ecosystem imbalance (Sedmak and Eleršek 2005). Potential toxic risks, to both animal and humans, may cause problems to local fisheries and to touristic and recreational activities (Chorus and Bartram 1999, Dokulil and Teubner 2000, Briand et al. 2003).

Environmental conditions promoting bloom-forming cyanobacterial growth

The mechanism of CB occurrences is very complex as they are not caused by a single environmental driver but rather by multiple factors occurring simultaneously (Dokulil and Teubner 2000, Heisler et al. 2008). Environmental conditions promoting growth of most common potentially toxic cyanobacteria in European stagnant waters are shown in Tab. 1. Onset of development and proliferation of CBs are closely associated with eutrophication and climatic conditions. Cyanobacteria can occupy almost all kinds of aquatic habitats as they are able to use different forms of carbon (C), nitrogen (N), phosphorus (P), and sulphur (S), they grow well in shade, are resistant to grazing and release allelochemicals to out-compete other organisms (Sharma et al. 2010). Cyanobacteria possess certain unique adaptations that make them a successful competitor. These include their ability to grow in warm waters, to utilize low total N (TN) to total P (TP) ratio, to access low dissolved CO_2 concentration (in form of bicarbonate), and their ability of N fixation (Sharma et al. 2010).

Table 1: Recorded occurrencies of common potentially toxic cyanobacteria in freshwaters in Europe and environmental conditions promoting their growth.
Tabela 1: Pojavljanje najpogostejših potencialno toksičnih cianobakterij v evropskih celinskih vodah in okoljski dejavniki, ki spodbujajo njihovo rast.

Taxa	Trophic state of water body	Optimal temperature	Optimal light intensity	Occurrence	Additional info	Country	Source
Aphanizomenon flos-aquae Ralfs ex Bornet and Flahault	mesotrophic stagnant waters, reservoirs	20 °C - 28 °C	100 - 110 mmol phot. $m^{-2}s^{-1}$	Fresh and salty waters, common in plankton, sometimes creates blooms	Coexists with *M. aeruginosa*	Britain Denmark France Germany Netherlands Poland Portugal Romania Slovenia Spain Sweden Turkey (Europe)	Skuja (1948), Alvarez-Cobelas and Gallardo (1988), Aboal (1996), Kosi (1999), Dokulil and Teubner (2000), Tsujimura et al. (2001), Whitton (2002), Karlsson-Elfgren and Brunberg (2004), Aboal and Puig (2005), Dean and Sigee (2006), Ersanli and Gönülol (2006), O'Brien et al. (2006), Willame et al. (2006), Carrasco et al. (2007), Leao et al. (2009), Pérez et al. (2009), Kokocinski et al. (2010), Täuscher (2011), Caraus (2012)
Aphanizomenon gracile Lemmermann	mesotrophic-eutrophic stagnant waters (ponds, reservoirs)	20 °C - 28 °C	100 - 110 mmol phot. $m^{-2}s^{-1}$	Freshwater, planktic, common in stagnant waters (ponds, reservoirs)		Belgium France Germany Luxembourg Poland Romania Spain	Caraus (2002), Whitton (2002), Willame et al. (2006), Carrasco et al. (2007), Kokocinski et al. (2010), Täuscher (2011), Caraus (2012), Mehnert et al. (2012)
Chrysosporum ovalisporum (Forti) E.Zapomelová, O.Skácelová, P.Pumann, R.Kopp and E.Janecek syn. *Aphanizomenon ovalisporum* Forti, *Anabaena ovalisporum* Forti	eutrophic and mesotrophic-eutrophic reservoirs	26 °C – 30 °C		Mostly in Mediterranean Europe, Middle East, North America and Australia		Greece Italy Poland Spain Turkey (Europe)	Alvarez-Cobelas and Gallardo (1988), Bazzichelli and Abdelahad (1994), Gkelis et al. (2005), Ersanli and Gönülol (2006), Carrasco et al. (2007), Kokocinski and Soininen (2012), Sukenik et al. (2013)
Cuspidothrix issatschenkoi (Usachev) Rajaniemi, Komárek, Willame, Hrouzek, Ka syn. *Aphanizomenon issatschenkoi* (Usacev) Proshkina-Lavrenko	mesotrophic-eutrophic stagnant waters (ponds, reservoirs)			Freshwater, planktic in lakes and ponds in Europe and Asia		Britain France Germany Hungary Poland Portugal Romania Spain	Caraus (2002), Whitton (2002), Willame et al. (2006), Carrasco et al. (2007), Leao et al. (2009), Kokocinski et al. (2010), Täuscher (2011), Caraus (2012), Horváth et al. (2013)

Taxa	Trophic state of water body	Optimal temperature	Optimal light intensity	Occurrence	Additional info	Country	Source
Cylindrospermopsis raciborskii (Woloszynska) Seenayya and Subba Raju	mesotrophic-eutrophic stagnant waters (ponds, reservoirs)	29 °C - 31 °C	80 - 120 mmol phot. $m^{-2}s^{-1}$	Tropical and subtropical, but appears to be invading temperate regions (as far north as Vienna)		Germany Poland Portugal	Dokulil and Teubner (2000), Saker et al. (2004), Stuken et al. (2006), Wiedner et al. (2007), Carneiro et al. (2009), Leao et al. (2009), Kokocinski et al. (2010), Täuscher (2011), Mehnert et al. (2012), Kokocinski and Soininen (2012)
Dolichospermum circinale (Rabenhorst ex Bornet and Flahault) P.Wacklin, L.Hoffmann and J.Komárek syn. *Anabaena circinalis* Rabenhorst ex Bornet and Flahault	hypertrophic fishponds, mesotrophic-eutrophic stagnant waters (ponds, reservoirs)	20 °C - 28 °C		Freshwater, planktic, often forming heavy water blooms; cosmopolitan distribution with exception of subpolar regions; massive populations known mainly from Central Europe, South America and Australia		Britain Czech Republic France Germany Romania Slovenia Spain Sweden Turkey (Europe)	Skuja (1948), Alvarez-Cobelas and Gallardo (1988), Caraus (2002), Whitton (2002), Karlsson-Elfgren and Brunberg (2004), Ersanli and Gönülol (2006), Täuscher (2011), Zapomelová et al. (2011), Caraus (2012), Database of Slovenian Environment Agency
Dolichospermum crassum (Lemmermann) P.Wacklin, L.Hoffmann and J.Komárek syn. *Anabaena crassa* (Lemmermann) Komark.-Legn. and Cronberg	hypertrophic fishponds, mesotrophic-eutrophic stagnant waters (ponds, reservoirs)	20 °C - 28 °C		Freshwater, planktic in ponds and reservoirs, in temperate zones of both hemispheres, up to subtropical regions		Czech Republic Germany Luxembourg Slovenia Spain	Willame et al. (2006), Carrasco et al. (2007), Täuscher (2011), Zapomelová et al. (2011), Sukenik et al. (2013), Database of Slovenian Environment Agency
Dolichospermum flos-aquae (Brébisson ex Bornet and Flahault) P.Wacklin, L.Hoffmann and J.Komárek syn. *Anabaena flos-aquae* Brébisson ex Bornet and Flauhault	hypertrophic fishponds, mesotrophic-eutrophic stagnant waters (ponds, reservoirs)	20 °C - 28 °C		Freshwater, common in plankton of stagnant waters, cosmopolitan species with exception of subpolar regions; tropical populations less frequent; often creates blooms		Britain Czech Republic Denmark Germany Romania Slovenia Spain Sweden	Skuja (1948), Álvarez-Cobelas (1982), Alvarez-Cobelas and Gallardo (1988), Kosi (1999), Caraus (2002), Whitton (2002), Dean and Sigee (2006), Sigee et al. (2007), Täuscher (2011), Zapomelová et al. (2011), Caraus (2012)

Taxa	Trophic state of water body	Optimal temperature	Optimal light intensity	Occurrence	Additional info	Country	Source
Dolichospermum lemmermannii (Ricter) P.Wacklin, L.Hoffmann and J.Komárek syn. *Anabaena lemmermannii* P. Richter	hypertrophic fishponds, mesotrophic-eutrophic stagnant waters (ponds, reservoirs)	20 °C - 28 °C		Freshwater, common in plankton of reservoirs in whole temperate zone (distinct water blooms); never found in tropical regions	One of the dominant species in cyanobacterial mass occurrences in boreal lakes, even in relatively oligotrophic lakes	Czech Republic Denmark Finland Germany Romania	Caraus (2002), Olli et al. (2005), Täuscher (2011), Zapomelová et al. (2011), Caraus (2012)
Dolichospermum planctonicum (Brunnth.) Wacklin, L.Hoffm. and Komárek syn. *Anabaena planctonica* Brunnthaler	hypertrophic fishponds, mesotrophic-eutrophic stagnant waters (ponds, reservoirs)	20 °C - 28 °C	prefers moderate light intensities	Freshwater, common in plankton of stagnant waters		Belgium Britain Czech Republic Germany Italy Luxembourg Romania Slovenia Spain	Bruno et al. (1994), Caraus (2002), Whitton (2002), Willame et al. (2006), Carrasco et al. (2007), Täuscher (2011), Zapomelová et al. (2011), Caraus (2012), Database of Slovenian Environment Agency
*Dolichospermum solitarium (*Klebahn) Wacklin, L.Hoffmann and Komárek syn. *Anabaena solitaria* Klebahn	oligotrophic stagnant waters (mountain lakes, quarries) and mesotrophic stagnant waters, reservoirs	20 °C - 28 °C		Freshwater, common in plankton of stagnant waters		Czech Republic Finland Germany Romania Slovenia	Willen and Mattsson (1997), Caraus (2002), Kastovsky et al. (2010), Täuscher (2011), Caraus (2012), Database of Slovenian Environment Agency
Dolichospermum spiroides (Kleb.) Wacklin, L.Hoffm. and Komárek syn. *Anabaena spiroides* Klebahn	hypertrophic fishponds, mesotrophic-eutrophic stagnant waters (ponds, reservoirs)	20 °C - 28 °C	prefers moderate light intensities	Freshwater, common in plankton of stagnant and slowly running waters, mainly from May to October		Belgium Britain Czech Republic France Germany Romania Slovenia Spain Sweden Turkey (Europe)	Skuja (1948), Alvarez-Cobelas and Gallardo (1988), Kosi (1999), Caraus (2002), Whitton (2002), Ersanli and Gönülol (2006), Willame et al. (2006), Täuscher (2011), Zapomelová et al. (2011), Caraus (2012)
Gloeotrichia echinulata P.Richter	mesotrophic stagnant waters, reservoirs			Freshwater, common in plankton of stagnant and slowly running waters, sometimes creates blooms		Britain Germany Romania	Whitton (2002), Täuscher (2011), Caraus (2012)

Taxa	Trophic state of water body	Optimal temperature	Optimal light intensity	Occurrence	Additional info	Country	Source
Limnothrix redekei (van Goor) M.-E. Meffert	mesotrophic-eutrophic and mesotrophic stagnant waters, reservoirs, also wetlands, pools, furrows, usually with water plants			Freshwater, planktic, widely distributed in temperate zone throughout the whole year (distinct populations occur in winter season); common in Northern and Central Europe	often together with *Planktothrix agardhii*	Czech Republic Germany Poland Romania Slovenia	Chorus and Bartram (1999), Kastovsky et al. (2010), Kokocinski et al. (2010), Täuscher (2011), Caraus (2012), Database of Slovenian Environment Agency
Microcystis aeruginosa (Kützing) Kützing	eutrophic water bodies (lakes, fishponds, reservoirs)	28 °C - 32 °C		Fresh and brackish waters, planktic, sometimes forming heavy water blooms, common; cosmopolitan with exception of polar and subpolar regions		Britain Germany Portugal Romania Slovenia Spain Sweden Turkey (Europe)	Skuja (1948), Alvarez-Cobelas and Gallardo (1988), Kosi (1999), Nalewajko and Murphy (2001), Caraus (2002), Carrillo et al. (2003), Whitton (2002), Martín et al. (2004), Bárbara et al. (2005), Ersanli and Gönülol (2006), Jöhnk et al. (2008), Paerl and Huisman (2008), Young et al. (2008), Leao et al. (2009), Metcalf et al. (2009), Pérez et al. (2009), Pérez et al. (2010), Täuscher (2011), Caraus (2012)
Microcystis ichtyoblabe (Kunze) Kützing	mesotrophic or slightly eutrophic, but not polluted lakes	28 °C - 32 °C		Freshwater, planktic, sometimes forming water blooms; more in northern regions of the temperate zone, probably not occurring in tropical countries		Belgium Czech Republic Germany Slovenia	Nalewajko and Murphy (2001), Willame et al. (2006), Jöhnk et al. (2008), Paerl and Huisman (2008), Kastovsky et al. (2010), Täuscher (2011), Database of Slovenian Environment Agency

Taxa	Trophic state of water body	Optimal temperature	Optimal light intensity	Occurrence	Additional info	Country	Source
Microcystis flos-aquae (Wittrock) Kirchner	mesotrophic and slightly eutrophic water bodies	28 °C - 32 °C		Freshwater, planktic, usually together with other planktic algae and cyanoprokaryotes, sometimes part of water blooms, cosmopolitan in the whole temperate zone, particularly in northern regions		Britain Germany Romania Turkey (Europe) Slovenia	Nalewajko and Murphy (2001), Caraus (2002), Whitton (2002), Ersanli and Gönülol (2006), Sigee et al. (2007), Jöhnk et al. (2008), Paerl and Huisman (2008), Täuscher (2011), Caraus (2012), Database of Slovenian Environment Agency
Microcystis viridis (A.Braun) Lemmermann	slightly eutrophic lakes and ponds	28 °C - 32 °C		Freshwater, planktic, sporadical, sometimes forming water blooms; cosmopolitan		Germany Romania Slovenia Spain Sweden	Skuja (1948), Alvarez-Cobelas and Gallardo (1988), Nalewajko and Murphy (2001), Caraus (2002), Jöhnk et al. (2008), Paerl and Huisman (2008), Eleršek (2009), Täuscher (2011), Caraus (2012), Scholz and Liebezeit (2012)
Nodularia spumigena Mertens ex Bornet and Flahault	eutrophic ponds, lakes and reservoirs	20 °C - 30 °C	high tolerance of ultraviolet radiation	Mostly in salty/brackish waters, also in fresh waters, planktonic, common, often forms blooms in lagoons and estuaries		Britain Ireland Poland Romania Spain Turkey	Guiry (1978), Alvarez-Cobelas and Gallardo (1988), Calvo and Bárbara (2002), Caraus (2002), Moisander et al. (2002), Whitton (2002), Bárbara et al. (2005), Akcaalan et al. (2009), Jodlowska and Latala (2010), Caraus (2012)
Planktothrix agardhii (Gomont) Anagnostidis and Komárek	mesotrophic-eutrophic stagnant waters (ponds, reservoirs), hypertrophic fishponds	10 °C - 25 °C	prefers low light intensities inhibited above 180 $\mu E\ m^{-2}s^{-1}$	Freshwater, planktic in lakes and ponds, often forming water blooms, widely distributed in temperate zones; less in tropical regions	Never forms scums	Belgium Germany Luxembourg Poland Romania Slovenia Spain	Chorus and Bartram (1999, Kosi (1999), Dokulil and Teubner (2000), Willame et al. (2006), Quesada et al. (2006), Willame et al. (2006), Carrasco et al. (2007), Oberhouse et al. (2007), López Rodríguez et al. (2009), Kokocinski et al. (2010), Täuscher (2011), Caraus (2012), Kokocinski and Soininen (2012)

Taxa	Trophic state of water body	Optimal temperature	Optimal light intensity	Occurrence	Additional info	Country	Source
Planktothrix rubescens (De Candolle ex Gomont) Anagnostidis and Komárek	mesotrophic and eutrophic large lakes and stagnant waters	cold water form (10 °C - 14 °C)	prefers low light intensities	Freshwater, planktic, in large lakes and stagnant waters, forming red water blooms; in several regions in northern temperate zone with obligatory distribution, outside of these areas occasionally over the whole temperate zone	Usually does not form scums during the bathing season	France Germany Italy Romania Slovenia Spain Switzerland	Kosi (1999), Dokulil and Teubner (2000), Almodóvar et al. (2004), Grach-Progrebinsky et al. (2004), Viaggiu et al. (2004), Willame et al. (2006), Carrasco et al. (2007), Holland and Walsby (2008), Täuscher (2011), Caraus (2012)
Woronichinia naegeliana (Unger) Elenkin syn. *Coelosphaerium naegelianum* Unger, *Gomphosphaeria naegeliana* (Unger) Lemmermann	eutrophic lakes and ponds			Freshwater, common in plankton of lakes and ponds, sometimes forming water blooms, in temperate zones, in Europe and North America up to northern regions		Britain Czech Republic Germany Luxembourg Romania Slovenia Spain Sweden	Skuja (1948), Alvarez-Cobelas and Gallardo (1988), Cronberg et al. (1999), Whitton (2002), Rajaniemi-Wacklin et al. (2006), Willame et al. (2006), Täuscher (2011), Caraus (2012), Database of Slovenian Environment Agency

Nitrogen and phosphorous

Because CBs often develop in eutrophic lakes, it was originally assumed that they require high P and N concentrations. However, in late summer, when CBs mostly occur, concentrations of dissolved phosphate tend to be the lowest. Experimental data showed that the affinity of many cyanobacteria for N or P is higher compared to other photosynthetic organisms meaning that they can out-compete other phytoplankton organisms under conditions of P or N limitation (Chorus and Bartram 1999). In most freshwater systems P is considered to be prime limiting nutrient (Xu et al. 2010) and small changes in P levels may influence the growth and toxin production of cyanobacteria (Sivonen 1990, Chorus and Bartram 1999). Cyanobacteria usually uptake P in orthophosphate form (PO_4^{3-}), however they are also able to uptake other phosphate forms like polyphosphates (Mukherjee et al. 2015). According to Downing et al. (2001) high concentration (30-100 µg/L) of TP promotes CB formation. Because of their high affinity for P, cyanobacteria can store substantial amount of P during P-sufficient conditions. Excess P-loading (luxury consumption) may facilitate growth of other phytoplankton groups leading to increased turbidity, which additionally favours cyanobacterial growth (Chorus and Bartram 1999). Besides P alone, P in combination with other nutrients may regulate cyanobacterial dominance in bloom environment. High TN:TP ratio is indicative for P limitation and vice-versa (Pinckney et al. 2001). However, according to Downing et al. (2001), TP is better predictor of cyanobacterial dominance than TN:TP ratios.

Light intensity

Turbid, low irradiance conditions promote growth of non-heterocystous cyanobacteria

(e.g. *Oscillatoria, Lyngbia, Planktothrix rubescens*) causing their domination in the phytoplankton community, which is attributed mainly to their ability to maintain net growth at low underwater irradiance (Havens et al. 2003). However, cyanobacteria which form surface blooms (e.g. *Cylindrospermopsis raciborskii*) have a higher tolerance for high light intensities most probably due to an increase in carotenoid production, which protects the cells from photoinhibition (Paerl et al. 1983, Wiedner et al. 2007, Carneiro et al. 2009). In moderately deep, stratified eutrophic lakes typically N_2-fixing cyanobacteria such as *Anabaena* and *Aphanizomenon* (Paerl et al. 2001) are present.

Temperature

CBs in stagnant waters are correlated, to a considerable degree, with weather conditions and consequently with climate conditions in a given area (Zhang et al. 2012). Cyanobacteria usually dominate phytoplankton assemblages in temperate freshwater environments during the warmest period of the year, particularly in eutrophic systems (Paerl 2008, Paerl and Huisman 2008). Optimum temperatures for cyanobacteria are in general higher than for green algae and diatoms (e.g. 25 °C or higher for species from genera *Microcystis*), which may explain why, in addition to the lower nutrient levels in epilimnion, in temperate and boreal water bodies most CBs occur during summer (Chorus and Bartram 1999, Dokulil and Teubner 2000, Paerl and Huisman 2008, Jöhnk et al. 2008, Mehnert el at. 2010). However, some species such as *Planktothrix rubescens* and *Aphanizomenon flos-aquae* have low temperature preference or tolerance and thus bloom during late autumn and winter (Tsujimura et al. 2001). According to Lürling et al. (2013) intensification of CBs in warmer climate is not attributed to their higher growth rates compared to other phytoplankton species, but rather to their ability to migrate vertically and prevent sedimentation in warmer and more strongly stratified waters and to their resistance to grazing.

Water column stability

CBs are promoted by calm, vertically stratified conditions with adequate nutrient supplies and weak wind mixing (Paerl and Millie 1996, Kanoshina et al. 2003, Huisman et al. 2004, Sharma et al. 2010). In the case of wind- or flow induced destratification cyanobacteria may lose their competitive advantage, which together with cell and filaments damages due to increased turbulence (Moisander et al. 2002) can cause, if such conditions persist, rapid degradation of CBs. However, when intermittent weak stratification occurs during favourable growth periods (summer), blooms can quickly re-emerge. Non-disruptive, low-level turbulence can promote localized nutrient cycling, alleviate certain forms of nutrient limitation (e.g. PO_4^3, trace metals), and enhance cyanobacterial growth.

pH

Alkaline conditions favour CB formation (Havens 2008). Cyanobacteria capacity for photosynthesis in environments with low CO_2 concentrations (by using bicarbonate (HCO^{3-}) as their carbon source (Kaplan et al. 1991)) and high pH is an important characteristic giving cyanobacteria advantage over other phytoplankton organisms in water environments with high pH values, a general characteristic of eutrophic lakes (Dokulil and Teubner 2000, Kardinal and Visser 2005).

Salinity

Increased salination (e.g. summer droughts, rising sea levels, increased use of freshwater for agricultural irrigation) has major impacts on freshwater plankton communities with repercussions for water quality and use (Paerl and Huisman 2009). One such impact is increased vertical density stratification, which benefit buoyant cyanobacteria (Walsby et al. 1997, Huisman et al. 2004). In addition, some species of the common cyanobacterial genera *Anabaena, Anabaenopsis, Microcystis* and *Nodularia* are sometimes more salt tolerant than eukaryotic freshwater phytoplankton species (Moisander et al. 2002, Tonk et al. 2007). Thus, increased salination of freshwater and brackish waters can favour cyanobacteria over other freshwater phytoplankton species exposing other aquatic organisms and human users of these waters to elevated concentrations of cyanobacterial toxins (Paerl and Huisman 2009). The high salt

tolerance of freshwater cyanobacteria is reflected by increasing numbers of CBs in brackish waters, for example, in the Baltic Sea in Scandinavia (Kanoshina et al. 2003, Suikkanen et al. 2007) and in the Küçükçekmece Lagoon in Turkey (Albay et al. 2005).

Bioactive substances

Cyanotoxins

Healthy CBs produce little extracellular toxin, while cell-bound concentrations are several orders of magnitude higher (Li et al. 2009). Very often, different strains of the same cyanobacteria species with similar growth rate produce different amounts of the same types of toxins (Watanabe and Oishi 1985, Sivonen 1990). External factors, including chemical conditions, modify not only cyanobacteria growth and toxin production but also affect cell longevity and the leakage of toxins to the environment which, in natural conditions, occurs mainly as the result of cell damage, death, lysis and decomposition of the aging cells. Thus, concentration of dissolved toxins may be much higher in ageing or declining CBs compared to healthy young CBs. However, toxin excretion from the cells can be promoted also by high temperature, high salination, high light intensities, low concentrations of P and chemical treatment for the eradication of cyanobacteria (especially use of algicides) (Sivonen 1990, van Apeldoorn et al. 2007, Rapala et al. 1997). Not all toxigenic species or toxic CBs will be toxic at all times (Carmichael 2001). Factors influencing formation and toxicity of toxic CBs include i) genetics as there are distinct toxin and non-toxin producing strains, and ii) good growth conditions together with optimum conditions for toxin production. Toxicity of CBs depends also on the ratio of toxin to non-toxin producers and the factors that lead to surface scums production (Carmichael 2001).

The freshwater cyanotoxins fall into three broad groups of chemicals: i) cyclic peptides (hepatotoxic microcystins and nodularins); ii) alkaloids (neurotoxic anatoxin-a, anatoxin-a(S), saxitoxins and hepatotoxic cylindrospermopsins); and iii) lipopolysaccharides (potentially irritant) (van Apeldoorn et al.2007). General features of the cyanotoxins occurring in freshwaters in Europe and their effects on human health are shown in Tab. 2.

Hepatotoxic cyclic peptides are the most frequently found cyanobacterial toxins in CBs from fresh and brackish waters and pose a major challenge for the production of safe drinking water from surface waters containing cyanobacteria with these toxins. In mouse bioassays, which traditionally have been used to screen toxicity of field and laboratory samples, cyanobacterial hepatotoxins (liver toxins) cause death by liver haemorrhage within a few hours of the acute doses (Chorus and Bartram 1999). The cyclic peptide microcystins and nodularins are specific liver poisons in mammals. Following acute exposure to high doses, they cause death from liver haemorrhage or from liver failure, and they may promote the growth of liver and other tumours following chronic exposures to low doses (Chorus and Bartram 1999).

Microcystins

Microcystins (MC) are the most frequently reported cyanobacterial toxins. 248 MC analogues have been reported to date (Spoof and Catherine, 2017). The amount of MC production by a cyanobacterial population in culture is directly proportional to its growth rate, no matter what environmental factor is limiting the growth (van Apeldoorn et al. 2007). While variants of MC produced by a particular strain are rather constant, the ratio of individual MC may change with time, temperature and light intensity. According to van Apeldoorn et al. (2007) at high P levels hepatotoxic cyanobacterial strains produced more toxins. Non-nitrogen fixing species such as *Microcystis* and *Oscillatoria* produce more toxins under N rich conditions (van Apeldoorn et al. 2007). MC are intracellular toxins, and whilst contained only in living cells, they are degraded slowly. MC are only released into the water by senescence or cell death or through water treatment processes such as pre-chlorination or algicide application. The study of Zastepa et al. (2014) demonstrated that MCs can persist well beyond the disappearance of the bloom. Dissolved MC-LA declined more slowly and persisted longer than particulate (cell-bound) MC-LA with *in situ* half-lives (total 1.5–8.5 days) shorter than *in vitro* (total 6.8–60.0 days).

Table 2: General features of the cyanotoxins occurring in freshwaters in Europe and their effects on human health. Adapted from Chorus and Bartram (1999), Chorus et al. (2000) and van Apeldoorn et al. (2007).
Tabela 2: Splošne značilnosti cianotoksinov, ki se pojavljajo v evropskih celinskih vodah, in njihovi učinki na zdravje ljudi. Povzeto po Chorus in Bartram (1999), Chorus in sod. (2000) in van Appeldoorn in sod. (2007).

Toxin group	**Primary target organ in mammals**	**Reported effects on human health**	**Taxa**	**LD_{50} of pure toxin (mouse bioassay)**
CYCLIC PEPTIDES				
Microcystins (MC)	Liver	Short term: gastroenteritis, liver damage, acute liver failure, birth defect, Haff disease, blistering of lips, allergic reactions (contact dermatitis, asthma, hay fever, conjunctivitis), vomiting, diarrhoea, abdominal pain, sore throat, pneumonia. Long term: hepatocellular carcinoma.	*Microcystis, Anabaena, Planktothrix (Oscillatoria), Nostoc, Hapalosiphon, Anabaenopsis Woronichinia Limnothrix Gloeotrichia Aphanizomenon*	MC in general: 45-1000 µg/kg MC-LR: 60 (25-125 µg/kg) MC-YR: 70 µg/kg MC-RR: 300-600 µg/kg
Nodularins	Liver	no human poisonings recorded, only reports of skin rashes	*Nodularia*	30-50 µg/kg
ALKALOIDS				
Anatoxin-a	Nerve synapse	no data till date	*Anabaena, Planktothrix (Oscillatoria), Aphaniziomenon Dolichospermum Microcystis aeruginosa*	250 µg/kg
Anatoxin-a(S)	Nerve synapse	no data till date	*Anabaena Dolichospermum Raphidiopsis mediterranea*	40 µg/kg
Cylindrospermopsins	Liver	hepatoenteritis, acute tender liver enlargement, constipation, vomiting and headache, diarrhoea, dehydration	*Cylindrospermopsis, Aphanizomenon, Umezakia Dolichospermum Cuspidothrix Chrysosporum*	2100 µg/kg/d 200 µg/kg/5-6 d
Saxitoxins	Nerve axons	no data till date of human poisonings	*Anabaena, Aphanizomenon, Dolichospermum Lyngbya, Cylindrospermopsis*	10-30 µg/kg
LIPOPOLYSACCHARIDES	Potentially irritant; affects any exposed tissue	can cause skin irritation	All	

Decline of MC was accelerated by higher temperature and irradiance, both of which are considered the most important environmental factors in MC degradation. MC can accumulate in aquatic organisms, such as zooplankton, phytoplankton, gastropods, mussels, clams and fish, and thus enter the food chain and pose possible threat to human health. The oral intoxication route is the most important as it involves not only the drinking of water containing cyanobacterial toxins but also the consumption of toxin-containing animal or plant tissues (Spoof 2005, van Apeldoorn et al. 2007).

Many reported worldwide cases demonstrate that MC cause both acute and chronic effects on humans (Ueno et al. 1996, WHO 1998, Zhou et al. 2002). Acute intoxication by MC coincides frequently with the lysis of the bloom-forming cells (by natural senescence or water treatment processes) and liberation of toxins to the water. The inhalation of dry cyanobacteria cells or contaminated water is more dangerous than oral ingestion of contaminated water indicating the hazardous potential of practising aquatic sports in recreational waters that suffer a microcystin producing bloom (WHO 2003). Chronic exposure to low concentrations of microcystins in drinking water can be a serious problem to public health, contributing to promotion of cancer in humans. Epidemiological studies have already related the presence of MC in drinking water to an increase in the incidence of colorectal cancer (Zhou et al. 2002) and primary liver cancer (Ueno et al. 1996). Recent studies show that toxic responses of MC may also be seen in kidney, heart, reproductive system, brain and lungs (Milutinovič et al. 2006, Wang et al. 2008, Chen et al. 2016, McLellan and Manderville 2017).

Nodularins

Nodularins are cyclic pentapeptides found in *Nodularia spumigena* (Chorus and Bartram 1999, Spoof 2005). To date, approximately 10 variants have been discovered, among which nodularin-R is the most abundant (Chen et al. 2013). The occurrence of *N. spumigena* blooms is determined by water temperature, light intensity, and nutrients concentration, among which levels of N and P are critical (Mazur et al. 2003). Nodularins tend to accumulate in mussels, clams and fish (van Apeldoorn et al. 2007) and have been implicated in the deaths of wild and domestic animals (Chen et al. 2013). No guidelines have been set for nodularins by the World Health Organization (WHO), and their toxicity can currently only be estimated from MC, which have been reported to have similar toxicity to nodularins (Paerson et al. 2010). Since nodularins generally occurs in brackish waters, accidental swallowing of water during recreational activities and seafood consumption could be the major routes with regard to human exposure.

Alkaloids

Anatoxins

Anatoxins are a group of neurotoxic alkaloids which includes anatoxin-a, homoanatoxin-a and anatoxin-a(S). Anatoxins exposure and effects on humans or aquatic biota have not been fully determined yet also no clear evidence of human poisoning from anatoxins exists (Osswald et al. 2007, van Apeldoorn et al. 2007, EPA 2015). Anatoxin-a (ANTX-a) is produced by certain species of *Anabaena* (*A. planctonica*, *A. flos-aquae*, *A. spiroides* and *A. circinalis*), *Planktothrix* (*Oscillatoria*), *Cylindrospermum, Aphanizomenon,* and in minor amounts *M. aeruginosa* (e.g. Agnihotri, 2014). ANTX-a is a potent postsynaptic depolarizing neuromuscular blocking agent and causes death in laboratory animals within minutes to a few hours (Stevens and Krieger 1991, Fitzgeorge et al. 1994, van Apeldoorn et al. 2007). According to Chorus and Bartram (1999) P levels have no effects on ANTX-a production. ANTX-a differs from other cyanotoxins (like microcystins) in that it undergoes rapid photochemical degradation in sunlight even in the absence of cell pigments. Stevens and Krieger (1991) found that the degradation of ANTX-a is dependent on the light intensity and/or pH, with higher pH favouring degradation reactions. ANTX-a has been widely identified in surface waters in North America and Europe used for recreation, and hence a risk exists for ANTX-a poisoning of recreational water users (Chorus et al. 2000). Homoanatoxin-a was reported to be produced by *Planktothrix formosa,* by Norvegian strain of *O. formosum* (*Phormidium formosum*), some unidentified *Anabaena* species from Ireland and *Raphidiopsis mediterranea* (Chorus and Bartram 1999, Furey et al. 2003, Watanabe et al.

2003). ANTX-a(S), which chemical structure is un-related to ANTX-a, is produced by *Anabaena flos-aquae, A. lemmermannii*, *A. spiroides* and *A. crassa* (Chorus and Bartram 1999, Becker et al. 2010, de Abreu et al. 2013).

Saxitoxins

Saxitoxins (STX) are a group of carbamate alkaloid neurotoxins which are either non-sulphated (saxitoxins - STX), singly sulphated (gonyautoxins - GTX) or doubly sulphated (C-toxins). In addition, decarbamoyl derivatives (dc) and several new toxins (Lyngbya-wollei toxins, LWTXs) have been identified in some cyanobacterial species (van Apeldoorn et al. 2007). STX and its analogues are produced by *Anabaena circinalis* (Chorus and Bartram 1999); very low concentrations were detected also in two other *Anabaena* species: *A pertubata* and *A. spiroides* (Velzeboer et al. 2000). In a few Danish lakes containing STX, *A. lemmermannii* was the dominant cyanobacterium (Kass and Hendriksen 2000). Also *A. flos-aquae* from Portugal and *Planktothrix* sp. FP1 from Italy were reported to produce STX (Molica et al. 2002). All saxitoxins act in the same way: they block nervous transmission causing muscle paralysis (Briand et al. 2003). Till date no reports of human poisonings due to STX presence in freshwater environments are known (Chorus and Bartram 1999).

Cylindrospermopsin

Cylindrospermopsin (CYN) is a tricyclic alkaloid, possessing a tricyclic guanidine moiety combined with hydroxymethyluracil produced by *Cylindrospermopsis raciborskii*, *Umezakia natans* and *Aphanizomenon ovalisporum* (van Apeldoorn et al. 2007). CYN like microcystins, primarily affects the liver, although causes considerable damage also to other major organs e.g. kidneys, spleen, thymus and heart. CBs which caused both liver and kidney damage due to the CYN (and possibly related cyanotoxins) have been reported in Australia, Japan, Israel and Hungary (Chorus and Bartram 1999). Chorus and Bartram (1999) and Falconer (2001) reported health problems associated with presence of CYN in drinking water supplies in Australia. Patients suffered from an unusual hepatoenteritis, acute tender liver enlargement, constipation, vomiting and headache, followed by diarrhoea and dehydration. Chonudomkul et al. (2004) pointed out that *C. raciborskii* is not only an ongoing invasive species but also a species with different physiological strains or ecotypes and temperature tolerance.

Volatile organic compounds and other bioactive substances

Cyanobacteria can produce various compounds causing off-flavour, also known as volatile organic compounds (VOC). 2-Methylisoborneol (2-MIB) and geosmin are among the most important odorous compounds in cyanobacteria and are often cited as sources of unpleasant earth-like and musty odour, especially in various aquatic environments (Fujise et. al. 2010). VOC are primarily produced by different prokaryotic and eukaryotic benthic and pelagic aquatic microorganisms (e.g. Streptomyces, fungi). Many of the known cyanobacterial producers of VOC are nonplanktic (approx. 30%), while the remainder are benthic or epiphytic. According to Jüttner and Watson (2007) geosmin and 2-MIB production is limited to filamentous cyanobacteria and is unknown among chorococcalean taxa. According to Milovanović et al. (2015) growing conditions have significant impact on production of VOC in cyanobacteria, and altering these conditions may be useful in obtaining cyanobacterial biomass with favourable sensory properties for potential use in formulation of food and feed products.

Beside toxins and VOC, cyanobacteria produce also other very heterogeneous biologically active substances, such as peptides, retinoids, alkaloids, lactones, phospholipids (Sychrova et al. 2012, Wu et al. 2012a). Some of these metabolites are also potentially toxic to mammals, as they can cause inhibition of enzymes in key metabolic pathways, skin irritation, signalling and hormonal disruption, cytotoxicity, reproductive disorders, and neurological damage, or act as a tumour promotors. Also, they can influence CB physiology and their blooming capacity (Sukenik et al. 2002, Schatz et al. 2007). Bioactive substances produced by cyanobacteria can be divided in following groups a) aeruginosins and spumigins (Ersmark et al. 2008, Fewer et al. 2009); b) anabaenopeptins (Harada et al. 1993, Bubik et al. 2008); c) biogenic amines (MLA 2001, EFSA 2011); d) depsipeptides (Blom et al. 2006, Bubik et al. 2008); e) endocrine

disruptors and novel tumour promoters (Bláha et al. 2010, Nováková et al. 2013); f) microginins (Neumann et al. 1997); and g) microviridins (Murakami et al. 1995).

Most common bloom-forming cyanobacterial taxa of European freshwaters

Occurrence and reported observations of the most common potentially toxic bloom-forming cyanobacteria in European freshwaters are shown in Tab. 1. Ecology of the most common cyanobacterial taxa occurring in European freshwaters is shown in Tab. 3.

Control of cyanobacterial blooms

Several approaches are available to control CBs in water bodies such as minimizing nutrient load, using chemical, biological, and/or physical treatment. Nutrient removal can have positive long-term effects leading to the reduction in the trophic state of the water body and thus to the reduction of CBs, but is almost impossible for most areas across the world due to economical limitations (Jančula and Maršálek 2011).

CBs can be efficiently reduced by addition of chemicals to water such as copper-based algaecides, herbicides, photosensitizers, and chemical flocculants (e.g. Surosz and Palinska, 2004; Jančula and Maršálek 2011). However, chemical treatment has several disadvantages: (1) toxicity against non-target organisms; (2) generation of secondary pollutants; (3) introduction of heavy metals to the water and their accumulation in the environment (Jančula et al. 2014). Chemical treatment of CBs especially using potassium permanganate or chlorine may indirectly effect other organisms due to the sudden release of cyanotoxins from cyanobacteria cells as a consequence of cell lysis (Mahvi and Dehgani 2005). Dissolved cyanotoxins can enter water supplies and pose potential risk for human health (van Apeldoorn 2007, Rajasekhar et al. 2012). In such cases, additional treatment of water by activated carbon, powerful oxidants such as ozone and/or intense ultraviolet light are needed to inactivate or degrade dissolved toxins (Chorus and Bartram 1999; Jančula and Maršálek 2011). Copper-based algaecides and chemical flocculants are commonly used to control CBs, but may be harmful to aquatic life by generating secondary pollutants (Mahvi and Dehgani 2005, McNeary and Erickson 2013, Jančula et al. 2014) and large amount of algae sludge (Xu et al. 2006). More sustainable treatment method is using clay minerals as flocculation agents, where dense clay particles attach to the cyanobacteria cells and promote conglomeration and sinking of the cells, despite their buoyancy (McNeary and Erickson 2013). Also, low concentrations of hydrogen peroxide (HP) have shown promising potential to act as specific cyanocide for *Planktothrix agardhii*, *Anabaena*, *Aphanizomenon* and *Microcystis*, both in the laboratory and in whole-lake treatments. HP acts very fast and there are no lasting chemical traces of the added HP (sustainability), nor toxic substances including released cyanotoxins or particulate organic matter from dead cyanobacteria retained in the water body (Matthijs et al. 2016).

Biological removal of CBs such as natural grazing by phytoplanktivorous fish (Jančula et al. 2008) or biomanipulation by introduction of new cyanobacteria eating species to the water body (Lacerot et al. 2013) is gaining importance due to its environmental friendliness compared to chemical treatment. Biomanipulation is faster than natural establishment of cyanobacteria eating communities and can selectively affect only target organisms (Guo et al. 2015).

Hydrodynamic and acoustic cavitation are the main physical methods for CBs control. Although the effects of acoustic cavitation on CBs removal have been studied more extensively compared with hydrodynamic cavitation (Dular et al. 2016), both techniques are still in the research phase. According to Xu et al. (2006) hydrodynamic cavitation is causing the collapse of gas vesicles and the destruction of thylakoid together with the changes in the structures of phycocyanins and chlorophyll *a* in *M. aeruginosa* cells, eventually resulting in the death of the cells. Wu et al. (2012b) studied combined effects of hydrodynamic cavitation and ozone treatment on growth of *M. aeruginosa* assuming that mechanical forces affect the cyanobacteria by damaging the cell wall and make them more sensitive to ozone treatment. 99% reduction of cyanobacteria was achieved

Table 3: Ecology of the most common cyanobacterial taxa occurring in European freshwaters. Chl *a* = chlorophyll *a*, N = nitrogen, P = phosphorus, PAR = photosynthetically active radiation, CB = cyanobacteria.
Tabela 3: Ekologija najpogostejših cianobakterijskih taksonov, ki se pojavljajo v evropskih celinskih vodah. Chl *a* = klorofil *a*, N = dušik, P = fosfor, PAR = fotosintetsko aktivno sevanje, CB = cianobakterije.

Species	Blooms typically found in	Advantages	Importance	Sources
***Microcystis* spp.**	• Warm, turbid, slow-moving waters, high in nutrients • Waters deeper than 3 m, but also in shallower lakes (temperate regions) • Bodies with chl *a* concentrations of 20-50 µg/L and Secchi transparency of 1-2 m • Spring and summer	• Less sensitive to high light intensities (capable of buoyancy regulation)	• *M. aeruginosa* one of the most damaging species. Prevalence in bodies with varying nutrient loading. High toxicity to aquatic and terrestrial organisms. • Rapid reproduction triggered the most by P runoff. • High nutrient levels favour the growth of toxic over nontoxic strains.	Prasath et al. 2014, Chorus and Bartram 1999, Lehman et al. 2005, Vezie et al. 2002
Planktothrix agardhii	• Turbulent, low radiance waters • First few meters of the water column in shallow waters • Greatly dependent on high-frequency phosphate availability • Summer (temperate regions)	• Ability to absorb sufficient energy from the entire PAR spectrum • Resistance to photoinhibition • Tolerant to continuous mixing of water column • High P storage capacity • Buoyancy regulation • Tolerant to shade and temperature variation	• One of the most common toxic bloom-forming species.	Budzyńska et al. 2009, Scheffer et al. 1997, Oberhaus et al. 2007, Dokulil and Teubner 2000, Padisak and Reynolds 1998, Bonilla et al. 2012, Catherine et al. 2008, Crossetti and Bicudo 2008, Kokocinski et al. 2010, Aubriot et al. 2011
Aphanizomenon flos-aquae	• Higher latitudes (less frequent at lower latitudes) • Grows independently of dissolved N resources and also under P limitation • Late autumn and winter	• Low temperature preference • Ability of autonomous fixation of atmospheric N • Able to grow in unfavourable conditions (forms akinetes) • Substantial storage capacity for P	• May appear as plankton in eutrophic waters where other CB are almost undetectable. • Its dynamic affected by co-occurring CB like *Microcystis* spp.	Tsujimura et al. 2001, Yamamoto 2009, Preussel et al. 2006, Takano and Hino 2000
***Anabaena* spp.**	• Lake environment	• N fixing abilities • Toxin production	• Widely diversified group with around 80 morphospecies. • Dominant long term populations • *A. flos-aquae* usually appears during summer (max N starvation and PAR inputs).	Zapomelova et al. 2010, Dean et al. 2008, Agnihotri 2014, Paerl 1979

compared to less than 15% removal of cyanobacteria by hydrodynamic cavitation and 35% by ozone treatment alone. According to Jančula et al. (2014) and Dular et al. (2016) hydrodynamic cavitation is more effective on removal of buoyant cyanobacteria by disintergrating their gas vesicles than other planktonic algae without gas vesicles (e.g. green microalgae), which indicates good potential of hydrodynamic cavitation for selective cyanobacterial removal from water bodies.

Acoustic cavitation has similar effects on cyanobacteria as hydrodynamic cavitation (Jančula et al. 2014, Li et al. 2014), namely reducing the growth rate of cyanobacteria by collapsing the gas vesicles, inhibiting cell division, and/or inflicting immediate damage on photosynthetic activities (Nakano et al. 2001, Ahn et al. 2003, Mahvi and Dehgani 2005, Zhang et al. 2006a). Acoustic cavitation is known to cause cell lysis and thus releasing the intracellular materials in water column (Zhang et al. 2006b, Rajasekhar et al. 2012). On the other side it is also effective in degrading the cyanotoxins (Song et al. 2005). Acoustic cavitation has potential to reduce algal capacity to float and thus reducing their concentration near the surface of water bodies, which is consequently inhibiting their growth and survival (Mahvi and Dehgani 2005). Effects of acoustic cavitation on cyanobacteria removal depends on frequency, intensity and time of sonication (Rajasekhar et al. 2012). Beside acoustic cavitation, a low intensity ultrasound without cavitation can be used for CBs control; in fact several such technologies are already available on the market. Low intensity ultrasound is appropriate solution for aquaculture systems, natural ponds or drinking water reservoirs, since it is not damaging the cyanobacteria cells and the toxins are not released from the cells (Krivograd Klemenčič and Griessler Bulc 2010). Furthermore, it is affecting cyanobacteria selectively by collapsing gas vacuoles causing cyanobacteria cells to sink at the bottom of the water body, where cells in deep water bodies die due to lack of light necessary for photosynthesis (Krivograd Klemenčič and Griessler Bulc 2010). The disadvantage of low intensity ultrasound technology is relatively long contact time with cyanobacteria to affect their buoyancy (Williams 2014).

In 2014 the European Commission funded the research project (7FP Dronic, http://dronicproject.com) developing the monitoring and ultrasonic treatment robotic system that can localize and treat hotpots of CBs in large water bodies. Because of the direct and localized treatment, the Dronic system is environmentally friendly, with a minimal impact on the ecology of the water body. The Dronic system is equipped with ultrasound acoustic system that uses two different types of ultrasound. The first type precipitates the cyanobacteria by directly affecting their buoyancy, while the second type is neutralizing the cyanotoxins by cavitation. The Dronic system is still in the research phase. However, if it proves successful it will be the first system that can autonomously locate and localized treat CBs only at the part of the water body, which is experiencing CB.

Povzetek

Ob ugodni temperaturi, svetlobnih pogojih in zadostni količini hranil v površinskih vodah lahko pride do cvetenja cianobakterij, ki je pogost problem evtrofnih vodnih teles v Evropi in po svetu. Pričakovano je, da se bo s klimatskimi spremembami problem še poglobil, kar prestavlja nove izzive za upravljalce voda.

Cianobakterije v splošnem cvetijo pozno poleti in zgodaj jeseni, ko so rekreacijske aktivnosti na vodnih telesih v porastu. Cvetenje cianobakterij poveča motnost vode, zavira rast makrofitov in vpliva na nevretenčarje in ribe v vodnem okolju. Poleg tega nekatere cianobakterijske vrste proizvajajo toksine, škodljive ljudem in živalim. Cvetenje toksičnih vrst cianobakterij pogosto povzroči zmanjšanje biodiverzitete, porušenje trofičnih verig in ravnotežja v ekosistemih.

Mehanizem pojavljanja cianobakterijskega cvetenja je kompleksen, ker do tega pride ob hkratnem delovanju več dejavnikov. Posamezne cianobakterijske vrste posedujejo vrsto prilagoditev na različne okoljske dejavnike, kar jim omogoča prednost pred tekmeci in naselitev večine vodnih habitatov. Med najpogostejšimi cianobakterijskimi vrstami, ki se pojavljajo v evropskih celinskih vodah, so *Microcystis* spp., *Planktothrix agardhii*, *Aphanizomenon flos-aquae* in *Anabaena* spp.

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Invertebrates as a study model of anaerobic infections

Nevretenčarski modeli za proučevanje anaerobnih infekcij

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Abstract: Experiments with invertebrates have recently gained increased attention as a practicable substitute to traditional mammalian models in the study of host-bacterial interactions. Using an invertebrate study model has a number of advantages over traditional mammalian model including simple growth condition, short life-time, can be easily maintained, infected without anesthesia and with a smaller extent of ethical limitations. From a microbiological viewpoint, importance of anaerobic bacteria as agents for various diseases remains an interesting field for research. The study of the interaction between invertebrate model host and anaerobic bacteria therefore provides insights into the mechanisms underlying pathogen virulence and host immunity and complements or even compensates the use of mammalian model in assay for infectious disease. This review offers to consider about the appropriate invertebrate model select for the study of particular aspects of anaerobic bacterial pathogenesis.

Keywords: invertebrate model, anaerobic bacteria, virulence factors, disease

Izvleček: Poskusi na nevretenčarjih so lahko odlični nadomestni model za proučevanje interakcij med gostiteljem in bakterijo. Uporaba nevretenčarjev ima številne prednosti pred uporabo sesalskih živalskih modelov za raziskovalne poskuse. Pogoji za življenje so enostavni, življenjska doba je kratka, vzdrževanje je enostavno, izvajanje poskusov ne vključuje anestezije, kot tudi uporaba nevretenčarjev za raziskovalne namene je manj etično sporna. Anaerobne bakterije in bolezni, ki jih povzročajo, so vedno zanimivo področje raziskovanja. Novi pristopi pri proučevanju možnih negativnih učinkov anaerobnih bakterij in virulenčnih dejavnikov bi lahko postali tudi nevretenčarski modeli. Prispevek opisuje uporabnost nevretenčarskih modelov, že opisane povezave med patogenimi učinki virulenčnih dejavnikov anaerobnih bakterij pri nevretenčarskih modelih in o novih pristopih izbire nevretenčarjev kot model za proučevanje patogeneze.

Ključne besede: nevretenčarski modeli, anaerobne bakterije, virulenčni dejavniki, bolezen

Introduction

The molecular basis of the pathogenicity of infectious agents, and of the corresponding mechanisms of host defence can be studied using model systems (Couillault and Ewbank 2002). There is a continuing need for the development of a simple animal model for the study of host pathogen interactions (Finlay 1999).

A number of different invertebrate host model systems have been described in the past few years that allow multidisciplinary studies of host–bacterial interactions from the perspectives of both the pathogen and the host. Consequently, many researchers have turned to invertebrates as effortless, practicable, simple, and inexpensive hosts to model a variety of human infectious diseases. It is important to select the model host that is best suited for testing aspecific hypothesis (Mylonakis et al. 2007) including ethical, procedural and financial characteristics.

A number of different model systems, including amoeba, nematodes, crustaceans and insects, have been introduced, and it was observed that different bacteria responded in different ways to presumptive alternate hosts, and specific model systems might be more or less advisable for a defined pathogen (Ott et al. 2012). Aerobic and obligate anaerobic bacteria are successfully isolated but to isolate anaerobic bacteria from invertebrate models is often impracticable (Bergan 1984), even though, anaerobic bacteria in invertebrates cannot be excluded. The subsequent isolation of strictly anaerobic bacteria resulting in anaerobic microniches within an oxic environment. This finding of anaerobic microniches presents microhabitat of various microbes, despite the fact of its obviously inappropriate environment (König 2006).

This review presents five model hosts, the amoeba *Acanthamoeba polyphaga*, the nematode *Caenorhabditis elegans* (*C. elegans*), the fruit fly *Drosophila melanogaster* (*D. melanogaster*), the greater wax moth *Galleria mellonella* (*G. mellonella*) and the isopod *Porcellio scaber* (*P. scaber*). Predominant empirical advantages of each model are well developed genetic, biochemical and biological functions, the precision evaluation of an assay, conserved innate immune response, handling experiments at 37°C and ease of inoculation of an explicit amount of pathogen (Borner 2016).

Anaerobic bacteria are significant clinical pathogens

Anaerobes and their pathogenicity factors can affect common hosts and hosts with compromised resistance of harmed tissue. Their complex metabolism, the capability to produce pathogenicity components like extracellular toxins, superoxide dismutase, catalase, the abscess inducing capsular polysaccharide, proteases, lipases, heparinase, nucleases hyaluronidase, haemolysin, lipolysin, and neuroaaminidase, enzymes inactivating antibiotics, and resistance against phagocytosis, are responsible for local and systemic expansion of the endogenous bacterial infection during antimicrobial therapy (Bergan 1984, Brook 2011, Dorer and Isberg 2006). Avoidance and early healing treatment of circumstances that can lead to anaerobic infection can reduce their amount.

Anaerobic bacteria are found on the skin, on mucosal surfaces, in the mouth, pharynx and intestinal tract or genital tract as a part of the normal microbiota. Additionally, anaerobes can be isolated in all types of anaerobic infection including respiratory infection, subcutaneous and soft-tissue infections, endogenous infections in the central nervous system, oral cavity, head and neck, chest, abdomen, pelvis, skin, and soft tissue (Brook 2016). Infections results when anaerobes and other bacteria of the normal flora weaken and deceive immune system to avoid detection (Borner 2016), or permeate integumentary barriers. The infections are often polymicrobial, with other anaerobes, facultative anaerobes, and aerobes (Brook 2016). Several important diseases, botulism, tetanus, gas gangrene, food poisoning, and pseudomembranous colitis are caused by anaerobic *Clostridium* species from the environment or from normal flora (Brook 2016, Brooks et al. 2010). Their individual pathogenicity factors serum-independent chemotactic factors that attract polymorphonuclear cells, superoxide dismutase, catalase, capsular structures, proteases, lipases, heparinase, and nucleases, exotoxins of histotoxic clostridia and the ability of different anaerobes to produce enzymes inactivating antibiotics has become well established. Pathogenicity factors like hyaluronidase, haemolysin, lipolysin, and neuraaminidase have been isolated (Brooks et al. 2010). Inspite of all that, the importance of

other virulence factors that may contribute to the pathogenicity of the anaerobic bacteria remains unclear (Brooks et al. 2010, Harding et al. 2013).

Important anaerobes that may cause human infection and/or are isolated in polymicrobial anaerobic infections are: i. Gram-negative bacilli *Bacteroides* spp., *Prevotella* spp., *Porphyromonas* spp., *Fusobacterium* spp., *Bilophila* spp. and *Sutterella* spp., ii. Gram-negative cocci mainly *Veillonella* spp. (Brook, 2011), iii. Gram-positive cocci *Peptostreptococcus* spp., *Anaerococcus* spp., *Finegoldia* spp., *Parvimonas spp.*, and *Peptoniphilus* spp. (Murphy and Frick, 2013), iv. Gram-positive spore forming *Clostridium* spp., and no spore-forming bacilli *Actinomyces* spp., *Propionbacterium* spp., *Eubacterium* spp., *Lactobacillus* spp., *Bifidobacterium* spp., *Eggerthella* spp., *Arachnia* spp. (Brook 2011). The isolation and identification of anaerobic bacteria associated with specific infection are important as well as characterization of their virulence factors (Brook 2016, Murphy and Frick 2013).

Invertebrate as a model host for studying virulence of anaerobic bacteria

Invertebrate models have gained increased attention as a viable alternative to traditional mammalian models of infection (Mowlds et al. 2008, Renwick et al. 2006) and are increasingly being used to study a number of important human pathogens. Using of invertebrate models have number of advantages over traditional mammmalian models, as invertebrates are not subject to the ethical limitations of mammalian models (Harding et al. 2013). No invertebrate model hosts reproduces all aspects of mammalian infection and any particular invertebrate is likely to have specific advantages. The selection of a model system for studying virulence of anaerobic bacteria is largely dependent on the specific pathogen virulence related factors, the specific host innate immune responses of interest, and the scientific question asked. If the goal is to study innate immune responses, the choice most likely will require the selection of a multicellular model genetic organism such as *D. melanogaster* or *C. elegans*. If the goal is to study phagocytosis and/or the outcome of ingestion, the choices include unicellular organisms such as amoebae and slime mold or invertebrates such as insects with phagocytic cells (Mylonakis et al. 2007). If the goal is to study gut microbe homeostasis and gut infection by the human pathogen anaerobic bacteria, a model with increasing evidence for a reciprocal relationship between beneficial and pathogenic bacteria in the gut and the intestinal immune system with suitable environment for developing of resident and anaerobic microbiota must be found and practiced (Glavis-Bloom et al. 2012).

Manipulating with alimentary, physiological and behavioral characteristics of different invertebrate models might play an important role with an optimal adaptation of anaerobic bacteria to invertebrate‘s environment through a completely different mechanism of interactions ranging from pathogenesis to obligate mutualism.

Acanthamoeba polyphaga as a study model for anaerobic bacteria

Protozoa are frequently used in laboratories as experimental organisms for studies of cell locomotion (*Amoeba proteus*), nonmuscle contractile systems (*Acanthamoeba*), and the effects of removing and transplanting nuclei (Brusca and Brusca 2004).

Amoebae species are well established model systems for a number of pathogenic bacteria. Amoebae have been used as model organism to study the pathogenicity of bacterial strain, such as *Pseudomonas aeruginosa* (Pukatzki et al. 2002), as a biological tool for isolation of several amoeba-resisting intracellular microorganisms (Adekambi et al. 2004, Greub et al., 2004, La Scola et al. 2004) but it has not been an appropriate study model for anaerobic bacteria yet. *Clostridium frigidicarnis* was demonstrated to be lytic for amoebae (Pagnier et al. 2008).

Caenorhabditis elegans as a study model for anaerobic bacteria

The soil-living small size nematode *C. elegans* with rapid life cycle and transparent body, fully sequenced genome, and physiological and anatomical simplicity is a model host with excellent potential for studying cell biology and pathogenicity

(Brusca and Brusca 2004, Glavis-Bloom et al. 2012). Both, aerobic and anaerobic metabolic pathways are found and worm is able to switch from one pathway to the other according to environmental oxygen concentrations. Facultative anaerobiosis is evidently meaningful in parasitic nematodes and those that live in additional anoxic environments (Brusca and Brusca 2004).

Bacteria that infect *C. elegans* are both Gram-negative and Gram-positive bacteria. *Salmonella typhimurium*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, *Burkholderia cepacia*, *Burkholderia cenocepacia*, *Yersinia pestis*, *Yersinia pseudotuberculosis* are Gram-negative bacteria that infect *C. elegans* and *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Microbacterium nematophilium*, *Enterococcus faecalis*, *Bacillus thuringiensis* are Gram-positive bacteria (Borner 2016).

Practical advantages, the particular bioinformatics approach and biological processes have increased the use of *C. elegans* in toxicological studies (Boyd et al. 2012). *C. elegans* is killed by many pathogens and many virulence factors produced by *pathogens* that contribute to the pathogenicity in humans have been shown to be important for disease in *C. elegans*, including persistent infection of the intestine, colonisation with biofilm formation on the worm cuticle, and killing by botulinum toxin, hydrogen cyanide or hydrogen peroxide (Kaletta and Hengartner 2006).

The genetically tractable nematod *C. elegans* has been extensively used to study bacterial virulence and offers many advantages (Mahajan-Miklos et al. 1999) as a convenient host for studies of pathogen infections (Balla and Troemel 2013). This small hermaphroditic animal has been the object of intense study for more than 20 years (Brillard 2001). From a microbiological standpoint, *C. elegans* is an attractive model for a broad range of host processes from the molecular level to the whole organism level. Genetic tractability and convenience, small size and simplicity, ease of culture, transparency and short lifespan all contribute to making *C. elegans* a useful laboratory organism for conducting large-scale studies of host microbe interactions (Clark 2012). A large number of human diseases have been investigated using *C. elegans* (Kaletta and Hengartner 2006). Several Gram-negative human pathogens have been shown to kill *C. elegans* when presented to the nematodes as a source of food (Aballay et al., 2000; Darby et al. 1999, Mahajan-Miklos et al. 1999, Tan et al. 1999). Gram-positive human pathogens also kill *C. elegans.* Literature is demonstrating that a range of aerobic and opportunistic bacterial pathogen is involved in virulence in *C. elegans* as well an anaerobically grown *Enterococcus faucium*. *E. faucium* kills the nematode via the production of hydrogen peroxide, which also poses an oxidative stress to nematodes (Bolm et al. 2004, Borner 2016, Jansen et al. 2002, Moy et al. 2004). Notable research was made in 2014, where worms treated with botulinum toxin A of *Clostridium botulinum* showed slight paralysis and the toxin treatment resulted in the increase of yolk protein concentration in embryos (Kim et al. 2014). *C. elegans* has become an assisting model to probe vital biological and physiological processes and molecular mechanisms involved in many human disesaes. It has served as a model for Parkinson‘s, Alzheimer‘s and Huntington‘s disease, diabetes, cancer, immune disorders, and the development and testing of therapeutics agents (Bier and McGinnis 2008, Wilson-Sanders 2011).

C. elegans may subsequently be the model of anaerobic microbial processes and toxicity.

Introducing bacteria to worms is quite simple, but laboratory standing conditions differ with the conditions in its natural soil habitat. The nematode absences a variety of mammalian anatomical structures and the reproductive fitness of *C. elegans* strongly depends on the effects of air composition, habitat structure, and bacterial food availability. Owing to these not all diseases and immune responses can be testified (Glavis-Bloom et al. 2012) or have to be carefully interpreted by concern to their natural consequences (Freyth et al. 2010).

Galleria mellonella as a study model for anaerobic bacteria

Unique advantages of *G. mellonella* as a model host for studying pathogen virulence mechanisms and the efficiency of potential antimicrobial compounds are its ability to survive at 37°C when studying pathogenic temperature-sensitive virulence and production of microbial toxins. *G. mellonella* can be stored at room temperature,

likewise is straightforwardly and practically obtained in sizes large enough to be inoculated by pathogens (Glavis-Bloom et al. 2012). A reliable and inexpensive experimental infection model is convenient to differentiate between virulent and non-virulent isolates, for the identification of presumed virulence genes through comparative genomics studies and the identification of novel molecular targets for antimicrobial therapy and vaccine development. *G. mellonella* was recently established as a suitable host model to study the pathogenesis of bacterial and yeast species causing diseases in humans, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Cryptococcus neoformans*, and *Candida albicans* (Altincicek et al. 2012). Conversely, *G. mellonella* cannot replace well-established and more physiological *in vivo* experimental vertebrate models in the assessment of pathogenic mechanisms associated human diseases (Giannouli et al. 2014, Glavis-Bloom et al. 2012).

Drosophila melanogaster as a study model for anaerobic bacteria

The fruit fly *D. melanogaster* has many of the similar advantages as *C. elegans*. Small size, short generation time, a fully sequenced genome, and pre-existing libraries of genetic mutants, genes and pathways similar to those found in mammals completed *D. melanogaster* as an excellent model host (Frenzel et al. 2015). In particular, *D. melanogaster* is useful model for the study of mammalian intestinal bacterial infections (O'Callaghan and Vergunst 2010) and their effects on undifferentiated and matured enteric epithelial cells in the initial stages of intestinal cancer, for the study of intestinal infection with *Pseudomonas aeruginosa*, a human opportunistic bacterial pathogen (Glavis-Bloom et al. 2012) and as a model for the innate immune response to pathogens (Borner 2016) such as antimicrobial peptide production, phagocytosis and melanization reactions (Charroux and Royet 2012). Pathogens that infect *D. melanogaster* are *Salmonella* Typhimurium, *Pseudomonas aeruginosa*, *Mycobacterium marinum*, *Mycobacterium fortuitum*, *Staphylococcus aureus*, *Serratia marcescens*, and *Vibrio cholerae* (Borner 2016, O'Callaghan and Vergunst 2010).

The fruit fly *D. melanogaster* is an excellent model organism to research intestinal homeostasis, the gut microbiota, pathways that regulate intestinal stem cell signaling, innate immune reactions and disease. About 20 diverse species, including main dominant species of the genus *Acetobacter* and *Lactobacillus* and with few anaerobes were found in the intestine of *D. melanogaster*. No noteworthy anaerobic bacteria had been found in the *D. melanogaster* gastrointestinal tract (Charroux and Royet 2012) before the anaerobe *Clostridium perfringens* was isolated. The promoting effect on the growth and development of *D. melanogaster* were detected (Wei et al. 2016). It is a primary organism used in developing biology (Wilson--Sanders 2011) considering that *D. melanogaster* indirect-flight-muscle actin was ADP-ribosylated by *Clostridium botulinum* C2 toxin and *Clostridium perfringens* iota toxin (Just et al. 1993).

C. perfringens is the most frequently isolated histotoxic clostridia and produces several necrotizing extracellular toxins correlated with tissue necrosis, hemolytic anemia and renal failure and *C. botulinum* infections can result in intestinal toxemia, food poisoning and wound infections containing a highly potent neurotoxin (Brook 2016). Appropriate management of *D. melanogaster* for identifying and understanding anaerobes that are presented in human diseases could contribute to biological discoveries.

Drosophila served as a model for host-parasite relationships, is established model for the study of neoplastic diseases (Tipping and Perrimon 2014, Wang et al. 2014, Wilson-Sanders 2011), has been used to study cellular defenses against fungal pathogens (Arvanitis et al. 2013, Fuchs and Mylonakis 2006), it has been proven as excellent system for studying the normal function of human genes and pathways linked to neurodegenerative diseases (Allan et al. 2014, Liang et al. 2013, Mhatre et al. 2013). and is a simple model organism for studying diseases caused by viruses (Bier and Guichard 2012, Panayidou et al. 2014).

Porcellio scaber as a study model for anaerobic bacteria

The terrestrial crustacean, soil dwelling isopod *Porcellio scaber* is likely to be dependant on microorganisms associated with the gut (Kostanjšek

et al. 2004, Wang et al. 2004, Zimmer 2002). The digestive tract is complete with a well-developed, cuticle-lined, stomodeal foregut and proctodeal hindgut, connected by an entodermally derived midgut. A characteristic feature is a permeable peritrophic membrane to protect the delicate midgut epithelium from abrasion (Bier and McGinnis 2008).

Terrestrial isopods have an inherent and multiplicity gut microflora (Drobne 1995). Therefore, slight changes in the animal fitness might have efects on the microbial community in the intestinal tract (Guarner and Malagelada 2003; Loker et al. 2004). Due to their significant environmental aspect and their complete digestive tract a considerable amount of experimentation was focused on *P. scaber*. Anaerobic bacteria from *P. scaber* hindgut were identified. Further, obligate anaerobic bacteria of genus *Bacteroides* and *Enterococcus* species were isolated. Additionally, bacteria from the genus *Desulfotomaculum* were isolated from gut wall and cultivated under anaerobic conditions (Kostanjšek et al. 2004). Bacteria from the genus *Desulfotomaculum* were isolated from gut wall and cultivated under anaerobic conditions (Kostanjšek et al. 2004). Nothing can be concluded about the changed structure or function of the entire gut bacterial community. Gut microflora toxicity studies are a promising way to get applicable facts on terrestrial environments.

Conclusion

Importance of invertebrates as reservoirs of multihost pathogens often plays a crucial role of various infections. The invertebrates appeared for a long time to be an unsuitable environment for growth of anaerobic bacteria. The finding of anaerobic bacteria reveals that a unique microbial environment remains an interesting field for further microbiological research (König 2006) focusing on genotoxicity and other virulence factors, inflammation, host defences modulation, and bacterial derived metabolism. A better understanding of the interactions between the invertebrate host and anaerobe pathogenicity depends on further functional studies and findings in almost every area of biology and medicine (Gagniere et al. 2016).

A very few anaerobic bacteria have been isolated in only two invertebrate models, *D. melanogaster* and *P. scaber*. Although anaerobic bacteria are unimpressive found in gastrointestinal tract of invertebrates, inflammatory diseases of the intestine arise from imbalanced interactions between the host gut epithelia and resident or ingested anaerobic microbes. Developing *in vivo* disease models with well characterized development and simple immunity (Chamilos et al. 2007) can help to explicate the basic mechanism underlying disease (Mhatre et al. 2013) start with a similary important pathogenic role and life-threatenig infections in invertebrates. The intestinal niche is also challenged continuosly by numerous environmentally derived bacteria because of its exposed anatomy that is accessible to the external environment (Lee and Lee 2014).

Invertebrate model hosts represent valuable tools for the study of host-pathogen interactions because they facilitate the identification of bacterial virulence factors and allow the discovery of novel components involved in host innate immune responses (Miyata et al. 2003, König 2006). As well as facilitating the identification and study of virulence mechanisms (Mahajan-Miklos et al. 2000), simple model system may also permit direct genetic approaches for the study of host defenses (Ewbank 2002). The finding that diverse bacteria are pathogenic to invertebrate models opens the prospect of using this experimentally simple model to identify genes that are necessary not only for pathogenesis in study model but also for virulence or symbiosis in other hosts (Aballay et al. 2000, König 2006).

To study the pathogenesis in mammalian models is complicated by difficulties of handling, long reproductive cycles, small brood sizes, physiological and anatomical complexity, regulatory requirements, high cost, and ethical considerations. Workers in the field of pathogenesis have the opportunity to select from several invertebrate animal model systems in their studies. An understanding of the unique strengths and limitations associated with each model host is necessary, as particular virulence characteristics are not equally important in all systems and genetic tractability is not available in all model hosts (Mylonakis et al. 2007) and especially when a presence of the anaerobic pathogens is looking for. A better model

systems may be identified and fully characterized in the future, like feeding-based infections, to use pathogens that invade the lumenal side of epithelial cells (Balla and Troemel 2013) or to study systemic infections by microbial injection into the hemolymph (Panayidou et al. 2014). By using and trying different experimental techniques and protocol details, observed the progression of infection in real-time by light microscopy, by fluorescence microscopy and by electron microscopy (Shu et al. 2011) and with well-established genetic, molecular and biochemical analyses of invertebrate animal models, a research model to facilitate maintenance of virulence by anaerobic bacteria could be created.

Genetic screening, the RNAi technique in the genetically tractable invertebrate model organisms have been proved to be a powerful and valuable tool for understanding of fundemental principles of bacterial resistance to infection and may be useful in screening for potential neurotoxicity (Abnave et al. 2015, Altincicek et al. 2007). Inflammatory diseases of the intestine, gastrointestinal cancer and gut-associated pathologies arise from imbalanced interactions between the host gut epithelia and resident or ingested microbes, interactions that are still poorly understood at the molecular level. *D. melanogaster* has been a very powerful model to study development and diseases (Charroux and Royet 2012).

The models reviewed are relatively inexpensive, easy to work with, have short lifespans, and often have very well characterized and stereotypical development and behaviour. Invertebrate models could serve as references for scientists concerned in alternatives to vertebrate animals (Lehner and Lee 2008) and could be a challenge for studying the pathogenesis of infections caused by anaerobic bacteria.

Povzetek

Preučevanje interakcij med nevretenčarji in anaerobnimi patogenimi bakterijami je na vseh področjih biologije in medicine pomembno, saj lahko vsaka odkrita medsebojna odvisnost pomembno vpliva na nastanek in razvoj bolezni (Gagniere in sod. 2016).

Število mikroorganizmov se pri nevretenčarjih nenehno spreminja, med drugim tudi zaradi okolja povezanega z gostiteljem, preproste anatomije in zato anaerobnih bakterij skoraj ni mogoče kultivirati (Lee in Lee, 2014). Teh mikrobov je malo. Prav izpostavljenost anaerobnim bakterijam pa poveča tveganje za kolonizacijo s sevi, ki so specifični za številne črevesne in izvenčrevesne okužbe. Zapisi o izoliranih anaerobnih bakterijah so pri dveh nevretenčarskih modelih, *D. melanogaster* (Charroux in Royet 2012) in *P. scaber* (Kostanjšek 2004, König 2006).

Nevretenčarji so bili dolgo časa neprimerni za rast in razmnoževanje anaerobnih bakterij, saj organizem ne ustvarja pogojev zanje. Znanstveniki pa so z izolacijo anaerobnih bakterij pri nevretenčarjih potrdili, da se lahko zaradi prilagodljivosti v okolju ustvarijo tudi rastni pogoji za pritrditev in razmnoževanje anaerobnih bakterij. Prav ta ugotovitev je vzbodbudila raziskovalce za nadaljevanje mikrobioloških raziskav v tem edinstvenem mikrobnem okolju nevretečarjev (König 2006).

Dejavniki, ki vplivajo na mnoga bolezenska stanja izvirajo iz sestave črevesnih bakterij, njihovega vpliva na delovanje imunskega sistema in njegovega ravnovesja ter izražanje genov. Znanstveni pristopi pri raziskavah sestave anaerobnih bakterij pri nevretenčarjih so lahko v dosedanji praksi delno uporabljeni za obvladovanje različnih bolezenskih sprememb (Mhatre in sod. 2013, Chamilos in sod. 2007). Prepoznavanje simptomov bolezni, poznavanje vrste in vloge anaerobov, dovzetnosti za posamezne bolezni so odraz na ravni gostitelja in mikroba. Njihov vpliv na fiziologijo črevesja, regulacijo metabolizma, razvoj in aktivacijo imunskega sistema so raziskovali v primerjalnih študijah na nevretenčarskih modelih (Aballay in sod. 2000, Miyata in sod. 2003, König 2006). Veliko je še neodkritega na tem področju. A z razvojem sodobnih molekularnih metod in eksperimentalnih tehnik (Shu in sod. 2011, Abnave in sod. 2015, Altincicek in sod. 2007) je mogoče prepoznavanje in opredeljevanje posamezne vrste bakterije in za preučevanje vloge genov so izjemno priročni tudi nevretenčarski modeli (Mahajan-Miklos in sod. 2000, Ewbank 2002, König 2006). Prav zaradi tega predstavljajo dragoceni model za preučevanje virulenčnih dejavnikov anaerobnih bakterij, saj omogočajo

tudi identifikacijo komponent, ki so vključene v imunski odziv (Aballay in sod. 2000).

Nevretenčarski modeli so enostavni za delo, njihova življenska doba je kratka, izvajanje poskusov je ponovljivo in uporaba je manj etično sporna. Vsak nevretenčarski model ima poznane prednosti in slabosti ter poznane vse pomembne dejavnike, ki so lahko povezani z nastankom in razvojem bolezni (Mylonakis in sod. 2007, Balla in Troemel 2013, Panayidou in sod. 2014). Zato so lahko kot alternativa živalskim poskusom (Lehner in Lee 2008) in nov izziv v raziskovanju patogeneze okužb, ki jih povzročajo anaerobne bakterije.

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Conflicts of interest

The authors had no conflicts of interest to declare in relation to this article.

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The effect of selenium and iodine on selected biochemical and morphological characteristics in kohlrabi sprouts (*Brassica oleracea* L. var. *gongylodes* L.)

Vpliv selena in joda na izbrane biokemijske in morfološke lastnosti pri kalicah kolerabice (*Brassica oleracea* L. var. *gongylodes* L.)

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Abstract: Selenium (Se) and iodine (I) are essential elements for humans and animals, while their essential role for plants has not been established yet. There is also very little information about the interaction between selenium and iodine in plants. The aim of our research was to determine the effect of different forms of Se, I and their combinations on selected biochemical and morphological characteristics of the kohlrabi sprouts (*Brassica oleracea* L. var. *gongylodes* L.). Sprouts were grown from seeds, which were soaked in different solutions of selenite, selenate, iodide, iodate and their combinations. We measured the content of chlorophyll *a* and *b*, carotenoids, anthocyanins, and UV-A and UV-B absorbing substances. We also measured potential photochemical efficiency of photosystem II (PS II). At the end of the experiment the weight and height of the sprouts were measured. In order to compare the results the entire experiment was carried out twice. Different chemical forms of Se and I, and combinations did not significantly affect the number of sprouts that germinated from seeds. The various chemical forms of Se and I, and combinations differently affected on the amount of pigments in the kohlrabi sprouts. Potential photochemical efficiency of PS II was close to theoretical maximum 0.83.

Keywords: kohlrabi, sprouts, selenium, iodine

Izvleček: Selen (Se) in jod (I) sta esencialna elementa za ljudi in živali, medtem ko njuna esencialna vloga za rastline še ni dokazana. Obstaja tudi zelo malo podatkov o interakciji med Se in I pri rastlinah, zato je pomembno, da preučujemo hkraten vpliv obeh elementov na rastline, ki jih uporabljamo za prehrano ljudi. Z raziskavo smo želeli ugotoviti, ali različne oblike Se in I posamezno ali v kombinaciji vplivajo na izbrane biokemijske in morfološke lastnosti pri kalicah kolerabice (*Brassica oleracea* L. var. *gongylodes* L.). Kalice smo vzgojili iz semen, ki smo jih namočili v osem različnih raztopin z različnimi kombinacijami in oblikami Se in I ter v kontrolno raztopino. Ostale raztopine so poleg dH_2O vsebovale posamezno dodan selenit (SeO_3^{2-}) oz. selenat (SeO_4^{2-}) s koncentracijo 10 mg Se/L, jodid (I^-) oz. jodat (IO_3^-) s koncentracijo 1.000 mg I/L in kombinacije različnih oblik Se in I (SeO_3^{2-}+ I^-, SeO_3^{2-}+ IO_3^-, SeO_4^{2-} + I^-, SeO_4^{2-} + IO^{3-}). Selen je bil dodan v obliki natrijevega selenita (Na_2SeO_3) oz. natrijevega selenata (Na_2SeO_4), I pa v obliki kalijevega jodida (KI) oz. kalijevega jodata (KIO_3). Različne kemijske oblike Se in I ter njune kombinacije niso statistično značilno vplivale na

število kalic, ki so vzklile iz semen. Različne kemijske oblike Se in I ter njune kombinacije so različno vplivale na koncentracijo barvil pri kalicah kolerabice. Potencialna fotokemična učinkovitost fotosistema II je bila blizu teoretičnega maksimuma 0,83.

Ključne besede: kolerabica, kalice, selen, jod

Introduction

Selenium (Se) and iodine (I) are essential elements for humans and animals, while their essential role for plants has not been established yet (Hasanuzzaman et al. 2014). There is also scarce information about the interaction between selenium and iodine in plants. It is therefore important to study the combined effect of these two elements on plants which can be used for human consumption. Slovenia is a country with iodine deficiency, because of that fortification of salt with potassium iodide increased in 1999 to 25 mg KI per kg of salt. Later on recommended gradual decrease of salt in nutrition reduces this nutritional source of iodine. Lack of selenium in Slovenia soils is known as well (Pirc in Šajn 1997), that results to reduced selenium content in plants to the values below optimal to assure adequate nutritional supply from food of crop origin. Approximately 2/3 of the world's population has health problems associated with insufficient intake of Se and I with diet. One of the easiest ways to combat this problem is biofortification or enrichment of crops with Se and I, to increase the transfer of Se and I into the food chain (White and Broadley 2009). The primary rationale for this is that Se is essential for I metabolism in the thyroid. It was discovered that the deiodinase enzymes, which convert T4 (thyroxin) into T3 (triiodothyronine) and also T3 into T2 and thereby degrading it, are selenium enzymes. Plant roots can take up Se as selenate, selenite or organoselenium compounds, such as selenocysteine and selenomethionine. Plants are one of the main dietary sources of Se for humans and animals (Schiavon et al. 2017). Selenium is known to increase the tolerance of plants to UV-induced oxidative stress, regulate water status of drought exposed plants, delay senescence and promote the growth of ageing seedlings (Kuznetsov et al. 2003, Xue et al. 2001). In most soils, I is present in solution as iodide, although iodate can also be present under oxidizing conditions. The effect of I on biochemical and physiological process, has been scarcely evidenced (Blasco et al. 2011, Landini et al. 2011, Jerše et al. 2017). There is little data on combined effects of Se and I on physiological and biochemical characteristics and yield of plants (Zhu et al. 2004, Smolen et al. 2015, 2016). Our aims were to investigated the effect of addition of Se, I and I+Se on growth and physiological and biochemical characteristics of kohlrabi sprouts.

Materials and methods

Kohlrabi seeds were soaked in solution for 8 h in 200 mL of distilled water (MilliQ) (control), or in solutions contained selenite (SeO_3^{2-}) or selenate (SeO_4^{2-}) with a concentration of 10 mg Se/L, iodide (I-) or iodate (IO_3-) with a concentration of 1000 mg I/L, and their combinations (SeO_3^{2-}+ I^-, SeO_3^{2-}+ IO_3^-, SeO_4^{2-} + I^-, SeO_4^{2-} + IO_3^-). Selenium was applied in the form of sodium selenite (Na_2SeO_3) and sodium selenate (Na_2SeO_4), respectively. Iodine was applied in the form of potassium iodide (KI) and potassium iodate (KIO_3), respectively. After soaking seeds were distributed in plastic trays. Sprouts were grown in controlled conditions in the growth chamber with constant temperature 19°C and 60 % relative air humidity, and 160 µM $m^{-2}s^{-1}$ PAR, 16 h : 8 h. Measurements were done after 14 days of growing sprouts.

Contents of chlorophyll *a* and *b* and carotenoids were measured using a UV/VIS Spectrometer System (Lambda 12, Perkin-Elmer, Norwalk, CT, USA). Chlorophyll content was determined as described in Lichtenthaler and Buschmann (2001a, 2001b). Content of anthocyanins was determined as proposed by Khare and Guruprasad (1993) and Drumm and Mohr (1978). Anthocyanins were extracted from weighed sprouts by homogenizing in a mortar and extracting with HCl:methanol = 1:99 (v/v). Absorbances of extracts were measured at 530 nm with a UV/VIS spectrometer (Lambda 25, Perkin-Elmer, Norwalk, CT, USA). Content of anthocyanins was expressed in relative units.

Content of UV-absorbing compounds was determined according to Caldwell (1968). Fluorescence of chlorophyll was performed on the cotyledons of randomly selected sprouts using the fluorometer (PAM 2500 Portable Chlorophyll Fluorometer, WALZ). Samples were dark adapted for 20 min prior to measurements. The fluorescence parameters that were recorded included minimal (F_0) and maximal (Fm) chlorophyll fluorescence and were provided by dark-adaptation clips. Fv is the variable fluorescence. Fv/Fm (Fv/Fm = Fm – Fo/Fm) ratio is common parameter used in fluorescence which reflects the capacity to trap electrons by the photosystem (PS) II reaction centre (Schreiber et al. 1995).

Results

The percentage of germination of the individual treatments was 67 – 68%. It was in the same range in control and treated sprouts in both experiments (data not shown).

Sprouts from seeds, soaked in Se(VI), had in the first experiment statistically significantly lower concentration of chlorophyll *a* comparing to sprouts from seeds, soaked in Se(IV). Sprouts from seeds, soaked in I(-I), had statistically significantly lower concentration of chlorophyll *a* comparing to sprouts from seeds, soaked in I(V). Sprouts from seeds, soaked in Se(VI) and I(-I), had statistically significantly lower concentration of chlorophyll *a* comparing to sprouts from seeds, soaked in Se(VI)+I(V) and Se(IV)+I(-I). Sprouts from seeds, soaked in I(V), had statistically significantly higher concentration of chlorophyll *a* comparing to sprouts from seeds, soaked in Se(VI), I(-I) and Se(VI)+I(-I).

In the second experiment we determined in sprouts from seeds, soaked in Se(VI) and I(-I), statistically significantly lower concentration of chlorophyll *a* comparing to sprouts from seeds, soaked in Se(VI)+I(V), Se(IV)+I(-I) and I(V). Sprouts from seeds, soaked in Se(VI), had statistically significantly lower concentration of chlorophyll *a* comparing to sprouts from seeds, soaked in Se(VI)+I(-I) (Fig. 1).

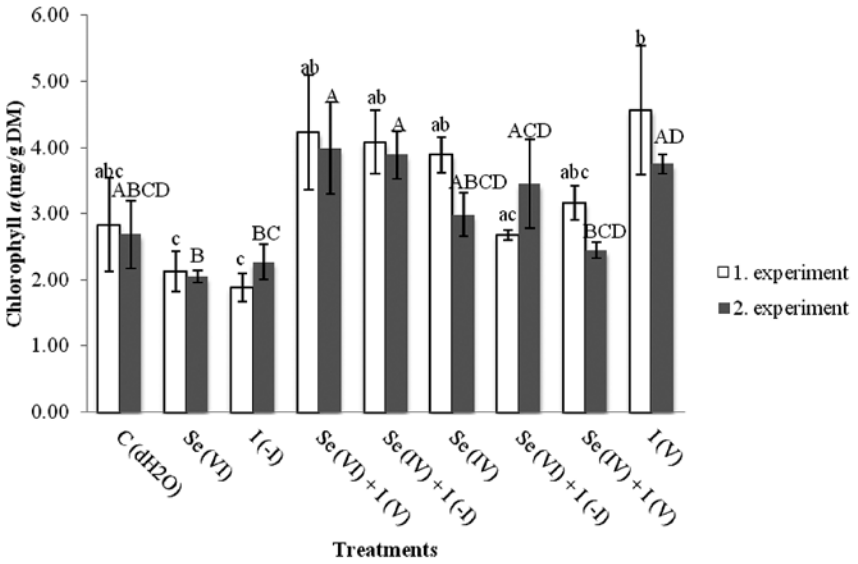

Figure 1: Concentration of chlorophyll *a* per DM in kohlrabi sprouts. Mean ± SE, n = 4, C - control. Mean values, marked with the same letter, are not significantly different at $p \leq 0.05$.

Slika 1: Koncentracija klorofila *a* na SM v kalicah kolerabice. Predstavljene so povprečne vrednosti ± SE (n = 4). C – kontrolne kalice. Stolpci, označeni z različnimi črkami, se med seboj statistično značilno razlikujejo pri $p \leq 0{,}05$.

In the first experiment had sprouts from seeds, soaked in Se(VI), statistically significantly lower concentration of carotenoids from sprouts from seeds, soaked in Se(IV). Sprouts from seeds, soaked in I(-I), had statistically significantly lower concentration of carotenoids comparing to sprouts from seeds, soaked in I(V). Sprouts from seeds, soaked in Se(VI) and I(-I) had statistically significantly lower concentration of carotenoids comparing to sprouts from seeds, soaked in Se(VI)+I(V) and Se(IV)+I(V). Concentration of carotenoids was similar in control and all treatments in the second experiment (Fig. 2).

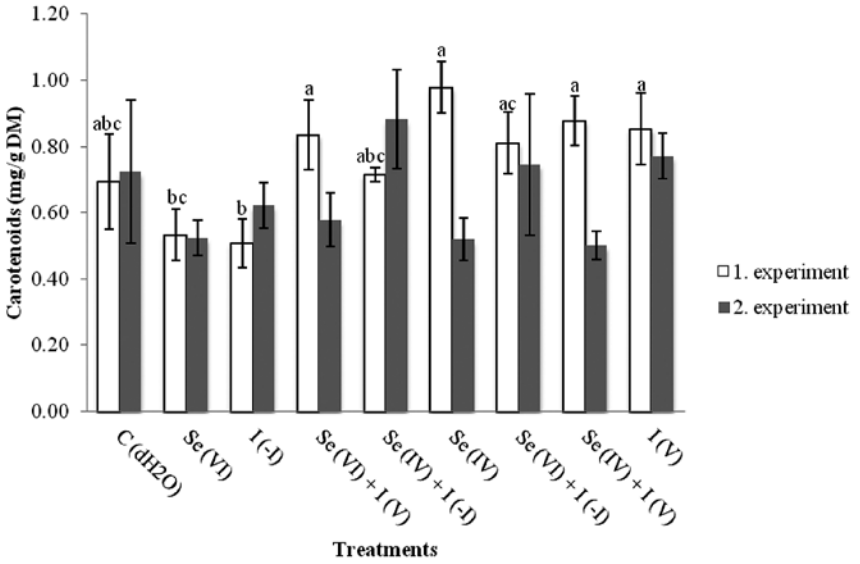

Figure 2: Concentration of carotenoids per DM in kohlrabi sprouts. Mean ± SE, n = 4, C - control. Mean values, marked with the same letter, are not significantly different at $p \leq 0.05$.

Slika 2: Koncentracija karotenoidov na SM v kalicah kolerabice. Predstavljene so povprečne vrednosti ± SE (n = 4). C – kontrolne kalice. Stolpci, označeni z različnimi črkami, se med seboj statistično značilno razlikujejo pri $p \leq 0{,}05$.

In the first experiment sprouts from seeds, soaked in Se(VI), had statistically significantly higher concentration of anthocyanins comparing to sprouts from control and from seeds soaked in other treatments.

In the second experiment had sprouts from seeds, soaked in Se(VI), statistically significantly lower concentration of anthocyanins comparing to sprouts from seeds, soaked in Se(IV). Sprouts from seeds, soaked in I(-I), had statistically significantly higher concentration of anthocyanins comparing to seeds, soaked in Se(IV) (Fig. 3).

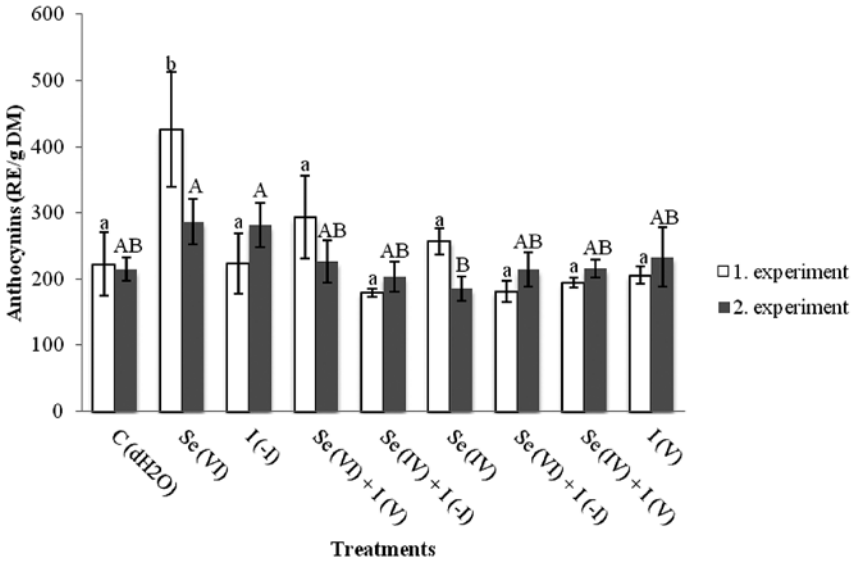

Figure 3: Concentration of anthocyanins per DM in kohlrabi sprouts. Mean ± SE, n = 4, C - control. Mean values, marked with the same letter, are not significantly different at $p \leq 0.05$.

Slika 3: Koncentracija antocianov na SM v kalicah kolerabice. Predstavljene so povprečne vrednosti ± SE (n = 4). C – kontrolne kalice. Stolpci, označeni z različnimi črkami, se med seboj statistično značilno razlikujejo pri $p \leq 0{,}05$.

Concentration of UV-B absorbing compounds was similar in control and treated sprouts in the first experiment.

In the second experiment had sprouts from seeds, soaked in I(V), statistically significantly higher concentration of UV-absorbing compounds comparing to sprouts from seeds, soaked in Se(IV)+I(-I) and Se(VI)+I(-I) (Fig. 4).

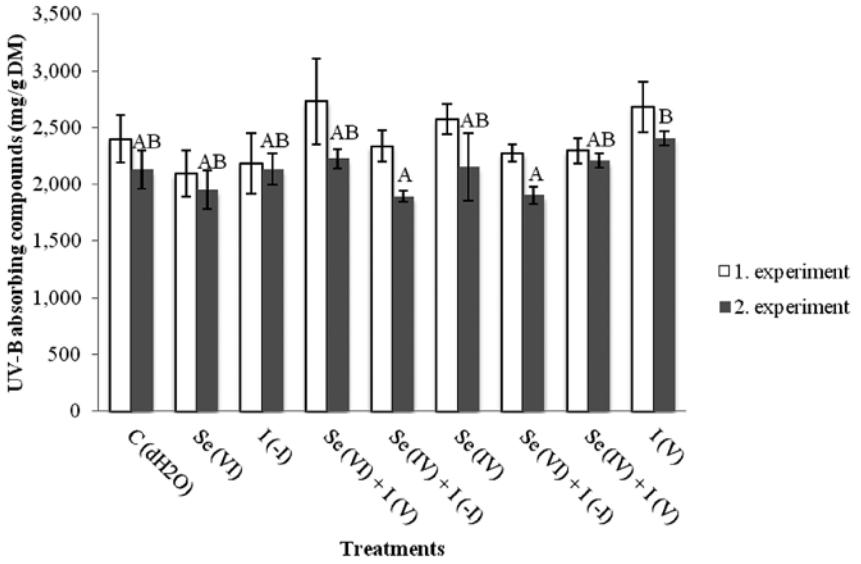

Figure 4: Concentration of UV-B absorbing compounds per DM in kohlrabi sprouts. Mean ± SE, n = 4, C - control. Mean values, marked with the same letter, are not significantly different at $p \leq 0.05$.

Slika 4: Koncentracija UV-B absorbirajočih snovi na SM v kalicah kolerabice. Predstavljene so povprečne vrednosti ± SE (n = 4). C – kontrolne kalice. Stolpci, označeni z različnimi črkami, se med seboj statistično značilno razlikujejo pri $p \leq 0,05$.

Potential photochemical efficiency of PS II was similar in control and treated sprouts in the first experiment.

In the second experiment had sprouts from seeds, soaked in Se(VI)+I(-I), statistically significantly higher potential photochemical efficiency of PS II from sprouts from seeds, soaked in I(V). (Fig. 5).

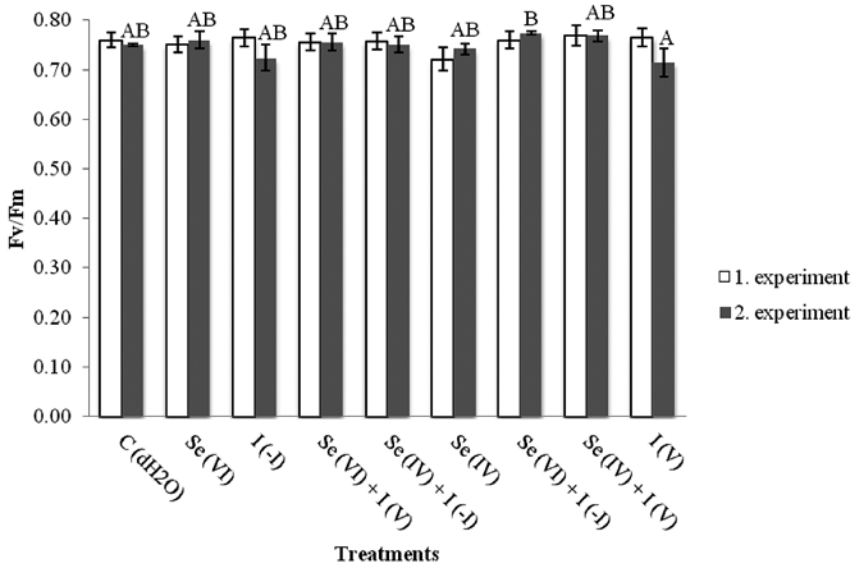

Figure 5: Potential photochemical efficiency of PS II in kohlrabi sprouts. Mean ± SE, n = 4, C - control. Mean values, marked with the same letter, are not significantly different at $p \leq 0.05$.

Slika 5: Potencialna fotokemična učinkovitost FS II v kalicah kolerabice. Predstavljene so povprečne vrednosti ± SE (n = 4). C – kontrolne kalice. Stolpci, označeni z različnimi črkami, se med seboj statistično značilno razlikujejo pri $p \leq 0{,}05$.

In the first experiment control sprouts showed statistically significantly lower dry mass comparing to sprouts from seeds, soaked in Se(VI), I(-I), Se(IV)+I(-I) and Se(VI)+I(-I). Sprouts from seeds, soaked in Se(IV)+I(-I), had statistically significantly higher dry mass comparing to sprouts from seeds, soaked in Se(VI), Se(VI)+I(V), Se(IV), Se(VI)+I(-I), Se(IV)+I(V) and I(V). Sprouts from seeds, soaked in I(-I), had statistically significantly higher dry mass comparing to sprouts from seeds, soaked in Se(VI)+I(V) and Se(IV)+I(V).

In the second experiment control sprouts showed statistically significantly lower dry mass comparing to sprouts from seeds, soaked in Se(VI)+I(V), Se(IV)+I(-I), Se(IV) and Se(IV)+I(V). Sprouts from seeds, soaked in Se(VI)+I(V) and Se(IV)+I(-1) had statistically significantly higher dry mass comparing to sprouts from seeds, soaked in Se(VI), I(-I), Se(VI)+I(-I) and I(V). Sprouts from seeds, soaked in Se(IV) and Se(IV)+I(V) had statistically significantly higher dry mass comparing to sprouts from seeds, soaked in Se(VI)+I(-I) and I(V) (Fig. 6).

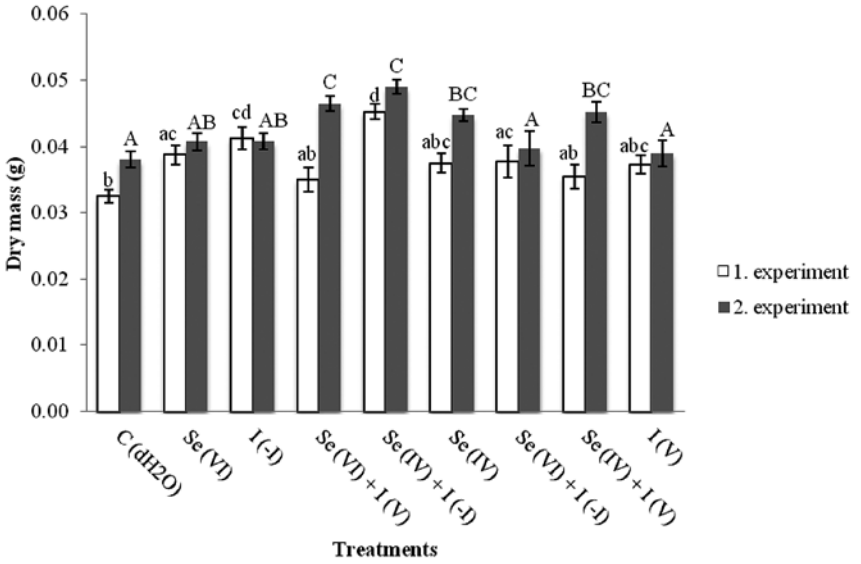

Figure 6: Dry mass of kohlrabi sprouts. Mean ± SE, n = 4, C - control. Mean values, marked with the same letter, are not significantly different at $p \leq 0.05$.

Slika 6: Suha masa kalicah kolerabice. Predstavljene so povprečne vrednosti ± SE (n = 4). C – kontrolne kalice. Stolpci, označeni z različnimi črkami, se med seboj statistično značilno razlikujejo pri $p \leq 0,05$.

Discussion

Seed germination is a critical stage in the plant life cycle. It starts with the imbibition, which means uptake of water of dry seed embrio (Herman et al. 2007). In our experiment different treatments did not affect germination of kohlrabi sprouts. Even though processes in seed germination and sprout development depend on environment factors and may be negatively affected by abiotic stress, such as high concentrations of minerals in soaking solution (Pongrac et al. 2016).

Increased chlorophyll levels indicate a greater potential for photosynthesis. In our study sprouts, treated with Se(VI), produced lower concentration of chlorophyll *a* and carotenoids comparing to sprouts developed from seeds which had been treated with Se(IV). Germ et al. (2015) conducted experiment with common buckwheat with the same concentrations and forms of Se and I as in the present study. Similarly as in the present study they found out that sprouts developed from seeds which had been soaked in Se(VI) had lower concentration of chlorophyll *a* and carotenoids comparing to sprouts from seeds, soaked in Se(IV). Sprouts from seeds, soaked in I(-I), had statistically significantly lower concentration of chlorophyll *a* comparing to sprouts from seeds, soaked in I(V). There are scarce information about the effect of iodine on the concentration of chlorophyll. In the study Krzepilko et al. (2016) found out that in comparison with the control, KI did not affect chlorophyll content of lettuce seedlings.

In the first experiment sprouts produced from seeds, soaked in Se(VI), had statistically significantly higher concentration of anthocyanins comparing to control seeds and seeds, soaked in other treatments. Results are in line with Hawrylak-Nowak (2008) who found out that in maize, selenate treatments at concentrations 7.9 mg Se/L increased the content of anthocyanins.

Potential photochemical efficiency of photosystem II was close to theoretical maximum (0.83) (Schreiber et al. 1995) in both control and treated groups. None of the treatments presented stress conditions for experimental plants.

Se and I added in any form and combination did not affect the synthesis of UV- absorbing compounds, the concentrations of which were similar in control and treated sprouts. Addition of Se may

reduce the negative effects of UV-B radiation on seedlings of wheat possibly by increasing the amount and activity of the antioxidant enzymes (Yao et al. 2010, Yao et al. 2011) and by increasing the amount of anthocyanins and phenolic compounds (Yao et al. 2010), which also have an antioxidant effect.

Hajiboland and Keivanfar (2012) investigated the influence of Se(VI) in oilseed rape (*Brassica napus*). Oilseed rape plants were 19 weeks foliarly sprayed with the Se(VI) at different concentrations: 0 (control), 0.01, and 0.02 mg Se/L. They found that the dry weight of the pods and seeds was significantly higher for the plants treated with Se(VI) compared with control plants. Foliar spraying with Se(VI) had no effect on the dry weight and height of above ground parts and on the dry mass of roots and seeds of two varieties of common buckwheat (Tadina et al. 2007). In contrast, foliar spraying of potatoes with Se(VI) at a concentration of 10 mg Se/L lowered the weight of the tubers (Germ et al. 2007). On the other hand in our experiment in the first experiment sprouts from Se(VI) treated seeds had a positive impact on the dry weight of sprouts. Addition of I(-I) in the form of KI at concentration of 10 mg I/L, significantly reduced the biomass of lettuce plants in comparison with plants from the control treatment, whereas the addition of I(V), in the form of KIO_3, increased biomass of plants, which reached a maximum value at a dose of 2.5 mg I/L (Blasco et al. 2011). In our experiment the soaking of seeds in I(V) solution did not affect the dry mass of sprouts. Foliar spraying radish plants (*Raphanus sativus*) with I(-I) respectively I(V) had no effect on the dry weight of leaves and roots (Strzetelski et al. 2010). Blasco et al. (2008) found that treatment of lettuce with I(-I) ($\geq$ 10 mg I/L) had toxic effects on the growth of plants due to excessive accumulation of this element in the plant tissue.

Smoleń et al. (2014) found out that treatment of lettuce plants with Se(VI)+I(V) had no effect on the average weight of the heads. Zhu et al. (2004) added Se(VI) and I(V) to the nutrient solution, where they grow spinach plants. Se(VI) was added in concentrations of 0.8, 1.6 and 4 mg Se/L, while the I(V) was added in concentrations of 1.25, 2.5 and 6.25 mg I/L. It has been found that the addition of Se (VI) at a concentration of 0.8 and 1.6 mg Se/L and I(V) at a concentration of 1.25 and 6,25 mg I/L respectively lowered the biomass of roots.

However in our second experiment I(V) treatment did not have any effect on the dry mass of sprouts in either of experiments.

Conclusions

The aim of our research was to determine the effect of different forms of Se, I and their combinations on selected biochemical and morphological characteristics of the kohlrabi sprouts (*Brassica oleracea* L. var. *gongylodes* L.). Response of sprouts to different chemical forms of Se and I, and combinations thereof differed between the measured parameters. Higher concentration of Se and I would probably have a greater impact on the kohlrabi sprouts.

Povzetek

Selen (Se) in jod (I) sta nujno potrebna elementa za ljudi in živali, medtem ko njuna esencialna vloga za rastline še ni dokazana. Cilj raziskave je bil ugotoviti, ali različne oblike Se in I posamezno ali v kombinaciji vplivajo na izbrane biokemijske in morfološke lastnosti pri kalicah kolerabice (*Brassica oleracea* L. var. *gongylodes* L.). Poskus je bil sestavljen iz devetih obravnavanj. Kalice smo vzgojili iz semen, ki so bila namočena v različne raztopine z različnimi kombinacijami in oblikami Se in I. Raztopine so vsebovale selenit (SeO_3^{2-}) oz. selenat (SeO_4^{2-}) s koncentracijo 10 mg Se/L, jodid (I^-) oz. jodat (IO_3^-) s koncentracijo 1.000 mg I/L in kombinacije različnih oblik Se in I (SeO_3^{2-}+ I^-, SeO_3^{2-}+ IO_3^-, SeO_4^{2-} + I^-, SeO_4^{2-} + IO^{3-}). Merili smo koncentracijo klorofila *a* in *b*, karotenoidov, antocianov, UV-A absorbirajočih snovi, UV-B absorbirajočih snovi ter potencialno fotokemično učinkovitost fotosistema II (FS II). Ugotavljali smo delež kaljivosti semen. Po koncu poskusa smo izmerili še maso kalic. Različne kemijske oblike Se in I ter njune kombinacije niso statistično značilno vplivale na število kalic, ki so vzklile iz semen. Različne kemijske oblike Se in I ter njune kombinacije so različno vplivale na koncentracijo barvil pri kalicah kolerabice. Potencialna fotokemična učinkovitost fotosistema

II je bila blizu teoretičnega maksimuma 0,83, kar kaže, da kalice niso bile izpostavljene stresnim razmeram. Predvidevamo, da bi imele večje koncentracije preučevanih elementov večji vpliv na rast in fiziološke lastnosti kalic.

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Variability of testes morphology and the presence of testis-ova in the European blind cave salamander (*Proteus anguinus*)

Variabilnost v morfologiji testisov in prisotnost jajčnih celic v testisih pri proteusu (*Proteus anguinus*)

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Abstract: The European blind cave salamander, *Proteus anguinus,* is a neotenic, permanently aquatic amphibian with a very long life span, late sexual maturity, and the ability to reproduce for at least 30 years. *Proteus* is considered to be vulnerable species, and yet very little is known about its reproductive biology. The objective of this study is to describe the detailed morphology of the testes of adult *Proteus* and determine the maturation state of the gonads and gametogenesis with respect to body size and seasonality. This research showed that testis size increases with adult male body length, but the shape and meiotic condition of the testes are highly variable and independent of the length of the specimen. The testis of *Proteus* has a simple cystic type of organization in which cysts are enclosed in lobules, with synchronous maturation of the germ cells within each cyst. Spermatogenesis progresses in a caudo-cephalic direction within the testis, as in other salamanders, and appears to be seasonal, despite the fact that *Proteus* is a cave animal living in stable environmental conditions. Surprisingly, the testes of approximately one third of the specimens, regardless of their morphology or meiotic condition, contained testis-ova located randomly among groups of spermatogonia and spermatocytes. These testes-ova contained germinal vesicles with lampbrush chromosomes, and thus correspond to primary oocytes. The presence of testis-ova may be related to a sex-chromosome turnover involving X-Y translocation that was recently discovered in *Proteus*.

Keywords: testis, morphology, spermatogenesis, proteus, *Proteus anguinus*

Izvleček: *Proteus anguinus* je neotenična jamska dvoživka z zelo dolgo življenjsko dobo, pozno spolno zrelostjo in reproduktivno sposobnostjo najmanj 30 let. Proteus ima status ranljive vrste, vendar o njeni reproduktivni biologiji pravzaprav ne vemo veliko. Cilj raziskave je podrobno opisati morfologijo testisov odraslih živali proteusa in določiti zrelostno fazo gonad glede na velikost telesa in sezono. Raziskava je pokazala, da se velikost testisov povečuje s telesno dolžino živali, vendar sta oblika in zrelost testisov zelo variabilni in popolnoma neodvisni od velikosti živali. Testisi proteusa imajo preprost cistični tip organizacije, s številnimi lobuli, ki vključujejo ciste s sinhronim zorenjem spolnih celic v vsaki od njih. Tako kot pri ostalih repatih dvoživkah, spermatogeneza poteka v kavdalno - cefalični smeri testisa. Navkljub, da

je proteus jamska žival, ki živi v okolju s stabilnimi abiotskimi dejavniki, je videti, da je spermatogeneza v korelaciji s sezono. Presenetljivo je tudi to, da so v testisih proteusa prisotne jajčne celice (testis-ova), in sicer kar pri tretjini proučevanih osebkov ter popolnoma neodvisno od morfologije in zrelosti testisov. Jajčne celice so v lobulih testisa med spermatogoniji ali pa spermatociti. Po morfologiji in krtačastih kromosomov v jedrih ustrezajo primarnim oocitom v jajčnikih proteusa. Prisotnost oocitov v testisih povezujemo z nedavno odkrito preureditvijo spolnih kromosomov proteusa, ki vključuje translokacijo kromosoma Y na kromosom X.

Ključne besede: testis, morfologija, spermatogeneza, cave salamander, *Proteus anguinus*

Introduction

The European blind cave salamander, *Proteus anguinus*, is endemic to underground waters in the Dinaric karst of the Balkan region of Europe. Because of its subterranean habitats, *Proteus* is thought particularly vulnerable to environmental degradation, and thus we need solid baseline studies especially on its reproductive biology. The species is divided into two subspecies, the strongly troglomorphic (i.e. cave-adapted) "white" proteus, *P. a. anguinus*, and the recently discovered (1986), troglobitic but non-troglomorphic, "black" proteus, *P. a. parkelj* (Sket and Arntzen 1994). *Proteus a. anguinus* is geographically the more wide-spread subspecies and is distributed from Italy in the northwest to Montenegro in the southeast. Altogether, almost 250 locations are known, most of them in Slovenia (Sket 1997). The general troglomorphic characteristics include specialization of the sensory organs (e.g. mechano-, electro-, and chemoreceptors), especially of the head, elongation of individual body parts, asymmetric growth of the head and body, degenerate eyes, and skin depigmentation (Bulog et al. 2000, Langecker 2000, Schlegel et al. 2009, Bulog and Bizjak Mali 2014). *Proteus a. parkelj* is different from *Proteus a. anguinus* in having small but otherwise well-developed larval eyes (Kos in Bulog 2001) and dark pigmented skin, and is known only from an area of less than 2 km^2 in the region of Bela Krajina, southeastern Slovenia (Sket 1997, Gorički et al. 2017). Both subspecies of *Proteus anguinus* are obligate neotenes, and their inability to metamorphose is presumably due to the lack of response of target tissues to thyroid hormones (Langecker 2000). Neotenic characteristics are retained in adults, e.g. three pairs of external gills, two pairs of gill slits, an integument with many larval characteristics, and a typical larval visceral skeleton with no maxillary bones (Noble 1931, Langecker 2000). *Proteus* is the longest-lived amphibian, with an estimated lifespan of over 100 years in captivity (Voituron et al. 2011). *Proteus* also has a very low metabolic rate and can cope with long-term starvation (Briegleb 1962, Vandel and Bouillon 1959, Vandel 1965, Hervant et al. 2001, Bizjak Mali et al. 2013). Some of these characteristics of *Proteus*, such as low metabolic rate, large cells, and low rates of growth and development, may be related to its large genome size (Gregory 2001, 2005) which, at approximately 49 billion base pairs, is about 16 times that of humans and one of the largest among salamanders.

Proteus reproduction has been studied over many years in the Cave-laboratory of Moulis, France, revealing extremely long reproductive cycles and delayed sexual maturity in comparison with other amphibians. Females become sexually mature after 15 years at 11-12ºC (or even later, after 17 years, at lower temperature) (Juberthie et al. 1996). Males mature earlier than females, at 11 years. Both mature at a total body length of 140 to 180 mm, but they do not start to reproduce until they reach 200 to 240 mm total length (Durand and Delay 1981) These studies estimate that a *Proteus* female lays eggs at intervals of 6 to 12.5 years, with a total reproductive period that lasts 30 years or even more (Jubertie et al. 1996). *Proteus* has a sex ratio of nearly 2:1 in favor of females (our own observation based on a sample over 100 individuals) but, unlike most other salamanders, *Proteus* males and females

are indistinguishable by external morphological criteria, which hampers demographic studies using non-invasive techniques. As in many other salamanders, *Proteus* reproduces via internal fertilization, with the male producing spermatophores (Briegleb 1961). Females lay eggs at any time of the year with a slight preference for winter-time (Jubertie et al. 1996). The large, yolky eggs, each approximately 4-5 cm in diameter, are laid in small clutches of 35 -70 eggs, and are constantly guarded by the female. Embryonic development is slow and lasts 130 days to hatching at 11-12ºC, and the embryonic mortality rates are quite high (≥ 50%) (Juberthie et al. 1996).

We have performed studies of the gonads and gametogenesis in *Proteus* to gain better base-line knowledge of their reproductive biology, with potential applications in conservation issues as well as in the management of their reproduction in captivity. Several of our previous studies were focused on the detailed morphology of the ovaries of *Proteus*, including descriptions of the developmental stages of the oocytes (Bizjak Mali and Bulog 2010, Bizjak Mali et al. 2010, Bizjak Mali and Bulog 2011, Bizjak Mali et al. 2013). These studies showed that ovarian maturation is not seasonal, although it is positively correlated with body length and mass (Bizjak Mali et al. 2010, 2013). However, except for the research of Kezer (1962), who worked on meiotic chromosomes from testes of *Proteus* from the summer season and briefly mentioned testis condition, the detailed external and internal morphology and process of meiosis of the testes of *Proteus* have never been described. The objective of this study was therefore to perform a detailed analysis of the morphological and histological variation of the testes of adult specimens of *Proteus,* and to determine the maturation state of the gonads, including the processes of spermatogenesis and spermiogenesis. From previous studies of the ovaries of female *Proteus* (Bizjak Mali et al. 2010, 2013), I predicted that the condition of the testes, especially size, would be positively correlated with body size (a proxy for age), and that there would be no correlation between reproductive state of the testes and season of the year, as is seen in other urodelan species living in constant temperature environments (Chan 2003, Ogielska and Bartmanska 2009). Our results confirm some of these predictions but not others, and also include the unexpected discovery of a high frequency of testis-ova in male *Proteus*. The significance of these findings is discussed.

Materials and methods

The testes of 16 adult male *Proteus anguinus* were examined. Specimens included both subspecies of *Proteus anguinus* (*P. a. anguinus,* N=11 and *P. a. parkelj,* N=5) collected at different seasons of the year. Seasons of the year were defined as Fall (September –November), Winter (December –February), Spring (March – May), and Summer (June – August). No animals were available for April to June. Six specimens of *P. a. anguinus* were from southwest Slovenia (Planina), and five specimens were from southeast Slovenia (Otovec, Krupa and Grčarske Ravne). Specimens of *P. a. parkelj* were from Jelševnik (southeast Slovenia). The total body lengths of the animals from all localities ranged from 200 to 360 mm, and body weight ranged from 11.4 to 76.9 g.

The testes of *Proteus* analysed for this study were from an archived collection of specimens and tissues of the Department of Biology, University of Ljubljana. The testes had been fixed in 10% buffered formalin, rinsed with water, and stored in 70% ethanol. Some of the testes were already embedded in Paraplast®. The animals were collected for other research purposes with permission of the Ministry of the Environment and Spatial Planning of the Republic of Slovenia. (35701-81/2004-9 and 35601-1/2010-6). Using archived specimens allowed me to perform this study without sacrificing additional animals.

The total length, and the diameter at the widest part of testes were measured. A gonadosomatic index (GSI = [testis weight/total body weight] x 100) was calculated for six archived specimens for which data on testis weight were available (ranging nearly 40-fold from 0.016 to 0.63 g).

Gross morphology and histological structure of the testes were analyzed using stereo and light microscopes. For histology, testes fixed in 10% buffered formalin and stored in 70% ethanol were dehydrated through graded alcohols, cleared in xylene, and embedded in Paraplast®. Serial 5μm-thick sections were stained by Weigert hema-

toxylin–eosin or Masson Trichrome methods, or Feulgen staining (Humason 1979, Kiernan 1990, Presnell and Schreibman 1997). Meiotic cells were staged according to the size of the nuclei and condensation of chromatin (Uribe 2003). Stages of meiosis were identified as primary or seconday spermatogonia (SgI and SgII), primary or secondary spermatocytes (ScI and ScII), spermatids (Sd), and spermatozoa (Sz). The slides were examined by light microscopy using a Zeiss OPTON-Axioskop and images were captured by a DFC290 HD digital camera (Leica) and LAS 4 program (Leica).

Results

External Anatomy of the Testes

The testes are paired, small, and non-pigmented organs lying parallel to the ventral side of the kidneys, adjacent to the mesonephric or Wolffian ducts and *paramesonephric ducts* (rudimentary Müllerian ducts, a pair of embryonic ducts parallel to the Wolffian ducts that in female develop into the oviducts), and are attached to the dorsal body wall with a dorsal mesorchium. Anteriorly, the testes are attached to the lung via mesentery (Fig. 1A). The position of the testes in *Proteus* is always asymmetrical, with the right testis positioned more anteriorly than the left one.

The testes showed extensive variability in their size and shape. At least four testis morphologies were distinguished in our study (Figs. 1A - D): **1)** simple narrow testis (SNT; n = 8 specimens) (Fig. 1A), which is mostly uniformly narrow, 14 ± 3.5 mm long and 1 ± 0.4 mm wide; in three cases the caudal part was slightly wider (1.25 mm in diameter); **2)** narrow single-lobed testis (NLT; n = 4 specimens) (Fig. 1B), is widest in the posterior half and is narrower at the cephalic and extreme caudal ends with the mean total length 13.3 ± 3.6 mm with a diameter of 3.4 ± 1.0 mm; **3)** broad single-lobed testis (BLT; n = 2 specimens) (Fig. 1C), is uniformly wide over the whole length with short narrow parts at the cranial and caudal end. The mean length of the BLT testes was 14.4 ± 0.6 mm with a diameter of 4.6 ± 1.5 mm; **4**) multilobed testis (MLT; n = 2 specimens) (Fig. 1D), has two, well-developed lobes connected by a narrow region. MLT testes also have narrow regions at the cephalic and caudal ends of the testis, and a much smaller third lobe at the caudal end. The mean length was 30 ± 7.1 mm and a diameter of 3.8 ± 0.4 mm.

The SNT and NLT testes were found in specimens of both subspecies of *Proteus anguinus*, BLT testes were found only in the white subspecies *P. a. anguinus*, and MLT testes were found only in the black subspecies *P. a. parkelj* (Table 1).

Table 1: The morphological forms and stages of spermatogenesis of the testis in both subspecies of *Proteus anguinus*. The Table also includes locality, total body length, months of the capture of animals and presence of testis-ova.

Tabela 1: Morfološke oblike testisov in faze spermatogeneze pri obeh podvrstah proteusa *Proteus anguinus*. Tabela vključuje tudi lokaliteto, dolžino telesa, mesece ulova živali in prisotnost oocitov v testisih.

Locality	Body length (mm)	Month / season	Testis forms	Testis-ova	SgI	SgII	ScI	ScII	Sd	Sz
Pap-SE-J	211	jan (W)	SNT^a	yes	x	x				
Paa-SW-P	246	jul (Su)	SNT^a	yes	x	x				
Paa-SE-O	210	aug (Su)	SNT^a	/	x	x				
Paa-SE-K	241	nov (F)	SNT^a	/	x	x				
Paa-SE-G	240	nov (F)	SNT^b	/	x	x	x			
Paa-SW-P	248	oct (F)	SNT^b	/	x	x	x			
Pap-SE-J	200	nov (F)	SNT^b	/	x	x	x			
Pap-SE-J	253	dec W)	SNT^b	/	x	x	x			
Paa-SE-O	265	mar (Sp)	NLT	/	x	x	x			
Paa-SW-P	282	mar (Sp)	NLT	/	x	x	x			
Paa-SW-P	210	dec (W)	NLT	yes	x	x	x			
Paa-SW-P	250	sept (F)	NLT	yes	x	x	x	x	x^1	
Paa-SE-O	280	aug (Su)	BLT	/	x	x	x	x	x^2	
Paa-SW-P	255	dec (W)	BLT	yes	x	x	x	x	x	x
Pap-SE-J	360	jan (W)	MLT	/	x	x	x			
Pap-SE-J	247	sept (F)	MLT	/	x	x	x			

Legend: Paa – *P.a.anguinus*, Pap – *P.a.parkelj*; SW – south west Slovenia, SE – south east Slovenia; G – Grčarske Ravne, J – Jelševnik, K – Krupa, O – Otovški breg, P – Planina cave; Sp – Spring, Su – Summer, F – fall, W- Winter; BLT – broad single-lobed testis, NLT – narrow single-lobed testis, MLT – multilobed testis, SNT^a – uniformly narrow simple narrow testis, SNT^b – simple narrow testis with slightly wider posterior part; SgI – primary spermatogonia, SgII – secondary spermatogonia, ScI – primary spermatocytes in pachyten I, ScII – secondary spermatocytes, Sd – spermatids, Sz – spermatozoa; ^{1} – early spermiogenesis, ^{2} – late spermiogenesis.

Legenda: Paa – *P.a.anguinus*, Pap – *P.a.parkelj*; SW – jugozahodna Slovenija, SE – jugovzhodna Slovenija; G – Grčarske Ravne, J – Jelševnik, K – Krupa, O – Otovški breg, P – Planinska jama; Sp – pomlad, Su – poletje, F – jesen, W- zima; BLT – širok testis, NLT – ozek testis z razširitvijo, MLT – testis z več razširitvami, SNT^a – enakomerno širok ozek testis, SNT^b – ozek testis z rahlo razširjenim posteriornim delom; SgI – primarni spermatogoniji, SgII – sekundarni spermatogoniji, ScI – primarni spermatociti v pahitenu I, ScII – sekundarni spermatociti, Sd – spermatide, Sz – spermatozoji; ^{1} – zgodnja spermiogeneza, ^{2} – pozna spermiogeneza.

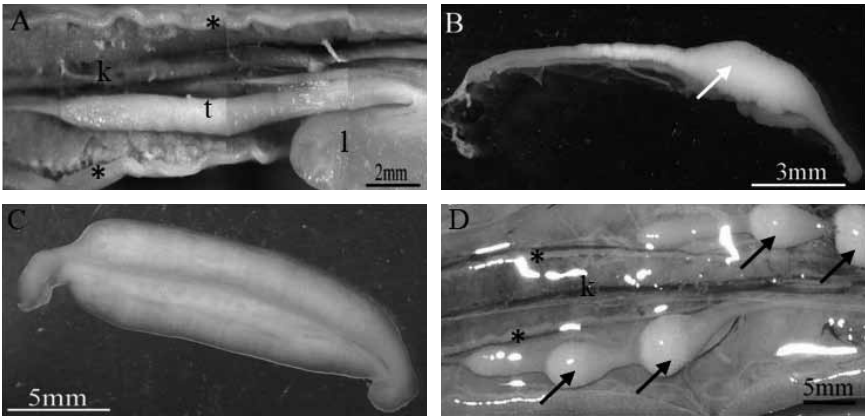

Figure 1: Diversity in the morphology of testes in *Proteus anguinus*. **A** – Simple narrow testis (SNT) of *P. a. anguinus*. **B** – Narrow single-lobed testis (NLT) of *P. a. anguinus*. **C** – Broad single-lobed testis (BLT) of *P. a. anguinus*. **D** –Multilobed testis (MLT) of *P. a. parkelj*. Cranial part of testes in all Figures is on the right. Arrow – lobe, asterisk – Müllerian duct, l – posterior region of lung, k – kidney.

Slika 1: Raznolikost v morfologiji testisov pri proteusu *Proteus anguinus*. **A** – Preprost ozek testis (SNT) pri *P. a. anguinus*. **B** – Ozek testis z razširitvijo (NLT) pri *P. a. anguinus*. **C** – Širok testis (BLT) pri *P. a. anguinus*. **D** –Testis z več razširitvami (MLT) pri *P. a. parkelj*. Na vseh slikah je kranialni del testisov na desni strani. Puščica – razširitev, zvezdica – Müllerjev vod, l – posteriorni del pljuč, k – ledvica.

Testis surface area appears to be positively correlated with total body length (Fig. 2A), although sample sizes are quite small. It is possible that the data points fit a logistic growth curve. The correlation between testis weight with body length is not as pronounced (Fig. 2B), and could also represent a growth curve. Animals less than about 250 mm in length all had very low testis mass and testes were 30-40 times heavier in larger animals.

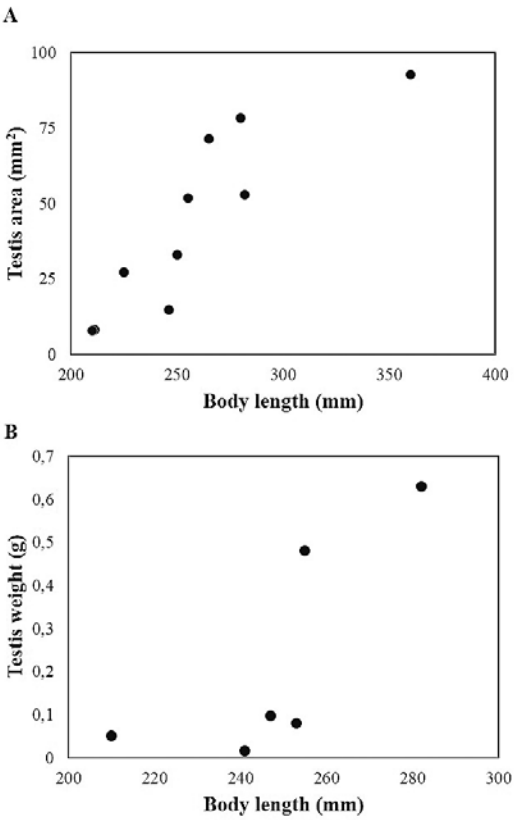

Figure 2: Testis area vs total body length (**A**), and testis weight vs total body length (**B**) in specimens of *Proteus anguinus*.

Slika 2: Površina testisa proti dolžini telesa (**A**), in teža testisa proti dolžini telesa (**B**) pri proteusu *Proteus anguinus*.

Histology of testis and germ cells

The testis is surrounded by a thin epithelium (mesothelium) and connective tissue (tunica albuginea). The internal testis arrangement consists of numerous lobules which communicate with the system of intratesticular ducts. The lobules are delimited by thin walls of loose connective tissue and contain groups of germinal cells within cysts in which spermatogenesis occurs. All of the germ cells within a particular cyst are at the same stage of development.

Spermatogonia

Primary spermatogonia (SgI) are the largest germ cells visible in the testes, around 35 -40 µm in diameter, with very large nuclei. They are round cells with light cytoplasm and round or irregular shaped nuclei with a diameter of approximately 25 µm and containing diffuse chromatin (Fig. 3A). SgI cells are found in groups or individually in the connective tissue at the base of the lobules, located near the midline in the testis. Secondary spermatogonia (SgII) form small clusters of cells, are smaller than SgI, and have heterochromatic (i.e. darkly stained) nuclei (20 µm) (Fig. 3B).

Spermatocytes

Primary (ScI) and secondary spermatocytes (ScII) are recognizable on the basis of nucleus size and condensation of the chromatin. The ScI are spherical and similar in size to SgII. Their nuclei are smaller with a diameter of about 17 µm. ScI

can usually be identified by different stages of prophase I (Figs. 3C-D) and very rarely in the other stages of meiosis I (Fig. 3 E). The ScI at pachytene stage are the most abundant, reflecting the fact that this is a very long-lasting stage, whereas the other prophase I stages (leptotene, zygotene, diplotene) are rarely observed. ScII are smaller than ScI (Fig. 3F), with nuclear diameters of 13 to 15 μm, and they are scarce in sections, reflecting the fact that the stages of the second meiotic division, culminating in spermatids, are of relatively short duration.

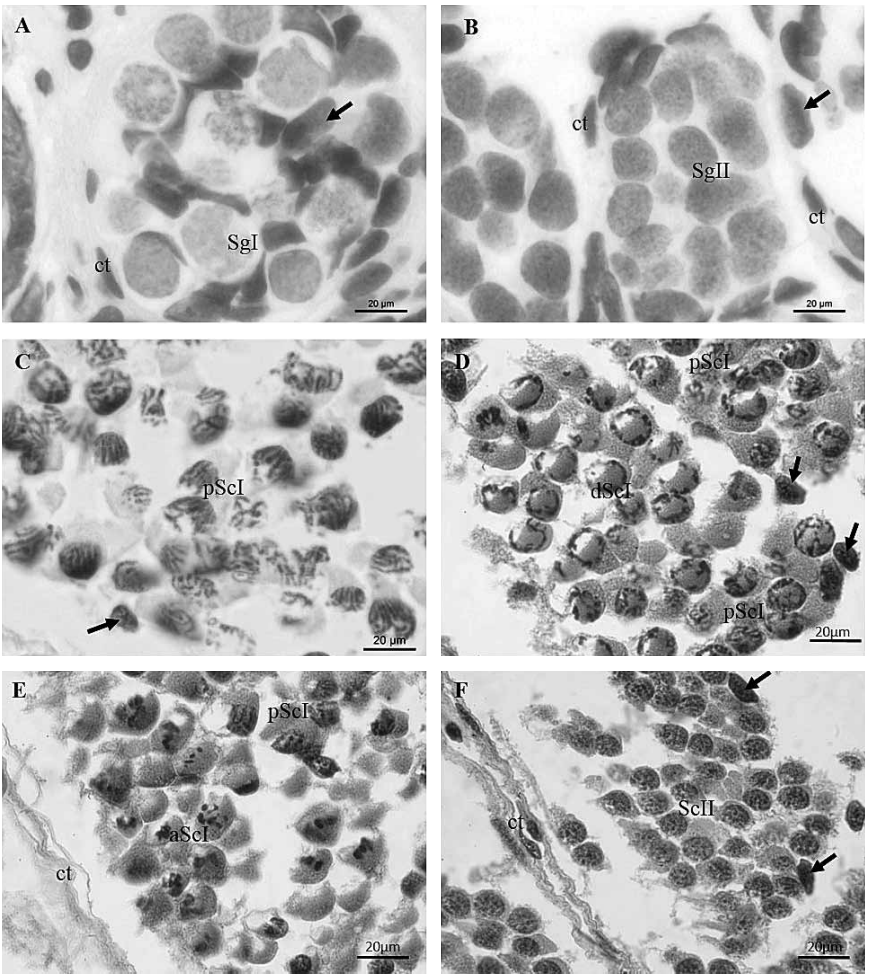

Figure 3: Spermatogonia and spermatocytes in the testis of *Proteus anguinus*. **A –** Group of primary spermatogonia (SgI) in connective tissue of the testis. **B –** Cluster of secondary spermatogonia (SgII) in cyst. **C – D -** Primary spermatocytes in pachytene (pScI, **Fig. C**) and diplotene stage of prophase I (dSc, **Fig. D**). **E** - Primary spermatocytes in anaphase I (aScI). **D** – Secondary spermatocytes (ScII). Arrow – nucleus of Sertoli cell, ct – the nuclei of the connective tissue between lobules. Feulgen staining (**A–C**) and H&E staining (**D-F**).

Slika 3: Spermatogoniji in spermatociti v testisu proteusa *Proteus anguinus*. **A –** Skupek primarnih sprermatogonijev (SgI) v vezivnem tkivu testisa. **B –** Gruča sekundarnih spermatogonijev (SgII) znotraj ciste. **C – D –** Primarni spermatociti v pahitenu profaze I (pScI, **Fig. C**) in v diplotenu profaze I (dSc, **Fig. D**). **E** – Primarni spermatociti v anafazi I (aScI). **D** – Sekundarni spermatociti (ScII). Puščica – jedro Sertolijeve celice, ct – vezivno tkivo med lobuli. Barvanje Feulgen (**A–C**) in barvanje H&E (**D-F**).

Spermatids

Early spermatids (Sd), immediately following telophase II, have small round, haploid nuclei (10 - 11 μm) and densely packed chromatin (Fig. 4A). The amount of cytoplasm is minute. They gradually transform during spermiogenesis and become progressively elongated with long, thin nuclei (Figs. 4B-D). The centrosome are clearly visible at the posterior end of the nuclei, at the site of emerging flagella (Fig. 4C). The mature spermatids are arranged in bundles and are attached to the Sertoli cells by their apical parts (Figs. 4E-F). During early stages the Sd are enclosed within a cyst, while in later stages the cyst wall disintegrates and spermatids are distributed throughout the lobules.

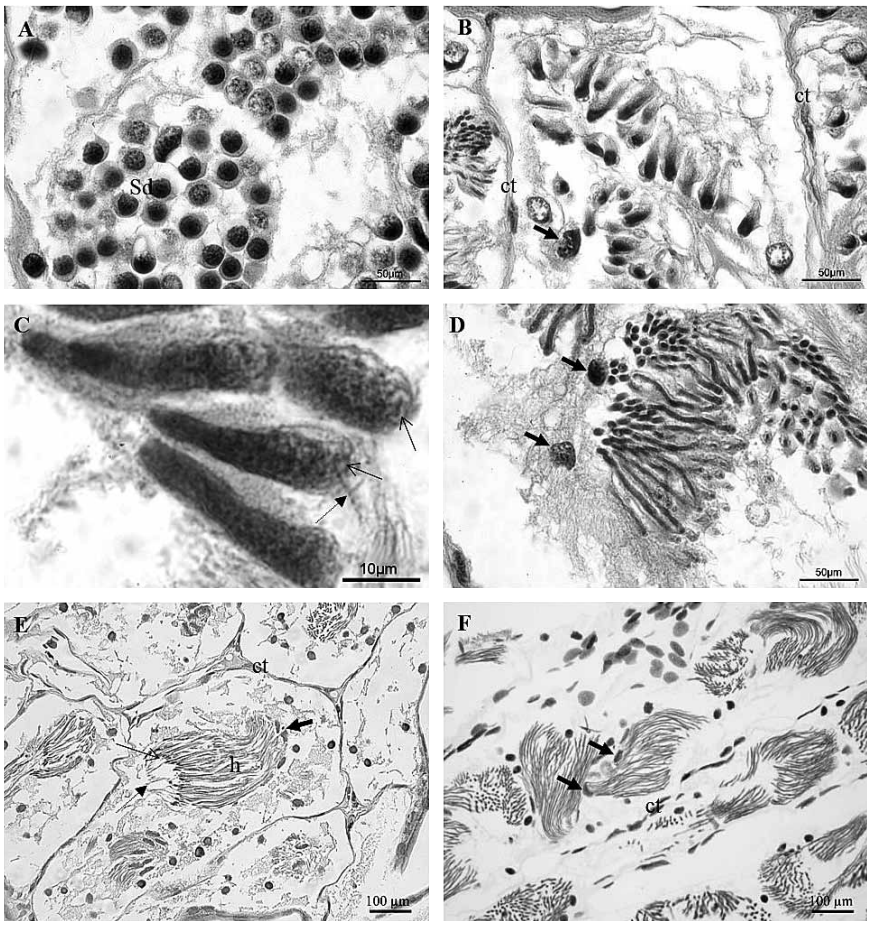

Figure 4: Spermatides in the testis of *Proteus anguinus.* **A –** Early spermatids (Sd). **B –** Stage of elongation of spermatids. **C**- Sd in the elongation process at higher magnification with lighter colored centrioles (open arrow) and protruding flagella at the proximal end of nucleus (closed arrow). **D** – Elongated Sd. **E** – A bundle of mature spermatids. **F** – Feulgen stained nuclei of mature spermatids. Thick arrows – nucleus of Sertoli cell, open arrows – centrosome, closed arrow – flagella, ct – connective tissue between lobules, h – heads of mature spermatids. H&E staining (**A-E**).

Slika 4: Spermatide v testisu proteusa *Proteus anguinus.* **A –** Zgodnje spermatide (Sd). **B –** Faza podaljševanja spermatid. **C**- Spermatida v fazi podaljševanja pod večjo povečavo s svetleje obarvanim centriolom (odprta puščica) in bičkom na proksimalnem koncu jedra (zaprta puščica). **D** – Podaljšane spermatide. **E** – Zrele spermatide. **F** – Jedra zrelih spermatid barvana po Feulgenu. Debele pu**ščice** – jedro Sertolijeve celice, odprte puščice – centrosom, zaprta puščica – biček, ct – vezivno tkivo med lobuli, h – glave zrelih spermatid. Barvanje H&E (**A-E**).

Spermatozoa

Early spermatozoa (Sz) have a distinct, elongated head, midpiece and tail region (Fig. 5A). The darkly stained nuclei are extremely thin and elongated. Spermatozoa belonging to one former cyst are oriented with the heads in the same direction towards the Sertoli cells (Fig. 5A) and gradually elongate and form tighter bundles (Fig. 5B).

The heads of mature sperm are very long (~ 350 µm) and extremely thin. Staining with Feulgen stain showed increased concentration of chromatin at the base of the nuclei (Fig. 5C), and Trichrome staining indicated the presence of a long acrosome at the apical end of the spermatozoan head (Fig. 5D).

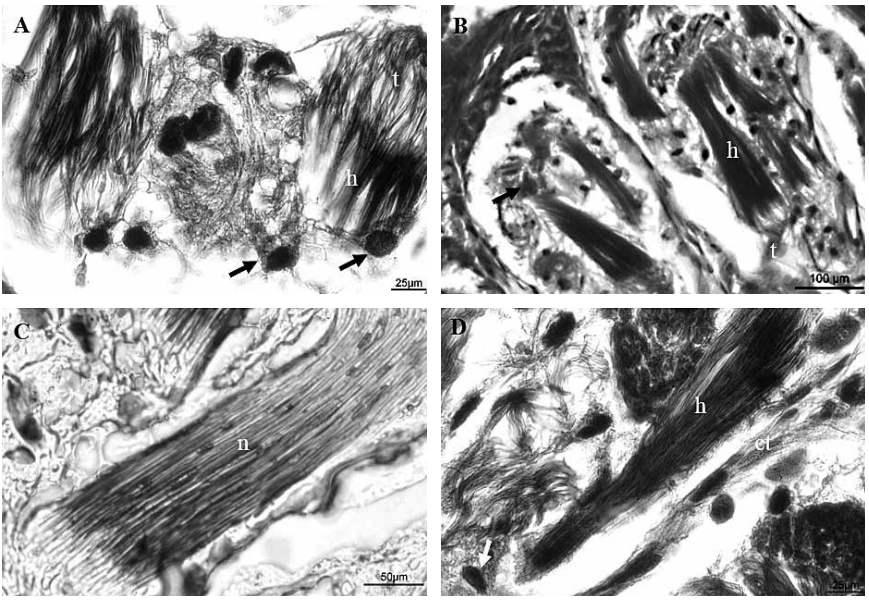

Figure 5: Spermatozoa in the testis of *Proteus anguinus.* **A –** Early spermatozoa. **B –** Mid-stage elongated spermatozoa. **C –** Feulgen stained nuclei of mature spermatozoa. **D –** Apical parts of mature spermatozoa heads with acrosomal vesicles darkly stained with Trichrome staining. Arrows – nucles of Sertoli cell, ct – connective tissue between lobules, h – heads of spermatozoa, n – nuclei, t - tails of spermatozoa. Trichrome staining (**A, B**).

Slika 5: Spermatoziji v testisu proteusa *Proteus anguinus.* **A –** Zgodnji spermatozoji. **B –** Podaljšani spermatozoji. **C –** Jedra zrelih spermatozojev barvana po Feulgenu. **D –** Apikalni deli zrelih spermatozojev s temneje obarvanimi akrosomskimi vezikli (rdeče). puščice - jedro Sertolijeve celice, ct – vezivno tkivo med lobuli, h – glave spermatozojev, n – jedra, t – repi spermatozojev. Trikromno barvanje (**A, B**).

Meiotic Condition of Different Morphological Forms of Testis

The different morphological forms of testes (SNT, NLT, MLT and BLT) have different meiotic conditions (Table 1), which is reflected in their external and internal morphology. Otherwise, all of the testes show the same general histological organization.

In the simple narrow testes (SNT) only the early stages of spermatogenesis, primary and secondary spermatogonia (SgI and SgII), are present, and occasional mitosis is observed among the SgI (Fig. 6A). Lobules are uniform throughout the whole SNT testis. The lobules are small, similar in size, and closely apposed one to the other. The number of cells in the cysts is larger in the caudal part of testis as compared to the cephalic part. In the SNT testes with a wider posterior part, the lobules in this region are larger and a lumen is already formed (Fig. 6B). The walls of the lobules contain very young cysts including

SgII, and the lobules are larger with an increased number of SgII in the cysts at the most caudal part of the enlargement (Fig. 6C). In a few cases the lobules also include spermatocytes (Sc). SgI always lie in the midline of the testis around the intratesticular ducts of the SNT testes.

The narrow single-lobed testes (NLT) and multilobed testes (MLT) contain pachytene stage ScI at the posterior region of the lobe, the lobules are wider, and cysts are not well defined (Fig. 7A). The slightly narrower anterior region of the lobe contains SgII and scarce Sc, and the lobules are elongated with SgI at the base of each lobule (Fig. 7B). The narrow parts at either end in NLT and MLT testes, as well as the regions between the lobes in MLT testes, have the same appearance of lobules as SNT testes (Fig. 6A), and contain only spermatogonia (SgI and SgII).

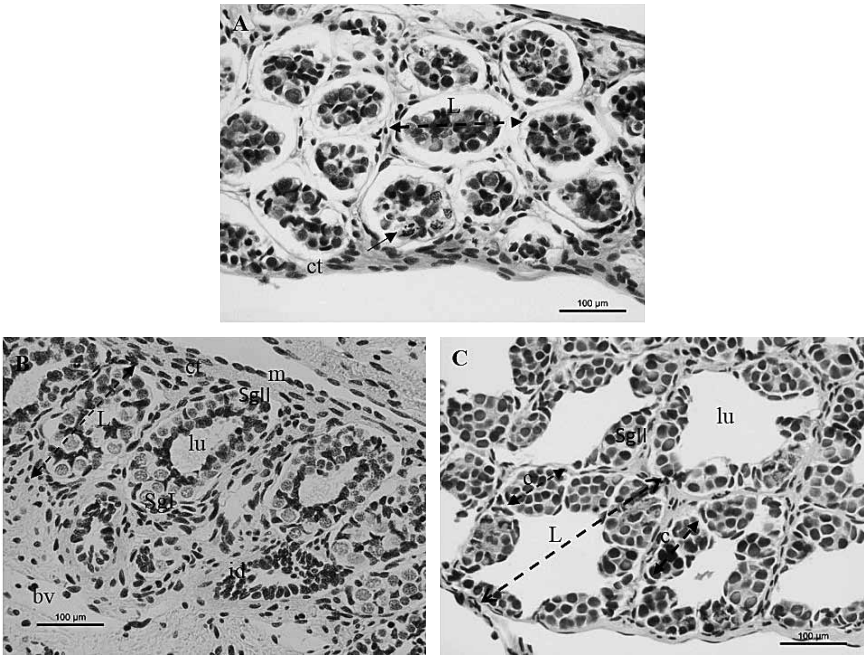

Figure 6: Histology of the simple narrow (SNT) testis of *Proteus anguinus.* **A** – Lobules of an evenly wide SNT testis with early stages of spermatogenesis. **B** - Lobules with cavity in an SNT testis with wider posterior part. **C** – Lobules of the caudal region of an SNT testis with wider posterior part. arrow – mitotic figures in Sg, bv – blood vessel, c – cyst, ct – conective tissue of tunica albuginea, it - intratesticular ducts, L – lobule, lu – lumen of lobule, m - mesothelium. H&E staining.

Slika 6: Histologija preprostega ozkega testisa (SNT) pri proteusu *Proteus anguinus.* **A** – Lobuli z zgodnjimi fazami spermatogeneze v enakomerno širokem testisu (SNT). **B** – Lobuli z lumnom v SNT testisu s širšim posteriornim delom. **C** – Lobuli kavdalne regije SNT testisa s širšim posteriornim delom. „ – mitotične figure v Sg, bv – krvna žila, c – cista, ct –tunica albuginea, it – intratestikularni vodi, L – lobul, lu – lumen lobula, m – mezotelij. Barvanje H&E.

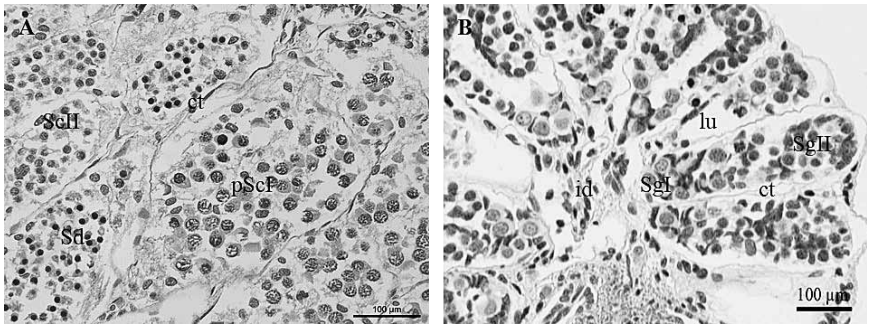

Figure 7: Histology of the lobed testes (NLT and MLT) of *Proteus anguinus.* **A –** Posterior region of a lobe of the testis with pachytene spermatocytes (pScI), secondary spermatocytes (ScII) and early spermatids (Sd). **B** – Anterior region of a lobe of the testis with spermatogonia (SgI and SgII). ct – connective tissue of the lobule, id - intratesticular ducts, lu – lumen of the lobule. Feulgen staining (**A**) and H&E staining (**B**).

Slika 7: Histologija testisov z razširitvami (NLT in MLT) pri proteusu *Proteus anguinus.* **A –** Posteriorna regija razširitve testisa s spermatociti v pahitenu I (pScI), sekundarnimi spermatociti (ScII) in zgodnjimi spermatidami (Sd). **B** – Anteriorna regija razširitve testisa s spermatogoniji (SgI and SgII). ct – vezivno tkivo med lobuli, id - intratestikularni vodi, lu – lumen lobula. Barvanje Feulgen (**A**) in barvanje H&E (**B**).

Broad single-lobed testes (BLT) contain the full range of meiotic stages including spermatids (Sd) and spermatozoa (Sz) (Figs. 8A-C). Spermatogenesis evidently progresses in a caudal-cephalic direction. The cephalic region of the testis contains mostly pachytene ScI (Fig. 8A), the middle region of the testis contains Sc at different stages of meiosis (Fig. 8B), and the caudal region of the testis is packed with cells undergoing spermiogenesis with progressive elongation of spermatids (Fig. 8C). The BLT testes of one animal also contained mature spermatozoa.

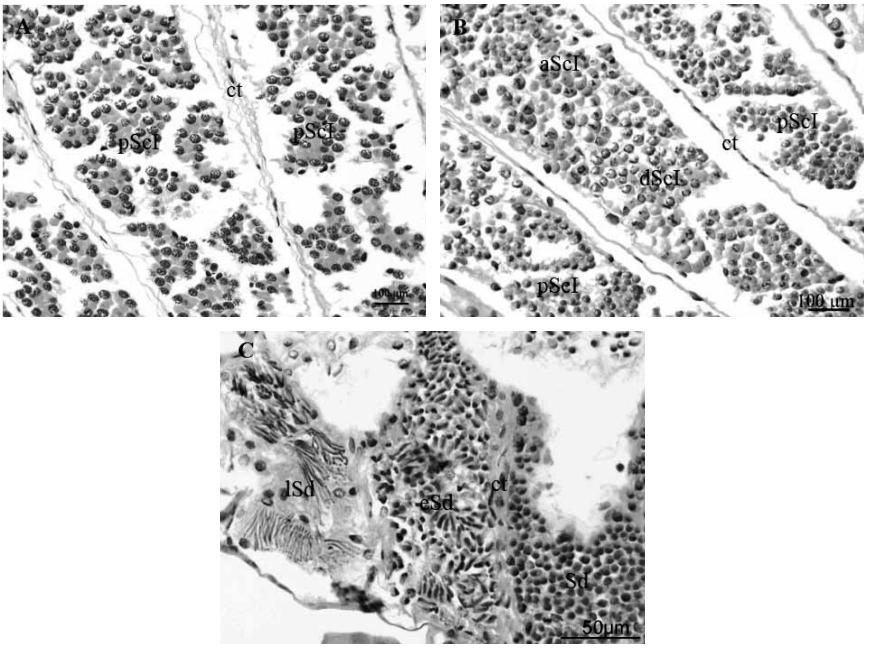

Figure 8: Histology of a broad single-lobed (BLT) testes of *Proteus anguinus.* **A -** The cephalic region of the testis with cysts of pachytene spermatocytes (pScI). **B –** Middle region of the testis with diplotene (dScI) and anaphase spermatocytes (aScI), as well as with first spermatids (Sd). **C**- The caudal region of the testis with evident spermiogenesis. Individual lobules include early spermatids (Sd), elongated spermatids (eSt), late spermatids (ISt). ct – connective tissue between lobules. H&E staining.

Slika 8: Histologija širokega testisa (BLT) pri proteusu *Proteus anguinus.* **A –** Cefalična regija testisa s spermatociti v pahitenu I (pScI). **B –** Osrednja regija testisa z diplotenimi (dScI) in anafaznimi spermatociti (aScI), ter maloštevilnimi spermatidami (Sd). **C**- Kavdalna regija testisa z lobuli v spermatogenezi. Posamezni lobuli vključujejo zgodnje spermatide (Sd), podaljšane spermatide (eSt) in zrele spermatide (ISt). ct – vezivno tkivo med lobuli. Barvanje H&E.

The relative mass of the testes (n = 6), expressed as gonadosomatic index (GSI = [testis weight/total body weight] x 100), appears to be positively correlated with meiotic stages (Fig. 9A); low GSI corresponds to early meiotic stage testes and high GSI to late meiotic stages. Although sample size is very small, this relationship suggests that testis mass increases as a proportion of total body mass as meiosis progresses.

A comparison of meiotic stages in the testes from specimens collected from different seasons of the year (Fig. 9B, Table 1) shows that individuals with immature (SNT) testes containing only Sg stages, were found in all seasons of the year except for early Spring (March). However, no animals were available for late Spring (April and May) and early Summer (June). Individuals with testes containing later stages of spermatogenesis (NLT, MLT, BLT) were found from sequentially later times of the year: spermatocytes were found only in individuals collected from Fall to Spring, and spermatids were found only in individuals collected from Summer to Fall. In our sample, only one individual, collected in Winter, had fully mature (BLT) testes containing spermatozoa (Table 1, Fig. 9B).

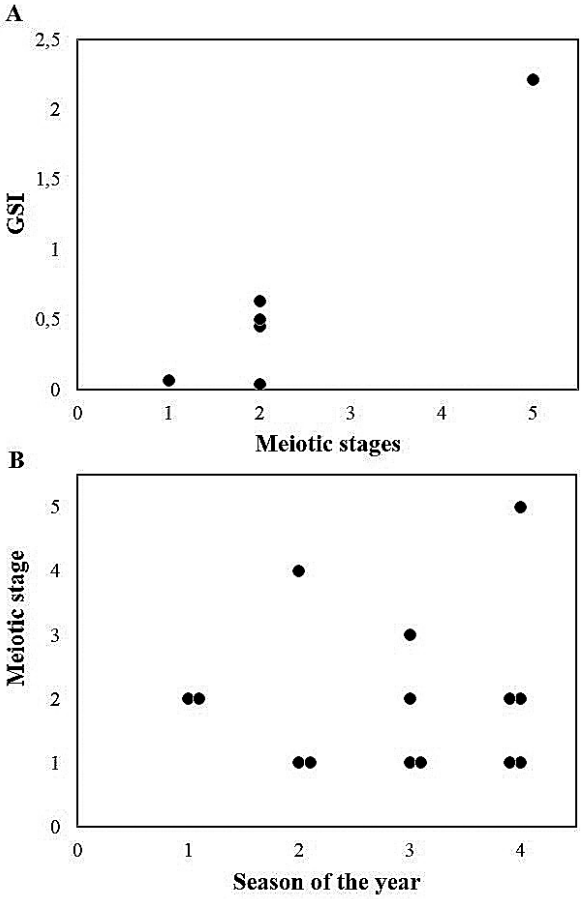

Figure 9: **A -** Correlation of gonadosomatic index (GSI) with meiotic stage in testes of *Proteus anguinus*. **B** – Comparison of meiotic stages of testes of *Proteus* with different seasons of the year. Meiotic stages: 1 – primary spermatogonia, 2 – spermatocytes, 3 – early spermatids, 4 – late spermatids, 5 – spermatozoa. Seasons of the year: 1 - Spring, 2 - Summer, 3 - Fall, 4 – Winter.

Slika 9: **A –** Korelacija med gonadosomatskim indeksom (GSI) in fazami mejoze v testisih proteusa *Proteus anguinus*. **B** – Primerjava faz mejoze v testisih proteusa z sezonami leta. Faze mejoze: 1 – primarni spermatogoniji, 2 – spermatociti, 3 – zgodnje spermatide, 4 – zrele spermatide, 5 – spermatozoji. Letni časi: 1 - pomlad, 2 - poletje, 3 - jesen, 4 – zima.

Testis-ova

Individual oocytes were observed in the testes of *Proteus* regardless of the morphology or meiotic condition of the testes. These testis-ova were found in approximately 30% of the sampled testes (Table 1). They were located randomly among spermatogonia or spermatocytes inside the lobules (Fig. 10A). The testis-ova were at diplotene stage with distinctly visible lampbrush chromosomes and numerous nucleoli (Fig. 10B), and with total cellular diameter betwen 58 and 120 µm, which is consistent with Stage III (early vitellogenic) oocytes in the normal ovary of *Proteus* (Bizjak Mali et al. 2013, 2015).

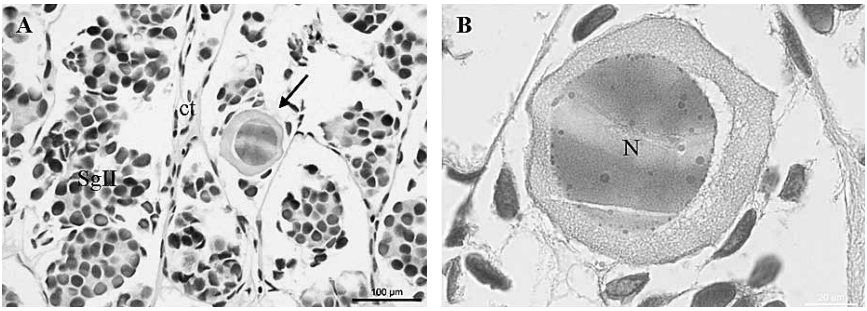

Figure 10: Oocytes in the testis of *Proteus anguinus*. **A –** A testis-ovum (arrow) in diplotene stage of meiosis among the groups of secondary spermatogonia (SgII). **B** – A testis ovum at higher magnification. N – nucleus with lampbrush chromosomes and nucleoli. H&E staining.

Slika 10: Oociti v testisih proteusa (*Proteus anguinus*). **A –** Oocit (puščica) v diplotenu mejoze I med sekundarnimi spermatogoniji (SgII). **B** – Oocit pri večji povečavi. N – jedro s krtačastimi kromosomi in jedrci. Barvanje H&E.

Discussion

The goals of this study of *Proteus* testes were three-fold, first to perform a detailed description of the morphology of the testis and meiotic stages, secondly to determine whether there is any correlation between testes morphology and body size in this long lived salamander, and third, to determine whether spermatogenesis in this cave animal shows any seasonal patterns. Since body length may reflect sexual maturity (age) in salamanders, as we found in female *Proteus* (Bizjak Mali et al. 2010, 2013), I expected to find a positive correlation between testis size and maturation state with body length in *Proteus*. Furthermore, since *Proteus* lives in stable cave environments with no extreme fluctuation in temperature, I also expected that the mature testes would show a non-seasonal pattern of spermatogenesis, as seen in other amphibian species living in constant temperature environments (Chan 2003, Ogielska and Bartmanska 2009), including female proteus (Bizjak Mali et al. 2010, 2013).

Testis morphology and meiotic condition are obviously highly variable in *Proteus*. Even though the sample size is small, the data show that testis size (length and width) is positively correlated with total body length of the animal. However, the meiotic state of the testis (Table 1) is not correlated with body length, and immature testes were found in even one of the larger animals (246 mm in total length). Nor is testes morphology or meiotic state correlated with locality (Table 1). The positive correlation seen between the meiotic state of the testis and testis mass, expressed as proportion of total body mass (GSI), indicates simply that testes increase in mass relative to overall body mass as meiosis progresses.

The basic internal structure of the testes of *Proteus* is similar to that described for other urodele amphibians (Humphrey 1922, De Sa and Berois 1986, Callard 1992, Pierantoni et al. 2002, Uribe 2003, 2009, Flament et al. 2009, Uribe and Mejía-Roa 2014). The interior of the testis of *Proteus* is divided into numerous lobules which includes cysts, with synchronous maturation of the germ cells within each cyst. A single cyst is the primary germ unit of the amphibian testis and is established when the Sertoli cell engulfs two daughter cells derived from a single primary spermatogonium SgI (Callard 1992, Pierantoni et al. 2002). SgI multiply mitotically to give rise to secondary Sg which eventually differentiate more-or-less synchronously to produce meiotic spermatocytes. The ScII then form mature haploid spermatids which elongate and transform into spermatozoa through the process of spermiogenesis. As in other urodeles, spermatogenesis in *Proteus* testes progresses in a caudo-cephalic »wave« along the length of the testis, i.e. the earlier stages of meiosis are found in the more cranial lobules while the later stages are present in more caudal lobules. The reason for

this universal pattern is not currently understood. Spermatogenesis in *Proteus* testes also shows a medial-lateral pattern of differentiation in relation to internal collecting ducts in the midline of testes, such that cells form a differentiation series from Sg in the center of the testes to more mature stages found in the peripheral parts. This explains expansion of the testis in diameter over time. Finally, when the spermatogenic process in other urodeles nears completion, most of the testis becomes full of the mature stages of spermatogenesis (spermatozoa). Unfortunately, we do not know if this happens in *Proteus* since the most mature testes we have examined contain these mature stages, including spermatozoa, only at the most posterior part while the middle part is full of Sc in prophase I.

Generally, the spermatozoa of urodeles are longer, including proportionately longer heads, than those of other vertebrates, including other amphibians (Scheltinga and Jamieson 2003, Uribe and Mejía-Roa 2014). The size of the head of *Proteus* spermatozoa is in the range of the sizes of spermatozoa heads in *Necturus maculosus* (338-366 μm) which, with the total length of 1mm, are the longest spermatozoa among the urodeles (Scheltinga and Jamieson 2003). The very large size of the sperm heads in proteid salamanders is probably directly related to chromosome and genome size, which are large in both even compared to other salamanders (Macgregor and Walker 1973, Sessions 2008, Sessions et al. 2016).

In most urodeles, spermatogenesis is initiated cyclically, closely correlated with the seasons of the year (Uribe 2003), and changes in testis size reflect spermatogonial activity with spermatozoa present only before breeding. On the contrary, amphibians living in constant temperature environments show continuous spermatogenesis that does not correlate with seasons of the year (Chan 2003, Ogielska and Bartmanska 2009). In these species, breeding activity is potentially continuous and spermatozoa are present in the testes all year round. Results of this study suggest that, contrary to my prediction, meiotic activity in male *Proteus* shows a seasonal pattern (Table 2), but this pattern is partly obscured by the variability of the meiotic stages seen in the testes between individuals in the sample. Adult males with immature testes full of spermatogonia were found from Summer through Winter. Individuals with spermatocytes in their testes were only found from Fall through Spring, and individuals going through spermiogenesis were found from Summer through Fall. Only one individual, collected in December, had spermatozoa in its testes. Thus, despite this seasonal trend, at any given time of the year it is possible to find adult males with testes representing a wide range of maturation (as noted also by Kezer, 1962). It seems likely that part of the reason for this overlap in meiotic condition is that the rate of spermatogenesis could be very slow in *Proteus*, with at least a two-year cycle in which early stages are always present and later stages only occur from later summer to

Table 2: Interpretation of the sequential meiotic condition of the sampled testes of *Proteus anguinus* correlated with seasons over two years. One meiotic cycle extends from summer of the first year to winter of the second year.

Tabela 2: Interpretacija zaporedja faz mejoze v dvoletnem ciklu pri vzorčenih živalih proteusa *Proteus anguinus*. En cikel mejoze poteka od poletja prvega leta do zime naslednjega leta.

Meiotic stage	Summer	Fall	Winter	Spring	Summer	Fall	Winter
Sg	■	■	■				
Sc		■	■	■			
Sd					■	■	
Sz							■

Legend: Sg – spermatogonia, Sc – spermatocytes, Sd – spermatids, Sz – spermatozoa.
Legenda: Sg – spermatogoniji, Sc – spermatociti, Sd – spermatide, Sz – spermatozoji.

mid-winter (Table 2). Possible explanations for this pattern include low temperature and limited nutrient availability in cave environments, but also its large genome size, which is known to slow down cell cycles including gametogenesis (Gregory 2001). To my knowledge, meiotic rate has never been examined in *Proteus*, but it might help explain why reproductive cycles are so slow in this species.

Detection of seasonality in *Proteus* testes is surprising given the fact that *Proteus* lives in a relatively stable habitat with environmental conditions that are conducive to non-seasonal reproductive cycles. Also, we have already reported that oogenesis in female *Proteus* appears to be non-seasonal (Bizjak Mali et al. 2010, 2013), suggesting that gametogenesis in male and female *Proteus* is only loosely synchronous. This may indicate that courtship and insemination occur weeks or months before oviposition. Fertilization in most species of salamanders, including *Proteus* (Briegleb 1961), is internal via a spermatophore (a package of spermatozoa, produced by the male's cloaca, which is picked up by the female during courtship). In many species, including its closest living relative the North American mudpuppy, *Necturus maculosus*, the sperm may be stored by the female for long periods of time before actual egg laying (Shoop 1965, Sever 2002, Bruce 2003). These issues underline how incompletely we still understand reproduction in this enigmatic animal.

The testes of most urodeles are either simple with a single lobe (NLT), or multilobed (MLT) with a series of enlargements separated by narrow bridges containing only early germ cells (SgI and SgII) (Humphrey 1922, Sever 1974, Pudney 1995, Pierantoni et al. 2002, Uribe 2003, Exbrayat 2009, Flament et al. 2009, Uribe 2009, Uribe and Mejía-Roa 2014). Multilobed testes in urodeles are known in two other salamander familes, the Salamandridae and Plethodontidae. A similar testis structure has been reported for some species of Gymnophiona (De Sa and Berois 1986, Wake 1986, Smita et al. 2004). In urodeles, the lobes develop successively during adult life and each lobe actually has the structural organization of a miniature testis, and the lobes are morphologically and functionally similar (Humphrey 1922, Uribe 2003, 2009). The formation of the lobes of multilobed testes has been studied in detail in the salamandrid, *Salamandra salamandra* (Humphrey 1922), where the number of lobes is positively correlated with age of the salamander. Old males of *Salamandra* can have up to six testis lobes on each side. Likewise, adult specimens of *Triturus vulgaris* may have up to three to four lobes on each side, and a new lobe is developed every second year.

The results of this study show that *Proteus* has both types of testes described for other urodeles, single-lobed testes (NLT) as well as multilobed testes (MLT) (seen only in *P.a.parkelj* in this study, and in *P.a.anguinus* by Kezer, 1962). Such intraspecific variability in the morphological types of testes is also common for other urodeles (Sever 1974), but their interpretation is not always clear. In *Proteus*, the simple narrow testis (SNT) is clearly an immature testis where spermatogenesis has just started (Sg1 and Sg2 and in few cases also early Sc) while the other morphological forms of testis (NLT, BLT, and MLT) represent different maturation states with different stages of spermatogenesis and/or spermiogenesis.

It is interesting that these different testis morphologies, presenting different meiotic states, are not correlated with body length (age) in male *Proteus*. Working at the Moulis cave lab with *Proteus*, Durand and Delay (1981) reported that males become sexually mature at a total body length of 140 to 180 mm, but that they do not start to reproduce until they reach 200 to 240 mm total length. I examined the testes of one of their smaller specimens of known age that was born in that cave lab (151 mm total length, 8 years old) and it had a well-developed NLT testes full of pachytene ScI. The males with immature, SNT testes in my research were substantially larger, in the range of body length between 210 do 253 mm, but their testes were obviously just at the beginning of the spermatogenetic process. These results demonstrate that body size is a poor predictor of reproductive state in *Proteus* males. Instead, we conclude that the testes of *Proteus* males contain the earliest stages of spermatogenesis regardless of body size.

NLT testes with a single lobe were also reported by Kezer (1962) for white proteus and we found them in both subspecies of *Proteus*. The BLT testes, found only in white proteus from two different populations sampled (SW and SE

Slovenia), are similar in overall appearance to the fully formed testes of the other member of the family Proteidae, the mudpuppy *Necturus maculosus* (Pudney and Callard 1984), but much smaller. The simplest explanation for these two sub-types of single-lobed testis (NLT and BLT) in *Proteus* is that they represent early and late stages, respectively, in the normal progression of testis development. In other words, the NLT testes simply enlarge to become BLT testes as the germ cells proliferate and differentiate so that, at least in the white proteus, the testes form a maturation series of SNT-NLT-BLT. The two-lobed MLT testis, which we found only in *P. a. parkelj*, suggests that the series in the black subspecies is ST-MLT. But MLT testes were also reported in white proteus by Kezer (1962), which makes interpretation more difficult. Do the lobes represent individual differences that vary over time, or do the lobes eventually fuse somehow to generate a single-lobed testis? It does not seem plausible that they fuse since each lobe contains an independent, caudal-cephalic series of meiotic stages. On the other hand, fusion of lobes was described for some species of Gymnophiona (Exbrayat 2009, Wake 1986). A better understanding of these differences in morphological forms of testes will require examination of additional specimens of both subspecies of *Proteus*.

Perhaps the most remarkable discovery in this study is the high frequency of testis-ova in the testes of *Proteus*. The morphology of the testis-ova and the presence of lampbrush chromosomes and nucleoli in their nuclei confirm that these are viable, developing oocytes at the diplotene stage of maturation. The testis-ova were observed in 30% of the testes, as well as in all morphological forms of testes described (SNT, NLT, MT, BLT), and were usually located among groups of spermatogonia and spermatocytes. Testis-ova have been described for developing testes in the juvenile stages of some anuran species (Kobayashi and Iwasawa 1976, Ogielska and Bartmanska 1998, 2009, Kobayashi et al. 2014, Lambret et al 2015, Griffing et al. 2017). The presence of testis-ova in testes are usually interpreted as the result of dysfunction of hormonal control (Ogielska and Bartmanska 2009) and has even been linked to environmental endocrine disruptors (Hayes et al 2002, 2011, Hecker et al. 2006, Kosai et al. 2011, Kobayashi et al 2014, Lambret et al 2015). We consider that in *Proteus* these abnormalities might be related to current evidence that *Proteus* has undergone a sex-chromosome turnover involving an X-Y translocation (Sessions et al. 2016). Such a translocation could disrupt the function of genes involved in sex determination through "position effects" (Dimitri and Pisano 1989). In other words, an X-Y translocation can disrupt sex determination and cause gender ambiguity. In clinical cases in humans (Ferguson-Smith 1966), for example, it leads to the same kinds of abnormalities in the ovaries and especially the testes that we are seeing in *Proteus*, especially testis-ova and hermaphrodites (Bizjak Mali, unpublished observation).

In conclusion, considering the vulnerable status of *Proteus*, we need solid baseline studies, especially on its reproductive biology, in order to detect abnormalities that could be induced by environmental degradation. Further work should be done on archived collections to generate additional information about the significance of morphological variation in the testes in *Proteus*, especially in regard to the multi-lobed testes and whether gametogenesis in *Proteus* is really seasonal. It will also be important to determine if there are differences between *P. a. anguinus* and *P. a. parkelj*, since the phylogenetic relationship between these subspecies is not fully understood (Gorički and Trontelj 2006). Finally, in anticipation of the eventual need for a captive breeding program for this endangered species, these data could lead to the development of protocols to induce spermatogenesis and reproduction in *Proteus*.

Povzetek

V raziskavi smo se osredotočili na morfologijo gonad odraslih samcev proteusa *Proteus anguinus*, in zrelost gonad glede na velikost živali in sezono. Raziskava je vključevala testise osebkov obeh podvrst proteusa, troglomorfne bele podvrste *P. a. anguinus* in ne-troglomorfne podvrste *P. a. parkelj*, z razponom dolžine telesa od 210 do 360 mm. Vzorec je vključeval tudi različne populacije bele podvrste *P.a.anguinus,* in sicer iz jugozahodnega dela Slovenije, kot tudi različnih lokalitet jugovzhodnega dela (Tabela 1).

Raziskava je razkrila, raznoliko morfologijo testisov pri proteusu, ki je popolnoma neodvisna od velikosti živali. Zastopane so vsaj štiri morfološke oblike testisov (SNL, NLT, BLT in MLT) (Slika 1A-D, Tabela 1), ki predstavljajo različne zrelostne faze gonad. Preprosti ozki testisi (SNL) so nezrele gonade z zgodnjimi fazami spermatogeneze (primarni in sekundarni spermatogoniji). Imeli so jih tako manjši kot tudi večji samci. Ostale morfološke oblike (NLT, BLT in MLT) so odrasla oblika gonad na različnih stopnjah spermatogeneze. Ozki testisi z enim režnjem oziroma razširitvijo (NLT) in testisi z več razširitvami (MLT) so vključevali večinoma spermatocite in tudi spermatide, široki testisi (BLT) pa so vključevali spermatide in tudi spermatozoje. Vse tri oblike testisov (NLT, BLT in MLT) so bile zastopane pri različnih dolžinah telesa.

Osnovna notranja zgradba testisov proteusa je podobna ostalim repatim dvoživkam. Notranjost testisa je predeljena v številne lobule, ki vključujejo ciste s sinhrono zoritvijo spolnih celic v vsaki od cist. Spermatogeneza poteka v kavdalno – cefalični smeri testisa, z zrelejšimi fazami v kavdalnem delu testisa in zgodnjimi fazami mejoze v cefaličnem delu. Različne morfološke oblike testisov (SNL, NLT, BLT in MLT) se razlikujejo glede na zastopanost faz mejoze, kar se odraža tudi v organizaciji in velikosti lobulov v testisih, in nenazadnje v velikosti in zunanji morfologiji testisov.

Morfološki obliki testisov SNT in NLT smo našli pri obeh podvrstah *P. a. anguinus* in *P. a. parkelj*, in BLT testis samo pri podvrsti *P. a. anguinus.* Testis MLT pa so imeli le osebki črne podvrste *P.a.parkelj*, vendar slednjo omenja tudi Kezer (1962) pri beli podvrsti *P. a. anguinus*. Različne morfološke oblike testisov pri proteusu so lahko odraz progresivnega procesa zoritve spolnih celic. Predvidevamo, da se NLT testis v procesu proliferacije celic in njihove diferenciacije postopamo poveča, kar vodi v oblikovanje BLT testisa. Zaporedje razvoja testisa od SNT preko NLT do BLT je zastopano vsaj pri beli podvrsti *P. a. anguinus*. Pri črni podvrsti *P. a. parkelj* pa je videti, da je zaporedje razvoja in zoritve gonad od SNT do MLT testisa. Zelo verjetno, sta testisa BLT in MLT različna morfološka tipa testisov pri proteusu. Variabilnost v morfologiji testisov v okviru iste vrste je poznana tudi za nekatere druge urodele.

Nasprotno od našega predvidevanja, da spermatogeneza ni v korelaciji s sezono, smo pri samcih našli sezonski vzorec mejotske aktivnosti oziroma spermatogeneze. Nezrele gonade z zgodnjimi fazami spermatogeneze (SgI in SgII) so imeli osebki poletnega obdobja pa vse do zime, spermatociti (Sc) so bili v testisih osebkov jesenskega obdobja pa vse do pomladi, spermiogenezo (proces zoritve spermatid v zrele spermatozoje) so imeli osebki poznega poletnega in jesenskega obdobja, pri osebku iz zimskega obdobja pa so testisi vključevali tudi spermatozoje (Slika 9B). Mejotična aktivnost v testisih preučevanih samcev je zelo raznolika, vendar tudi izrazito prekrivajoča, kar je lahko nenazadnje odraz upočasnjene spermatogeneze z najmanj dve letnim ciklom zoritve spolnih celic (Tabela 2). Slednje bi lahko razložili z nizkimi temperaturami v jamskem okolju, omejeno razpoložljivostjo hrane, vlogo pri tem pa ima najverjetneje tudi velikosti genoma proteusa (slednji je med večjimi v primerjavi z ostalimi urodeli), ki dodatno upočasnjuje celične cikle (Gregory 2001), vključno z gametogenezo. Sezonska aktivnost spermatogeneze pri proteusu je sicer presenetljiva, glede na to, da živijo v okolju z dokaj stabilnimi abiotskimi dejavniki, prav tako pa se zastavlja vprašanje sinhronosti gametogeneze samcev in samic. Možno je, da se paritev in oploditev pri proteusu dogodi tedne ali pa celo mesece pred odlaganjem jajčec, kot je to značilno za njegovega najbližnjega sorodnika severno ameriškega nektura *Necturus maculosus*, in mnoge druge repate dvoživke.

Presenetljivo je tudi odkritje oocitov v testisih proteusa, ki so zastopani pri tretjini preučevanih živali, neodvisno od morfologije in zrelosti testisa. Oociti ali testis-ova so v lobulih testisa bodisi med spermatogoniji ali pa spermatociti. Po morfologiji in krtačastih kromosomih v jedrih ustrezajo diplotenim oocitom v jajčnikih samic in so videti popolnoma viabilne celice. Testis-ova navajajo predvsem za razvijajoče testise juvenilnih osebkov pri nekaterih brezrepcih in jih povezujejo s hormonskim neravnovesjem in endokrinimi motilci v okolju. Pri proteusu predvidevamo, da je za oocite v testisih odgovorna translokacija kromosoma Y na kromosom X, ki je bila nedavno odkrita (Sessions et al. 2016). Translokacija lahko moti delovanje genov za determinacijo spola preko t.i. »pozicijskega učinka«. Npr. pri kliničnih

primerih pri človeku povzroča najasnost spola in razvoj različnih abnormalnosti gonad, predvsem prisotnost oocitov v testisih in hermafroditizem, ki je bil nenazadnje najden tudi pri proteusu (Bizjak Mali, neobjavljeno).

Dejstvo je, da je poznavanje reproduktivne biologije pri tej enigmatični dvoživki še vedno nepopolno, in da so nadaljne raziskave neobhodno potrebne, tudi zaradi degradacije njegovega življenskega okolja in prepoznavanja anomalij, saj je proteus zaradi svojih specifičnih prilagoditev na jamsko okolje izredno občutljiva in ranljiva vrsta živali.

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Vzorec osifikacije skeleta pri ličinkah navadne krastače *Bufo bufo*

Ossification patterns of the skeleton in the larvae of the common European toad *Bufo bufo*

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Izvleček: Z uporabo presvetlitvene tehnike in barvanja skeleta za hrustančno in kostno tkivo smo spremljali vzorec osifikacije skeleta pri navadni krastač *Bufo bufo*, katerih ličinke imajo izredno hiter razvoj. Osredotočili smo se na osifikacijo hrbtenice, oplečja in okolčja ter okončin, s poudarkom na osifikaciji njihovih distalnih delov. Ugotovili smo, da so hrustančne zasnove skeleta ob koncu premetamorfoznega obdobja (faza 34) že oblikovane. Prve pokostenitve se pojavijo v prometamorfozi (faza 39) in sicer, sočasno v nevralnih lokih, v diafiznem delu proksimalnih elementov sprednjih in zadnjih okončin in v proksimalnem delu črevnic okolčja. Oplečje je v tej fazi še v celoti hrustančno. Osifikacija je med prometamorfozo bolj intenzivna in postopoma napreduje v kranio – kavdalni smeri vzdolž hrbtenice in od proksimalnih v distalne dele okončin, prav tako se širi iz osrednjega dela dolgih kosti okončin proti epifiznim koncem. Osifikacija prstov poteka v posteriorno – anteriorni smeri glede na os okončine. Tako kot pri večini brezrepcev, je skelet krastače ob zaključku metamorfoznega klimaksa (faza 46) skoraj popolnoma osificiran. Hrustančni ostajajo osrednji dorzalni del hrbtenice in distalni konci prečnih odstavkov vretenc, kavdalni del postsakralne regije, epifizni konci dolgih kosti okončin in karpus ter metakarpus, ter nadlopatici oplečja in sramnici okolčja. Rezultati raziskave so potrdili konzervativnost splošnega vzorca osifikacije skeleta dvoživk, navkljub da je razvoj ličink krastače v primerjavi z ostalimi brezrepci bistveno hitrejši.

Ključne besede: skelet, osifikacija, *Bufo bufo*, presvetlitvena tehnika in barvanje skeleta

Abstract: We used a clearing and staining method for cartilage and bone to analyse the pattern of ossification in the skeleton of the common European toad, *Bufo bufo*, a species with rapid larval development. We focused on the ossification of vertebrae, pectoral and pelvic girdles, and limbs with an emphasis of the ossification in their distal parts. We found that the cartilage primordia of the skeleton were formed by the end of premetamorphosis (Stage 34). The first ossifications appeared in early prometamorphosis (Stage 39) and occurred concurrently in the neural arches, diaphyses of proximal elements of front and hind limbs, and in the proximal part of the ileum. The pectoral girdle was still completely cartilaginous at this stage. Ossification intensifies during prometamorphosis and gradualy progresses in a cranial - caudal direction along the

spine and from proximal to distal parts of the skeleton, as well as to the epiphyses of the long bones. Fingers ossified in a posterio-anterior direction according to the main limb axis. As in most other anurans, the skeleton of *Bufo* is almost completely ossified by the end of the metamorphic climax (Stage 46). At this time, cartilage remains in the dorsal midline of the spine, in the distal parts of transverse processes of the vertebrae, the caudal part of the post-sacral region, as well as in the epiphyses, and carpals and metacarpals of the limbs, and in the suprascapula and the pubis of the girdles. These results show a conserved pattern of ossification in *B. bufo* common to other anurans despite its rapid rate of larval development.

Keywords: skeleton, ossification, *Bufo bufo*, clearing and staining method for skeleton

Uvod

Skelet vretenčarjev se razvije iz treh različnih linij embrionalnega tkiva: somiti paraksialnega mezoderma so zasnova za osni skelet, mezoderm lateralne plošče za skelet okončin in kranialni nevralni greben za škržne loke in obrazni del lobanje (Gilbert 2000). Poznana sta dva glavna načina tvorbe kosti oz. osteogeneze, ki vključujeta transformacijo mezenhimskega tkiva v kostno tkivo ter rezultira v podaljševanju in debelitvi kosti. Neposredna pretvorba mezenhimskega tkiva v kost je t.i. dermalna (intramembranska) osifikacija. Na ta način nastajajo ploščate kosti lobanje in del oplečja (ključnici in klejtruma). Pri endohondralni osifikaciji pa se mezenhimske celice najprej diferencirajo v hrustanec, ki ga postopoma nadomesti kostno tkivo. Z endohondralno osifikacijo nastajajo nevrokranium, splanhnokranium ali visceralni skelet, vretenca, del oplečja (lopatici, krokarnici), okolčje in okončine (Gilbert 2000).

Dolge kosti okončin vretenčarjev kostenijo s perihondralno osifikacijo (pod perihondrijem, ki obdaja hrustančno zasnovo) in z endohondralno osifikacijo. Perihondralna osifikacija poteka pred ali pa sočasno z endohondralno (Carter in sod.1998). Pri sesalcih in ptičih je razvoj dolgih kosti okončin predvsem rezultat endohondralne osifikacije, pri kateri hrustanec služi kot začetni skeletni element, ki ga kasneje nadomesti kostno tkivo (Simsa in Monsonegro Ornan 2007). Pri kuščarjih perihondralna in endohondralna osifikacija potekata sočasno (Carter in sod.1998). Pri dvoživkah prevladuje perihondralna osifikacija okončin (Felisbino in Carvahlo 2002). V tem primeru se zunanji sloji hrustančnih zasnov diferencirajo v pokostnico, ki nalaga kostnino. Tako kost imenujemo periostalna kost. Kostno tkivo se najprej nalaga na centralnih delih diafiz, nato nalaganje med razvojem počasi napreduje proti epifizam, ki dokončno mineralizirajo šele po četrti hibernaciji (Rozenblut in Ogielska 2005). Pri ličinkah brezrepcev se prične osifikacija hrustančnih primordijev okončin ob koncu premetamorfoze (faza 34 po Gosnerju, 1960), je najbolj intenzivna v času prometamorfoze (faze 36-41) in se zaključi v obdobju metamorfoznega klimaksa (faze 42-46) (Rozenblut in Ogielska 2005). Ta tip osifikacije pri dvoživkah napreduje hitreje kot endohondralna osifikacija pri ostalih skupinah vretenčarjev (Gilbert 2000). Ob formaciji periostalne kosti hondrociti hrustančne zasnove hipertrofirajo in so nepravilnih oblik, sledi njihova degeneracija in oblikovanje mezgovne votline, v kateri začne nastajati kostni mozeg (Çiçek in sod. 2011). V kostnem tkivu se oblikujejo prehranjevalni kanali in krvne žile. Diafize se podaljšujejo z longitudinalno rastjo, kar je posledica aktivnosti posebnega dela pokostnice v epifizah, ki nalaga kostno tkivo na svojih terminalnih delih (Rozenblut in Ogielska 2005). Sočasno se kost tudi debeli z nalaganjem kostnega tkiva z robnega dela pokostnice vzdolž celotne dolžine periostalne kosti.

Za razliko od večine vretenčarjev, pri katerih proces osifikacije poteka v fetalnem obdobju, poteka osifikacija pri dvoživkah v postembrionalnem obdobju, torej med razvojem ličink. Navkljub konzervativizmu ontogenetskega razvoja pri vretenčarjih, prihaja do časovnih razlik in razlik v hitrosti dejanskega poteka dogodkov med razvojem (Rieppel 1994). To velja tudi za postembrionalno osifikacijo skeleta brezrepcev, kjer sta časovni potek in hitrost osifikacije skeleta vrstno specifična, medtem ko je osnovni vzorec

osifikacije skeleta ohranjen med različnimi vrstami dvoživk (Kemp in Hoyt 1969, Duellman in Trueb 1986, Rozenblut in Ogielska 2005, Çiçek in sod. 2011, Yildirim in Ugor 2014). Osifikacija skeleta brezrepcev se prične v proksimalnih delih skeleta in postopoma napreduje proti distalnim delom. Pri večini dvoživk osifikacija nastopi proti koncu premetamorfoze in je bolj izrazita v prometamorfozi ter se zaključi pred koncem metamorfoze in prehodom na terestrični način življenja (Dunlap in Sanchiz 1996, Hass 1999, Yildirim in Ugur 2014).

Skelet ličink je kompleksna struktura, ki zagotavlja pomembno informacijo o filogenetskih odnosih brezrepcev (Pugener et al. 2003). Raziskave razvoja in osifikacije skeleta imajo pomembno vlogo pri razumevanju raznolikosti dvoživk in njihovi evoluciji (Yildirim in Ugur 2014). V raziskavi smo se osredotočili na vzorec osifikacije skeleta navadne krastače *Bufo bufo*, saj je razvoj ličink v primerjavi z večino brezrepcev izredno hiter (28 do 31 dni pri sobni temperaturi) (Semlitsch 1994). Npr. pri žabi krempljarki *Xenopus laevis* poteka razvoj ličink okoli 58 dni (Nieuwkoop in Faber 1956) in pri žabi *Rana pipiens* 75 do 90 dni (Taylor in Kollros 1946). Namen raziskave je opisati zaporedje in časovni potek osifikacije skeleta ličink navadne krastače in primerjati s poznanimi podatki za ostale brezrepce, s poudarkom na osifikaciji distalnih delov okončin, saj v literaturi za to vrsto dvoživke ni poznanih podatkov.

Metode in materiali

Potek osifikacije skeleta smo preučili v različnih razvojnih fazah ličink navadne krastače *Bufo bufo,* ki smo jih določili v skladu s tabelo faz razvoja za brezrepce po Gosnerju (1960). Embrionalni razvoj vretenčarjev in razvoj ličink dvoživk namreč sledi zaporedju dogodkov, ki so ohranjeni med osebki iste vrste, kot tudi med sorodnimi vrstami (Wake in Roth 1989). Na osnovi konzervativnosti razvoja pa so oblikovane tabele razvojnih stadijev, ki temeljijo na zunanjih morfoloških lastnostih.

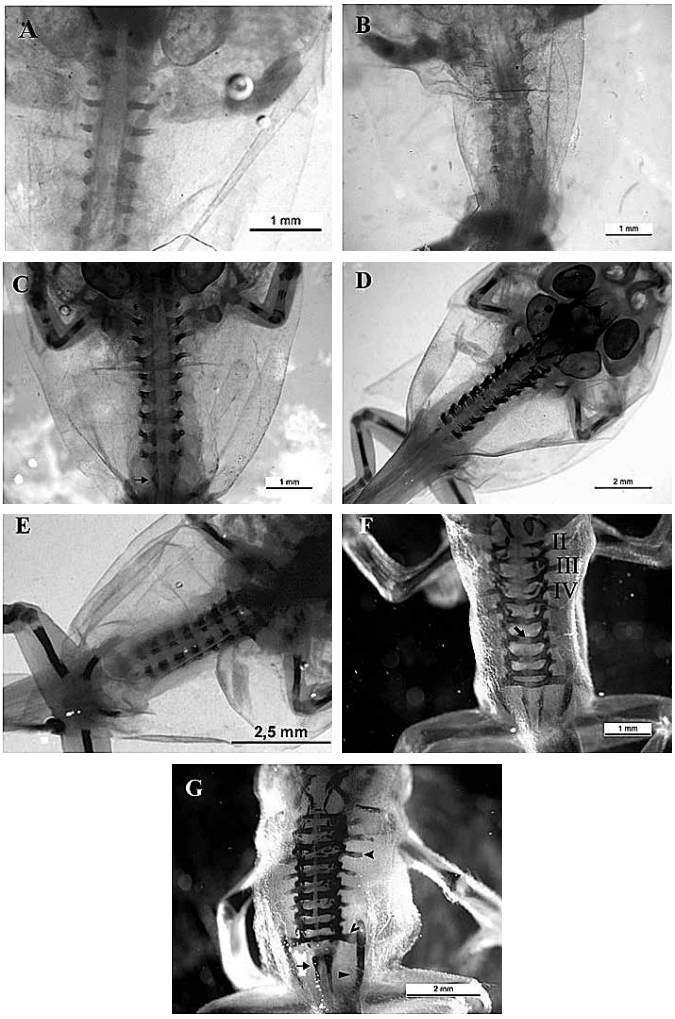
Skelet smo analizirali z metodo presvetlitve tkiv in barvanja hrustančnega in kostnega tkiva (Hanken in Wassersug 1981), ki omogoča vizualizacijo skeleta v organizmu, brez predhodnega odstranjevanja okolnih mehkih tkiv. Velika prednost metode je, da lahko sledimo osifikaciji skeleta med razvojem, prav tako omogoča prostorsko predstavo pozicije skeletnih elementov ter njihovo medsebojno povezovanje. V raziskavo smo vključili ličinke premetamorfozne faze (faza 34), štiri faze v prometamorfoznem obdobju (faza 36, 39, 40, 41) in tri faze v obdobju metamorfoznega klimaksa (faza 42, 44, in 46). Skupno smo pregledali 16 živali, in sicer po dve živali vsake od izbranih razvojnih faz. Material je bil fiksiran v 10% formaldehidu v PBS pufru in shranjen v 70% etanolu. Živalim smo odstranili visceralne organe in jih za 24 ur inkubirali v barvilu alcian modro, ki obarva hrustanec modro. Sledilo je enourno izpiranje odvečnega barvila z raztopino etanola in ocetne kisline (v razmerju 50 : 50) in s 100% etanolom čez noč ter rehidracija v vodi za 24 ur. Kožo smo razbarvali s 3% vodikovim peroksidom. Tkiva smo nato razmehčali v raztopini 30% natrijevega borata in tripsina (24 ur). Sledilo je barvanje tkiva z barvilom alizarin rdeče v 0,5% KOH za 24 ur, ki obarva kostno tkivo rdeče in izpiranje odvečnega barvila z 0,5% KOH. Postopoma (v razmiku nekaj dni) smo KOH nadomeščali z glicerolom v naslednjih razmerjih: 2:1, 1:1, 1:2 in 100% glicerol.

Za analizo osifikacije skeleta osebkov smo uporabili stereolupo MZ FLIII (Leica). Slike smo zajeli s pomočjo digiralne kamere DFC 290 HD (Leica) in programom Las V4.0 (Leica).

Rezultati

Hrbtenica

Hrbtenico gradijo tri regije: presakralna, sakralna in postsakralna. Presakralna regija je iz 8 vretenc, sakralno vretence je eno samo in se povezuje z okolčjem, postsakralna vretenca pa so medsebojno zlita in oblikujejo enotno strukturo, imenovano urostil. Prve hrustančne zasnove hrbtenice so oblikovane v fazi 34 prometamorfoze (slika 1A) in so očitneje razvidne v fazi 36 (slika 1B). V fazi 39 je vidna prva osifikacija v predelu zigapofiz nevralnih lokov vretenc in sicer intenzivneje v kranialnem delu hrbtenice (slika 1C). Izraziti so tudi hrustančni zametki prečnih odstavkov vretenca II do IV (slika 1C). Prvo vretence nima odstavkov. Hrustančni zametki post-



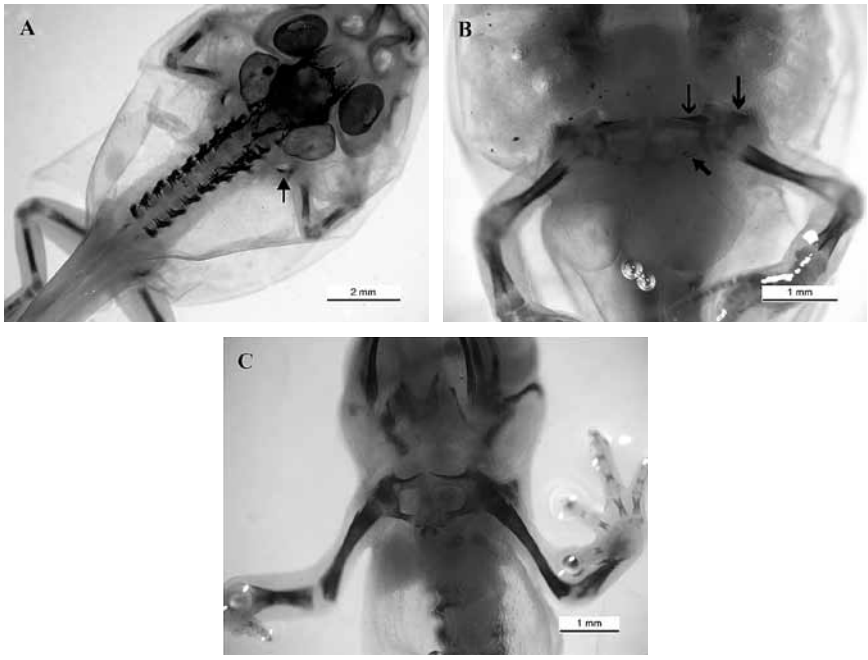
Slika 1: Vzorec osifikacije hrbtenice pri navadni krastači *Bufo bufo*. **A** – Faza 34, zgodnje hrustančne zasnove hrbtenice. **B -** Faza 36, hrustančne zasnove vretenc so izrazitejše. **C -** Faza 39, osifikacija v zigapofizah nevralnih lokov vretenc, ki je intenzivnejša v kranialnem delu hrbtenice. Postsakralna regija je hrustančna (puščica). **D -** Faza 40 (dorzalno), osifikacija v bočnih delih nevralnih lokov. **E –** Faza 40 (ventralno), osifikacija v bočnih delih centrumov. **F -** Faza 44, osifikacija v dorzalnih delih nevralnih lokov in v prečnih odstavkih vretenc II - IV. Prvo vretence je brez odstavkov. **G -** Faza 46, vretenca so osificirana, vključno s križnim vretencem (odprta glava puščice) in kranialnim delom urostila (puščica). V kavdalnem delu urostila je osifikacija šibka. Centralni deli nevralnih lokov in distalni deli prečnih odstavkov (glava puščice) so hrustančni.

Figure 1: Ossification pattern in the backbone of *Bufo bufo*. **A** – Stage 34, early cartilaginous primordial for vertebrae. **B –** Stage 36, cartilaginous vertebrae are more pronounced. **C –** Stage 39, ossification in zigaphophyses of the neural arches of vertebrae, which is more intense in cranial part of the spine. The postsacral region is cartilaginous (arrow). **D –** Stage 40 (dorsal), ossification in lateral parts of neural arches of vertebrae. **E -** Stage 40 (ventral), ossification in lateral parts of vertebral centra. **F –** Stage 44, ossification in dorsal parts of neural arches and in transverse processes of vertebrae II - IV. First vertebra is without of processes. **G –** Stage 46, vertebrae are ossified, including sacral vertebra (open arrow head) as well as cranial part of urostil (arrow).The ossification in the caudal part of urostil is weak. Central parts of neural arches and distal parts of transverse processes (arrow head) are cartilaginous.

sakralne regije so v tej fazi tudi bolj izraziti (slika 1C). V fazi 40 osifikacija postopoma napreduje proti bočnim delom nevralnih lokov in v prečne odstavke vretenc (slika 1D - dorzalno). Proces osifikacije je viden tudi v bočnih delih ventralno ležečih centrumov vretenc (slika 1E - ventralno). V fazi 44 so nevralni loki trupne regije osificirani tudi v dorzalnem delu, medtem ko njihov centralni del ostaja hrustančen (slika 1F). Osifikacija je tudi že obsežnejša v prečnih odstavkih kranialne regije (slika 1F). Prečna odstavka sakralnega vretenca sta hrustančna in večja od ostalih. Vidna je tudi šibka osifikacija v hrustančnih zasnovah postsakralne regije (slika 1F). V fazi 46 je večina hrbtenice osificirana, vključno s kranialnim delom postsakralne regije, ki že oblikuje kratek urostil (slika 1G). V kavdalnem delu postsakralne regije je osifikacija šibka. Centralni del nevralnih lokov hrbtenice ostaja hrustančen, kot tudi distalni deli prečnih odstavkov vretenc (slika 1G). Hrustančni so tudi stiki med centrumom in nevralnim lokom vsakega posameznega vretenca.

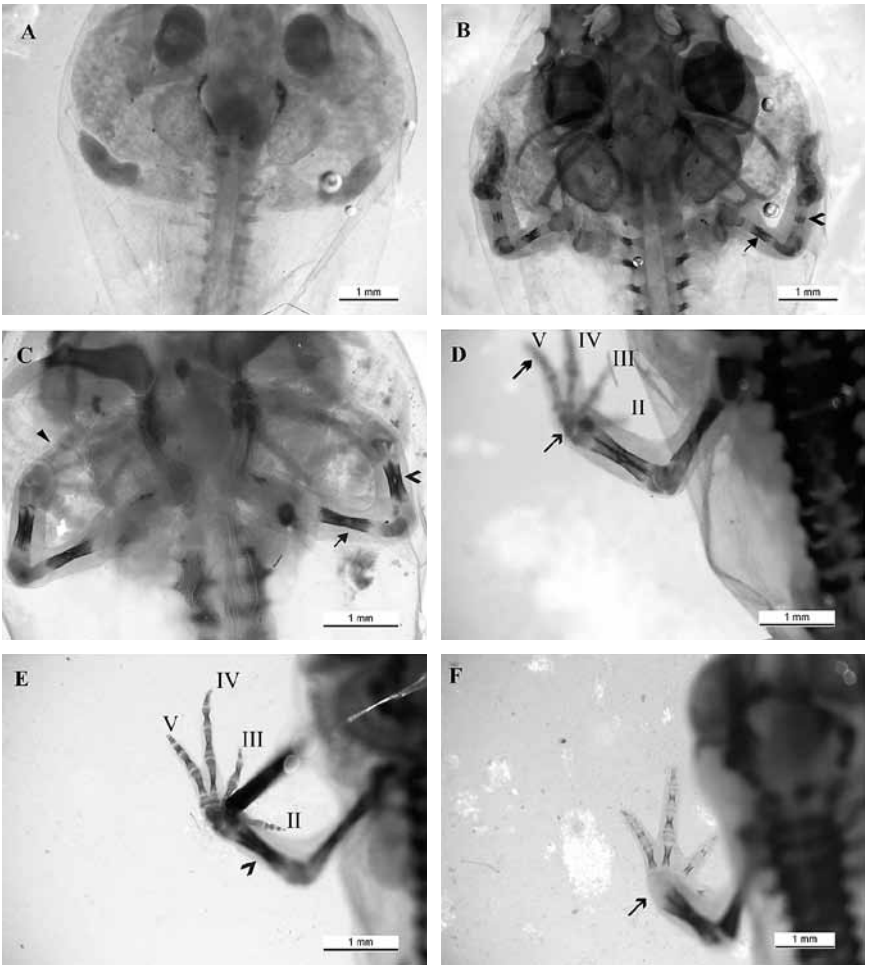
Oplečje in sprednje okončine

Oplečje leži neposredno za glavo in ga gradi parna ključnica ali klavikula, krokarnica ali korakoid, lopatica ali skapula in nadlopatica ali supraskapula s kljetrumom. V fazi 39 je ramenski obroč še popolnoma hrustančen. Prva osifikacija se pojavi v fazi 40, v področju klejtrumov, ki sta na kranialnem robnem delu nadlopatic (slika 2A). V fazi 42 je vidna izrazita osifikacija lopatic in ključnic ter šibka osifikacija v osrednjem delu krokarnic (slika 2B). Osifikacija elementov oplečja je skoraj popolnoma zaključena v fazi 46 (slika 2C), hrustančni ostaneta nadlopatici in stiki med krokarnico in ključnico. Hrustančna je tudi prsnica, s katero sta v stiku ključnici in krokarnici (slika 2C).



Slika 2: Vzorec osifikacije oplečja pri navadni krastači *Bufo bufo*. **A -** Faza 40 – šibka osifikacija v klejtrumu (puščica). **B -** Faza 42, izrazita osifikacija v lopatici (debela puščica) in ključnici (tanka puščica) ter šibka v krokarnici (kratka puščica). **C -** Faza 46, oplečje je osificirano, hrustančni ostaneta nadlopatici in prsnica.

Figure 2: Ossification pattern in the pectoral girdle of *Bufo bufo*. **A –** Stage 40 – weak ossification in cleithrum (arrow). **B –** Stage 42, strong ossification in scapula (thick arrow) as well as in clavicle (thin arrow) and weak in coracoid (short arrow). **C –**Stage 46, pectoral girdle is ossified, sub-scapula and sternum remain cartilaginous.



Slika 3: Vzorec osifikacije sprednjih okončin pri navadni krastači *Bufo bufo*. **A -** Faza 34, zasnove sprednjih okončin so podaljšane in hrustančne, nakazana sta dva prsta. Okončine so znotraj škržnih komor. **B -** Faza 39, osifikacija je izrazita v diafizi nadlahtnice (puščica) in šibka v podlahtnici in koželjnici (glava puščice). **C -** Faza 40, osifikacija je napredovala proti epifizam nadlahtnice (puščica) in podlahtnice s koželjnico (odprta glava puščice). Izoblikovane so tudi že vse hrustančne prstnice prstov (glava puščice). **D -** Faza 42, sprednje okončine niso več v škržnih komorah, osifikacija v proksimalnih delih okončine je obsežnejša. Vidna je tudi šibka osifikacija v dlančnicah prsta IV in V. Zapestnice (odprta puščica) in prstnice prstov (zaprta puščica) so hrustančne. **E -** Faza 44, podlahtnica in koželjnica sta združeni v enotno kost (glava puščice). Osificirane in podaljšane so tudi vse prstnice prstov, razen v prstu II. **F -** Faza 46 –Okončine so osificirane, hrustančni ostajajo epifizni konci in zapestnice (puščica).

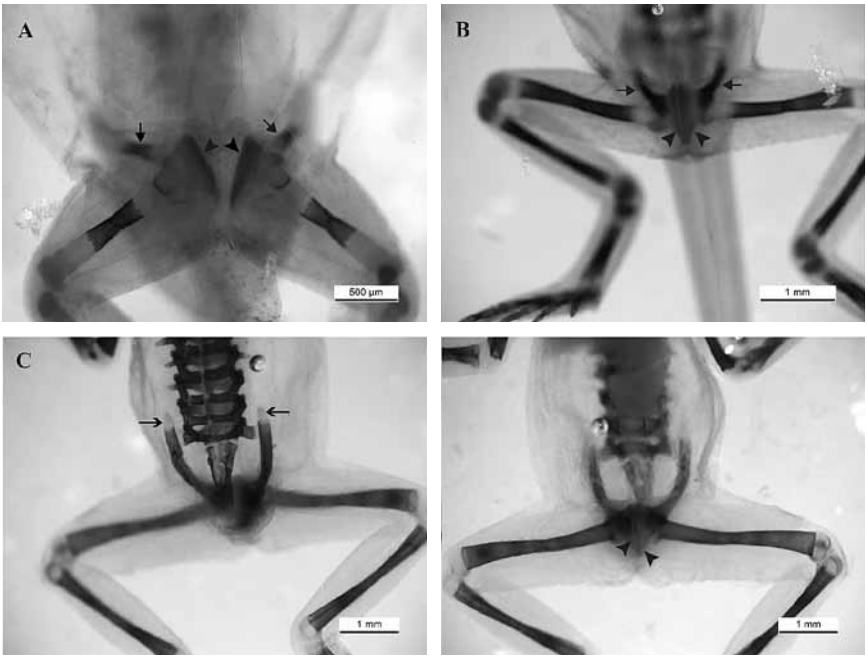
Figure 3: Ossification pattern in the front limbs of *Bufo bufo.* **A –** Stage 34, the forelimb buds are elongated and they are cartilaginous. The first two fingers are barely visible. The limbs develop inside the gill chambers until the stage 42. **B –** Stage 39, ossification is strong in diaphysis of the humerus (arrow) and weak in ulna and radius (arrow head).**C –** Stage 40, ossification progresses towards the epiphyses of the humerus (arrow), ulna and radius (open arrow head). All cartilaginous phalanges are formed (closed arrow head). **D –** Stage 42, the forelimbs are no longer in the gill chambers, the ossification of the proximal parts of forelimbs is extensive. The ossificaton in metacarpus of finger IV and V is weak. Carpus (closed arrow) and phalanges (open arrow) are cartilaginous. **E –** Stage 44, ulna and radius are already fused in one bone (arrow head). All phalanges, except in finger II are ossified and elongated. **F –** Stage 46 –Forelimb are ossified, epiphyses and carpus (arrow) remain cartilaginous.

Sprednje okončine gradi nadlahtnica ali humerus in zliti podlahtnica in koželjnica ali radioulna, sledijo elementi zapestja in dlani (karpalne in metakarpalne kosti) ter prstnice štirih prstov (II – V). V pre- in prometamorfoznih fazah (slika 3A-C), so zasnove sprednjih okončin še znotraj telesa, natančneje znotraj škržnih komor. V fazi 34 sta okončini hrustančni in podaljšani, oblikovani sta zasnovi za dva prsta (slika 3A). V fazi 39 je izrazita osifikacija v diafizah nadlahnic, vidna pa je tudi šibka osifikacija v centralnih delih diafiz podlahtnic in koželjnic (slika 3B). Oblikovani so vsi štirje prsti, ki so še hrustančni. V fazi 40 je osifikacija napredovala proti epifizam nadlahtnic in podlahtnic s koželjnicama. Izoblikovane so tudi že vse prstnice prstov, ki so še hrustančne (slika 3C). V fazi 42, ki predstavlja začetek metamorfoznega klimaksa, sta sprednji okončini že vidni, pred tem je njun razvoj potekal znotraj škržne komore. Osifikacija proksimalnih delov okončin je v tej fazi že obsežnejša, širi pa se tudi v distalne dele okončin (slika 3D). Osificirane so tudi že prve dlančnice, in sicer prsta IV in V. Zapestnice in prstnice prstov so hrustančne. V fazi 44 (slika 3E) so dlančnice in prstnice podaljšane in osificirane, z izjemo II. prsta, ki ima hrustančne prstnice. V fazi 46 (slika 3F) so osificirani skoraj vsi elementi zadnjih okončin. Hrustančno ostane zapestje, in epifizni konci vseh kosti okončine.

Okolčje in zadnje okončine

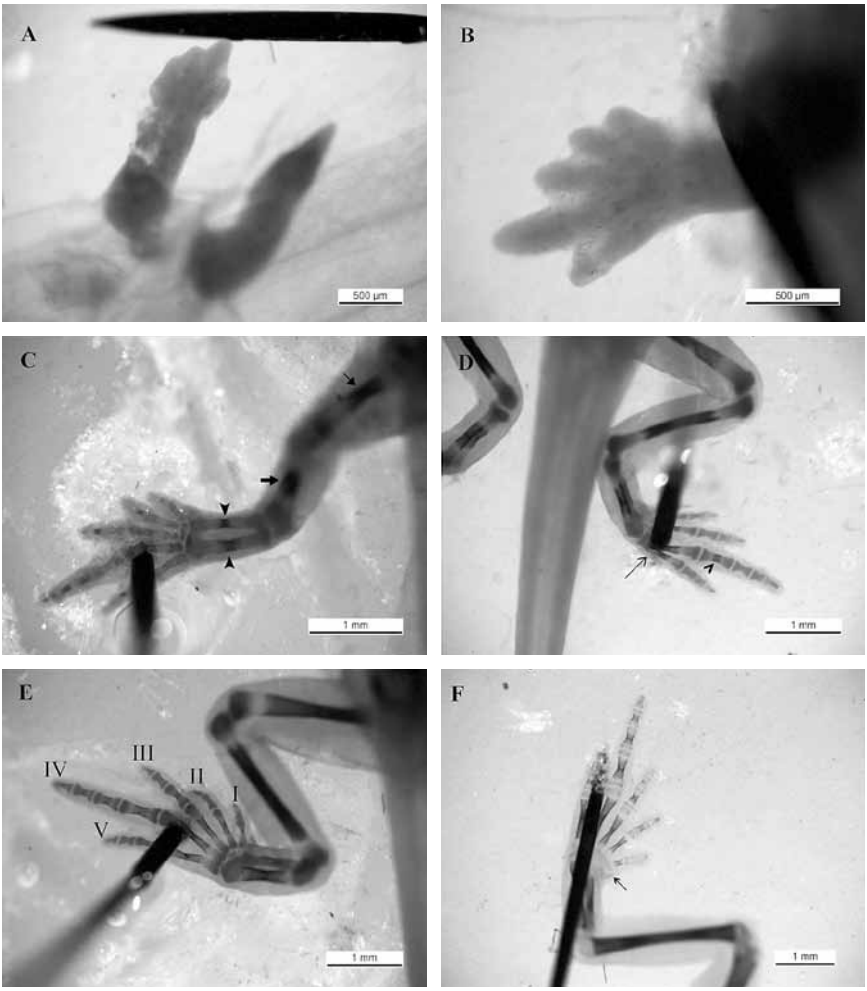
Okolčje je zgrajeno iz treh parnih elementov (črevnice ali iliuma, sednice ali ishiuma in sramnice ali pubisa), ki se združujejo v osrednjem kavdalnem delu hrbtenice in oblikujejo jamico (acetabulum) za sklepno vezavo stegnenic. V fazi 39 (slika 4A) so parne zasnove za sednico in sramnico še hrustančne, medtem ko v črevnicah že poteka osifikacija, in sicer v njunem posteriornem delu. Lega črevnic je še horizontalna, prav tako parni elementi sednice in sramnice še niso združeni. Združevanje poteče v fazi 44 (slika 4B). Osifikacija črevnic je v tej fazi izrazitejša, prav tako je njuna lega že bolj vertikalna. V fazi 46 je glavnina okolčja osificirana, hrustančni ostajata sramnici in distalna konca črevnic (slika 4C). Črevnici sta že postavljeni paralelno s hrbtenico in se povezujeta z osificiranima prečnima odstavkoma (diafizama) križnega vretenca.

Zadnje okončine gradi stegnenica ali femur, ki je v stiku z okolčjem, ter zraščeni golenica in mečnica ali tibiofibula. Sledita parna in podaljšana proksimalna tarzalna elementa ter preostale tarzalne kosti, podaljšane nartnice ali metatarzalni elementi in prstnice prstov. Zadnja okončina ima pet prstov. V fazi 34 in 36 so zadnje okončine še v celoti hrustančne (slika 5A, B). Vseh pet prstov je izoblikovanih v fazi 36, vendar posamezne prstnice še niso vidne. V fazi 39 je že dobro vidna osifikacija v diafizah stegnenice in tibio-fibule, ki je v tem obdobju še iz ločenih zasnov (slika 5C). V proksimalnih tarzalnih elementih je vidna šibka osifikacija. Distalni del okončine je še hrustančen. Prsti so podaljšani, razvidne so tudi že posamezne hrustančne prstnice (slika 5C). V fazi 40 je razvidno napredovanje osifikacije proti epifiznim predelom pri stegnenici, tibio-fibuli (zasnovi sta že združeni) in tarzusu (slika 5D). Vidna je tudi šibka osifikacija v nartnicah vseh štirih prstov in v prvi prstnici najdaljšega prsta (slika 5D). V fazi 44 so dolge kosti proksimalnega dela okončin že osificirane, epifize ostajajo hrustančne (slika 5E). Osifikacija je vidna v proksimalnih prstnicah prstov od III do V ter druga prstnica prsta III. V fazi 46 je skoraj celotna okončina koščena, hrustančne so epifize in distalni elementi gležnja (slika 5F).



Slika 4: Vzorec osifikacije okolčja pri navadni krastači *Bufo bufo*. **A -** Faza 39, osifikacija v proksimalnem delu črevnic (puščica). Sramnici (glava puščice) sta hrustančni. **B** - Faza 44, osifikacija črevnic (puščica) je obsežnejša. Parni hrustanci sramnice (glava puščice) in sednice so v osrednjem delu hrbtenice. **C** - Faza 46, okolčje je osificirano, razen v distalnih koncih črevnic (puščica) (slika levo z dorzalne strani) in v predelu sramnic (glava puščice) (slika desno z ventralne strani).

Figure 4: Ossification pattern in the pelvic girdle of *Bufo bufo*. **A –** Stage 39, ossification in the proximal part of ileum (arrow). Pubic bones (arrow head) are cartilaginous. **B –** Stage 44, ossification of the ileum is more extended (arrow). Dual cartilaginous elements of pubic bones (arrow head) and ischium are moved in central part of the spine. **C –** Stage 46, pelvic girdle is ossified except in distal parts of the ileum (arrow) (left figure from dorsal side) and in the pubic bones (arrow head) (right figure from ventral side) which are cartilaginous.



Slika 5: Vzorec osifikacije zadnjih okončin pri navadni krastači *Bufo bufo*. **A -** Faza 34, zasnove za okončini so podaljšane in hrustančne. **B** - Faza 36, izoblikovanih je vseh pet prstov. **C** - Faza 39, izrazita osifikacija v diafizah stegnenice (tanka puščica) in tibio-fibule (debela puščica) ter šibkejša v diafizah proksimalnih kosti gležnja (glava puščice). Izoblikovane so tudi že vse hrustančne prstnice. **D** - Faza 40, osifikacija je napredovala proti epifizam v stegnenici, tibio-fibuli in proksimalnih kosteh gležnja. Vidna je tudi šibka osifikacija v nartnicah (puščica) vseh štirih prstov in v prvi prstnici najdaljšega prsta (glava puščice). **E -** Faza 44, obsežna osifikacija v vseh dolgih kosteh okončine, razen v najbolj distalnih prstnicah, ki so hrustančne. **F** - Faza 46, osificirane so vse kosti okončine, vključno s prstnicami. Epifizni konci in distalni elementi gležnja (puščica) so hrustančni.

Figure 5: Ossification pattern in the hind limbs of of *Bufo bufo*. **A –** Stage 34, limb buds are elongated and cartilaginous. **B**. – Stage 36, all five fingers are formed. **C –** Stage 39, strong ossification in diaphysis of femur (thin arrow) and in tibiofibula (thick arrow), and weak ossification in diaphyses of proximal tarsus bones (arrow head). All cartilaginous phalanges are formed. **D –** Stage 40, ossification progresses towards the epiphyses of femur and tibiofibula as well as to the epiphyses of proximal tarsal bones. The ossification in metatarsus of all four fingers (arrow) and in first phalange of the longest finger (arrow head) is weak. **E -** Stage 44, extensive osiffication in all long bones of the limb, except in the most distal phalanges which are cartilaginous. **F –** Stage 46, all limb bones are ossified including phalanges. The epiphyseal ends and distal tarsal elements (arrow) are cartilaginous.

Zaporedje in časovni potek osifikacije skeleta pri ličinkah navadne krastače *B. bufo* povzemamo v Tabeli 1. Prva osifikacija skeleta nastopi v zgodnjem prometamorfoznem obdobju (faza 39) in se postopoma širi v kranialno - kavdalni smer vzdolž hrbtenice, ter v proksimalno – distalni smeri v okončinah, oplečju in okolčju. Ob zaključku metamorfoze (faza 46) je skelet skoraj popolnoma osificiran.

Tabela 1: Vzorec osifikacije skeleta navadne krastače *Bufo bufo.*
Table 1: Skeleton ossification pattern in common European toad *Bufo bufo*.

Razvojne faze po Gosnerju	**Osni skelet**	**Sprednje okončine in oplečje**	**Zadnje okončine in okolčje**
39	zigapofize nevralnih lokov I-VIII*	nadlahtnica podlahtnica s koželjnico	stegnenica tibio-fibula proksimalni tarzalni kosti proksimalni del črevnice
40	bočni deli nevralnih lokov I-VIII* in IX bočni deli centrumov I-VIII*	klejtrum	nartnice proksimalna prstnica prsta IV
42	dorzalni deli nevralnih lokov I-III * prečni odstavki II-III* kranialni del post-sakralne regije	lopatica ključnica krokarnica dlančnice prstov IV-V	
44		dlančnice prstov II-III prstnice prstov III-V	proksimalne prstnice prstov III in V druga prstnica prsta III
46	prečni odstavki IV-VII prečna odstavka vretenca IX kavdalni del post-sakralne regije	prstnici prsta II	sednica distalne prstnice prstov I–V

*– osifikacija intenzivnejša v sprednjem delu hrbtenice
Skeletni elementi so navedeni glede na najbolj zgodnji pojav osifikacije, ki smo ga zasledili s pomočjo barvanja kostnega tkiva z barvilom alizarin rdeče.
*- ossification is intense in cranial part of the spine
Skeletal elements are listed according to the earliest appearance of ossification process, which was detected by using the alizarin red stain for bone tissue.

Diskusija

V raziskavi smo analizirali postembrionalno osifikacijo skeleta pri navadni krastači *Bufo bufo*, ki imajo izredno hiter razvoj ličink. Osredotočili smo se na zaporedje in časovni potek osifikacije skeletnih delov v hrbtenici, oplečju in okolčju ter okončinah, s pudarkom na distalnih delih okončin, saj v literaturi za navadno krastačo ni podanih podrobnih opisov. Tako kot pri ostalih brezrepcih (Kepm in Hoyt 1969, Duellman & Trueb 1986, Dunlap in sod. 1996) se hrustančne zasnove skeleta krastače oblikujejo v obdobju premetamorfoze in so v fazi 34 tudi že jasno razvidne. Prva osifikacija skeleta nastopi v prometamorfoznem obdobju (faza 39), in sicer sočasno v hrbtenici, proksimalnih delih okončin in črevnicah okolčja, ter se postopoma širi v kranialno - kavdalni smeri vzdolž hrbtenice ter od proksimalnih v distalne dele okončin, okolčja in oplečja, kot tudi od osrednjega dela dolgih kosti okončin proti njihovim epifiznim koncem.

Zasnove vretenc nastanejo iz mezenhimskih celic embrionalnega sklerotoma, ki se diferencirajo v hondrocite in obdajo hrbtenjačo in hrbtno struno (Gilbert 2000). V dorzalnem delu se oblikujejo ločene parne zasnove nevralnih lokov, ki zaščitijo hrbtenjačo, zasnove okoli hrbtne strune oblikujejo centrume vretenc, ki embrionalno hrbtno struno v celoti nadomestijo (Liem in sod. 2001). Prva

osifikacija hrbtenice pri navadni krastači je opazna v zigapofizah nevralnih lokov vretenc presakaralne regije v obdobju prometamorfoze (faza 39). Vzorec osifikacije ločenih zasnov nevralnih lokov poteka podobno kot opisujejo za nekatere druge brezrepce (Haas 1999, Ročkova in Roček 2005, Yirdirim 2014), in sicer se postopoma širi iz proksimalnega dela leve in desne polovice nevralnih lokov proti njihovemu centru. Osifikacija hrbtenice poteka v kranialno – kavdalni smeri, kar se odraža tudi v intenzivnejši osifikaciji elementov hrbtenice kranialne regije, vključno s prečnimi odstavki vretenc. Slednji v kavdalni regiji presakralnih vretenc osificirajo šele v metamorfoznem klimaksu, njihovi distalni konci pa so ob zaključku metamorfoze še vedno hrustančni. Hrustančni distalni konci odstavkov vretenc pri krastači so zasnove embrionalnih reber, ki pri dvoživkah ostajajo kratka in zlita z odstavki in ne tvorijo rebrne košare, kot je značilno za ostale vretenčarje (Liem in sod. 2001). Ob zaključku metamorfoze ostaja pri krastači hrustančen tudi osrednji dorzalni del nevralnih lokov, ki najverjetneje osificira po metamorfozi. Pri nekaterih vrstah brezrepcev se obe polovici nevralnih lokov na področju sprednjih vretenc ne združijo, vendar je pri večini vrst lok popoln in koščen (Duellman in Trueb 1986). Pri večini dvoživk poteka sočasna osifikacija centrumov in nevralnih lokov vretenc (Haas 1999, Ročkova in Roček 2005, Yirdirim 2014). To naj bi veljalo tudi za navadno krastačo (Dunlap in Sanchiz 1996), vendar se je v našem primeru osifikacija centrumov pojavila kasneje (faza 42). Vse do faze 42 so centrumi še hrustančni, medtem ko je v nevralnih lokih že intenzivna osifikacija. Podobno kot v nevralnih lokih, tudi v predelu centrumov poteka osifikacija najprej v bočnih delih hrustančnih centrumov, in se nato širi v proti osrednjemu delu posameznih centrumov vretenc.

Postsakralna vretenca so pri brezrepcih zlita v enotno osrednje ležečo stukturo ali urostil, ki je pomembna vzmet pri skakajočem načinu lokomocije, blažilec pri doskoku, nanj se pripenjajo mišice, ki omogočajo rotacijo okolčja (Kemp 1969, Duellman in Trueb 1986, Pough in sod. 2013). Do zlivanja postsakralnih elementov pri brezrepcih prihaja ob koncu metamorfoze in se nadaljuje tudi po zaključku metamorfoze (Duellman in Trueb 1986). V raziskavi smo ločene hrustančne zametke postsakralne regije hrbtenice opazili v fazi 39, prvo osifikacijo pa v kranialnem delu postsakralne regije, v fazi 42, torej na začetku metamorfoznega klimaska. V fazi 46 je kranialni del postsakralne regije že zlit v strukturo imenovano urostil in tudi že osificiran, medtem ko je v kavdalnem koncu zastopana šele šibka osifikacija. Proces mineralizacije urostila pri krastači in ostalih brezrepcih se zaključi po metamorfozi (Dunlap in Sanchi 1996).

Oplečje brezrepcev v osnovi gradita dve dermalni kosti, ključnica ventralno in klejtrum z dorzalne strani ter lopatica z nadlopatico dorzalno in korakoid z ventralne strani, ki so endohondralnega nastanka (Duellman in Trueb 1986). Obe polovici oplečja na trebušni strani povezujejo elementi prsnice. Vzorec osifikacije oplečja ličink krastače *B. bufo* je podoben, kot ga opisujeta Dunlap in Sanchiz (1996) za različne vrste rodu *Bufo*, in je značilen tudi za ostale brezrepce (Duellman in Trueb 1986,). Prva osifikacija oplečja je bila opazna v klejtrumu (faza 40). Ta se nato širi v proksimalni del lopatice. Sočasno poteka osifikacija korakoida, kar sovpada z zaključkom osifikacije nadlahtnice sprednjih okončin. Sledi širjenje osifikacije v distalni konec lopatice in na področje ključnice (faza 42). Ob zaključku metamorfoze (faza 46) je oplečje pri krastači koščeno, razen nadlopatic, ki sta v celoti hrustančni. Obseg osifikacije nadlopatice je vrstno specifičen, npr. pri vrstah iz družine pravih žab (Ranidae) obsega približno 2/3 celotne nadlopatice (Duellman in Trueb 1986).

Okolčje brezrepcev je v primerjavi z repatimi dvoživkami zelo modificirano, parni elementi okolčja so namreč združeni v osrednjem delu okolčja, tako da je sklepna vezava zadnjih okončin na okolčje v centralni osi hrbtenice (Duellman in Trueb 1986, Liem 2001, Pough in sod. 2013). Podaljšani sta tudi črevnici in ležita vzporedno s hrbtenico in se pripenjata na diafizo križnega vretenca. Posteriorna dela črevnic sta razširjena in oblikujeta anteriorno polovico kolčnične ponvice, ki je mesto sklepne vezave stegnenice zadnje okončine (Duellman in Trueb 1986). Preostali del ponvic oblikujeta sednici in sramnici. Čeprav je lega parnih sednic in sramnic okolčja odraslih brezrepcev v centralni osi hrbtenice, se elementi okolčja razvijejo iz parnih hondrifikacijskih centrov na vsaki strani hrbtenice (Ročkova in Roček 2005, Yirdirim 2014), kar je tudi lepo razvidno v našem

primeru pri ličinkah krastače. Parne hrustančne zasnove so jasno razvidne v fazi 34 premetamorfoze in ležijo na vsaki strani hrbtenice. Prvo osifikacijo okolčja smo opazili v prometamorfozi (faza 39), in sicer v posteriornem delu črevnic sočasno s stegnenico in tibiofibulo. Osifikacija sednice nastopi šele ob koncu metamorfoznega klimaksa, medtem ko pubisa ostajata hrustančna. Vzorec razvoja okolčja je pri vseh brezrepcih podoben, časovno različno pa je zlivanje obeh polovic okolčja, ki se pri nekaterih rodovih brezrepcev pojavi predno črevnici dosežeta svojo končno dolžino (npr. *Discoglossus, Bombina* in *Xenopus*), pri drugih (npr. *Bufo, Pelobates* in *Rana*) pa se pojavi kasneje, čeprav sta črevnici že podaljšani (Ročkova in Roček 2005). V našem primeru prihaja do združevanja ločenih parnih zasnov sednice in sramnice v fazi 44 metamorfoznega klimaksa. Dunlap in Sanchi (1996) za *Bufo bufo* tega podatka ne navajata. Osifikacija okolčja krastače se pojavi v prometamorfozi(faza 39) v posteriornem delu črevnic sočasno s stegnenico in tibiofibulo, medtem ko nastopi osifikacija sednice šele ob koncu metamorfoznega klimaksa. Razen sramnic, ki ostajata hrustančni, je okolčje koščeno ob zaključku metamorfoznega klimaksa. Dunlap in Sanchi (1996) za krastačo *Bufo bufo* sicer navajata zgodnejšo osifikacijo črevnic (faza 37) in sednic (faza 44) ter popolno osifikacijo okolčja pred zaključkom metamorfoze (faza 45).

Tako kot je značilno za ostale dvoživke (Duellman in Trueb 1986), se osifikacija v okončinah krastače *B. bufo* pojavi najprej v proksimalnih delih in se nato postopoma širi v distalne dele. Proksimalni deli okončin krastače osificirajo v zgodnjem prometamorfoznem obdobju, saj so v fazi 39 že intenzivno osificirani, kar se sklada z opisom od Dunlap in Sanchi (1996). Prav tako nastopi proces osifikacije najprej v zadnjih okončinah, saj je bilo obarvanje kostnega tkiva v stegnenicah intenzivnejše in obsežnejše. Zanimiv je vzorec osifikacije distalnega dela okončin (t.i. autopodija), ki ga Dunlap in Sanchi (1996) pri svojem opisu razvoja in osifikacije skeleta pri *B. bufo* ne podajata. Osifikacija nartnic zadnjih okončin (faza 40) se pojavi pred dlančnicami sprednjih okončin (faza 44). Sočasno z nartnicami poteka tudi osifikacija proksimalne prstnice IV. prsta, medtem ko nastopi osifikacija v preostalih proksimalnih prstnicah bistveno kasneje, in sicer proti koncu metamorfoznega klimaksa (faza 44). Osifikacija distalnih prstnic vseh petih prstov zadnje okončine se pojavi šele ob zaključku metamorfoze. Sočasno z dlančnicami sprednje okončine poteka osifikacija proksimalnih prstnic vseh prstov sprednje okončine, z izjemo II. prsta (faza 42). Proksimalna prstnica prsta II sprednje okončine osificira sočasno z distalnimi prstnicami preostalih prstov (faza 44). Prst I je v evoluciji brezrepcev reduciral, tako da ima sprednja okončina štiri prste (Liem in sod. 2001). Pri zadnji okončini sočasno z nartnicami osificira le proksimalna prstnica prsta IV (faza 40), proksimalne prstnice preostalih prstov osificirajo kasneje (faza 44). Razen proksimalno – distalne osifikacije v okončinah je zaslediti tudi posteriorno – anteriorno osifikacijo prstov, ki pravzaprav sledi postopnemu izraščanju prstov med razvojem okončin paglavcev (Badawy in sod. 2012). Ob zaključku metamorfoze (faza 46) so okončine pri krastači koščene, z izjemo epifiznih koncev ter karpalnih in metakarpalnih elementov. Tudi pri večini ostalih brezrepcev karpalni in metakarpalni elementi pokostenijo šele po metamorfozi (Kemp in Hoyt 1969, Duellman in Trueb 1986, Dunlap in Sanchi 1996, Hass 1999). Epifizni konci pa so pri večini brezrepcev popolnoma mineralizirani šele po četrti hibernaciji (Rozenblut in Ogielska 2005).

Pri večini vrst brezrepcev je relativni čas osifikacije posameznih enot skeleta (lobanja, hrbtenica in okončine) dokaj podoben (Hass 1999), prav tako se osifikacija najprej pojavi v lobanjskem delu, ki ji sledi osifikacija v hrbtenici, nato v zadnjih okončinah in nazadnje še v sprednjih okončinah. Običajno se osifikacija v okončinah dvoživk pojavi tudi z večjim časovnim zamikom glede na lobanjo in hrbtenico. V primeru navadne krastače *B. bufo* se prva osifikacija okončin pojavi sočasno z osifikacijo hrbtenice. Rezultati raziskave pri navadni krastači *B. bufo* kažejo tudi na to, da je osnovni vzorec osifikacije skeleta dvoživk ohranjen, neglede na to, da je razvoj ličink krastače v primerjavi z ostalimi brezrepci bistveno hitrejši.

Summary

Ossification is a process in which pre-existing mesenchymal tissue or cartilage transforms into bone. In amphibia, this process occurs in the post-

embryonic period during larval development. The basic pattern of ossification is strongly conserved among closely related species, but the precise timing of skeletal ossification is species-specific.

In our research we analyzed the pattern of skeletal ossification in larvae of the common European toad, *Bufo bufo*, a species of anuran with very rapid larval development. We focused on post-embrional ossification of the axial skeleton with an emphasis on the distal parts of the limbs. We used a clearing and staining method for cartilage and bone. The developmental stages of the larvae were identified using the Gosner (1960) Staging System for Anurans. Our results suggest that at the end of the premetamorphosis stage (Stage 34), the skeleton is entirely cartilaginous. The first visible ossification by the applied method is evident in prometamorphosis (Stage 39), and occurs simultanously in the zigapohyses of the neural arches of the spine, in the diaphyses of proximal parts of the forelimbs (humerus, radius-ulna) and hind limbs (femur and tibio-fibula), as well as in the proximal part of the ileum of the pelvic girdle. The process intensifies during prometamorphosis and gradualy progresses in a cranial - caudal direction along the spine and from proximal to distal parts of the limbs, and in the pelvic and pectoral girdles, as well as to the epiphyses of the long bones. Fingers ossified in a posterio-anterior direction. Ossification of the postsacral vertebrae begins at the onset of the metamorphic climax (Stage 42), while their distal ends are still cartilaginous at the end of metamorphosis and, only weak ossification is visible. The retardation of ossification in the postacral region is common also for other anuran species.

In most amphibians, ossification takes place simultanously in vertebral centra and neural arches of the vertebrae. We noticed that ossification of vertebral centra began later (Stage 40) in *B. bufo* than the neural arches. The ossification process in neural arches occurs in prometamorphosis (Stage 39) with the ossification of zygapophyses.

Overall, the basic ossification pattern in the limbs and girdles of *B. bufo* is also similar to that reported for other amphibian species and begins in the diaphyses of the proximal parts of limbs and then gradually progresses to the distal parts, as well as from the diaphyse to the epiphyses of the long bones. The first evidence of ossification in the pectoral girdle is in the cleithrum (Stage 40) which then gradually continues to the other parts of the girdle (scapula, clavicle, coracoid). The ossification of the pelvic girdle occurs before the pectoral girdle, in prometamorphosis (Stage 39), with the ossification of the posterior part of the ileum, and simultaneously with the femur and tibia-fibula. By the start of the metamorphic climax (Stage 44), the dual and laterally placed cartilaginous elements of the girdle, the ischium and pubis, have moved to the central part of the spine, where, together with the ileum, they form the articulation site (acetabulum) for hind limbs. At the end of metamorphosis (Stage 46), the ischium begins the ossification process while the pubis remains cartilaginous.

At the end of the metamorphic climax (Stage 46), the skeleton of *B. bufo* is still not complety ossified. At this stage, cartilage remains in the dorsal midline of the spine, in the distal parts of the transverse processes of the vertebrae, as well as in the epiphyses, the mesopodial elements (carpals and metatarsals) of the limbs, and in the suprascapula and pubis of both girdles. Although all of these elements (except the suprascapula) are fully ossified in amphibians by the end of metamorphosis, the carpals in the front limbs and metatarsals in the hind limbs usually remain cartilaginous.

Most Anuran species are similar in the relative timing of ossification of the major skeletal units (cranium, vertebral column, hind and front limbs); the very first ossification appears in the cranium, shortly followed by ossifications of the vertebral column, then the hindlimb, and last the forelimb (Haas 1999). Usually hind- and forelimbs start ossification with a clear delay relative to the cranium and vertebral column. In the case of common toad *B. bufo*, it appears that the vertebral column, hind and front limbs begin to ossify simultaneously. In most other respects, however, the results of the research also show that despite rapid larval development, the patterns of ossification seen in *B. bufo* are very similar to those of other anuran species, underlining the fact that they are strongly conserved.

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Lower secondary school students' interest and emotions regarding dissection in schools - a pilot study

Interes in čustva osnovnošolcev v povezavi s seciranjem v šoli - pilotna študija

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Abstract: In the present study, we investigated lower secondary school student's interest and emotions regarding dissection in schools. Self-reported interest and emotions of fear and disgust toward dissection were assessed. In addition to well-known gender differences from prior studies, the author also assessed if age, participation in home cooking of meat and fish or participation in the home slaughtering and butchering of livestock has an effect on these mentioned dependent variables. The results show, that situational interest was predominantly influenced by the students' age. Older students displayed higher interest in school dissections. Individual interest was influenced by grade, gender and by participation in home slaughtering. Emotions concerning dissection were significantly influenced by gender. Situational interest was predicted both by personal interest and negative emotions. From the results, it can be concluded that participation in home cooking and home slaughtering of livestock has no effect on students' interest and emotions regarding dissection, which is somehow contradictory to the statements of other authors who argue that repeated exposure to dissections raises student's interest and lowers negative emotions regarding dissections. Perhaps spatial and temporal dimensions ought to be considered in addition to the level of students' involvement in such activities.

Keywords: animals, dissection, interest, emotions, secondary school students

Izvleček: Namen raziskave je bil ugotoviti interes in čustva osnovnošolcev glede seciranja pri pouku naravoslovja in biologije. Učenci so v ta namen v obliki samoporočila ocenili svoj interes za seciranje ter nivo strahu in gnusa, ki bi ga občutili ob tem. V predhodnih študijah so različni avtorji ugotovili, da na ocene interesa in čustev v povezavi s seciranjem v pretežni meri vpliva spol učencev. V raziskavi smo zato ugotavljali tudi, v kolikšni meri se čustva in interes v povezavi s seciranjem spreminjajo glede na starost učencev, njihovo vključenost v gospodinjska opravila in sodelovanje pri domačih kolinah. Rezultati raziskave so pokazali, da na interes učencev za seciranje vpliva predvsem starost učencev. Starejši učenci so izkazali višji interes za seciranje kot mlajši učenci. Na osebni interes učencev za učenje o zgradbi in delovanju organizmov vplivajo starost in spol učencev ter njihovo sodelovanje pri domačih kolinah. Spol učencev vpliva na izražanje čustev v povezavi s seciranjem. Interes za seciranje je povezan tako z osebnim interesom kot negativnimi čustvi posameznika. Iz rezultatov

lahko sklepamo, da vključenost učencev v gospodinjska opravila in sodelovanje pri domačih zakolih ne vplivata na interes učencev za seciranje in oblikovanje njihovih čustev. Naša ugotovitev je v nasprotju z razmišljanji in ugotovitvami drugih avtorjev, ki trdijo, da ponavljajoča se izpostavljenost seciranju vzbuja interes in znižuje negativna čustva ob tem. V prihodnjih študijah bi bilo vredno upoštevati tudi prostorske in časovne dimenzije vključenosti učencev v zunajšolske aktivnosti, ki so povezane s seciranjem živali ter stopnjo njihove vključenosti pri teh dejavnostih.

Ključne besede: živali, seciranje, interes, čustva, osnovnošolci

Introduction

Biology is essentially the study of life and one can hardly imagine a biology classroom without the presence of living organisms (National Association of Biology Teachers [NABT] 2008).

On the other hand, one of the most "controversial" topics of biology are the topics of animal anatomy and physiology that rely on dissection of animals and animal parts.

Dissection however represent the traditional teaching method for animal anatomy, and it has been used in schools since the early 20th Century (Kinzie et al. 1993). Whether to use dissection in schools, particularly in primary and secondary schools is an ongoing debate (DeVilliers and Monk 2005, Hug 2008). While some authors argue that dissection is a viable option for medical students (Cho and Hwang 2013, Houwink et al. 2004, Rizzolo and Stewart 2006), some believe that it must be drastically (if not completely) removed from schools and replaced with alternatives (Hug 2008, Maloney 2005). A lot of alternatives have been developed, mainly due to ethical concerns regarding dissections (Hug 2008). Such alternatives include the use of preserved specimens, three-dimensional anatomical models, books, charts, slides, photographs, films, computer-based simulations, and interactive simulations (DeVilliers and Monk 2005, Oakley 2012).

Studies that favour alternatives to dissection state their beneficial outcomes such as low time, costs (one time usability of animals), better control over own learning, better structure clarity, and absence of odour and body fluids (what can lead to higher emotional responses and also confusion and frustration) (DeVilliers and Monk 2005, Oakley 2012, Predavec 2001). On the other hand, those who defend the use of dissection in schools often state that the experience of alternatives is not the same compared to real dissections (Offner 1993). In alternatives, students cannot learn practical dissection skills, sensory experience is not the same, visual-spatial thinking is limited and they lack realism (in DeVilliers and Monk 2005).

Allchin (2005) for example argues that no computer guided alternative is appropriate to teach anatomy. Oversimplification or idealized observed structures and diagrams are considered cheating, because not all structures are visible (or are missing) as opposed to the "real thing". By his opinion, one cannot teach the students virtual respect for life, as the emotions expressed through dissection are directly linked to self-understanding and understanding of the meaning of death and dying. Without that, a full understanding of life is incomplete.

In the American biology classrooms, for example, more than 80 % of middle and high school teachers report using dissections as their selected form of teaching (Osenkowski et al. 2015). In the mentioned study, 48 % of interviewed students would not use an alternative to dissection, 37 % would use an alternative and 15 % were undecided. The high number of students that would use an alternative to dissection should not be neglected. Therefore, it is no wonder that some authors argue that dissection should be an optional rather than compulsory component of the curriculum (Barr and Herzog 2000). Špernjak and Šorgo (2017) on the other hand found, that less than 15 % of Slovenian students, regardless of gender and school level, would like to opt-out from dissection practice. Furthermore, most of the students would like to conduct more dissections.

Disgust and interest concerning dissection are two topics that were until recently scarcely researched (Fančovičová et al. 2013, Holstermann 2009, Randler et al. 2012). Dissection can be regarded as a form of practical work where emotions

of both disgust and fear are present. Either whole bodies, body parts and bodily fluids or excretions cause a certain level of aversion in students.

Disgust is a basic emotion (Ekman 1999) related to avoidance of certain animals, ill humans, faeces, vomit, sexual substances and other harmful events (Rozin et al. 2008). It can be measured as a trait – trait disgust – or as a state – state disgust (Tolin et al. 2006). Disgust sensitive students display higher level of state disgust within dissection activities what can lower their interest in dissection. This can lead to lowered knowledge acquisition and learning skills. On the other hand, repeated exposure to dissections can increase interest and lower the experience of disgust (Randler et al. 2012).

Interest is defined as a psychological state, which in later phases of development, is also a predisposition to reengage content. This applies to in-school and out-of-school learning and to young and old alike (Hidi and Renninger 2006). We can discern between situational and individual (personal) interest (in Abrahams 2009, Palmer et al. 2016). Whilst individual interest is a relatively enduring predisposition to reengage particular contents, situational interest is described as focused attention and affective reaction, triggered in the moment by environmental stimuli, which may or may not last over time (Hidi and Renninger 2006). For the purpose of the present study, situational interest was regarded as a students' reported interest in dissection activities and their approval of such activities. Individual interest was regarded as a personal view about the importance of anatomy and physiology knowledge and skills for (later) life.

Several studies have already examined the role of disgust on students' achievement, anxiety and knowledge with regard to dissection activities. Holstermann et al. (2009) found that students who experience higher level of state disgust during dissection, also perceive their own performance as less good than their counterparts. The same was observed for interest.

When controlling for gender, females display higher level of disgust toward dissection than males, what is not necessarily true for the interest (Holstermann et al. 2012). Females also show less support for dissection activities than boys (Lock 1995; Holstermann et al. 2012, Fančovičová et al. 2013). Regardless of gender, students who own pets are less supportive of dissections (Fančovičová et al. 2013).

Purpose of the study

With the change of state curricula for lower secondary school science and biology in 2011, which shifted from ecosystem approach to molecular approach, topics of animals' internal structures came into focus. Dissection of live animals (vivisection) and killing of animals for dissection purpose are prohibited in Slovenian schools. Nevertheless, teachers are allowed to use animals and animal parts that can be bought in stores, for dissection (i.e. fish, sea molluscs, animal organs such as pigs heart, kidney). Knowing that more than 40 % of lower secondary school biology teachers are not using dead animals or animal parts in instruction and would rather use alternatives (Nedižavec 2009), the author was interested how students would report their:

- situational interest toward dissection;
- individual interest in anatomy and physiology;
- emotions that accompany dissection activities (i.e. fear and disgust).

Allchin (2005) stated that in a culture where anyone is helping in slaughtering and butchering of animals or anyone who wears a leather and is also helping in skinning of animals, dissection in a school classroom might be redundant. To test this notion the author has, in addition to grade and gender, also assessed if reported home experiences (cooking meat or fish and participating in home slaughter of livestock) influence before mentioned dependent variables.

Material and methods

Sample of research

A total of 113 lower secondary school students from grades seven (N = 55) and nine (N = 58) participated in the study. Seventh and nine grade students were selected because seventh grade students were learning about ecosystems where majority of the topics were about animals while nine grade students were learning about human

anatomy. Therefore, in mentioned grades, teachers can meaningfully incorporate dissection activities into their teaching. The mean age of seventh grade students was 12.6 (SD = 0.50) years and ninth grade students 14.4 (SD = 0.49) years. The proportion of male (N = 54) and female (N = 59) students was slightly different between grades (χ^2 = 3.963, df = 1, p = 0.047). More than 65 % of students reported that they are participating in cooking meat or fish and 44 % of students reported participating in home slaughter of livestock. More male (59 %) than female (30 %) students reported participating in home slaughter (χ^2 = 9.447, df = 1, p = 0.002). There were no other statistically significant differences in distributions found (all $p > 0.05$).

Instruments and procedures

Participating students completed a questionnaire about (1) their situational interest for dissection, (2) their individual interest in anatomy and physiology and (3) emotions that accompany dissection activities (fear and disgust). The self-constructed questionnaire included fourteen 5-point Likert-type items ([1] *strongly disagree*, [2] *disagree*, [3] *neither agree nor disagree*, [4] *agree* and [5] *strongly agree*). Items covering the domain of emotions and perceived health issues concerning dissection were negatively worded and were subsequently reversed.

In statistical analysis, first Principal Component Analysis (PCA) with Direct oblimin rotation

Table 1: Principal Component Analysis with Direct oblimin Rotation of the questionnaire items.
Tabela 1: Analiza glavnih component z uporabo poševnokotne rotacije (Direct oblimin).

Item	**Principal component**		
	I	**II**	**III**
I would not mind dissecting in a classroom.	0.845		
Dissection is interesting.	0.736		
I think I would perform well when dissecting.	0.706		
I would learn a lot through dissection.	0.706		
I would like to dissect organisms in my classroom.	0.600		
Knowledge about my body will be useful for my life.		0.802	
I like to learn about structure and functioning of my body.		0.791	
Knowing the animals' anatomy is important for me.		0.749	
Seeing blood makes me sick. R			0.801
When dissecting, I am afraid of harming myself (e.g. cutting myself). R			0.728
Dissection is a disgusting practice. R			0.691
I would be scared if I would have to touch a dead animal or an animal organ. R			0.515
When dissecting, I would be scared of getting infected. R			0.458
Dissection in classroom is unacceptable. R			0.418
Cronbach α	0.84	0.72	0.76
Eigenvalue	4.71	2.22	1.28
% of total variance	33.64	15.86	9.13

R – reversed items

was used in order to extract meaningful principal components (hereafter PCs) (Tab. 1). The KMO (Kaiser-Mayer-Olkin) index of the sampling adequacy test (0.861) and Bartlett's test for sphericity (χ^2 = 569.4, df = 91, $p < 0.001$) suggested that factor analysis was appropriate for this dataset. PCs with eigenvalues > 1.0 were considered in further analyses. In order to test the reliability of extracted PCs, Cronbach's α coefficients were calculated and all shown not to be below the accepted limit of 0.69 (Leech 2005). Cronbach's α of the whole questionnaire was 0.83.

According to PCA, three principal components were interpreted. Situational interest about dissection items loaded highest on PC I (5 items). On PC II, items about students' individual interest in learning about anatomy and physiology were placed (3 items). And on PC III, affective factors that accompany dissection activities (fear and disgust related items) were placed (6 items). All three PC explained 58.63 % of the results' variability.

Data analysis

First, Principal component analysis (PCA) was applied in order to reduce the number of dependent variables. Next, the effect of selected independent variables on individual dependent variable was calculated. For that purpose, GLM univariate statistical procedure was used.

As all independent variables were dichotomous, Mann-Whitney U test was used and the effect sizes were calculated using formula $r = z/\sqrt{N}$.

Spearman's correlations (r_s) between individual PCs were assessed, in order to find correlations between students' interest for dissection, their individual interests in human and animal anatomy and physiology and emotional factors that govern their acceptance of dissection.

All the data were analysed using the SPSS for Windows 21.0.0 statistical software.

Results

Results are presented in three parts. First, the results of univariate statistics are shown, followed by the effects of individual independent variable on students' ratings. Lastly, the correlations between individual PCs are presented.

Results of univariate statistics for individual principal component

Results of univariate statistics (Tab. 2) show that grade (age) produces the largest differences in students' ratings on situational interest (PC I). On the other hand, students' individual interest (PC II) is influenced by several factors. While gender and grade produced medium sized effects on students' ratings, participation in a home slaughter had a marginal effect. Interaction between all independent variables was also found for that PC. Students' emotional response toward dissection (PC III) was strongly influenced by gender only.

Table 2: GLM univariate analysis of the effect of independent variables on students' ratings for individual principal component.
Tabela 2: Univariatna analiza vpliva neodvisnih spremenljivk na izbrane odvisne spremenljivke.

Attitude dimension	Type III Sum of Squares	df	Mean Square	F	*p*	Partial η^2
PC I: Situational interest						
Gender	0.950	1	0.950	0.813	0.370	0.008
Grade	6.891	1	6.891	5.898	**0.017**	**0.057**
Cooking	0.623	1	0.623	0.533	0.467	0.005
Slaughter	0.583	1	0.583	0.499	0.482	0.005
PC II: Individual interest						
Gender	5.417	1	5.417	5.900	**0.017**	**0.057**
Grade	5.876	1	5.876	6.400	**0.013**	**0.062**
Cooking	1.723	1	1.723	1.877	0.174	0.019
Slaughter	3.540	1	3.540	3.856	**0.052**	**0.038**
Gender * Grade * Cooking * Slaughter	6.644	1	6.644	7.236	**0.008**	**0.069**
PC III: Emotions						
Gender	14.923	1	14.923	22.182	**<0.001**	**0.186**
Grade	0.572	1	0.572	0.850	0.359	0.009
Cooking	0.146	1	0.146	0.217	0.643	0.002
Slaughter	0.148	1	0.148	0.220	0.640	0.002

The effect of individual independent variables on students' ratings

Gender had the highest effect on students' ratings about emotional perception of dissection (PC III; Fig. 1A), where females showed significantly more aversion than boys ($r = 0.48$). Females also perceived the importance of human (animal) anatomy and physiology knowledge for life to be more important than males (PC II, Fig. 1A). But the effect of gender on this principal component was low ($r = 0.19$). **Grade** produced statistically significant differences on all three principal components (Fig. 1B). Ninth graders expressed higher situational interest for dissection (PC I, $r = 0.35$; medium effect) and higher individual interest for usefulness of the anatomy and physiology knowledge (PC II, $r = 0.20$; low effect) than seventh graders. Also, seventh graders perceived dissection emotionally more demanding than ninth graders (PC III, $r = 0.19$; low effect). The only difference in ratings between students with regard to reported participation in cooking meat or fish was on individual interest dimension (PC II, Fig. 1C). Students with reported participation displayed higher individual interest ($r = 0.19$; low effect). No statistically significant differences were found in students' ratings according to participation in home slaughter of livestock on any PC (Fig. 1D).

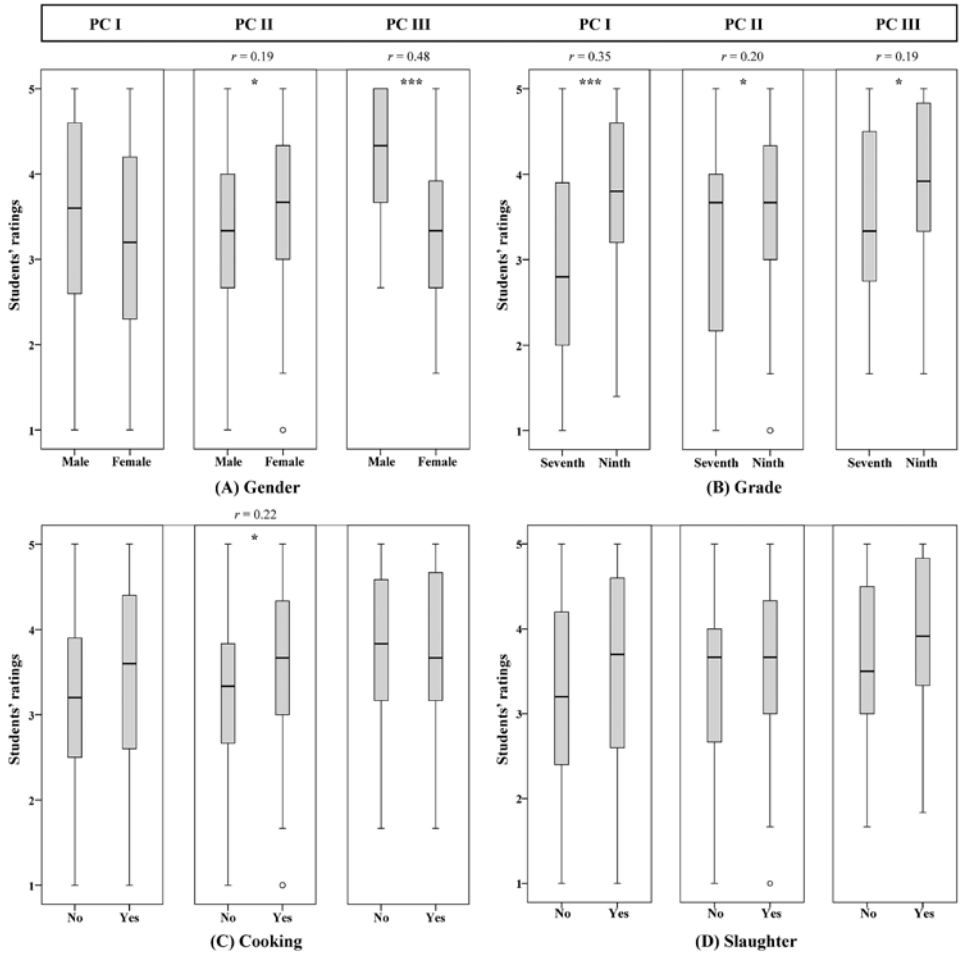

Figure 1: Students ratings on principal components according to **A** - gender, **B** - grade, **C** - reported cooking experiences with meat or fish and **D** - reported participation in home slaughter of livestock (statistical significance was assessed with Mann-Whitney U test; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.01$; r – effect size).

Slika 1: Ocene učencev za posamezno osnovno komponento glede na **A** - spol, **B** - razred, **C** - izkušnje pri kuhanju mesa ali rib in **D** - sodelovanje na domačih kolinah (statistična pomembnost razlik računana z Mann-Whitney U preizkusom; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.01$; r – velikost učinka).

Correlations between individual principal components

Spearman's *r* correlations were calculated between dependent variables (Tab. 3). Results show the highest correlation to be between students' situational interest for dissection and their expressed emotions. Due to reversed items, the correlation number is positive, meaning the higher the students' situational interest is the less negative is their emotional response. Low correlation was also found between situational and individual interest ratings. No significant correlation was found between individual interest and emotions.

Table 3: Correlations between individual principal components.
Tabela 3: Povezave med posameznimi osnovnimi komponentami.

	PC II: individual interest	PC III: emotions
PC I: situational interest	0.283**	0.572***
PC II: individual interest	-	0.077

Spearman's r: ***$p < 0.001$, **$p < 0.01$; PC III: EMOTIONS column values are positive due to reversed statements.

Discussion

Present study shows, that many lower secondary school students are willing to study anatomy through means of dissection. The findings correspond with the study of Špernjak and Šorgo (2017), where only a minority of students chose to opt out from dissections. This information should be disseminated to teachers, because many of them do not use dissection activities in their classrooms mainly due to their unfamiliarity with local legislation (Nedižavec 2009) what should also be worked on in the future. Still, a relatively high proportion of students in the present study stated their disapproval of dissection in the classroom. These students also expressed higher levels of negative emotional responses linked to situational interest regarding dissection. Items that assessed students' perceived emotional response were linked to fear and disgust (for example: "*Dissection is a disgusting practice.*" and "*When dissecting, I am afraid of harming myself (e.g. cutting myself).*" Similar results were obtained by Holstermann et al. (2009) and Randler et al. (2012) via assessing the effect of actual dissection activities. The authors argue that disgust eliciting stimuli should be reduced during dissection in order to achieve high interest, as it is known that interest has an important influence on cognitive, motivational and affective processes (see also Hidi and Renninger 2006).

Older students displayed higher interest in school dissections than younger ones, which can be explained by the younger students' preference for studying live animals (Prokop and Tunnicliffe 2010) and their lower interest in internal structures of animals. The author speculates that older student's higher interest of animals' anatomy and physiology is a result of their development. Specifically, the changes in their bodies during puberty, shifts their interest toward studying animal's and their own internal structures and their functioning. It was also found that interest in dissections remains high through the upper high school level (Špernjak and Šorgo 2017).

Although correlations between students' interest for dissections and their general interest about anatomy and physiology were observed, they were low, as opposed to higher correlations between students' interest in dissections and their emotional response. Nevertheless, the former correlation is not to be neglected. As noted by Abrahams et al. (2009), repeated elicitation of situational interest could lead to formation of one's individual interest. Practical work in schools has an effect only if it is well structured and with clear goals what students should gain, which is of particular relevance for dissection activities. Renninger and Hidi (2011) argue that students may experience situational interest when they are exposed to experiences that relate to their individual interests. In this study, students' interest in dissection activities can be

linked to students' personal interest in learning anatomy and physiology. Palmer et al. (2016) found that substantial situational interest can occur if the presented topic is of personal relevance, if it carries a novelty and/or students successfully learn something new. From a constructivist point of view, mentioned can be considered as meaningful learning (Mintzes et al. 1998). But because the correlation between individual and situational interest was low, the statement cannot be completely confirmed.

And lastly, to comment on Allchin's (2005) statement "*that in a culture where anyone is helping in slaughtering and butchering of animals or anyone who wears a leather and is also helping in skinning of animals, dissection in a school classroom might be redundant*". This study found almost no differences between students' ratings regarding situational interest for dissection, nor in the expressed emotions regarding dissection. This finding is somehow contradictory to statements of other authors, who argue that repeated exposure to dissection might raise students' interest and lower their negative emotions regarding dissection (Holstermann et al. 2009, Randler et al. 2012). Perhaps spatial and temporal dimensions ought to be considered in future studies, in addition to assessing of the levels of students' involvement in such activities.

Limitations of the study

The results of this pilot study have confirmed that a link between students' interest in dissections and their emotional response to dissections exists. Furthermore, the link between students' situational interest regarding dissection and their personal interest for anatomy was found. It appears that students' out-of school experiences regarding cooking meat or fish and participating in home slaughtering of livestock do not influence their interest in dissection. However, the later should be reassessed, using a larger sample of students, joint with a more in-depth analyses of students' experiences.

Povzetek

S prenovo učnih načrtov naravoslovja in biologije se pri obeh predmetih obravnavajo tudi teme s področja anatomije in fiziologije. Učenci se pri pouku naravoslovja v osnovni šoli tako bolj podrobno učijo o notranji zgradbi živali. Pri pouku biologije v srednjih šolah in gimnazijah pa je poudarek na razumevanju strukture in funkcije. Ena od učnih metod, preko katere učenci spoznavajo zgradbo in delovanje živali, je seciranje. Slednje se učitelji ne poslužujejo pogosto, kar lahko med drugim pripišemo tudi slabemu poznavanju zakonodaje s tega področja. Vivisekcija ali usmrtitev živali za namen sekcije sta prepovedana, kar pa ne izključuje uporabe materialov živalskega izvora ali živali pri pouku naravoslovja in biologije. Omenjene materiale lahko učitelji kupijo v mesnicah ali drugih živilskih trgovinah in jih uporabijo za seciranje. Ob tem velja poudariti, da lahko čustvi, kot sta strah in gnus, ki ju učenci močno doživljajo ob seciranju, vplivata na kvaliteto njihovega učenja. Predhodne raziskave so pokazale, da je večina učencev naklonjena seciranju v šoli. Sekciji bi se izognilo le 15 % slovenskih učencev in dijakov. Nekateri avtorji menijo, da lahko sekcijo nadomestimo z alternativnimi viri za učenje (3d modeli, interaktivnimi gradivi, knjigami in drugim), ali pa med sekcijo omilimo dražljaje (npr. vonj), na katere bi se lahko učenci odzvali z negativnimi čustvi.

Namen raziskave je bil ugotoviti interes in čustva osnovnošolcev glede seciranja pri pouku naravoslovja in biologije. Učenci so v ta namen v obliki samoporočila ocenili svoj interes za seciranje ter nivo strahu in gnusa, ki bi ga občutili ob tem. V predhodnih študijah so različni avtorji ugotovili, da na ocene interesa in čustev v povezavi s seciranjem v pretežni meri vpliva spol učencev.

V raziskavi smo zato ugotavljali tudi, v kolikšni meri se čustva in interes v povezavi s seciranjem spreminjajo glede na starost učencev, njihovo vključenost v gospodinjska opravila (obdelava mesa in rib) in sodelovanje pri domačih kolinah. Rezultati raziskave so pokazali, da na interes učencev za seciranje vpliva predvsem starost učencev. Starejši učenci so izkazali višji interes za seciranje kot mlajši učenci. Na osebni interes učencev za učenje o zgradbi in delovanju organizmov vplivajo starost in spol učencev ter

njihovo sodelovanje pri domačih kolinah. Spol učencev vpliva na izražanje čustev v povezavi s seciranjem. Interes za seciranje je povezan tako z osebnim interesom kot negativnimi čustvi posameznika. Iz rezultatov lahko sklepamo, da vključenost učencev v gospodinjska opravila in sodelovanje pri domačih zakolih ne vplivata na interes učencev za seciranje in oblikovanje njihovih čustev. Naša ugotovitev je v nasprotju z razmišljanji in ugotovitvami drugih avtorjev, ki trdijo, da ponavljajoča se izpostavljenost seciranju vzbuja interes in znižuje negativna čustva ob tem. V prihodnjih študijah bi bilo vredno upoštevati tudi prostorske in časovne dimenzije vključenosti učencev v zunajšolske aktivnosti, ki so povezane s seciranjem živali ter stopnjo njihove vključenosti pri teh dejavnostih.

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Dan očarljivih rastlin

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»Rastline - stotine milijonov let samostojnega razvoja na Zemlji, desetine milijonov let skupnega razvoja z opraševalci, deset tisoč let v službi človeka, dva tisoč let odkrivanja njihove edinstvenosti in očarljivosti. Le nekaj desetletij pa je minilo od spoznanja, da rastline niso le ozadje živalskemu svetu, da živijo izjemno zanimivo zasebno življenje, da se na raznolike načine sporazumevajo med seboj in z drugimi živimi bitji in da so nam v svojih odgovorih na notranje in zunanje okolje celo zelo podobne«

S temi besedami je prof. dr. Marina Dermastia 18. maja 2012 vabila na predavanje ob prvem Dnevu očarljivih rastlin, na katerem so navzoči spoznali, kaj dela rastline tako fascinantne kljub temu, da se tako redko zavedamo njihovega pomena. Običajno smo zanje slepi, našo pozornost največkrat pritegnejo z izrazitimi barvami cvetov in jesenskega lista.

Pobuda za Dan očarljivih rastlin ima korenine v evropski organizaciji za raziskave rastlin (ang. European Plant Science Organisation – EPSO), ki združuje okoli 30.000 raziskovalcev iz Evrope in širše. Glavna naloga EPSO je izboljšati vpliv in prepoznavnost znanosti o rastlinah. Ravno s tem namenom je 18. maja 2012 prišlo do mednarodne akcije, imenovane Fascination of Plants Day, ki smo jo v Sloveniji poimenovali Dan očarljivih rastlin (http://www.plantslo.org/dan_rastlin/index.php). Svoje aktivnosti je takrat prijavilo skoraj 600 ustanov iz 39 držav in vseh celin sveta. V Sloveniji se je kot glavni koordinator pobudi pridružilo Slovensko društvo za biologijo rastlin, ki kot prostovoljno znanstveno in strokovno združenje deluje v javnem interesu in vključuje strokovnjake in znanstvenike, ki delujejo na različnih področjih, povezanih z rastlinami, kot so biologija, agronomija, gozdarstvo, kemija, farmacija idr.

V mednarodnem merilu tako vsaki dve leti okoli 18. maja številne ustanove, kot so univerze, inštituti, botanični vrtovi, muzeji, društva, šole, podjetja in drugi gostijo različne vrste dogodkov, ki so povezani s temeljnimi in uporabnimi raziskavami rastlin, kmetijstvom, ohranjanjem okolja, biodiverziteto, izobraževanjem in umetnostjo. V letu 2017 je bilo takih prireditev 710 v 52 državah. V Sloveniji je bil odziv tako izvajalcev aktivnosti kot obiskovalcev že od prvega leta dalje izjemen, pa čeprav so nas včasih ledeni možje in uscana Zofka pošteno namočili. Zlasti osnovne šole so prepoznale Dan očarljivih rastlin kot priložnost za celovit vpogled v rastlinski svet, saj so rastline v žarišču predmeta Naravoslovje v 6. razredu. Zato smo se odločili, da bomo v Sloveniji organizirali Dan očarljivih rastlin vsako leto. V šestih letih, odkar organiziramo Dan očarljivih rastlin, se je naših prireditev udeležilo okoli 3300 obiskovalcev, med katerimi so gotovo tudi bodoči znanstveniki in naravoslovci. S široko zasnovo predstavitev pa seveda želimo izboljšati odnos vseh, mladih in starejših, do rastlin in okolja.

Osrednji dogodek je sprva potekal v idiličnem in zelenem okolju ljubljanskega botaničnega vrta, zadnji dve leti pa smo ga umestili v Biološko središče, kjer domujeta Oddelek za biologijo Biotehniške fakultete in Nacionalni inštitut za biologijo, in odziv je bil ravno tako navdušujoč.

Letos smo se zbrali 19. maja in na 23 stojnicah so potekali praktični prikazi, delavnice, igre in kvizi, vsi v povezavi z rastlinami. Obiskovalci so lahko izdelali herbarijsko polo, spoznali zgradbo zelnatih in lesnatih rastlin, njihove življenjske procese (kalitev, fotosintezo, prenos vode), interakcije rastlin z okoljem in prilagoditve na posebne rastne razmere (mesojedke, kaktusi), invazivne tujerodne rastline, alge, gozdni ekosistem, rastlinske opraševalce in škodljivce, uporabne rastline (zdravilne rastline, oljnice, stročnice,

citruse, začimbe, sadje in zelenjavo v prehrani, med) in njihovo gojenje ter pomen gensko spremenjenih rastlin. Posamezne aktivnosti so tako kot v preteklih letih vodili strokovnjaki in znanstveniki iz sodelujočih inštitucij, kot so Biotehniška fakulteta Univerze v Ljubljani, Nacionalni inštitut za biologijo, Gozdarski in Kmetijski inštitut, Prirodoslovni muzej, v velikem številu pa so se nam pridružili tudi študenti Društva študentov biologije, Biotehniške in Pedagoške fakultete UL in celo dijaki Biotehniškega izobraževalnega centra Ljubljana. Obiskalo nas je preko 650 ljudi, poleg osnovnošolcev tudi skupina nadobudnih gimnazijcev in naključni obiskovalci vseh starosti.

Z Dnevom očarljivih rastlin povezani dogodki so potekali tudi drugod po Sloveniji. Revija Pil je organizirala nagradni literarni natečaj Zelena čudesa, v Trenti je odprl vrata Alpski in botanični vrt Julijana, na Raščici so se botanično sprehodili od Kobiljega curka do Lehnjaka, očarljivost rastlin je bila tudi del 19. Belarjevih dni v organizaciji Triglavskega narodnega parka in Prirodoslovnega muzeja Slovenije. K medijski prepoznavnosti nam je pomagal tudi častni pokrovitelj National Geographic Slovenija.

Slovensko društvo za biologijo rastlin

Dan očarljivih rastlin

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**), a hyphen and followed by the 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 international abbreviation for 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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