

Alizarin red S staining of the crustacean cuticle: implementation in the study of *Porcellio scaber* larvae

Histokemijska analiza kutikule rakov z barvilom alizarin rdeče S: uporaba v proučevanju ličink raka enakonožca vrste *Porcellio scaber*

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Abstract: Exoskeletal cuticle of crustaceans is a chitinous matrix, produced apically by epidermis and stiffened by sclerotization and calcification. Embryos of terrestrial isopod crustacean *Porcellio scaber* develop within the female brood pouch, marsupium, and after hatching larvae mancae continue their development in the marsupium for another week. This study was performed to reveal at the histochemical level whether the exoskeletal cuticle of marsupial mancae is already calcified. Fifteen different procedures of histochemical staining with alizarin red S (ARS), established for calcified tissue localization primarily in vertebrate histology, were evaluated on mancae and adult *P. scaber* specimens. The best differential staining of the exoskeletal cuticle was obtained with neutral buffered formaldehyde fixation, followed by paraffin sections staining with ARS 1 (pH 9) or ARS 2 (pH 6.4) or ARS 3 (pH 4.8) solution. Clear differential staining was achieved also in cryosections of formaldehyde fixed samples, stained with ARS 1 solution (pH 9). Our results suggest that prominent calcification of exoskeletal cuticle is present during postembryonic development of *P. scaber* mancae in the marsupium. Exoskeleton hardening is likely important also for body movements, that we observed in mancae before they were released from marsupium. The proposed procedures of ARS method are presumed to be applicable for histochemical studies of other calcified chitinous matrices.

Keywords: calcification, histochemistry, larval development, terrestrial isopods, Crustacea

Izveček: Eksoskeletna kutikula rakov je hitinski matriks na apikalni strani epidermisa. Trdnost kutikule je posledica sklerotizacije in kalcifikacije organskega matriksa. Embriji kopenskega raka enakonožca *Porcellio scaber* se razvijajo v vrečastem valilniku samice, marzupiju, kjer po izleganju nadaljujejo svoj razvoj približno en teden tudi ličinke manke. S histokemijsko metodo smo ugotavljali, ali je eksoskeletna kutikula marzupijskih mank že kalcificirana. Na vzorcih mank in odraslih rakov *P. scaber* smo ovrednotili petnajst različnih postopkov histokemijske analize z barvilom alizarin rdeče S (ARS), ki se uporabljajo za lokalizacijo kalcificiranega tkiva v histologiji vretenčarjev. Eksoskeletna kutikula se je izrazito diferencialno obarvala v primeru fiksacije z nevtralno raztopino formaldehida, ki ji je sledilo

barvanje parafinskih rezin z eno izmed raztopin ARS: ARS 1 (pH 9), ARS 2 (pH 6.4) ali ARS 3 (pH 4.8). Jasno lokalizacijo smo dosegli tudi z barvanjem zamrznjenih rezin vzorcev, fiksiranih v formaldehidu in barvanih z raztopino ARS 1 (pH 9). Rezultati kažejo na znatno kalcifikacijo eksoskeletne kutikule že v postembrionalnem razvoju mank *P. scaber* v marzupiju. Trdnost eksoskeleta je najverjetneje pomembna tudi za gibanje mank, ki smo ga opazili pred sprostitvijo iz valilnika. Pričakujemo, da bodo postopki, ki jih predlagamo, uporabni tudi za histokemijska proučevanja kalcifikacije drugih hitinskih matriksov.

Ključne besede: kalcifikacija, histokemija, razvoj ličink, kopenski raki enakonožci, Crustacea

Introduction

Mineralized organic matrices constitute many morphologically and functionally diverse structures in different organisms ranging from bacteria to humans. Their unique feature is a prominent mineral component, which is closely connected with the organic matrix. Mineralization increases matrix strength and hardness that provides protection against environmental pressures and support for muscle attachment. A common type of mineralization in living organisms is calcification, the deposition of different calcium minerals in the organic matrices (Bonucci 2007). Well known examples of calcified organic matrices in vertebrates are bones and teeth, whose basic organic component is collagen. Exoskeleton of crustaceans or exoskeletal cuticle is a representative example of a calcified matrix based on chitinous organic scaffold.

The exoskeletal cuticle is a complex hierarchically structured extracellular matrix, consisting of the polysaccharide chitin, proteins, lipids and also minerals. It is produced by a single-layered epidermis during embryonic development and it is renewed periodically during molting in adults. The ultrastructure of exoskeletal cuticle in adult isopod *Porcellio scaber* has been described in detail (Ziegler 1997, Hild et al. 2008, Seidl and Ziegler 2012). It comprises the outermost epicuticle, exocuticle and the inner endocuticle. Thin epicuticle is composed mainly of lipoproteins and consists of thinner 5-layered outer epicuticle and thicker inner epicuticle. Exocuticle and endocuticle are calcified and comprise sublayers of chitin-protein fibers arranged in a characteristic helicoidal pattern (Fig. 1).

Cuticle in adults is calcified due to deposition of crystalline calcium carbonate (calcite), amorphous calcium carbonate (ACC) and amorphous calcium phosphate (ACP) (Ziegler 1994, Ziegler 1997, Becker et al. 2005, Luquet 2012). Recent studies have shown defined spatial distribution of both polymorphs of calcium carbonate in different layers of adult isopod cuticle (Hild et al. 2008, Hild et al. 2009, Neues et al. 2011, Seidl et al. 2011). In *P. scaber* the exocuticle contains both calcite and ACC, whereas the endocuticle is calcified only by ACC (Hild et al. 2008). There are a few data on the structure and composition of the exoskeletal cuticle in embryonic and larval stages in crustaceans. The central focus of this study is to show at the histochemical level whether the larval cuticle in *Porcellio scaber* is calcified or not.

The specimens of *Porcellio scaber* belong to a group of terrestrial isopod crustaceans (Oniscidea). The embryonic development takes place in the fluid-filled brood pouch (marsupium) on the ventral side of female body, that has likely been of a great adaptive significance in the colonization of land by crustacean species (Hornung 2011, Warburg 2011). Intramarsupial development of *P. scaber*, from released fertilized eggs to embryos and marsupial larvae mancae, lasts approximately 35 days in laboratory conditions and it was described morphologically with twenty progressive stages (Wolff 2009, Milatovič et al. 2010). After hatching of embryo from two egg envelopes (chorion and vitelline membrane), larva termed manca stays in the marsupium for a week and is subsequently released to the external environment (Supplementary fig. 1, supplementary figures are available in online edition).

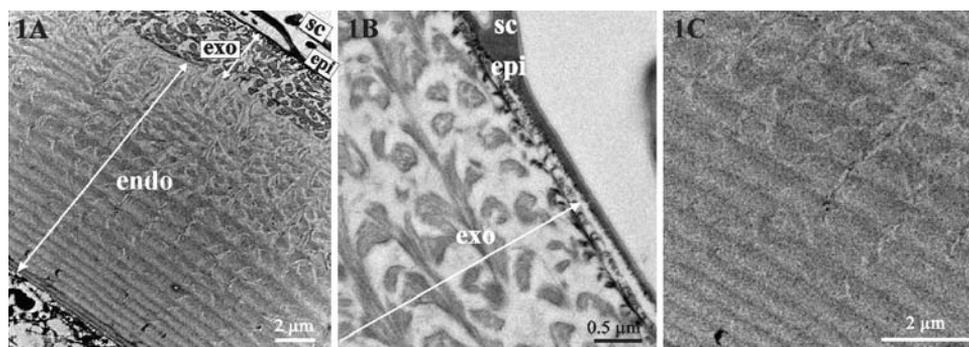


Figure 1: Ultrastructure of the exoskeletal cuticle in adult *P. scaber*. A – Cuticle is organized in distinct horizontal layers: epicuticle (epi), exocuticle (exo) and endocuticle (endo). B – Epicuticle (epi) is a thin and electron dense layer with prominent scales (sc). Exocuticle (exo) comprises chitin-protein fibers, arranged in a characteristic pattern. C – Endocuticle comprises lamellar chitin-protein sublayers.

Slika 1: Ultrastruktura eksoskeletne kutikule odraslega raka *P. scaber*: A – Kutikulo sestavljajo različni sloji: epikutikula (epi), eksokutikula (exo) in endokutikula (endo), kot si sledijo od zunanjega proti notranjemu delu. B – Epikutikula (epi) je tanek, elektronsko gost sloj z izrazitimi luskami na površini (sc). Eksokutikula (exo) vsebuje hitinsko-proteinska vlakna, ki so urejena v značilen vzorec. C – Endokutikula vsebuje lamelarne podслоje hitinsko-proteinskih vlaken.

Our previous study shows that the cuticle in *P. scaber* marsupial mancae exhibits main ultrastructural features of the adult crustacean cuticle (Mrak et al. 2012). Cuticle of marsupial mancae is composed of three layers, the outermost thin electron dense epicuticle, the middle exocuticle and the innermost endocuticle. The characteristic pattern of chitin-protein fiber arrangement in the exocuticle is already present and sublayers are evident in the endocuticle (Fig. 2). The thickness of cuticle is up to 3 μm . Some morphological features suggesting cuticle renewal are observed occasionally in marsupial mancae, such as cuticle detachment from the epidermis, partly disintegrated inner portion of the endocuticle, assembling of the new cuticle and apical protrusions of epidermal cells with electron dense tips (Fig. 2B).

The data on mineralization of chitin matrix in mancae larvae is very limited. Mancae, released from the marsupium (postmarsupial mancae), were investigated in this respect by Hadley and Hendricks (1987) using energy dispersive X-ray spectroscopy (EDS) in the isopod *Porcellionides pruinosus*. They detected calcium in the cuticle of mancae already released to the external environment, but in much lower quantities compared to the adult cuticle levels. Concerning marsupial mancae, exoskeleton calcification is still an open question.

Localization and characterization of calcified tissues can be performed by different methods, each of them focused to address specific questions regarding tissue composition and structure. Identification of inorganic components can be achieved using different biophysical and morphological methods (Bonucci 2007). The advantage of histochemical techniques is simple and quick performance that is important when screening of many samples is needed to gain preliminary information about the presence of mineralized tissue. On the basis of these results selected samples can be further analysed in detail with advanced and highly demanding biophysical techniques, like: X-ray, neutron and electron diffraction for studying the structure of crystals; energy dispersive X-ray elemental analysis (EDS) and electron energy-loss spectroscopy (EELS) for analysing the presence of specific elements and their distribution in the tissue; infrared and Raman spectroscopy for revealing details about molecular structure and especially mineral forms in the tissue. A commonly used histochemical method to demonstrate calcified tissue, applied also in our study, is alizarin red S (ARS) staining. According to Virtanen and Iso-tupa (1980) and Lievreumont et al. (1982), alizarin red S molecules react with calcium ions via its sulfonate and hydroxyl groups, forming brick-red

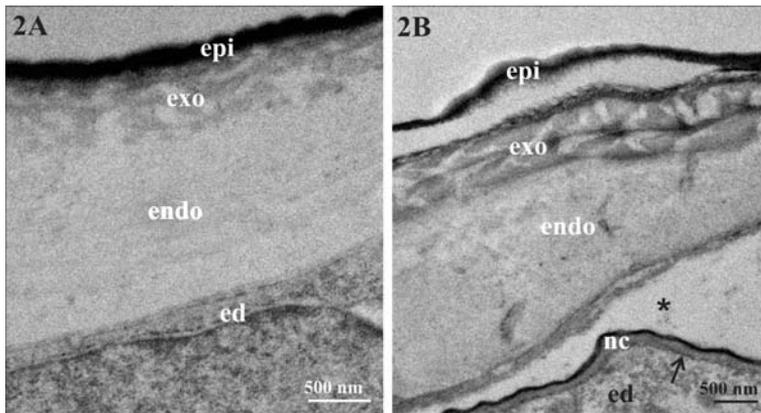


Figure 2: Ultrastructure of *P. scaber* marsupial manca cuticle. Differentiation in epicuticle (epi), exocuticle (exo) and endocuticle (endo) is evident. The micrograph B shows some features of cuticle renewal: cuticle detachment from the epidermis, ecdysal space (*) between the old and the new cuticle, partial disintegration of inner portion of endocuticle, newly assembling cuticle (nc) and protrusions with electron dense tips (arrow) on apical plasma membrane of epidermal cell (ed).

Slika 2: Ultrastruktura kutikule pri marzupijskih mankah raka *P. scaber*. Diferenciacija v epikutikulo (epi), eksokutikulo (exo) in endokutikulo (endo). Mikrografija B prikazuje nekatere značilnosti obnavljanja kutikule, kot so: odstopanje kutikule od epidermisa, levitveni prostor (*) med staro in novo kutikulo, delna razgradnja notranjega dela endokutikule, nastajanje nove kutikule (nc) in izrastki z elektronsko gostimi konicami (puščica) na apikalni plazmalemi epidermalnih celic (ed).

precipitates (ARS-calcium salts, complexes and chelates). Other cations (like magnesium, barium, copper, zinc, iron, aluminium) react with ARS as well, but normally only calcium is present in biological tissues in sufficient quantities for demonstration (McGee-Russell 1958, Puchtler et al. 1969, Lievreumont et al. 1982). ARS is also an anionic dye and as such causes non-specific pink background staining. According to the literature, that reports several modifications in procedure of the staining technique, the histochemical result is greatly influenced by the fixation method and pH of staining solution. Fixation method has a significant effect on preservation of calcium in tissue, while pH of staining solutions effects staining sensitivity and staining specificity. As this method has relatively low sensitivity, small differences in calcium concentrations can not be distinguished and low calcium concentrations can not be detected.

This study, based on alizarin red S staining, a histochemical approach for calcified tissue localization, was performed to show whether

cuticle is calcified already during intramarsupial larval development in isopod crustacean *Porcellio scaber*. Considering many modifications of this method, particularly regarding fixation and staining pH, we have evaluated several procedures for estimating the presence of cuticle calcification in *P. scaber* marsupial mancae. This method is expected to be useful also in the studies of calcification of other chitinous matrices.

Materials and methods

In this study the histochemical localization of calcified tissues by alizarin red S (ARS) was implemented to estimate the presence of exoskeleton calcification in marsupial mancae of the species *Porcellio scaber* Latreille, 1809 (Crustacea: Isopoda). Adult animals were maintained in a laboratory culture, in soil and leaf litter, at 25°C, high relative humidity and a 12-h light/12-h dark cycle. Gravid females with mancae in the marsupium were selected from laboratory culture. Thirteen mancae were isolated from the marsupia, anesthetized and fixed. They were carefully perforated with a thin needle, enabling better infiltration of the fixative and/or the embedding medium. Adult animals that were without any signs of molting were used as positive controls for histochemical reaction. Animals were anesthetized, transversely cut in two pieces and fixed. Subsequently they were always processed simultaneously with the samples of marsupial mancae in all procedures applied. Negative controls of histochemical

reaction were the sections of adult and mancae samples, preincubated in decalcification solution (10% ethylenediamine-tetracetic acid disodium salt (EDTA), pH 7.4, 5 minutes) before ARS staining.

Fixation was performed in five ways (Table 1): (a) three different chemical fixations, (b) chemical fixation followed by freezing or (c) freezing. In the procedure (a) – chemical fixation, three different fixatives were used, recommended by several authors: (a1) Carnoy fixative (absolute ethanol-chloroform-glacial acetic acid, 6:3:1), (a2) 3.7% formaldehyde in 0.1 M cacodylate buffer (pH 7.2) or (a3) 70% ethanol. Formaldehyde is a widely recommended fixative, also for studies of calcium localization (McGee-Russell 1958, Bancroft and Gamble 2008, Kiernan 2008), although some authors prefer Carnoy fixative and describe formaldehyde as unsuitable fixative for studies of calcium deposits as it could remove some calcium during prolonged fixation (Puchtler et al. 1969, Presnell and Schreiber 1997). Alcoholic fixatives are widely recommended for calcified tissue localization as they preserve calcium in tissue (Puchtler et al. 1969, Presnell and Schreiber 1997, Bancroft and Gamble 2008, Kiernan 2008), though it is a poor fixative for preservation of tissue structure. After fixation, samples were dehydrated through an ascending series of ethanol and in xylene, infiltrated with paraffin wax at 60°C overnight and embedded afterwards. Transversal sections (10 µm) were cut with a Leica RM2265 microtome, transferred to water on microscope slides and stretched and dried on a hot plate.

Samples prepared for paraffin sectioning were exposed to aqueous solutions several times, which could affect the tissue by removing amorphous calcium. Several authors demonstrated high solubility of ACC in aqueous solutions (Brečević and Nielsen 1989, Gal et al. 1996, Meiron et al. 2011). To avoid calcium loss from the tissue, cryosectioning was performed, which minimizes exposure to aqueous solutions. Specimens destined for cryosectioning were either fixed in 3.7% formaldehyde in 0.1 M cacodylate buffer (pH 7.2) and frozen afterwards (procedure b) or directly frozen without any pretreatment (procedure c). They were embedded in tissue freezing medium (Jung). Sections (10 µm) were cut with a Leica

CM1850 cryostat at –20°C. Cryosections were transferred directly to microscope slides and dried at room temperature.

According to the literature, ARS staining is greatly influenced by pH of staining solution. Alkaline ARS solution liberates less calcium and has lower sensitivity but enables more precise localization of calcified tissue (Puchtler et al. 1969, Kiernan 2008). Acidity of ARS solution leads to releasing of more calcium ions from tissue and consequently calcium localization appears more dispersive and sensitivity for calcified tissue demonstration appears higher. Paraffin sections and cryosections were stained by three different staining solutions of ARS, that were recommended by several authors (McGee-Russell 1958, Puchtler et al. 1969, Presnell and Schreiber 1997, Bancroft and Gamble 2008, Kiernan 2008). ARS solution 1 (purple-red coloured) was 0.5% solution in 0.2 M Trihydroxymethyl aminomethane (Tris)-HCl buffer, pH 9. ARS solution 2 (dark red coloured) was 1% aqueous solution, adjusted to pH 6.4 with 10% NH₄OH. ARS solution 3 (brown-red coloured) was 1% aqueous solution, adjusted to pH 4.8 with 10% ammonium hydroxide (NH₄OH). Before staining, paraffin sections were deparaffinized and rehydrated in a descending series of ethanol to distilled water and after staining, they were dehydrated and mounted in synthetic resin Pertex. Cryosections were stained directly and after staining they were mounted in glycerol jelly. Staining duration was 20–30 seconds for paraffin sections and 10 seconds for cryosections, followed by a quick rinse in distilled water. The applied procedures are summarized in Table 1.

Sections were imaged by Zeiss AxioImager Z.1 light microscope, equipped with a HRc AxioCam camera and Axiovision software. Cryosections were inspected immediately after mounting. Intensity of the histochemical reaction with ARS was classified semiquantitatively in four categories: (i) no staining, (ii) light staining, (iii) moderate staining and (iv) intense staining.

Results

In this study the calcification of exoskeleton in marsupial mancae of *Porcellio scaber* Latreille, 1809 (Crustacea: Isopoda) was estimated at the

Table 1: Summary of the fixation and alizarin red S staining procedures, used in this study on *Porcellio scaber* marsupial mancae.

Tabela 1: Povzetek postopkov fiksacije in histokemijske lokalizacije z barvilom alizarin rdeče S, uporabljenih v tej študiji marzupijskih mank raka enakonožca *Porcellio scaber*.

(a) chemical fixation			(b) chemical fixation followed by freezing	(c) freezing at -20°C
(a1) Carnoy fixative	(a2) neutral buffered 3.7% formaldehyde	(a3) 70% ethanol	neutral buffered 3.7% formaldehyde	
	↓ dehydration			↓ tissue freezing medium embedding and freezing
	↓ paraffin embedding			↓
	↓ paraffin sectioning			↓ cryosectioning
	↓ deparaffinization and rehydration			
			↓ staining in alizarin red S solution 1 (pH 9) or 2 (pH 6.4) or 3 (pH 4.8)	
			↓ quick rinse in distilled water	
	↓ dehydration			↓
	↓ mounting in resin			↓ mounting in glycerol jelly

histochemical level by alizarin red S (ARS) method for calcified tissues localization. Several methods have been suggested for ARS staining and here we tested five different fixations in combination with three different staining solutions, altogether fifteen different procedures, on *P. scaber* marsupial mancae. We analysed the staining intensity of mancae cuticle in comparison to other tissues (background) and regarding positive and negative controls of histochemical reaction.

In Carnoy fixed specimens (procedure a1) no differential staining of the cuticle was obtained, neither in adults nor in mancae sections (Table 2, Supplementary fig. 2). ARS staining of Carnoy fixed specimens resulted in light red staining of all tissues with basic ARS solution (ARS 1). ARS 2 (pH 6.4) and ARS 3 (pH 4.8) solutions resulted in deeper red staining, displaying intensely stained cuticle and moderately stained other tissues, i.e. connective tissue and muscles (Table 2, Supplementary fig. 2).

ARS staining of neutral buffered formaldehyde fixed specimens (procedure a2) resulted in

clearly differential staining of exoskeleton (Table 2, Supplementary fig. 3). The exoskeletal cuticle in adults and mancae was intensely red, while other tissues like glands and muscles were not stained. Negligible background was visible with acid ARS staining solution (ARS 3, pH 4.8). In specimens pretreated in decalcification solution (EDTA) for negative controls, the cuticle was not red stained with ARS and only in the case of ARS 2 and ARS 3 staining solutions negligible background was observed (Supplementary fig. 3).

The overall histological structure of specimens fixed in 70% ethanol (procedure a3) was not so well preserved in comparison to specimens fixed in formaldehyde (Supplementary fig. 4). We did not obtain any undoubtedly differential staining of the exoskeleton in ethanol fixed samples (Table 2, Supplementary fig. 4). In all mancae and adult sections cuticle and other tissues were stained nearly with the same intensity, except for the more intensely stained adult cuticle with basic ARS (ARS 1). In negative controls (pretreated in

Table 2: Summary of Alizarin red S staining results of *Porcellio scaber* marsupial mancae and adults: Specimens were fixed by five different methods: (a1) Carnoy fixative, (a2) 3.7% neutral buffered formaldehyde, (a3) 70% ethanol, (b) 3.7% neutral buffered formaldehyde, followed by freezing and cryosectioning or (c) freezing and cryosectioning. Staining was performed with one of the following Alizarin red S solutions: ARS 1 (pH 9), ARS 2 (pH 6.4) or ARS 3 (pH 4.8). Intensity of ARS staining is classified as: ○ – no staining; * – light staining; ** – moderate staining; *** – intense staining. 'Diffusion' marks diffusion artifacts. Where different staining intensities were obtained for mancae and adults, both results are presented separately: mancae / adults. Gray labeled fields mark the procedures that resulted in clearly differential staining of exoskeletal cuticle in comparison to other tissues, indicating their suitability for calcified exoskeleton localization.

Tabela 2: Povzetek rezultatov histokemijske reakcije z barvilom alizarin rdeče S pri marzupijskih mankah in odraslih rakih enakonožcih *P. scaber*: Vzorce smo fiksirali na pet različnih načinov: (a1) s fiksativom Carnoy, (a2) s 3.7 % nevtralnno raztopino formaldehida, (a3) s 70 % etanolom, (b) s 3,7 % nevtralno raztopino formaldehida in zamrzovanjem ali (c) samo z zamrzovanjem. Za barvanje smo uporabili eno od naslednjih raztopin barvila alizarin rdeče S: ARS 1 (pH 9), ARS 2 (pH 6.4) ali ARS 3 (pH 4.8). Intenziteta ARS barvanja je označena z naslednjimi kategorijami: ○ – brez obarvanja; * – rahlo obarvanje; ** – zmerno obarvanje; *** – intenzivno obarvanje. 'Diffusion' označuje difuzijski artefakt. Kjer je bila intenziteta obarvanja različna pri mankah in odraslih, sta navedena oba rezultata ločeno na način: manke / odrasli. Sivo obarvana polja označujejo postopke, pri katerih se je eksoskeletalna kutikula izrazito diferencialno obarvala v primerjavi z drugimi tkivi, kar kaže na to, da so primerni za lokalizacijo kalcificiranega eksoskeleta.

procedure	(a) chemical fixation and paraffin sectioning					
	(a1)		(a2)		(a3)	
	Carnoy fixative		3.7% formaldehyde		70% ethanol	
staining solution	exoskeletal cuticle	other tissues	exoskeletal cuticle	other tissues	exoskeletal cuticle	other tissues
ARS 1 (pH 9)	*	*	***	○	***	**
ARS 2 (pH 6.4)	***	**	***	○	***	***
ARS 3 (pH 4.8)	***	**	***	○	***	***

procedure	(b) 3.7% formaldehyde, freezing and cryosectioning		(c) freezing and cryosectioning	
	exoskeletal cuticle	other tissues	exoskeletal cuticle	other tissues
ARS 1 (pH 9)	***	*	***	**/**
ARS 2 (pH 6.4)	***	*/**	**/**	*/**
ARS 3 (pH 4.8)	diffusion		diffusion	
	***	*/**	**/**	*/**
	diffusion		diffusion	

EDTA) a very faint red staining was noticeable (Supplementary fig. 4).

In cryosections of neutral buffered formaldehyde fixed and frozen samples (procedure b) a clearly differential staining of exoskeletal cuticle in adults and mancae was obtained with ARS 1 solution (pH 9) (Table 2, Supplementary fig. 5). Staining in ARS 2 (pH 6.4) and ARS 3 (pH 4.8) resulted in intensely red exoskeleton in adults and mancae, but in the sections of adult specimens

considerable staining of other tissues was also evident. In addition, a diffuse red staining in the close vicinity of exoskeletal cuticle was observed in all samples subjected to ARS 2 or ARS 3 solutions. Diffusion of stain occurs as a consequence of calcium salts solubility in staining solutions and is termed diffusion artifact. Negative controls (pretreated in EDTA) showed no red staining in ARS 1 and a very faint colouring in ARS 2 and ARS 3 (Supplementary fig. 5).

In the specimens which were not chemically fixed and were frozen only (procedure c), staining was not clearly differential, except for the basic ARS staining (ARS 1) of adult cuticle, where exoskeleton was intensely red and only a light background was visible (Table 2, Supplementary fig. 6). In all other stainings applied to frozen only sections various difficulties regarding histochemical reaction were encountered: (i) staining of other tissues, (ii) diffusion artifacts and (iii) nonuniform staining of different slides or sections that was evident especially in mancae. Negative controls displayed no staining, only in sections of adults pretreated with EDTA and exposed to acid staining solution a faint colouring was visible (Supplementary fig. 6).

Discussion

Histological demonstration of calcified tissues is commonly performed by alizarin red S (ARS) and von Kossa's methods (Bancroft and Gamble 2008). Although a great variety of other more advanced and sophisticated methods for calcium demonstration is available, histological methods are beneficial for examination of numerous samples to gain preliminary coarse information about the calcified tissue localization. Von Kossa's method is not specific for the calcium cations, but depends on the presence of the salt anion (carbonate, phosphate, oxalate), while alizarin red S (sodium alizarin sulphonate) reacts with calcium (McGee-Russell 1958, Bancroft and Gamble 2008). It reacts also with other metallic cations like copper, magnesium, barium, zinc, iron, aluminium, etc, but generally they are not present in biological structures in sufficient quantities for histochemical demonstration (Puchtler et al. 1969, Lievremont et al. 1982). A great variety of alizarin red S method modifications is reported in the literature. Modifications involve mainly differences in tissue fixation and in pH of staining solution. Fixation methods and pH of staining solution have an effect on calcium preservation in tissue, staining sensibility and specificity. Acidity of ARS solution leads to releasing of more calcium ions from tissues (Puchtler et al. 1969, Kiernan 2008). Consequently calcium localization appears more dispersive and sensitivity for calcified tissue demonstration

appears higher. Basic ARS solution has lower sensitivity but enables more precise localization of calcified tissue. ARS usually slightly stains the background tissue as well as it is an anionic dye (Puchtler et al. 1969, Kiernan 2008). Previous studies that include method of ARS staining are particularly based on vertebrates calcified tissues, while systematic evaluations of this method for other calcified biological systems rarely occur. Here we show a comparison of fifteen different modifications of ARS method applied to mancae and adult isopod crustaceans, to establish a quick, simple and inexpensive procedure appropriate to screen a large number of samples to estimate the presence of cuticle calcification.

The best differential staining of the exoskeletal cuticle in marsupial mancae and in adults as positive control was achieved with 3.7% formaldehyde fixation (2 days) and paraffin sections staining with ARS 1 (pH 9), ARS 2 (pH 6.4) or ARS 3 (pH 4.8) solution. The exoskeletal cuticle was specifically stained in all samples and the background remained unstained. Samples kept in neutral formaldehyde solution for a month, did not give positive staining of mancae cuticle (data not shown). Although formaldehyde is a widely recommended fixative, some authors described it as unsuitable fixative for studies of calcium deposits as it could remove some calcium during prolonged fixation (Puchtler et al. 1969, Presnell and Schreibmann 1997).

Alcoholic fixatives are widely recommended for calcified tissue localization as it is said they preserve calcium in tissue (Puchtler et al. 1969, Presnell and Schreibmann 1997, Bancroft and Gamble 2008, Kiernan 2008), though less adequate preservation of tissue structure could be caused by dehydrating effect. Since ARS staining of 70% ethanol fixed specimens in our study was not clearly specific, with intense or moderate staining of the background, we consider that 70% ethanol is not a suitable fixative for differential calcified cuticle localization. Staining of Carnoy fixed specimens was also not clearly differential, as in addition to exoskeletal cuticle all other tissues were stained too. We included this fixation in our study as it was recommended for vertebrate calcified tissues by Puchtler et al. (1969) and by Presnell and Schreibmann (1997). Our results also showed that all Carnoy fixed tissues were less intensely

stained in basic ARS solution in comparison to other two ARS solutions. We conclude that Carnoy fixative is not suitable for calcified cuticle identification.

Next, we performed cryosectioning to minimize exposure of samples to aqueous solutions, that could cause loss of amorphous forms of calcium from tissue as it is known that amorphous calcium carbonate has high solubility in water (Brečević and Nielsen 1989, Gal et al. 1996, Meiron et al. 2011). These methods keep most of the tissue components intact and are considered a better choice to study tissue composition, in spite of the fact that tissue structures appear poorly resolved. These methods are also less time consuming than conventional histological methods. Cryosections of samples fixed in neutral buffered formaldehyde and stained by basic ARS solution (ARS 1, pH 9) resulted in evidently differential staining of exoskeletal cuticle in marsupial mancae and in adults (a positive control). In all other procedures applied to specimens frozen after chemical fixation and to frozen only specimens, the histochemical reaction was either not differential or other imperfections were encountered, like diffusion artifacts and nonuniform staining. Diffusion artifacts, observed after staining in neutral and acid ARS solutions, were described also by Puchtler et al. (1969) in human tissues. Diffusion artifacts presumably appear due to the higher solubility of calcium salts in acid solutions in comparison to basic solutions. Nonuniform staining of sequential slides or sequential cryosections on the same slide, that we observed in the specimens of frozen only mancae, were possibly due to minimal variations in washing after staining.

Our results showed that cuticle of marsupial mancae was intensely and differentially stained by four different alizarin red S histochemical procedures, which also resulted in the differential staining of the exoskeletal cuticle in adults. These results suggest that prominent calcification of exoskeletal cuticle is present during postembryonic development of *P. scaber* mancae in the marsupium. Calcification provides hardness of exoskeleton that enables its protective role and mobility of the animal. Our findings show an importance of cuticle calcification for exoskeleton rigidity in mancae before they leave the marsupium. These findings support previous suggestions made by

Surbida and Wright (2001) and by Ouyang and Wright (2005), that do not give direct evidence of cuticle calcification since the aims of these studies were focused to other issues, like investigations of osmotic tolerance and total calcium concentration in developmental stages. Surbida and Wright (2001) presume that wide osmotic tolerance of *Armadillidium vulgare* marsupial mancae is a consequence of calcification of their cuticle. Ouyang and Wright (2005) suggest that cuticle calcification starts in the stage of marsupial manca, as they observed the increase of total calcium concentration in isopod *Armadillidium vulgare* late-stage marsupial manca. In order to address the issue of calcium forms in marsupial mancae additional analytical methods for demonstration of mineral forms should be performed.

Conclusions

Exoskeletal cuticle of marsupial mancae and adults of *P. scaber* was differentially stained by the following varieties of the histochemical alizarin red S method:

- (a) in paraffin sections of formaldehyde fixed samples, stained with alizarin red S solutions ARS 1 (pH 9), ARS 2 (pH 6.4) or ARS 3 (pH 4.8) and
- (b) in cryosections of samples fixed in formaldehyde, stained with basic ARS solution (ARS 1, pH 9).

This study suggests that prominent calcification of exoskeletal cuticle occurs already in marsupial mancae of isopod crustacean *P. scaber*. Exoskeleton hardening is likely important also for body movements, that we observed in mancae before they were released from marsupium.

Alizarin red S procedures that resulted in distinct differential staining of exoskeletal cuticle in this study are expected to be applicable for localization of calcified chitinous matrices in other species.

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Povzetek

Eksoskeletna kutikula rakov je apikalni zunaj-celični matriks epidermisa, osnovan na hitinskem organskem ogrodju in utrjen s sklerotizacijo in kalcifikacijo. Tvorba nove kutikule poteka v zgodnjem razvoju in ob vsaki levitvi pri odraslih osebkih. Pomemben proces v formiranju nove kutikule je tudi kalcifikacija, nalaganje kalcijevih mineralov v organski matriks. Embriji kopenskega raka enakonožca vrste *Porcellio scaber* se razvijajo v vodnem okolju valilnika (marzupija), ki je vrečasta struktura na trebušni strani samice. Embriji se izležejo v ličinke manke, ki nadaljujejo razvoj v valilniku še približno teden dni. Predhodno smo ugotovili, da kutikula marzupijskih mank kaže osnovne ultrastrukturne značilnosti kutikule odraslih živali, kot so organizacija v glavne sloje – epikutikulo, eksokutikulo in endokutikulo ter razporeditev hitinsko – proteinskih vlaken v značilen vzorec (Mrak in sod. 2012). V tej študiji smo ugotavljali, ali je eksoskeletna kutikula pri marzupijskih mankah že kalcificirana. V ta namen smo uporabili histokemijsko tehniko za lokalizacijo kalcificiranega tkiva z barvilom alizarin rdeče S (ARS), ki omogoča preprost in hiter pregled večjega števila vzorcev in je osnova za nadaljne bolj zahtevne in natančne tehnike. Glede na to, da je v literaturi predlaganih več modifikacij te metode, ki se primarno uporablja v histologiji vretenčarjev, smo izvedli pet različnih načinov fiksacije tkiva: (a1) v fiksativu Carnoy, (a2) v 3,7 % nevtralni raztopini formaldehida ali (a3) v 70 % etanolu, (b) fiksacija v 3,7 % nevtralni raztopini formaldehida in zamrzovanje ali (c) samo zamrzovanje. Barvali smo s tremi različnimi raztopinami

barvila: ARS 1 (pH 9), ARS 2 (pH 6,4) ali ARS 3 (pH 4,8). Za pozitivno kontrolo smo uporabili barvanje eksoskeletne kutikule odraslih živali, za negativno kontrolo pa predhodno inkubacijo rezin odraslih živali in mank v dekalifikacijski raztopini EDTA. Eksoskeletna kutikula marzupijskih mank in odraslih živali se je izrazito diferencialno obarvala pri vzorcih fiksiranih v nevtralni raztopini formaldehida, vklopljenih v parafin in barvanih z eno izmed raztopin barvila alizarin rdeče S: ARS 1 (pH 9), ARS 2 (pH 6,4) ali ARS 3 (pH 4,8). Da bi se čimbolj izognili vodnim raztopinam, v katerih so amorfne oblike kalcijevih soli dobro topne, smo v študijo vključili fiksacijo vzorcev z zamrzovanjem in barvanje kriostatnih rezin. Pri tej tehniki se je eksoskeletna kutikula diferencialno obarvala v primeru zamrznjenih rezin vzorcev, predhodno fiksiranih v formaldehidu, ki smo jih barvali z bazično raztopino ARS 1 (pH 9). Postopki histokemijske lokalizacije z barvanjem ARS, ki so se izkazali kot primerni, bodo predvidoma uporabni tudi pri študijah kalcifikacije drugih hitinskih matriksov.

Eksoskeletna kutikula marzupijskih mank *P. scaber* se je izrazito diferencialno obarvala s štirimi različnimi postopki metode ARS, pri katerih smo enako diferencialno obarvanje dobili tudi v primerih eksoskeletne kutikule pri odraslih (pozitivne kontrole). Ti rezultati kažejo na znatno kalcifikacijo eksoskeletne kutikule že v razvojnem obdobju pred sprostitvijo v zunanje okolje. Eksoskelet se torej oblikuje in kalcificira že pri ličinkah mankah v marzupiju, kar je najverjetneje pomembno tudi za gibanje mank, ki smo ga opazili pred sprostitvijo iz valilnika. Za ugotavljanje oblik kalcijevih soli v eksoskeletu marzupijskih mank bi bilo v nadaljevanju dela potrebno uporabiti analitske metode za identifikacijo mineralnih oblik, kot sta npr. infrardeča in Raman spektroskopija.

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Supplementary material

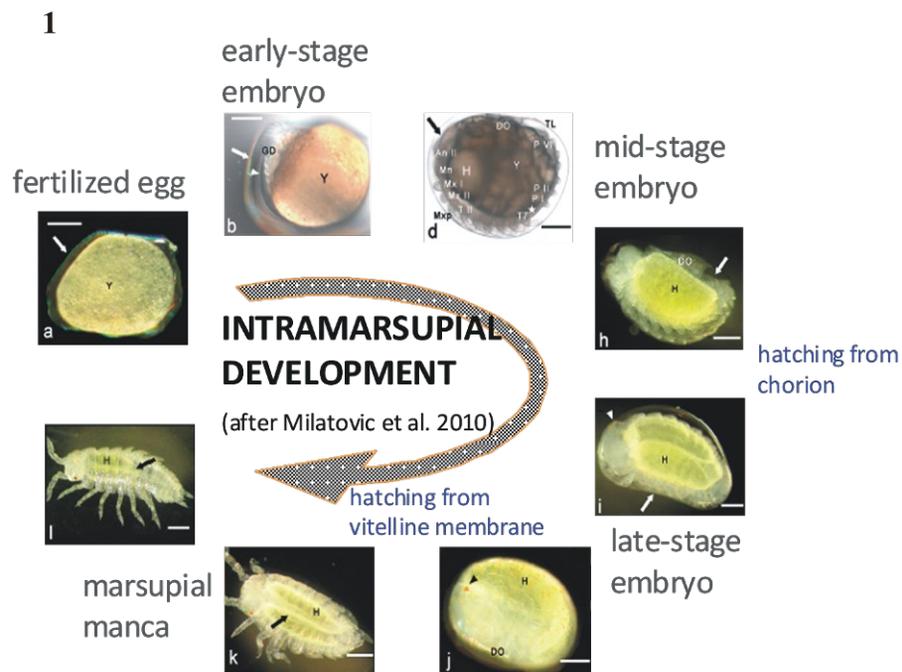


Figure 1: Principal stages of *P. scaber* intramarsupial development, from fertilized egg, early-, mid- and late-stage embryos to marsupial manca (adapted from staging system in Milatović et al. (2010)).

Slika 1: Glavni stadiji razvoja raka *P. scaber* v marzupiju, od oplojenega jajčeca, zgodnjega, srednjega in poznega embrija do marzupijske manke (povzeto po razvojnem sistemu v Milatović in sod. (2010)).

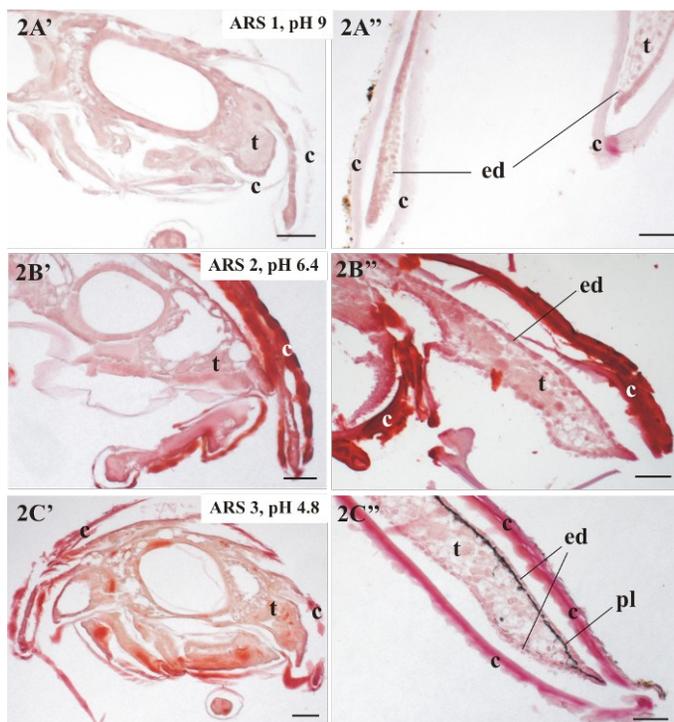


Figure 2: Alizarin red S staining of *P. scaber* specimens, fixed in Carnoy fixative and paraffin embedded (procedure a1). A', A'' - Staining solution ARS 1 (pH 9). B', B'' - Staining solution ARS 2 (pH 6.4). C', C'' - Staining solution ARS 3 (pH 4.8). A', B', C' - Marsupial mancae, cross sections. A'', B'', C'' - Positive controls - adults, cross sections. Staining of exoskeletal cuticle was not differential, neither in mancae nor in adults. c - cuticle; ed - epidermis; pl - pigment layer; t - tissue. Bars: 50 μ m.

Slika 2: Histokemijska lokalizacija z barvilom alizarin rdeče S - barvanje vzorcev rakov enakonožcev *P. scaber*, fiksiranih v fiksativu Carnoy in vklapljenih v parafin (postopek a1). A', A'' - Barvanje v raztopini ARS 1 (pH 9). B', B'' - Barvanje v raztopini ARS 2 (pH 6.4). C', C'' - Barvanje v raztopini ARS 3 (pH 4.8). A', B', C' - Marzupijske manke, prečni prerezi. A'', B'', C'' - Pozitivne kontrole - odrasle živali, prečni prerezi. Tako pri mankah kot pri odraslih eksoskeletalna kutikula ni bila diferencialno obarvana. c - kutikula; ed - epidermis; pl - pigmentni sloj; t - tkivo. Merila: 50 μ m.

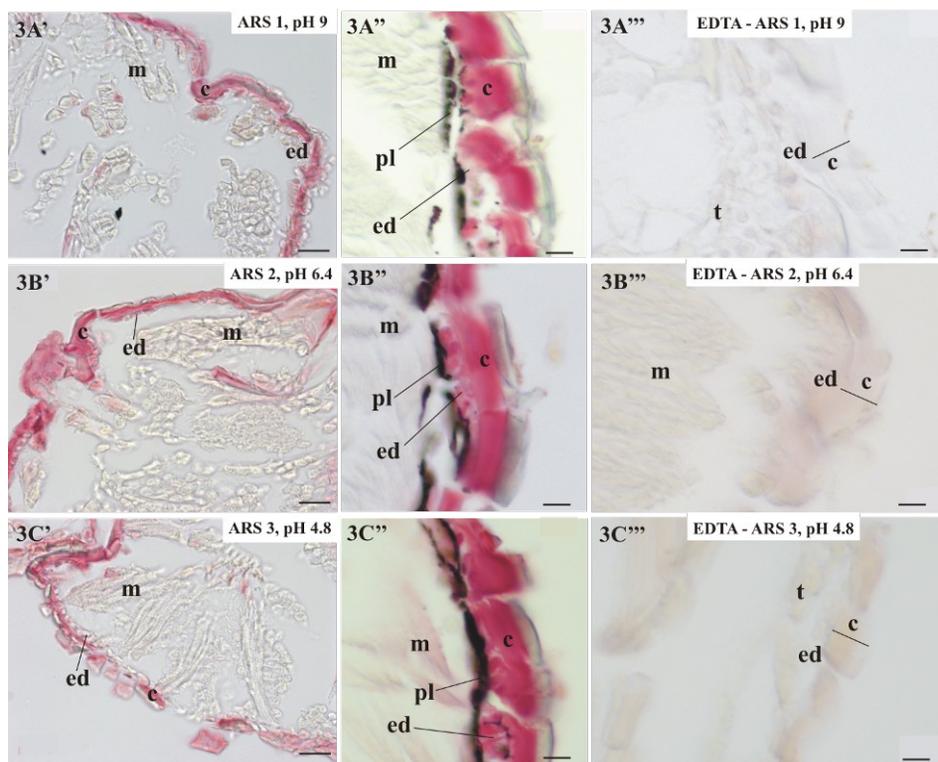


Figure 3: Alizarin red S staining of *P. scaber* specimens, fixed in neutral buffered formaldehyde and paraffin embedded (procedure a2). A', A'', A''' - Staining solution ARS 1 (pH 9). B', B'', B''' - Staining solution ARS 2 (pH 6.4). C', C'', C''' - Staining solution ARS 3 (pH 4.8). A', B', C' – Marsupial mancae, cross sections. A'', B'', C'' – Positive controls - adults, dorsal regions of animals cross sections. Exoskeletal cuticle was clearly differentially stained in mancae and adults with all three staining variations. C', C'' - Negligible colouring of other tissues was visible in ARS 3 staining. A''', B''', C''' - Negative controls in adults (EDTA) resulted in negligible or no staining. c – cuticle; ed – epidermis; m – muscles; pl – pigment layer; t – tissue. Bars: A', B', C' – 20 μ m; A'', A''', B'', B''', C'', C''' – 10 μ m.

Slika 3: Histokemijska lokalizacija z barvilom alizarin rdeče S - barvanje vzorcev rakov enakonožcev *P. scaber*, fiksiranih v nevtralni raztopini formaldehida in vklopljenih v parafin (postopek a2). A', A'', A''' - Barvanje v raztopini ARS 1 (pH 9). B', B'', B''' - Barvanje v raztopini ARS 2 (pH 6.4). C', C'', C''' - Barvanje v raztopini ARS 3 (pH 4.8). A', B', C' - Marzupijske manke, prečni prerezi. A'', B'', C'' - Pozitivne kontrole - odrasle živali, dorzalne predeli živali v prečnem prerezu. Eksoskeletalna kutikula se je diferencialno obarvala pri mankah in odraslih pri vseh treh različnih barvanja. C', C'' - Pri barvanju z raztopino ARS 3 so se zanemarljivo obarvala tudi druga tkiva. A''', B''', C''' - Negativne kontrole odraslih (EDTA) se niso obarvale ali pa je bilo obarvanje zanemarljivo. c – kutikula; ed – epidermis; m – mišice; pl – pigmentni sloj; t – tkivo. Merila: A', B', C' – 20 μ m; A'', A''', B'', B''', C'', C''' – 10 μ m.

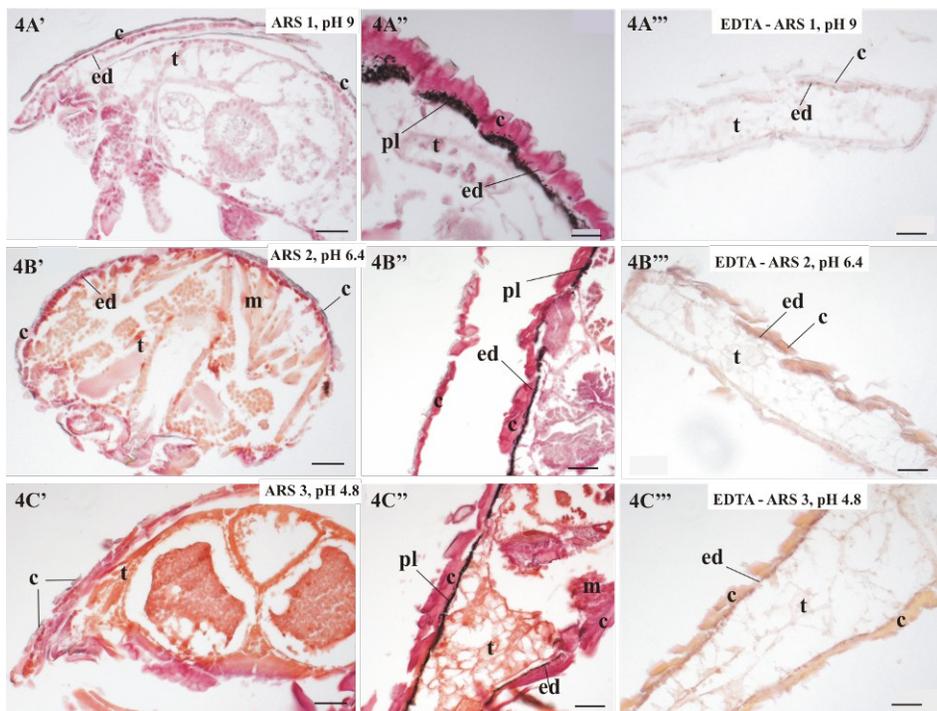


Figure 4: Alizarin red S staining of *P. scaber* specimens, fixed in 70% ethanol and paraffin embedded (a3). A', A'', A''' - Staining solution ARS 1 (pH 9). B', B'', B''' - Staining solution ARS 2 (pH 6.4). C', C'', C''' Staining solution ARS 3 (pH 4.8). A', B', C' - Marsupial mancae, cross sections. A'' B'', C'' - Positive controls - adults, cross sections. In ARS 1 staining of adults, exoskeletal cuticle was more intensely stained than other tissues (A''). In all other samples exoskeletal cuticle and other tissues were similarly stained. A''', B''', C''' - Staining of negative controls in adults (EDTA) resulted in a very faint red colouring. c - cuticle; ed - epidermis; m - muscles; pl - pigment layer; t - tissue. Bars: 50 μ m.

Slika 4: Histokemijska lokalizacija z barvilom alizarin rdeče S - barvanje vzorcev rakov enakonožcev *P. scaber*, fiksiranih v 70% etanolu in vključenih v parafin (postopek a3). A', A'', A''' - Barvanje v raztopini ARS 1 (pH 9). B', B'', B''' - Barvanje v raztopini ARS 2 (pH 6.4). C', C'', C''' - Barvanje v raztopini ARS 3 (pH 4.8). A', B', C' - Marzupijske manke, prečni prerezi. A'' B'', C'' - Pozitivne kontrole - odrasle živali, prečni prerezi. A''', B''', C''' - Negativne kontrole odraslih (EDTA) so se obarvale zelo šibko. c - kutikula; ed - epidermis; m - mišice; pl - pigmentni sloj; t - tkivo. Merila: 50 μ m.

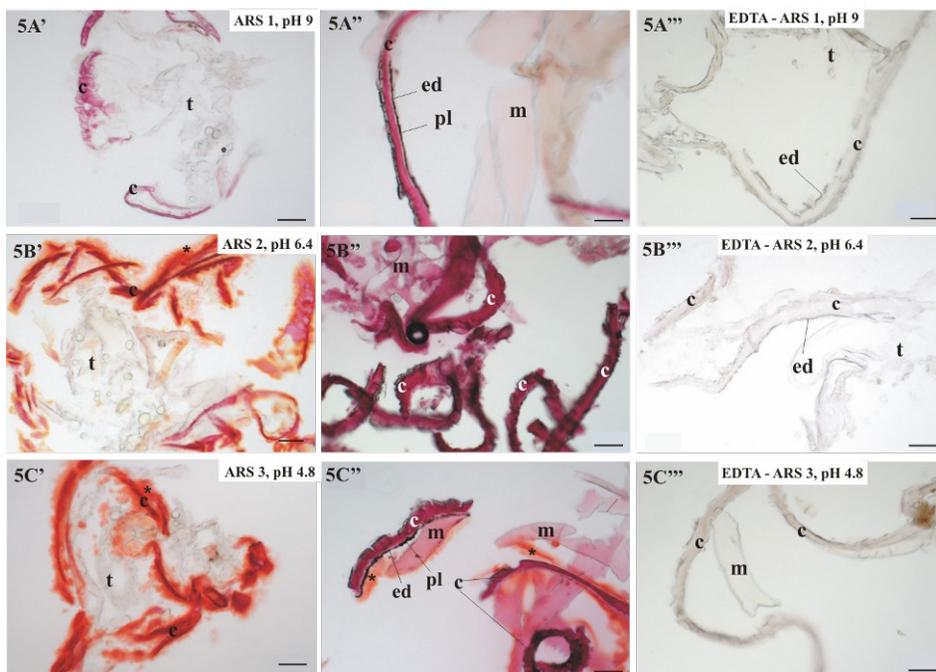


Figure 5: Alizarin red S staining of *P. scaber* specimens, fixed in neutral buffered formaldehyde and cryosectioned (procedure b). A', A'', A''' - Staining solution ARS 1 (pH 9). B', B'', B''' - Staining solution ARS 2 (pH 6.4). C', C'', C''' - Staining solution ARS 3 (pH 4.8). Structure of the tissues was not clearly distinctive, but cuticle and muscles were recognizable. A', B', C' – Marsupial mancae, cross sections. A'', B'', C'' – Positive controls – adults, cross sections. In ARS 1 solution exoskeletal cuticle of mancae and adults was clearly differentially stained (A', A''). In ARS 2 (B', B'') and ARS 3 (C', C'') solutions exoskeletal cuticle of mancae and adults was intensely red stained and diffusion artifacts (*) were present. In adults other tissues were stained also (B'', C''). A''', B''', C''' - Negative controls in adults (EDTA) were not red stained, only a faint colouring was noticeable with ARS 2 and ARS 3 staining. c – cuticle; ed – epidermis; m – muscles; pl – pigment layer; t – tissue. Bars: 50 µm.

Slika 5: Histokemijska lokalizacija z barvilom alizarin rdeče S - barvanje zamrznjenih vzorcev rakov enakonožcev *P. scaber*, ki so bili predhodno fiksirani v nevtralni raztopini formaldehida (postopek b). A', A'', A''' – Barvanje v raztopini ARS 1 (pH 9). B', B'', B''' – Barvanje v raztopini ARS 2 (pH 6.4). C', C'', C''' – Barvanje v raztopini ARS 3 (pH 4.8). Struktura tkiv ni bila povsem razločna, prepoznavne so bile kutikula in mišice. A', B', C' – Marzupijske manke, prečni prerezi. A'', B'', C'' – Pozitivne kontrole - odrasle živali, prečni prerezi. V ARS 1 raztopini se je eksoskeletna kutikula mank in odraslih diferencialno obarvala (A', A''). Pri barvanjih z ARS 2 (B', B'') in ARS 3 (C', C'') raztopinama se je kutikula mank in odraslih intenzivno rdeče obarvala. Opazni so bili difuzijski artefakti (*). Pri odraslih so se obarvala tudi druga tkiva (B'', C''). A''', B''', C''' - Negativne kontrole odraslih (EDTA) niso bile rdeče obarvane, opazno je bilo le šibko obarvanje v raztopinah ARS 2 in ARS 3. c – kutikula; ed – epidermis; m – mišice; pl – pigmentni sloj; t – tkivo. Merila: 50 µm.

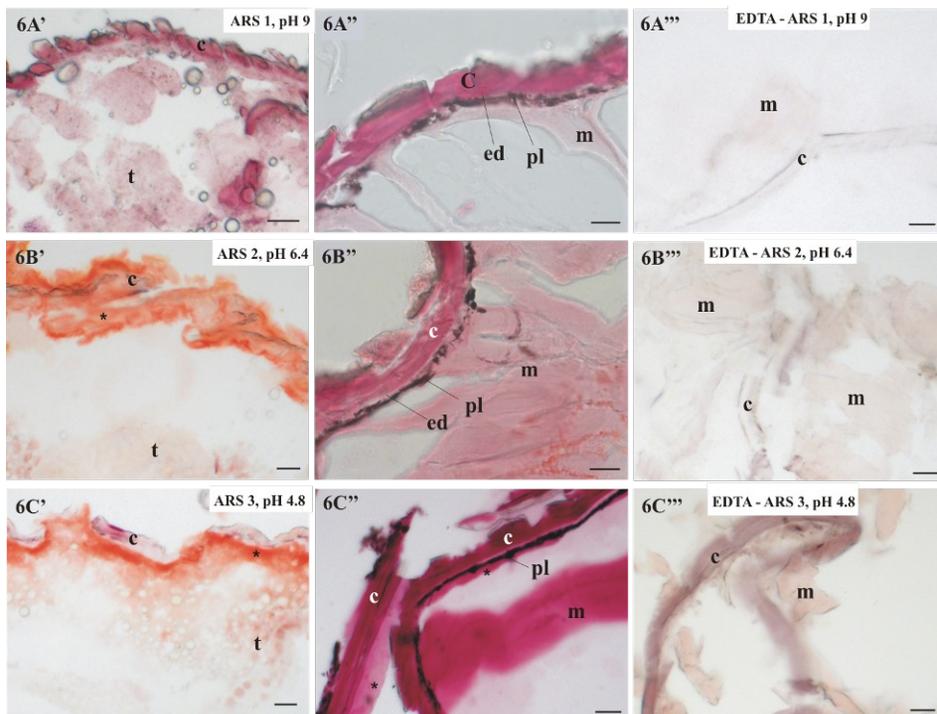


Figure 6: Alizarin red S staining of *P. scaber* specimens, frozen and cryosectioned (procedure c). A', A'', A''' - Staining solution ARS 1 (pH 9). B', B'', B''' - Staining solution ARS 2 (pH 6.4). C', C'', C''' - Staining solution ARS 3 (pH 4.8). Histological structure was not well preserved, although cuticle and muscles were recognizable. A', B', C' – Marsupial mancae, longitudinal sections. A'', B'', C'' – Positive controls - adults, cross sections. In ARS 1 staining of adults the exoskeletal cuticle was differentially stained (A''). In ARS 2 (B', B'') and ARS 3 stainings (C', C'') diffusion artifacts (*) were present in all samples and considerable background was evident in adults. In mancae a nonuniformity of staining was also observed. A''', B''', C''' - Negative controls of adults (EDTA) resulted in no staining, only a faint colouring was visible in ARS 3 staining. c – cuticle; ed – epidermis; m – muscles; pl – pigment layer; t – tissue. Bars: 20 μ m.

Slika 6: Histokemijska lokalizacija z barvilom alizarin rdeče S - barvanje zamrznjenih vzorcev rakov enakonožcev *P. scaber* (postopek c). A', A'', A''' – Barvanje v raztopini ARS 1 (pH 9). B', B'', B''' – Barvanje v raztopini ARS 2 (pH 6.4). C', C'', C''' – Barvanje v raztopini ARS 3 (pH 4.8). Struktura tkiv je bila histološko slabo ohranjena, prepoznavne so bile kutikula in mišice. A', B', C' – Marzupijske manke, vzdolžni prerezi. A'', B'', C'' – Pozitivne kontrole - odrasle živali, prečni prerezi. Pri ARS 1 barvanju odraslih živali se je eksoskeletna kutikula diferencialno obarvala (A''). Pri barvanju v raztopinah ARS 2 (B', B'') in ARS 3 (C', C'') so se pri vseh vzorcih pojavili difuzijski artefakti (*). Pri odraslih živalih je bilo ozadje precej obarvano. Pri barvanju mank je bila intenziteta barvanja neenakomerna. A''', B''', C''' – Negativne kontrole odraslih (EDTA) se niso obarvale, opazno pa je bilo šibko obarvanje pri ARS 3 raztopini. c – kutikula; ed – epidermis; m – mišice; pl – pigmentni sloj; t – tkivo. Merila: 20 μ m.