ACTA BIOLOGICA SLOVENICA

VOL. 55 ŠT. 1 LJUBLJANA 2012

prej/formerly BIOLOŠKI VESTNIK

ISSN 1408-3671
UDK 57(497.4)

izdajatelj/publisher
Društvo biologov Slovenije

ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2011 Vol. 55, Št. 1: 1–48

Acta Biologica Slovenica
Glasilo Društva biologov Slovenije – Journal of Biological Society of Slovenia

Izdaja – Published by
Društvo biologov Slovenije – Biological Society of Slovenia

Naslov uredništva – Adress of Editorial Office
Acta Biologica Slovenica, Večna pot 111, SI-1001 Ljubljana, Slovenija
http://bijh.zrc-sazu.si/abs/

Oblikovanje – Design
Žare Vrezec

ISSN 1408-3671 UDK 57(497.4)

Natisnjeno – Printed on: 2011
Tisk – Print: Tiskarna Pleško d.o.o., Ljubljana
Naklada: 400 izvodov

Cena letnika (dve številki): 15 € za posameznike, 42 € za ustanove

Številka poslovnega računa pri Ljubljanski banki: 02083-142508/30

Publikacijo je sofinancirala Javna agencija za knjigo Republike Slovenije

Acta Biologica Slovenica je indeksirana v – is indexed in: CAB Abstracts, Ulrichsweb

ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2012 Vol. 55, Št. 1: 3–8

Why do aquatic carnivorous plants prefer growing in dystrophic waters?

Zakaj vodne karnivore rastline rastejo v distrofnih vodah?

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Abstract: The majority of aquatic carnivorous plants (ACPS; *Aldrovanda, Utricularia*) usually grow in shallow dystrophic waters. In these habitats, rootless ACPs usually grow together with rooted aquatic non-carnivorous plants (N-ACPs). Yet species diversity of rooted N-ACPs in dystrophic lakes is relatively poorer than that of abundant ACPs. If generally true, why do rootless ACPs prefer growing in shallow dystrophic waters and why is the occurrence of rooted N-ACPs in these waters limited? These questions are elucidated on the basis of different specific adaptive traits of both functional groups and a different treatment of external habitat factors on both plant groups.

Keywords: aquatic carnivorous plants, *Aldrovanda, Utricularia,* submerged rooted plants, free CO_2, humic acids, pH, potential species pool, water dystrophy

Izvleček: Večina vodnih karnivorih rastlin (VKR; *Aldrovanda, Utricularia*) navadno uspeva v plitvih, distrofnih vodah. V takšnih habitatih, VKR brez korenin rastejo skupaj z ukoreninjenimi ne karnivorimi vrstami (N-VKR). Vrstna pestrost ukoreninjenih N-VKR v distrofnih jezerih je manjša kot pestrost množično zastopanik VKR. Če je to res, zakaj VKR brez korenin rastejo vplitvih distrofnih vodah in zakaj je pojavljanje ukoreninjenih N-VKR omejeno? Odgovori na ti dve vprašanji sta podani na osnovi potez prilagajanja obeh funkcionalnih skupin in različnim obravnavanjem obeh skupin rastlin z zunanjimi habitatnimi parametri.

Ključne besede: vodne karnivore rastline, *Aldrovanda, Utricularia,* potopljene ukoreninjene rastline, prosti CO_2, huminske kisline, pH, potencialna prisotnost vrst, distrofna voda

Introduction

The functional group of aquatic carnivorous plants (ACPs) comprises the species *Aldrovanda vesiculosa* L. (Droseraceae) and about 50 species of the genus *Utricularia* L. (Lentibulariaceae) (Taylor 1989, Adamec 1997, 2011, Guisande et al. 2007). The majority of these plants usually grow in shallow dystrophic (humic) waters and most of them are considered rare and strongly or critically threatened (Casper and Krausch 1981, Murphy 2002). In their recent minireview, Ellison and Adamec (2011) have thoroughly compared ecophysiological traits and cost-benefit trade-off of rooted terrestrial and rootless aquatic

carnivorous plants. They have concluded that the ecophysiological differences between these functional groups within carnivorous plants are greater than those between ACPs and rooted submerged aquatic non-carnivorous plants (N-ACPs) and also between terrestrial carnivorous and non-carnivorous plants. In their shallow dystrophic habitats, rootless ACPs usually grow together with rooted N-ACPs. Yet, on the basis of literature (e.g. Kamiński 1987, Murphy 2002), Ellison and Adamec (2011) have stated that species diversity of rooted N-ACPs in dystrophic lakes is relatively poorer than that of abundant ACPs. If generally true, why do rootless ACPs prefer growing in shallow dystrophic waters and, in contrast, why is the occurrence of rooted N-ACPs in these waters limited? Furthermore, are these reasons based more on different specific adaptive traits of both functional groups or on a different treatment of external (unfavourable) ecological habitat factors on both plant groups?

The characterization of dystrophic waters

Dystrophic or humic waters are usually characterized primarily by increased concentration of humic acids (+ tannins), causing the water to have a brownish colour, and by lower pH values (Hansen 1962). However, such a characterization is quantitatively rather vague as there are many types of dystrophic waters (e.g. peat bogs, fen lakes, forest pools, reed-dominated lake littorals). These types differ greatly from each other in the organic sediment composition, dominant vegetation and, also, water chemistry. Generally, it is difficult to determine where dystrophy starts. It is thus reasonable to differentiate the degree of dystrophy (*sensu* Chmiel 2010). The other associated symptoms commonly occurring in dystrophic waters are low electric conductivity (i.e. soft waters) and low concentration of mineral forms of N and P (sometimes also K^+) within the range of oligo-mesotrophy (Kamiński 1987, Adamec 2007, 2008, Guisande et al. 2007); this results in the waters exhibiting low biological productivity. Due to slow decomposition of loose organic sediment (composed of mosses, reed or sedge litter), the usually low concentration of dissolved oxygen in dystrophic waters is accompanied by high concentration of free CO_2 (Adamec 1997, 2007). Summarily, the unfavourable factors affecting rooted submerged vascular vegetation are high concentration of humic acids and tannins, dark water associated with very steep temperature gradient (overheating at the surface, cold at the bottom) and considerable light attenuation and shift of light spectrum in deeper water, low pH values, hypoxia in the free water column and anoxia in the partly decomposed, loose organic bottom sediment (litter), which is unsuitable as a rooting medium for rooted submerged plants. The only advantage (high [CO_2]) cannot evidently prevail over the unfavourable factors (Adamec 1997, 2007, 2008, Murphy 2002). As an exact definition of dystrophic waters is difficult for the above hydrochemical reasons, the opposite phytosociological approach considering the typical dystrophic vegetation may be used for denoting dystrophic waters (Murphy 2002). An analysis of the main water chemistry factors, using both published and unpublished data for 307 microsites of 11 ACP species on four continents, shows clearly that the amplitudes of all estimated parameters are extremely broad (Fig. 1). Yet the means, medians and quartiles for all parameters characterize truly the essence of shallow dystrophic waters. Fifty % of the waters have high [CO_2] within 0.14–0.92 mM.

The ecophysiological characterization of aquatic carnivorous plants

ACPs are always rootless and float freely below the water surface or are weakly attached to loose sediments, submerged or even amphibious. Most ACPs have a linear and modular shoot structure consisting of nodes with filamentous leaves and tubular internodes. The majority of species have homogeneous green shoots bearing traps. Several species have dimorphic shoots differentiated into green photosynthetic and pale carnivorous (trapping) ones attached to sediments (Taylor 1989). Moreover, ACPs show very rapid apical shoot growth of 1.0–4.2 nodes d^{-1} (see Adamec 2011) but their basal shoot segments die at about the same rate ("conveyer-belt" growth system); the new biomass is allocated into branching and flowering only. Thus, due to the special growth form and the

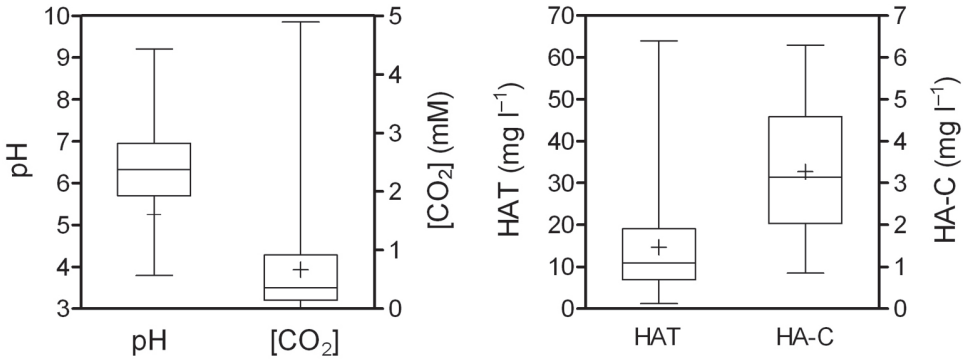

Figure 1: Analysis of main water chemistry factors at world sites of aquatic carnivorous plants from literature data and unpublished results (n = 34-307). Boxes show median (central horizontal line), upper and lower quartiles (limits of boxes), and range of values (horizontal bars delimiting vertical lines). Asterisks show mean. Mean pH was calculated via $[H^+]$. HAT, sum of humic acids and tannins; HA-C, carbon of humic acids.

Slika 1: Analiza glavnih parametrov kemizma vode na različnjih svetovih rastiščih karnivorih rastlin (podatki iz literature in neobjavljeni podatki; n=34-307). Srednja črta v škatli je mediana, zgornja in spodnja meja 1. in 2. kvartil in ročaji 3. in 4. kvaril. Zvezdice označujejo srednjo vrednost. Srednji pH smo izračunali na podlagi $[H^+]$. HAT, vsota huminskih kislin in taninov; HA-C, ogljik v huminskih kislinah.

absence of roots, photosynthetic shoots of ACPs can always float at or near the water surface at a relatively high irradiance even in frequently oscillating water levels, while rooted N-ACPs with slower apical growth are firmly anchored in the bottom growing in the deep shade. If ACP species with dimorphic shoots are attached to the bottom by their pale carnivorous shoots, due to shoot plasticity and rapid apical growth, their photosynthetic shoots can reach the water surface faster (Adamec 2007). In spite of possible generative reproduction of most ACPs (Taylor 1989), this way is probably limited mostly to colonisation of new sites and/or restoration of sites after drying out. Most ACP species propagate at their sites mainly vegetatively by shoot branching and separation of branches (Kamiński 1987, Adamec, 2011). Numerous European N-ACP species of the genera *Potamogeton*, *Callitriche* and *Ranunculus* regularly set larger and germinating seeds and their proportion of vegetative propagation may be obviously much lower. As seed germination in dystrophic habitats occurs under unfavourable conditions (for small seedlings) of hypoxia/anoxia, deep shade and low temperature at the bottom, the generative recovery of both functional plant groups is greatly impaired. Yet rootless ACP seedlings sprout at the water surface under favourable conditions – this difference could also favour the occurrence of ACPs. Similarly, most temperate ACPs form turions (overwintering buds) which usually overwinter at the bottom of water bodies but germinate and sprout at the water surface under favourable conditions again, while the not-so-common turions of N-ACPs (*Potamogeton* spp.) are either permanently attached to the bottom or exclusively sprout there (Adamec 2010). So, this adaptive trait of ACPs also supports their growth in dystrophic waters.

Under favourable conditions, ACPs exhibit very rapid growth: the relative growth rate ranges between 0.035–0.15 d^{-1} (Adamec 2011, Ellison and Adamec 2011). Frequent shoot branching is a symptom of such a rapid growth. The rapid growth of rootless ACPs in nutrient-poor dystrophic waters requires several ecophysiological adaptations: high photosynthetic rate, prey capture, efficient nutrient re-utilization from senescent shoots and high nutrient uptake affinity from the ambient water (Adamec 2011). All ACP species are strict CO_2 users and their high net photosynthetic rates at $[CO_2]$ >0.2 mM are among the highest values found in N-ACPs (Adamec 1997, 2011). Thus, high $[CO_2]$ occurring at most sites of ACP

(usually >0.1 mM; Fig. 1) together with a medium irradiance at the water surface (>*c.* 100 μmol m^{-2} s^{-1} PAR) are the first prerequisites for attaining high photosynthetic rates and, consequently, high growth rates. Based on only several ACP species, it is evident that N and P are re-utilized from aged shoots very efficiently but all K^+ is lost (Adamec 2011). Similar information is lacking for N-ACPs. Prey capture in rootless ACPs in dystrophic waters can cover even a considerable proportion of their seasonal N and P gain, giving them definite advantage over rooted N-ACPs, but many nutrient-poor dystrophic waters exhibit very low prey availability minimising this advantage (Richards 2001, Adamec 2008, 2011, Peroutka et al. 2008). It is therefore possible to assume that ACP shoots have a very high uptake affinity for mineral nutrients from the ambient water.

The potential occurrence of ACPs and N-APCs in dystrophic waters

The comparison of data on the relative abundance of both functional plant groups from the literature and unpublished data from110 microsites in six Central European countries (Poland, Slovakia, Czech Republic, Germany, Switzerland, NE France with ACPs (mostly *Aldrovanda, U. vulgaris, U. australis*) was performed (Table 1). The potential community species pool at these sites comprised seven ACPs (all Central European species, i.e., *A. vesiculosa* +6 *Utricularia* species, cf. Casper and Krausch 1981) and 44–52 vascular lowland submerged N-ACP species (depending on the country; Casper and Krausch 1981) mostly of the genera *Potamogeton, Ranunculus, Callitriche, Myriophyllum* (including rootless *Ceratophyllum* spp. and amphibious *Pilularia globulifera, Hottonia palustris, Luronium natans, Eleocharis acicularis, Juncus bulbosus, Sparganium natans* and floating-leaved *Potamogeton natans,* but excluding montane *Isoëtes* spp., *Ranunculus fluitans* growing strictly in streams and partly emergent *Stratiotes aloides*), which are usually rooted and have the dominant part of submerged plant biomass. Although the mean species number of N-ACPs (2.12 ± 0.26) was significantly greater (*t* test; $P < 0.05$) than that of ACPs (1.49 ± 0.07), the relative diversity of N-ACPs expressed in % of the potential species pool (4.24 ± 0.51) was highly significantly lower ($P < 0.0001$) than that of ACPs (21.3 ± 1.0). Although this approach is rather simplified and biased and one can discuss what is the real potential species pool of submerged plants in dystrophic waters in each country and at each site studied, the analysis shows clearly that the relative diversity of N-ACPs is limited. Only 24 species of N-ACPs out of the potential species pool were found at these sites. Moreover, the most common co-occurring N-ACP species were *Potamogeton natans, Juncus bulbosus* and *Lemna trisulca* which are not strictly submerged. *P. natans* has a good deal of natant foliage, *J. bulbosus* is ecologically an extremely plastic amphibious species and *L. trisulca* is an amphibious non-rooting species. It is also possible to assume that N-ACPs tend to grow rather in waters at a lower degree of dystrophy. However, due to unsufficient data, this cannot be proven. Comparing the occurrence of both functional groups in dystrophic waters, different phylogenetic constraints should be mentioned for both groups of plants. All ACPs, except for the monotypic genus *Aldrovanda*, are all confined to sections of the single genus *Utricularia* (Taylor 1989) and, thus, must be highly phylogenetically constrained; this fact suggests their relatively good adaptation to growing in dystrophic waters. On the contrary, submerged N-ACPs are phylogenetically (and also ecologically) a very diverse group (see e.g. Casper and Krausch 1981) and consist of subgroups such as rooted in the bottom, rootless, amphibious, rooted with partly floating leaves. The great diversity of N-ACPs predetermines also a diversity of the degree of their adaptations to shallow dystrophic waters.

Generally, these data support the above view that rooting in the bottom is not beneficial for submerged plants in dystrophic waters and that the prevailing strategy of aquatic plants in these waters is to reach or follow the (oscillating) water surface. The question raised above may be answered in that specific adaptive traits of ACPs (no roots, rapid apical growth, turions, high photosynthetic rate, high nutrient uptake affinity, carnivory) mitigate the impact of some unfavourable ecological factors of dystrophic waters (anoxic organic bottom, dark water, low nutrient concentration) on ACPs when compared with N-ACPs. The unfavourable water chemistry factors (humic acids and tannins,

Table 1: Analysis of the occurrence of aquatic carnivorous plants (ACPs) and aquatic submerged non-carnivorous vascular plants (N-ACPs, usually rooted species) at sites of ACPs in six Central European countries from literature data and unpublished results (n = 110). Potential community species pool is expressed in % as the proportion of each functional group to the potential maximal species number (7 for ACPs; 44-52 for N-ACPs).

Tabela 1: Analiza pojavljanja vodnih karnivorih rastlin (VKR) in vodnih ne-karnivorih rastlin (N-VKR, navadno neukoreninjenih) na mestih z VKR v šestih srednje evropskih državah na podlagi literaturnih in neobjavljenih podatkov (n=110). Potencialna zastopanost vrst v združbi je izražena v % kot delež vsake funkcionalne skupine glede na potencialno maksimalno število vrst (7 za VKR; 44-52 za N-VKR).

Plant group	Species number Mean ± SE	Potential species pool (%)		
		Mean ± SE	Median	Quartiles
ACPs	1.49 ± 0.07	21.3 ± 1.0	14.3	14.3; 28.6
N-ACPs	2.12 ± 0.26	4.24 ± 0.51	2.27	0.0; 6.0

low pH) affect both functional plant groups in the same way but ACPs seem better adapted to tolerate these factors.

To understand better the specific adaptive traits of ACPs for growing in dystrophic waters, ACP tolerance of high concentrations of humic acids and tannins (excessive to most N-ACPs) should preferentially be studied as the key factor. Are they essential for ACP growth? What are their concentration limits for single species? Are humic acids able to cover a part of seasonal N gain? Our knowledge of this subject is still very fragmentary (see Kamiński 1987). Another mystery associated with mineral nutrition of ACPs is K^+ economy. Dystrophic waters are commonly very poor in K^+ but shoot K content in ACPs is relatively high (median 1.6% dry weight; Ellison and Adamec 2011). Moreover, animal prey is considered a rather poor K^+ source and, thus, the total K^+ uptake from prey is very limited (Adamec 2011). However, zero re-utilization of K^+ from senescent shoots was reported in two ACP species (Adamec 2011). Do these facts indicate a very high K^+ uptake affinity of ACP shoots from the ambient water? Traps of many aquatic *Utricularia* species live close to great amounts of organic detritus (Adamec 2007) which is frequently aspirated into the traps. How important can the utilisation of organic detritus for the seasonal N, P and K gain in aquatic *Utricularia* species in dystrophic waters be?

Conclusions

The ability of ACPs to easily follow the favourable water surface conditions both during the growing season and after overwintering together with their carnivory confers a great ecological advantage over rooted N-ACPs when growing in dark dystrophic waters. The great potential of high [CO_2] occurring in these waters can be fully exploited. Thus, the ecological cost/benefit relationships for growing in shallow dystrophic waters are much more optimised in ACPs than rooted N-ACPs. The possibility of capturing animal prey together with high CO_2 availability were obviously the key favourable ecological factors which "drove" the adaptive evolution of ACP ancestors to living in dystrophic waters.

Acknowledgements

The study was partly supported from the Czech long-term research development project No. RVO 67985939. Sincere thanks are due to B. G. McMillan for correction of the language and to J. Klimešová for valuable comments.

References

Adamec, L., 1997. Photosynthetic characteristics of the aquatic carnivorous plant *Aldrovanda vesiculosa*. Aquat. Bot., 59, 297–306.

Adamec, L., 2007. Investment in carnivory in *Utricularia stygia* and *U. intermedia* with dimorphic shoots. Preslia, 79, 127–139.

Adamec, L., 2008. Mineral nutrient relations in the aquatic carnivorous plant *Utricularia australis* and its investment in carnivory. Fundam. Appl. Limnol., 171, 175–183.

Adamec, L., 2010. Tissue mineral nutrient content in turions of aquatic plants: does it represent a storage function? Fundam. Appl. Limnol., 176, 145–151.

Adamec, L., 2011. Ecophysiological Look at Plant Carnivory: Why Are Plants Carnivorous? In: Seckbach J., Dubinski Z. (eds.): All Flesh Is Grass. Plant-Animal Interrelationships. Cellular Origin, Life in Extreme Habitats and Astrobiology Vol. 16. Springer Science + Business Media B. V., Dordrecht, Heidelberg, London, pp. 455–489.

Casper S.J., Krausch H.D., 1981. Süsswasserflora von Mitteleuropa, Band 24: Pteridophyta and Anthophyta. VEB Gustav Fischer Verlag, Jena.

Chmiel, S., 2010. Hydrochemical evaluation of dystrophy of the water bodies in the Łęczna and Włodawa area in the years 2000–2008. Limnol. Rev., 9, 153–158.

Ellison, A.M., Adamec, L., 2011. Ecophysiological traits of terrestrial and aquatic carnivorous plants: are the costs and benefits the same? Oikos, 120, 1721–1731.

Guisande, C., Granado-Lorencio, C., Andrade-Sossa, C., Duque, S.R., 2007. Bladderworts. Funct. Plant Sci. Biotechnol., 1, 58–68.

Hansen, K., 1962. The dystrophic lake type. Hydrobiologia, 19, 183–190.

Kamiński, R., 1987. Studies on the ecology of *Aldrovanda vesiculosa* L. I. Ecological differentiation of *A. vesiculosa* population under the influence of chemical factors in the habitat. Ekol. Pol., 35, 559–590.

Murphy, K.J., 2002. Plant communities and plant diversity in softwater lakes of northern Europe. Aquat. Bot., 73, 287–324.

Peroutka, M., Adlassnig, W., Volgger, M., Lendl, T., Url, W.G., Lichtscheidl, I.K., 2008. *Utricularia*: a vegetarian carnivorous plant? Algae as prey of bladderwort in oligotrophic bogs. Plant Ecol., 199, 153–162.

Taylor P., 1989. The Genus *Utricularia*: A Taxonomic Monograph. Kew Bulletin Additional Series XIV, HMSO, London, 724 pp.

ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2012 Vol. 55, Št. 1: 9–15

Response of two submersed macrophytes *Ceratophyllum demersum* and *Myriophyllum spicatum* to selenium in water

Odziv dveh potopljenih vrst makrofitov *Ceratophyllum demersum* in *Myriophyllum spicatum* na selen v vodi

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Abstract: Two submersed macrophytes (*Ceratophyllum demersum* and *Myriophyllum spicatum*) were exposed to water containing 10 mg Se(IV) L^{-1}, later transferred to water without Se and exposed again to 10 mg Se(IV) L^{-1} with the aim to observe the recovery of plants. After each transplantation trial, potential photochemical efficiency of photosystem II, respiratory potential and the amount of photosynthethic pigments and anthocyanins were measured. Photochemical efficiency was similar in all three trials. Electron transport system (ETS) activity increased drastically in *C. demersum* plants that were transferred from the water with Se to the water without Se, while ETS activity strongly increased in *M. spicatum* specimens, when the second time transferred to water containing Se. Alternation in the concentration of Se in the growth media demanded metabolic changes in studied plants. The amount of chlorophylls was higher in plants of *M. spicatum* growing in water without Se than in exposed plants, while the amount of carotenoids and anthocyanins decreased in the same species grew in water without Se. The amount of Se was higher in plants exposed to Se, while plants that grew in water without Se had lower amount of Se in the tissues.

Keywords: *Myriophyllum spicatum*, *Ceratophyllum demersum*, selenium, photochemical efficiency, respiratory potential

Izvleček: Potopljeni vrsti *Ceratophyllum demersum* in *Myriophyllum spicatum* sta bili najprej izpostavljeni koncentraciji 10 mg Se(IV) L^{-1}, kasneje smo rastline prestavili v vodo brez dodanega Se, nato pa ponovno v vodo, ki je vsebovala 10 mg Se(IV) L^{-1} z namenom, da bi ugotovili, ali se stanje rastlin, ki rastejo v vodi brez Se, izboljša. Po vsaki presaditvi in nekaj dnevih izpostavljenosti rastlin, smo izmerili fotokemično učinkovitost fotosistema II, dihalni potencial ter vsebnost fotosinteznih barvil in antocianov. Vrednosti fotokemične učinkovitosti so bile v vseh obravnavanjih podobne. Menjavanje koncentracij Se v vodi, kjer so uspevale rastline, je povzročilo spremembe v metabolizmu rastlin, kar smo izmerili s pomočjo meritev aktivnosti elektronskega transportnega sistema (ETS). Vsebnost klorofilov je bila višja pri rastlinah vrste *M. spicatum*, ki je bila izpostavljena Se, medtem ko je bila vsebnost karotenoidov in antocianinov nižja v rastlinah, ki so uspevale v vodi brez dodanega Se. Vsebnost Se je bila višja v rastlinah obeh vrst, ki so bile izpostavljene Se, medtem ko so rastline, ki so rastle v vodi brez dodanega Se vsebovale manj Se.

Ključne besede: *Myriophyllum spicatum*, *Ceratophyllum demersum*, selen, fotokemična učinkovitost, dihalni potencial

Introduction

Selenium (Se) is a naturally occurring trace element which is toxic at high concentrations, but it is also an essential element for many organisms (Fan et al., 2002). It is found in the Earth's crust, soils, minerals, in freshwater, seawater, and in sediments. In aquatic systems Se can be found mostly in the form of selenite and selenate (Canton and Van Derveer, 1997) and these forms are potentially toxic to aquatic organisms.

Se pollution in the environment arises from both natural and anthropogenic sources. Se is found in aquesous discharge from electric power plants, coal ash leakages, oil refinery effluents, industrial wastewater, as well as in agricultural drainage water for irrigation (Fan et al., 2002; Lemly, 2004). The addition of Se to feed stuffs and soil fertilizers is a common practice. Part of this added Se is used by animals and part is spilled or secreted and passed to the environment. Se pollution is a worldwide problem and there is a tremendous demand for clean-up of Se-contaminated water (Dhote and Dixit, 2009).

Macrophytes are aquatic plants which have been used as indicators of trace element pollution since the early seventies (Phillips, 1977). Some macrophyte species are suitable for wastewater treatment because they have a tremendous capacity for absorbing nutrients and other substances from the water (Boyd, 1970) and hence reduce the pollution. Some aquatic plants can take up trace elements through their roots whereas in submersed plants such are *Myriophyllum alternifolium*, *Vallisneria spiralis*, *Chara carolina* and *Veronica aquatica* (Delmail et al., 2011; Rai et al., 1995; Robinson et al., 2006), leaves as well as roots take part in uptake. In previous studies it was evidenced that *Myriophyllum spicatum* and *Ceratophyllum demersum* took up a large amount of trace elements (Rai et al., 1995; Robinson et al., 2006; Mechora et al., 2011).

The purpose of our study was to investigate the difference in response of *M. spicatum* L. and *C. demersum* L. growing in water with Se(IV) and water without Se. We also want to observe the recovery of the plants. To reach these aims, we measured physiological and biochemical parameters of plants, namely the photochemical efficiency, ETS, content of photosynthetic pigments and the concentration of Se in the plants tissues.

Materials and Methods

Plants and growth conditions

Experiments were conducted under natural conditions at Ljubljana, Slovenia. *Myriophyllum spicatum* was obtained from Lake Bohinj (547 m asl, 46º17´ N, 13º54´ E, Slovenia) and planted on 15 April 2011, while *Ceratophyllum demersum* was obtained from a pond in the Botanical Garden (Ljubljana: 320 m asl, 46º35´ N, 14º55´ E, Slovenia) and placed in containers on 18 April 2011. Both species were placed in two separate containers of size 120 cm x 52 cm x 54 cm, containing 160 L of tap water and layer of soil and sand.

After two weeks of plant acclimatization, sodium selenite (Na_2SeO_3) was added to the experimental containers. One container had water without Se, while the second contained 10 mg Se L^{-1}. During the experiment the concentration of Se in water was measured and maintained at the desired levels.

After 5 days of an exposure to Se, we measured physiological and biochemical parameters (photochemical efficiency of photosystem II, respiratory potential and the amount of photosynthetic pigments and anthocyanins) and then transferred the plants in the container with water without added Se. After 10 days of exposure, the selected parameters were measured again and then plants were transferred back to 10 mg Se L^{-1} solution. After 5 days of exposure selected parameters were measured again in plants, transferred again to water containing 10 mg Se L^{-1}. At the end of an experiment, plants were harvested, washed, lyophilized and milled for further analysis of Se.

Photochemical efficiency

Chlorophyll fluorescence was measured *in situ* on ten vital plants from each container using a fluorometer (PAM 2100 Chlorophyll Fluorometer, Heinz Walz GmbH, Germany). The potential quantum yield was evaluated in terms of the ratio F_v/F_m. Measurements of minimal (F_0) and maximal (F_m) chlorophyll fluorescence were made after 10 min of darkness, provided by dark-adaptation clips. Fluorescence was excited with a saturating beam of "white light" (PPFD = 8 000 µmol m^{-2} s^{-1}, 0.8 s).

Electron transport system (ETS) activity

The respiratory potential of mitochondria was measured as terminal electron transport system (ETS) activity in plants (Packard, 1971). 0.2–0.4 g of leaves from four plants from container with added Se and without added Se were homogenized using ice-cold homogenization buffer and sonication with an ultrasound homogenizer (40W, 4710, Cole-Parmer, Vernon Hills, IL, USA). The homogenate was then centrifuged (8500 x g, for 4 min, 0 ºC) in a top-refrigerated ultracentrifuge (Sigma 2–16 PK, Germany). We added 1.5 mL of substrate solution and 0.5 mL of iodo-nitrotetrazolium chloride (INT) to triplicates of the supernatant (0.5 mL), and incubated at 20 ºC for 40 min. INT instead of oxygen was reduced to formazan during incubation. After stopping the reaction with formaldehyde and phosphoric acid (1:1), the formazan absorption at 490 nm was measured. ETS activity was determined as the rate of tetrazolium dye reduction and conversion to oxygen equivalents (Kenner and Ahmed, 1975).

Photosynthetic pigments

For content of chlorophylls *a* and *b* and carotenoids, leaves of four plants from container with added Se and without Se were selected. Chlorophylls and carotenoids were extracted with 90 % acetone. Extracts were centrifuged in a refrigerated ultracentrifuge (2K15, Sigma, Osterode, Germany) at 4000 rpm for 4 min at 4 ºC. Absorbance was measured with a UV/VIS Spectrometer System (Lambda 12, Perkin-Elmer, Norwalk, CT, USA) at 470nm, 644nm and 662 nm. The amounts of pigment were determined as described by Lichtenthaler and Buschmann (2001a and 2001b). The total anthocyanin content was measured as described by Drumm and Mohr (1978).

Determination of total Se concentration in plants

Three plants from container with added Se and three from the container without Se were analyzed for Se content. To 0.200 g of homogenized and lyophilized sample, acids were added and heated for 24 h at 80 °C. H_2O_2 and 0.1 mL 40% HF were added to the cooled solution. After heating and cooling the samples again, 0.1 mL of V_2O_5 in H_2SO_4 was added. Se (VI) was reduced to Se (IV) by the addition of concentrated HCl and heating at 90 °C. The method is described in detail by Smrkolj and Stibilj (2004). The solution was diluted before determining the Se content, which was carried out by hydride generation atomic fluorescence spectrometry (Smrkolj and Stibilj, 2004). Each sample was analysed at least in triplicate.

Statistical analysis

The significance of the difference between mean values was determined by the analysis of variance with LSD test. Differences at $p < 0.05$ were considered as statistically significant.

Results and discussion

Measurements of physiological and biochemical parameters can show the status of the plants. Photochemical efficiency, respiratory potential measured as electron transport system (ETS) activity as well as photosynthetic pigments can be a good indicator of stress. The values of F_v/F_m around 0.8 indicate that plants are in good condition (Schreiber et al., 1995). In plants, exposed to water with 10 mg Se L^{-1}, the values of photochemical efficiency were around 0.36 for *C. demersum* and 0.48 for *M. spicatum* (Fig. 1). These values indicated that Se exposed plants were under stress. Photochemical efficiency in plants slightly increased, when plants were transferred to water without Se, but there was no statistically significant difference (Fig. 1). On the other hand F_v/F_m was

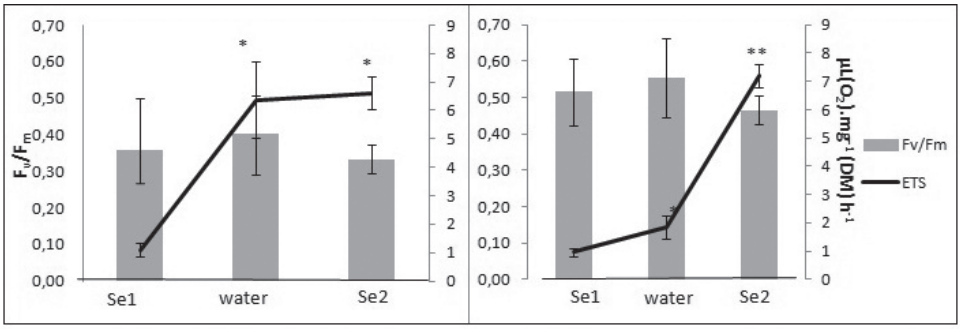

Figure 1: Photochemical efficiency and ETS activity in *Ceratophyllum demersum* (left) and *Myriophyllum spicatum* (right).
Each parameter was tested separately for each species. Results with * were statistically different from the others at $p < 0.05$. Se1 – exposed to 10 mg Se L^{-1} for the first time; Se2 – exposed to 10 mg Se L^{-1} for the second time.

Slika 1: Fotokemična učinkovitost in aktivnost ETS pri vrsti *Ceratophyllum demersum* (levo) in *Myriophyllum spicatum* (desno).
Vsak parameter je bil testiran posebej za vsako vrsto. Rezultati označen z * je statistično značilno različen od drugih pri $p < 0.05$. Se1 – izpostavljene prvič 10 mg Se L^{-1}; Se2 – izpostavljene drugič 10 mg Se L^{-1}.

higher in *M. spicatum* and *C. demersum*, exposed to 10 mg Se(VI) L^{-1} comparing to untreated plants (Mechora et al., 2011). After transferring the plants to Se solution the values of F_v/F_m slightly decreased again, the values being 0.33 in *C. demersum* and 0.46 in *M. spicatum* (Fig. 1).

ETS activity was the lowest in plants exposed to Se at the beginning of the experiment (Fig. 1). ETS activity increased drastically in *C. demersum* that were transferred to the water without added Se. In the case of *M. spicatum* ETS activity strongly increased in plants, which once again grew in water containing Se. Any changes in environmental parameters request metabolic adaptation (Larcher, 2003). This is in concordance to our results in the transplantation experiment.

The content of chlorophyll *a* in *M. spicatum* increased, when plants were transplanted into water without Se, while the content of chlorophyll *b* decreased (Table 1), but there was no statistically significant difference. In other study, the addition of Se had no effect on the amount of chlorophylls in *M. spicatum* (Mechora et al. 2011), while Cd lowered the amount of chlorophylls in *M. alternifolium* (Delmail et al., 2011) and *M. spicatum* (Sivaci et al., 2004). In *C. demersum* the amount of chlorophylls was the lowest in water without Se (Table 1), however the results were

Table 1: The amount of pigments in macrophytes growing in water and Se solution (n = 4).
Tabela 1: Vsebnost barvil v makrofitih, ki so rastli v vodi brez in z dodanim Se (n = 4).

	Ceratophyllum demersum			***Myriophyllum spicatum***		
	10 mg L^{-1}	water	10 mg L^{-1}	10 mg L^{-1}	water	10 mg L^{-1}
chlorophyll *a*	0.82±0.12	0.44±0.13	0.84±0.22	0.52±0.26	1.12±0.13*	0.81±0.17
chlorophyll *b*	0.59±0.12	0.27±0.11	0.64±0.40	0.83±0.45	0.54±0.08	0.33±0.10
carotenoids	0.44±0.05	0.20±0.07	0.33±0.04	0.90±0.21*	0.37±0.06	0.38±0.03
anthocyanins	97±23	37±15	220±45	160±10*	59±36	130±71*

Each parameter was tested separately for each species. Results with * were statistically different from the others at $p < 0.05$.

not statistically significant. This could suggest that Se did not affect the synthesis of chlorophylls in *C. demersum*.

The content of carotenoids and anthocyanins in *M. spicatum* and in *C. demersum*, grew in water without Se (Table 1) was lower comparing to plants, grew in Se solution. On the contrary, a negative effect of Cd on the amount of carotenoids was observed in *M. alternifolium* (Delmail et al., 2011) and *M. spicatum* (Sivaci et al., 2004). Carotenoids and anthocyanins can start to accumulate in plants, when they are in stress (Winkel-Shirley, 2002) that is in line with the present results (Table 1).

The amount of Se in plants, exposed to Se solution, was 319 and 436 µg Se g^{-1} DM in *C. demersum* and *M. spicatum*, respectively (Fig. 2). When plants were transferred to the water without Se, the amount of Se in the tissues decreased. The content of Se in plants, which were once again exposed to Se, was higher comparing to plants, exposed to Se at the beginning of the experiment. To our knowledge there is no study dealing with the response of aquatic plants when transferred from water with Se and water without added Se. However, Robinson et al. (2006) made an experiment with arsenic (As). In that study *M. propinquum* released around 20% of accumulated As to the ambient water (Robinson et al., 2006) therefore the content of As in tissues were lower.

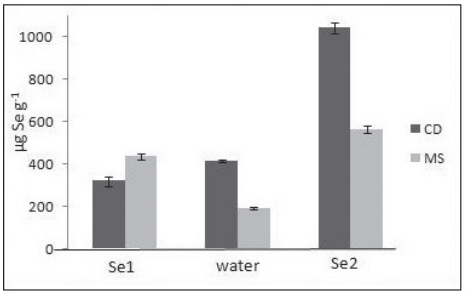

Figure 2: The amount of Se in tissues of macrophytes. CD – *C. demersum*, MS – *M. spicatum*. Se1 – exposed to 10 mg Se L^{-1} for the first time; Se2 – exposed to 10 mg Se L^{-1} for the second time.

Slika 2: Vsebnost Se v tkivih makrofitov. CD – *C. demersum*, MS – *M. spicatum*. Se1 – izpostavljene prvič 10 mg Se L^{-1}; Se2 – izpostavljene drugič 10 mg Se L^{-1}.

One explanation for the lower amount of Se in plants growing in water could be that some small amount of Se was only adsorbed on the surface of the plants and was released to the water.

Conclusions

Photochemical efficiency was similar in macrophytes exposed to 10 mg Se(IV) L^{-1}, in water without Se and in water, containing 10 mg Se(IV) L^{-1} once again. Alternation in the concentration of Se in the water in transplantation experiment demanded metabolic changes in studied plants, which were evidenced by the measurement of ETS activity. The amount of chlorophylls was higher in plants of *M. spicatum* growing in water without Se in comparison to plants growing in water with Se, while the amount of carotenoids and anthocyanins decreased in water without Se for this species. The recovery of the plants, grew in water, was not observed. The amount of Se was higher in plants exposed to Se, while in plants that grew in water without Se, the amount of Se decreased for both studied species.

Povzetek

Makrofiti so bili izpostavljeni 10 mg Se(IV) L^{-1}, kasneje smo jih prestavili v vodo brez dodanega Se, nato pa ponovno v vodo, ki je vsebovala 10 mg Se(IV) L^{-1}. Vrednosti fotokemične učinkovistosti so bile v vseh obravnavanjih podobne. Menjavanje koncentracij Se v mediju, kjer so rastline rastle, je povzročilo spremembe v metabolizmu rastlin, kar se je pokazalo pri meritvah aktivnosti ETS. Vsebnost klorofilov je bila višja v vrsti *M. spicatum*, ki je bil izpostavljen Se v primerjavi z rastlinami, ki so rastle v vodi brez dodanega Se, medtem ko je bila vsebnost karotenoidov in antocianinov nižja pri rastlinah, ki so uspevale v vodi brez dodanega Se. Pri rastlinah v vodi brez dodanega Se ni bilo opaznega izboljšanja stanja rastlin. Vsebnost Se je bila višja v rastlinah, ki so bile izpostavljene Se, medtem ko so rastline, ki so rastle v vodi brez dodanega Se, vsebovale manj Se pri obeh vrstah.

Acknowledgements

The authors would like to thank Martin Vrhovšek for technical support. This research was financed by the Ministry of Higher Education, Science and Technology of the Republic of Slovenia through the program "Young researchers" (32059) and "Biology of plants" (P1–0212).

References

Boyd, C.E., 1970. Production, mineral nutrient accumulation and pigment concentration in *Typha latifolia* and *Scirpus americanus*. Ecology, 51, 285–290.

Canton, S.P., Van Derveer, W.D., 1997. Selenium toxicity to aquatic life: An argument for sediment-based water quality criteria. Environmental Toxicology and Chemistry, 16, 1255–1259.

Delmail, D., Labrousse, P., Houdrin, P., Larcher, L., Moesch, C., Botineau, M., 2011. Ohysiological, anatomical and phenotypical effects of a cadmium stress in different-aged chlorophyllian organs of *Myriophyllum alternifolium* DC (Haloragaceae). Environmental and Experimental Botany, 72, 174–181.

Dhote S., Dixit S. 2009. Water quality improvement through macrophytes – a review. Environmental Monitoring and Assessment, 152, 149–153.

Drumm, H., Mohr, H., 1978. Mode of interaction between blue (UV) light photoreceptor and phytochrome in anthocyanin formation of *Sorghum* seedling. Photochemistry and Photobiology, 27, 241–248.

Fan, T.W.M., Teh, S.J., Hinton, D.E., Higashi, R.M., 2002. Selenium biotransformations into proteinaceous forms by foodweb organisms of selenium-laden drainage waters in California. Aquatic Toxicology, 57, 65–84.

Germ, M, Gaberščik, A., 2003. Comparison of aerial and submerged leaves in two amphibious species, *Myosotis scorpioides* and *Ranunculus trichophyllus*. Photosysntheitca, 41, 91–96

Kenner, R.A., Ahmed, S.I., 1975. Measurements of electron transport activities in marine phytoplankton. Marine Biology, 33, 119–127.

Larcher, W., 2003: Physiological plant ecology, 4th ed. Berlin: Springer, 513 pp.

Lemly, A.D., 2004. Aquatic selenium pollution is a global environmental safety issue. Ecotoxicology and Environmental Safety, 59, 44–56.

Lichtenthaler, H.K., Buschmann, C., 2001a. Extraction of photosynthetic tissues: chlorophylls and carotenoids. In: Wrolstad, R.E. et al. (Eds.): Current Protocols in Food Analytical Chemistry, John Wiley & Sons Inc., New York, F.4.2.1 – 4.2.6.

Lichtenthaler, H.K., Buschmann, C., 2001b. Chlorophylls and carotenoids: measurement and characterisation by UV-VIS. In: Wrolstad, R.E. et al. (Eds.): Current Protocols in Food Analytical Chemistry, John Wiley & Sons Inc., New York, F.4.2.1 – 4.2.6.

Mechora, Š., Cuderman, P., Stibilj, V., Germ, M., 2011. Distribution of Se and its species in *Myriophyllum spicatum* and *Ceratophyllum demersum* growing in water containing Se (VI). Chemosphere, 84, 1636–1641.

Packard, T.T., 1971. The measurement of respiratory electron-transport activity in marine phytoplankton. Journal of Marine Research, 29, 235–243.

Phillips, D.J.H., 1977. The use of biological indicator organisms to monitor trace metal pollution in marine and estuarine environments. A review. Environmental Pollution, 13, 281–317.

Rai, U.N., Sinha, S., Tripathi, R.D., Chandra, P., 1995. Wastewater treatability potential of some aquatic macrophytes: Removal of heavy metals. Ecol. eng. 5, 5–12.

Robinson, B., Kim, N., Marchetti, M., Moni, C., Schroeter, L., van den Dijssel, C., Milne, G., Clothier, B., 2006. Arsenic hyperaccumulation by aquatic macrophytes in the Taupo Volcanic Zone, New Zealand. Environmental and Experimental Botany, 58, 206–215.

Schreiber, U., Bilger, W., Neubauer, C., 1995. Chlorophyll fluorescence as a nonintrusive indicator for rapid assessment of *in vivo* photosynthesis. In: Schulze, E.D., Caldwell, M.M. (Eds.): Ecophysiology and Photosynthesis, Springer-Verlag, Berlin – Heidelberg – New York, pp. 49–70.

Sivaci, E.R., Sivaci, A., Sökmen, M., 2004. Biosorption of cadmium by *Myriophyllum spicatum* L. and *Myriophyllum tryphyllum* orchard. Chemosphere, 56, 1043–1048.

Smrkolj, P., Stibilj, V., 2004. Determination of selenium in vegetables by hydride generation atomic fluorescence spectrometry. Analitica Chimica Acta, 512, 11–17.

Winkel-Shirley, B., 2002. Biosynthesis of flavonoids and effects of stress. Current opinion in Plant Biology, 5, 218–223.

Zayed A., Lytle C.M., Terry N. 1998. Accumulation and volatilization of different chemical species of selenium by plants. Planta 206, 284–292.

ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2012 Vol. 55, Št. 1: 17–27

Preliminary multispecies test of a model for non-lethal estimation of metabolic activity in freshwater crayfish

Preliminarni test modela za oceno metabolne aktivnosti pri več vrstah potočnih rakov

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Abstract: We tested the applicability of electron transport system (ETS) derived from a single leg as a tool for non-lethal assessment of metabolic activity in freshwater crayfish. ETS activity of the whole body and of a leg was measured in four crayfish (Arthropoda, Crustacea, Decapoda) species: two European (*Astacus astacus*, *Austropotamobius torrentium)*, and two North American (*Orconectes limosus, Pacifastacus leniusculus)*. Mass scaling of whole body ETS activity (ETS_{whole}) and leg ETS activity (ETS_{leg}) was not significantly different for the European *A. astacus* and the American *O. limosus.* Therefore common models were constructed and tested on the remaining two species. The ratio ETS_{whole}/ETS_{leg} was significantly positively related to body mass. In the first model (model 1) ETS_{whole} was calculated from ETS_{leg} multiplied by the ratio estimated from the known body mass. ETS_{whole} of *A. torrentium* was underestimated by this model, because they mature at smaller body size than the larger species. A direct relation between ETS_{leg} and ETS_{whole} was therefore proposed as a general model (model 2), since they are correlated similarly in the studied species. The results show that model 2 is suitable for estimating the whole body ETS activity from leg ETS activity for the four investigated decapods.

Keywords: electron transport system (ETS) activity, crayfish, size scaling, method

Izvleček: V raziskavi smo testirali uporabnost modela za oceno metabolne aktivnosti pri potočnih rakih z merjenjem aktivnosti elektronskega transportnega sistema (ETS) le na eni nogi. Aktivnost ETS smo merili na celih osebkih in na nogi pri štirih vrstah potočnih rakov: dveh evropskih (*Astacus astacus*, *Austropotamobius torrentium)*, in dveh severnoameriških (*Orconectes limosus, Pacifastacus leniusculus)*. Ker se spreminjanje aktivnosti ETS celega telesa (ETS_{whole}) in aktivnosti ETS noge (ETS_{leg}) ni razlikovalo med jelševcem *A. astacus* in trnavcem *O. limosus,* smo naredili skupni model, ki smo ga testirali na preostalih vrstah. Razmerje ETS_{whole}/ETS_{leg} je bilo v pozitivnem razmerju z maso telesa. Pri prvem modelu (model 1) smo ETS_{whole} izračunali iz ETS_{leg} tako, da smo jo pomnožili z razmerjem, ki smo ga ocenili iz znane mase rakov. Izkazalo se je, da smo s tem modelom podcenili ETS_{whole} pri koščaku *A. torrentium,* verjetno zaradi nastopa zrelosti pri manjši velikosti kot pri večjih vrstah. Tako smo predlagali kot splošni model neposredno povezanost med ETS_{leg} in ETS_{whole}, ki sta pri vseh vrstah rakov v podobnem razmerju. Rezultati so pokazali, da na podlagi modela

2 lahko na osnovi izmerjene aktivnosti ETS pri nogi napovemo aktivnost ETS pri vseh testiranih vrstah rakov.

Ključne besede: aktivnost elektronskega transportnega sistema (ETS), potočni raki, velikost, metoda

Introduction

Freshwater crayfish are the largest freshwater macroinvertebrates. They are becoming recognized increasingly for their importance in the natural elimination of the dead organisms (Covich et al. 1999, Nyström 2002). In Europe, the increasing loss of freshwater habitats, coupled with the spread of non-indigenous North American crayfish species, and by the infection of *Aphanomyces astaci* that selectively kills the European species, populations of European crayfish has dramatically reduced (Holdich et al. 2009). The ecology of the native and introduced species and interactions between them have been studied intensively (e.g. Tamkevičiene 1988, Firkins and Holdich 1993, Holdich et al. 1995, Gil-Sánchez and Alba-Tercedor 2002, Paglianti and Gherardi 2004, Hudina et al. 2011). However, our knowledge about the role of crayfish species in the ecosystem in regard to energy flux and nutrient cycling through their metabolic activity is very limited.

Crayfish are key energy transformers among the different trophic levels since animals show omnivorous feeding character. They make major sources of energy (detrital material, decaying wood, dead organisms and periphyton) available to higher trophic levels at a more rapid rate than any other consumers (Momot et al. 1978). An estimate of crayfish metabolic activity could provide useful information for ecophysiological studies that deal with energy flow through ecosystems with crayfish populations. However, many crayfish species are threatened with population decline or extinction (Taylor 2002), so the number of experimental animals that can be taken from the wild is usually limited. Therefore, an elaboration of a new method that could provide samples of crayfish without killing them, or even without taking them from the wild, would be revolutionary.

Most comparative physiological studies on crayfish have been conducted on adult specimens (e.g. Demers et al. 2006, Styrishave et al. 2007). However, the effect of environmental pollution in the early development stages could be very different. For the study of this, the survey of the intra- and inter-specific, and size-dependent physiological reactions is very necessary. Knowledge of the metabolic activity of different sized crayfish would serve as a basis for such other ecophysiological population studies, in which the metabolic activity of entire crayfish populations has to be estimated, taking into account age and size. Most studies on the relationship between metabolic rate and body size in crustaceans have used respiration rate as a measure of metabolic activity (Buikema 1972, Ivleva 1980, Wheatly 1989, Glazier 1991, Marshall et al. 2003), which is impractical when dealing with large and endangered crayfish species. However, if organisms are basically similar in body size, size-related changes in most biochemical and physiological processes should parallel the scaling of metabolism (Peters 1983). Therefore, the use of enzyme activity to assess metabolic rates appears to be a better approach, as demonstrated in studies on several crustacean species which also took into account the body size effects (Berges and Ballantyne 1991, Berges et al. 1990, 1993, Simčič and Brancelj 2003).

The test of the enzymatic respiratory electron transport system (ETS) activity is a useful tool for estimating metabolic potential in aquatic organisms, since the result indicates the amount of oxygen would be consumed if all enzymes functioned maximally (Muskó et al. 1995). The method is simple, rapid and sensitive and has been used extensively on zooplankton (e.g. Bamstedt 1980, 1988, Borgmann 1978, James 1987, G.-Tóth and Drits 1991, G.-Tóth et al. 1995a; Simčič and Brancelj 1997, 2004), various amphipod and isopod species (e.g. Muskó et al. 1995, Simčič and Brancelj 2006, 2007, Simčič et al. 2005, 2010, Mezek et al. 2010) and fish (G.-Tóth et al. 1995b). Nevertheless, it has been rarely used in

decapods. Borgmann (1977) studied ETS activities in various tissues of the crayfish *Orconectes propinquus* (Girard, 1852), while Simčič et al. (2012) used whole animal homogenization to measure the ETS activity of the whole animal (ETS_{whole}) in the noble crayfish *Astacus astacus* (Linnaeus, 1758) in order to relate it to oxygen consumption. To avoid killing animals and to circumvent the impractical whole body homogenization procedure in further crayfish studies, Simčič et al. (2012) proposed a new approach to estimate the metabolic activity of whole crayfish based on measuring ETS activity of a leg (ETS_{leg}). The leg can easily be removed in the field and regenerates afterwards without harmful effects on the animal. The method was however tested only for a single species, while it is essential to know whether a general model could be established and used for different crayfish species.

The four species included in the present study belong to two crayfish families with natural distribution ranges in Europe (EU) and North America (NA): Astacidae (EU: *Astacus astacus* (Linnaeus, 1758), *Austropotamobius torrentium* (Schrank, 1803) NA: *Pacifastacus leniusculus* (Dana, 1852)), and Cambaridae (NA: *Orconectes limosus* (Rafinesque, 1817)). All species are distributed in lentic and lotic freshwater ecosystems in temperate regions (Holdich 2002). The aims of the present study were to determine size scaling of the relationship between ETS_{whole} and ETS_{leg} in two crayfish species, in order to establish a general model that can be used for estimating the metabolic activity of a whole crayfish on the basis of measured ETS_{leg}. The proposed model has been tested on additional two crayfish species.

Material and Methods

Four crayfish species were included in the study. Two species are indigenous to Europe, *Astacus astacus* (AA; n = 35), *Austropotamobius torrentium* (AT; n = 6), and two to North America, *Orconectes limosus* (OL; n = 12) and *Pacifastacus leniusculus* (PL; n = 5). One European (AA) and one North American (OL) species were used for model construction and the other two for validation of the models.

All specimens used in the laboratory tests were sampled in natural water bodies in Slovenia and Italy in 2009 and 2010. Protected and endangered species were collected under special licence No. 35601-135/2010-10 (issued by the Slovenian Environment Agency) in limited numbers. Live crayfish were transported to the laboratory in thermo-isolated bags in order to reduce stress effects. In the laboratory all specimens were maintained, prior to use, in aerated dechlorinated tap water for three weeks at 10°C and photoperiod light:dark = 16:8 hours. They were fed a commercial food (Sera crabs natural) *ad libitum*. Before measurements, the animals were weighed to the nearest 0.1 mg.

Electron transport system (ETS) activity was measured using the method originally proposed by Packard (1971) and improved by G.-Tóth (1999). The third walking leg or the whole crayfish was homogenized in liquid nitrogen using a mortar. A weighed amount (50 – 90 mg wet mass) was sonicated in 4 ml of ice-cold homogenization buffer (0.1 M sodium phosphate buffer pH = 8.4; 75 µM $MgSO_4$; 0.15% (w/v) polyvinyl pyrrolidone; 0.2% (v/v) Triton-X-100) for 20 sec (4710; Cole-Parmer) and centrifuged at 8500 x g for 4 min at 0ºC (Centrifuge Sigma). Three 0.5 ml samples from each homogenate were incubated for 30 min at 10 °C in 1.5 ml substrate solution (0.1 M sodium phosphate buffer pH = 8.4; 1.7 mM NADH; 0.25 mM NADPH; 0.2% (v/v) Triton-X-100) with 0.5 ml 2.5 mM 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) solution. The reaction was ended by addition of 0.5 ml of stopping solution (formalin: H_3PO_4 conc = 1:1 v/v). Blanks (1.5 ml substrate solution and 0.5 ml INT solution) were incubated and treated as for the samples, followed by addition of 0.5 ml of homogenate. Formazan production was determined spectrophotometrically from the absorbance of the sample at 490 nm against the control blank within 10 min of stopping the reaction (WTW PhotoLabSpektral). ETS activity was calculated according to Kenner and Ahmed (1975).

Since body protein content measured in homogenates of whole animals is generally a constant fraction of organism composition and therefore often used in size scaling studies (Berges and Ballantyne 1991), we measured the protein concentration in whole body ($PROT_{whole}$) and legs

($PROT_{leg}$), using a commercial BCA Protein assay kit (Pierce). 0.1 ml of homogenate, from either the leg or the whole body, was pipetted into a test tube with 2.0 ml of reagent and mixed well. Samples were incubated at 37 °C for 30 min. Absorbance was measured at 562 nm against the blank (distilled water) within 10 min and the protein concentration determined from a calibration curve constructed with bovine albumin as standard.

For model construction we used two species, one indigenous (AA) and one non-indigenous (OL), for each of which we obtained a sufficiently large amount of data to distinguish between general and species-specific patterns. The effect of sex on body mass, ETS_{whole}, ETS_{leg}, $PROT_{whole}$, $PROT_{leg}$, and on the ratio ETS_{whole}/ETS_{leg} was tested with separate paired t-tests for each species. Since there was no significant effect ($P > 0.05$), both sets of data were pooled in further analyses. Least-squares regression analyses were performed to establish the mass scaling of the ETS activity and protein content variables. Regressions were performed with a power model of the form $y = ax^b$, where y is ETS activity or protein content, x is wet body mass of a crayfish and *a* and *b* are regression coefficients. We tested whether a single mass scaling equation can be used for both AA and OL. The improved fit obtained by taking into account species identity was evaluated by multiple regression in which species identity was added with a binary dummy variable A. The variables were *ln* transformed as necessary to fit a linear regression model: $lny = ln(a)+blnx+cA+d(A \times lnx)$. The coefficient *c* tests the significance of the difference in intercepts while *d* tests for the difference in slopes. Separate models were deemed necessary if either slope or intercept, or both, differed significantly between species. If not, a joint equation was calculated from the pooled AA and OL data. The statistically significant equations are shown in the Figures 1–4.

Pearson correlation between ETS activity and protein content was calculated separately for whole bodies and for legs. The relationship between ETS activity and protein content was established, by power regression, separately for whole bodies and for legs. The difference between the regression models for AA and OL was tested as for mass scaling.

We tested two models for predicting ETS_{whole} from ETS_{leg}. The first model involves the use of the ratio ETS_{whole}/ETS_{leg}, estimated from wet mass (WW). ETS_{whole} is thus obtained by multiplying the ETS_{leg} value with the estimated ratio. The final model is:

$$ETS_{whole} = aWW^b\ ETS_{leg} \quad \text{(model 1)}$$

The alternative model predicts ETS_{whole} directly from ETS_{leg}:

$$ETS_{whole} = aETS_{leg}^{\ b} \quad \text{(model 2)}$$

Body mass was excluded from model 2, since it did not contribute significantly to the fit ($P > 0.05$). We tested for the difference between the models for AA and OL by including the dummy

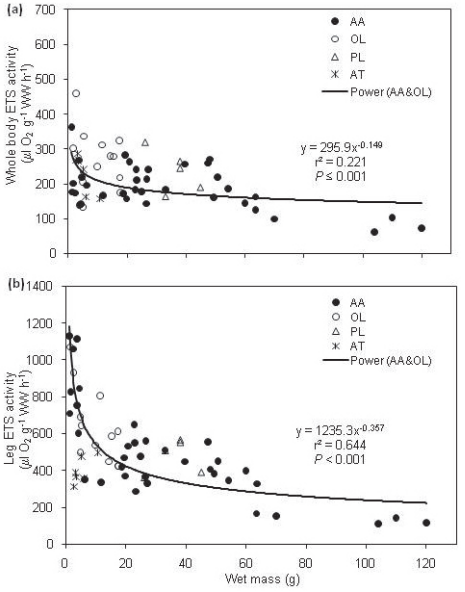

Figure1: The relationship between wet mass and (a) electron transport system (ETS) activity of whole body and (b) ETS activity of a leg in different crayfish species (OL – *Orconectes limosus*, AA – *Astacus astacus*, PL – *Pacifastacus leniusculus,* AT – *Austropotamobius torrentium*). The function was derived based on AA and OL data only.

Slika 1: Razmerje med svežo maso in (a) aktivnostjo elektronskega transportnega sistema (ETS) celega telesa in (b) aktivnostjo ETS noge za različne vrste potočnih rakov (OL – *Orconectes limosus*, AA – *Astacus astacus*, PL – *Pacifastacus leniusculus,* AT – *Austropotamobius torrentium*). Funkcija je bila narejena le na osnovi podatkov za AA in OL.

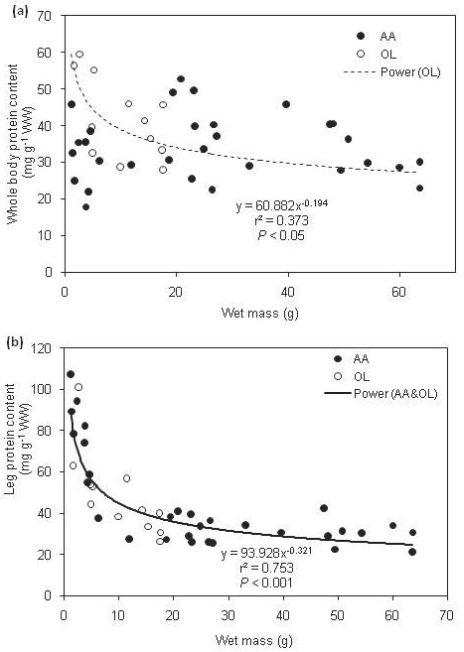

Figure 2: The relationship between wet mass and (a) protein content of whole body and (b) protein content of a leg in *Orconectes limosus* (OL) and *Astacus astacus* (AA).

Slika 2: Razmerje med svežo maso in (a) vsebnostjo proteinov v celem telesu in (b) vsebnostjo proteinov v celem telesu pri *Orconectes limosus* (OL) in *Astacus astacus* (AA).

variable A. Both models ultimately contained the same number of parameters to be estimated, so they could be compared on the basis of the adjusted r^2 values.

Finally we evaluated the applicability of the joint AA and OL models for two other crayfish species (PL, AT). Values of ETS_{whole} measured in these species were compared with those predicted from the two joint AA and OL models with paired t-tests. The same method was used to test whether mass scaling equations for protein content and ETS activity constructed with AA and OL data function also for the other two crayfish species. All statistical analyses were conducted in SPSS 13.0.

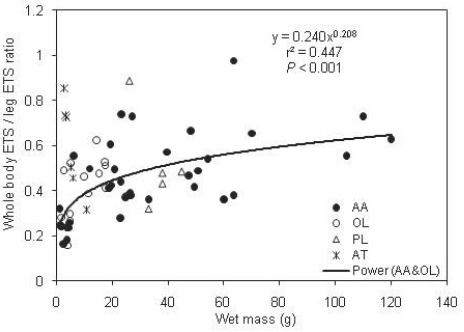

Figure 3: The relationship between wet mass and the ratio of electron transport system (ETS) activity of whole body to that of a leg, in different crayfish species (OL – *Orconectes limosus*, AA – *Astacus astacus*, PL – *Pacifastacus leniusculus,* AT – *Austropotamobius torrentium*). The function was derived based on AA and OL data only.

Slika 3: Odnos med svežo maso in razmerjem med aktivnostjo elektronskega transportnega sistema (ETS) celega raka in noge pri različnih vrstah potočnih rakov (OL – *Orconectes limosus*, AA – *Astacus astacus*, PL – *Pacifastacus leniusculus,* AT – *Austropotamobius torrentium*). Funkcija je bila narejena le na osnovi podatkov za AA in OL.

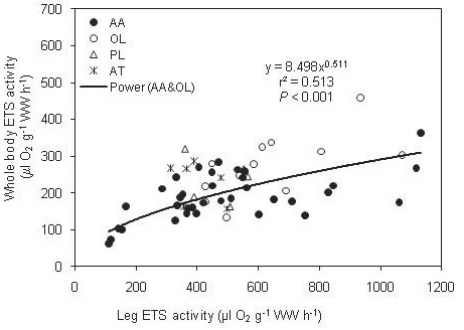

Figure 4: The relationship between electron transport system (ETS) activity in a leg and that of whole body in different crayfish species (OL – *Orconectes limosus*, AA – *Astacus astacus*, PL – *Pacifastacus leniusculus,* AT – *Austropotamobius torrentium*). The function was derived based on AA and OL data only.

Slika 4: Razmerje med aktivnostjo elektronskega transportnega sistema (ETS) noge in celega raka pri različnih vrstah potočnih rakov (OL – *Orconectes limosus*, AA – *Astacus astacus*, PL – *Pacifastacus leniusculus,* AT – *Austropotamobius torrentium*). Funkcija je bila narejena le na osnovi podatkov za AA in OL.

	Intercept		Slope	
Relationship	t	*P*	t	*P*
ETS_{whole} – wet mass	0.655	0.516	0.218	0.829
ETS_{leg} – wet mass	0.339	0.736	0.574	0.569
$PROT_{whole}$ – wet mass	2.758	0.009	2.081	0.044
$PROT_{leg}$ – wet mass	0.068	0.946	0.366	0.716
ETS_{whole}/ETS_{leg} – wet mass	1.200	0.237	0.426	0.672
ETS_{whole} – ETS_{leg}	0.720	0.476	0.835	0.408

Results

Size-structured samples (based on their body mass) of 58 crayfish specimens were used in laboratory experiments. Models were constructed on the basis of measurements on 47 specimens (AA, OL), and later evaluated on the basis of 11 specimens (AT, PL).

The mass of specimens did not differ significantly between sexes in AA and OL ($P > 0.05$), so differences in investigated parameters between sexes were tested using t-test. Since sex had no effect on values of ETS_{whole}, ETS_{leg}, $PROT_{whole}$, $PROT_{leg}$, or on the ratio ETS_{whole}/ETS_{leg} in AA and OL (t-tests, $P > 0.05$), the data for both sexes were pooled for further analysis.

Values of mass scaling of ETS_{whole} for AA (0.877) and OL (0.907) did not differ significantly (Table 1), so a single equation for pooled data was established (Fig. 1a). Insignificant differences were also observed between the two species in the regression of ETS_{leg} and wet mass (Table 1; Fig. 1b). Both ETS_{whole} and ETS_{leg} decreased with increasing wet mass.

The regression models for mass scaling of $PROT_{whole}$ for AA and OL differed significantly (Table 1). $PROT_{whole}$ of AA was independent of body mass ($r^2 = 0.005$, df = 28, $P > 0.05$), whereas that of OL decreased with body mass (Fig. 2a). On the other hand, $PROT_{leg}$ in AA and in OL decreased similarly with wet mass according to a power law (Fig. 2b). ETS activity correlated significantly with $PROT_{whole}$ (r = 0.788, n = 42, $P < 0.001$) and in $PROT_{leg}$ (r = 0.870, n = 42, $P < 0.001$). Similar scaling exponents *b* were observed for AA and OL (0.973 and 1.117) when the ETS_{whole} was expressed in relation to protein mass of crayfish with a common scaling exponent of 0.961.

The ratio ETS_{whole}/ETS_{leg} scaled similarly in AA and OL (Table 1) and showed a significant positive correlation with wet mass (Fig. 3). The common model (model 1), predicting ETS_{whole} from ETS_{leg} with the help of this estimated mass ratio, explained 45% of the variation in whole body ETS activity of AA and OL:

$$ETS_{whole} = 0.240\ WW^{0.208}\ ETS_{leg} \quad \text{(model 1)}$$

ETS_{whole} was estimated directly from ETS_{leg} using a power equation (Fig. 4). The regression coefficients for AA and OL did not differ significantly, so a common model (model 2) was constructed:

$$ETS_{whole} = 8.498\ ETS_{leg}^{0.511} \quad \text{(model 2)}$$

This model explained 51% of the variance in ETS_{whole} of OL and AA.

Mass scaling of ETS_{whole} was similar in all the crayfish species in this study. The observed values of ETS_{whole} of PL (t-test, t = 2.388, df = 4, $P > 0.05$), and AT (t-test, t = 0.305, df = 5, $P > 0.05$) did not differ significantly from those predicted with the mass scaling equation established from the AA and OL data. Similar results were obtained for ETS_{leg}, except for the ETS_{leg} of AT, where the observed and predicted values differed significantly (t-test, t = 4.270, df = 5, $P < 0.01$), indicating different mass scaling of ETS_{leg} in AT from those in OL and AA.

The observed values of ETS_{whole} of PL did not differ significantly from the predicted values obtained from model 1 (t-test, t = 0.062, df = 4, $P > 0.05$). However, the predicted values of ETS_{whole} of AT (t-test, t = 2.768, df = 5, $P < 0.05$) differed significantly from the observed values when model 1 was used.

Predicted values of ETS_{whole} obtained with model 2 for PL (t-test, t = 1.248, df = 4, $P > 0.05$), and AT (t-test, t = 1.840, df = 5, $P > 0.05$) did not differ significantly from the measured ETS_{whole}.

Discussion

It seems that estimating the metabolic activity of crayfish from a single leg only is an adjustable method and that the model for the relationship between ETS_{whole} and ETS_{leg} can be applied to different crayfish species. The relation between ETS activity and wet mass is in agreement with the findings in previous investigations that ETS activity varies with body mass according to a power law (Cammen et al. 1990, Muskó et al. 1995, Simčič and Brancelj 1997, 2003; Simčič et al. 2012). In two crustaceans, *Chirocephalus croaticus* (Steuer, 1899) and *Gammarus fossarum* Koch, 1835, it was shown that ETS activity is related to body size in a manner typical of a metabolic function with the scaling exponent *b* being 0.787 and 0.651, respectively (Simčič and Brancelj 2000, 2003). Since mass scaling of ETS activity did not differ significantly between AA and OL, a common equation for pooled data was proposed. The established *b*-value was in the range reported for the metabolic rate of crustaceans in general (Wolvekamp and Waterman 1960, Ivleva 1980). Comparison of intra- versus inter-specific *b* exponents for oxygen consumption in aquatic crustaceans showed that the intraspecific slope was approximately 0.1 less than the slope of the overall collected data (Wheatly 1989). In crayfish we have found even smaller differences between intra- and inter-specific *b* exponents for ETS activity. Berges and Ballantyne (1991) reported that intra- and inter-specific exponents for whole body maximal enzyme activities in aquatic crustaceans, i.e. *Macrobrachium rosenbergii* (De Man, 1879), *Artemia franciscana* (Kellogg, 1906) and *Daphnia magna* (Straus, 1820), were similar for enzymes such as citrate synthase, but significant differences between species were found for enzymes associated with pathways other than aerobic metabolism. However, several authors have assigned the decrease in mass-specific metabolic activity to an increasing proportion of metabolically inert mass as the animals grow (Glazier 1991, Simčič and Brancelj 2003). Thus, the proportion of metabolically inactive tissue differs with species and varies with size and developmental stage of the same species.

The protein content per gram of whole crayfish mass did not change with increasing wet mass in AA, but decreased significantly in OL (Fig. 2a). However, when the ETS activity was expressed in relation to protein mass, similar exponents were observed for AA and OL. The common scaling exponent for ETS activity of the two species was 0.961, similar to the exponents reported for decapod species *M. rosenbergii* for a variety of enzymes, where whole animals were homogenized and the enzyme activity was expressed in relation to protein mass (Berges and Ballantyne 1991). A closely similar *b*-value (0.955) was found for the relationship between the amount of protoplasm and ETS activity per individual in *G. fossarum* (Simčič and Brancelj 2003). Berges and Ballantyne (1991) reported that an exponent close to 1.0 is characteristic of enzymes capable of functioning catabolically or anabolically. Moreover, for larger crustaceans, such as *M. rosenbergii*, that rely increasingly on anaerobic metabolism as body size increases, scaling exponents closer to 1.0 are expected for the enzymes that function in both anaerobic and aerobic processes. The exponent close to 1.0 obtained for ETS_{whole} in the present study was in accord with the findings of Berges and Ballantyne (1991), since ETS activity measures both aerobic and anaerobic metabolism (Packard 1985).

ETS_{whole} and ETS_{leg} exhibited different mass scaling exponents (Fig. 1). This was expected, because different tissues contribute to the sample for ETS measurements in the two cases. Borgmann (1977) found that ETS activities differed in the various tissues of the crayfish *Orconectes propinquus*. Thus, ETS_{whole} reflects the activity of the mixture of the large number of different metabolically active tissues, body storage materials and exoskeleton, while in a leg sample muscle tissue and exoskeleton material predominate. Moreover, the proportion of protein material in a leg decreased up to 10 g of body mass, while in larger animals it was relatively constant (Fig. 2b). The decrease of $PROT_{leg}$ with similar exponents in the two species could mean a lower variability in $PROT_{leg}$ than in $PROT_{whole}$. The reason for higher variability in the relation between protein content and body mass probably lies in variable amounts of different storage materials and other metabolically inert tissues in the crayfish during their inter-annual life history, as well as in their age. However, the ETS_{whole} and ETS_{leg} correlated

well with their protein content, indicating that the latter plays a key role in ETS activity.

Since ETS_{whole} and ETS_{leg} scaled differently with body mass (Fig. 1), the ratio ETS_{whole}/ETS_{leg} showed a significant, positive power relationship with body mass (Fig. 3). Similar scaling of the ETS_{whole}/ETS_{leg} ratio in AA and in OL indicates a greater influence of body size than of species-specific properties on the relationship between whole body and leg metabolic potential. Therefore estimation of the whole crayfish metabolic potential can be estimated from ETS_{leg} using a common ratio for both species, but this ratio depends on body mass. Thus, calculation of the ETS_{whole}/ETS_{leg} ratio for a crayfish of a given body mass on the basis of the equation in Fig. 3 provides a factor that can be used for the estimation of ETS_{whole} from ETS_{leg} (model 1). The utility of this model for estimating ETS_{whole} in different crayfish species was tested by comparing observed and predicted values in two crayfish species not used in the model construction. For PL, observed did not differ from predicted values, but the ETS_{whole} of AT was underestimated by this model (Fig. 3). The reason probably lies in the different developmental stage of 2–5 g AT from that of other crayfish of this size. Wheatly (1989) reported that the comparison of organisms at different developmental stages is problematic due to the different physiology of immature and adult individuals. Small-sized crayfish species such as AT attain their maturity at a mass of 2 to 5 g, while large-sized species become mature at a mass of more than 5 g (Souty-Grosset et al. 2006). It means that the specimens of AT were actually in a mature developmental stage, but that the same sized individuals of other species were still immature, and therefore possessed different physiological characteristics. Berges et al. (1990) found that the activity per unit mass of the primary anabolic enzyme nucleoside diphosphate kinase (NDPK) decreases with size and that enzyme scaling is affected by differences in growth rate. Thus, different development stages probably contribute to the relatively low ETS_{leg} in AT.

To minimize the potential effect of different development stages on estimated ETS activity, we explored a model relating ETS_{whole} directly to ETS_{leg}. A common model (model 2) was constructed for both species, since the species-specific models did not differ significantly (Fig. 4). High variability in physiological and biochemical variables, especially due to different moulting stages, reproduction cycle and fitness, resulted in scattered data and, consequently, low coefficients of determination. Nevertheless, the results confirmed our expectation that the metabolic potentials of a whole crayfish and of a leg scale similarly in different crayfish species, since the whole body ETS activity estimated from a leg did not differ significantly from the observed value in all species investigated.

The results of this study suggested that the metabolic potential of whole crayfish could be estimated on the basis of the ETS activity measurement in a single leg, using a general model. Due to the non-lethal approach, the new method could allow larger sample sizes to be incorporated into metabolic activity studies on crayfish, which is essential in conducting studies on multi-population and interspecific levels. Direct measurement of respiration is time-consuming and impractical and may subject the animal to stress before and during measurement. The slow response of ETS activity to short-term variations in environmental factors or stress makes the method superior to direct respiratory measurements on incubated animals (Bamstedt 1980). Moreover, the results of the present study also revealed that the two species-specific models did not provide significantly better estimates of whole crayfish metabolic activity than the joint ones. Model 2, by which ETS_{whole} was related directly to ETS_{leg}, seems the most appropriate for an approximate estimation of whole body metabolic activity in all tested species. Further studies taking into account more species and larger samples could contribute to a clearer picture of the generality of model use. The proposed model, in which only population density, size structure (as body mass) and ETS_{leg} are needed for estimating whole population metabolic activity, could be applied, in particular, for the estimation of metabolic activity in endangered and rare crayfish species.

Acknowledgements

We thank Andrej Kapla and Martina Jaklič for assistance, Dr. Bruno Maiolini for providing the experimental animals and Dr. Roger Pain for linguistic improvement of the manuscript. We acknowledge the financial support of the Slovenian Research Agency (Project L1-2169).

References

Bamstedt, U. 1980. ETS activity as an estimator of respiratory rate of zooplankton populations. The significance of variations in environmental factors. J. Exp. Mar. Biol. Ecol., 42, 267–283.

Bamstedt, U. 1988. Ecological significance of individual variability in copepod bioenergetics. Hydrobiologia, 167/168, 43–59.

Berges, J. A., Ballantyne, J. S., 1991. Size scaling of whole-body maximal enzyme activities in aquatic crustaceans. Can. J. Fish Aquat. Sci., 48, 2385–2394.

Berges, J. A., Roff, J. C., Ballantyne, J. S., 1990. Relationship between body size, growth rate, and maximal enzyme activities in the brine shrimp, *Artemia franciscana*. Biol. Bull., 179, 287–296.

Berges, J. A., Roff, J. C., Ballantyne, J. S., 1993. Enzymatic indices of respiration and ammonia excretion: relationships to body sizes and food levels. J. Plankton Res., 15, 239–254.

Borgmann, U. 1977. Electron transport system activity in *Daphnia* and crayfish. Can. J. Zool., 55, 847–854.

Borgmann, U., 1978. The effect of temperature and body size on electron transport system activity in freshwater zooplankton. J. Zool., 56, 634–642.

Buikema, A. L. Jr., 1972. Oxygen consumption of the cladoceran, *Daphnia pulex*, as a function of body size, light and light acclimatization. Comp. Biochem. Physiol., 42A, 877–888.

Covich, A. P., Palmer, M. A., Crowl, T. A., 1999. The role of benthic invertebrate species in freshwater ecosystems. BioScience, 49, 119–127.

Cammen, L. M., Corwin, S., Christensen, J. P., 1990. Electron transport system (ETS) activity as a measure of benthic macrofaunal metabolism. Mar. Ecol. Prog. Ser., 65, 171–182.

Demers, A., Souty-Grosset, C., Trouilhe, M.-C., Füreder, L., Renai, B., Gherardi, F., 2006. Tolerance of three European native species of crayfish to hypoxia. Hydrobiologia, 560, 425–432.

Firkins, I., Holdich, D. M., 1993. Thermal studies with three species of freshwater crayfish. Freshwater Crayfish, 9, 241–248.

Gil-Sánchez, J. M., Alba-Tercedor, J., 2002. Ecology of the native and introduced crayfishes *Austropotamobius pallipes* and *Procambarus clarkii* in southern Spain and implications for conservation of the native species. Biol. Conserv., 105, 75–80.

Glazier, D. S., 1991. Separating the respiration rates of embryos and brooding females of *Daphnia magna*: Implications for the cost of brooding and the allometry of metabolic rate. Limnol. Oceanogr., 36, 354–362.

G.-Tóth, L., Drits, A. V., 1991. Respiratory energy loss of zooplankton in Lake Balaton (Hungary) estimated by ETS-activity measurements. Verh. Internat. Verein. Limnol., 24, 993–996.

G.-Tóth, L., Carrillo, P., Cruz-Pizarro, L., 1995a. Respiratory electron transport system (ETS)-activity of the plankton and biofilm in the high-mountain lake La Caldera (Sierra Nevada, Spain). Arch. Hydrobiol, 135, 65–78.

G.-Tóth, L., Szabó, M., Webb, D. J., 1995b. Adaptation of the tetrazolium reduction test for the measurement of the electron transport system (ETS)-activity during the embryonic development of medaka. J. Fish. Biol., 46, 835–844.

G.-Tóth, L., 1999. Aktivität des Elektronentransportsystems, In: von Tümpling W. and G. Friedrich (eds): Biologische Gewässeruntersuchung. Methoden der Biologischen Wasseruntersuchung 2, Gustav Fischer Verl., Jena, Stuttgart, Lübeck, Ulm, pp. 465–473.

Holdich, D. M. (ed.), 2002. Biology of freshwater crayfish. Blackwell Sciences Ltd, Oxford.

Holdich, D. M., Reader, J. P., Rogers, W. D., Harlioglu, M., 1995. Interactions between three species of crayfish (*Austropotamius pallipes, Astacus leptodactylus* and *Pacifastacus leniusculus*). Freshwater Crayfish, 10, 46–56.

Holdich, D. M., Reynolds, J. D., Souty-Grosset, C., Sibley P. J., 2009. A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. Knowl. and Manag. Aquat. Ec., 394–395, 11.

Hudina, S., Galić, N., Roessink, I., Hock, K., 2011. Competitive interactions between co-occuring invaders: identifying asymmetries between two invasive crayfish species. Biol. Invasions, 13, 1791–1803.

Ivleva, I. V., 1980. The dependence of crustacean respiration rate on body mass and habitat temperature. Int. Rev. Gesamten Hydrobiol., 65, 1–47.

James, M. R., 1987. Respiratory rates in cladoceran *Ceriodaphnia dubia* in lake Rotiongaio, a monomictic lake. J. Plankton Res., 9, 573–578.

Kenner, R. A., Ahmed, S. I., 1975. Measurements of electron transport activities in marine phytoplankton. Marine Biology, 33, 119–127.

Marshall, D. J., Perissionotto, R., Holley, J. F., 2003. Respiratory responses of the mysid *Gastrosaccus brevifissura* (Peracarida: Mysidacea), in relation to body size, temperature and salinity. Comp. Biochem. Physiol., 134A, 257–266.

Mezek, T., Simčič, T., Michael, T. A., Brancelj, A., 2010. Effect of fasting on hypogean (*Niphargus stygius*) and epigean (*Gammarus fossarum*) amphipods: a laboratory study. Aquat. Ecol., 44, 397–408.

Momot, W. T., Gowing, H., Jones, P. D., 1978. The dynamics of crayfish and their role in ecosystems. Am. Midl. Nat., 99, 10–35.

Muskó, I. B., G.-Tóth, L., Szábo, E., 1995. Respiration and respiratory electron transport system (ETS) activity of two amphipods: *Corophium curvispinum* G. O. Sars and *Gammarus fossarum* Koch. Pol. Arch. Hydrobiol.,42, 547–558.

Nyström, P., 2002. Ecology, pp. 190–235. In: Holdich, D. M. (ed.), Biology of Freshwater Crayfish. Blackwell Science, Oxford, UK.

Packard, T. T., 1971. The measurement of respiratory electron- transport activity in marine phytoplankton. J. Mar. Res., 29, 235–244.

Packard, T. T., 1985. Measurement of electron transport activity of microplankton, pp. 207–261. In, Jannas, H. and P. J. LeB. Williams (eds), Advances in aquatic microbiology 3, Academic Press, London.

Paglianti, A., Gherardi, F., 2004. Combined effects of temperature and diet on growth and survival of young-of year crayfish: A comparison between indigenous and invasive species. J. Crustacean Biol., 24, 140–148.

Peters, R. H., 1983. The ecological implications of body size. Cambridge University Press, Cambridge.

Simčič, T., Brancelj A., 1997. Electron transport system (ETS) activity and respiration rate in five *Daphnia* species at different temperatures. Hydrobiologia, 360, 117–125.

Simčič, T., Brancelj, A., 2000. Energy exploitation in *Chirocephalus croaticus* (Steuer, 1899) (Crustacea:Anostraca): survival strategy in an intermittent lake. Hydrobiologia, 437, 157–163.

Simčič, T., Brancelj, A., 2003. Estimation of the proportion of metabolically active mass in the amphipod *Gammarus fossarum*. Freshwater Biol., 48, 1093–1099.

Simčič, T., Brancelj, A., 2004. Respiratory electron transport system (ETS) activity as an estimator of the thermal tolerance of two *Daphnia* hybrids. J. Plankton Res., 26, 525–534.

Simčič, T., Lukančič, S., Brancelj, A., 2005. Comparative study of electron transport system activity and oxygen consumption of amphipods from caves and surface habitats. Freshwater Biol., 50, 494–501.

Simčič, T., Brancelj, A., 2006. Effects of pH on electron transport system (ETS) activity and oxygen consumption in *Gammarus fossarum*, *Asellus aquaticus* and *Niphargus sphagnicolus*. Freshwater Biol., 51, 686–694.

Simčič, T., Brancelj, A., 2007. The effect of light on oxygen consumption in two amphipod crustaceans – the hypogean *Niphargus stygius* and the epigean *Gammarus fossarum*. Mar. Fresh. Beh. Physiol. ,40, 141–150.

Simčič, T., Pajk, F., Brancelj, A., 2010. Electron transport system activity and oxygen consumption of two amphibious isopods, epigean *Ligia italica* Fabricius and hypogean *Titanethes albus* (Koch), in air and water. Mar. Fresh. Beh. Physiol., 43, 149–156.

Simčič, T., Pajk, F., Vrezec, A., Brancelj, A., 2012. Size scaling of whole-body metabolic activity in the noble crayfish (*Astacus astacus*) estimated from measurements on a single leg. Freshwater Biol., 57, 39–48.

Souty-Grosset, C., Holdich, D. M., Noël, P. Y., Reynolds, J. D., Haffner, P., 2006. Atlas of Crayfish in Europe. Muséum National d'Histoire Naturelle, Paris.

Styrishave, B., Bojsen, B. H., Witthøfft, H., Andersen, O., 2007. Diurnal variations in physiology and behaviour of the noble crayfish *Astacus astacus* and the signal crayfish *Pacifastacus leniusculus*. Mar. Fresh. Beh. Physiol., 40, 63–77.

Tamkevičiene, E. A., 1988. Growth and development of juveniles of the native and introduced species of freshwater crayfish. Freshwater Crayfish, 7, 396–400.

Taylor, C. A., 2002. Taxonomy and conservation of native crayfish stocks, pp. 236–257. In: Holdich, D. M. (ed.), Biology of freshwater crayfish. Blackwell Science, Oxford.

Wheatly, M. G., 1989. Standard rate of O_2 uptake and body size in the crayfish *Pacifastacus leniusculus* (Dana, 1852) (Decapoda: Astacidae): Intra- versus interspecific relations in crustaceans. J. Crustacean Biol., 9, 212–216.

Wolvekamp, H. P. I., Waterman T. H., 1960. Respiration, pp. 35–100. In: Waterman, T. H. (ed.): Physiology of Crustacea, Vol 1. Academic Press, New York.

Table 1: Results of statistical testing (for details see methods) of the differences in intercept and slope between *Orconectes limosus* and *Astacus astacus* for the specified relationships (ETS_{whole} – whole body ETS activity, ETS_{leg} – leg ETS activity, $PROT_{whole}$ – whole body protein content, $PROT_{leg}$ – protein content of a leg).

Tabela 1: Rezultati statističnega testiranja (za podrobnosti glej metode) razlik v odseku in naklonu med vrstama *Orconectes limosus* in *Astacus astacus* za podana razmerja (ETS_{whole} – aktivnost ETS celega telesa, ETS_{leg} – aktivnost ETS noge, $PROT_{whole}$ – vsebnost proteinov v celem telesu, $PROT_{leg}$ – vsebnost proteinov v nogi).

ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2012 Vol. 55, Št. 1: 29–33

Survey of the *Lynx lynx* distribution in the French Alps: 2005–2009 update.

Spremljanje razširjenosti risa v francoskih Alpah: 2005–2009

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Abstract: As part of the survey of the pan-alpine population of Eurasian Lynx, the French national network of large carnivores experts collected N = 301 data, out of which 159 (n_1 = 2 C1, n_2 = 62 C2, n_3 = 95 C3) were regarded robust enough from a technical point of view to evidence the presence of lynx (compared to 224 data in the previous pentad). Such a rejection rate (46%) significantly differed from that (24%) observed elsewhere in the Lynx area during the same period, but not from that during the previous pentad in the Alps (43%). The rejection rate was dependent on data type: hair and faeces samples were significantly more often rejected than other presence signs (78% vs. 41%). Among other presence signs, prints were more often rejected (55%) than expected, and sightings were less rejected (35%) than expected. Preys were rejected according to expectations given sample sizes. As noted during the previous pentad, a north-south gradient was evidenced in presence signs collected: C1+C2 were more often encountered north to Grenoble than in the southern part of the lynx area, contrary to C3. Using a modelling approach of the trend in the presence area detected, area with regular presence was increasing then stable, whereas a declining trend was noticed in the area newly colonized during the last years.

Keywords*: Lynx lynx*, France, Alps, presence signs, population trend.

Izvleček: V okviru spremljanja pan-alpske popoulacije evrazijskega risa je Francoska nacionalna mreža strokovnjakov za velike zveri zbrala N = 301 podatek o znakih prisotnosti risa, od tega je bilo 159 (n1 = 2 C1, C2 n2 = 62, N3 = 95 C3) podatkov dovolj zanesljivih s tehničnega vidika, da smo jih lahko vključili v analize (primerjalno; v prejšnji pentadi so zbrali 224 podatkov). Takšna zavrnitvena stopnja (46 %) se bistveno razlikuje od (24 %) stopnje v drugih državah na območju prisotnosti risa v istem obdobju, ne pa tudi od pretekle pentade v francoskih Alpah (43 %). Zavrnitvena stopnja je bila odvisna od vrste podatkov: vzorci dlake in iztrebkov so bili precej pogosteje zavrnjeni kot drugi znaki prisotnosti (78 % proti 41 %). Med drugimi znaki prisotnosti, so bile sledi pogosteje zavrnjene (55 %) kot je bilo pričakovano, neposredna opažanja pa so bila zavrnjena redkeje (35 %) kot je bilo pričakovano. Zavrnitvena stopnja pri ostankih plena je bila v skladu s pričakovanji glede na dano velikost vzorca. Kot je navedeno v poročilu za prejšnjo pentado je tudi za to pentado značilen gradient pogostosti znakov sever-jug, kar je razvidno iz zbranih znakov prisotnosti: C1 + C2 so se pogosteje pojavljali severno od Grenobla kot v južnem delu območja prisotnosti risa, v nasprotju s C3, ki so bili pogostejši na jugu. Z modeliranjem trendov

spreminjanja območja prisotnosti risa se je pokazalo, da je trend velikosti območja z redno prisotnostjo risa naraščal nato pa je bil stabilen, medtem ko je bil trend upadanja opazen v na novo koloniziranih območjih v zadnjih letih.

Ključne beside: *Lynx lynx*, Francija, Alpe, znaki prisotnosti, populacijski trend

Introduction

Most of the European large carnivore populations are by nature transboundary and should be monitored as such (Linnell et al. 2008). The alpine Lynx metapopulation is periodically evaluated based on standardized and common protocols as a follow-up of implementing the pan-alpine conservation strategy for the Lynx (Molinari-Jobin et al. 2001, 2003). The present work details how the "French" sub-unit has been changing during the last pentad (2005–2009), providing some insights in the data at hand, and showing how trends in the distribution area could be modelled.

Methods

Data collection

Presence signs are gathered by a national network of field lynx-experts (ca. 1000 people), who have been trained to collect them and describe their technical characteristics according to a standardized protocol and corresponding forms. Signs are further evaluated to check for their robustness and rejected where needed according to methods in Vandel and Stahl (2005). The validated signs were further converted to SCALP criteria (C1, C2, C3) following Molinari-Jobin et al. (in press).

Data analysis

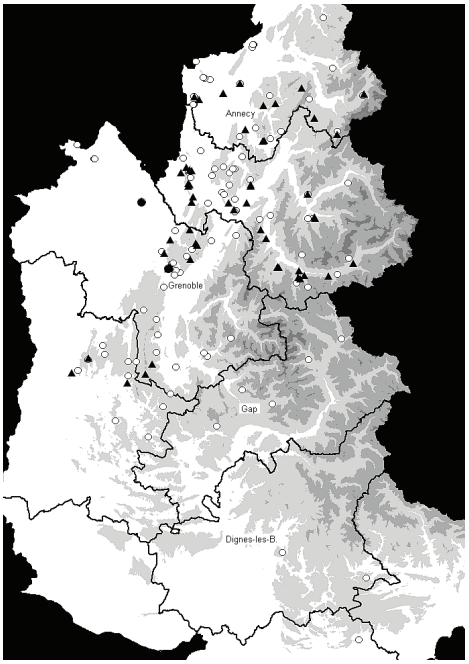
Validation / rejection rates according to data type or period were compared using Chi-square statistics. Validated data were converted into presence area based on Vandel and Stahl (2005). Trends in the area were modelled using log-linear Poisson regression implemented in TRIM (Pannekoek and Van Strien 1998). The method provides trend estimates together with a Goodness-of-Fit test (deviance, DEV) that can be used into an information-theoretic approach, based on Akaike Information Criteria (AIC = DEV + 2 np; see Anderson and Burnham 2002, for a review). AIC has been elaborated to help choosing among candidate models the one best supported by data at hand (e.g. no trend vs. linear trend vs. break points and changing trend). DEV is a measure of the discrepancy between data and the model; the larger the number of parameters (np) in the model, the better it fits to the data, but variance in estimated parameters will be large. Basically one has to solve a bias-variance trade-off, e.g. by selecting the model(s) with the lowest AIC value(s) to balance errors of over- vs. under-fitting.

Results

Data collected

During the 2005–2009 pentad, N = 301 data were collected, out of which 159 (n_1 = 2 C1, n_2 = 62 C2, n_3 = 95 C3) were validated from a technical point of view (compared to 224 data in the previous pentad, Table 1). Such a rejection rate (46%) significantly differed from that (24%) observed elsewhere in the Lynx area during the same period ($\chi^2 = 67.0$, 1 d.f., $p < 0.01$), but not from that during the previous pentad in the Alps (43%, $\chi^2 = 1.1$, 1 d.f., $p = 0.31$). The rejection rate was dependent on data type: hair and faeces samples were significantly more often rejected than other presence signs (78% vs. 41%, $\chi^2 = 27.1$, 3 d.f., $p < 0.01$). Among other presence signs, tracks were more often rejected (55%) than expected, and sightings were less rejected (35%) than expected ($\chi^2 = 5.9$, 2 d.f., $p = 0.05$). Wild ungulate kills were rejected according to expectations given sample sizes.

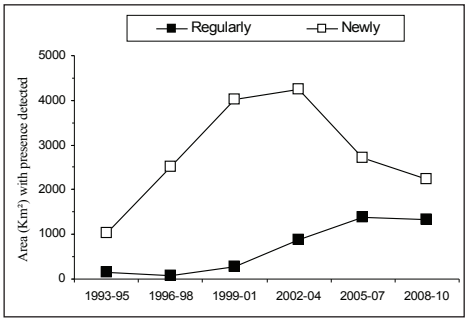
Because the colonizing process of the French Alps seems orientated north to south (Marboutin et al. 2006), data collected north to Grenoble were



Slika 1: Razporeditev ovrednotenih znakov prisotnosti risa (● = C1, ▲ = C2, o = C3) zbranih od 2005 do 2009 v francoskih Alpah; senčena območja predstavljajo višinske pasove (temnejše je višje).

Figure 1: Distribution of validated lynx signs (● = C1, ▲ = C2, o = C3) collected from 2005 to 2009 in the French Alps; shaded areas represent altitudinal patterns (the darker, the higher).

compared to those collected south to this area (Figure 1, Table 2). The most robust presence signs (C1 + C2) were significantly encountered more frequently in the northern range of the species contrary to C3 ($\chi^2 = 6.3$, 1 d.f., $p = 0.01$).



Slika 2: Trend v velikosti območij kjer je prisotnost risa zaznana redno proti območjem kjer se je ris pojavljal na novo v posameznih triletnih obdobjih.

Figure 2: Trend in the area with lynx presence detected regularly vs. newly during the corresponding 3-year periods.

Trend in the distribution area

Different trends were noticed in the area with regular presence of lynx, and in the area newly colonized (Fig. 2). Three different models were fitted to these data: model 1 assumed no trend in the data; model 2 assumed a linear trend; model 3 assumed changing points and related trends (Table 3). Based on minimum AIC, model 3 with one changing point and two slopes best described the data at hand regarding both changes in the area with regular detection of lynx and in area newly colonized. The area with regular presence has been first increasing (1993 – 2007) then stabilizing (2008–2010); the area newly colonized increased (1993–2004), and then decreased (2005–2010).

Tabela 1: Številčnost ovrednotenih znakov prisotnosti risa po SCALP kategorijah v francoskih Alpah.
Table 1: Numbers of lynx presence signs, according to SCALP categories, validated over the French Alps.

Categories	1990–94	1995–99	2000–04	2005–09	Total
C1	2	0	3	2	7
C2	5	7	92	62	166
C3	24	62	128	95	309
Total	31	69	224	159	483

Preglednica 2: Neuravnovešena številčnost znakov prisotnosti risa po SCALP kategorijah (C1+C2 proti C3) in prostorski razporeditvi.
Table 2: Unbalanced numbers of lynx presence data, according to SCALP categories (C1+C2 versus C3) and geographical location.

Categories	North to Grenoble	South to Grenoble
C1+C2	55	9
C3	65	30

Discussion

Despite a strong rejection rate of presence signs collected, which suggests that most spurious data may have been discarded, a strange geographical pattern still occurred in validated data. Robust lynx presence signs (C1+C2) were still located mostly north to Grenoble, as already mentioned in Marboutin et al. (2006). The southernmost robust presence signs were collected in the Chartreuse and Vercors massif, and in the Maurienne valley. Many places in the whole area of the Alps are actively monitored for wolf presence too, using systematic surveys of e.g. tracks in winter; an unknown – but possibly large – part of C3 may therefore correspond to "phantom" lynx (sensu Molinari et al. in press), specially in those places where only C3 are obtained. Based on trends observed in the detected presence area, the French Alpine lynx sub-population is likely stabilizing. Overall,

Tabela 3: Modeliranje trendov zaznanega prostorskega obsega populacije z uporabo programa TRIM.
Table 3: Modelling of trends in the detected population range using TRIM software.

Area with Presence detected	Model structure	Slope estimates	A.I.C.
regularly	 no time effect	0.00	304.7
	 linear trend 1 slope	0.49	41.0
	 1 changing point 2 slopes	0.69 -0.07	**13.1**
	 2 changing points 3 slopes	0.00 0.96 0.13	27.2
newly	 no time effect	0.00	296.3
	 linear trend 1 slope	0.07	271.3
	 1 changing point 2 slopes	0.38 -0.42	**51.4**

the estimated regular population range covers less than 1350 km², which may hardly correspond to more than 10 – 15 resident adults. The area newly colonized may be a mixture of actual dispersers and phantom lynx (wrong positive detection of the species). This suggests a conservative approach is needed, i.e. not considering such areas in the population status assessment as long as they do not turn to regular presence areas. Combining an analysis of the spatial recurrence in species detection, together with an analysis of the influence of species misidentification (Molinari-Jobin et al. in press), may help reaching the right balance between the risks of under- versus over-estimating the changes in population range.

Acknowledgments

This work was possible thanks' to data being collected by the network of field lynx-experts. The authors are indebted to A. Molinari-Jobin, SCALP coordinator, for animating the pan-alpine scientific network.

References

Anderson, D., Burnham, K., 2002. Avoiding pitfalls when using information-theoretic methods. Journal of Wildlife Management, 66, 912 – 918.

Marboutin, E., Duchamp, C., Rouland, P., Léonard, Y., Boyer, J., Catusse, M., Migot, P., Vandel, J.M., Stahl, P., 2006. Survey of the Lynx distribution in the French Alps: 2000 – 2004 population status analysis. Acta Biologica Slovenica, 49, 19–26.

Molinari-Jobin, A., Zimmermann, F., Breitenmoser-Würsten, C., Capt, S., Breitenmoser, U., 2001. Present status and distribution of the lynx in the Swiss Alps. Hystrix, 12, 17–28.

Molinari-Jobin, A., Molinari, P., Breitenmoser-Würsten, C., Woelfl, M., Stanisa, C., Fasel, M., Stahl, P., Vandel, J.M., Rotelli, L., Kaczensky, P., Huber, T., Adamic, M., Koren, I., Breitenmoser, U., 2003. Pan-Alpine Conservation Strategy for the Lynx. Nature and environment 130, Council of Europe Publishing, 20 pp

Molinari-Jobin, A., Kéry, M., Marboutin, E., Molinari, P., Koren, I., Fuxjäger, C., Breitenmoser-Würsten, C., Wölfl, S., Fasel, M., Kos, I., Wölfl, M., Breitenmoser, U. Monitoring in the presence of species misidentification: the case of the Eurasian lynx in the Alps. Animal Conservation, in press.

Linnell J., Salvatori, V., Boitani, L., 2008. Guidelines for population level management plans for large carnivores in Europe. A Large Carnivore Initiative for Europe report prepared for the European Commission (contract 070501/2005/424162/MAR/B2).

Pannekoek, J., van Strien A., 1998. TRIM 2.0 for Windows. (Trends and Indices for Monitoring data). Statistics Netherlands, Voorburg.

Vandel, J.M., Stahl, P., 2005. Distribution trend of the Eurasian lynx (*Lynx lynx*) populations in France. Mammalia , 69, 145–158.

ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2012 Vol. 55, Št. 1: 35–41

Status and distribution of the lynx (*Lynx lynx*) in the Italian Alps 2005–2009

Status in razširjenost risa (*Lynx lynx*) v italijanskih Alpah 2005–2009

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Abstract: To assess the status of lynx we analysed lynx signs of presence within the range of the Italian Alps from 2005 to 2009. A total of 268 signs have been collected, compared to 411 signs during the previous pentad. The distribution of the confirmed signs of lynx presence is confined to three concise areas: the North-eastern Alps of Friuli VG, the Trentino province and the Ossola valley in the Piedmont region. Occupancy modelling revealed a decrease of the lynx range by one third: The estimated number of occupied 100 km^2 cells decreased from 34 (pentad: 2000–2004) to 21 (pentad: 2005–2009). Less than 10% of the Italian Alps are colonized. We estimated the number of lynx present in all the Italian Alps at less than 15 individuals. Therefore, the persistence of lynx in the Italian Alps highly depends on immigration from neighbouring countries.

Keywords: Alps, distribution, Italy, *Lynx lynx*, monitoring, occupancy

Izvleček: Z namenom opredeliti status risa v italijanskih Alpah smo analizirali znake prisotnosti v obodobju 2005 do 2009. Skupno je bilo zbranih 268 znakov prisotnosti, primerjalno v pretekli pentadi pa 411 znakov. Razporeditev potrjenih znakov prisotnosti risa je omejeno na tri omejena, ločena območja: SV Alpe Julijske krajine, province Trentino ter doline Ossola v regiji Piedmont. Modeliranje zasedenosti prostora je pokazalo zmanjšanje razširjenosti risa za eno tretjino: ocenjeno število poseljenih 100 km^2 celic se je zmanjšalo iz 34 (pentada:2000–20004) na 21 v pentadi 2005–2009. To predstavlja manj kot 10% površine Itlijanskih Alp. Ocenjujemo, da je na celotnem območju italijanskih Alp prisotnih manj kot 15 risov. Tako je obstoj risa na tem območju močno odvisen od imigracije živali iz sosednjih držav.

Ključne besede: Alpe, razširjenost, Italija, *Lynx lynx*, monitoring, zasedenost

Introduction

Lynx spread into north-eastern Italy at the beginning of the 1980s as a consequence of reintroduction projects in Austria and Slovenia (Guidali et al. 1990, Molinari 1998, Bologna & Mingozzi 2003). This established a population nucleus in north-eastern Italy with a connection to the Slovenian lynx occurrence. A second, isolated lynx occurrence was reported from the Southern Dolomites in the Trentino region (Ragni et al. 1998). Besides, some scattered observations were registered from the Val d'Aosta and the Piedmont region close to the Swiss border (Molinari et al. 2001, Bologna & Mingozzi 2003). The trend of the two most prominent lynx occurrences in Italy varied greatly. The one in Trentino was considered extinct by 1999 (Molinari et al. 2001), while the one in the north-eastern Alps persisted (Molinari et al. 2006).

In the frame of the SCALP (Status and Conservation of the Alpine Lynx Population, Molinari-Jobin et al. 2012), each Alpine country updates the status and distribution of lynx in the respective territory in a 5-year intervals. The first status reports for Italy summarized the data from the reintroductions until 1995 (Molinari 1998, Ragni et al. 1998). The data from 1995 to 1999 were analysed by Molinari et al. (2001), and those from 2000 to 2004 by Molinari et al. (2006). Here, we give an overview on the development of the status and distribution of lynx in Italy summarizing data from 2005–2009. In fact, we compared the distribution of lynx signs in the period 2000–2004 with those of 2005–2009 by means of site-occupancy models (MacKenzie et al. 2006). These models jointly estimate the probability of occurrence and detection and therefore correct the distribution estimate for detection probability, i.e., the probability to detect the presence of a species at a site where it occurs.

Methods

We used a stratified approach to monitor lynx: the information for the whole Italian Alps is based on collected lynx signs of presence, in the north-eastern Alps camera traps were used to identify individual lynx and finally two male lynx were fit with GPS-GSM collars.

Signs of presence were collected analogous to the previous pentad (2000–2004, Molinari et al. 2006) by a network of people, mainly game wardens and foresters, who have attended special training courses. The number of trained people varied regionally: 3 Liguria, 44 Piemonte, 28 Val d'Aosta, 16 Lombardia, 58 Trentino Alto Adige, 26 Veneto, 54 Friuli V.G. (229 in total). Whenever possible, these "lynx experts" verified the signs of presence reported to them by the general public. Within each region, one or two persons were responsible for the centralisation of the data. By the end of the year the data were transferred to a common database. We distinguished three levels of reliability in accordance with the SCALP guidelines (Molinari-Jobin et al. 2012) and the possibility to verify the collected data:

C1: Confirmed "hard facts", verified and undisputable records of lynx presence such as (1) dead lynx, (2) captured lynx, (3) good-quality and geo-referenced lynx photos (e.g., from camera traps), and (4) samples (e.g. excrements, hair) attributed to lynx by means of scientifically reliable analyses.

C2: Records confirmed by a lynx expert (e.g. trained member of the network) such as (1) killed livestock or (2) wild prey, and (3) lynx tracks or other assessable field signs.

C3: Unconfirmed observations (kills, tracks, other field signs too old or badly documented, where however the description conforms to a lynx sign) and all observations such as sightings and calls which by their nature cannot be verified.

A dynamic site-occupancy model (MacKenzie et al. 2002, MacKenzie et al. 2003, MacKenzie et al. 2006, Royle & Kéry 2007, Kéry & Schaub 2011) was used to compare Alpine lynx distributions between two periods (2000–2004 and 2005–2009). This model jointly estimates the probability of occurrence and detection. It corrects the distribution estimate for detection probability, i.e. the probability to detect a specimen, where it indeed occurs. We compared two periods: 2000–2004 with 2005–2009. For this purpose we covered the Italian Alps with a grid of 756 squares of 100 km^2 each. Our analysis is based on two assumptions (Molinari-Jobin et al. 2012): (1) Lynx distributions remained unchanged within the two periods. This assumption may have been violated to some degree. As a consequence, our estimate

of occupancy may refer to the area of use rather than the permanent presence of lynx (MacKenzie et al. 2006). (2) Owing to the large number of persons and organisations that collaborate in the Alpine lynx monitoring, we assumed that there is a non-zero chance of detecting a lynx in every occupied 100 km^2 cell in each year (i.e. no cell was devoid of any monitoring efforts). Only if this assumption is met we can treat years without a lynx record as a zero rather than as a missing value in the detection history fed into the site-occupancy model. We strongly believe that this assumption holds for the vast majority of cells in our study area. If invalid, our probabilities of detection are underestimated, while probabilities of occupancy are overestimated.

We defined a multi-season occupancy design and modelled as data seasons of four-months (January to April, May to August, September to December) out of the five years in which lynx records were obtained in a 100 km^2 cell. Hence, within a season we ignored more than one record per cell and simply distinguished between cells and seasons in which no lynx was recorded (yielding a "0") and those with at least one record (yielding a "1"). The dynamic site-occupancy model is a state-space model, i.e., it distinguishes a latent (only partly observed) ecological process, which produces a state of occurrence or non-occurrence, and a dependent observation process, which produces the actual detection/nondetection observations. The ecological process is defined by the occurrence probability (= occupancy) in the first year (ψ) and the dynamic parameters of survival (also called extinction), ε, and of colonisation, γ. The observation process is defined by the annual detection probability p_t. We fitted a separate model to the two periods (2000–2004, 2005–2009) using only the confirmed data (C1 and C2). We assumed that the probabilities of first-year occupancy (ψ) and of extinction (ε) and colonisation (γ) were constant over the 756 100 km^2 cells. We also assumed that the detection probability differed only by season, but not among cells nor among years. We performed the occupancy analyses using the program PRESENCE (Hines 2006).

The site-occupancy model yields a detection-corrected estimate of the species distribution based on the number of occupied 100 km^2 cells. For comparison with the previous status report (Molinari et al. 2006), we buffered the location of each point with a 5 km radius, resulting in an area of approx. 80 km² for each record. This area corresponds roughly to an average female lynx home range size in the Alps (Breitenmoser-Würsten et al. 2001). All maps were drawn in ArcGIS 9.3.1 (Environmental Systems Research Institute, Inc. Redlands, CA).

Results

From 2005–2009, a total of 268 signs of lynx presence have been collected (Table 1), compared to 411 signs during the previous pentad (Molinari et al. 2006). The number of reported presence signs decreased steadily from 2005 to 2009. The distribution of the confirmed signs of lynx presence is confined to three concise areas: the North-eastern Alps of Friuli VG, the Trentino province and the Ossola valley in the Piedmont region (Fig. 1). Unverified signs origin in the Belluno province, South Tyrol and in the Western Alps where a few records are reported close to the French border. For the years 2005 to 2009, no signs of reproduction have been reported.

We photographed lynx four times on passages, and three times at kills in the north-eastern Alps during this pentad. The same lynx was pictured all the time, with one exception. Besides, in March 2007 a male lynx was captured and fit with a GPS/GSM collar in the Carnic Prealps of Friuli VG. The home range of this lynx covered 120 km^2 (Nadalini et al. 2010). Additionally, another male lynx was caught in February 2008 in Switzerland that dispersed to the Trentino region (Haller 2009), where he used a home range of 327 km^2 (Groff et al. 2011).

The area occupied by lynx (estimates of 5 km radius buffer) ranged from 731 km^2 (C1 data) to 1868 km^2 (C2 data) and to 4185 km^2 (C3 data). The area covered with C1 data increased based on intensified use of camera traps, while the area covered with C2 and C3 data decreased compared to the previous pentad (C2: 2491 km^2, C3: 6534 km^2, Molinari et al. 2006). The Italian Alps comprise an area of 51.052 km^2. Lynx signs of presence were recorded on less than 5% of the Italian Alps, considering confirmed data (C1 and C2) only, and less than 10% considering all data.

Occupancy modelling revealed differences between the two pentads in the occupied area: the number of cells recording lynx decreased from 30 (2000–2004 period) to 19 (2005–2009 period, Table 2). The estimated number of occupied cells decreased from 34 (first pentad) to 21 (second pentad). The area occupied by lynx had decreased by more than one third. The probability that lynx colonize a previously unoccupied cell was 0 for both periods. The probability of local extinction decreased from 0.13 (2000–2004 period) to 0 (2005–2009 period). Given a cell is occupied by lynx, the probability of detecting lynx was low and varied between periods and seasons (Table 2). In our analysis, the best season for detecting lynx was from January to April.

Discussion

Lynx signs of presence have decreased from the previous (2000–2004) to the current (2005–2009) pentad. The occupied area diminished by one third. Lynx presence was confirmed only in three out of seven Alpine regions: Friuli VG, Trentino Alto Adige and Piedmont. The distribution of signs of presence indicates a continuous occurrence in Friuli. Only single individuals are suspected to occur in Trentino Alto Adige and Piedmont. An underestimation of the lynx range is possible. However, the network of trained people did not decrease, i.e. contacts remained the same as in the previous pentad and more people have been trained especially in the Piedmont region. Moreover, we used occupancy modeling to take the observation process into account.

The occupancy model estimated the probability of local extinction at 0.13 for the 2000–2004 period and at 0 for the 2005–2009 period. This may indicate that the distribution has stabilized at the presently low level, with less than 10% of the Italian Alps colonized. The persistence of lynx in the Italian Alps highly depends on immigration from neighbouring countries. However, the number of lynx is estimated at up to 5 individuals in the Slovenian Alps with a decreasing trend (Kos et al. this volume). There is a high chance that lynx from the Swiss population will immigrate, as Switzerland is the only Alpine country from which regular reproduction is reported (Zimmermann et al. 2011). However, the aim of connecting the north-western and the south-eastern lynx subpopulations (Molinari-Jobin et al. 2003) is far from being reached. The south-eastern Alpine occurrence seems to have lost its expansion potential.

Four individual lynx were identified in the Italian Alps in 2009: two by means of radio-tracking and two by means of camera traps. Three of these lynx were identified in Friuli VG and one in the Trentino province where there is still no prove that other lynx frequent the area. We estimated the number of lynx present in all the Italian Alps at less than 15 individuals.

Acknowledgements

We thank all the game wardens, foresters and other people who have reported lynx observations and all national, regional and provincial institutions for the support of the monitoring programme. In particular the lynx experts who are responsible for the monitoring programme at regional level: B. Bassano, R. Benet, S. Bornei, K. Bliem, S. Borney, M. Catello, R. Colloredo, O. DaRold, F. De Bon, D. De Martin, P. De Martin, E. Ferroglio, S. Filacorda, A. Gagliardi, P. Gavagnin, L. Gerstgrasser, A. Martinoli, A. Mosca, P. Oreiller, R. Pontarini, M. Rodolfi, G. Sommavilla, G. Tormen, C. Vuerich and C. Wedam. We also thank to Accattino E., Bocca M., Bonzani F., Brondolin G., Brugnoli A., Bulfon P., Buzzi A., Buzzi E., Buzzi I., Buzzi W., Chaulet R., Cobai S., De Bortoli M., De Crignis D., Della Mea F., Della Mea S., Del Pedro M., Dorigatti E., Druidi F., Festa, M., Garanzini A., Garanzini P., Ianner G., Imboden M., Kammerer A., Kurschinski F., Mariolini P., Martino L., Maurino L., Mayr S., Molin C., Molinari S., Mosca A., Mosini A., Partel P., Passalacqua C., Paulon A., Pezzetta G., Picco L., Preschern V., Ribetto G., Sascor R., Sommavilla, G., Stoffella A., Tacchi I., Taffi P., Tolazzi F., Tolosano A., Vairoli P., Venturato A., Volcan G. and Zeni M. Marc Kéry and Benedikt Schmidt gave advice on occupancy modelling. We also thank Holger Frick for comments on an earlier draft. Special thanks for financial support and/or the sponsorship to following institutions and organisations: Ufficio Caccia e Pesca – Provincia Autonoma di Bolzano / Amt für Jagd und Fischerei – Autonome, Pro-

vinz Bozen, Corpo Forestale dello Stato – U.T.B. Foresta di Tarvisio, Parco Nazionale del Gran, Paradiso, Provincia di Udine, Provincia di Belluno – Servizio di Vigilanza Ambientale, Provincia di Torino – Servizio Tutela della Fauna e della Flora, Provincia Autonoma di Trento – Servizio Foreste e Fauna, Provincia di Savona, Regione Friuli Venezia Giulia, Ente di Gestione Parco Naturale dell'Alpe Veglia e Devero, Parco Naturale delle Prealpi Giulie, Parco Nazionale delle Dolomiti Bellunesi, Parco Naturale Dolomiti d'Ampezzo, Ufficio Parchi Naturali dell'Alto Adige.

References

Bologna, M., Mingozzi, T., 2003. *Lynx lynx* (Linnaeus, 1758). Distribuzione geografica. In: Boitani, L., Lovari, S., Vigna Taglianti, A. (eds). Fauna d'Italia, Mammalia III Carnivora Artiodactyla. Calderini, Bologna, 226–234.

Breitenmoser-Würsten, C., Zimmermann, F., Ryser, A., Capt, S., Laass, J., Siegenthaler, A., Breitenmoser, U., 2001. Untersuchungen zur Luchspopulation in den Nordwestalpen der Schweiz 1997–2000. KORA-Bericht, **9**, 1–88.

Haller, H., 2009. Ein Jungluchs auf Reisen. Cratschla, 1, 4–13.

Groff, C., Dalpiaz, D., Frapporti, C., Rizzoli, R., Zanghellini, P., 2011. Rapporto Orso 2010. Servizio Foreste e fauna della Provincia Autonoma di Trento, 1–68.

Guidali, F., Mingozzi, T., Tosi, G., 1990. Historical and recent distributions of Lynx *Lynx lynx* L., in Northwestern Italy, during the 19th and 20th centuries. *Mammalia*, 54, 587–596.

Hines, J.E., 2006. Program PRESENCE (Version 2). http://www.mbr-pwrc.usgs.gov/software/doc/presence/presence.html.

Kéry, M., Schaub, M., 2011. Bayesian population analysis using WinBUGS – A hierarchical perspective. Academic Press, Burlington, MA.

Kos, I., Potočnik, H., Koren, I., Krofel, M., 2012. Status and distribution of lynx (Lynx Lynx L.) in Slovenia from 2005 to 2009. Acta Biologica Slovenica, 55 (1), xx–xx.

MacKenzie, D.I., Nichols, J.D., Lachman, G.B., Droege, S., Royle, J.A., Langtimm, C.A., 2002. Estimating site occupancy rates when detection probability rates are less than one. Ecology, 83, 2248–2255.

MacKenzie, D.I., Nichols, J.D., Hines, J.E., Knutson, M.G., Franklin, A.B., 2003. Estimating site occupancy, colonization, and local extinction when a species is detected imperfectly. Ecology, 84**,** 2200–2207.

MacKenzie, D.I., Nichols, J.D., Royle, J.A., Pollock, K.H., Hines, J.E., Bailey, L.L., 2006. Occupancy estimation and modeling: inferring patterns and dynamics of species occurrence. Elsevier, San Diego.

Molinari, P., 1998. The lynx in the Italian South-eastern Alps. Hystrix, 10, 55–64.

Molinari, P., Rotelli, L., Catello, M., Bassano, B., 2001. Present status and distribution of the Eurasian lynx (*Lynx lynx*) in the Italian Alps. Hystrix, 12, 3–10.

Molinari, P., Bionda, R., Carmignola, G., Catello, M., Cetto, E., Filacorda, S., Gavagnin,P., Mingozzi, T., Rodolfi, M., Molinari-Jobin, A., 2006. Status of the Eurasian lynx (*Lynx lynx*) in the Italian Alps: an overview 2000–2004. Acta Biologica Slovenica, 49, 13–18.

Molinari-Jobin, A., Molinari, P., Breitenmoser-Würsten, Ch., Wölfl, M., Stanisa, C., Fasel, M., Stahl, P., Vandel, J.M., Rotelli, L., Kaczensky, P., Huber, T., Adamic, M., Breitenmoser, U., 2003. Pan-Alpine Conservation Strategy for Lynx. Nature and Environment, 130, 1–20.

Molinari-Jobin, A., Kéry, M., Marboutin, E., Molinari, P., Koren, I., Fuxjäger, C., Breitenmoser-Würsten, Ch., Wölfl, S., Fasel, M., Kos, I., Wölfl, M., Breitenmoser, U., 2012. Monitoring in the presence of species misidentification: the case of the Eurasian lynx in the Alps. Animal Conservation 15: 266–273.

Nadalini, R., Cerne, R., Filacorda, S., Gentili, S., Tropea, F., Dal Pra, S., 2010. Presenza e distribuzione della lince ed uso dell'habitat nel Friuli Venezia Giulia e nella zona Slovena di confine. In: E. Randi, B. Ragni, L. Bizzarr i, N. Agostini, G. Tedaldi, (eds.). 2010 – Biologia e conservazione dei Felidi in Italia. Atti del convegno – Santa Sofia (FC) 7–8 Novembre 2008. Ente Parco Nazionale Foreste Casentinesi.

Ragni, B., Possenti, M., Mayr, S., Carrer, M., Zangrando, E., Catello, M., Dorigatti, E., Di Lorenzo, M., Mosca, A., Fattor, M., Lombardi,G., 1998. The lynx in the Italian Alps. Hystrix, 10, 31–38.

Royle, J.A., Kéry, M., 2007. A Bayesian state-space formulation of dynamic occupancy models. Ecology, 88, 1813–1823.

Zimmermann, F., Molinari-Jobin, A., Ryser, A., Breitenmoser-Würsten, Ch., Pesenti, E., Breitenmoser, U., 2011. Status and distribution of the lynx *Lynx lynx* in the Swiss Alps 2005–2009. Acta Biologica Slovenica, 54, 73–80.

Table 1: Number of lynx records collected per year and category.
Tabela 1: Število zabeleženih znakov prisotnosti po letih in kategorijah.

Category 1	2005	2006	2007	2008	2009	Total
Photo	5	2		1	2	10
Scats	6	1				7
Total	11	3		1	2	17
Category 2						
Wild prey remains	14	14	1	1	2	32
Tracks	36	32	5	6	13	92
Total	50	46	6	7	15	124
Category 3						
Wild prey remains	5	6	5	2	1	19
Tracks	15	5	7	2	2	31
Sightings	21	17	11	11	11	71
Vocalisations	1	1		1	1	4
Scats		2				2
Total	42	31	23	16	15	127
Total all categories	103	80	29	24	32	268

Table 2: Observed number of occupied 100 km^2 cells and parameter estimates under the site-occupancy model (posterior means and standard deviations are shown).
Tabela 2: Opaženo število zasedenih 100 km^2 celic in ocena parametrov po modelu zasedenosti (site-occupancy model) (prikazane so ocenjene srednje vrednosti in standardne deviacije).

Metric of distribution	2000-2004	2005-2009
Number of occupied cells	30	19
Initial occupancy (ψ)	34.47 ± 6.20	21.17 ± 4.91
Ratio observed/estimated number of occupied cells	0.87	0.90
Probability of extinction (ε_i)	0.13 ± 0.05	0
Probability of colonisation (γ_i)	0	0
Detection probability (p) by season		
January – April	0.35 ± 0.05	0.22 ± 0.04
May – August	0.11 ± 0.03	0.04 ± 0.02
September – December	0.27 ± 0.04	0.16 ± 0.04

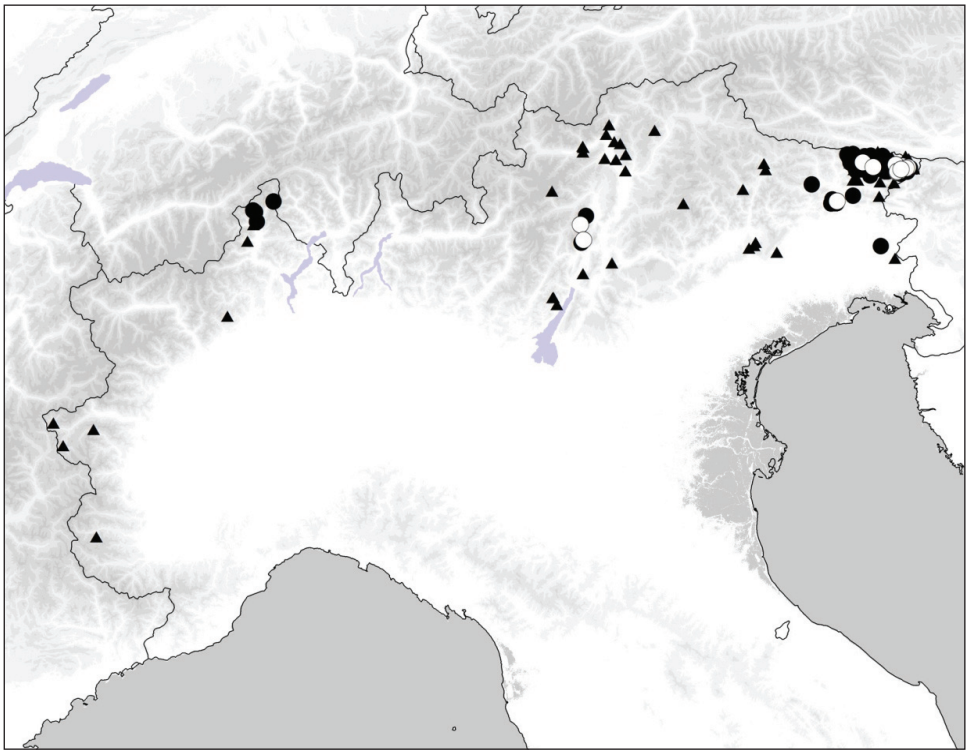

Figure 1: Distribution of lynx signs of presence in the Italian Alps for the five-year period 2005-2009 (white points = confirmed hard fact data C1; black points = confirmed data C2; black triangles = unconfirmed data C3).

Slika 1: Razporeditev znakov prisotnosti risa v italijanskih Alpah v petletnem obdobju 2005-2009 (bele točke = potrjeni neposredni dokazi prisotnosti C1; črne točke = potrjeni podatki C2; črni trikotniki = nepotrjeni podatki C3).

ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2012 Vol. 55, Št. 1: 43–44

Signs of lynx presence in Liechtenstein: 2005 – 2009.

Znaki prisotnosti risa v Lihtenštajnu: 2005 – 2009

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Abstract: Signs of lynx presence were recorded three times between 2005 and 2009.

Keywords: *Lynx lynx*, Alps, Liechtenstein, monitoring, distribution

Izvleček: Znaki prisotnosti risa so bili zaznani trikrat med leti 2005 in 2009.

Ključne besede: *Lynx lynx*, Alpe, Liechtenstein, monitoring, razširjenost

The lynx (*Lynx lynx*) disappeared in Liechtenstein over 100 years ago. The main causes were hunting, trapping and the overexploitation of forests and game by men. The first indicators for the presence of lynx in Liechtenstein were documented in January 2004 and January 2005 (Fasel 2006). Three more records occurred since. They can be classified as C2 and C3, respectively using SCALP categories. Tracks of lynx were found in December 2007 and November 2009 in Gafadura and Alpila, respectively (Fig. 1). Another documented record is a sighting by a private person on Guschgfiel in June 2008. The area of these records concurs with signs of presence mentioned in 2006 in Liechtenstein (Fasel 2006) and Vorarlberg, Austria (Laass et al. 2006). These findings indicate a single individual that most probably dispersed from the Eastern Swiss Alps occurrence (Zimmermann et al. 2012), crossed the river Rhine and found suitable hunting grounds in the border area between Liechtenstein and Austria. Additional regular sightings and prey carcasses were recorded from the same area but could not be associated with lynx unambiguously. A camera trap was therefore set and game wardens, foresters and hunters are urged to report any signs of lynx to the local authorities for validation.

References

Fasel, M., 2006. The lynx in Liechtenstein. Acta Biologica Slovenica, 46 (1), 53-54.

Laass, J., Fuxjäger, C., Huber, T., Gerstl, N., 2006. Lynx in the Austrian Alps 2000 to 2004. Acta Biologica Slovenica, 46 (1), 43-49.

Zimmermann, F. Status and distribution of the lynx (*Lynx lynx*) in the Swiss Alps 2005-2009

Status in razširjenost risa (*Lynx lynx*) v Švicarskih Alpah 2005-2009, Acta Biologica Slovenica, 54 (2), 73-81.

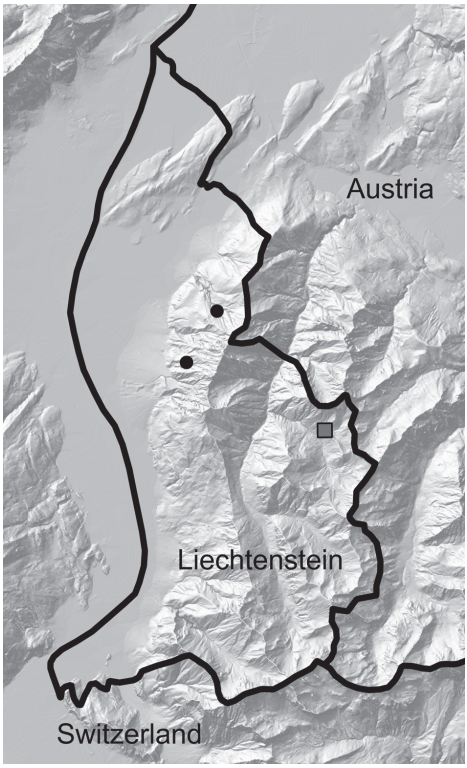

Figure 1: The three records of lynx between 2005 and 2009 are indicated as black dots (C2, tracks from 2007 and 2009) and a grey square (C3, sighting in 2008).

Slika 1: Tri lokacije kjer so bili zaznani znaki prisotnosti risa med 2005 in 2009 (črne točke = C2, sledi leta 2007 in 2009; sivi kvadrat = C3, opažen ris 2008).

ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2012 Vol. 55, Št. 1: 45–48

Lack of introns in putative parasitism factor gene, expansin (*exp*B2) from pale potato cyst nematode *Globodera pallida*

Odsotnost intronov v genu za ekspanzin (*exp*B2), verjetnem parazitskem dejavniku, pri beli krompirjevi ogorčici *Globodera pallida*

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Abstract: Expansins are a group of plant cell wall loosening proteins. In animals, functional expansin (EXPB1) has been discovered in the golden potato cyst nematode *Globodera rostochiensis*. In plant-parasitic nematodes expansins act as the parasitism factors or effectors. Molecular variability of another expansin (*exp*B2) gene was evaluated in the diverse populations of the *G. rostochiensis*. Comparison of the *exp*B2 gene structure in the two potato cyst nematode species, *G. rostochiensis* and *G. pallida*, revealed lack of all four introns in *exp*B2 gene of *G. pallida* species. Possible loss of introns in Gp-*exp*B2 is discussed.

Keywords: Cell wall degradation, *Globodera pallida,* effectors, expansin, intron, parasitism factor, plant-parasitic nematode, potato cyst nematode.

Izvleček: Ekspanzini so skupina proteinov, ki zrahlja rastlinsko celično steno. Pri živalih so odkrili funkcionalni ekspanzin (EXP1) pri vrsti rumena krompirjeva ogorčica, *Globodera rostochiensis*. Pri rastlinsko parazitskih ogorčicah ekspanzini delujejo kot parazitski dejavniki oz. efektorji. Pri *G. rostochiensis* je bila ovrednotena molekulska raznolikost dodatnega ekspanzinskega gena (*exp*B2). Primerjava strukture gena *exp*B2 pri dveh vrstah krompirjevih ogorčic, *G. rostochiensis* in *G. pallida*, je razkrila odsotnost vseh štirih intronov pri vrsti *G. pallida* v primerjavi z vrsto *G. rostochiensis.* Predvidevamo možnost izgube intronov pri Gp-*exp*B2.

Ključne besede: degradacija celične stene, efektorji, ekspanzin, intron, parazitizem, rastlinski parazitski nematodi, krompirjeva ogorčica

Introduction

Expansins are a group of proteins that operate by loosening non-covalent interactions between components of the plant cell wall making the individual components vulnerable to attack by other cell wall degrading enzymes (Cosgrove et al. 2000). These proteins were thought to be specific to plants; however an active expansin EXPB1 has unexpectedly been identified in the plant-parasitic nematode, golden potato cyst nematode *Globodera rostochiensis* (Woll.) Behrens (Qin et al. 2004). The potato cyst nematodes (PCN) *G. rostochiensis* and *G. pallida* (Stone) Behrens are plant-parasitic nematodes which parasitize different Solanaceous plant species. PCN pose a serious threat to potato production worldwide, and they are subject to strict quarantine regulations in many countries. In Slovenia *G. rostochiensis* has spread in the last decade (Širca et al. 2010), while *G. pallida* has

been first detected in Slovenian soil just recently (Širca et al. 2012).

When PCN invade a plant, they produce a mixture of lytic enzymes and expansins in their oesophageal glands and secrete them through the stylet into the plant. These proteins assist in the migration of infective juveniles through the host plant's tissues, and in the feeding site formation. Additionally, the host plant's own expansin genes are up-regulated upon nematode infection (Fudali et al. 2008). Expansins B1 and B2 were determined in the EST analysis of *G. rostochiensis,* while only expansin B1 was found in the *G. pallida* EST database (Popeijus et al. 2000, http://www.nematodes.org/nembase4/overview.shtml). Molecular variability of *exp*B2 gene was evaluated in the diverse populations of the *G. rostochiensis* (Gerič Stare et al. 2012).

The aim of this study was to check for the possible presence of the *exp*B2 in *G. pallida* and to determine its structure.

Materials and methods

DNA was extracted from 10 cysts of the *G. pallida* population. DNA extraction, amplification of *exp*B2 gene, cloning of the amplicon, isolation of pDNA, sequencing and sequence analysis were performed as previously described in detail by Gerič Stare et al. (2012) for the orthologous *exp*B2 gene in *G. rostochiensis* (*Gr-expB2*). Primers were designed based on the *Gr-expB2* mRNA sequence (AJ311902) coding for the putative functional expansin.

Results

The primer set designed for the *expB2* gene of *G. rostochiensis* yielded a much shorter PCR amplicon with the *G. pallida* genomic DNA (543 bp in *G. pallida* vs. 1.129 – 1.153 bp in *G. rostochiensis*). Sequence analysis revealed high homology to previously determined Gr-*exp*B2 precursor (AJ311902) by BLASTN with E value 0.0. Alignment of this *G. pallida* sequence with the previously determined genomic sequences of Gr-*exp*B2 (FJ705444, GQ152151 – GQ152166, GQ152168 – GQ152288) revealed lack of all four introns. Due to the high homology of the coding region of the Gr-*exp*B2 gene, this sequence was designated Gp-*exp*B2. Gp-*exp*B2 shared 99.6 % identity with Gr-*exp*B2 cDNA (GQ152150). Further, no highly homologous sequence to Gp-*exp*B2 could be found in the *G. pallida* genome sequence (http://www.sanger.ac.uk/resources/downloads/helminths/globodera-pallida.html), although there are several sequences similar to expansin, except for including introns. The sequences Gp-*exp*B2 reported here was deposited at Genbank with the accession number GQ152167.

Discussion

In the database of *G. pallida* EST sequences (http://www.nematodes.org/nembase4/index.shtml) there are no expansin B2 related sequences. Nonetheless, we have determined orthologous sequence using the primer set developed for Gr-*exp*B2 in a PCR with *G. pallida* gDNA. Gp-*exp*B2 sequence showed high similarity to exons of determined Gr-*exp*B2 sequences but lack of all four introns found in *G. rostochiensis*. Closely related species usually possess conserved introns, but there are also examples where introns are present in one species but not in closely related one (Kent and Zahler, 2000). From an alignment of two sequences it is not possible to tell whether the introns are being lost or gained.

One hypothesis is that introns may be lost during repair of double-stranded breaks in DNA helix in a repair mechanism involving reverse transcription of mRNA and reintegration of the synthesized cDNA into the genome by homologous recombination. Another example of a gene where this process might have happened is a gene for β-1,4-endoglucanases in *Heterodera glycines* (Hg-*eng5*), a parasitic factors of plan-parasitic nematodes that contains no introns (Gao et al. 2004, Kyndt at al. 2008). On the other hand, a recent horizontal gene transfer event from a prokaryote was suggested for lack of introns in Hg-*eng*5 (Gao et al. 2004). Furthermore, formation of genes without intron from paralogous genes with introns in eukaryotes might arise from gene duplication process involving reverse transcription of mRNAs with recombination of the synthesized cDNA in the genome (Boudet et al. 2001).

Conclusion

Comparison of orthologous *exp*B2 gene in potato cyst nematodes revealed lack of all four introns in *G. pallida* compared to *G. rostochiensis*.

Povzetek

Ekspanzini so proteini, ki z zrahljanjem nekovalentnih vezi pomagajo pri razgradnji rastlinske celične stene. Pri živalih so odkrili funkcionalni ekspanzin (EXP1) pri vrsti rumene krompirjeve ogorčice *Globodera rostochiensis* (Nematoda). Pri rastlinsko parazitskih ogorčicah ekspanzini delujejo kot parazitski dejavniki oz. efektorji. Pri *G. rostochiensis* je bila ovrednotena molekulska raznolikost dodatnega ekspanzinskega gena (*exp*B2). Z uporabo začetnih oligonukleotidov predhodno razvitih za Gr-*exp*B2 smo določili prisotnost gena *exp*B2 tudi pri sorodni vrsti bele krompirjeve ogorčice *G. pallida*. Primerjava strukture gena *exp*B2 je razkrila odsotnost vseh štirih intronov pri Gp-*exp*B2 v primerjavi Gr-*exp*B2. Do izgube intronov pri Gp-*exp*B2 bi lahko prišlo na različne načine. Prvi je mehanizem popravljanja poškodb dvoverižne DNA, ki vključuje obratno prepisovanje mRNA v cDNA in reintegracijo le te v genom s homologno rekombinacijo. Drug možen mehanizem je nedaven horizontalni prenos gena iz prokariontov. Tretja možnost nastanka genov brez intronov pri evkariontih je z podvojitvijo paralognih genov z introni v procesu, ki vključuje obratno prepisovanje mRNA in rekombinacijo tako nastale cDNA v genom.

Acknowledgements

This work was supported by the Slovenian Research Agency and the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (V4-0324 and BI-FR/07-08-INRA-003). This work benefited from links funded via COST 872 action.

References

Boudet, N., Aubourg, S., Toffano-Nioche, C., Kreis, M., Lecharny, A., 2001. Evolution of intron/exon structure of DEAD helicase family genes in *Arabidopsis*, *Caenorhabditis*, and *Drosophila*. Genome Research, 11, 2101–2114.

Cosgrove, D.J., 2000. Loosening of plant cell walls by expansins. Nature, 407, 321–326.

Fudali, S., Sobczak, M., Janakowski, S., Griesser, M., Grundler F.M.W., Golinowski. W., 2008. Expansins are among plant cell wall modifying agents specifically expressed during development of nematode-induced syncytia. Plant Signaling & Behavior, 3 (11), 969–971.

Gao, B.L., Allen, K.D., Davis, E.L., Baum, T.J., Hussey, R.S., 2004. Developmental expression and biochemical properties of a beta-1,4-endoglucanase family in the soybean cyst nematode, *Heterodera glycines*. Molecular Plant Pathology, 5, 93–104.

Gerič Stare, B., Lamovšek, J., Širca, S., Urek, G., 2012 Assessment of sequence variability in putative parasitism factor, expansin (*exp*B2) from diverse populations of potato cyst nematode *Globodera rostochiensis*. Physiological and Molecular Plant Pathology, in press (doi:10.1016/j.pmpp.2012.04.002)

Kent, W.J., Zahler, A.M., 2000. Conservation, regulation, synteny, and introns in a large-scale *C. briggsae-C. elegans* genomic alignment. Genome Research, 10, 1115–1125.

Kyndt, T., Haegeman, A., Gheysen, G., 2008. Evolution of GHF5 endoglucanase gene structure in plant-parasitic nematodes: no evidence for an early domain shuffling event. BMC Evolutionary Biology, 8, 305.

Popeijus, H., Blok, V.C., Cardle, L., Bakker, E., Phillips, M.S., Helder, J., Smant, G., Jones, J.T., 2000 Analysis of genes expressed in second stage juveniles of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* using the expressed sequence tag approach. Nematology, 2000, 2 (5), 567–574.

Qin, L., Kudla, U., Roze E.H.A., Goverse, A., Popeijus, H., Nieuwland, J.,
Overmars, H., Jones, J.T., Schots, A., Smant, G., Bakker, J., Helder, J., 2004. A nematode expansin acting on plants, Nature, 427, 30.
Širca, S., Gerič Stare, B., Strajnar, P., Urek, G., 2010, PCR-RFLP diagnostic method for identifying Globodera species in Slovenia. Phytopathologia Mediterranea, 49, 361–369.
Širca, S., Gerič Stare, B., Strajnar, P., Urek, G., Miklič Lautar, I., 2012. First report of the pale potato cyst nematode *Globodera pallida* from Slovenia. Plant disease, 96, 773.
http://www.nematodes.org/nembase4/overview.shtml