

**Four decades of multidisciplinary studies on isopods:
a tribute to Pavel Ličar**

Štiri desetletja interdisciplinarnih raziskav rakov enakonožcev (Crustacea:Isopoda):
v spomin Pavlu Ličarju

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Abstract: In this paper we review the research on aquatic and terrestrial isopods during the last four decades at the Chair of Zoology, Department of Biology, Biotechnical Faculty, University of Ljubljana. Isopods have attracted substantial attention from our research team in the following areas: functional morphology and developmental biology, host-microbiota specific interactions, ecotoxicology, and systematics and evolution. We present the rationale for using two isopod species as our central model organisms: the waterlouse (*Asellus aquaticus*) and the woodlouse (*Porcellio scaber*). We summarize the most important and interesting findings about the structure and function of the integument and digestive systems of several amphibious and terrestrial woodlice species during molting and developmental stages, the importance of *P. scaber* as a model organism in the study of arthropod-microbe interactions, and its central role as a test model in terrestrial ecotoxicity studies. We highlight the role that *A. aquaticus* has played in studying the evolution of subterranean biodiversity and in the evolution of troglomorphies. In addition to the retrospective view on our research with isopods we also present the scope of our future research, and the importance for zoology (biology). We wish to dedicate this work to our late co-worker, Prof. Dr. Pavel Ličar, who devoted much of his research into studying the digestive system of freshwater asellids (Isopoda: Asellota).

Keywords: isopods, terrestrial, aquatic, functional morphology, developmental biology, host-microbiota interactions, terrestrial isopod, aquatic isopod, ecotoxicology, systematics, evolution

Izvešček: V članku predstavljamo raziskave biologije rakov enakonožcev, ki že nekaj desetletij potekajo na Katedri za zoologijo Oddelka za biologijo Biotehniške fakultete Univerze v Ljubljani. Rake enakonožce proučujemo na različnih raziskovalnih področjih, kot so funkcionalna morfologija in razvojna biologija, študij interakcij med gostiteljem in mikroorganizmi, ekotoksikologija ter sistematika in evolucija. V prispev-

ku navajamo razloge za preučevanje dveh vrst rakov enakonožcev: navadnega prašička (*Porcellio scaber*) in vodnega oslička (*Asellus aquaticus*), ki sta odlična modelna organizma za omenjene raziskave. V prispevku povzemamo glavne ugotovitve raziskav zgradbe in delovanja integumenta in prebavnega sistema med levitvijo in razvojem amfibijskih in kopenskih mokric, pomen vrste *P. scaber* kot modelnega organizma za študij specifičnih interakcij z mikrobi ter njegovo osrednjo vlogo v kopenski ekotoksikologiji. Izpostavljamo tudi vlogo vrste *A. aquaticus* kot modelnega organizma za študij evolucije jamske pestrosti in troglomorfi. Poleg zgodovinskega pregleda raziskav na posameznih področjih, predstavljamo tudi najpomembnejše rezultate najnovjših raziskav, njihov pomen za zoologijo (biologijo) ter pomen za prihodnost. Članek je posvečen lani preminulemu sodelavcu prof. dr. Pavlu Ličarju, ki je raziskoval predvsem prebavila vodnih rakov enakonožcev iz skupine Asellota.

Ključne besede: raki enakonožci, kopenski raki, vodni raki, funkcionalna morfologija, razvojna biologija, interakcija gostitelj-mikroorganizmi, ekotoksikologija, sistematika, evolucija

Introduction

Research on peracarid crustaceans, mostly amphipods and isopods at the Chair of Zoology, has a long history, dating back to 1965 with the first publications on taxonomy of *Asellus aquaticus* (Sket 1965a,b, 1967) and has so far resulted in 319 publications of Prof. Sket and his co-workers. Ličar (1970) and, Ličar and Sket (1971) published their first papers on the stomach morphology of water lice in the journal *Acta Biologica Slovenica* (formerly *Biološki vestnik*). Since then, aquatic and terrestrial isopods have attracted substantial interest from different researchers at the Chair of Zoology, Department of Biology, Biotechnical Faculty, University of Ljubljana. In this paper we review our research on isopods undertaken in the last four decades to commemorate Professor Ličar's work.

Pavel Ličar devoted his academic career to aquatic isopods, mostly to *Asellus aquaticus* (Isopoda:Asellota). He investigated the structure and function of the isopod digestive system. His main scientific questions were related to morphological characteristics of the isopod stomach and its possible role as an identification key in taxonomy. From a morphological viewpoint, the stomach is by far the most complicated part of the digestive tract of isopod crustaceans. The structure of the stomach varies between species but in many there is a dorsal groove into which indigestible material is channelled and a ventral part which transports filtered food to the diges-

tive caeca, the hepatopancreas. He described the morphological characteristics of the stomach of *A. aquaticus* with emphasis on the fine structure of filters. In general, the isopod stomach is composed of an anterior primary filter, the adjacent masticatory areas, the posterior secondary filter on the lateral sides of the protuberations, called inferomedianum, and the inferolateralia. Ingested food can be filtered twice: first via the primary filter and then the secondary filter, both of which are composed of finely branched cuticular spines and plumose setae which can separate particles of different sizes. In the following years a detailed descriptions of the stomachs of different species of Asellidae were presented (Ličar 1976). He contributed significantly towards the understanding of the mechanical properties of primary filters (Ličar 1977, Ličar et al. 1979). It was stated, that the stomach structure of different genera of Asellidae is rather uniform, even similar to that of Janiridae, while Stenasellidae are remarkably varied and diverse; in specialized species the masticatory parts are vestigial.

A new approach in phylogenetic studies was research into the reproductive cycles of epigean and hypogean species of *A. aquaticus* which was an exciting topic for young researchers in his lab (Štrus and Blejec 1983). Ličar's work on stomach ultrastructure was the basis for international cooperation with researchers at the Institute of Zoology, University of Heidelberg (Germany) (Štrus et al. 1985, Storch and Štrus 1989, Drobne et al. 1991a, Štrus and Storch 2004)

and the University of Reading, United Kingdom (Drobne and Hopkin 1994). Studies on the stomach structure of terrestrial isopods were extended to ecotoxicological studies on digestive glands of the model organism *Porcellio scaber* (Drobne and Hopkin 1995). The structure of the digestive system of strictly terrestrial isopods was compared with the digestive system of amphibious species *Ligia italica* and *Ligidium hypnorum* (Štrus et al. 1995), both important species in the phylogenetic studies of woodlice.

A significant moment in the research of Prof. Pavel Ličar was when a scanning electron microscope (SEM; Stereoscan 600, Cambridge Instruments, UK) was installed at the Department of Biology in 1975, only a decade after the first commercial SEM appeared on the market. In the following years, his ideas and work expanded from the functional morphology of the stomach in aquatic isopods to also encompass amphibious and strictly terrestrial isopod species as a basic approach for phylogenetic studies of isopod crustaceans (Drobne et al. 1991b). It was confirmed that the mastication of food is an adaptation to terrestrial food sources, evidenced by masticatory structures in the stomach which are not so obvious in aquatic species. Amphibious and terrestrial isopods are fully adapted for life on land. Woodlice, suborder Oniscidea, are the only truly terrestrial group of crustaceans and show various adaptations to life on land. Research on the structure and function of the stomach was expanded towards other parts of the digestive system and other organic systems including integument and respiratory structures.

In 1999, the Department of Biology celebrated the 80th anniversary of the foundation. Almost 30-years of academic research of Professor Pavel

Ličar was summarised in the book of abstracts. Together with his close collaborators Jasna Štrus and Damjana Drobne, past, present and future research directions and perspectives were presented (Drobne et al. 1999a). Almost 20 years later, aquatic and terrestrial isopods still attract a lot of attention from different directions including structure and function of digestive system, integument and molting, embryology, immunology, ecotoxicology, and nanobiology. Here, the achievements of the last four decades and perspectives in research on isopods at the Department of Biology are presented.

Presentation of two key model isopod species

The terrestrial isopod Porcellio scaber (*Isopoda: Oniscidea*)

More than one third of described isopod species (approximately 9000) belong to terrestrial Oniscidea or woodlice, also known as “*slaters, sowbugs and pillbugs*” (Schmalfuss H. 2003). Terrestrial isopods are widely distributed, occurring from Iceland to South America and South Africa (Harding and Sutton 1985). They play an important role in plant litter decomposition by breaking down organic materials such as fallen leaves into smaller fragments (Hassall et al. 1987), they affect the soil by physical transportation of litter materials, and alter microbial activity. The terrestrial isopod *Porcellio scaber* (Latreille, 1804) (Slovenian “navadni prašiček”) (Fig. 1 A) is ubiquitous and common on tree trunks and walls, on waste ground, and in gardens and grassland (Harding and Sutton et al. 1985).

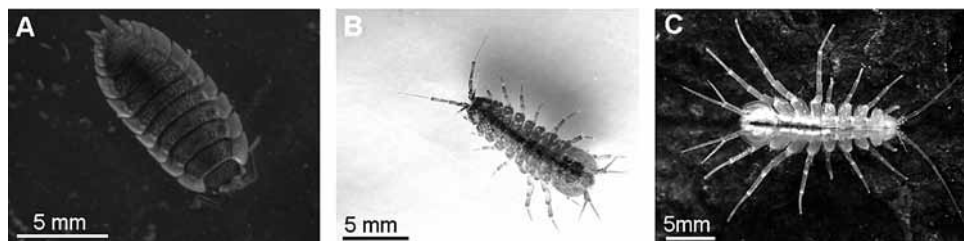


Figure 1: The terrestrial and aquatic isopods: (A)-The terrestrial isopod *Porcellio scaber* and (B)-aquatic isopods *Asellus aquaticus aquaticus*: the surface ecomorph and (C)-*A. a. cavernicolus*: the cave ecomorph.

Slika 1: Kopenski in vodni raki enakokožci. (A)- Kopenski rak enakonožec vrste *Porcellio scaber* in (B)-vodni osliček *Asellus aquaticus aquaticus*: kopenski ekomorf in (C)- *A. a. cavernicolus*: jamski ekomorf.

The aquatic isopod Asellus aquaticus (Isopoda: Asellota)

Asellus aquaticus (Linné, 1758) (Slovenian “vodni osliček”) is one of the most studied and well-known aquatic macroinvertebrates in Europe (Fig. 1B). Its common names include “water slater, waterlouse, aquatic sowbug and water hoglouse”. Its range reaches across the entire continent except for the Pyrenean Peninsula and some smaller Mediterranean areas (Birštejn 1951, Williams 1962, Argano 1979, Sket 1994, Henry and Magniez 1983, 1995, Henry et al. 1996). This generalist species is able to thrive in all types of fresh to slightly brackish surface waters. Its tolerance to organic pollution ranks *A. aquaticus* as an pollution indicator and a successful member of the ‘ α -mesosaprobic’ community (Liebmann 1962). While most of its range is inhabited by the nominotypical *A. a. aquaticus*, extensive racial diversification is present in S and SE Europe (Sket 1965a, Prevorčnik et al. 2004, Verovnik et al. 2003, 2004, 2005). Moreover, in two comparatively small karstic areas (E Romania and NW Dinaric karst) a number of local populations have invaded and adapted to subterranean fresh waters (Sket 1994, Henry et al. 1996, Turk et al. 1996, Turk-Prevorčnik and Blejčec 1998, Prevorčnik et al. 2004) (Fig. 1 C).

Isopods as key experimental models

Functional morphology of the integument and the digestive system of woodlice during molting and development

Woodlice are crustaceans that are well adapted to terrestrial life. Coping with the restrictions of living life on land has resulted in many important adaptations and changes to the animals nutrition, digestion, gas/water balance and reproduction strategies. The functional morphology of the digestive system was studied in different woodlice species and described thoroughly in the model species *P. scaber* (Lane 1988, Hames and Hopkin 1989, Štrus et al 2008). Comparative studies of the digestive system in amphibious woodlice *Ligidium hypnorum* (Drobne et al. 1991a), and *Ligia italica* (Štrus et al. 1995) showed important

differences in the structure and function of the stomach, alimentary canal and in the presence or absence of endodermal midgut. Woodlice are decomposers but can feed on almost anything. Food is mechanically and enzymatically broken down in the foregut, efficiently filtered in the stomach (Fig. 2A) and transported to the midgut glands where the secretion of digestive juices and absorption of nutrients take place (Štrus et al. 1985). The ultrastructure of masticatory regions and filters in stomachs of different isopod crustaceans was described in both asellids (Ličar 1976) and woodlice (Storch 1987). Undigested material is transported to the hindgut which is entirely covered by cuticle and composed of the anterior chamber, with absorptive and transport functions, and the osmoregulatory papillate region with the rectum, where water is resorbed and fecal pellets form. Microbiota which are associated with the cuticle surface are important as a food source but can also aid in food digestion (Kostanjšek et al. 2006). A description of the role of microbiota in the digestive system of woodlice is presented in the subsequent chapter.

Growth and reproduction in woodlice is governed by frequent molting which demands constant synthesis of new cuticles of the integument and alimentary canal. Molt cycles of amphibious and terrestrial woodlice have been described (Zidar et al. 1998) and cuticle ultrastructure and calcification were studied with various microscopic and analytical techniques implemented at the Chair of Zoology and in cooperation with labs worldwide (Štrus and Blejčec 2001, Štrus et al. 2008, Žnidaršič et al. 2010, Mrak et al. 2012, Vittori et al. 2013). The exoskeleton of woodlice is a multilayered calcified chitinous-proteinaceous matrix (Figs. 2B,C) which varies in its elasticity/rigidity depending on the body region, between individual animals and degree of species terrestrialization (Vittori and Štrus 2014). Cuticle is secreted during both embryonic development and molting in adults. In premolt animals, both old and new cuticles can be visualized due to apolysis and subsequent deposition of preecdysal cuticular layers (Fig. 2D). Resorption of organic and mineral components from the detached cuticle and formation of spherules in the ecdysal space are the most prominent features observed in the exoskeleton of premolt animals (Fig. 2E). Due to frequent

biphasic molting (Fig. 2F) and different cuticle types woodlice are excellent models for studying extracellular matrix secretion and calcification.

Embryonic and postembryonic development of crustaceans has been gaining an increasing amount of research interest in functional morphology and evolutionary developmental biology (Browne et al. 2005, Hejnol et al. 2006, Scholtz et al. 2009, Mittmann et al. 2014, Alwes and Scholtz 2014, Stamatakis and Pavlopoulos 2016). In our research group the morphogenesis of the digestive system and integument has been studied in the isopod *P. scaber* building on previous expertise and knowledge of the anatomy, ultrastructure and development of this organism (Milatovič et al. 2010). In laboratory rearing conditions the development from the released fertilized eggs till embryo hatching lasts about 25 days and after that marsupial mancas continue to develop within the female brood pouch (marsupium) for approximately 10 days (Figs. 3A and 3B). Based on morphological data, 19 sequential embryonic stages and 3 stages of marsupial mancas can be distinguished (Milatovič et al. 2010, Mrak et al. 2012). The microscopic anatomy of the digestive system in embryos and mancas was investigated by Štrus et al. (2008). Differentiation of the ectodermal stomodeum and proctodeum into the complex foregut and hindgut respectively, was revealed at the histological and ultrastructural levels. Morphogenesis of the endodermal midgut gland tubes was explained. In the early embryos the midgut gland primordia that contain yolk and lipid globules are visible. In late embryogenesis the midgut gland epithelium consists of two cell types, as is characteristic for adults. The ultrastructural aspects of the hindgut cuticle differentiation during development were analysed by Mrak et al. (2015). The hindgut in late embryos of stages 16 and 18 is lined by a precuticular matrix, structurally similar to that of

the epidermis. The first hindgut cuticle formation is evident in stages 18 and 19, before hatching (Fig. 3C). The hindgut cuticle in marsupial mancas is thinner in comparison to adults, the electron dense epicuticular layer in particular, and the structural differences between the cuticle in the anterior chamber and papillate region are not evident yet. The stomach in late marsupial mancas is already differentiated in complex cuticular structures (Fig. 3D). Exoskeletal cuticle differentiation was studied by Mrak et al. (2014). The ultrastructural organization and composition of precuticular matrices and exoskeletal cuticle in embryos and marsupial mancas were revealed. Two successively formed precuticular matrices were evident in mid-stage and late-stage embryos. The precuticular matrix is composed of the outermost ruffled lamina and subjacent loose material with no lamellar pattern characteristic for the cuticle and differs from the cuticle also regarding its organic scaffold composition as shown by wheat germ agglutinin labelling. In late embryos of stages 18 and 19 the first cuticle formation was observed (Fig. 3E), including elaboration of the surface scales and differentiation of characteristic cuticular layers. In marsupial mancas a new exoskeletal cuticle is secreted and according to the progression of development gradually becomes more similar to the cuticle of adults in respect to the ultrastructure, organic scaffold and calcification (Fig. 3F). An integrative part of cuticle differentiation is also the establishment of connections between cuticle and muscles via tendon cells. Tendon cells ultrastructure, including the linkages to cuticle at the apical membrane and myotendinous junctions to underlying muscle cells, is established in the prehatching embryos and marsupial mancas (Žnidaršič et al. 2012).

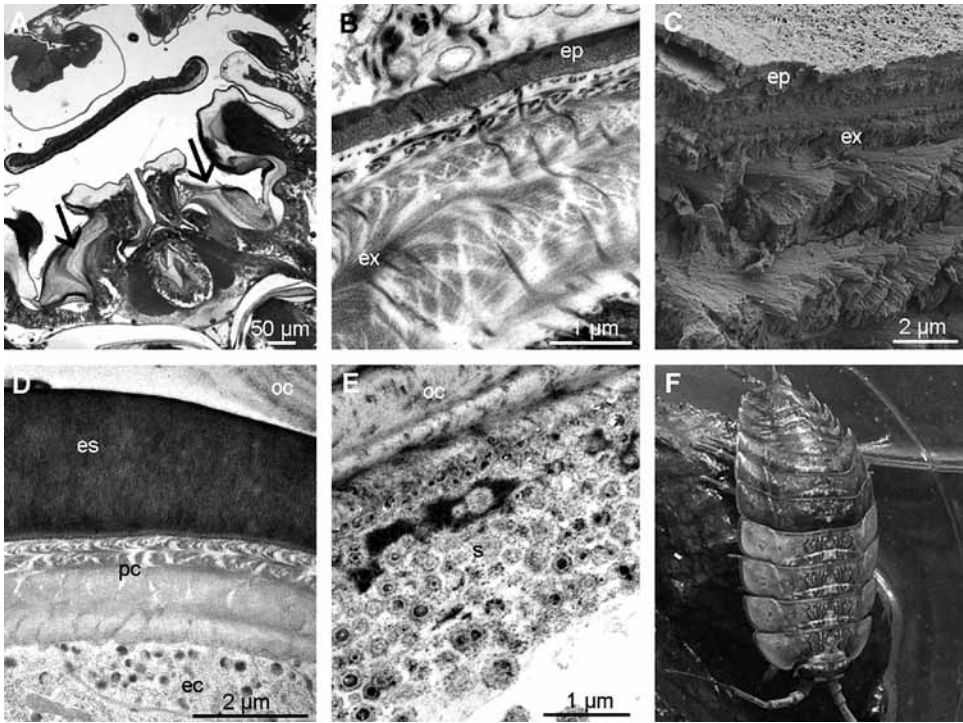


Figure 2: Cuticle structure and function in terrestrial isopods. (A)-Histological section of stomach lined by cuticle with prominent filtering areas (arrows) in adult *Porcellio scaber*. (B)-TEM micrograph of preecdysal exoskeletal cuticle ultrastructure in *Ligia palassii* showing the epicuticle (ep) and exocuticle (ex). (C)-SEM micrograph of preecdysal exoskeletal cuticle in *Ligia palassii* with layered epicuticle (ep) and exocuticle (ex) composed of helicoidally arranged chitin-protein fibers. (D)-TEM micrograph of exoskeletal cuticle in premolt *Ligia italica*; during apolysis old cuticle (oc) is detached and the preecdysal cuticle (pc) with epicuticular scale (es) is formed by epidermal cells (ec). (E)-TEM micrograph of resorbing lamella of the old cuticle (oc) with spherules (S) containing electron dense material. (F)-An intramolt male of *Ligia palassii* with shed posterior cuticle (animal size approximately 3 cm).

Slika 2: Struktura in funkcija kutikule pri kopenskih rakih enakonožcih: (A)- Histološki prerez želodca z izrazitimi kutikularnimi filtri (puščici) odrasle živali *Porcellio scaber*. (B)-TEM mikrofografija nove kutikule eksoskeleta v fazi predlevitve, ki prikazuje epikutikulo (ep) in eksokutikulo (ex) pri vrsti *Ligia palassii*. (C)-SEM mikrofografija kutikule eksoskeleta pred levitvijo prikazuje slojevito epikutikulo (ep) in eksokutikulo (ex) iz helicoidno urejenih hitinsko-proteinskih vlaken pri vrsti *Ligia palassii*. (D)-TEM mikrofografija kutikule eksoskeleta v fazi predlevitve pri vrsti *Ligia italica*; med apolizo se stara kutikula (oc) odmakne od epitela, epidermalne celice (ec) pa tvorijo novo kutikulo (pc) z epikutikularnimi luskami (es). (E)-TEM mikrofografija stare kutikule (oc) med resorpcijo in sferul (S), ki vsebujejo elektronsko gost material. (F)-Samec vrste *Ligia palassii* v fazi medlevitve, ko je že odvrgl posteriorno kutikulo (velikost živali je približno 3 cm).

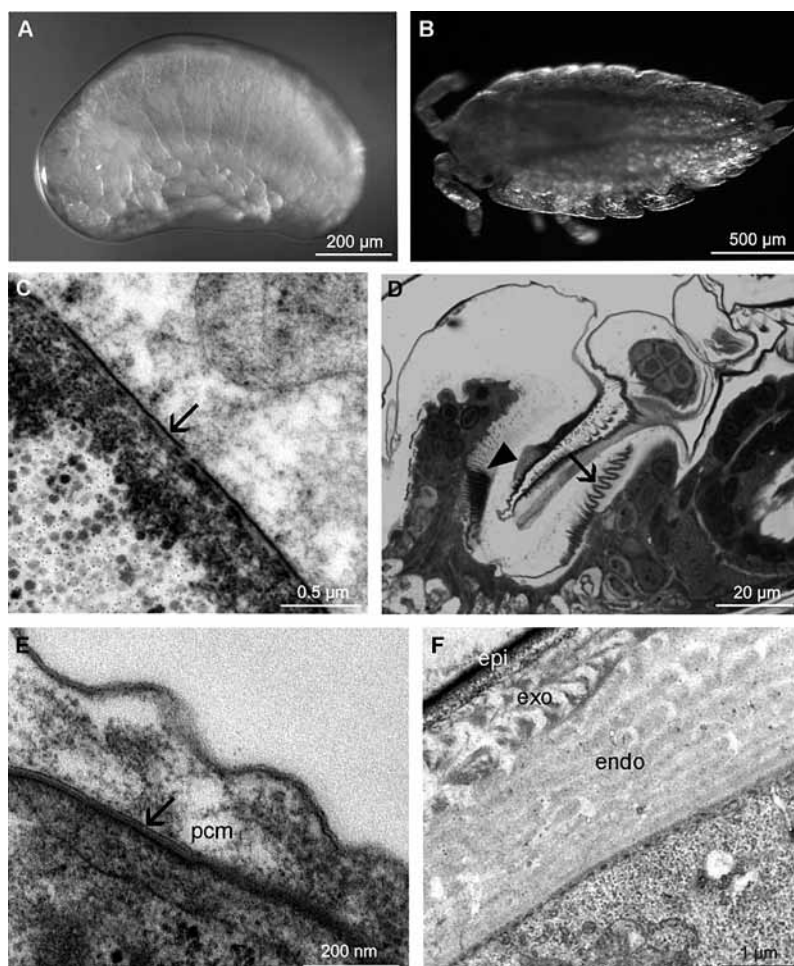


Figure 3: Morphogenesis of the gut and exoskeletal cuticle in embryos and marsupial manca of *Porcellio scaber*. (A)-Late embryo of the stage 18, enclosed in the vitelline membrane. Appendages, eye and midgut glands with yolk are distinguished. (B)-Mid-stage marsupial manca. (C)-Hindgut cuticle in the late embryo is initially deposited in segments (arrow) that form a continuous layer in subsequent development. (D)-Primary cuticular filters (arrow) and setae of laterallia (arrowhead) in the stomach of late marsupial manca. (E)-The first exoskeletal cuticle is formed beneath the precuticular matrix (pcm) in the late embryo. Deposition of the multilayered epicuticle (arrow) is evidenced in the image. (F)-Exoskeletal cuticle of the mid-stage marsupial manca, differentiation into epicuticle (epi), exocuticle (exo) and endocuticle (endo) is evident.

Slika 3: Morfogeneza kutikule prebavnega trakta in eksoskeletne kutikule pri embrijih in marzupijskih mankah vrste *Porcellio scaber*. (A) Pozni embrij v stadiju 18, obdan z vitelinsko membrano. Razvidne so okončine, oko in prebavne žleze z rumenjacom. (B) Posnetek ličinke marzupijske manke v srednjem stadiju. (C) Pri poznem embriju se začne kutikula zadnjega črevesa nalagati v segmentih (puščica), ki se med razvojem povežejo v neprekinjen sloj. (D) Primarni kutikularni filtri (puščica) in sete (glava puščice) v želodcu pozne marzupijske manke. (E) Prva kutikula eksoskeleta se tvori pod prekutikularnim matriksom (pcm) pri poznem embriju. Na sliki je razvidna večslojna epikutikula (puščica). (F) Eksoskeletna kutikula pri srednji marzupijski manki je diferencirana v epikutikulo (epi), eksokutikulo (exo) in endokutikulo (endo).

Interactions between isopods and their microbiota

Although important, the role of isopods as decomposers in terrestrial ecosystems is mainly indirect. It includes the promotion of microbial activity by fragmentation of the substrate, increasing the number of some of the ingested microbes in their gut and the distribution of microorganisms in the terrestrial ecosystem (Zimmer 2002), while data on their direct role as decomposers remains scarce. Since the cellulolytic capability of herbivorous animals has been traditionally assigned to symbiotic microbiota, the presence of the latter has also been assumed to be in the digestive system of terrestrial isopods.

The idea on the resident microbiota being potentially involved in the digestion process was initiated by observations of filamentous bacteria attached to the inner surface of the hindgut in *P. scaber* (Drobne 1995) (Fig. 4A). At the same time, the description of pathogenesis and the presence of intracellular bacteria in the digestive glands of the same isopod species (Drobne et al. 1999b) indicated a wide array of specialized associations between the isopod hosts and bacteria. Research focused on the microbial associations of isopods had started in the group at the end of 90's, with a goal to extend our knowledge on isopod-bacteria symbiosis by microscopic observations and molecular techniques.

In collaboration with the group of Prof. Dr. Gorazd Avguštin from the Department of Animal Science at the University of Ljubljana we provided the first characterization of the resident microbiota of the gut of *P. scaber* based on 16S rRNA gene analysis (Kostanjšek et al. 2002); the filamentous bacteria attached to the gut wall were identified as commensal '*Candidatus Bacilloplasma*' (Kostanjšek et al. 2007) and the intracellular pathogen was *Rhabdochlamydia por-*

cellionis (Kostanjšek et al. 2004). Investigations into the latter continued in collaboration with the Department of Microbial Ecology, University of Vienna (Austria) and currently involves research into genome analysis and distribution of these pathogens in other arthropods.

Although the cellulolytic activity associated with the isopod digestive system has been assigned to endogenous cellulases, rather than to indigenous microbiota (Kostanjšek et al. 2010), our studies extended the knowledge on specialized bacterial symbionts of isopods. The descriptions of specific attachment structures of commensal *Bacilloplasma* and *R. porcellionis* as the first described chlamydia in arthropods, provide evidence that isopods may be an important, yet overlooked evolutionary playground for the development of novel bacterial groups including emerging pathogens (Corsaro and Venditti 2004). Further studies on *R. porcellionis* provided the protocol for their isolation in cell lines (Sixt et al. 2013), a detailed description of pathology in their natural host and their immune response to infection (Kostanjšek and Marolt 2015a) (Fig. 4B, C).

Recent descriptions of a heterogeneous bacterial community colonizing calcium bodies, the paired organs in the body cavity specialized for calcium storage in trichoniscid isopods represents yet another example of a unique association between woodlice and bacteria (Fig. 4E-G) (Vittori et al. 2012, Vittori et al. 2013). Despite its diversity and close affiliation to soil bacteria, the bacterial community of calcium bodies has the ability to accumulate poly-phosphate (Kostanjšek et al. 2015b). Although not fully understood, the unique symbiosis in the calcium bodies represents the first evidence of polyphosphate-accumulating bacterial symbionts in animal tissues and the first description of symbiosis confined to a specialized organ in isopod crustaceans.

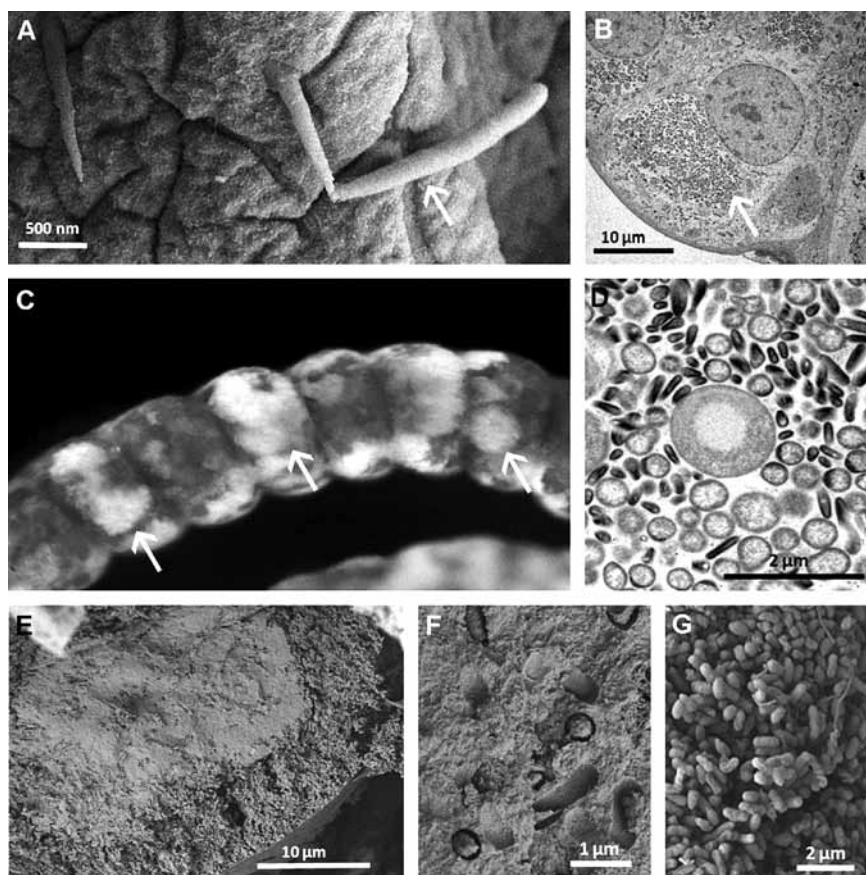


Figure 4: Associations between terrestrial isopods and bacteria. (A)-Scanning electron micrograph of a bacilloplasma (arrow) attached to a cuticular thorn in the hindgut of *Porcellio scaber*. (B)- Ultrastructure of hepatopancreatic cells of *P. scaber* infected with *R. porcellionis*. The cytoplasm of infected cells contains vacuoles (arrow) with numerous rhabdochlamydiae. (C)-White spots (arrows) in the hepatopancreas of *P. scaber* heavily infected with *Rhabdochlamydia porcellionis*. (D)-Higher magnification of rhabdochlamydiae within an infected cell. (E)-Scanning electron micrograph of a fractured calcium body of *Hyloniscus riparius*, containing a large bacterial population. (F)-Higher magnification of the mineral concretion with a calcium body of *H. riparius*, showing bacterial casts in the mineral. (G)-Diverse bacterial population consisting of rod-shaped, filamentous and helicoidal bacteria fills the lumen of a calcium body of *Titanethes albus*.

Slika 4: Povezave med kopenskimi enakonožci in bakterijami. (A)-Vrstična elektronska mikrofografija bacilloplazme (puščica), pritrjene na kutikularni trn v zadnjem črevesu pri enakonožcu *Porcellio scaber*. (B)- Ultrastruktura celice hepatopankreasa *P. scaber*, okužene z *R. porcellionis*. Citoplazma okužene celice vsebuje vakuole (puščica) s številnimi rabdoklamidijami. (C)-Bele lise (puščice) v hepatopankreasu *P. scaber*, okuženim z bakterijo *Rhabdochlamydia porcellionis*. (D)-Večja povečava rabdoklamidij v okuženi celici. (E)-Vrstična elektronska mikrofografija prelomljenega kalcijevega telesa pri enakonožcu *Hyloniscus riparius*, ki vsebuje številčno bakterijsko populacijo. (F)- Večja povečava mineralnega skupka v kalcijevem telescu *H. riparius*; v mineralu so vidni odtisi bakterij. (G)-Pestra bakterijska populacija, ki jo sestavljajo morfološko pestre bakterije, zapolnjuje lumen kalcijevega telesa vrste *Titanethes albus*.

Ecotoxicology

Terrestrial isopods have a long history as test organisms in ecotoxicology. This is due to their ecological relevance, their typical routes of exposure (soil and food), life history characteristics, ability to assimilate large amounts of metals, and the possibility to determine toxicity endpoints (Van Gestel 2012). According to the Thompson Reuters Web of Science™ one of the first ecotoxicity related papers on *P. scaber* was published already in 1978 reporting the accumulation of lead in these animals (Beeby 1978). In the 1980's the first papers on the assimilation of different metals in this species emerged (Hopkin et al. 1986, Dallinger and Prosi 1988, Donker et al. 1990) and ever since *P. scaber* has become a central terrestrial test organism in ecotoxicology.

Terrestrial ecotoxicology research at the Department of Biology began with the established cooperation with Steve Hopkin's group at the University of Reading (UK). As a result, the "standard laboratory test" with isopods was introduced based on studies with Zn (Drobne and Hopkin 1995, Drobne 1997). Further studies revealed that the molt frequency of isopods is influenced by the degree of Zn pollution in the food (Drobne and Štrus 1996). As molting also influences feeding and metal accumulation, a simple, brief, non-harmful identification of molt stages was proposed (Zidar et al. 1998). At that time, the majority of work at the department was focused on the assimilation of different metals in the whole body and digestive glands (Bibič et al. 1997, Zidar et al. 2003). Subsequent ecotoxicology research in the group followed the international progress in this field. The research efforts were aimed in two directions: (i) to assess the adverse effects of novel emerging pollutants and (ii) to study the effects on isopods investigated at different levels of biological organisation. In this respect, *P. scaber* has served as a central test object to investigate the effects of mercury (Hg) (Nolde et al. 2006), organophosphorus pesticides (Stanek et al. 2003), neonicotinoid pesticides (Drobne et al. 2008), natural insecticides (Zidar et al. 2012); veterinary medicines (Kolar et al. 2010, Žižek and Zidar 2013) and nanomaterials (Jemec et al. 2008, Novak et al. 2012a,b). Adverse effects of pollutants were studied on antioxidant enzyme

activities and the cholinergic system (Jemec et al. 2008), cell membrane integrity (Novak et al. 2012a), lysosomal integrity (Nolde et al. 2006), whole biomolecular profile of digestive gland (Novak et al. 2013), metallothionein-like proteins (Žnidaršič et al. 2005), ultrastructure of the digestive glands (Žnidaršič et al. 2003, Lešer et al. 2008), isopod gut microbiota (Lapanje et al. 2008), and feeding behaviour (Kaschl et al. 2002, Zidar et al. 2004).

Taking a retrospective view of the achievements made during the last 20 years of intensive terrestrial ecotoxicology research, we have confirmed that *P. scaber* is indeed a suitable test model providing a great deal of information regarding the adverse effects of pollutants and we have promoted the use of this organism in ecotoxicology widely at national and international levels. We have shown that pollutants induce a number of changes on the digestive mid-gut gland (hepatopancreas): Hg was shown to increase the permeability of the lysosomal membranes (Nolde et al. 2006), Hg and pesticide imidacloprid induced the thinning of epithelium (Lapanje et al. 2008, Drobne et al. 2008), copper decreased the digestive gland cell membrane permeability (Valant and Drobne 2012), and imidacloprid and TiO₂ nanoparticles altered the activity of antioxidant enzymes (Jemec et al. 2008, Drobne et al. 2008). It has been revealed that some pollutants affect the gut bacterial community structure (Lapanje et al. 2008), and organophosphate diazinon decreases the activity of the enzyme acetylcholinesterase in the whole body, resulting in decreased locomotory activity (Stanek et al. 2003). A strain of studies has been devoted to investigate the behavioural response of isopods to pollutants. We have commonly observed that isopods consume less polluted food when compared to the control exposure (Kaschl et al. 2002, Zidar et al. 2004, Drobne et al. 2008, Jemec et al. 2016). A number of investigations that included video tracking of foraging behaviour have revealed that isopods successfully discriminate between polluted and unpolluted food and soil (Zidar et al. 2003, 2004, 2005, 2012). The recognition of the isopods ability to detect chemicals in food or soil by using their chemoreceptors was a basis for establishing avoidance behaviour tests protocols with isopods. In the last 10 years we have been involved in stud-

ies investigating the interactions of nanomaterials with isopods. In this respect we have successfully joined the international nanotoxicity community. In the scope of this research we have shown that isopods substantially transform the nanoparticles inside the digestive tract. Namely, the dissolution of nanoparticles was substantially increased inside

the animal (Golobič et al. 2012, Romih et al. 2012). We have shown that nanoparticles do not internalise in the digestive gland, but only their dissolved ions do (Novak et al. 2012b, 2013). In Fig. 5 we present some photos obtained in different ecotoxicity assays with *P. scaber*.



Figure 5: Photos obtained in different ecotoxicity assays with *P. scaber*. (A)–Organism during the toxicity test. (B)–*P. scaber* after molt probably consuming the anterior exuvium (C)–Cross section of hepatopancreas; large B-cells include large lipid vesicles (arrow).

Slika 5: Slike pridobljene v različnih ekotoksikoloških testiranjih s *P. scaber*. (A)– Raki med testom strupenosti. (B)–*P. scaber* po levitvi sprednjega dela. (C)–Prečni prerez prebavne žleze hepatopancreas, velike B-celice imajo velike lipidne kaplje (puščica).

Systematics and evolution

The presence of morphologically diverse populations of ecologically important *A. aquaticus* in different types of freshwater habitats has challenged several authors to try to clarify the systematics of this species. In addition, the species is interesting from a phylogeographic perspective for several reasons: it is common throughout most of its range; it is present in several hydrographically separated Mediterranean drainages and all major drainages in central Europe; its current range has not been greatly affected by human impacts; it is tolerant to organic pollution and most of its range is inhabited by morphologically uniform populations of the nominotypical subspecies, while morphologically distinct subspecies with limited ranges occur in the S and SE of its range.

The first attempts at presenting racial differentiation in *A. aquaticus* were made on the basis of a small number of samples, specimens, and morphometric characters (Karaman 1952, Sket 1965a). Sket (1994) summarized the taxonomy of the species, listing as many as 10 subspecies and suggesting high probability of extensive racial diversification, especially in Slovenia. His supposition was confirmed by the results of multivariate

statistical analyses of geographic variation in an extensive set of morphometric characters run on large series of populations (Prevorčnik et al. 2004). High variation in characters within the surface populations resulted in their partial overlap, while the analysed highly troglomorphic populations were clearly analyzed separated not only from the surface ones, but also from each other. Some inconsistencies in formerly applied classification appeared; namely, the Romanian population from sulphurous mesothermal waters had already been described as *A. a. infernus* (Turk-Prevorčnik et Blejcek, 1998), while three populations from NW Dinaric karst were treated as a single subspecies (*A. a. cavernicolus* Racovitza, 1925) at the time.

To overcome the shortcomings of morphology-based taxonomy, molecular studies have been used as a promising taxonomic tool. The first studies focused on the relationships of the cave to surface populations using randomly amplified polymorphic DNA (RAPD; Verovnik et al. 2003) and nuclear and mitochondrial DNA sequences (Verovnik et al. 2004) as genetic markers. The results supported the observed morphological separation, showing that the invasions of the cave populations were spatially as well as temporally independent. Subsequently, DNA sequences were also used for the assessment

of the colonization history of the species (Verovnik et al. 2005). These analyses suggested that the European continent was colonized from the SE during late Miocene. Mitochondrial DNA still shows the pattern of several deeply subdivided clades, while much more uniform nuclear DNA sequences and geographical mixing of mitochondrial DNA groups speak in favour of extensive recent gene flow. As the original description of the nominate species was inadequate and the type material of Linné appeared to be lost, it was imperative to designate a neotype for *A. aquaticus* and provide its proper description (Verovnik et al. 2009).

Cave populations tend to be genetically isolated from nearby surface populations and thus potentially represent independent, albeit young species. The cave population from Grotta di Trebiciano/Labodnica near Trieste/Trst (lower part of the Reka River drainage, NW Italy) showed the highest degree of genetic independence among all subterranean populations. Therefore, it was described as the first cave species of the genus in Europe under the name *Asellus kosswigi* (Verovnik et al. 2009) (Fig. 6).

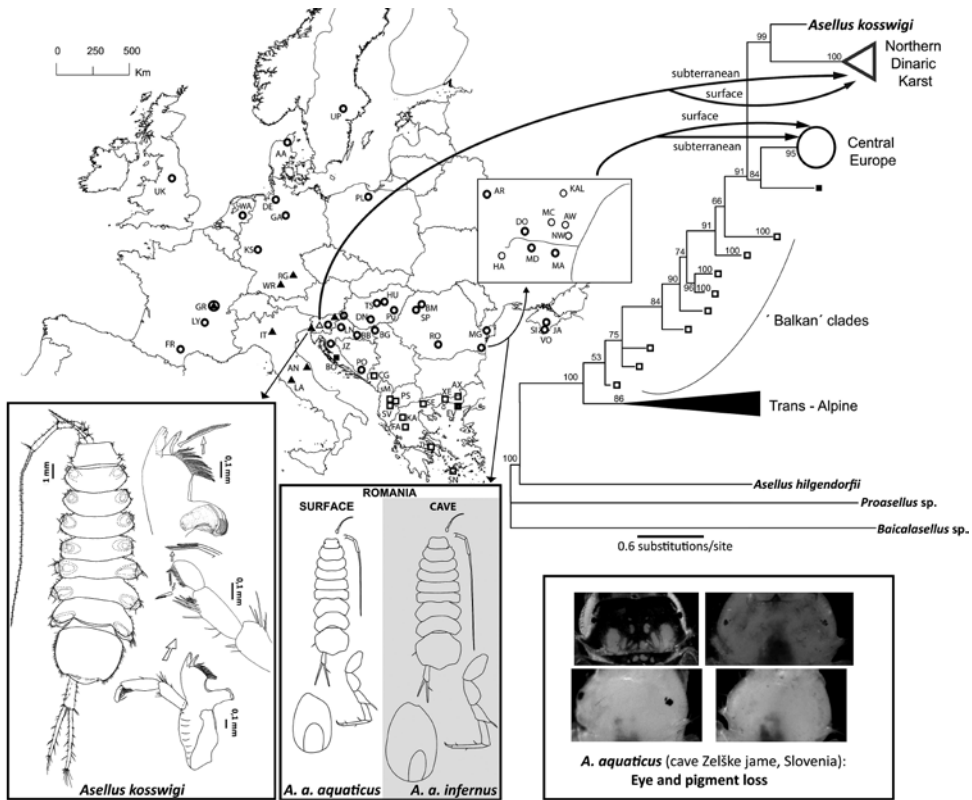


Figure 6: Systematics and evolution of *Asellus aquaticus*. The global phylogeography of the species is based on a Bayesian phylogeny of mitochondrial (COI) haplotypes (modified from Konec et al. 2015). Pictures show the recently described *A. kosswigi* from the subterranean Reka River (modified from Verovnik et al. 2009), the Romanian surface/cave ecomorph pair, and the variability of eye and pigment loss on the head in one of the Slovenian cave populations.

Slika 6: Sistematika in evolucija vodnega oslička, *Asellus aquaticus*. Globalna filogeografija vrste na podlagi Bayesove filogenije mitohondrijskega (COI) gena (prirejeno iz Konec et al. 2015). Slike prikazujejo še: nedavno opisanega troglobionta *A. kosswigi* iz reke Reke (prirejeno iz Verovnik et al. 2009), romunski površinski in jamski ekomorf ter različne stopnje izgube pigmenta in oči pri eni od slovenskih jamskih populacij.

Just recently, this species has also been discovered in the upper (Slovenian) parts of the same drainage (Konec et al. 2015). A number of other subterranean populations are known from caves in the Ljubljana and Krka River drainages. These populations are even younger invaders into the subterranean realm than *A. kosswigi* and therefore genetically less differentiated. Their genetic structure and evolutionary history was studied using microsatellite DNA, which is more variable than mitochondrial DNA sequences. In the unique hydrological setting of the cave Planinska jama (Slovenia) with the subterranean confluence of two sinking rivers, an equally unique diversity of subterranean *Asellus* populations was discovered. Two highly troglomorphic and genetically distinct populations each dwelling in its own channel of the cave, are isolated from each other as well as from the surrounding surface populations (Konec et al. 2016). Only at the confluence they form a narrow hybrid zone, representing the first known case of secondary sympatry between two subterranean sister lineages in a cave.

In the last decade, *A. aquaticus* became one of the few invertebrate model species for the study of troglomorphic evolution. It possesses several attributes expected in such species: its cave populations have evolved many troglomorphic traits for which the direction of change is known (since they were derived from surface ancestors); trait changes can be attributed to a specific environmental factor (absence of light and reduced/absent primary productivity); their extant surface ancestors provide a dual system of both forms; they are relatively abundant in nature, ability to rear in the laboratory and they produce large numbers of easily accessible embryos (Protas and Jeffery 2012). Pairs of surface and descendant subterranean *Asellus* populations from different habitats and different geographic areas were examined to address questions of evolutionary parallelism and convergence. Surprisingly, it turned out that only one third of all changes were convergent (Konec et al. 2015), and that reduction and loss of both eyes and body pigment is caused by two or more independent genetic pathways (Protas et al. 2011). Recently, ethological observations of *Asellus* habitat selection have started in an attempt to elucidate the acquisition and maintenance of reproductive isolation between surface and cave-dwelling populations in the absence of geographical barriers.

Over decades, numerous collaborations with domestic and foreign experts led to a number of important findings and *Asellus* has certainly contributed to the recognition of the research group within the speleobiological and wider scientific community.

Conclusions and prospective view

In this paper, we have presented the research relating to two isopod model species, terrestrial *Porcellio scaber* and aquatic *Asellus aquaticus*, undertaken at the Chair of Zoology, Department of Biology during the last 45 years.

P. scaber has been successfully used as a model species in studies on functional morphology of integument and digestive system during molting and development. Within this line of research future studies will be aimed at ultrastructural and cytochemical studies of different extracellular matrices in order to establish structural and functional features of natural materials under different conditions and thus provide the basic knowledge for designing bio-inspired materials used in new technologies. Despite considerable potential in agriculture, ecology, healthcare and pest control, the current exploitation of interactions between bacteria and arthropods remains restricted to a handful of associations (Bourtzis and Miller 2003). With a wide array of specialized bacterial associations, *P. scaber* represents an appropriate and much needed model organism for studies into arthropod-microbe interactions, with a strong potential for an extension of current knowledge and a better understanding of these associations. *P. scaber* has become a central test model in terrestrial ecotoxicity studies offering an array of possibilities to investigate the effects of pollutants on several organisational levels, from single molecules to populations. We envisage that our future ecotoxicity research will continue to follow the emerging environmental threats and contribute to the understanding of potential risks to terrestrial environments.

A. aquaticus has become one of the most important organisms for the study of evolution of subterranean biodiversity and the evolution of troglomorphies. To reach this stage, substantial fundamental research on taxonomy, population

genetics, morphology and ecology was performed in the past four decades. New exciting findings are within reach in the fields of evolutionary developmental genomics, population genomics, and the biology of speciation of subterranean species. The newly revealed taxonomic complexity of what has been until recently considered as a single species, prompted the description of the first subterranean species with at least two new ones to follow. In that way, *A. aquaticus* with its descendant subterranean populations fosters the tradition of speleobiological research at the Department of Biology and contributes its share to the subterranean biodiversity of the Dinaric Karst, which is known to be the richest in the World.

The scientific studies of terrestrial and aquatic isopods at the Department of Biology have been fostered since Pavel Ličar's first reports in the early 70's. The present paper brings strong evidence on the diversity, importance and scientific potential of our research proving isopods are an excellent model organism for future research. Perhaps, even more than Professor Pavel Ličar had ever imagined.

Povzetek

V članku predstavljamo študije rakov enakonožcev, obsežne skupine višjih rakov (Malacostraca: Peracarida: Isopoda), ki se ji na Katedri za zoologijo posvečamo že nekaj desetletij z raziskavami na področjih funkcionalne morfologije in razvojne biologije, specifičnih interakcij med gostiteljem in mikroorganizmi, ekotoksikologije ter sistematike in evolucije. V prispevku navajamo razloge za preučevanje dveh modelnih rakov enakonožcev: navadnega prašička (*Porcellio scaber*) in vodnega oslička (*Asellus aquaticus*). Poleg zgodovinskega poteka raziskav na posameznih področjih, predstavljamo tudi najpomembnejše rezultate novodobnih raziskav, njihov pomen ter načrte za prihodnost. Kopenski raki so, zaradi splošne razširjenosti, uspešnih prilagoditev življenju na kopnem, enostavnega vzdrževanja v laboratoriju in učinkovite reprodukcije odlični model za raziskave na področju funkcionalne morfologije in razvojne biologije. Pogoste levitve v zvezi z rastjo in razmnoževanjem in velika raznolikost v zgradbi eksoskeleta

so priložnost za študij sinteze in razgradnje zunajceličnega matriksa in procesov biomineralizacije, predvsem dinamike kalcija v epitelijih, ki izločajo kutikulo. Eksoskelet kopenskih rakov enakonožcev je večslojni matriks iz hitina in proteinov, ki je pri amfibijskih in terestričnih skupinah v različni meri kalcificiran. Struktura, kemijska sestava in mehanske lastnosti kutikule se razlikujejo med vrstami in tudi glede na telesne regije pri isti vrsti. Z raziskavami morfogeneze prebavnega sistema in integumenta pri vrsti *P. scaber* se vključujemo v širše področje raziskav embrionalnega in postembrionalnega razvoja rakov, ki v zadnjem času hitro napreduje in obsega vse več študij tako z vidika funkcionalne morfologije kot z vidika evolucijske razvojne biologije. Na osnovi morfoloških kriterijev smo opisali 19 embrionalnih stadijev in tri stadije marzupijskih mank. Razložili smo oblikovanje ektodermalnega in endodermalnega dela prebavnega sistema. Pojasnili smo tudi ultrastrukturne značilnosti diferenciacije eksoskeletne kutikule in kutikule v črevesu med razvojem embrijev in marzupijskih mank. V nadaljevanju te raziskave nadgrajujemo s študijem diferenciacije epitelnih celic integumenta in črevesa, diferenciacije povezav z mišicami in študijem zgodnjih procesov kalcifikacije kutikule eksoskeleta. Študij navedenih vsebin je izrazito interdisciplinaren, saj povežemo raziskave na področju anatomije, histologije, celične biologije, razvojne biologije in znanosti o materialih, kar je nujno za ustvarjanje novega znanja in njegovo uporabo v sorodnih področjih. Raziskave sekrecije in mineralizacije različnih tipov zunajceličnega matriksa med embrionalnim razvojem modelnega organizma *P. scaber* so pomembne za razumevanje vloge matriksa v diferenciaciji epitelijev prebavil in integumenta nevretenčarjev. Prisotnost raznolikih mikroorganizmov na površini kutikule prebavila je verjetno povezana s sestavo in dinamiko zunajceličnih matriksov med embrionalnim razvojem, zato raziskave nadaljujemo tudi v tej smeri. Odkrili smo številne in raznolike interakcije med *P. scaber* in mikroorganizmi v prebavilu. Raziskave interakcij med členonožci in mikroorganizmi so pomembne tudi z vidika razgradnje organske snovi in nastajanja prsti, zato so kopenski enakonožci pomemben model za ugotavljanje procesov dekompozicije. Tudi s slednjega stališča so kopenski raki *P. scaber*

pomemben osrednji model na področju kopenske ekotoksikologije. Tekom naših raziskav smo dokazali veliko število vplivov različnih vrst onesnaževal, od kovin, pesticidov, veterinarskih zdravil do nanomaterialov na organizem *P. scaber*. Vplive smo proučevali na različnih nivojih organizacije: celičnem (spremembe aktivnosti antioksidativnih encimov, holinergični sistem), tkivnem (struktura in funkcija prebavne žleze) ter prehranjevalnega vedenja živali s sledenjem in opisom.

Visoka morfološka pestrost populacij ekološko pomembnega vodnega oslička (*A. aquaticus*) je in še predstavlja taksonomski izziv. V raziskovalni skupini za speleobiologijo smo se ga lotili z morfolometričnimi in molekulskimi analizami velikega števila vzorcev in osebkov. Z vsemi analizami smo dokazali jasno ločitev podzemeljskih populacij od površinskih. Še več, podzemeljske so se močno razlikovale tudi med seboj. *A. aquaticus* je izjemno primeren model za filogeografske raziskave zaradi splošne razširjenosti v medsebojno ločenih evropskih povodjih, sorazmerne odpornosti proti onesnažilom ter velike rasne diferenciacije, zlasti na jugu in jugovzhodu območja razširjenosti. Z analizo mitohondrijske in jedrne DNA smo ugotovili, da naj bi do poselitve Evrope prišlo v poznem Miocenu, podzemeljske populacije pa so podzemlje očitno poselile večkrat neodvisno. Na osnovi neskladja z dotlej veljavno klasifikacijo smo poskrbeli za dopolnjen opis nominotipske vrste in opisali prvo evropsko podzemeljsko vrsto, *A. kosswigi* iz italijanske jame Labodnica. Pred kratkim smo jo našli tudi v Sloveniji, kjer na opis čakata še dve pravi podzemeljski vrsti,

ki med drugim živita v dveh rokavih Planinske jame. Ravno morfološko visoko specializirane (troglomorfne) jamske populacije, pri katerih je genetski pretok s predniškimi površinskimi populacijami zanemarljiv ali prekinjen, omogočajo poglobitev razumevanja procesov populacijske diferenciacije, speciacije in evolucije troglomorfij. Zato se je v zadnjih letih *A. aquaticus* uveljavil tudi kot ključni model v evolucijskih študijah, ki raziskujejo prilagajanje živali ob vselitvi v novo okolje. Nepričakovano se je izkazalo, da je pri osličkih le tretjina sprememb na račun življenja v podzemlju (troglomorfij) konvergenca, poleg tega pa do izgube pigmenta in oči privedeta dva različna genetska mehanizma. Pred kratkim smo se v skupini lotili tudi prvih vedenjskih poskusov, s katerimi poskušamo pojasniti nastanek reproduktivne bariere med površinskimi in podzemeljskimi populacijami. V raziskovalni skupini smo z izsledki taksonomskih, filogenetskih, filogeografskih in evolucijskih raziskav na vodnem osličku pomembno prispevali k splošnemu znanju o biodiverziteti Dinarskega Krasa, s podzemeljskim živalstvom najbogatejšega območja sveta.

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