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Achenes of common ragweed (*Ambrosia artemisiifolia)* in packages of sunflower achenes for outdoor birds

Plodovi pelinolistne žvrklje *(Ambrosia artemisiifolia)* v paketih sončničnih plodov za prostoživeče ptice

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Abstract: Common ragweed (*Ambrosia artemisiifolia*) is a highly invasive annual plant, introduced to Europe from North America. One of the important spreading vectors of *Ambrosia* is trading with contaminated sunflower achenes. We analysed the content of ragweed achenes in the packages of sunflower achenes for outdoor birds, which are available in Slovenian shops and markets. The ragweed achenes were found in 29 % of the 28 analysed packages. In three packages, the mass percentage of *Ambrosia* achenes per kg of animal feed exceeded the maximum content (50 mg/kg) allowed by EU law. The analysis showed that it is not possible to infer the presence or absence of *Ambrosia* achenes in a sample from the amount of impurities in the package of sunflower achenes for bird feed.

Keywords: *Ambrosia artemisiifolia*, *Helianthus annuus*, invasive species, ragweed, Slovenia, sunflower fruits

Izvleček: Pelinolistna žvrklja (*Ambrosia artemisiifolia*) je invazivna tujerodna enoletnica, ki je bila v Evropo prinesena iz Severne Amerike. Pomemben vektor širjenja vrste predstavlja transport in trgovanje s plodovi sončnic, ki vsebujejo primešane plodove žvrklje. V raziskavi smo preverjali prisotnost plodov žvrklje v paketih sončničnih plodov za hranjenje prostoživečih ptic. Plodovi žvrklje so bili prisotni v 29 % od 28 paketov hrane za ptice, ki smo jih kupili v slovenskih trgovinah. V treh paketih je količina plodov presegala največjo določeno količino plodov žvrklje, kot jo določa evropska zakonodaja (največ 50 mg/kg). Ugotovili smo tudi, da količina nečistoč v paketu ni povezana s prisotnostjo plodov žvrklje.

Ključne besede: *Ambrosia artemisiifolia*, *Helianthus annuus*, invazivna tujerodna vrsta, pelinolistna žvrklja, Slovenija, sončnični plodovi

Introduction

Common ragweed (*Ambrosia artemisiifolia*) is an annual plant from the family *Asteraceae*. It was introduced to Europe from North America in the 19th century (Csontos et al. 2010, Hegi 1979). In Europe it is considered an invasive alien species. It populates fields (corn, sunflower, pumpkin …), river banks, and disturbed places such as railways and road edges (Hegi 1979). It is detrimental to human health as it produces highly allergenic pollen, which causes late summer hay fever (Kofol Seliger 2001, Taramarcaz 2005).

The first records of the presence of *Ambrosia artemisiifolia* in Slovenia are from Leskovec near Krško in 1950 (Wraber 1983). In next years some new localities were discovered, and Wraber (1983) marked this species as casual in Slovene flora, dispersed with human activities. The first data about naturalisation of the species are from Prekmurje (Wraber 1983). In the last years the species has become present in almost all lowlands in Slovenia, and it is now known as one of most invasive alien plant species in Slovene flora (Jogan and Vreš 1998, Jogan 2001, Jogan et al. 2009).

Ambrosia artemisiifolia is an annual plant, 0.2–2.5 m high. One or more male flowers are grouped in numerous much reduced capitula, which include one or more male flowers. Male capitula are grouped in racemes at the end of branches, while female flowers are located at the bases of upper leaves. *Ambrosia artemisiifolia* is a short-day plant, and its flowering time begins in late summer (Allard 1943). Its fruits are achenes, each containing one seed. That is the reason for the simplified naming of ragweed achenes as seeds in non-botanical general use (newspapers, media …). Achenes usually drop off the mother plant, and they become part of soil seed bank, but they may also be dispersed by water or by human activities. Seeds have primary and secondary dormancy (Baskin and Baskin 1980), and can remain viable for 40 years (Darlington 1922, Telewski and Zeevaart 2002).

One of the important spreading vectors of *Ambrosia* is trading with contaminated sunflower seeds (more precisely: fruits or achenes) (Jehlik and Hejny 1974, Wraber 1983, Essl et al. 2009). Researchers in Austria have noticed that *Ambrosia* became frequent in habitats associated with bird feeding during the period 1950–1979 (Essl et al. 2009). In the international European research performed in years 2005 to 2009, which involved institutes from Switzerland, Germany, Denmark and Slovenia, *Ambrosia* achenes were found in a large proportion of the analysed sources of bird feed (Frick et al. 2011).

The spread of *Ambrosia* in Slovenia is controlled by two legally adopted regulations. The first is Commission Regulation (EU) No 574/2011 of 16 June 2011, concerned with undesirable contents of animal feed, including *Ambrosia* seeds (Anon. 2011). The limit of intervention is 50 mg of *Ambrosia* seeds per kg of animal feed (Anon. 2011). The second is the Slovenian regulation "Odredba o ukrepih za zatiranje škodljivih rastlin iz rodu *Ambrosia*" (Decree on measures to suppress harmful plants of genus *Ambrosia*), that obliges every citizen to remove all *Ambrosia* plants from his property, and he has to prevent the plants from developing flowers and fruits (Anon. 2010).

The aim of our research was to check if the packages of sunflower seeds for outdoor birds available in Slovene shops and markets contain ragweed achenes, to assess the extent of this contamination and to compare it with legally adopted regulations.

Materials and methods

In autumn of 2011 we collected 35 samples of packages of sunflower seeds for bird feed from various shops and markets in Slovenia for analyses of ragweed contamination. The samples were planned to cover: (1) different parts of Slovenia, (2) different shops and suppliers, and (3) different package sizes. Every package was numbered, and the bills from the shops were stored together with the package to keep the information about the date and locality of the purchase.

The following data have been collected from package labels: package ID, commercial name, size (weight), serial number or LOT number, country of sunflower fruit production, date of packaging, type of sunflower nuts (black, white, striped, mixed), and data about the purchase: location of shop, shop name, and date.

The first step in separating the ragweed achenes from sunflower achenes was hand-sieving. For this purpose, a special sieve with 1.5–2.3 mm wire mesh size was made. Prior to examination of the samples, the sieve was tested with artificial (prepared) mixture of sunflower and ragweed achenes. Since no ragweed achenes stayed on the sieve, and only a few sunflower achenes were smaller than the mesh size, we considered the hand-made sieve suitable enough for fractioning the samples.

Secondly, the small fraction that presumably contained the *Ambrosia* achenes was weighted and stored in a plastic bag. The fraction with sunflower achenes was weighted as well.

A small fraction was further analysed using a stereomicroscope (20 × magnification) to sort out ragweed achenes. Achenes were weighted, counted and stored in a small plastic bag.

There are two other species from genus *Ambrosia* present in Slovenia: *A. psilostachya* and *A. trifida*, but *A. artemisiifolia* is the most common one (Buttenschøn et al. 2008–09, Jogan 2001). There is a minor possibility that some of the ragweed fruits we found in bird feed belonged to other *Ambrosia* species, since no detailed species determination was made based on *Ambrosia* achenes properties. According to the frequency of all *Ambrosia* species in Europe and Slovenia, we assumed that the majority of achenes belonged to *Ambrosia artemisiifolia*.

Results

35 packages of sunflower seeds for bird food were collected in different Slovene shops. Seven packages were excluded from the analysis because it was not possible to isolate ragweed achenes by using the described methods.

Altogether, 2 packages of 5 kg, 24 packages of 1 kg, one package of 0.6 kg, and one package of 0.5 kg of bird feed were analysed (Table 1).

We included 13 different producers or importing companies of bird feed, but it is possible that they had the same source of raw material. The data about the origin of the sunflowers were absent from the majority of the packages labels. They were written only on 8 packages (23%).

The results of the analysis are shown in Table 2. Ragweed achenes were found in 8 packages of bird feed (29%). In three packages, the mass percentage of *Ambrosia* seeds per kg of animal feed exceeded the maximum content (50 mg) allowed by EU law (Anon. 2011). Producers of these three packages were Agrolit, Semenarna Ljubljana and AnimaPet.

We have included more than one sample of bird feed from the same producer (Erdtmanns, Agrolit, Tukano, and Semenarna Ljubljana), and the same series of raw material in our analysis (Table 3). It is apparent, that there are big differences in contents of ragweed achenes in packages within the samples of the same producer, and even within the same series of the raw material (0 or 35 ragweed achenes in 1 kg of bird feed in the case of Semenarna Ljubljana, 105-PC, Table 3).

The analysis of correlation between the number of ragweed achenes and the content of the small fraction (impurities) in the sample showed that there is no clear connection between these parameters. Therefore we cannot infer the presence or absence of *Ambrosia* seeds in a sample just from the amount of impurities in the package of sunflower achenes for bird feed.

Discussion

Ragweed achenes were found in one third of the 28 analysed packages of bird feed. In three packages, the mass percentage of *Ambrosia* seeds per kg of animal feed exceeded the maximum content (50 mg) allowed by EU law (Anon. 2011). Thus, these packages should not be sold. From a biological point of view, all packages containing one or more seeds of *Ambrosia* are problematic. Only one successfully germinated seed that develops into a flowering and afterwards fruiting plant is enough to establish a new population of *Ambrosia*. Although a smaller number of the seeds reduces the possibility of such event, it still makes it possible.

The National Veterinary Institute conducted a similar investigation in 2007/08 and 2008/09, analysing 10 samples of bird feed each year (Frick et al. 2011). *Ambrosia* achenes were found in 7 and 5 samples, respectively, with the highest amount of 292 *Ambrosia* achenes per kg of bird feed. In our samples from 2011, the contamination with *Ambrosia* achenes was lower (33% of contaminated samples with maximum 198 *Ambrosia* achenes

Table 1: Information about the packages of sunflower fruits for bird feed used in the analysis. Packages no.: 2, 6, 7, 10, 11, 19 and 22 (grey shading) were excluded from the analysis (explanation in the text). × – no data, *unpacked sunflower achenes with no commercial name bought on a food market.

Tabela 1: Informacije o paketih sončničnih plodov za hranjenje zunanjih ptic, ki smo jih zbrali za analizo. Pakete 2, 6, 7, 10, 11, 19 in 22 (siva podlaga) smo iz analize izključili (razlaga v besedilu). × – ni podatkov, *nepakirani sončnični plodovi kupljeni na tržnici.

Sample number	Commercial name	Producer/Importing company	Lot/Series	Package size (kg)	Country of origin of sunflowers
1	Sonnenblumen Kerne	Erdtmanns	210910	1	x
2	Bonami	Semenarna Ljubljana	105-PC	1	x
3	Bela sončnična semena	Versele_Laga	B-9800	0.6	Belgium
4	Sončnice pisane	Roko	480-43486	1	Hungary
5	Hobby Vit	Tukano	21300040	1	Bulgaria
6	Menu Complete	Friskies	EAN	0.4	x
7	Lojna pogača	Vogel pick	21305065	0.2	x
8	Sončnična semena	Tukano	21300042	1	Bulgaria
9	Bonami, Sončnice pisane za ptičjo hrano	Semenarna Ljubljana	460-PC	5	x
10	Bonami, Hrana za zunanje ptice	Semenarna Ljubljana	P1330	5	x
11	Bonami, Mešanica semen	Semenarna Ljubljana	70507015	1	x
12	Bonami, Semena sončnic	Semenarna Ljubljana	460-PC	1	x
13	Bonami, Sončnice Mešane	Semenarna Ljubljana	P1279	1	x
14	Hobby Vit	Tukano	M1/17:21:29 /10/10/11	1	x
15	Sončnična semena	Roko	480-43486	1	x
16	Bonami	Semenarna Ljubljana	105-PC	1	x
17	Sončnice Mešane	Semenarna Ljubljana	P1278	5	x
18	Sončnice pisane	Zoo hoby Škofja Loka	0013	1	x
19	Hobby Vit	Tukano	11246	1	x
20	Sonnenblumen Kerne	Tierell	772013	1	x
21	Bonami, Mešanica semen	Semenarna Ljubljana	P1331	1	x
22	Bonami, Semena sončnic	Semenarna Ljubljana	460-PC	1	x
23	Sončnična semena	Agrolit	19. 9. 2011	1	x
24	Bonami, Semena sončnic	Semenarna Ljubljana	105-PC	1	x
25	Hobby Vit	Tukano	21300042	1	Bulgaria
26	Sončnična semena	Roko	11/210	1	Hungary
27	Sončnična semena	Agrolit	4. 12. 2010	1	EU
28	Sončnična semena	Agrolit	19. 9. 2011	1	x
29	Sonnenblumen Kerne	Erdtmanns	210910	1	x
30	Sonnenblumen Kerne	Erdtmanns	210910	1	x
31	Futter Boy	Erdtmanns	210829	0.5	x
32	Hesa Saaten	Tierell	CHA70	1	x
33	Sončnična semena	Hobby program	21300042	1	x
34	Sončnična semena 1kg	AnimaPet	598.10	1	EU
35	x*	food market	x	1	x

Table 2: Presence of ragweed achenes in the analysed packages of the bird feed.
Tabela 2: Prisotnost plodov žvrklje v pregledanih paketih ptičje hrane.

Sample number	**Content of ragweed achenes (mg/kg)**	**Number of ragweed achenes**	**Mass % of the small fraction (including ragweed)**
24	**198**	35	1.9
23	**61**	11	0.5
34	**51**	9	0.7
28	**45**	8	0.5
29	**34**	6	0.3
14	**6**	1	0.3
22	**6**	1	0.3
17	**1**	1	0.1
1	**0**	0	0.3
3	**0**	0	0.4
4	**0**	0	0.2
5	**0**	0	6.8
8	**0**	0	0.4
9	**0**	0	0.1
12	**0**	0	0.3
13	**0**	0	0.5
15	**0**	0	0.2
16	**0**	0	0.4
18	**0**	0	0.2
20	**0**	0	0.4
25	**0**	0	0.4
26	**0**	0	0.2
27	**0**	0	2.2
30	**0**	0	0.3
31	**0**	0	0.7
32	**0**	0	0.4
33	**0**	0	0.7
35	**0**	0	0.4

per kg of bird feed), which may indicate a gradual decrease of the contamination level in bird feed available on Slovene market.

In Slovenia, every citizen is obliged to remove all *Ambrosia* plants from his property and he has to prevent the plants from developing flowers (because of the allergenic pollen) and fruits (to limit their dispersal) (Anon. 2010). Therefore it is very important to inform people that it is highly possible to introduce *Ambrosia* into gardens with contaminated bird feed. In Slovenia this is already in progress. Informations about *Ambrosia* and the related problems were already published in public journals (Jogan 2011, Bačič 2012, Strgulc Krajšek 2013), project publications (for example: Buttenschøn et al. 2008–09), and on different websites (for example: Project Thuja: http://www.tujerodne-vrste.info, DOPPS: http://www.ptice.si).

Povzetek

Pelinolistna žvrklja (*Ambrosia artemisiifolia*) je ena najbolj razširjenih invazivnih tujerodnih rastlinskih vrst na območju Slovenije (Jogan et al. 2001). Gre za severnoameriško vrsto, ki je bila v 19. stoletju nehote prinesena v Evropo in

Table 3: Differences in number of ragweed achenes among the packages of bird feed of the same producer, or the same series of the raw material.
Tabela 3: Razlike v prisotnosti plodov žvrklje med paketi istega proizvajalca in iste serije paketov sončničnih plodov.

Producer, Series	Sample number	Package size (kg)	Mass of small fraction (including ragweed) (g)	Number of ragweed achenes
Erdtmanns, 210910	1	1	2.9	0
	29	1	3.2	6
	30	1	3.0	0
Agrolit, 19. 9. 2011	23	1	5.5	11
	28	1	5.0	8
Tukano, 21300042	33	1	6.8	0
	25	1	3.6	0
	8	1	4.4	0
Semenarna Ljubljana, 105-PC	24	1	18.9	35
	16	1	3.9	0
Semenarna Ljubljana, 460-PC	9	5	4.8	0
	22	1	3.1	1
	12	1	3.5	0

se širi predvsem ob železnicah, cestah, ruderalnih mestih in poljih (Jogan 2009). Problematična je zaradi alergenega peloda, ki povzroča poznopoletni seneni nahod. Eden od pomembnejših vektorjev razširjanja je prenos plodov žvrklje skupaj s sončničnimi plodovi, ki jih prodajajo za hranjenje prostoživečih ptic (Essl et al. 2009, Frick et al. 2011).

V okviru raziskave smo pregledali pakete sončničnih plodov za hranjenje ptic, ki so naprodaj v trgovinah v Sloveniji. Naš namen je bil ugotoviti, v kolikšni meri se v njih pojavljajo plodovi (oreški) pelinolistne žvrklje. Pakete hrane za ptice smo nakupili jeseni leta 2011. Zbrali smo 35 paketov, od katerih jih 7 nismo pregledali, ker se z izbranimi metodami iz njih ni dalo ločiti plodov žvrklje. Za izolacijo plodov žvrklje smo uporabili metodi sejanja skozi sito in ročnega prebiranja vzorca. Izmed 28 pregledanih paketov ptičje hrane, smo plodove žvrklje našli v 8 paketih, kar predstavlja 29 % pregledanih vzorcev. V treh paketih je količina plodov žvrklje presegala zakonsko predpisano zgornjo dovoljeno mejo masnega deleža plodov pelinolistne žvrklje (Anon. 2011), kar ustreza 9 plodovom pelinolistne žvrklje na kilogram ptičje krme. Ugotovili smo veliko raznolikost vsebnosti plodov žvrklje med paketi istega proizvajalca (uvoznika) in celo znotraj iste serije pakiranja sončničnih plodov, kar je verjetno povezano z načinom pakiranja plodov. Količina nečistoč, ki jih predstavlja drobna frakcija po sejanju vzorca skozi sito, v paketu ni povezana s količino plodov žvrklje.

Na slovenskem tržišču so torej v prodaji paketi sončničnih plodov za hranjenje zunanjih ptic, ki vsebujejo plodove žvrklje, celo s količinami, ki presegajo zakonsko določeno zgornjo mejo. Nujno je obveščanje potrošnikov o tem problemu, saj lahko zaradi priljubljenega zimskega hranjenja ptic posamezniki nehote razširjajo to alergeno rastlino in jo celo zanesejo na svoje vrtove, od koder pa jo morajo od v skladu z odredbo iz leta 2010 odstranjevati (Anon. 2010).

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The synergy of xenobiotics in honey bee *Apis mellifera*: mechanisms and effects

Sinergizem ksenobiotikov v medonosni čebeli *Apis mellifera*: mehanizmi in učinki.

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Running title: Glavan, Božič: The synergy of xenobiotics in honey bee

Abstract: During foraging activities honeybees are frequently exposed to different xenobiotics, most of them are agrochemical pesticides and beehive chemicals. Many pesticides are applied together and synergism is likely to occur in different organisms. The risk of synergisms is neglected and relatively few studies were performed concerning the effects and synergy mechanism of different xenobiotic combinations in honeybees. The understanding of synergy mechanisms between xenobiotics is very important for the control of defined mixtures use and also for the prediction of potential toxicity of newly developed substances in agriculture and apiculture. This review is focused on the effects, mechanisms and molecular targets of xenobiotics in honeybees and possible complex mechanisms of their synergisms. The main threat for honeybees are insecticides which primary molecular targets are few neuronal molecules therefore causing the impairment of neuronal system that have a profound effect on honeybee behavior, cognitive functions and physiology. However, the majority of synergistic effects observed in honeybees were ascribed to the inhibition of detoxifying midgut enzymes P450 involved in xenobiotic metabolism since most of studies were done with the mixtures xenobiotic/P450 inhibitor. The main inhibitors of P450 enzymes are specific compounds used to prolong the effects of pesticides as well as some fungicides. Some insecticides can also interact with these enzymes and influence the xenobiotis. Although the primary mechanisms of action of individual xenobiotics especially insecticides are well known and there are possible interactions in honeybees at their primary target sites, this issue is underestimated and it warrants further investigation.

Keywords: synergism, xenobiotic, A*pis mellifera*, mechanism, pesticide, P450

Izvleček: Medonosne čebele so med iskanjem hrane pogosto izpostavljene različnim ksenobiotikom, večinoma so to fitofarmacevtska sredstva in panjske kemikalije. Številna fitofarmacevtska sredstva se uporablja skupaj in znano je, da lahko pride do sinergističnih interakcij v organizmih. Tveganje za nastanek sinergizmov je podcenjeno in narejenih je relativno malo študij na čebelah o učinkih in mehanizmih sinergizmov različnih kombinacij ksenobiotikov. Razumevanje mehanizmov sinergizmov ksenobiotikov je zelo pomembno za nadzor nad uporabo definiranih mešanic in napovedovanje potencialne toksičnosti novih ksenobiotikov v kmetijstvu in čebelarstvu. Pregledni članek se osredotoča na učinke, mehanizme in molekulske tarče ksenobiotikov v medonosnih čebelah in osvetljuje morebitne primere ter mehanizme nastanka sinergizmov. Najbolj nevarni za čebele so insekticidi, katerih primarne tarče so nekatere molekule živčnih celic,zato le-ti motijo delovanje živčnega sistema. Insekticidi zato lahko močno vplivajo na vedenje, kognitivne funkcije in fiziologijo čebel. Kljub temu raziskovalci večino sinergijskih učinkov v čebelah razlagajo z inhibicijo črevesnih detoksifikacijskih encimov P450, ki presnavljajo ksenobiotike, saj je bila večina študij narejena z mešanicami ksenobiotik/zaviralec encimov P450. Glavni zaviralci encimov P450 so specifični inhibitorji za podaljšanje učinka fitofarmacevtskih sredstev ter nekateri fungicidi. Tudi nekateri insekticidi lahko vplivajo na delovanje encimov P450 in tako vplivajo na interakcije med ksenobiotiki. Čeprav so primarni mehanizmi delovanja posameznih ksenobiotikov, še posebej insekticidov, precej znani in so sinergizmina ciljnih tarčah pri čebelah možni, je to področje podcenjeno in neraziskano.

Ključne besede: sinergizem, ksenobiotik, *Apis mellifera*, mehanizem, pesticidi, P450

Introduction

In addition to gathering nectar to produce honey, honey bees carry out another crucial function: pollination of agricultural crops, home gardens, orchards and wildlife habitat. A substantial decline of honey bee populations so called colony collapse disorder was observed in the last 15 years in many countries in Europe and in North America (vanEngelsdorp and Meixner 2010).Colony numbers in Europe for example decreased from over 21 million in 1970 to about 15.5 million in 2007 (FAO, 2009),a severe decline occurred after 1990. Many factors such as diseases, parasites, xenobiotics (pesticides and veterinary products), the environment, and socio-economic factors probably influence managed bee population, working alone or in combinations (vanEngelsdorp and Meixner 2010).

Honey bees may frequently become exposed to xenobiotics, environmental chemicals as a consequence of their foraging activities. Most of them are agrochemical pesticides and beehive or veterinary products, many of them of insecticide action, used against parasitic honey bee mites: *Acarapis sp.*, *Varroa destructor* and *Aethina tumida* (Thompson 2012). The use of pesticides to control weeds, fungi and arthropod pests seems inevitable in modern agriculture which seeks for the highest yields of the produces. Nectar foraging bees are likely to experienced highest exposure to both sprayed and systemic seed and soil treatments compounds followed by nurse and brood-attending bees. The residues of pesticides were found in pollen, wax and nectar within colonies, pollen and nectar residues from plants, in pollen loads on bees returning to the hives and in adult workers (Thompson 2012). Pesticide regulations so far focused mainly on protection of bees against direct poisoning (Thompson and Wilkins 2003, Desneux et al., 2007). The direct poisoning is now regulated and prevented by the implementation of European Council Directive 91/414 in Europe, and the Federal Insecticide, Fungicide and Rodenticide Act in the US (Desneux et al. 2007, vanEngelsdorp and Meixner 2010). The standard approaches for determination of acute pesticide toxicity in bees are the calculation of the LD50 (median lethal dose) or LC50 (median lethal concentration) of a given substance with respect to adult bees or larvae. In spite of more or less controlled protection against direct poisoning, massive dying of honey bees is still present. For this reason many studies are focusing to the chronic sub-lethal exposure of xenobiotics causing a variety of sub-lethal effects on bees (reviewed by Desneux 2007) which are physiological and behavioral, affecting the honeybee colony as a whole, resulting in the perturbations of learning and communication ability. Even more, as many pesticides are applied together, scientists are arguing for years that toxic exposures to pesticides should be measured as they would normally occur, in combination with one another. The most intriguing or concerning aspect of pesticide mixtures is the opportunity for complex interactions such as a synergy when the administration of one chemical increases the toxicity of another. There are relatively few experimental data regarding synergistic effects of pesticides on honeybees, but in some cases pesticide mixtures, particularly with insecticides, have been shown to be synergistic, with reported increases in toxicity of up to100-fold (Thomson 1996). However, the effects of pesticide exposure on colony health is not systematically monitored, and the Environmental Protection Agency (EPA) does not require data on sub-lethal or synergistic effects for pesticide registration (NAS, 2009) therefore this specific issue warrants special attention.

This review focuses on the mechanisms and molecular targets of xenobiotics in honeybees which could be the basis of their synergism, especially insecticides which are the most potentially dangerous for honeybees. Since of greater potential to cause underestimation of the risk posed to the honeybee colonies decline the modes of synergisms of xenobiotics known so far are emphasized and summarized in this section of our review. The aim is to exemplify the possible complex mechanisms of their interaction.

Mechanisms and effects of xenobiotics

Agrochemical pesticides

The agrochemical pesticides affecting honey bee colonies are fungicides, herbicides and insecticides, applied to crops (Johnson et al. 2010). The large number of commercial pesticides used worldwide, whether based on natural products or being entirely of synthetic origin, act on relatively few, perhaps 95 biochemical targets in pest insects, weeds, and destructive fungi (Casida 2009). Herbicides in general are blocking photosynthesis, carotenoid synthesis, or aromatic and branched chain amino acid synthesis essential in plants. Many fungicides inhibit ergosterol (the fungal sterol) or tubulin biosynthesis or cytochrome c reductase. Others disturb basic cellular functions (Casida 2009).

The pesticides that represent a main threat to the honeybees are **insecticides**. Many of the most effective insecticides in current use act on the insect nervous system (Narahashi 1992, Bloomquist 1996).Others are insect growth regulators (Tasei 2001, Thomson et al. 2005). The growth regulating insecticides are functioning as juvenile hormone analogues (fenoxycarb), chitin synthesis inhibitors (diflubenzuron), ecdysteroid synthesis inhibitors (azadirachtin) and ecdysteroid analogues (tebufenozide) (Tasei 2001, Thomson et al. 2005).The main nerve targets of current insecticides are voltage-gated sodium channels, an enzyme acetylcholinesterase (AChE) and receptors for neurotransmitters: L-glutamate-gated chloride channels working as glutamate receptors (GluRs), ionotropic γ-aminobutyric acid (GABA) receptors(GABARs) binding GABA and nicotinic cholinergic receptors (nAChRs) stimululated by acetylcholine (ACh) (Coats 1990, Fukuto 1990, Zlotkin 1999, Bloomquist 2003, Raymond-Delpech et al. 2005, Wolstenholme and Rogers 2005, Davies et al. 2007, Jeschke and Nauen 2008).Voltage-gated sodium channels are molecular targets for three big groups of insecticides, **pyrethroid, DDT-type** and **organ chlorine** insecticides (Coats 1990). These channels mediate the transient wave of sodium entry spreading along the nerve axons and dendrites, carrying the action potential along these structures and are ubiquitous in the honeybee nervous system (Sattelle and Yamamoto 1988, Narahashi 1992). All three groups of insecticides cause death due to hyperexcitation of the nerves, but in slightly different way. Pyrethroid pesticides by binding to voltage-gated sodium channels induce hyperexcitation that results from prolongation of the open phase of sodium gate function results in neurotoxic effects such as tremors and convulsions. The DDT-type insecticides, DDT (dichloro diphenyl trichloroethane) and DDT analogues (N-alkylamides, dihydropyrazoles), act primarily on the peripheral nervous system (Coats 1990). The mechanism of DDT is the prevention of the deactivation or closing of that gate after activation and membrane depolarization. The result is a persistent leakage of Na+ ions through the nerve membrane, creating a destabilizing negative afterpotential. The hyperexcitability of the nerve is the consequence of trains of repetitive discharges in the neuron after a single stimulus and/or occur spontaneously (Coats 1990).The acute toxic effects in animals of organ chlorine insecticides are also due to hyperexcitation in the nervous system and death is frequently recognized as respiratory failure after the disruption of nervous system function (Coats 1990).

Organophosphate and **carbamate** insecticides are inhibiting the action of AChE (Fukuto 1990). AChEis an enzyme that terminates the synaptic actions ofACh, the important neurotransmitter of sensory neurons and interneurons of insect brain which is necessary for sensory-input processing and learning in honey bee (Massoulie et al. 1993, Homberg 1994, Weinberger 2006). AChE is widely distributed in the insect brain, the thoracic and abdominal segments and the abdominal ganglia (Kreissl in Bicker 1989, Thany et al. 2010).The potential target sites for organophosphate and carbamate insecticides in the honeybee brain are the optic lobes, antennal afferents projecting into the dorsal lobe, fibers connecting the two brain hemispheres, and within the protocerebrum and the mushroom bodies where AChE is highly expressed (Kreissl in Bicker 1989).AChE was found also in the compound eye and ocelli (Kral 1980, Kral and Schneider 1981).The inhibition of AChE by organophosphate and carbamate insecticides causes irreversible blockage leading to accumulation of the enzyme which results in overstimulation of cholinergic receptors (Fukuto 1990). As ACh is a major neurotransmitter of insect nervous system (Homberg 1994) the inhibition of AChE could cause a systemic failure in the insect body. Widely used organophosphate as hive varroacides is coumaphos.

Insecticides that act selectively on insect nAChRs as potent agonists are **neonicotinoids** (Jeschke and Nauen 2008). Among ionotropic receptors affected by insecticides, nAChRs are the most abundant excitatory postsynaptic receptors (Sattelle 1980).The central nervous system of insects is rich in nAChRs more so than any other organism (Jones and Sattelle 2010).They are located postysinaptically and directly activated by ACh, released from presynaptic cholinergic neurons facilitating fast excitatory synaptic transmission (Thany et al. 2010).In the honeybee brain the highest binding site densities for nAChR are localized in the suboesophageal ganglion, the optic tubercles, optic lobes medulla and lobula, antennal lobes, dorsal lobes and the α-lobes of the mushroom bodies (Scheidler et al. 1990). Neonicotinoids cause excitation of the neurons and because of a high concentration of nACh receptors in honeybees the eventual paralysis could be very profound occurring at low concentration of neonicotinoids, leading to death.

The insecticides that interfere with GABARs are **pyrethroids and phenylpyrazole insecticides** (Raymond-Delpech et al. 2005, Davies et al. 2007). In insects GABARs are associated with neurotransmitter GABA mediating inhibitory synaptic transmission in the nervous system and at nerve-muscle junctions (Homberg 1994). In the central nervous system of honeybees the neurotransmitter GABA is generally present in neuropil, especially in structures that are associated with learning and memory, such as antennal lobe and the mushroom body and the optic lobe (Schafer and Bicker 1986, El Hassani et al. 2009). The presence of the neurotransmitter GABA in the honeybee brain was shown mainly for local interneurons and less in the projection neurons. In the brain and subesophageal ganglion only minority of neurons contained GABA (Bicker et al. 1985, Meyer et al. 1986, Schafer and Bicker 1986). By targeting the GABARs which are chloride channels pyrethroids and phenylpyrazole insecticides disrupt normal neuronal influx (e.g., passage of chloride ions) and, at sufficient doses, causing excessive neural excitation, severe paralysis, and death (Cole et al. 1993, Gunasekara et al. 2007). Most known representative of phenylpyrazole insecticides is fipronil and widely used pyrethroid as hive varroacides is tau-fluvalinate. Pyrethroids are very complex group regarding the molecular mechanisms of their functioning, because they don’t bind only to GABA-gated chloride channel, but they can also interfere with other molecules such as calcium regulation. They could inhibit both Ca-ATPase and Ca-Mg ATPase (Coats 1990). In this respect direct effects on neurotransmitter release have been observed, as well as the inhibition of Ca2+ uptake. However, they have also various secondary targets such as signal transduction pathways by altering the protein phosphorylation cascade that may result, among other things, in programmed cell death (Ray and Fry, 2006). In mammals a variety of different effects of pyrethroids were discovered like modulation of protein phosphorylation, voltage-gated sodium channels, voltage-gated chloride channels, noradrenaline release, membrane depolarization, GABA-gated chloride channels, nicotinic receptors, mitochondrial complex I, apopotosis induction, voltage-gated calcium channels, lymphocyte proliferation, volume-sensitive anion channels, calcium ATP-ase, intercellular gap junctions and chromosomal damage, but many of these effects were not shown for insects (Ray and Fry 2006).

The insecticides that activate GluRs which bind neurotransmitter L-glutamate are **avermectin** and **milbemycin** (Raymond-Delpech et al. 2005, Wolstenholme and Rogers 2005). The distribution of GluRs in the nervous system of honeybees is not known but they probably modulate excitability in the nervous system and muscle cells as neurotransmitter L-glutamate is enriched in these tissues (Cully et al. 1996). Studies performed by Maleszka et al. (2000) and Locatelli et al. (2005) suggested that glutamatergic neurons in the honeybee brain, in particular those found in the mushroom bodies, may be part of the circuitry involved in processing of olfactory memory. In the honeybee, a high level of a glutamate transporter is present in the optic lobes and in restricted areas of the mushroom bodies corresponding to the Kenyon cells of the calyces (Kucharski et al. 2000). GluRs are permeable to chloride ions and the activation of these receptors with insecticides avermectin and milbemycin causes a very long-lasting hyperpolarization or depolarization of the neuron or muscle cell and therefore blocking further function leading to paralysis and death (Wolstenholme and Rogers 2005).

Insecticides have various neural effects in honeybees that were in details reviewed by Belzunces et al. (2012). They impair cognitive functions, including learning and memory, habituation, olfaction and gustation, navigation and orientation. They affect also behavior, including foraging and physiological functions, including thermoregulation and muscle activity.

Acaricides

Commonly used in hive varroacides are amitraz, coumaphosand tau-fluvalinate (Johnson et al. 2010). Amitraz is a formamidine pesticide. The mode of action of formamidine pesticides such in insects is believed to be the toxic effects on a G protein-coupled receptor for a neuromodulator octopamine, working as octopaminergic agonists (Evans and Gee 1980, Dudai et al. 1987). High levels of octopamine in the honey bee brain are associated with increased foraging behavior (Schulz and Robinson 2001). Forager honey bees treated with octopamine increased the reported resource value when communicating via the dance language (Barron et al. 2007). However, the effects of amitraz on foraging activity of honeybees were not investigated, but the acute toxicity of this compound was shown in larvae where it increases apoptotic cell death in the midgut (Gregorc and Bowen 2000). Another popular in hive varroacide is tau-fluvalinate which was initially very effective at controlling Varroa mites by blocking voltage-gated sodium channels (Davies et al. 2007). Tau-fluvalinate was quite promising since it is tolerated by bees in high concentrations due to rapid detoxification by cytochrome P450 monooxygenases, but many Varroa populations are now resistant (Lodesaniet et al. 1995, Johnson et al. 2009). However, tau-fluvalinate is not completely harmless, high doses could affect queens to grow smaller and drones to dieuntil reaching sexual maturity (Rindereret et al. 1999, Haarmann et al. 2002). As the efficacy of tau-fluvalinate against Varroa was beginning to decrease, coumaphos, an organophosphate pesticide, was starting to be used (Elzen andWestervelt 2002). Although honey bees can tolerate similar to tau-fluvalinate therapeutic doses of coumaphos, probably as a result of detoxicative P450 activity (Johnsonet et al. 2009), negative effects from coumaphos exposure were observed. Queens exposed to coumaphos were smaller, suffered higher mortality and were more likely to be rejected when brought into a colony (Haarmann et al. 2002, Collins et al. 2004, Pettis et al. 2004). Drone sperm viability was lower in stored sperm collected from drones treated with coumaphos (Burley et al. 2008). Coumaphos also affects food transfer between workers of honeybee (Bevk et al. 2011). Fenpyroximate is a pyrazole acaricide that presumably kills mites through the inhibition of electron transport in the mitochondria at complex I, thereby interfering with energy metabolism (Motoba et al.1992). It was found that chronic exposure to fenpyroximate causes the increased generation of reactive oxygenspecies (Sherer et al. 2007).

Two monoterpenoid components of plant-derived essential oils, thymoland menthol, are used for control of Varroa and tracheal mites. They were found to be among the most toxic of all terpenoids tested when applied to honey bees as a fumigant (Ellis and Baxendale 1997).The thymol molecular targets include binding to octopamine receptors (Enan 2001) and AChE (Priestley et al. 2003), but also insect tyramine and GABA receptors (Blenau et al. 2011).Receptor activation leads to changes in the concentration of intracellular second messengers such as cAMP or InsP3/Ca2+. Thymol could affect honeybees inducing brood removal (Marchetti and Barbattini 1984, Floris et al. 2004) and the increase of queen mortality (Whittington et al. 2000). Exposure to thymol was shown to decrease phototactic behavior in the honeybee (Bergougnoux et al. 2013).

Among organic acids, formic acid and oxalicacid are used as varroacides. Formic acid is inhibiting electron transport in the mitochondria binding of cytochromec oxidase in mites and may produce a neuroexcitatory effect on arthropod neurons (Keyhani and Keyhani 1980, Song and Scharf 2008). Formic acid can reduce worker longevity (Underwood and Currie 2003) and harming brood survival (Fries 1991).The mode of action of oxalic acid against Varroa is unknown, but in mammals it interferes with mitochondrial electron transport leading to increased production of reactive oxygen species and to kidney toxicity (Cao et al. 2004, Meimaridou et al. 2005). Repeated treatment of colonies with oxalic acid can result in higher queen mortality and a reduction in the amount of sealed brood (Higes et al. 1999). The midguts of honey bees fed oxalic acid in sugar water exhibited an elevated level of cell death (Gregorc and Smodisskerl 2007).Recent studies are focusing on molecular mechanisms underlying the sub-lethal effects of in-hive acaricides on honey bees. Using a gene expression profiling Boncristiani et al. (2012) found that thymol, coumaphos and formic acid are able to alter detoxification gene expression pathways, components of the immune system responsible for cellular response and developmental genes. This study indicates that these acaricides could significantly influence the health of individual honey bees and entire colonies (Boncristiani et al. 2012).

Mechanisms and factors influencing the synergy of xenobiotics applied to honeybees

Understanding the toxicity and synergy of chemicals in organisms requires considering the molecular mechanisms involved as well as the relationships between exposure concentration and toxic effects with time (Tennekes and Sánchez-Bayo 2013).In addition, the relevance of synergy of xenobiotics is a subject to understanding the routes of application, the way of transportation to target molecules in the tissue and the metabolism of pesticides in the target organism, all having a profound influence on the concentration and chemical structure of active substances at target sites. The analysis of the studies when monitoring the residues in honeybees following in-hive treatments or pesticide applications revealed that the highest exposure routes were sprayed and systemic seed and soil compound treatments to which preferentially foraging bees are exposed during collecting contaminated nectar and the direct exposure to acaricides used in beehives (Thomson 2012). This is probably due to the availability of relatively high concentration of agricultural pesticides and in-hive compounds, but also the time between pesticide application to crops and bee exposure is very important as many pesticides degrade or dilute in the environment. The importance of other routes of exposure such as dusts produced during sowing of treated seeds, water from puddles or guttation droplets and beeswax might be relevant but data about these are limited. The final actions of xenobiotics are greatly dependent on the mode of exposure, acute, sub-chronic and chronic, defining the nature and the intensity of their effects. Metabolism of xenobiotics elicited by intrinsic enzymes is remarkably important as it could result in the elevation or decrease of their toxicity or it could produce different effects. Chemical interactions between xenobiotics in the mixture are also possible, causing the changes in chemical structures of particular substance. There are also other factors such as physiological states of the organisms including age, the season and the capacity of immune system that have impact on synergism (Thomson 2012). For example, the immune system of honeybees could be profoundly affected by various pathogens, bacterial, fungal and viral pathogens as well as ecto- and endoparasites that in many cases elevate the toxicity of xenobiotics. Most of the studies in honeybees have focused on the synergisms at the level of midgut enzymes when certain xenobiotic inhibit the detoxifying ability of these enzymes and potentiate the toxicity of another substance, but the synergism at target site is poorly investigated.

The synergism at the level of midgut detoxifying enzymes

Probably the most frequent way of the transfer of xenobiotics into honeybee tissue is the consummation of contaminated nectar and absorption in the midgut thought the midgut wall into the hemolymph, but also passage thought cuticle and sometimes inhalation of vaporous compounds is possible. In the midgut of the honeybee xenobiotics are metabolized by enzymes glutathione-S-transferases (GSTs), cytochrome P450 monooxygenases (P450s) and carboxyl/cholinesterases (CCEs) (Scott and Wen 2001, Enayati et al. 2005, Wheelock et al. 2005). These enzymes metabolize pesticides by different mechanisms, but P450s are probably the most important for honeybees as they play a significant role in the detoxification of phytochemicals present in the nectar, honey and pollen that bees consume (Mao et al. 2009). They catalyze a range of reactions including oxidation and demethylation which decrease pesticide activity or produce active metabolites (Scott and Wen 2001).For example, they convert the thion to oxon forms of organophosphorus pesticides or change neonicotinoid thiamethoxam to clothianidin. P450s can also oxidaze aromatic rings of tau-fluvalinate and flumethrin used in varroa control (Ortiz de Motellano and De Voss 2005). GSTs in insects can metabolize insecticides by facilitating their reductive dehydrochlorination or by conjugation reactions with reduced glutathione, to produce water-soluble metabolites that are more readily excreted. In addition, they contribute to the removal of toxic oxygen free radical species produced through the action of pesticides (Enayati et al. 2005).Carboxylesterases (CaEs) are hydrolazes and catalyze the hydrolysis of carboxyl esters of three different classes of agrochemicals, pyrethroids, organophosphates and carbamates via the addition of water (Wheelock et al. 2005). The selective toxicity of xenobiotics is affected by the ratio and the levels of metabolizing enzymes which fluctuate in different insect species and also in individual organism. The level of enzymes could be affected also by the season, the study on winter honeybees demonstrated reduced levels of P450-mediated detoxification since the synergism between pyrethroid deltamethrin and the P450-inhibiting fungicide prochloraz was much reduced during winter periods (Meled et al. 1998).

By far the majority of the studies of pesticide synergism in honeybees have focused to P450 enzymes that are inhibited by specific pesticides mostly by monitoring the toxicity calculation of the LD50 or LC50 (Table 1.).The developers of insecticide synergists have often exploited inhibition of P450s activity to prolong the efficacy of pesticides which are otherwise rapidly detoxified. It was shown that P450-inhibitors elevated toxicity of pyrethroids (cyfluthrin, permethrinand tau-fluvalinate), neonikotinoid insecticides(imidacloprid, acetadimiprid, thiacloprid), and carbamate insecticide carbaryl (Georghiou and Atkins Jr. 1964, Yu et al. 1984, Hagler et al. 1989, Iwasa et al. 2004, Johnson et al. 2006). It was also found that the classic P450 inhibitors PBO synergize with varroacides tau-fluvalinate and coumaphos at high levels but other inhibitors have minor effect (Johnson et al., 2009, Johnson et al., 2013). Many examples of synergy have been reported between EBI (ergosterol biosynthesis inhibitor) fungicides such as prochloraz, propiconazole, epoxiconazole, carbendazimand insecticides due to the fungicide inhibitory action on P450s. This was the case with neonicotinoids (acetamiprid, thiacloprid, imidacloprid)and pyrethroid insecticides (deltamethrin, lambda-cyhalothrin, alphacypermethrin) (Pilling 1992, Meled et al. 1998, Vandame and Belzunces 1998a, Vandame and Belzunces 1998b, Papaefthimiou and Theophilidis 2001, Thompson and Wilkins 2003, Schmuck et al. 2003, Iwasa et al. 2004, Thompson 2013).The effects of EBI fungicides on the contact toxicity of the active ingredients of the pyrethroid varroacides flumethrin and tau-fluvalinate are synergized by the fungicides with relatively high increases in toxicities (Thompson and Wilkins 2003). Another EBI fungicide prochloraz which is also a P450s inhibitor elevated the toxicity of the acaricides coumaphos and fenpyroximate

(Johnson et al. 2013). The studies on synergism between insecticides in honeybees were rarely conducted, most of them between in-hive acaricides. Johnson et al. (2009) observed a large increase in the toxicity of tau-fluvalinate to bees that had been treated previously with coumaphos, and a moderate increase in the toxicity of coumpahos in bees treated previously with tau-fluvalinate. These compounds were chosen due to their low toxicity to honey bees which were attributed to rapid detoxification mediated by P450s. The synergisms occurred also between in-hive miticides coumaphos, thymol, amitraz, fenpyroximate and oxalic acid (Johnson et al. 2013). The observed synergism was explained as a result of competition between miticides for access to detoxicative P450s (Johnson et al. 2009).See the Table 1. for the list of synergisms of xenobiotics observed in honeybee.

The synergisms were found also for carbamate insecticides (carbaryl, carbofuran) and herbicide atrazine but the mechanism of this synergy is unknown (Sonnet et al. 1978). The synergy between monoterpenoid thymol and tau-fluvalinate or coumaphos was observed and was explained to be the consequence of the P450s inhibitory activity of thymol, but thymol inhibitory property was shown only in human liver microsomes but not for honeybee midgut (Johnson et al. 2013).

In other organisms, the synergisms were studied between insecticides and insecticide/herbicide at the level of detoxifying enzymes. The interactions such as a competition with metabolic enzymes esterases are possible that are maybe not very significant for honeybees since it was shown that the role of these enzymes participating in the detoxification of xenobiotics is minor. It was also shown that certain organophosphate insecticides could bind to the active site associated with esterase enzymes responsible for detoxification of pyrethroid-based insecticides and so organophosphate insecticides may be considered useful synergists for pyrethroids (Cloyd 2011).The synergisms at the level of detoxifying enzymes was described also for organophosphates and pyretroids, P450 activated by organophosphates decrease the organism's ability to detoxify pyretroids due to esterases inhibition, so greater than additive toxicity is often observed (Hernández et al. 2013). Recent studies have demonstrated the potentiating effects of triazine herbicides, such as atrazine to the toxicity of organophosphates when these herbicides stimulate P450 activity by increasing the rate of bioactivation of organophosphates resulting in the potentiation of the cholinesterase inhibiting property of organophosphates (Hernández et al. 2013).

It seems that the regulation of the P450s in honeybees is unique. Contrary to other insects, in the honey bee these enzymes are rarely induced by a substrate itself. The honeybee genome has substantially fewer protein coding genes for xenobiotic detoxifying enzymes than *Drosophila melanogaster* and *Anopheles gambiae* (Claudianos et al. 2006) and many researchers failed to demonstrate an increase of midgut detoxifying enzymes induced by xenobiotics (Yu et al. 1984). Even exposure to phenobarbital which is an inducer of P450s showed no alterations in the expression of many of P450 genes tested in honey bees (Mao et al. 2011). Only two studies indicated the increase in P450 activity in honeybees. Application of tau-fluvalinate and coumaphos elevated specific detoxifying P450 enzymes CYP9Q1, CYP9Q2, and CYP9Q3 and benzo(α)pyrene monooxidase activity in honey bee guts was induced by exposure to benzo(α)pyrene itself and by the in-hive acaricides tau-fluvalinate and cymiazole hydrochloride (Kezic et al. 1992, Mao et al. 2011).As it has been at first suggested that reduced diversity of detoxification enzymes may contribute to the sensitivity of honey bees to certain pesticides (Claudianos et al., 2006) the importance of midgut detoxifying enzymes P450 in honeybees was highlighted by the studies with specific P450-inhibitors. Two studies indicate that GSTs and CaEs are active detoxifying enzymes in honeybees but they play a relatively minor role in detoxification as compared to P450s (Johnson et al. 2009, Iwasa et al. 2004). The CaEs inhibitor DEF (S,S,S-tributylphosphorotrithioate) and GSTs inhibitor DEM (diethyl maleate) were shown to increase the toxicity of certain pyrethroids and neonicotinoids but this effect was significantly smaller that for the P450 inhibitor PBO (piperonyl butoxide) (Iwasa et al. 2004). Recently the interesting study performed by Johnson et al. (2012) suggested that regulation of honey bee P450s is affected by chemicals occurring naturally in the hive environment in the nectar, pollen and propolis since only quercetin, a common pollen

Table 1: The list of synergisms of xenobiotics in honeybee *Apis mellifera* and proposed mechanisms.
Tabela 1: Seznam sinergizmov med ksenobiotiki v medonosni čebeli *Apis mellifera* in predlagani mehanizmi.

Xenobiotic	Xenobiotic (P450 inhibitor)	Reference
Mechanism of synergy: inhibition of P450 detoxifying enzymes		
pyrethroid insecticides	***classical P450-inhibitor***	
cyfluthrin	piperonyl butoxide	(Johnson et al. 2006)
permethrin	piperonyl butoxide	(Hagler et al. 1989)
lambda-cyhalothrin	piperonyl butoxide	(Johnson et al. 2006)
tau-fluvalinate	piperonyl butoxide	(Johnson et al. 2006; Johnson et al. 2013)
neonicotinoid insecticides	***classical P450-inhibitor***	
imidacloprid	piperonyl butoxide	(Iwasa et al., 2004, Johnson et al. 2012)
acetadimiprid	piperonyl butoxide	(Iwasa et al. 2004)
thiacloprid	piperonyl butoxide	(Iwasa et al. 2004)
carbamate insecticide	***classical P450-inhibitor***	
carbaryl	piperonyl butoxide	(Georghiou and Atkins Jr. 1964)
hive varroacides	***classical P450-inhibitor***	
tau-fluvalinate	piperonyl butoxide	(Johnson et al. 2009, Johnson et al. 2013)
coumaphos	piperonyl butoxide	(Johnson et al. 2009, Johnson et al. 2013)
fenpyroximate	piperonyl butoxide	(Johnson et al., 2013)
neonicotinoid insecticides	***EBI (ergosterol biosynthesis inhibitor) fungicides***	
acetamiprid	epoxiconazole, propiconazole, triadimefon, triflumizole, uniconazole-P	(Iwasa et al. 2004)
thiacloprid	prochloraz, propiconazole, tebuconazole, triflumizole	(Schmuck et al. 2003, Iwasa et al. 2004)
imidacloprid	propiconazole, triflumizole	(Iwasa et al. 2004)
pyrethroid insecticides	***EBI (ergosterol biosynthesis inhibitor) fungicides***	
deltamethrin	difenoconazole+carbendazim, prochloraz, prochloraz+ difenoconazole 850	(Belzunces and Colin 1993, Colin and Belzunces 1992, Papaefthimiou and Theophilidis 2001, Vandame and Belzunces1998b, Vandame and Belzunces 1998a)
lambda-cyhalothrin	difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole, thiophanate-methyl	(Thompson and Wilkins 2003)
alphacypermethrin	difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole	(Thompson and Wilkins 2003)
hive varroacides	***EBI (ergosterol biosynthesis inhibitor) fungicides***	
coumaphos	prochloraz	(Johnson et al. 2013)
flumethrin	carbendazim, difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole, thiophanate-methyl	(Thompson and Wilkins 2003)

Xenobiotic	**Xenobiotic (P450 inhibitor)**	**Reference**
tau-fluvalinate	carbendazim, difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole, thiophanate-methyl, myclobutanil, metconazole, fenbuconazole,	(Thompson and Wilkins 2003, Johnson et al. 2013)
fenpyroximate	prochloraz	(Johnson et al. 2013)
hive varroacides	***hive varroacides***	
coumaphos	tau-fluvalinate	(Johnson et al. 2009, 2013)
thymol	tau-fluvalinate, coumaphos	(Johnson et al. 2013)
amitraz	tau-fluvalinate, coumaphos, fenpyroximate	(Johnson et al. 2013)
fenpyroximate	tau-fluvalinate, coumaphos	(Johnson et al. 2013)
Mechanism of synergy: increased oxidative stress		
hive varroacides	***Fungicides (mitochondrial inhibitors)***	
tau-fluvalinate	pyraclostrobin, boscalid	(Johnson et al. 2013)
fenpyroximate	pyraclostrobin	(Johnson et al. 2013)
Unknown mechanism of synergy		
oxalic acid	tau-fluvalinate, fenpyroximate, amitraz, thymol	(Johnson et al. 2013)
herbicide atrazine	carbamate insecticides (carbaryl, carbofuran)	(Sonnet et al. 1978)
thio and dithiophosphoric ester pesticides – ethyl parathion, dimethoate, dialifos	coumaphos varroacide	(Lienau 1990)
thiacloprid (neonicotionoid)	fungicides cyprodinil, tolyfluanid	(Schmuck et al. 2003)
alphacypermethrin, lambda-cyhalothrin	fungicide chlorothalonil fungicide chlorothalonil	(Thompson and Wilkins 2003)

and honey constituent, reduced tau-fluvalinate toxicity. Bees fed with extracts of honey, pollen and propolis showed elevated expression of three CYP6AS P450 genes. Non-naturally occurring inducers of cytochrome P450 enzymes did not alter the toxicity of certain xenobiotics and it seems that a wide range of synthetic pesticides do not induce in bees. It is now clear that certain substances found in bee products such as quercetin, p-coumaric acid, pinocembrin, and pinobanksin 5-methyl ether naturally elevate the levels of bee detoxifying enzymes P450 and probably helping bees to resist the toxicity of certain xenobiotics (Johnson et al. 2012, Mao et al. 2013).

The synergism of xenobiotics working at the same targets

Although the basic molecular mechanisms of most xenobiotics are more or less known, the possible mechanisms of their synergy at primary target sites in honeybees are unexplored. One of plausible mechanism of this synergy is that effects at the site of toxic action include increased response of the site (such as a receptor) following initial pesticide exposure and according to this direct synergistic effect could be predicted for substances that have similar targets (Thomson 1996). In this respect only one study in honeybees was performed, on semi-isolated heart (Papaefthimiou and Theophilidis 2001).In this study the synergistic effect was observed between EBI fungicide prochloraz and pyrethroid insecticide deltamethrin which rapidly decreased the frequency and the force of the cardiac contractions with marked effects at 0.01 μM, equivalent to internal doses of 4–5 μg kg^{-1} body weight. Prochloraz showed to be more cardiotoxic than deltamethrin, what seemed surprising since deltamethrin is a neurotoxic substance whereas prochloraz is an inhibitor and an inducer of detoxifying enzymes P450. So, authors concluded that there must be the neural basis of the deltamethrin prochloraz synergy. Belzunces et al. (2012) suggested that the basis of their synergy is the interaction of these two pesticides with shared molecular targets, such as ATPases, potassium and calcium channels.

The existence of the synergy of insecticides at primary target sites was demonstrated also in the cockroach *P. americana*, in the cercal-afferent giant-interneuron synapses of the terminal abdominal ganglion (Corbel et al. 2006). Authors demonstrated that pyrethroid permethrin and carbamate propoxur insecticides applied together increased drastically the ACh concentration within the synaptic cleft, which thereby stimulated a negative feedback of ACh release mediated by presynaptic muscarinic receptors causing the synergism. Johnson et al. (2013) demonstrated 5-fold increase in the toxicity of tau-fluvalinate by amitraz pretreatment in honeybees. Interactions between formamidines and pyrethroids are known in other insects and may be due to synergism at the target site through cooperative binding (Liu and Plapp 1992).It was shown that formamidine pesticides working as octopaminergic agonists change the binding properties of pyrethroid insecticides to nerve membrane sodium channels. This mechanism could be also the cause for the synergism of tau-fluvalinate and amitraz in honeybees. Thomson (1996) reported another possible mechanism of pesticide interaction, when esterases can act as irreversible binding sites for organophosphate and carbamate insecticides, reducing the levels available to bind to AChE within the brain. Thus, prior exposure to an organophosphate may result in a reduction in the number of available binding sites and an increase in the blood levels of free pesticide. All these mechanisms mentioned above are possible for honeybees, but they are not explored. The lack of similar studies suggests that the mechanisms of synergisms of xenobiotics at primary target sites in honeybees are very much ignored and underestimated and a need for additional studies is unavoidable.

Conclusions

Bees are very often exposed to mixtures of products applied to plants on which they forage such as fungicides, herbicides and insecticides and in addition very high levels of varroacides may be present within colonies. The very potential risk from most mixtures of these substances is the development of synergisms that can profoundly affect honeybee colonies and may significantly contribute to honeybee colony loss observed in the last 15 years. This risk is underestimated and relatively few relevant studies were performed concerning the effects and mechanisms of synergy of different xenobiotic combinations. The understanding the mechanisms of synergy between xenobiotics is very important for the restriction of the use of defined mixtures and also for the prediction of potential toxicity of newly developed substances in agriculture and apiculture. Many observable effects are induced by xenobiotics such as alternation of cognitive functions, behavior or integrity of physiological functions, many of them unambiguously explained by the mechanisms of xenobiotic actions at primary target sites. In spite of these physiological mechanisms of action of individual xenobiotics are more or less identified, especially for insecticides, the majority of synergistic effects observed in honeybees is ascribed to the inhibition of detoxifying midgut enzymes P450 involved in xenobiotic metabolism. Even more, as most of the studies focused on synergistic effect of mixure xenobiotic/P450 inhibitor, only few were performed on insecticide/insecticide interactions. Johnson et al. (2013) proposed that the synergistic interactions occur when the compounds work through different modes of action, but few experiments in insects studying the synergism of insecticides at target sites suggest that the synergism is also possible for substances working through the same mode of action, at least when they are working on the same system such as cholinergic synapse. Therefore, the aspect of mechanisms of synergism at the similar targets is underestimated since only one study was performed in honeybee and therefore this issue demands extra investigation. The improved knowledge of the mechanisms of pesticide and bee-hive compound interactions would prevent the negative impact on beneficial organisms like honeybees.

Povzetek

Medonosne čebele so med iskanjem hrane pogosto izpostavljene različnim ksenobiotikom, večinoma so to fitofarmacevtska sredstva in panjske kemikalije. Čebele v zadnjih 15 letih množično umirajo, vzrok za to naj bi bila tudi uporaba ksenobiotikov. Številna fitofarmacevtska sredstva se uporablja hkrati in znano je, da lahko pride do sinergističnih interakcij v organizmih. Tveganje za nastanek sinergizmov je podcenjeno in narejenih je relativno malo študij na čebelah o učinkih in mehanizmih sinergizmov različnih kombinacij ksenobiotikov. Razumevanje mehanizmov sinergizmov ksenobiotikov je zelo pomembno za nadzor nad uporabo definiranih mešanic in napovedovanje potencialne toksičnosti novih ksenobiotikov v kmetijstvu in čebelarstvu. Pregledni članek se osredotoča na učinke, mehanizme in molekulske tarče ksenobiotikov v medonosnih čebelah in osvetljuje primere mehanizmov nastanka sinergizmov. Opisani so tudi drugi dejavniki,ki vplivajo na njihov nastanek, okoljski in fiziološki, poudarek je na detoksifikacijskih encimih medonosne čebele. Najbolj nevarni za čebele so insekticidi, ki delujejo predvsem na nekaj različnih živčnih molekularnih tarč in tako motijo delovanje živčnega sistema, kar vpliva na vedenje, kognitivne funkcije in fiziologijo čebel. Glavne živčne tarče insekticidov so napetostno odvisni natrijevi kanalčki, encim acetilholinesteraza, glutamatni receptorji, receptorji za gama-aminomasleno kislino in nikotinski receptorji. Znane skupine insekticidov so piretroidi, DDT, DDT podobni insekticidi, organofosfati, karbamati, fenilpirazolni pesticidi ter neonikotinoidi. Kljub temu, da so tarče delovanja posameznih ksenobiotikov,še posebej insekticidov, precej znani, raziskovalci večino sinergijskih učinkov v čebelah razlagajo z inhibicijo črevesnih detoksifikacijskih encimov P450, ki presnavljajo ksenobiotike. Večina študij je bila namreč narejena z mešanicami ksenobiotik/zaviralec encimov P450. Glavni zaviralci encimov P450 so specifični inhibitorji, ki jih dodajajo fitofarmacevtskim sredstvom za podaljšanje učinka ter nekateri fungicidi. Študij na čebelah, s katerimi bi raziskovali sinergizem med insekticidi, skoraj ni. Čeprav so sinergizmi ksenobiotikov, še posebej insekticidov, na primarnih ciljnih tarčah pri čebelah možni, saj so bili prikazani pri drugih organizmih, je ta vidik podcenjen. Narejena je bila samo ena raziskava mehanizmov na tarčnem mestu pri medonosni čebeli, pa še to med insekticidom in fungicidom.

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Circadian rhythms and their role in living organisms

Cirkadiani ritmi in njihova vloga v živih organizmih

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Abstract: Numerous physiological processes in organisms as diverse as bacteria and man are regulated by a small molecular clock termed the circadian clock. It is present in virtually all cells of the body and enables various physiological processes to occur at specific times of the day and with a period of about 24 hours. It was not until recent years that the role of the circadian clock has become evident for normal physiology of humans as well as other mammals. Disruption of the normal circadian rhythms can lead to a number of metabolic disorders characteristic of modern lifestyle including diabetes, obesity and cancer. It is the aim of this review to give the reader a general overview of what circadian rhythms are, how they look at the molecular level and why they can influence various metabolic processes in the way they do.

Keywords: Biological rhythms, circadian rhythms, chronobiology, circadian clock

Izvleček: Številne fiziološke procese v raznolikih organizmih uravnava majhna molekularna ura, ki jo imenujemo cirkadiana ura. Nahaja se v skoraj vseh celicah telesa in omogoča, da različni procesi v telesu potekajo ob določenih delih dneva ter da se le ti ponovijo v periodi 24 ur. Šele v zadnjih nekaj letih je pomen cirkadiane ure postal jasen tudi za pravilno homeostazo telesa, tako človeka, kakor tudi drugih sesalcev. Motnje normalnega cirkadianega ritma lahko vodijo v razvoj metabolnih motenj, kot sta diabetes in prekomerna telesna teža, značilnih za sodoben način življenja. Namen preglednega članka je bralcu predstaviti osnove cirkadianih ritmov, njihove lastnosti na molekularnem nivoju ter njihovo prepletenost s procesi metabolizma.

Ključne besede: Biološki ritmi, cirkadiani ritmi, kronobiologija, cirkadiana ura

Biological rhythms

What would life on Earth look like if there were no biological rhythms? This might seem like an irrelevant question since obviously biological rhythms are not that important, or are they? If you look at various processes occurring in living organisms it becomes evident that biological rhythms are an integral part of life. We are all aware of our heart beat, yet we almost never truly comprehend it as a biological rhythm, despite the fact that the absence or perturbation of its rhythm is used in everyday medical practice to distinguish between life and death or illness and health. This is one simple example among many that shows how biological rhythms are not only important for the survival of an organism itself but also for the survival of species and the ecosystem in general.

Biological rhythms are defined as biological events or functions that reoccur in a repeated order and with a repeated interval (period) between occurrences (Aschoff 1981) and can be divided into three classes based on the duration of the phase (Fig. 1A). While the majority of efforts in both early studies as well as in recent years have been focused on circadian rhythms with a period of about 24 h, rhythms having longer or shorter periods are also important. Infradian rhythms are rhythms with periods longer than 28 hours. A well-known example is the menstrual cycle in women. In male subjects on the other hand the presence of such rhythms is still controversial. Very few studies have been conducted on man, mainly due to the lack of a distinct marker, such as monthly bleeding in women. The results although statistically significant have indicated several variables to have an infradian period, among them body weight, grip strength, estrogen and testosterone production, sexual activity and mood. The small number of subjects on which the studies have been performed necessitates additional research to confirm published results (Koukkari and Sothern 2006). Ultradian rhythms on the other end have periods shorter than 20 hours as shown in the example of the heart beat above. In

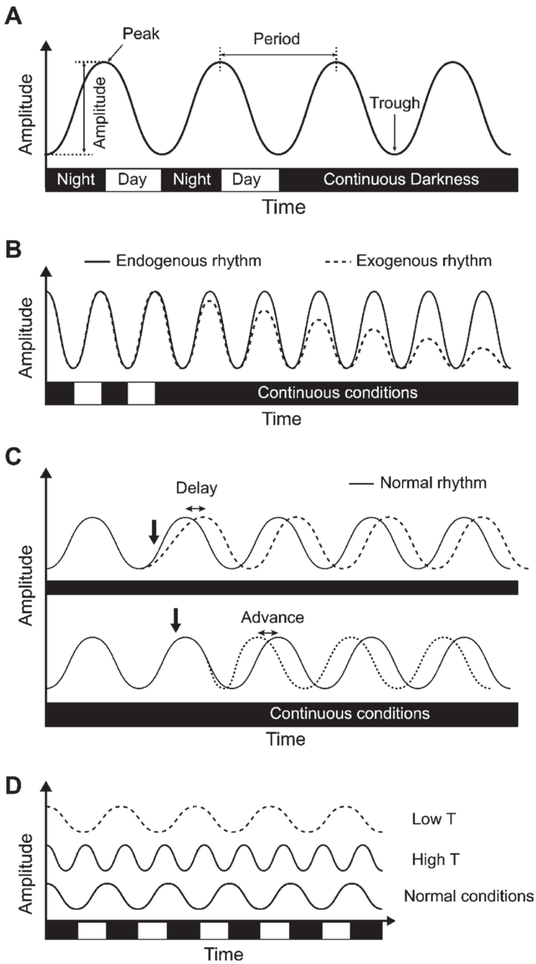

Figure 1: Characteristics of biological and circadian rhythms. A – An example of a circadian rhythm of hormone concentration in blood is shown. The difference between the maximum (peak) and minimum (trough) concentration is the *amplitude*. *Period* equals the interval between two time points. B – Endogenous rhythms persist with no dampening in constant conditions such as complete darkness. Contrary, exogenous rhythms dampen when put in constant conditions. C – Resetting of the circadian rhythm. Exposure to light (black arrow) can shift the rhythm either back (delay) or ahead (advance) depending when during the cycle it is presented. Normal rhythm is depicted by a full gray line. D – temperature compensation. If circadian clocks were not temperature compensated they would run faster at higher temperatures (high T, period < 24h) and slower in lower temperatures (low T, period > 24h) compared to normal conditions.

Slika 1: Lastnosti bioloških in cirkadianih ritmov. A – primer prikazuje cirkadiani ritem koncentracije hormona v krvi. Razlika med največjo koncentracijo (vrh) in najnižjo koncentracijo (dno) je enaka amplitudi. Perioda je enaka intervalu med dvema točkama. B – endogeni ritmi ohranjajo amplitudo tudi v primeru konstantnih pogojev, kot je popolna tema. V nasprotju pa začne amplituda pri eksogenih ritmih v konstantnih pogojih počasi upadati. C – ponastavitev cirkadianega ritma. Svetlobni pulz (črna puščica) lahko povzroči premik faze cirkadianega ritma nazaj (zamuda) ali naprej (napredovanje) v odvistnosti od tega kdaj v fazi cikla je bil pulz prisoten. Normalni ritem je prikazan s polno sivo črto. D – Temperaturna kompenzacija cirkadianih ritmov omogoča, da le-ti v primeru visokih temperature ne potekajo hitreje (perioda < 24h) in v primeru nizkih temperature počasneje (perioda > 24 h), kot pri normalni temperaturi.

Table 1: Examples of biological rhythms. The table shows examples of different biological rhythms belonging to three classes (ultradian, circadian and infradian), defined by the length of the period (based on (Koukkari and Sothern 2006).

Tabela 1: Primeri bioloških ritmov. Tabela prikazuje primere različnih bioloških ritmov iz vseh treh razredov (ultradiani, cirkadiani in infradiani), ki jih definira dolžina periode. Povzeto po (Koukkari and Sothern 2006).

Time	**Period**	**Variable**	**Organism**	**Source**
Seconds	< 1s	EEG activity (delta frequency)	Human (*Homo sapiens*)	(Kripke 1972)
	< 1s	ECG (depolarization of heart ventricles)	Human (*Homo sapiens*)	(Koukkari and Sothern 2006)
Minutes	2–4 min	Leaflet movement	Telegraph plant (*Desmodium gyrans*)	(Koukkari et al. 1985)
	15 min	Cortisol secretion	Horse (*Equus caballus*)	(Drake and Evans 1978)
	30 min	Transpiration	Ota (*Avena sativa*)	(Johnsson 1973)
	90–100 min	REM-NREM sleep	Human (*Homo sapiens*)	(Aserinsky and Kleitman 1953)
Hours	4 h	Enzyme activity	Euglena (*Euglena gracilis*)	(Balzer et al. 1989)
	12 h	Amylase activity	Alfalfa (*Medicago sativa*)	(Henson et al. 1986)
Day	24 h	Body temperature	Human (*Homo sapiens*)	(Aschoff et al. 1972)
	24 h	Sleep-wakefulness	Human (*Homo sapiens*)	(Kleitman 1963)
	24 h	Leaf movements	Alibizzia (*Alibizzia julibrissin*)	(Koukkari et al. 1974)
	24 h	Activity	Mouse (*Mus musculus*)	(Decoursey 1960)
Week	7 days	Oviposition (egg laying)	Spring Tail (*Folsomia candida*)	(Chiba et al. 1973)
	7 days	Organ transplant	Human (*Homo sapiens*)	(DeVecchi et al. 1981)
	7 days	Imbibition of seeds	Bean (*Phaseolus vulgaris*)	(Spruyt et al. 1987)
Month	27–34 days	Menstrual cycle	Human (*Homo sapiens*)	(Presser 1974)
	6 months	Ulcer perforation	Human (*Homo sapiens*)	(Svanes et al. 1998)
Year	1 year	Seed germination	Pole bean *(Phaseolus vulgaris)*	(Spruyt et al. 1988)
	1 year	Migration	Willow warbler (and others) (*Phylloscopus trochilus*)	(Gwinner 1977)
	1 year	Hibernation	Golden-mantled ground squirrel (*Citellus lateralis*)	(Pengelley and Fisher 1963)
	1 year	Gonadal weight	Purple sea urchin (*Strongylocentrotus purpuratus*)	(Halberg et al. 1987)
	8–10 years	Population	Ruffed Grouse (*Bonasa umbellus*)	(Gullion 1982)
	100–200 years	Flowering	Chinese bamboo (*Phyllostachys bamusoides*)	(Janzen 1976)

humans several ultradian rhythms are known both in males and females. Among them is the cycling of the human brain between REM and non-REM sleep (Kishi et al. 2011), regulation of body temperature (Lindsley et al. 1999), hormone release (Ho et al. 1988, Saad et al. 1998, Simon and Brandenberger 2002) and bowel action (Moore 1992). Some examples of different biological rhythms are shown in Table 1. Another important aspect of biological rhythms is whether they are endogenous or exogenous. Exogenous rhythms are simply responses of the organism to external cyclic stimuli, whereas endogenous rhythms are a product of the organism itself and are self-sustained (Fig. 1B) (Aschoff 1981). This review is intended to introduce the basic principles of circadian rhythms, their molecular structure and their role in normal physiology.

Circadian Rhythms

Although the first mention of daily rhythms dates all the way back to 4th century BC, when Androsthenes, a historian of Alexander the Great, described diurnal movements of leaves of several trees, the French astronomer Jean Jacques Ortous de Mairan, is regarded as the discoverer of circadian rhythms. In 1729 he was the first to describe the daily opening and closing of leaves of the mimosa plant (*Mimosa pudica*) even when put in complete darkness (Devlin 2002). However it was not until the 1950s that the field of circadian biology began to develop with studies on fruit flies and humans done by Colin Pittendrigh and Jürgen Aschoff respectively (Vitaterna et al. 2001).

As mentioned circadian rhythms are biological rhythms with a period of about 24 hours, which is implied by the term circadian derived from the Latin *circa*, meaning "around or approximately ", and *diem*, meaning "day". In order for a biological rhythm to be classified as circadian four criteria need to be meet (Vitaterna et al. 2001). First the biological process or function needs to repeat itself with a period of approximately 24 hours. Secondly, the rhythm has to have a characteristic of an endogenous cycle, meaning that it has to continue with a period of close to 24h even in constant conditions devoid of any external time-giving cues (Fig. 1B). Thirdly, the rhythm needs to maintain its period over a range of different temperatures, called temperature compensation. Temperature compensation is important because without it the clock would run faster at higher temperatures compared to lower temperatures due to higher thermal energy of molecular processes (Fig. 1D). Lastly, the rhythm has to have the ability to adapt to changes in the environment and synchronize itself to new conditions (Fig. 1C). This process called entrainment is achieved with the help of external time cues (Zeitgebers), the main one being the light-dark cycle produced by Earth's rotation around its axis (Vitaterna et al. 2001). Pittendrigh discovered that animals will respond differently to light depending on the phase of the cycle they are at (Pittendrigh 1960). For instance, if animals are exposed to light in the early part of their normal night, they will respond with

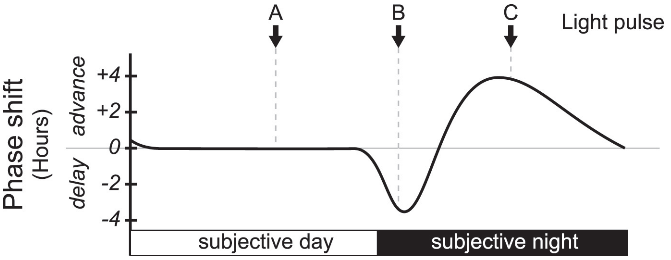

Figure 2: Phase response curve (PRC). A phase response curve shows in what way (advance or delay) the phase of a circadian function (e.g. locomotor activity) will respond, when an external stimuli is given at different times of the circadian cycle. The x-axis represent the time of day, the y-axis shows the amount of phase shift in hours. Light pulse A - (subjective day) won't have any effect on the phase of the circadian function; light pulse B - (beginning of subjective night) will induce a phase delay in the circadian function (also see Fig. 1C); light pulse C - (end of subjective night) will induce a phase advance in the circadian function (also see Fig. 1C).

Slika 2: Krivulja faznega odziva (KFO). Krivulja faznega odziva nam pove v katero smer (zamuda ali napredovanje) se bo premaknila faza cirkadianega procesa (npr. lokomotorna aktivnost), kot posledica odgovora na zunanji dražljajev (svetloba), ki ga dajemo ob različnih časih dneva. X os predstavlja čas dneva, y os predstavlja velikost zamika faze naprej ali nazaj v urah. Svetlobni signal A - (subjektivni dan) ne bo imel vpliva na fazo cirkadianega procesa; svetlobni signal B - (pričetek noči) bo povzročil zamik faze cirkadianega procesa (glej tudi Sl. 1C); svetobni signal C - (konec noči) bo povzročil napredovanje faze cirkadianega procesa (glej tudi Sl. 1C).

Table 2: Examples of circadian rhythms. Some examples of circadian rhythms present in a variety of organisms ranging from bacteria to humans.

Tabela 2: Primeri cirkadianih ritmov. Nekateri primeri cirkadianih ritmov prisotni pri različnih organizmih od bakterij do človeka.

Domain	Process	Organism	Source
Archea	Oxygen-dependent metabolism	*Halobacterium salinarum*	(Whitehead et al. 2009)
Bacteria	Cyclic surface variations during growth	*Pseudomonas putida*	(Soriano et al. 2010)
	N.D.	*Thermosynechococcus elongatus*	(Onai et al. 2004)
	Rhythms of nitrogen fixation	*Synechococcus sp.* RF-1	
Fungi	Growth patterns	*Neurospora crassa*	(Pittendrigh et al. 1959)
Plants	Several physiological processes	*Chlamydomonas reinhardtii*	(Mittag et al. 2005),
	Leaf movement rhythm, germination, growth, enzyme activity, stomatal movement and gas exchange, photosynthetic activity, flower opening, and fragrance emission	*Mimosa pudica,* *Arabidopsis thaliana,* bean *(Phaseolus vulgaris),* chestnut *(Castanea sativa) ,* pea *(Pisum sativum),* soybean *(Glycine max),* tail (*Brassica rapa),* tomato *(Solanum lycopersicum),* poplar (*Populus spp*.)*, papaya (*Carica papaya*)*, grape (*Vitis vinifera*)*	(McClung 2013), (McClung 2011)
Animals	Time of Eclosion, foraging and mating activities	fruit fly (*Drosophila melanogaster*)	(Panda et al. 2002)
	visit flowers to collect pollen and nectar in a rhythmic manner	honeybee *(Apis mellifera)*	(Moore et al. 1998)
	Timing of their mating flights	ant (*Camponotus compressus)*	(Sharma et al. 2004)
	Timing of migratory flights	Monarch butterflies *(Danaus plexippus)*	(Froy et al. 2003)
	Preparation for hibernation	Golden-mantled ground squirrel (*Callospermophilus lateralis*)	(Dunlap et al. 2004)
	Diving timing	loggerhead turtle *(Caretta caretta)*	(Oishi et al. 2010)
	Locomotor activity	Japanese grass lizard (*Takydromus tachydromoides*)	(Oishi et al. 2010)
	Body temperature and locomotor activity	*Iguana iguana*	(Oishi et al. 2010)
	Diurnal rhythms in hypothalamic/ pituitary AVT synthesis and secretion	*Oncorhynchus mykiss* (rainbow trout)	(Rodriguez-Illamola et al. 2011)
	Body temperature, blood pressure, metabolism, hormone synthesis etc.	Mouse (*Mus musculus*)	(Green et al. 2008; Tzameli 2012)
	Body temperature, blood pressure, metabolism, hormone synthesis etc.	Human (*Homo sapiens sapiens*)	(Green et al. 2008; Tzameli 2012)

* – clock genes have been found by genome wide analysis however functional assesments of the clock are still missing (McClung 2013).

a phase delay, whereas they will respond with a phase advance when they are exposed to light in the later part of their normal night (Fig. 1C, Fig. 2). The exact way an animal will respond to a zeitgeber at a specific time can be studied with the help of phase response curves. A phase response curve is constructed by determining whether a phase advance or delay of a certain circadian variable (e.g. locomotoric activity) is produced when the same zeitgeber is given at different times of the circadian cycle (Fig. 2) (Pittendrigh 1960, Golombek and Rosenstein 2010).

The importance of entrainment may not seem obvious at first, but simple mathematics shows how quickly a species can come out of synch with the day-night cycle if the phase of the rhythm changes by just a fraction. Let's assume a mouse's endogenous period would be a mere 10 minutes longer tha˝n 24 h. With no entrainment to external conditions, it would take only 6 days for the mouse to be 1h in advance of the normal day night cycle and in just a matter of 2 months it would become a diurnal instead of a nocturnal animal. This would have a significant negative impact on the fitness of an individual that would substantially reduce its success of survival and reproduction. For this reason if a mouse's active night period is too long and extends into morning hours, the light will trigger a phase advance (Fig. 2). As a consequence the active period will begin sooner in the coming day and also end before the morning, entraining the internal mouse clock to the environmental conditions.

In spite of these four restrictions a large fraction of today's organisms, ranging from bacteria to humans, display a clear circadian rhythm in various physiological and behavioral processes (some are listed in Table 2). Due to its almost ubiquitous presence, the circadian rhythm clearly has an evolutionary advantage. Anticipation of daily changes in the environment by an organism rather than just reacting to them seems to be one of the main ones (Ramsey et al. 2007). At least two studies in cyanobacteria and *D. melanogaster* have shown that wild-type strains are more successful in survival compared to their mutant ones when grown in the same test tube (Johnson et al. 1998, Klarsfeld and Rouyer 1998).

Genetics of the clock

Despite the discovery of the double helix in 1953 and the development of various genetic and molecular biology techniques thereafter, the first two decades of circadian rhythm research were devoted mainly to understanding the basic principles (Pittendrigh et al. 1959, Pittendrigh 1960) including resetting of the rhythm by light pulses (Bruce et al. 1960), construction of phase response curves (Aschoff 1965), temperature compensation (Zimmerman et al. 1968) etc. It was not until 1971 that the era of clock genetics began, when Ron Konopka and Seymour Benzer first described the existence of the *period* (*per*) locus in *Drosophilla melanogaster*. Using genetic screens of mutated fruit flies they discovered 3 mutants which significantly changed their 24h rhythm of both eclosion and locomotor activity: long period (28h rhythm), short period (19h rhythm) and arrhythmic (no rhythm) (Konopka and Benzer 1971). By the beginning of the 21th century, similar genetic screens were used in various model organisms to discover other clock related genes including: *per* and timeless (*tim*) in *D. melanogaster*; white collar 1 and 2 (*wc1* and *wc2*) and frequency (*frq*) in *N. crassa;* timing of crab (*TOC1*) in *A. thaliana* and *Clock, Bmal1*, *Per1, Per2, Cry1* and *Cry2* in mice (Takahashi 2004, Zhang and Kay 2010). Regardless of the fact that different organisms use different sets of genes the basic molecular mechanism behind all circadian clocks seems to be the same and can be described by a simple transcription-translation feedback loop (Roenneberg and Merrow 2002). While the transcription-translation feedback loop remains at the core of the circadian clock, the use of novel high-throughput technologies in the last decade showed that the clock is not a simple loop but is composed of multiple networks operating on different levels (Zhang and Kay 2010). It is not within the scope of this review to present any details about the molecular components of circadian clocks in various organisms. However, since the basic principle of how molecular clocks work is similar in all species, we will take a closer look at the molecular clock of mammals (Fig. 3).

The transcription-translation feedback loop of mammals is composed of a positive, represented by *Clock* and *Bmal1* and a negative limb, represented by *Per1*, *Per2*, *Cry1* and *Cry2*. During the day, CLOCK and BMAL1 proteins form a heterodimer that acts as a transcriptional factor, binding to E-box promoter regions of various genes, including *Pers* and *Crys*, and activating their transcription. The resulting PER and CRY proteins heterodimerize and translocate back to the nucleus where they inhibit the transcriptional activity of the CLOCK/

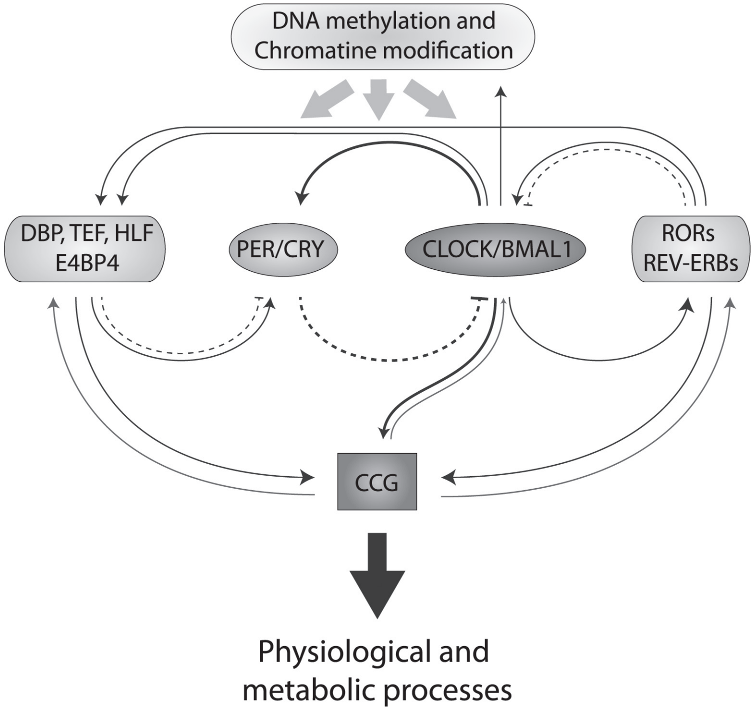

Figure 3: Molecular organization of circadian rhythms. Although different model organisms have different clock components the overall architecture of the transcriptional translational feedback loop is similar. The CLOCK/BMAL1 heterodimer presents the positive limb and activates transcription of various core clock and clock output genes. PER and CRY proteins represent the negative limb that inhibits CLOCK/BMAL1 transcriptional activation. PER and CRY degradation leads to a new round of CLOCK/BMAL1 initiated activation. Various other loops (D-box and RRE) can influence the core clock mechanism. In addition DNA methylation and chromatin modifications influence various components of the clock mechanism. Clock controlled genes (CCG) regulate circadian physiological processes and can also feedback information to the core clock mechanism.

Slika 3: Molekularna osnova cirkadianih ritmov. Kljub razlikam v sestavi genov in proteinov, ki tvorijo molekularno osnovo cirkadianih ur pri različnih organizmih, pa je arhitektura transkripcijsko translacijske povratne zanke pri vseh podobna. Heterodimer CLOCK/BMAL1 aktivira izražanje genov centralne cirkadiane ure in output genov. Proteina PER in CRY predstavljata negativno povratno zanko, ki inhibira transkripcijsko aktivnost heterodimera CLOCK/BMAL1. Proteolitska razgradnja PER in CRY proteinov povzroči ponovno aktivacijo transkripcije preko heterodimera CLOCK/BMAL1. Poleg opisanih, obstajajo še druge zanke, kot sta D-box in E-box zanka, ki lahko vplivajo na mehanizem centralne ure. Mehanizem centralne cirkadiane ure je podvržen tudi regulaciji preko DNA metilacije in modifikacije kromatina. CCG (*Clock controled genes*): geni, ki jih uravnava cirkadiana ura, omogočajo cirkadiano izražanje fizioloških procesov, hkrati pa lahko tudi posredujejo informacije nazaj k cirkadiani uri.

BMAL1 heterodimer. During the night however, the PER/CRY heterodimer is degraded enabling a new round of transcription by the CLOCK/BMAL1 dimer to start. This whole process takes about 24h to complete (Ko and Takahashi 2006). In addition to the core loop other loops exist that interact with the core loop. One such is the REV-response element (RRE) loop, which is composed of proteins belonging to the nuclear receptor family of transcriptional factors. By binding to the RRE element in promoter regions of *Bmal1* proteins such as *Rorα, Rorβ* and *Rorγ* or *Rev-erbα* and *Rev-erbβ* activate or repress its transcription respectively (Preitner et al. 2002). Similar to the RRE, the D-box loop represents the third feedback loop and is generated by transcription factors D-box binding protein (DBP), thyrotroph embryonic factor (TEF) and hepatic leukemia factor (HLF) as activators and E4 promoter-binding protein 4 (E4BP4) as a repressor (Fig. 3)(Takahashi et al. 2008). These additional loops are important because they provide (1) robustness of the clock, (2) enable the clock to receive entrainment signals from various sources and (3) provide several different clock output ways (Zhang and Kay 2010).

Regardless of the complexity of the loops mentioned above the circadian rhythm in cells is also controlled by other means. In mammals posttranslational modifications (PTM) play an important role by modulating protein half-life and their subcellular location. All of the core clock proteins in mammals (CLOCK, BMAL1, PERs and CRYs) are known to be modified by one or several modifications including phosphorylation (all), acetylation (BMAL1, PER2), ubiquitination (all) and sumoylation (BMAL1) (Bellet and Sassone-Corsi 2010). PTM are also important for epigenetic control of the clock next to DNA methylation and miRNA. Several studies have shown that chromatin remodeling is involved in expression of circadian genes as well as that chromatin modifications appear to follow a circadian pattern at different clock controlled genes (CCG) (Curtis et al. 2004, Doi et al. 2006, Bellet and Sassone-Corsi 2010).

It is evident that the control regulation of circadian clocks in cells is a complex process involving different levels of regulation ranging from transcriptional control all the way to epigenetic modifications. Likewise, because of the interaction between different molecular loops that feed information into the core circadian loop, the clock is well integrated with other physiological processes and vice versa. The exact interplay between the clock and cell physiology and metabolism is still a matter of research, but much has been learned in recent years.

Interplay between circadian rhythms and metabolism?

In multicellular organisms such as mammals light cannot reach every cell in the body and therefore cannot synchronize the clock in these cells directly. For this reason the circadian system evolved a hierarchical structure in which a master clock residing in the suprachiasmatic nuclei (SCN) of the hypothalamus synchronizes peripheral clocks in various tissues such as liver, adipose tissue, heart, intestine and adrenal gland (Fig.4).

The SCN receives light signals from the retina through the retinohypotalamic tract and thereby synchronizes its internal clock to the outside world (Reppert and Weaver 2002). It is responsible for driving various behavior rhythms (e.g. locomotor activity) as well as synchronizing circadian clocks in peripheral tissues, with the help of neural and humoral signals, to maintain proper phase relationships and prevent clocks in these tissues from dampening out (Dickmeis 2009). While the SCN is primarily entrained by light, peripheral tissues can in addition to SCN signals, also be entrained to various other stimuli among which feeding is the dominant zeitgeber (Damiola et al. 2000). There has been a lot of debate in recent years of whether the SCN can also be entrained by temperature fluctuations or not. While some publications have shown this to be true (Ruby et al. 1999, Herzog and Huckfeldt 2003) other have proven the opposite (Buhr et al. 2010). What has been show by all is the fact that a single SCN neuron can be affected by temperature fluctuations, however for the SCN as a whole this has not yet been proven and is still a matter of further research.

It was not until only recently that the influence of circadian clocks on metabolism became evident in mammals. With the use of DNA microarrays it was shown that between 5 % and 20 % of all transcripts in a particular tissue have circadian profiles of expression. Different tissues showed only limited overlap between rhythmic genes, suggesting that the expression is regulated in a tissue specific manner (Akhtar et al. 2002, Durgan et al. 2006, Zvonic et al. 2006, Kosir et al. 2012). Among genes shown to have rhythmic expression were transcripts involved in gluconeogenesis, glycolysis, lipid and cholesterol metabolism, steroid hormone synthesis and xenobiotic metabolism (Green et al. 2008, Acimovic et al. 2011, Zmrzljak and Rozman 2012, Kosir et al. 2013). It has also been discovered that different hormones regulating metabolism in mammals including glucagon, insulin, leptin, adiponectin and corticosterone

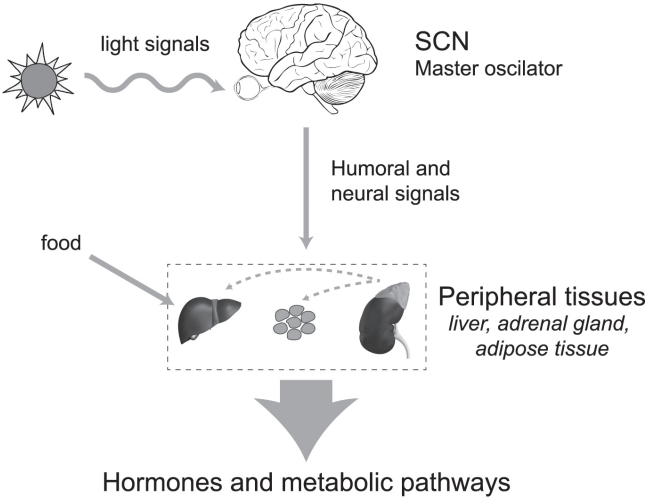

Figure 4: Anatomical organization of circadian rhythms in mammals. In mammals the circadian system is composed of a master oscillator located in the suprachiasmatic nucleus and is synchronized to the outside world by light pulses that reach it from the retina through the retinohypotalamic tract. The SCN controls peripheral clocks in tissues through various humoral and neural signals. In addition to SCN signals, food can also entrain some peripheral tissues especially liver. Adrenal glands excrete glucocorticoids in a circadian fashion that can also influence expression of genes in other tissues.

Slika 4: Anatomska struktura cirkadianih ritmov pri sesalcih. Pri sesalcih je cirkadiani sistem zgrajen iz glavne cirkadiane ure, ki se nahaja v suprahiazmatičnem jedru (SCN) v hipotalamusu, ter perifernih cirkadianih ur, ki se nahajajo v različnih organih. SCN se vsakodnevno sinhronizira z zunanjimi pogoji svetlobe in teme, preko retine in retinohipotalamičnega trakta. V nadaljevanju SCN preko živčnih ali hormonskih poti sinhronizira periferne cirkadiane ure. Periferne cirkadiane ure se lahko sinhronizirajo tudi z drugimi signali, neodvisno od SCN, kot je npr. hrana. Nadledvična žleza izloča tudi glukokortikoide, ki prav tako vpliva na izražanje genov v nekaterih perifernih tkivih. Koncentracija glukokortikoidov, kot je kortizol ali kortikosteron je v plazmi cirkadiana.

show circadian oscillations (Froy 2011). These examples clearly show a direct influence of the circadian clock on various metabolic processes, but there are several key metabolic factors which can also influence the core clock mechanism. We previously already mentioned REV-ERBa and RORa, that regulate the expression of *Bmal1* but are also important in adipocyte differentiation and regulation of lipogenesis respectively (Froy 2011). PPARa, another member of the nuclear receptor family, is important in lipid and glucose metabolism. It shows circadian rhythmicity but also activates the transcription of *Bmal1*, indicating yet another feedback loop of the clock (Canaple et al. 2006). Other molecules such as AMPK (AMP-activated protein kinase), PGC-1a (PPARg co-activator 1a) and SIRT1 (sirtuin I) have also been implicated in the regulation of clock genes either directly through transcription (PGC-1a) or indirectly through phosphorylation (AMPK) and deacetylation (SIRT1) (Canto and Auwerx 2009).

The importance of an intact circadian clock for normal homeostasis and metabolism has been well established and it has been show that disruption of circadian rhythms may lead to development of various forms of metabolic syndrome (Green et al. 2008, Froy 2011, Naik et al. 2013). The most compelling evidence comes from mouse models. Here both obesity and metabolic syndrome have been discovered in mice carrying mutations in core clock genes. For example *Bmal1* knock-out mice are completely arrhythmic and have disruptions in rhythmic levels of glucose and triglycerides. To see whether these disruptions are a consequence of the loss of rhythmicity of the SCN or of peripheral oscillators, *Bmal1* liver and pancreas specific knock-out mice we generated. Despite normal locomotor rhythm both tissue specific knock-outs

Table 3: Mouse experimental models. Examples of metabolic defects in mice with mutations or gene knock-outs of clock genes. Based on (Froy 2011, Sahar and Sassone-Corsi 2012).

Tabela 3: Eksperimentalni mišji modeli. Primeri metabolnih motenj, ki se pojavijo pri miših z mutacijami ali izbitimi geni cirkadiane ure. Povzeto po (Froy 2011; Sahar and Sassone-Corsi 2012).

Gene mutated or knocked-out	**Metabolic Consequence**
Clock	Hyperlipidemia, hyperleptinemia, hypoinsulinemic and hyperglycemia
Bmal 1	Abolished oscillations in plasma glucose and triglycerides impaired gluconeogenesis, hyperleptinemia, glucose intolerance, and dyslipidemia
Per 1	Increased urinary sodium excretion
Per 2	Altered lipid metabolism, lower body weight
Cry 1 and *Cry 2*	Hyperglycemia Salt-sensitive hypertension
Reverba	Increased serum triglycerides
Rora	Reduced plasma triglycerides and HDL Enhanced atherosclerosis
Pgc–1a	Increased sensitivity to insulin Altered thermogenesis
Nocturnin	Resistant to diet-induced obesity Altered lipid metabolism

displayed disturbances in blood glucose levels (Sahar and Sassone-Corsi 2012). Several other mouse models with mutations or deletions of core clock genes have been generated that display perturbation to normal metabolism (Tab. 3). In addition to mouse genetic models epidemiological studies on humans have identified a correlation between shift work and metabolic disorders. Humans that were active and eating during normal night were shown to have decreased leptin (adipose tissue specific hormone that promotes satiety) levels and increased insulin and glucose levels. Leptin levels were also found to be reduced in healthy patients that were subjected to only 4 hours of sleep in six consecutive nights (Spiegel et al. 2004). It is interesting to note that in the same time period that we have seen an increase in metabolic diseases and obesity we have also seen a decrease in the quality and duration of sleep.

Low quality and duration of sleep and disruptions of the normal circadian rhythm can also be related to another problem facing modern societies: light pollution. Light pollution is defined as artificial light (usually over illuminated streets, buildings, commercial ads etc.) present during the otherwise dark night. The effects of light pollution on various animal species have been well established unfortunately less research has been done on human subjects. Nevertheless a study done in Israel compared the level of artificial light at night and occurrence of breast cancer in 147 communities and discovered that women living in areas with high night light had a greater chance for developing breast cancer (Kloog et al. 2011). Several studies have shown that the production of the night hormone melatonin, by the pineal gland, is abruptly terminated when individuals are exposed to light during the night faze. Since melatonin is known for helping to regulate the body's biologic clock, it might be an important link between the disrupted circadian clock of the body and light pollution (Chepesiuk 2009).

Conclusion

The presence of circadian rhythms in almost all organisms ranging from bacteria and unicellular eukaryotes to multicellular organisms including humans clearly shows their importance and evolutionary advantage. While a lot has been learned in the six decades of circadian rhythm research it is only in the last few years that we began to appreciate their importance in human health. The alarming increase in the rate of hypertension, obesity, metabolic syndrome and cancer worldwide, especially in developed and developing countries, could well be related to a disrupted circadian rhythm caused by lifestyle changes. For this reason much research is needed to completely understand the intricate relationships between the circadian clock and metabolism as well as the circadian clock and cancer to eventually be able to reset the inner clock and prevent metabolic or cancer disorders from developing.

Povzetek

Cirkadiani ritmi so biološki ritmi, ki se ponavljajo s periodo okoli 24h in predstavljajo pomembno evolucijsko adaptacijo organizmov na ciklične spremembe v okolju, ki so posledica vrtenja Zemlje okoli svoje osi. Najdemo jih v skorajda vseh organizmih od bakterij pa vse do ljudi, kjer uravnavajo številne fiziološke in metabolne procese. Začetki obširnejših raziskav cirkadianih ritmov segajo v 50. leta 20. stoletja, ko sta Colin Pittendrigh in Jürgen Aschoff predvsem z opazovanjem sprememb obnašanja živali razkrila osnovne značilnosti cirkadianih ritmov in njihove lastnosti. Moderna doba raziskav cirkadianih ritmov, ki je vključevala tudi molekularne osnove ritma, pa se je pričela šele v 70 letih 20. stoletja. V tem času sta Ron Konopka in Seymour Benzer odkrila prve mutante lokusa *period* pri vinski mušici (*D. melanogaster*), ki so povzročile spremenjen cirkadiani ritem lokomotorne aktivnosti mušic. Kmalu so z uporabo različnih modelnih organizmov kot so *N. crassa, D. melanogaster, M. musculus* in še nekaterih drugih odkrili, da je osnovni molekularni mehanizem cirkadiane ure pri vseh organizmih zelo podoben. Osnova ritma je transkirpcijsko translacijska povratna zanka, ki je npr. pri sesalcih sestavljena iz aktivatorjev, kot sta *Clock* in *Bmal1,* ter represorjev, kot so družina genov period in kriptokrom. Proteina CLOCK in BMAL1 v heterodimeru delujeta kot transkripcijska faktorja, saj aktivirata izražanje represorjev kot tudi številnih drugih genov uravnavanih s cirkadiano uro. Proteini PER in CRY pa delujejo tako, da preprečijo traskripcijsko aktivnost heterodimera CLOCK/BMAL1 in ustavijo transkripcijo tako sebe kot drugih genov. Po določenem času se proteini PER in CRY razgradijo in tako omogočijo, da se aktivacija transkripcije s CLOCK/BMAL1 ponovno prične. Celoten cikel traja približno 24 ur da se ponovi. Predvsem pri višjih organizmih, kot so sesalci in človek, v zadnjih nekaj letih prihaja vedno bolj do izraza prepletenost cirkadiane ure in različnih fizioloških procesov ter metabolizma. Postalo je jasno, da lahko porušen cirkadiani ritem povzroči nastanek različnih metabolnih motenj, kot sta diabetes in prekomerna teža. Nadaljne raziskave bodo pripomogle k boljšemu razumevanju prepleta med cirkadiano uro in metabolizmom ter morda v prihodnosti omogočile izdelavo režima, s katerim bomo vzpostavili normalno delovanje, sicer porušene cirkadiane ure pri številnih bolnikih, in tako pripomogli k njihovemu zdravljenju.

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Nestling growth of Great Tits *Parus major* with comparison among altitudes

Rast mladičev velike sinice *Parus major* s primerjavo med nadmorskimi višinami

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Abstract: Fledgling mass can have great influence on individual's life history and reflects the availability and quality of food in the breeding territory. Thus growth curve is used to compare the difference in the quality between different ecological conditions. The aim of the study was to fill the knowledge gap on nestling growth in Great Tit in Slovenia, to determine the influence of altitude on it and to present a tool for estimating age of nestlings and key dates in breeding phenology of Great Tit. Great Tits young were monitored using nest-boxes at three locations between years 2010 and 2012. At two locations nest-boxes were placed on three separate altitudinal belts. Weight growth curve was compared with the curves from other parts of Europe. Growth curve from Slovenia differs in growth parameters from other European populations but falls within their range. Difference in growth parameters between separate populations probably comes from the difference in ecological conditions. When comparing three altitudes weight parameters of hatchlings were similar at lower and middle but different at upper altitudes indicating that weigh growth changes with the altitude. Lower food abundance that comes with rising altitude may be offset by lower competition through lower breeding density (hence similar growth parameters in lower and middle altitude), but not past certain altitude. Weight parameters are useful when comparing different populations, but wing-length is better in determining the age of young in the nest.

Keywords: Great Tit, hatchling growth, altitude, Slovenia,

Izvleček: Masa mladičev v gnezdu je pomemben dejavnik, ki ima močan vpliv na življenje osebka. Masa mladičev v gnezdu odseva dostopnost in kvaliteto hrane na gnezdišču in je zaradi tega dober pokazatelj stanja v okolju. Namen raziskave je bil zapolniti vrzel v znanju o rasti velike sinice na območju JV Evrope ter o pomenu nadmorske višine na rast mladičev. Hkrati bodo rezultati lahko koristili tudi kot orodje za določanje starosti mladičev velike sinice v gnezdu. Meritve mladičev velike sinice v gnezdu so bile opravljene na treh lokacijah med leti 2010 in 2012. Na dveh so bile gnezdilnice postavljene na treh ločenih nadmorskih višinah. Rastna krivulja za maso se razlikuje od krivulj iz ostalih delov Evrope vendar je znotraj njihovih meja. Razlike verjetno izhajajo iz različnih ekoloških razmer v okoljih. Rast mase je podobna med spodnjim in srednjim ter različna od zgornjega višinskega pasu. Podobnost na spodnjih višinah je verjetno posledica kompenzacije manjše količine plena z nižjo konkurenco zaradi nižje gostote gnezdečih parov. Kompenzacija pa je uspešna le do določene nadmorske višine, nad to pa se zmanjševanje količine hrane pozna na počasnejši rasti mladičev. Rast mase je uporabno orodje za primerjavo med populacijami, dolžina peruti pa je boljše orodje za določanje starosti mladičev v gnezdu.

Ključne besede: Velika sinica, rast mladičev, nadmorska višina, Slovenija

Introduction

Environmental conditions change considerably with altitude and the most prominent of all is the change in ambient temperature (Newton 1998). Individuals breeding at the limit of species distribution show markedly different breeding parameters than their counterparts in more optimal environments (Orell and Ojanen 1980, 1983, Veistola et al. 1994). While the onset of breeding within certain species consistently change along altitudinal gradient, with later start at higher elevations (Gil-Delgado et al. 1992, Belda et al. 1998, Fargallo 2004), other breeding parameters like breeding density (Schmid et al. 1998) or clutch size (Gil-Delgado et al. 1992, Fargallo 2004) can show contradictory trends between and also within species. For example Fargallo (2004) found that the clutches of Blue Tit *Cyanistes caeruleus* were smaller with increasing altitude while Gil-Delgado et al. (1992) found that they were similar. In fact the clutch size can differ more among habitats than does across altitudes (Gil-Delgado et al. 1992).

Fledgling mass has great influence on individual's survival (Tinbergen and Boerlijst 1990, Barba et al. 1993, Perrins and McCleery 2001) and fecundity (Perrins and McCleery 2001). Individual growth rate and fledgling mass is determined by the amount of food they receive (Gill 1994, Keller and van Noordwijk 1994). Since Great Tits are altricial nesters all food consumed by young is provided by the parents (Tanner et al. 2007). How much food they can provide is determined by the availability and quality of food in breeding territory (Gibb and Betts 1963) and can be further influenced by unfavourable conditions such as weather or competition (Minot 1981, Keller and Van Noordwijk 1994).

The growth curve can offer comparison between nestling development since it is defined mathematically by three values: initial size, growth rate and asymptotic value (Gill 1994). Difference between two environments can be reflected in fledging mass (Richner 1989), thus growth curve may be used for comparing the quality difference between breeding environments within the range of species (Janssens et al. 2003, Eeva et al. 2009). Also growth parameters can vary considerably between different populations (Barba et al. 1993), though this does not seem to be a general rule (King and Hubbard 1981). There are some studies that offer growth data of the Great Tit from other parts of Europe (Barba et al. 1993, Janssens et al. 2003, Eeva et al. 2009) but there hasn't been a study yet considering South-East Europe. In fact, apart from studies of Dolenec (2005) and Dolenec et al. (2005) remarkably little is known about Great Tit in this region. Scientific publications on Slovenian population of Great Tit are even scarcer. There are only a few reports about number of ringed birds per year and about exceptional recoveries (Božič 2009, Šere 2009).

Considering the altitude, there are some studies about influences on breeding phenology and clutch size of Tits in Europe (Gil-Delgado et al. 1992, Krementz and Handford 1984, Sanz 1998), but none that uses altitude as an influencing factor on growth parameters.

With the present study I aimed to fill the knowledge gap on Great Tits nestling growth in Slovenia. I also aimed to use weight of nestlings to compare influence of environmental conditions at different altitudes. Since conditions for nesting deteriorate with the rising altitude, e.g. less food (Hodkinson 2005), I hypothesise that this results also in slower nestling growth at higher altitudes. Presented data on growth curve of young Great Tits might also serve as a tool for other researchers to estimate key dates in breeding phenology of Great Tit (start of incubation, hatching time, age of nestlings, etc.) from body part measurements.

Methods and materials

Breeding biology of Great Tit using nest-boxes was studied at three locations in Slovenia between years 2010 and 2012. At the first location, Ljubljana (300 m asl), five nest-boxes were set to measure hatchlings weight and wing-length. Location 1 is a small forest fragment dominated by Beech *Fagus sylvatica*, White Fir *Abies alba* and Norway Spruce *Picea abies*. This location was chosen due to its easy accessibility and comparable altitude to study sites in other European countries (Barba et al. 1993). Hatchlings were measured two to four times a week. When their weight reached more than 10 g they were ringed and individual's size was monitored then after. The nest-box visiting frequency was high enough even before hatching, so the first measurements of all chicks have been taken within two days after hatching.

At locations 2 and 3, Mt. Krim and Mt. Pohorje respectably, nest-boxes were placed in similar habitat (mixed forest dominated by Beech, White Fir and Norway Spruce) on three altitudinal belts with an average altitude of 335 (Lower), 654 (Middle) and 1023 m asl (Upper belt). On each of the upper two belts 40 nest-boxes were placed while on the lower one 32 were placed. At locations 2 and 3 nest-boxes were checked once a week. Number of nest-boxes at each location has corresponded to our capability to survey them and was higher on higher altitudes due to lower breeding density of Great Tits there (Schmid et al. 1998).

Measurements from location 1 were used to produce logistic growth curves for wing-length and body-weight. This was done by calculating growth constant (k), which denotes relative speed of growing, and time it takes for hatchling to reach half their weight at fledging (t_{50}) (Ricklefs 1967, Tome 1995). Asymptotic value was estimated from individual chicks at the age of 17 days, since at about that age they leave their nest (Perrins and McCleery 2001) and the weight of chicks out off the nests levels of or may even drop after emergence (Tome 1995). Weight growth curve was than compared with the curves from other parts of Europe e.g. Spain, Finland, Great Britain, Germany and Netherland with the use of growth parameters published by Barba et al. (1993).

From the wing-length growth curve obtained from nestlings at location 1 age of hatchling from nests at locations 2 and 3 was estimated with an accuracy of ± 1 day. Then weight growth curves for chicks from all three altitudes were calculated separately (Ricklefs 1967, Tome 1995). Average weight of nestlings in individual nest on particular day from locations 2 and 3 was compared with the calculated value from nestlings in Ljubljana. The difference between these weights was used to compare nestling's condition among altitudes. Only data from nestlings at the age between 11 and 15 days was used, when growth is most constant and overlapping among estimated age classes is less likely. Statistical significance was tested with the use of nonparametric Kruskal-Wallis statistic test (PAST 2.03).

Results

Five nests and 35 hatchlings were monitored at location 1. One nest containing eight hatchlings failed and its measurement were later disregarded. At locations 2 and 3 altogether weekly measurements from 90 nests containing 644 hatchlings were gathered. Of those 369 hatchlings from 52 nest were from lower, 235 (31 nests) from middle and 40 (7 nests) from upper altitudinal belts.

The growth parameters from location 1 were for the wing-length: $k = 0.347$; $t_{50} = 9.252$; and the body weight: $k = 0.430$; $t_{50} = 5.928$. These two parameters were used for modelling wing-length and weight growth curves respectably (Fig. 1). Measurements and estimated values for wing-length at location 1 are presented in Tab. 1.

Weight growth curve of nestlings from Slovenia falls within the range of the curves from other parts of Europe (Fig. 2). Asymptotic weight is second only to that from Great Britain and only marginally higher than the one from Spain. Growth constant is highest and t_{50} is the second lowest behind Finland (Tab. 2).

Weight growth curves for location 1 and for lower and middle belts from locations 2 and 3 are similar (Fig. 3). Curve from the upper belt is somewhat lagging behind the rest. Nestlings at the upper belt had lower asymptotic value, lower growth constant and it took them longer to reach 50% of asymptotic value (Tab. 2). For 11-15 days old chicks weights at lower and middle altitudes were similar, but both were statistically heavier from those at the upper altitude (Kruskal-Wallis All: $H_3 = 7.18$, $p < 0.05$; Lower / Middle: $p = 0.905$; Lower / Upper: $p < 0.05$; Middle / Upper: $p < 0.01$).

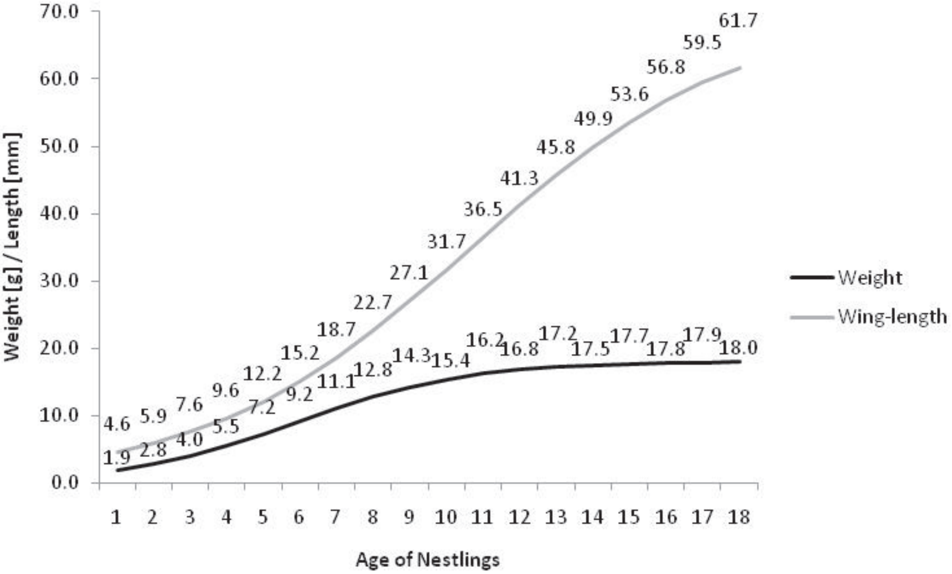

Figure 1: Calculated logistic growth curves for weight (asymptotic value: 18.1g) and wing-length (asymptotic value: 72mm) for Great Tit *Parus major* hatchlings in Ljubljana with values shown. Number of nestling monitored: 27.

Slika 1: Izračunana logistična rastna krivulja za maso (asimptotična vrednost: 18.1g) in dolžino peruti (asimptotična vrednost: 72mm) mladičev velike sinice *Parus major* v Ljubljani s prikazanimi vrednostmi. Število spremljanih mladičev: 27.

Table 1: Results of nestling measurements in Ljubljana with average, minimal and maximal measured wing-length compared with estimated wing-length gained from logistic curve. N represents number of measured nestling at particular age.

Tabela 1: Rezultati merjenj mladičev v Ljubljani s prikazano povprečno, minimalno in maksimalno izmerjeno dolžino peruti primerjano z izračunano dolžino peruti iz logistične krivulje. N predstavlja število izmerjenih mladičev za posamezno starost.

Age	Average wing-length (mm)	StD	Min – max wing-length (mm)	N	Estimated wing-length (mm)
0	4.3	0.5	4-5	6	4.6
1	5.8	0.5	5-6	4	5.9
2	7.1	0.7	6–8	7	7.6
3	9.6	0.9	8–11	13	9.6
4	12.4	1.1	11-14	16	12.2
5	14.7	1.3	13-16	11	15.2
6	19.5	1.6	15-21	15	18.7
7	24.0	1.4	21-26	20	22.7
8	29.1	1.6	26-31	15	27.1
9	33.1	1.4	30-36	15	31.7
10	37.5	1.3	34-39	19	36.5
11	42.3	2.0	38-45	12	41.3
12	46.1	1.9	43-50	19	45.8
13	47.9	2.1	44-52	14	49.9
14	50.8	1.4	48-53	8	53.6
15	55.9	0.8	55-58	14	56.8
16	60.0	0.7	59-61	9	59.5
17	62.3	1.3	61-44	4	61.7

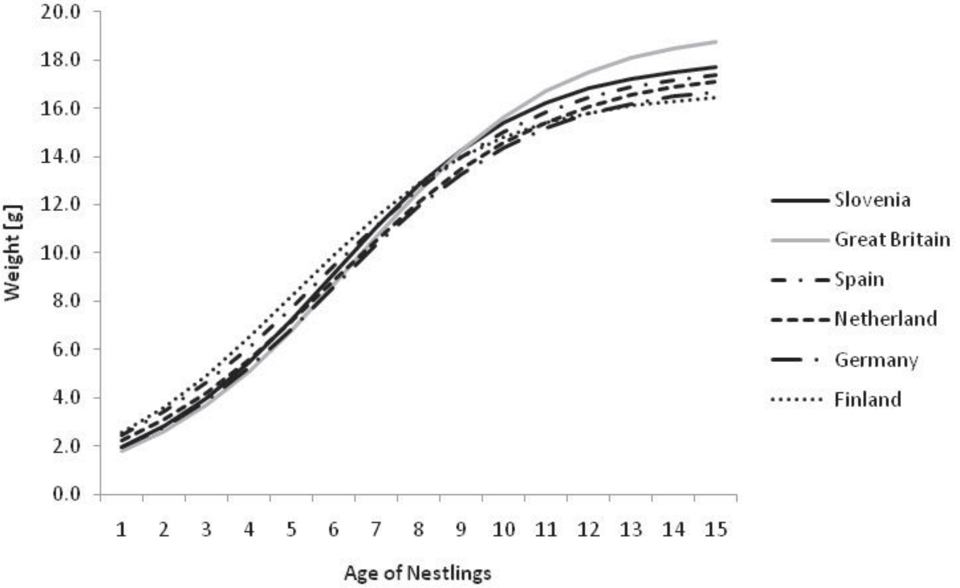

Figure 2: Calculated logistic growth curves for hatchlings weight of Great Tit *Parus major* from separate European populations. Curves from other parts of Europe are from Barba et al. (1993).

Slika 2: Izračunana logistična rastna krivulja za maso mladičev velike sinice *Parus major* iz ločenih evropskih populacij. Rastne krivulje za ostale dele Evrope so iz Barba et al. 1993.

Table 2: Growth parameters of Great Tit *Parus major* nestlings from different European populations and different altitudes. Growth parameters published by Barba et al. (1993) were used for other parts of Europe.

Tabela 2: Rastni parametri mladičev velike sinice *Parus major* z različnih delov Evrope in ločenih višinskih pasov. Rastni parametri objavljeni v Barba et al. (1993) so bili uporabljeni za ostale dele Evrope.

Population	**Asymptotic weight (g)**	**Growth constant (k)**	**t_{50}**
Great Britain	19.3	0.413	6.486
Spain	17.9	0.389	5.695
Netherland	17.6	0.390	5.970
Germany	17.1	0.412	5.964
Finland	16.7	0.418	5.075
Ljubljana	18.1	0.430	5.422
Lower	18.2	0.420	6.173
Middle	18.0	0.436	6.382
Upper	17.5	0.426	7.133

t_{50} – time it takes hatchlings to reach 50% of asymptotic weight

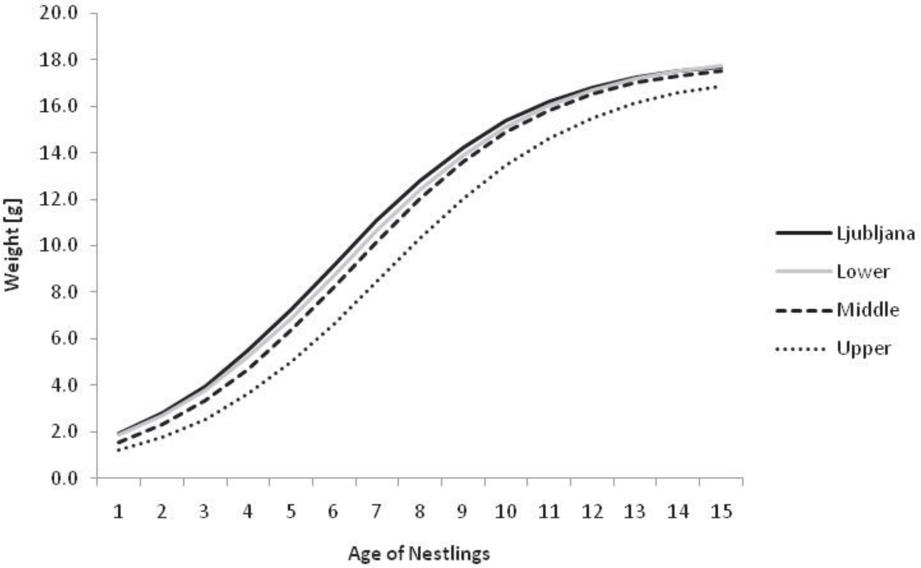

Figure 3: Calculated logistic weight growth curves for hatchlings of Great Tit from *Parus major* Ljubljana and separate altitudinal belts from Mt. Krim and Mt. Pohorje.

Slika 3: Izračunana logistična rastna krivulja za maso mladičev velike sinice *Parus major* iz Ljubljane in ločenih višinskih pasov na Krimu in Pohorju.

Discussion

Weigh growth of Great Tit nestlings changes with the altitude (Fig 3.) but it seems that this does not happen linearly. The nestlings from middle and lower altitudes have similar growth curve and similar weight at the age of 11 to 15 days. Both indicate similar food availability for the young. Although conditions usually deteriorate with altitude (Hodkinson 2005), lower food abundance in the field, one of the major factors influencing growth (Keller and van Noordwijk 1994), may be offset by the lower competition through lower breeding density (Schmid et al. 1998). This could explain similar results of growing parameters from lower and middle altitude on our study site – there were more nest-boxes occupied on lower as on middle altitude indicating higher breeding density. But this obviously has a limit. The upper altitude in our study site has even lower breeding density (7 compared to 31 nests at middle altitude with the same nest-box availability) but also lower weight growth rate of the nestlings. It seems that presumably larger home-ranges of pairs on the highest altitudes cannot offset lower food availability. Thus hypothesis that higher altitude reduces average nestlings weight can be accepted but only after the effect of altitude can no longer be offset by lower intraspecific competition.

Barba et al. (1993) found that the growth rates differ between populations of Great Tit (Table 1), and this also applies for Slovenian population, which is none the less within the range of other growth rates. What is more, growth rate differs also between habitats (Richner 1989) and climatic zones (Barba et al. 1993). Presented data shows that the same applies also for altitudes. Difference in growth parameters between separate populations probably comes from the difference in ecological conditions in which a particular population lives (Barba et al. 1993). Lower asymptotic weight in Finland population and higher growth constant is probably the response to the shorter breeding period (Veistola et al. 1994). On the other hand reasons affecting higher asymptotic weight in Great Britain population are harder to explain, but more stable and milder climatic conditions may suggest the answer.

Weight is useful for studies of environmental differences between populations as shown higher up, but is less so for determining age of young in the nest. Not only that the weight differs between birds from different climatic zones (Barba et al. 1993), altitudes (*this study*), along pollution gradient (Eeva et al. 2009) and habitats (Richner 1989), it also changes between years (Orell 1983) and differs between sex (due to different asymptotic weight; females are lighter) and the first and the second clutches (Orell 1983). Wing-length on the other hand is less variable parameter (Tome 1995, Tome 2007) and thus wing measurements for ageing young Great Tits in the nest seems a better choice.

Conclusions

Altitude influences weight-grow parameters in Great Tit nestlings, but only on the highest altitudes, while on the middle altitudes deteriorated environmental conditions are possibly offset by lower breeding density and hence lower competition.

Growth rates differ between populations of Great Tit, and this applies also for Slovenian population, which is none the less within the range of other growth rates.

Weight is useful for studies of environmental differences between populations, but wing-length is a better tool for determining the age of young.

Povzetek

Masa mladičev v gnezdu je pomemben dejavnik, ki ima močan vpliv na nadaljnjo življenje osebka. Pogosto imajo mladiči z večjo maso več potomcev. Masa mladičev v gnezdu odseva tudi dostopnost in kvaliteto hrane na gnezdilnem teritoriju staršev. Zaradi tega je rast mase v gnezdu dober pokazatelj stanja v okolju in jo lahko uporabimo za primerjavo med posameznimi populacijami. Namen raziskave je bil zapolniti vrzel v znanju o rasti velike sinice na območju JV Evrope ter o pomenu nadmorske višine, ki je slabo raziskana povsod po JV Evropi. Hkrati bodo rezultati uporabni tudi kot orodje za določanje starosti mladičev velike sinice v gnezdu, ter pomembnih gnezditvenih parametrov, ki iz tega izhajajo. Gnezditvena biologija velike sinice je bila spremljana na treh lokacijah med leti 2010 in 2012. Na dveh, Pohorje in Krim, so bile gnezdilnice postavljene na treh ločenih nadmorskih višinah (32 na spodnji in 40 na srednji in zgornji višini). Meritve mladičev v petih gnezdilnicah v Ljubljani so služile za določitev logistične rastne krivulje dolžine peruti in mase. Krivulja rasti peruti je bila uporabljena za določanje starosti mladičev na Krimu in Pohorju, krivuljo rasti mase smo primerjali s podatki iz Evrope. V Ljubljani smo dnevno merili 27 mladičev, na Krimu in Pohorju pa tedensko skupaj 644 mladičev (369 spodnja, 235 srednja, 40 zgornja višina). Rastna krivulja za maso se razlikuje od krivulj iz ostalih delov Evrope vendar je znotraj njihovih meja. Razlika med rastnimi krivuljami verjetno izhaja iz razlike v ekoloških pogojih, ki vladajo posameznim populacijam. Rast mase mladičev je podobna med spodnjim in srednjim vendar je različna od zgornjega višinskega pasu. Ocenjujem, da sta spodnji višini podobni med seboj zaradi kompenzacije manjše količine dostopnega plena na srednji višini z nižjo gostoto gnezdečih parov. Ta kompenzacija pa je uspešna le do določene nadmorske višine, saj je bila rast mladičev na zgornji nadmorski višini počasnejša kljub majhni gnezditveni gostoti. Masa je dobro uporabno za primerjavo med različnimi ekološkimi pogoji, dolžina peruti pa je boljše orodje za določanje starosti mladičev v gnezdu.

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Nest survival of Great Tit *Parus major* in spatial and temporal gradient

Preživetje gnezd velike sinice *Parus major* v prostorskem in časovnem gradientu

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Abstract: The aim of the study was to compare nest survival of Great Tit between different regions, altitudes and years to discern which has the highest influence. Temperature is known to be prominent for breeding parameters and it changes consistently with altitude. Our hypothesis was that altitude has greater influence on nest survival than the region and year. Breeding parameters were monitored with the use of nest-boxes over a three year period at three altitude belts in two regions. The main factor influencing nesting success was modelled using daily survival rate in the program MARK. We gathered data from 104 first nesting attempts of which 26 failed. Although nest failure increased with altitude this was significant only for one region. There was significant difference in nest failure between the regions but not between the years. Modelling showed higher support in data for regions than for altitudes and years thus rejecting the hypothesis. Some possible reasons for such results are discussed.

Keywords: Great Tit, nest survival, altitude, Slovenia

Izvleček: Namen raziskave je bil primerjati preživetje gnezd velike sinice med različnimi regijami, nadmorskimi višinami in leti ter tako ugotoviti, kateri dejavnik ima največji vpliv. Temperatura ima velik vpliv na gnezditvene parametre in se spreminja konsistentno z nadmorsko višino, zato je postavljena hipoteza, da ima nadmorska višina večji vpliv kot leto in lokacija. Gnezditveni parametri so bili z uporabo gnezdilnic spremljani v treh letih, na treh nadmorskih višinah in dveh lokacijah. Poglavitni dejavnik, ki vpliva na preživetje gnezd, je bil zmodeliran s pomočjo programa MARK. Skupaj je bilo spremljanih 104 prvih gnezd velikih sinic med katerimi jih je 26 propadlo. Čeprav je število propadlih gnezd naraščalo z nadmorsko višino, je bilo to statistično značilno samo na eni lokaciji. Je pa bila statistično značilna razlika v številu propadlih gnezd med obema regijama, ne pa tudi med posameznimi leti. Modeliranje je izkazalo večjo podporo podatkov regijam kot letom in nadmorski višini in tako zavrnilo postavljeno hipotezo. V diskusiji je omenjenih nekaj možnih razlogov za dobljene rezultate .

Ključne besede: Velika sinica, preživetje gnezd, nadmorska višina, Slovenija

Introduction

Nest survival is influenced by many factors such as breeding density (Verhulst 1995 Newton 1998), habitat (Riddington and Gosler 2008), food availability (Newton 1979), predation (Wesołowski 2002), parasites (Eeva et al. 1994), weather (Bionda and Brambilla 2012) and competition for resources (Högstedt 1980, Prokop 2004). It may also depend on nest site (Neal et al. 1993, McCleery et al. 1996, Naef-Daenzer et al. 2001, Strusis-Timmer 2009). Cavity nesting reduces, but does not exclude, threats from predators and weather (Kluijver 1951). In most studies of breeding failure in hole-nesting species, predation was found to be the most important factor (McCleery et al. 1996, Julliard et al. 1997, Naef-Daenzer et al. 2001, Wesołowski 2002), while weather was considered as less important (Kluijver 1951, Wesolowski et al. 2002, Radford and du Plessis 2003).

Great Tit is one of the most studied hole-nesting bird species (Bednekoff et al. 1994, Noordwijk et al. 1995, Cresswell and McCleery 2003, Nussey et al. 2005, Tanner et al. 2008, Eeva et al. 2009). It utilizes variety of habitats, natural or manmade (Gosler and Wilson 1997). It can breed from sea level up to the tree line (Schmid et al. 1998) and from tropics to the edge of tundra (Quader 1995). In Europe Great Tit is one of the birds most associated with studies on the influence of temperature change on breeding (Slagsvold 1976, Sather et al. 2003, Visser et al. 2006, 2009a, 2009b). While there have been some studies considering change of breeding parameters with latitude and across several years (Kluijver 1951, Slagsvold 1976, Sasvári and Orell 1992, Sanz et al. 2000, Sather et al. 2003, Silverin et al. 2008, Visser et al. 2009a) and also considering constraints they are facing at the northern limit of their distribution (Veistola et al. 1995, Rytkönen and Krams 2003), there were only few studies considering change in breeding parameters across elevation. They consider breeding distribution, species richness or altitudinal range of species (Klosius 2008). A few of them consider influence of altitude on breeding parameters (Slagsvold 1976, Krementz and Handford 1984, Belda et al. 1998, Sanz 1998), while none report about the influence on nest survival.

Our aim was to compare nest survival between Great Tits from different altitudes. To put the results into the perspective, we compared them with results of nest survival in different regions and in different years, to found out which of the three variables is more important. Since temperature is known to influence breeding parameters prominently (Neal et al. 1993, Visser et al. 2009b) and since it changes consistently with altitude (Begon et al. 1996) our hypothesis is that the altitude has greater influence on nest survival than does region or year.

Materials and Methods

Data were gathered at two sites in two different regions (Fig. 1) in central and in north-eastern Slovenia from 2010 to 2012. The first site, Mt. Krim (45°55'N, 14°28'E), is an 1107 m high mountain covered with extensive forest dominated by Beech *Fagus sylvatica*, White Fir *Abies alba* and Norway Spruce *Picea abies*. Three separate altitudinal belts 300-400, 600-750 and 900-1150 m a.s.l. were selected at this site. The second site, Mt. Pohorje (46°30'N, 15°34'E), is a 1543 m high mountain dominated by Beech and locally by Norway Spruce and White Fir. Again three altitudinal belts with same altitudinal ranges were selected. Mt. Krim has in general about 30% more rain than Mt. Pohorje and similar temperature (Tab. 1). Bedrock on Mt. Krim is consisted mainly from limestone and on Mt. Pohorje mainly from silicate rocks (Perko and Orožen Adamič 1998).

Great Tit breeding data were collected using 112 nest-boxes with dimensions 23x15x16cm and with an entrance-hole of 32mm. Nest-boxes made from wood-concrete material were placed in a line more than 50 m apart. On Mt. Krim 48 nest-boxes (16 at each altitudinal belt) were installed in 2009 and additional 16 (8 on each of the upper two altitudinal belts) in 2010. In 2010 48 (16 at each altitudinal belt) nest-boxes were installed on Mt. Pohorje. Nest-boxes were checked about once a week from mid-March to the end of June regardless whether they were empty, occupied or deserted in previous inspection. During each visit, occupancy of the nest-boxes was noted and the number of eggs / nestlings counted. To minimize the impact of human inspections nest-boxes were

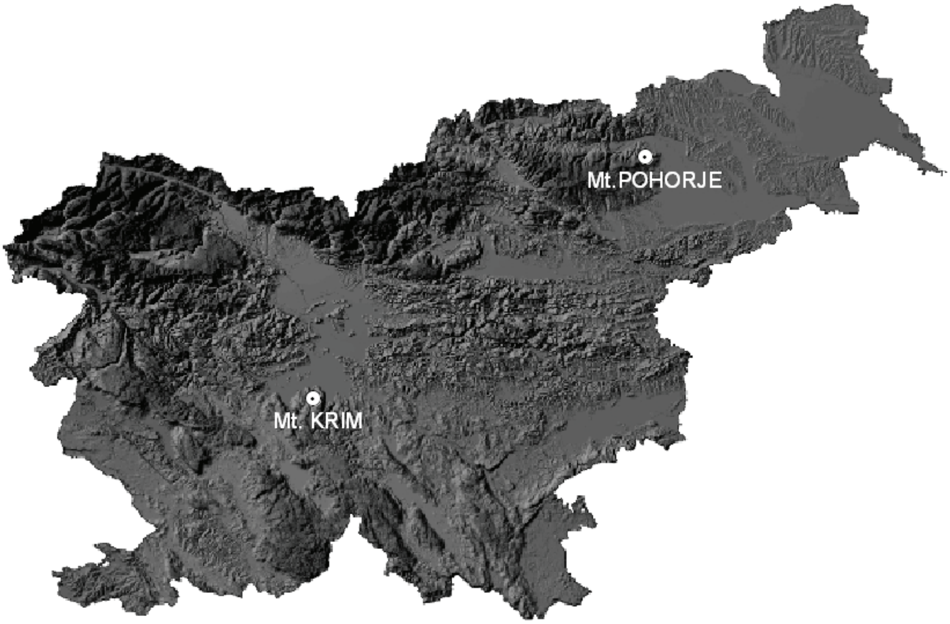

Figure 1: Location of study sites in Slovenia.
Slika 1: Lokaciji obeh raziskovanih območij v Sloveniji

checked quickly on dry, warm days only (Kania 1989, Keller and van Noordwijk 1994). Only the first nesting attempts in any year were used in the analysis of sources of variation in nest survival. We regarded as failed all nests where at least one egg was laid, but breeding did not end with any successfully fledged chicks.

Data on cumulative monthly rainfall for April and May (main breeding period for Great Tits in Slovenia; unpublished data) from 2010 to 2012 was obtained from the nearest meteorological station (within radius of 10 km from the study site) operated by the Environmental Agency of the Republic of Slovenia (for Mt. Krim: Pokojišče and Črna vas; for Mt. Pohorje: Maribor-Tezno). Temperatures were measured using temperature loggers (LogTag Trix-8 Temperature recorder, accuracy ±0.5°C) on both study sites on all altitudinal belts. From four daily measurements (at noon and then every six hours) average temperature was calculated for both regions.

Table 1: The average amount of rain and the average ambient temperatures in April and May 2010–2012 at two study regions.
Tabela 1: Povprečna količina dežja in povprečna temperatura okolja v Aprilu in Maju obdobja 2010–2012 na dveh lokacijah.

	Mt. Pohorje	**Mt. Krim**
Precipitation (mm)	151	233
Temperature (°C)	11.3	11.1

The program MARK was used to model the daily survival rate and to estimate true nest survival (equivalent for nest success) of the Great Tit nests (White and Burnham 1999, Rotella et al. 2004). The program is sensitive to date of nest failure – nests failed at the beginning of the breeding cycle reduce nest survival rate more than nests failed at the end of breeding cycle although none of them produced offspring at the end. Due to weekly nest inspections, nest failure was established with an accuracy of 7 days. Seven models (all possible additive combinations) were carried out from three sources of variance: 1) Region (R), 2) Altitude (A) and 3) Year (Y). Akaike weights (w_i) were used to select the model with the best support in the data, to find out which of the three variables influence nest failure more.

Chi square test was used for comparison of proportion of failed nests among altitudes, regions and years.

Results

Altogether 104 first nesting attempts of Great Tits were monitored, of which 26 failed (25%). Among years, regions and altitudes the percentage of failed nests was highest in 2010, on Mt. Krim and on upper belt (Tab. 2), but the difference was significant only for regions and for altitudes on Mt. Pohorje (Chi square; Region: $\chi^2_1 = 4.04$, $P < 0.05$; Year $_{\mathrm{Mt.Krim}}$: $\chi^2_2 = 3.04$, $P = 0.22$; Year $_{\mathrm{Mt.Pohorje}}$: $\chi^2_2 = 3.63$, $P = 0.16$; Altitude $_{\mathrm{Mt.Krim}}$: $\chi^2_2 = 2.21$, $P = 0.33$; Altitude $_{\mathrm{Mt.Pohorje}}$: $\chi^2_2 = 4.04$, $P < 0.05$).

Assuming constant survival for the Great Tit the daily nest survival was 0.989 and an estimated true nest survival rate was 0.706. Model with Region (R) as a source of variance had more support in the data than models with altitude (A) and year (Y) (Table 3). Also when considering all models that included any source of data, region had again more support (75.7%) than altitude (35.4%) and year (32.2%). Thus among the three sources of variance region seems to explain more variance in nest survival than the other two.

Table 2: Percentage of failed nests on different altitudes, years and in regions, with the number of nests in parenthesis.

Tabela 2: Delež propadlih gnezd po različnih nadmorskih višinah, letih in na lokacijah s prikazanim številom gnezd v oklepaju.

	Year	**Upper (%)**	**Middle (%)**	**Lower (%)**	**Total (%)**
Mt. Krim	2010	/	20 (5)	55 (11)	44 (16)
	2011	0 (1)	33 (9)	8 (12)	18 (22)
	2012	75 (4)	22 (9)	31 (16)	34 (29)
	All	60 (5)	26 (23)	31 (39)	31 (67)
Mt. Pohorje	2010	100 (1)	50 (2)	0 (4)	29 (7)
	2011	/	33 (6)	9 (11)	18 (17)
	2012	0 (1)	0 (2)	0 (10)	0 (13)
	All	50 (2)	30 (10)	4 (25)	14 (37)
Total		57 (7)	27 (33)	20 (64)	25 (104)

Table 3: The model selection for Great Tit *Parus major* nest survival. Models in bold with $\Delta\mathrm{AIC} \leq 2$ have the greatest support in data (Burnham and Anderson 2002).

Tabela 3: Izbor modelov uspešnosti gnezd velike sinice *Parus major*. Poudarjeni modeli imajo $\Delta\mathrm{AIC} \leq 2$ in imajo največjo podporo v podatkih (Burnham in Anderson 2002).

Model	**K**	**AICc**	**ΔAIC**	**Model likelihood**	**wi**	**w1/wj**
S(.R)	**2**	**173.6690**	**0.0000**	**1.0000**	**0.35294**	**1.00**
S(.R+A)	**3**	**175.1394**	**1.4704**	**0.4794**	**0.16920**	**2.09**
S(.R+Y)	**4**	**175.3752**	**1.7062**	**0.4261**	**0.15039**	**2.35**
S(.)	1	176.3623	2.6933	0.2601	0.09180	3.84
S(.R+A+Y)	5	176.5387	2.8697	0.2381	0.08405	4.20
S(.A)	2	177.0899	3.4209	0.1808	0.06381	5.53
S(.Y)	3	177.5583	3.8893	0.1430	0.05048	6.99
S(.A+Y)	4	178.1627	4.4937	0.1057	0.03732	9.46

R = region, A =altitude, Y = year, S. = basic model

Discussion

We confirmed significant influence of altitude on nest failure of Great Tit nest only in one of the two studied regions. While in both regions percentage of failed nests, increased with altitude, with greatest percentage just on the upper altitude (Table 2). Both indicate that altitude may influence nest success but this may be true only locally or it may be obscured by some other factors. Later is supported by significantly different proportion of failed nests between regions and our modelling results. These show that the effect of altitude was less important compared to the effect of region (Table 3), thus rejecting proposed hypothesis. Overall habitat on both regions are similar, with similar human presence factor (Perko and Orožen Adamič 1998) and ambient temperature (Table 1), but differs in number and density of some predator / competitor species (Kryštufek 1991, Mihelič et al. 2000, unpublished data), which could influence different predation or abandonment rate, in bedrock (Perko and Orožen Adamič 1998), which could influence reproduction through different calcium availability (Tilgar et al. 2002) and in rainfall (Table 1).

Predation is the most important reason for nest failure in birds (Wesolowski et al. 2002). Although there are no known predators specialised on Great Tits on either locality, some influence of bird and mammal predator generalists could be expected over apparent competition (Holt 1977). Apparent competitors (i.e. small mammals) can fluctuate greatly between years (Kryštufek and Flajšman 2007) and can support high population of predators which can increase predation pressure on alternative prey in years of low dominant prey population (Schmidt et al. 2008, Sotenšek 2012). But if this would be the case, we would expect year models to support the data better. Rather they have the lowest support in data of all three parameters.

We also argue that the storage of calcium in the bedrock is less likely the candidate for different proportion of failed nests between two regions, since other breeding parameters, such as breeding density and clutch size were virtually the same among them. Average percentage of all occupied nest-boxes on Mt. Pohorje was 31.3% and 31.7% on Mt. Krim, with average clutch size in both regions being 8.9.

Rain and temperature fluctuate through the season and both can have high influence on breeding success of open nesters (McDonald et al. 2004, Fairhurst and Bechard 2005, Denac 2006, Bionda and Brambilla 2012). Although it generally has lower effect on hole nesters (Wesolowski et al. 2002), in the absence of predation it is still the most important external cause of nest failure (Kluijver 1951, Wesolowski et al. 2002). The cause of weather related failure is a trade-off in energy allocation between incubation / brooding and feeding in adult birds. Bad weather increases on-bout time (Keller and van Noordwijk 1994, Radford et al. 2001), but also increases energetic expenditure, which can almost double during cold spells (Haftorn and Reinertsen 1985). Prey is also less visible or accessible in bad weather conditions, which prolongs feeding time (Keller and van Noordwijk 1994, Pasinelli 2001, Avery and Krebs 2008). Assumption that the rainfall, compared to the temperature, is more important cause of nest failure on breeding success of the Great Tits is supported by the fact that region in our study had greater influence than altitude and that there is about 30% more rain on Mt. Krim than on Mt. Pohorje, while at the same time the difference in average temperature between regions is minimal. So although altitude indeed had some influence on survival of Great Tit nests, probably indirectly through the availability of food, region can have even greater when sites lie in regions with markedly different precipitation regimes.

Povzetek

Razmere v okolju so povezane z geografskimi danostmi območij in tako določajo razširjenost in gnezditvene parametre ptic. Pri veliki sinici je bilo narejenih nekaj raziskav o razlikah v gnezditvenih parametrih po geografski širini in med različnimi leti, zelo malo pa je bilo narejenega na področju vpliva nadmorske višine. Namen raziskave je bil primerjati delež preživetja gnezd velike sinice med lokacijami, nadmorskimi višinami in leti ter ugotoviti, kateri od teh dejavnikov ima večji vpliv. Temperatura ima velik vpliv na gnezdenje ptic, zato smo postavili hipotezo, da ima nadmorska višina večji vpliv na preživetje gnezd kot regija ali pa leto. Podatki o gnezdenju so bili s pomočjo 112 gnezdilnic na treh višinskih pasovih zbrani na dveh lokacijah, Krim in Pohorje, med leti 2010 in 2012. S programom MARK je bila izračunana dnevna stopnja preživetja gnezd in izdelanih sedem modelov (vse aditivne kombinacije parametrov lokacija, nadmorska višina in leto). Šest vrst ptic je skupaj zasedlo 49.5% gnezdilnic. Od 104 prvih legel velike sinice jih je 26 propadlo. Največ jih je propadlo v letu 2010, na Krimu in na zgornji višini, vendar je bila razlika statistično značilna samo pri razlikah med lokacijami in med nadmorskimi višinami na Pohorju. Dnevno preživetje gnezd je bilo 0.989, gnezditveni uspeh pa 0.706. Na obeh lokacijah je delež propadlih gnezd naraščal z višino z največjim deležem na zgornji višini, kar kaže, da nadmorska višina verjetno ima vpliv na preživetje gnezd. Predpostavljamo, da vpliv deluje posredno preko razlik v temperaturi, ki se med višinami značilno razlikujejo. Po drugi strani rezultati modela kažejo, da je bil vpliv nadmorske višine manj pomemben od vpliva lokacije, s čimer je bila postavljena hipoteza zavrnjena. Ocenjujemo, da razlika med lokacijama verjetno ni bila posledica možnih razlik v sestavi plenilcev in kompetitiorjev, ki bi lahko vplivali na propad gnezd, niti v razliki količine plena, temveč v količini padavin. Krim je v času raziskave prejel okoli 30% več padavin kakor Pohorje.

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Development of the concept of cell division through biology education

Razvoj koncepta celične delitve skozi biološko izobraževanje

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Abstract: School practice shows that a cell and the related cell division represent many problems for students as well as teachers. Students often hold misconceptions which form in everyday life under the influence of people with whom students live, as well as the media. Teachers are confronted with the problem of how to most effectively introduce this subject in class. They should enable students to understand basics of the cell and its implications as part of scientific literacy in the contemporary world. This survey used a test to assess the knowledge of students about cells. It included students on three levels of education; 13 year-olds, 15 year-olds, and 21 year-olds. The results showed that the knowledge of both younger groups was insufficient. The knowledge of the oldest group was substantially better, but still not satisfactory. It also showed that many students learn isolated facts and do not see the relationships between those facts. We believe that active methods of work in the classroom which are taken from everyday life could improve these results.

Key words: cell, cell division, genetics, education

Izvleček: Šolska praksa kaže, da predstavljata celica in z njo povezana celična delitev veliko težav učencem in tudi učiteljem. Učenci imajo pogosto napačne predstave, ki so se izoblikovale v vsakdanjem življenju pod vplivom ljudi, med katerimi učenci živijo, in medijev. Učitelji se srečujejo s problemom, kako tako vsebino predstaviti na čim bolj razumljiv način, saj morajo učenci razumeti temeljno znanje o celici in njegove implikacije kot del naravoslovne pismenosti v sodobnem svetu. V raziskavi smo z metodo testiranja preverili znanje učencev o izbranih temah s področja celice. Vključenih je bilo 171 učencev treh stopenj izobraževanja: 13-letniki, 15-letniki in 21-letniki. Rezultati so pokazali, da je znanje o celici pri obeh mlajših skupinah skromno, znanje najstarejše skupine pa sicer bistveno boljše, vendar še vedno nezadovoljivo. Opaziti je, da se mnogi učenci učijo naravoslovje kot izolirane podatke in ne uvidijo medsebojnih odnosov, ki veljajo v naravi in svetu okoli njih. Domnevamo, da bi bilo to mogoče izboljšati z metodami dela, ki miselno aktivirajo učence in so povezane z vsakdanjim življenjem.

Ključne besede: celica, delitev celice, genetika, izobraževanje

Introduction

Understanding the basic concepts of biology is essential for the efficient scientific literacy of citizens (Venville et al. 2005) in the modern world. Therefore, various institutions around the world engaged in the renovation of teaching biology. They all strive to enable students to change their misconceptions and achieve high levels of expertise, which include understanding and the ability to apply acquired knowledge. Cell and the related cell division represent, as we know from school practice, many problems for students as well as teachers. For successful teaching we need teachers who are competent in the field of biology and possess educational skills. However, research has shown that prospective teachers of biology possess specific knowledge deficits (Dikmenli 2010, Šorgo and Ambrožič-Dolinšek 2009). Teachers tend to follow the traditional methods of teaching and the traditional sequence of learning content, and they use similar learning strategies (Watts and Jofili, 1998). Textbooks can be an important obstacle in learning biology as well. As showed the analysis by Knippels et al. (2005) biology textbooks failed to start on a phenomenon level and gradually descend to the lower levels. Besides, the conceptual relationships between interrelated chapters were not made explicit.

In accordance with the old biology curriculum (Verčkovnik et al. 2003) that was in use at the time of our study, students in Slovenia began to learn in detail the structure of cells and cell division at the age of 14. In 2011 a new curriculum came into force which requires that in school year 2012–2013 cell structure is taught to students three years younger, i.e. to 11 year-olds, and cell division to students one year younger, i.e. to 13 year-olds.

Recent research by Tomažič and Vidic (2011) showed that Slovenian students hold some misconceptions about the function of a cell. In our survey we wanted to establish what conceptions of the two scientific phenomena – the cell structure and the process of mitosis – are held by our students, and whether students have any false or alternative ideas about these topics. There has been a long debate in the science education literature about the extent to which the various conceptions held by students of scientific phenomena are to be seen as misconceptions or alternative conceptions. With rare exceptions, it is generally held that there is a single valid scientific conception so that alternative conceptions are misconceptions (Reiss et al. 2007).

Misconceptions serve people quite well. For example, they help reduce the burden of processing information. Nor do misconceptions necessarily hinder the implementation of various practical tasks. For example, a person can have false notions about how objects move, but that person is nonetheless able to catch a ball (Fisher, 1985). The problem with misconceptions is adjusting them to newer and correct notions. According to Lewis and Kattmann (Castro 2009), students, even when they use proper biological vocabulary, may not use the expressions correctly. Students get lost in technical terminology; they do not connect the expressions and therefore cannot fully grasp the mechanisms behind the processes.

Students have problems understanding and connecting biological knowledge (Castro 2009, Lewis and Kattmann 2004, Locke and McDermid 2005, Mbajiorgu et al. 2007, Venville and Treagust, 1998, Venville et al. 2005, Williams et al. 2012). Many students learn science topics as isolated facts and do not construct links between old and new knowledge. As a consequence they find it difficult to understand subsequent topics (Novak, 1988). BouJaoude (as cited in Cavallo, 1996) even stated, that students develop misconceptions about science if they strictly learn by memorization. Similar findings for Slovenian primary and secondary levels of education are given by Fošnarič et al. (2009), Jagodnik et al. (2009), Japelj Pavešić et al. (2012), and Štraus et al. (2007). Learning by memorization can make further science learning increasingly difficult and may deter many students from science in general (Novak, 1988).

Teachers must therefore strive to better understand how to improve students' understanding and their inferential skills, which will in turn lead to higher-quality knowledge.

Purpose of the Study

The purpose of our study was to assess the knowledge of cells and of cell division in students of three different age groups in Slovenia. In particular, we wanted to find out whether students have any misconceptions about the cellular structure of organisms and cell division, and what these were. It was hypothesized that: (1) the knowledge and understanding of basic concepts concerning cells and cell division increases with the age of students in our sample; and that (2) students finishing general elementary education do not possess useful knowledge concerning cells and cell division, as stated in our national curriculum (Verčkovnik et al. 2003).

Material and methods

Participants

In Slovenia, elementary school provides education from grades 1 to 9. The pupils are generally aged between 6 and 14. Our survey included 171 students at three levels of education; 13 year-olds, 15 year-olds, and 21 year-olds (Tab. 1). Such a sample was chosen in order to get a wider range of possible responses, and thus make it easier to evaluate how age and the increasing level of education affect development and deeper understanding of the concept of cellular structure of all living beings, and of cell division.

Table 1: Students involved in the survey.
Tabela 1: Učenci, vključeni v raziskavo.

Students			
Age	**Level of education in Slovenian school system**	**N**	**%**
13 years	8th grade of elementary school	58	34
15 years	1st year of high school	87	51
21 years	3rd year biology students (university)	26	15
Total		171	100

Knowledge test

The research method was quasi-experimental, which means that the participants in existing groups were compared by age or by level of education. As part of their regular classes the students completed a test with the following six questions:

1. Draw a cell and name its parts.
2. Which organisms are built from cells? (Multiple correct answers possible.)
   a. Bacteria
   b. Bee
   c. Human
   d. Oak
   e. Fungus
3. How do cells multiply? Draw a picture.
4. In which organisms do cells multiply? (Multiple correct answers possible.)
   a. In bacteria
   b. In humans
   c. In fungi
   d. In plants
   e. In animals
5. Why do cells in your body multiply?
6. When you cut your skin it eventually heals. Describe the process of healing.

The first four questions were given to all three groups of students, while the last two questions were only given to the 15 year-olds.

Students were first given three questions, in which we asked about basic knowledge that was explicitly stated in operational objectives in the curriculum for 14 year-olds (9th grade of elementary school in Slovenian school system) (Verčkovnik et al. 2003): students get to know the microscopic structure of a cell, students are able to draw a picture of a cell, and students get to know cell division. Than we asked them three additional questions in which they were expected to use basic knowledge and apply it in given situations. Cell structure and cell division are topics that were a part of the Slovenian curriculum (Verčkovnik et al. 2003) first covered in the 9th grade (14 year-olds), so we expected that the youngest group in our sample i.e. eighth-graders (13 year-olds) wouldn't know it very well. The wound healing process (question 6) was not discussed in regular classes in any of the groups of students in our study, so we expected that this subject would not be well mastered. What we wanted to know was what naive notions on wound healing students have and whether they were able to integrate knowledge about cells and cell division with wound healing.

Data analysis

The data was analysed with the SPSS 20 statistical program. The statistical significance of the differences between the responses of the three age groups was assessed by the Kruskal-Wallis test. The Jonckheere-Terpstra test was used to establish whether there is a significant trend in the data regarding the age of students. The effect size estimate r was also calculated.

Results and discussion

Results to two questions where students were asked to draw a picture (Question 1: Draw a cell and name its parts., Question 3: How do cells multiply? Draw a picture.) are based on a qualitative analysis, while others are based on a quantitative analysis.

Draw a cell and name its parts

The results we obtained clearly show that 13 year-olds only know the basic cell structure. More than half of them did not even answer the question and the drawings of cells by those who did were mostly simple. Next to the drawings they often indicated the parts they knew, which were the nucleus and the "envelope" or "membrane." Drawings of cells by 15 year-olds were at first sight not very different from those of 13 year-olds. It is interesting that most of them drew animal cells, while some 13 year-olds also drew plant cells. Another survey (Yorek, Sahin, and Ugulu 2010) conducted on students from 11 to 17 years old came to similar conclusion: An increase was observed in the proportion of students who drew an animal cell as the grade level increased, along with a decrease in the proportion of the students who drew a plant cell. The cells drawn by the 15 year-olds in our study almost always included the nucleus which was also indicated, as well as some other components. All this and the fact that almost all the students answered the question suggest that 15 year-olds have a better command of this subject than the 13 year-olds. Drawings of 13- and 15-year-olds represent the cell as two concentric circles, representing the cell membrane and the nucleus, representation for which Clément (2007) introduced the term the "fried-egg model." This representation is lost in 21 year-olds, mainly due to a larger amount of other organelles with which they are familiar. The group of 21 year-olds showed the best knowledge of cell structure, as expected. They all answered the question, giving a drawing as well as a written response. Their drawings are very detailed, correct, and contain much more information than the drawings of the 15 year-olds. These results are similar to those reported by Saka et al. (2006).

Which organisms are built from cells?

We found statistically significant differences among students of different ages regarding knowledge of oak, bees, and fungi (Kruskal-Wallis test; $p < 0.05$), while not of humans and bacteria (Kruskal-Wallis test; $p > 0.05$) (Fig. 1). The Jonckheere-Terpstra test revealed a significant trend in the data for oak, bees, and fungi: students in higher grades had more knowledge than students in lower grades ($p < 0.001$, $r = 0.29–0.44$).

We found very poor results for bacteria. However, we believe that this result does not reflect a genuine lack of knowledge but is due to a large proportion of students' literal understanding of the question that was stated in plural form (Which organisms are built from *cells*?). We therefore speculate that students actually showed knowledge here, as most of them answered that a single bacteria is not made of *cells*. Their responses therefore stress that bacteria is not a multicellular organism. This part of the survey should be repeated with a differently formulated question such as: "In which living beings is the cell the basic building unit?" to help us confirm or reject our assumption.

We established that 21 year-olds comprehend the concept of the cellular structure of all living beings, since almost all gave correct answers (96–100%). The lowest scores were achieved by the youngest group (13 year-olds), as was expected. This means that they have the lowest mastery of the concept of the cellular structure of living beings. However, to our surprise 13 year-olds answered

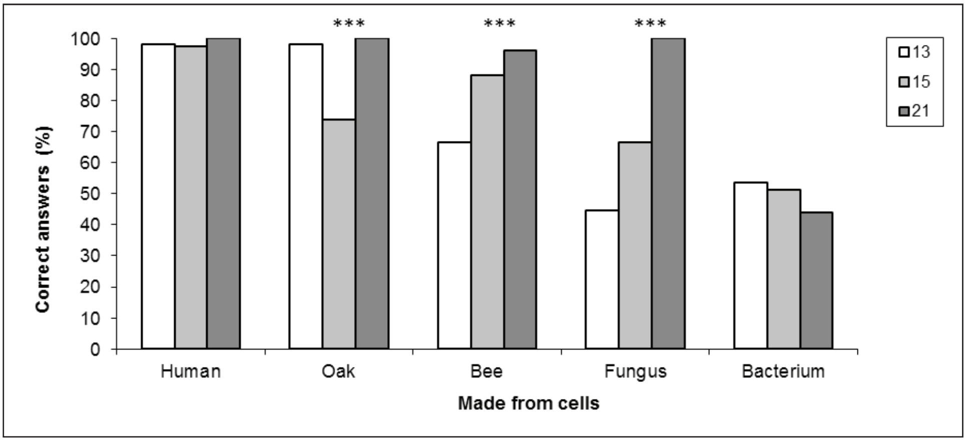

Figure 1: Percentages of 13 year-olds, 15 year-olds, and 21 year-olds who correctly answered the question: Which organisms are built from cells? (N = 171). Statistically significant differences, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Slika 1: Deleži 13-, 15- in 21-letnikov, ki so pravilno odgovorili na vprašanje: Kateri organizmi so zgrajeni iz celic? (N = 171). Statistično značilne razlike * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

much better than 15 year-olds, and almost as correct as 21 year-olds concerning the oak. This could be explained by the fact that in the time of our survey 13 year-olds were learning about the systematics of plants, therefore this topic was fresh and more familiar to them than it was to 15 year-olds. Low scores of 15 year-olds concerning oak can be explained by the fact, that more than a year had elapsed since they had learned this topic, so they may have forgotten it. The fact that 88% of 15 year-olds remembered that animals are made of cells, while only 74% think that plants are made of cells, and only 67% think that fungi are made of cells is probably due to the higher attractiveness of animals (Kinchin, 1999, Strgar 2007, Wandersee and Schussler 2001).

Our results are very similar to those reported by Banet and Ayuso (2000). 20–25% of secondary school students in their survey thought that plants are not made of cells, and 30% thought that fungi are not made of cells.

How do cells multiply? Draw a picture.

13 year-old students made very simple cell division drawings, some of them suggesting they knew the cell forms a cleavage furrow; some also drew nuclei, but the responses very rarely accompanied the picture. This is not surprising since this subject was not yet covered in school by 13 year-olds in our sample. In the 15 year-olds' drawings a qualitative conceptual leap was noticeable; it is clear that the students knew the basics of cell division, and they almost always drew the nucleus. Some even drew the chromosomes or the mitotic spindle. They drew both animal and plant cells. The results for 21 year-olds have shown that their conceptual understanding of cell division is relatively weak. They understand the basics of cell division, but surprisingly, there were no significant conceptual differences between their drawings and the drawings of 15 year-old students. These findings overlap with the findings of the study conducted by Dikmenli (2010) on a sample of 22 year-old, pre-service biology teachers. The drawings of 21 year-olds in our sample illustrated plant and animal cells, in part drawn simply and in part very precise. The drawings were mostly accompanied by text further explaining the processes.

In which organisms do cells multiply?

The results (Fig. 2) for all five groups of living beings show a comparatively similar picture regarding the notions of students at different age levels. Understanding that cells in humans, plants, and animals divide is poorest with 13 year-olds and then improves with student age. However, even in the oldest group (21 year-olds) not everybody answered correctly. 13 year-olds associate cell division with animals, fungi, and particularly with bacteria, but not as much with humans and plants. 15 year-olds and 21 year-olds associate cell division mainly with humans, plants, and animals, and less with bacteria and fungi. Misconceptions concerning plants were already found by Wood-Robinson (1994), who stated that students of different ages in different parts of the world often thought that plants do not reproduce sexually. Our results seem logical, taking into consideration that students at the secondary level of education are expected to be able to identify living beings and to understand their cellular structure at least at the elementary level before they can start to learn cell division and genetics (Banet and Ayuso 2000).

The best knowledge of all the organisms was shown by the oldest group (21 year-olds). However, statistically significant differences between different age groups of students were found for all groups of organisms (Kruskal-Wallis test; $p < 0.05$), except for bacteria (Kruskal-Wallis test; $p > 0.05$). The Jonckheere-Terpstra test revealed a significant trend in the data for humans, plants, animals, and fungi: students in higher grades had more knowledge than students in lower grades ($p < 0.001$, $r = 0.29–0.41$). This was expected, because cell division was first taught in school to 14 year-olds (Verčkovnik et al. 2003). The results of 15 year-olds therefore show that their knowledge about humans and plants had improved by almost 30% compared to 13- year-olds, and their knowledge about animals had improved by almost 20%.

To our surprise 15 year-olds were the ones who showed the least understanding of the fact that fungi and bacteria undergo cell division. This could be explained by possible development of alternative concepts, as year by year students gain new information. Also, as knowledge gets more complicated, and students forget some of the previously learned knowledge and may develop alternative concepts (Saka et al. 2006).

We observed obvious disparity of responses to the second question asking which living beings are built from cells and the fourth question asking in which organisms cells multiply (Fig. 3). This discrepancy indicates the troubling fact that students did not logically connect the individual learning sets. This means their knowledge remained fragmented and therefore not well understood. It may also be the case that students simultaneously have two different notions on the same subject, and the one they use depends on the situation in

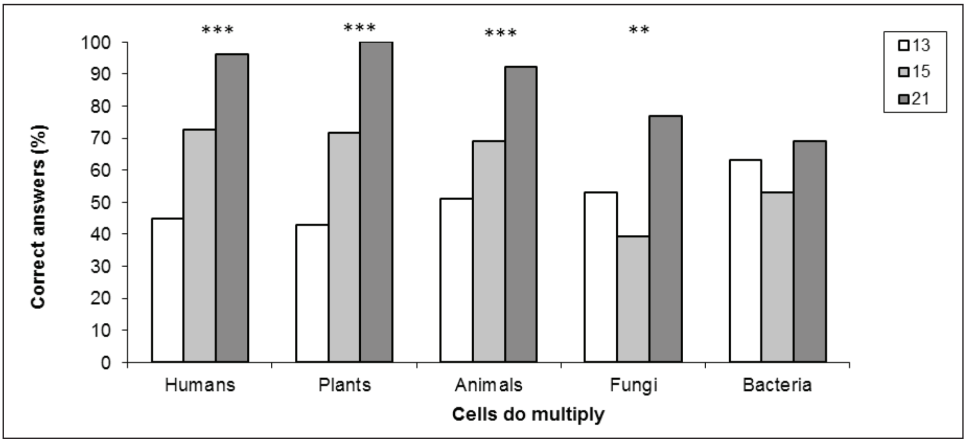

Figure 2: Percentages of 13 year-olds, 15 year-olds, and 21year-olds who correctly answered the question: In which organisms do cells multiply? (N = 171). Statistically significant differences, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Slika 2: Deleži 13-, 15- in 21-letnikov, ki so pravilno odgovorili na vprašanje: V katerih organizmih se celice delijo? (N = 171). Statistično značilne razlike * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

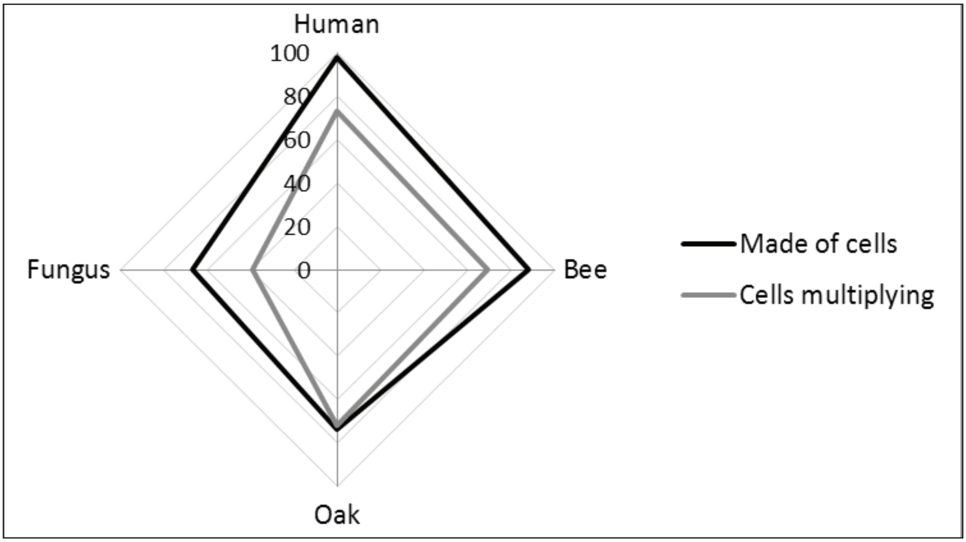

Figure 3: Percentages of 15 year-olds who know that the four given organisms are made of cells and percentages of 15 year-olds know that cells in these organisms multiply (N = 87).

Slika 3: Deleži 15-letnikov, ki vedo, da so štirje dani organizmi zgrajeni iz celic, in deleži 15-letnikov, ki vedo, da se celice v teh organizmih delijo (N = 87).

which they find themselves, as some research has also shown (Venville and Treagust, 1998).

Why do cells in your body multiply?

This question was only answered by 15 year-old students. Almost three quarters (72.7%) did not even attempt to answer, which lead to a conclusion that this subject is unfamiliar to them (Table 2). The percentage of correct answers was only 10.1%, for example “we grow,” “the body regenerates.” These responses were therefore correct, but not complete, as almost no one gave more than one reason for cell division.

Such results are not surprising since cell division was discussed in the 9th grade, but the emphasis was on the process and terminology, not on its purpose. In question 3, where students had to draw cell division, we found a satisfactory knowledge of process basics, but poor answers to question 5 indicate that a higher level of knowledge was not achieved. If students at lower levels of education learn these basic concepts meaningfully, they will build upon it successfully in their future education. It is hard to expect that students with a limited understanding of the basic concepts could develop an understanding of the more advanced topics (Saka et al. 2006).

Table 2: Responses of 15 year-olds to the question: Why do cells in your body multiply? (N = 87).

Tabela 2: Odgovori učencev na vprašanje: Čemu se celice v tvojem telesu delijo? (N = 87).

Answer	Number of answers (%)
Correct	10.1
Semi-correct	2.0
Incorrect	15.2
No answer	72.7

When you cut your skin it eventually heals. Describe the process of healing.

This question was only answered by the 15 year-olds. More than half (53.5%) did not even attempt to answer, while a quarter (25.3%) answered incorrectly. In semi-correct answers (12.1%) students did not explicitly link cell division with wound healing, but they noted, for instance:

"The skin is regenerating and with that the wound cicatrizes." "The skin cicatrizes." "The cells are cicatrizing over the wound."

A correct answer was given only by 9.1% of students, in terms of "the skin regenerates – skin cells multiply." We can therefore see that only a small part of students who finished elementary school (15 year-olds) have at least a basic understanding of wound healing, even though they encounter it almost daily. This is linked to the lack of fundamental knowledge, as some of the students don't know that all organisms are made of cells (Fig. 1 and Fig. 3). Even greater is the proportion of students who don't know that cells divide in all organisms (Fig. 2 and Fig. 3). The finding that students don't have sufficient fundamental knowledge of the cell, which is an obstacle for further work, was also established by Banet and Ayuso (2000).

Conclusions

All groups of students in our sample, regardless of the level of education, have some surprising misconceptions about individual living beings. The worrisome fact is that too many students do not possess basic biological knowledge (as is the concept of cellular structure of all living beings) when they finish elementary school. This is of special concern since many of these students will never learn biology again in their further education.

We accepted the first hypothesis saying that the knowledge and understanding of basic concepts concerning the cell and cell division increases with the age of students in our sample, since we found that the results of 21 year-olds were significantly better than the results of 13 year-olds and 15 year-olds. However, a too large percentage of students even at the university level failed to make a complete shift to a better understanding of the cell. This is particularly worrisome because those were third-year pre-service biology teachers, i.e. people who will, in a few years, teach this subject in elementary or secondary school.

We also accepted the second hypothesis saying that students finishing general elementary education do not possess useful knowledge regarding the cell and cell division, as stated in our national curriculum. We found that students finishing elementary school in Slovenia (15 year-olds) do not understand the basic mechanism of wound healing. This is linked to a lack of meaningful fundamental knowledge – a significant number of students don't know that all organisms are made of cells or that the cells divide in all organisms.

In Slovenia, we are now in the running-in period of the new curriculum for the elementary school level, and we are looking for solutions that would enable the general population to understand the basics of biology. Teachers are faced with the problem of: (1) how to present biological content in the most comprehensive manner; and (2) how to provide what students will need for everyday life, as well as a solid foundation for any further education.

We believe that a biology program for elementary schools should originate from the direct experience of students and, where possible, include useful knowledge for everyday life. This would make it more interesting for the students. Teachers should also be aware of the misconceptions that are widespread among students.

The results of our survey will serve as the basis to build a consistent sequence of teaching this topic. This will allow students to learn with understanding, which is an essential foundation for the acquisition of useful knowledge.

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Educational outcomes from summer camps on conservation of freshwater ecosystems

Vzgojno-izobraževalni učinki poletnih taborov o ohranjanju ekosistemov celinskih voda

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Abstract: The purpose of this paper is to analyze the educational outcomes of different activities organized through summer camps concerning the conservation of freshwater ecosystems. The six-day duration camps were organised in three different locations across Slovenia. The camps were attended by 15 males and 27 females, aged 11 to 18 years. Educational outcomes were measured on the fifth and sixth day of each camp, using a combination of qualitative and quantitative methods. Results showed that participants greatly enjoyed attending the camps. The respondents reported that they learned much about freshwater ecosystems, fauna and flora, different research methods and handling research equipment. They also showed a high awareness and understanding of problems related to freshwater ecosystem conservation.

Keywords: outdoor education, camp, adolescents, awareness raising, freshwater ecosystem

Izvleček: Namen prispevka je analizirati rezultate vzgojno-izobraževalnih aktivnosti, ki so potekale na poletnih taborih o ohranjanju ekosistemov celinskih voda. Na treh lokacijah v Sloveniji so bili organizirani šestdnevni tabori. Taborov se je skupaj udeležilo 15 fantov in 27 deklet, starih med 11 in 18 let. Izobraževalni rezultati so bili merjeni kvalitativno in kvantitativno. Rezultati kažejo, da so udeleženci na taborih uživali. Menijo, da so se veliko naučili o ekosistemih celinskih voda, živalih in rastlinah, ter o različnih raziskovalnih metodah in opremi. Kažejo tudi visoko ozaveščenost in razumevanje problematike ohranjanja ekosistemov celinskih voda.

Ključne besede: šola v naravi, tabor, adolescenti, ozaveščanje, ekosistemi celinskih voda

Introduction

Freshwater ecosystems, particularly wetlands, are the victims of human activities that have led to widespread habitat degradation, flow regulation, pollution, and water extraction, fisheries overexploitation, and alien species introductions (Strayer and Dudgeon 2010). National and international initiatives (e.g. Ramsar Convention, Conservation International) pay much attention to education and raising awareness about the importance of freshwater ecosystem conservation, but whether this will bring the necessary change is a key issue.

Kellert (1996) believes that education plays a crucial role in creating environmentally conscious citizens. Slovenian school curricula devote attention to freshwater ecology and freshwater ecosystem conservation, particularly in the last years of primary and in secondary schools.

One of the key questions is whether information is the locked door that prevents us, as a society, to change our behavior towards freshwater ecosystems. Knowing about something does not necessarily mean caring or conserving it. Educational programs that simply provide information often do not lead to the changes hoped for, except where the lack of information is a significant barrier to anticipated behaviour (Schultz 2002).

Environmental education has the potential to affect a wide range of individuals and provides an opportunity to promote pro-environmental behaviour. It focuses on people’s abilities to increase their understanding over the long term, affecting their attitudes, behaviour and worldviews in general (Clayton and Myers 2009). Authors emphasise that success of environmental education depends particularly on cognitive development and environmental knowledge (with special attention to knowledge of biology and ecology), affective and motivational factors (especially through connection with nature and feelings about one’s self ability to achieve effects in the world), and actual behaviour (participating, taking action and problem-solving).

Becker (2008) emphasized that talking about nature and being in natural surroundings are two different things. He pinpointed the importance of the practical and sensuous approaches to nature and its phenomena. Palmberg and Kuru (2000) found that children participating in different informal education programs such as hiking, adventure trails, field trips and camping, contributed to the development of definable emphatic relationship with nature, knowledge of, and values concerning, nature protection and a development of environmentally responsible actions and skills. Natural environments, nature centres and parks are places where people may develop an increased concern for nature through contact with natural environments and these places provide opportunities for learning about natural systems which may increase the awareness of how nature is threatened by human activity (Kola-Olusanya 2005). According to Kellert (2002) such direct encounters with nature can serve as powerful motivators and stimuli for learning and personal development.

In order for experiences to be individually meaningful, Palmer (2004) suggested that learning experiences must extend beyond just learning something; they should guide people’s perceptions of who they really are and what are they capable of. Orr (1994, p. 6) suggests that “we experience nature mostly through sight, sound, smell touch and taste – through a medley of sensations that play upon us in complex ways”.

The purpose of this study is to discuss educational outcomes from different activities organized on summer camps. The main goal of the camp was to educate youth about fauna and flora in freshwater ecosystems and about the impact of human activities on these ecosystems. Orr (1993, p. 17) wrote that experiential knowing is based on the assumption that “there is no way to separate feeling from knowledge, or object from subject; there is no good way to separate mind and body from its ecological and emotional context”. Putting this into the context of our work we expected that provided outdoor education experiences will have a positive influence on students’ understanding and awareness how freshwater ecosystems work and how are threatened by human activity. We were particularly interested at their research skills and attitudes gained by attending the summer camp.

Methodology

Description of summer camps

During the summer of 2009 three research summer camps for youth were organized at three different locations in Slovenia. Participation was voluntary. As Wang et al. (2004) highlighted, young adolescents are more motivated to participate in outdoor education programmes if they join them voluntarily. They should also be provided with a meaningful rationale for participation, given some autonomy for decision-making and to have enjoyable experience during the programme. Camps were held at Fokovci in Goričko, at Fara near the river Kolpa, and at Rakov Škocjan near the river Rak. Participants and mentors stayed in outdoor centres or local schools where food and accommodation was provided. Each camp was six-days in duration and mentors were present at the camps the entire time.

Every morning participants were divided into groups of four to five persons and each group was led by one mentor. The purpose of the research activities was not to educate the young people solely on one aspect of the freshwater ecosystem, but to provide a broader understanding; therefore, each day a particular group studied a different aspect of the environment and they changed mentors daily. They undertook a study on marsh and other aquatic plants, tracking animals, watching birds, analyzing chemical and ecological conditions of freshwater ecosystems, catching and determining species of amphibians and butterflies. Due to different research goals and methods, each mentor prepared a variety of research activities, but they tried to have the same pedagogical approach. To achieve this, mentors had previously agreed on how to work and every evening throughout the educational program they met and share their experiences and views. Mentors endeavoured to give the participants a clear and short introduction to the research goals and instructions how to use research equipment. Then they tried to "back up" and give participants a chance to explore and research nature on their own. Mentors thus had the role of consultants and facilitators. They pointed out some interesting and specific details or raised questions when this was necessary.

Every afternoon the camps were active with artistic workshops, orientation and sports games, which enriched the afternoons at the camps. A wide selection of workshops and games, attended by all the participants, offered the young people an opportunity to develop and discover their talents and to establish themselves among their peers and mentors. Mentors regularly participated in these activities. The purpose of the games was also to improve group dynamics among the participants; therefore, significant attention was given to these activities during the first few days in order to develop mutual respect among youth and between youth and mentors. It should be noted that much attention and time was devoted to order and mutual respect, which are prerequisites for the safe and efficient completion of the camp. Towards the end of the camp, more autonomy and responsibility for research activities, workshops and games were assumed by the participants themselves. In this context, group dynamics "builds" and develops from the first to the last day of the camp. These workshops were related to the freshwater ecosystems (for example, quizzes, treasure hunting, photographic exhibition...).

The participants spent the evenings in the company of guests, renowned researchers who have presented their own career histories that have led them to work on scientific research. We hosted researchers from various scientific fields such as forestry, nature conservation, ornithology, entomology, botany, physiology and geography together with some artists whose work is very connected to nature, such as photographers. The purpose of inviting renowned researchers was to give participants positive stimulus for their early future decisions about their future career paths.

At each camp we organized a long hike to a location of a natural or cultural heritage site (waterfall, castle, hill). We usually organized it on the fifth or sixth day of the camp when the participants had already had the chance to get to know each other and establish mutual trust and respect. We used the hike to discuss further education and careers, share impressions of the camp but also with some social group activities such as singing.

Participants

The camps were attended by 15 males and 27 females, aged 11 to 18 (*mean age* = 13). Summer camps were organized in June and July 2009. Participants were not selected, but they themselves signed up to attend the camp.

Qualitative and quantitative methods

Educational outcomes were measured on fifth and sixth day of each camp, using quantitative (structured questionnaire) and qualitative (focus groups discussion) methods.

A structured questionnaire was developed based on educational goals. For each statement respondents were asked to score their personal opinion on a scale ranging from strongly disagree (1); disagree (2); cannot say (3); agree (4); strongly agree (5) (Likert, 1932). A prototype of the questionnaire was tested with five 11 to 13-year old girls who, being in the same age class as the participants, were thought to represent a reasonable match with the sample population of participants. The questionnaire was completed on the last day of the 6-days long camp.

An analysis of participants' posters and *focus group discussions* was used to evaluate the camps' activities. On the fifth day each participant made a poster expressing their impressions of the activities that were organized during the camp. When making the posters, students could use images and texts from various magazines, catalogues, calendars and newspapers (all provided), and different coloured pens (Fig. 1). Posters were exhibited and presented in groups of between 7 to 15 participants, followed by a group discussion. This was the starting point for discussion that was developed on the activities that had taken place at the camp. The discussion was moderated by an experienced researcher and tape recorded. Respondents were informed about the use of tape recorder prior the discussion. All the outcomes of camp activities were also presented to their parents, including group discussion and the usage of tape records for research purposes.

Figure 1: An example of a poster.
Slika 1: Primer posterja.

Data analysis

Data entry and analysis of structured questionnaires was conducted using the Statistical Package for the Social Sciences (SPSS). The data were processed at the level of descriptive and inferential statistics. We used basic descriptive statistics of variables, and the Pearson correlation coefficient in order to analyze the relationships between mean scores for participants' self-evaluation of gained knowledge (Tab. 1), opinions about evening guests (Tab. 2), and awareness and understanding about conservation problems in freshwater ecosystems (Tab. 3). Asterisk (*) denotes items that were rotated to compute an average for each instrument. Cronbach's alpha coefficient for participants' opinions about research activities and learning outcomes was 0.87. For participants' opinions about the evening guests Cronbach's alpha was 0.86. Cronbach's alpha for instrument investigating participants' awareness and understanding about conservation problems in freshwater ecosystems was 0.68. Nunnaly (1978) reported, reliability is satisfactory, when alpha is minimally 0.70 and Fraser (1989) reported that alpha coefficients in the range 0.58 – 0.81 indicate that the instrument has satisfactory reliability.

Qualitative data in the form of images and text from posters were analyzed. Images with the same meaning were coded together. Low-frequency images with the same meanings as high-frequency images were subsumed under the more frequent (general) ones. For example, images of binocular and monocular were coded as research equipment. Images that appeared less than five times, and that could not be coded with others, were excluded. The images were categorized using a criterion of semantic relationship (a similar method is used for analyzing word associations, for example see Sato and James, 1999; Flogaitis and Agelidou, 2003) and the frequency of the images in each category was calculated. The text on the posters was transcribed. As there was little text on the posters this was not sorted into categories. Text was analyzed in combination with comments and explanations recorded in the group discussion following poster presentation. General comments and impressions are discussed in this paper.

Results and discussion

The questionnaire was completed by 42 participants. In the first section participants were asked to give their opinion on the research activities, research methods and learning outcomes (Tab. 1). We were interested to know if participants learned anything new about animals, plants and other natural phenomena in freshwater ecosystems and surrounding environments. The items in Tab. 1 measure participants' self-evaluation of gained knowledge. The results show, that the majority of participants agree that they learned new things. . They were now more familiar with the functions of rivers, lakes and streams in the wild. They agreed that they had opportunities to experience animals and touch them (animals like frogs, snakes, lizards, dragonflies, and butterflies). Kellert (2002) wrote that being in natural environments and confronted with natural phenomena

Table 1: Participants' opinions about research activities and learning outcomes.
Tabela 1: Mnenje udeležencev o raziskovalnih aktivnostih in učnih rezultatih.

Statement (strongly disagree (1) … strongly agree (5))	**Mean**	**SD**
I learned so many new things about plant species.	4.29	0.64
I learned so many new things about animal species.	4.33	0.61
I learned a lot about the main characteristics of fresh water.	4.02	0.78
I realized what the functions of rivers, lakes and streams in the wild are.	4.43	0.55
I had the opportunity to experience, touch different animals.	4.83	0.38
I did not like field work in the groups.*	1.45	0.63
I learned new methods of natural science research.	4.48	0.67
I learned how to better observe and distinguish between different animals and plants.	4.52	0.63
I learned to operate research equipment and facilities.	4.48	0.63
I learned to analyze and process the field data.	4.50	0.63

can provide positive stimulus for the educational process of individuals. Moreover, as suggested by Smith, Reynold, Donaldson and Masters (1972), the outdoor education experience maximizes the use of the natural physical environment as a learning laboratory. The learning took place at the primary source and not through secondary resource information (workbook, video material, teacher's experiences and knowledge etc.). Nevertheless, this educational situation does not necessarily guarantee the maximum learning outcomes; much depends on other factors such as a pedagogical approach and student motivation. However, outdoor education gives individuals the optimal starting point – being at the primary source for information (nature).

Participants were also asked to express their opinion about research methods and equipment used in the research activities. Results gathered in Tab. 1 show that participants were satisfied with the work in research groups and they learned much about research methodologies used in particular, how to handle equipment, how to analyze and process research data and how to distinguish different animal and plant species.

In Tab. 2 participants' opinions about the evening guests are presented. The purpose of presenting different renowned researchers (evening guests) was to motivate participants toward science and research activities. Results show participants liked evening guests and the diversity of their professions. They also reported learning more about nature and nature protection from evening guests than from their teachers in school. We were particularly interested to know if they saw evening guests as positive role models and stimulus for their future decisions in life. The result from the last statement indicated that evening guests, who are renowned researchers, made a positive impression on participants and they want to try to be like them in their future professions.

Table 2: Participants' opinions about evening guests.
Tabela 2: Mnenje udeležencev o večernih gostih

Statement (strongly disagree (1) … strongly agree (5))	**Mean**	**SD**
I did not like the evening guests.*	1.69	0.71
I met a variety of researchers and their work.	4.60	0.50
From the evening guests I learned more about the nature and nature conservation than from my teachers in school.	4.52	0.67
I was impressed how evening guests were dedicated to research work.	4.19	0.99
I also wish to be so successful and devoted to my profession, which I am going to chose.	4.61	0.79

In the last section of the questionnaire we investigated their awareness and understanding of some conservation problems in freshwater ecosystems (Tab. 3). We are aware that the results gathered after intervention (camp) were without knowing their opinions before entering the camp activities and thus may have limited scientific significance. Nevertheless, we were interested to see how they understand and feel about conservation problems in freshwater ecosystems. Results show that, in general, participants' were aware of the negative effects certain human activities have on freshwater ecosystems and they understand the important role trees have for rivers.

Pearson's product moment correlation coefficient was used to examine the relationships between mean scores for instruments presented in Tab. 1, Tab. 2 and Tab. 3. Participants' who rated research activities and learning outcomes of summer camps higher had also more positive opinions about evening guests ($r = 0.75$; $p < 0.01$). Participants' with positive opinions about evening guests were also more aware and better understood the conservation problems in freshwater ecosystems ($r = 0.34$; $p = 0.03$). Correlation between participants' mean score of research activities and learning outcomes and their awareness and understanding of conservation problems in freshwater ecosystems was almost significant ($r = 0.29$; $p = 0.06$).

On the fifth day of the research summer camps, the participants prepared posters to express their impressions, concerns for, and perceptions of, the camp activities they had experienced during the week. At their disposal there were

Table 3: Participants' awareness and understanding about conservation problems in freshwater ecosystems.
Tabela 3: Ozaveščenost in razumevanje problemov ohranjanja ekosistemov celinskih voda.

Statement (strongly disagree (1) … strongly agree (5))	**Mean**	**SD**
Walled banks of rivers and streams have adverse affect on aquatic organisms.	4.33	0.75
Landfill sites can be a threat to the nearby watercourse.	4.74	0.50
Trees along rivers are important as they prevent erosion of banks and shade the water surface.	4.26	0.94
Along rivers, lakes and streams are too many trees that are causing flooding.*	1.58	0.89
Ponds should be drained, because they set too many insects.*	1.71	0.97

different magazines, newspapers, catalogues and calendars with different content not linked only to nature. The purpose of creating posters was to present their newly acquired knowledge and experiences through the creativity and imagination and to present their view of camp activities. We analyzed the pictures used for making the posters and classified them into five categories: *animals, plants, environments, research* and *other activities*. Within the categories were determined individual elements or groups of elements that occurred in the posters more than five times. We analyzed 39 posters, with a total of 415 images or photographs and texts. Despite the wide range of offered options most pictures appearing on the posters represented animals (48.43%), followed by plants (17.83%), environments (14.22%), research activities (6.02%) and finally other activities (2.41%). Among the *animals* whose pictures had appeared more than five times the prevailing groups were birds (26.87%), butterflies (7.96%), flies (6.97%) and frogs (5.97%). *Plants* represented 17.83% of all pictures used, of those 20.72% represented aquatic plants, 9.46% trees and 6.76% orchids. In the category of *environment* pictures of natural environments (lake, forest, rocks, rivers, sea, marsh) as well as man-made environments (field, orchard, vineyard, city) were represented. Most of the images encountered depicted lakes (20.34%), grassland (13.56%), rocky masses (13.56%) and forests (13.56%). To the category of *research activities* were assigned images that illustrated nature observation, hunting, research equipment and computers. The category *other activities* contained pictures of games, singing, hiking, eating and pictures of workshops. The remaining 9.40% pictures were not categorized (eg. pictures of pregnancy, heart, clouds, feet, umbrella). Results clearly show how fascinated participants were when they were encountering animals and plants. The variety of images used on the posters suggests that participants, at the end of the camp, showed fascination with natural phenomena in general, not only freshwater ecosystems.

We also analyzed the text on posters and participants' poster presentations (focus group discussions). Mostly they reflect the impressions of the camps' activities and nature - the beauty of nature, miracles of nature, colours of nature, friendship at the camp, loyalty, food chain, the camp activities, animals seen at the camp etc. From the discussion following the poster activity we can generally conclude that participants learned a lot about fauna and flora in freshwater ecosystems. They also appreciated the opportunity to explore nature, learn about different professions in science and to talk to established scientists. Qualitative data obtained also show that the research-oriented program at summer camps allowed participants to develop their skills for autonomous learning. Participants feel more competent in expressing their views on the importance of preserving freshwater ecosystems and reflect a high motivation to learn about complexity of nature. Following Palmer's suggestions (2004) summer camp activities provided meaningful learning experiences to participants that go beyond just learning something; they influence on student's personality and competences. By giving them more opportunities, autonomy and responsibility for exploring the natural environment we positively influenced their confidence in personal abilities and motivation to experience nature in a more complex and ecological context.

Conclusions

The present study explored the attitudes of students, aged 11 to 18 years, attending summer camps on the conservation of freshwater ecosystems. The respondents reported that they learned much about freshwater ecosystems, animals and plants, different research methods and handling research equipment. They enjoyed meeting evening guests, who were renowned researchers; they want to try to be like them in their future professions. Participants also showed a high awareness and understanding of problems related to freshwater ecosystem conservation.

The model of outdoor education presented here may be very useful to others interested in working towards motivating more young people towards the sciences and research work. Youth surprised us with their enthusiasm and reasonable, yet childlike, view of the world in which we live. This view was natural, direct and an honest look at the good and bad, the true and quasi-decision we all make in our society. Adults often underestimate the young and do not impose upon them proper burdens of life. Conversations with participants showed that experience of such camps inspire them with certain degree of confidence in themselves and their abilities.

In the future, it would be very useful to recall knowledge and skills in an extended time period to explore if there is any sustained learning as a consequence of the activities experienced at the summer camp.

Povzetek

Ekosistemi celinskih voda, predvsem mokrišča, so pogosto ogroženi zaradi človekovih dejavnosti, kot so regulacije, onesnaževanje, črpanje vode za namakalne sisteme in vnos tujerodnih vrst. Prizadevanja za ohranjanje le teh potekajo tako na nacionalnem kot mednarodnem nivoju (npr. Ramsarska konvencija). Pozornost je namenja tudi vzgojno-izobraževalnim programom, ki bi dvignili znanje in zavest o pomenu ohranjanja ekosistemov celinskih voda. Številni strokovnjaki na področju vzgoje in izobraževanja izpostavljajo pomen zunajšolskih izobraževalnih programov, ki naj bi bolj celovito obravnavali kompleksna naravovarstvena vprašanja, kakršno je ohranjanje ekosistemov celinskih voda. V prispevku obravnavamo vzgojno-izobraževalne aktivnosti, ki so potekale v okviru poletnih taborov za učence in dijake, namenjenih dvigu zavesti o pomenu ohranjanja ekosistemov celinskih voda. Glavni namen taborov je bil mlade poučiti o raznolikosti favne in flore ekosistemov celinskih voda ter o vplivih človekovih aktivnosti na te ekosisteme. Udeleženci so se ob tem spoznavali z različnimi raziskovalnimi metodami. Imeli so tudi priložnost spoznati raziskovalno pot in dosežke uveljavljenih raziskovalcev, ki so mlade udeležence taborov obiskovali ob večerih. Šestdnevni tabori so bili organizirani na treh lokacijah v Sloveniji: Fokovci na Goričkem, Fara ob reki Kolpi in Rakov Škocjan ob reki Rak. Taborov se je skupaj udeležilo 15 fantov in 27 deklet, starih med 11 in 18 let. Vzgojno-izobraževalni rezultati so bili merjeni kvalitativno (posterji in fokusne skupine) in kvantitativno (anketa). Iz kvantitativnih rezultatov lahko zaključimo, da so se udeleženci na taborih veliko naučili o ekologiji celinskih voda in o različnih raziskovalnih metodah ter opremi. Kot pozitivna in navdihujoča ocenjujejo tudi večerna srečanja z uveljavljenimi raziskovalci. Ti so jim na zelo oseben način predstavili svoje raziskovalne začetke in raziskovalno področje, s katerim se ukvarjajo. Udeleženci v odgovorih izražajo tudi visoko ozaveščenost o problematiki ohranjanja ekosistemov celinskih voda. Izračunani Pearsonov koeficient korelacije kaže linearno povezanost med pozitivnimi mnenji udeležencev o pridobljenem znanju o ekologiji celinskih voda, različnih raziskovalnih metodah in opremi, ki so ga pridobili na taboru, ter njihovim pozitivnim mnenjem o večernih srečanjih z uveljavljenimi raziskovalci. Udeleženci, ki pozitivno ocenjujejo večerna srečanja z uveljavljenimi raziskovalci so bili tudi bolj ozaveščeni o problematiki ohranjanja ekosistemov celinskih voda. Analiza kvalitativno pridobljenih podatkov je pokazala, da je raziskovalno delo na taborih omogočalo udeležencem, da razvijajo svoje sposobnosti za raziskovalno učenje ter krepijo svoj pozitiven odnos do narave. Udeleženci se počutijo bolj kompetentne pri izražanju svojih stališč o pomenu ohranjanja ekosistemov celinskih voda in izražajo visoko motiviranost za spoznavanje narave in njene ekološke kompleksnosti.

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Secondary structures of *Potato spindle tuber viroid* variants detected in Slovenia

Sekundarna struktura v Sloveniji najdenih genotipov viroida vretenatosti krompirjevih gomoljev

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Abstract: A survey of *Potato spindle tuber viroid* was initiated in Slovenia in 2006. Until the end of 2010, 100 samples of ornamental plants were found to be infected. Analysis of 96 complete master sequences of Slovene samples revealed new genetic variants. Our sequence variants showed considerable variation in their predicted secondary structure. The variability was observed in the pathogenic, central and variable domains. In several cases even single mutations were sufficient for the change in predicted secondary structure.

Keywords: *Potato spindle tuber viroid,* PSTVd, pospiviroid, predicted secondary structure, sequence variants

Izvleček: Leta 2006 smo v Sloveniji uvedli posebni nadzor viroida vretenatosti krompirjevih gomoljev (*Potato spindle tuber viroid*). Do konca leta 2010 smo okužbo potrdili v 100 vzorcih okrasnih rastlin. Analizirali smo 96 celotnih prevladujočih nukleotidnih zaporedij in predvideli njihove sekundarne strukture. Odkrili smo nove genotipe in znatno variabilnost sekundarnih struktur in sicer v patogeni, osrednji in variabilni domeni viroida. V določenih primerih je že mutacija enega samega nukleotida povzročila spremembo predvidene sekundarne strukture viroida.

Ključne besede: *Potato spindle tuber viroid,* PSTVd, pospiviroid, sekundarna struktura, različice nukleotidnega zaporedja

Introduction

Viroids are circular, covalently closed RNA molecules with a high degree of self-complementation resulting in compact folding. Approximately 30 viroids are known today, many of which cause serious losses of important crops. *Potato spindle tuber viroid* (PSTVd) is the type species of the genus *Pospiviroid* and can cause losses up to 64% in potato and up to 45% in tomato (Singh et al. 2003). The members of genus *Pospiviroid* show a rod-like secondary structure which is divided into 5 structural domains: terminal left, pathogenic, central, variable and terminal right domain (Keese and Symons 1985). This secondary structure is assumed to be the key for biological activity (replication, processing, transport, and pathogenesis) by being functional as such or by providing binding signals to host factors (Tabler and Tsagris 2004, Verhoeven 2010).

Viroids propagate in their plant hosts as populations of closely related variants. In the family *Pospiviroidae* one genotype usually dominates the viroid population in host plant and is called the predominant genotype or master sequence (Verhoeven and Roenhorst 2010).

Materials and methods

In the years 2006-2010 389 samples of ornamental plants were tested for PSTVd infection as described in details by Viršček Marn et al. (2013). Direct sequencing (Macrogen, Korea) of RT-PCR amplification products using primer pairs of Shamloul et al. (1997) and of Di Serio (2007) was performed to obtain whole PSTVd sequences. Additionally, amplification products from three selected samples were purified, cloned into pGEM-T easy vector (Promega, WI, USA) and sequenced (Macrogen, Korea). Obtained sequences were analysed using the computer software BioEdit version 7.0.5.3 (Hall 1999). Thermodynamic prediction of RNA secondary structure was performed using computer software mfold 2.3 in circular mode at 25°C (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3; Zuker 2003).

Results and discussion

PSTVd infection was confirmed in 88 samples of *Solanum jasminoides*, 5 samples of *Solanum rantonnetii*, 3 samples of *Petunia* spp., 3 samples of *Solanum muricatum* and one sample of *Brugmansia cordata*. Ninety-six whole viroid sequences were obtained. Determined PSTVd variants are presented in Table 1. Some of these variants were detected in numerous samples and/or various hosts. A hotspot was found on position 65. Multiple peaks of similar height for G and U; U and A; or G and U and A were observed and/or sequences of the same sample amplified with different primers had different nucleotides (G, U or A) at position 65. The occurrence of the hotspot was confirmed by cloning tree samples showing ambiguity at this position. Apart from polymorphism at position 65, other substitutions or insertions were observed in 9 out of 33 clones (Table 1).

Slovene sequences showed differences in predicted secondary structure models, calculated at 25°C (Fig. 1). The variability was observed in three out of five structural domains: pathogenic, central and variable domain. The replacement of G with U at position 65 enlarged the loop around this site (loop 10). Loop enlargement was also observed in some other sequences. Clone 1F, which has an additional G at position 100, has an enlarged loop E (loop 16). The substitution at position 83 in clone 3M changed the secondary structure from loop 11 to loop 14. Drastically changed secondary structure was observed from loop 17 to 23 due to the substitution at position 136 in clone 2C. Other polymorphisms in the clone sequences do not affect the secondary structure. Master sequences HQ454933 and HQ454936 have the same secondary structure as the prevalent master sequence detected in Slovenia (HQ454914 – HQ454916). All of the Slovene master sequences with 360 nt have structural changes on the right side from the loop 17. These changes are the results of several mutations on positions 121, 124, 126, 127 and 238. In sequence variant HQ454937 the substitution on the position 241 contributes to the changes due to the before mentioned mutations, therefore the loop 18 is larger in this 360 nt long sequence. Differences in the position and the size of loop 10 were also observed among different 360 nt long sequence.

The study of secondary structures of genotypes detected in Slovenia on ornamental plants showed several cases where single nucleotide mutation significantly changed the predicted secondary structure of the viroid. Apart from the single-site mutations, multiple mutation events in the same domain also resulted in the secondary structure changes. Induced mutations, either of one or several nucleotides, have been used to study the role of different loops for PSTVd characteristics. It was determined that disruption of nearly every loop had an impact on either replication or systemic trafficking (Qi et al. 2004, Zhong et al. 2008). We have not tested the functional impact of variability in the secondary structure of PSTVd variants determined on ornamental plants in Slovenia, but these changes could have an impact on their characteristics.

Table 1: Characteristics of the Slovene PSTVd sequences. Differences that influence the predicted secondary structure are highlighted in bold. Mutations that influence the same domain of the secondary structure are shadowed.

Tabela 1: Lastnosti slovenskih nukleotidnih zaporedij PSTVd. Razlike, ki vplivajo na predvideno sekundarno strukturo, so označene z odebeljenim tiskom. Mutacije, ki vplivajo na isto domeno sekundarne strukture, so senčene.

Host	**Acc. No.**	**No. of sequences**	**Sequence name**	**No. of nt**	**Difference in comparison with HQ454914 on position of the relevant sequence**
Master sequences					
Solanum jasminoides	HQ454914	52	G-type	357	–
S. rantonnetii	HQ454915	2	G-type	357	–
Petunia sp.	HQ454916	1	G-type	357	–
S. jasminoides	HQ454917	10	U-type	357	**G → U on 65**
S. rantonnetii	HQ454918	2	U-type	357	G → U on 65
S. jasminoides	HQ454919	6	K-type	357	G → K on 65
S. jasminoides	HQ454920	3	W-type	357	G → W on 65
S. jasminoides	HQ454921	12	D-type	357	G → D on 65
S. jasminoides	HQ454933	1	B29, B140	357	A → U on 221
Petunia sp.	HQ454936	1	B141	357	A → U on 221
S. jasminoides	HQ454937	2	B14,B82	360	**U → A ob 121, G → A on 124, insertion of GA on 126-127, insertion of C on 238, U → C on 241**
S. jasminoides	HQ454934	1	B90	360	**U → A ob 121, G → A on 124, insertion of GA on 126-127, insertion of C on 238, C → A on 311, A → U on 63, U → A on 64**
S. muricatum	HQ454935	2	B313, B314	360	**U → A ob 121, G → A on 124, insertion of GA on 126-127, insertion of C on 238**
S. muricatum	HQ454932	1	B315	360	**U → A ob 121, G → A on 124, insertion of GA on 126-127, insertion of C on 238, G → U on 65**
Clones					
S. jasminoides	HQ454914	7	G-type	357	–
S. jasminoides	HQ454917	10	U-type	357	–
S. jasminoides	HQ454922	7	clone A	357	**G → A on 65**
S. jasminoides	HQ454923	1	clone 1J	357	A → G on 30
S. jasminoides	HQ454924	1	clone 1D	357	A → G on 90, U → C on 258
S. jasminoides	HQ454925	1	clone 1F	358	G → A on 65, **insertion of G on 100**
S. jasminoides	HQ454926	1	clone 1G	357	U → C on 35
S. jasminoides	HQ454927	1	clone 2C	357	**G → U on 65, U → C on 136**, U → C on 302
S. jasminoides	HQ454928	1	clone 2J	357	U → C on 331
S. jasminoides	HQ454929	1	clone 3M	357	**G → U on 65, U → C on 83**
S. jasminoides	HQ454930	1	clone 3J	357	**G → U on 65**, A → G on 122
S. jasminoides	HQ454931	1	clone 3L	357	**G → U on 65**, A → G on 100

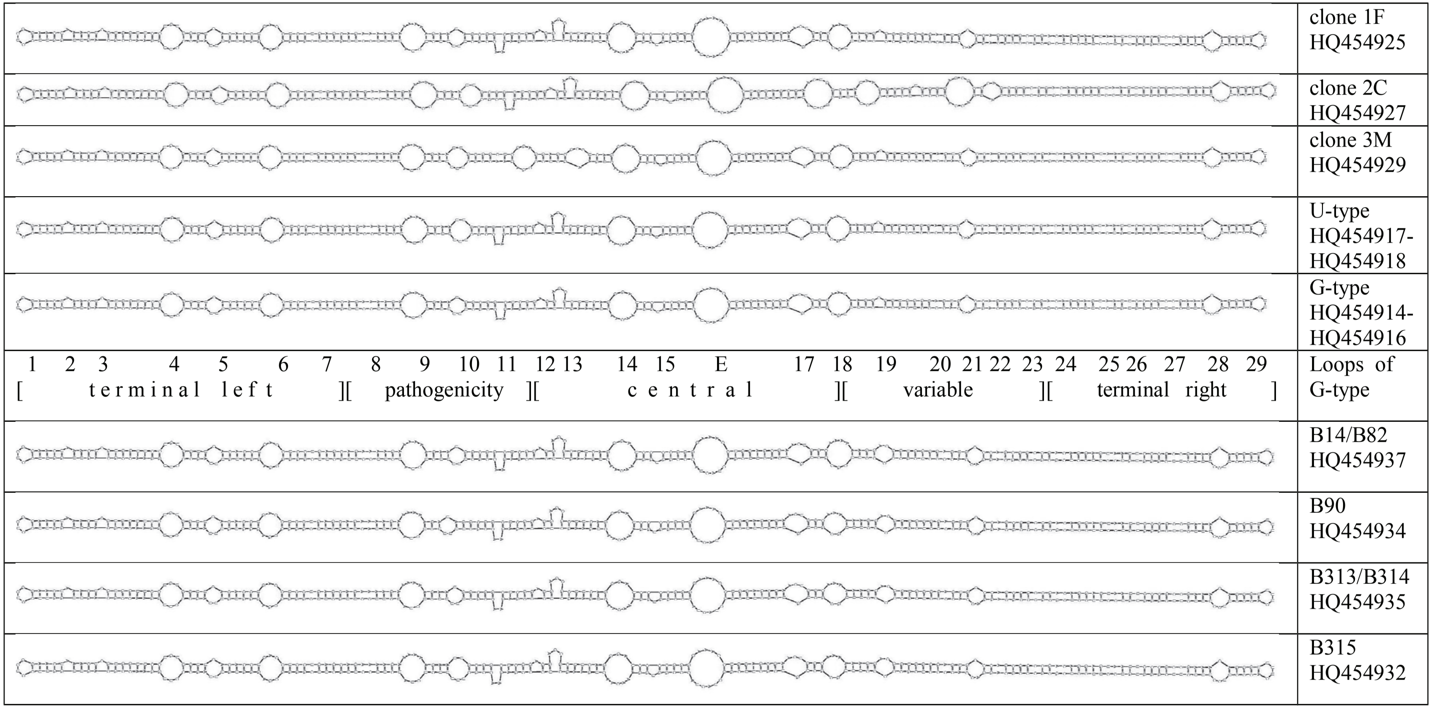

Figure 1: Thermodynamic predictions of secondary structure for PSTVd sequences detected on ornamental plants. Changed secondary structures in comparison with the most prevalent PSTVd sequence (G-type, HQ454914- HQ454916) are shown above and below G-type. The term loop is used for both loops and bulges.

Slika 1: Termodinamične napovedi sekundarnih struktur nukleotidnih zaporedij PSTVd potrjenih na okrasnih rastlinah v Sloveniji. Spremenjene sekundarne strukture v primerjavi z v Sloveniji najbolj pogosto najdenim nukleotidnim zaporedjem PSTVd (G-type, HQ454914- HQ454916) so prikazane nad in pod G-type nukleotidnim zaporedjem.

Conclusions

Predicted secondary structure of PSTVd sequences detected on ornamental host plants in Slovenia showed considerable variation. The variability was observed in the pathogenic, central and variable domains.

Povzetek

Leta 2006 smo v Sloveniji uvedli posebni nadzor viroida vretenatosti krompirjevih gomoljev (*Potato spindle tuber viroid*, PSTVd). Do konca leta 2010 smo analizirali 398 vzorcev večinoma okrasnih rastlin in okužbo potrdili v 88 vzorcih *Solanum jasminoides*, 5 vzorcih *Solanum rantonnetii*, 3 vzorcih *Petunia* spp., 3 vzorcih *Solanum muricatum* in enem vzorcu *Brugmansia cordata*. Določili in analizirali smo 96 celotnih prevladujočih nukleotidnih zaporedij. Nekatera od teh nukleotidnih zaporedij smo našli v številnih vzorcih in/ali v vzorcih iz različnih gostiteljskih rastlin. Na mestu 65 smo potrdili visoko pogostnost mutacij. Za termodinamično napoved sekundarne strukture RNA smo uporabili program mfold 2.3 pri 25 ^{o}C. Odkrili smo nove genotipe in znatno variabilnost sekundarnih struktur in sicer v patogeni, osrednji in variabilni domeni viroida. V določenih primerih je že mutacija enega samega nukleotida povzročila spremembo sekundarne strukture. Glede na objave drugih avtorjev o pomenu zank v strukturi PSTVd imajo lahko spremembe sekundarne strukture v Sloveniji najdenih nukleotidnih zaporedij PSTVd pomemben vpliv na njihove lastnosti.

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

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.

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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.

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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.

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

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

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

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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.