HISTORY AND BIOLOGY OF THE «BLACK PROTEUS« (*PROTEUS ANGUINUS PARKELJ* SKET & ARNTZEN 1994; AMPHIBIA: PROTEIDAE): A REVIEW

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ABSTRACT

History and biology of the «black proteus« (*Proteus anguinus parkelj* Sket & Arntzen 1994; Amphibia: Proteidae): a review

Proteus anguinus parkelj Sket & Arntzen 1994 is a black pigmented with developed eyes, non-troglomorphic, but yet an troglobiotic subspecies of *P. anguinus* Laurenti 1786. It is endemic to the karst of Bela krajina in southeast Slovenia. As a probable mimic of the epigean ancestor it is very informative regarding adaptation to subterranean environment. Since it was discovered only in 1986, relatively little is known about its biology. The purpose of this review paper is to summarize the published data about the circumstances of its discovery, its distribution, phylogeny, taxonomy and its biology, including environmental threats, conservation status, and actions that are being taken to ensure its survival. The exceedingly limited distribution of the black proteus makes it particularly vulnerable. It is endangered by environmental pollution as well as by various potential pathogens that are of global concern to amphibians. Populations of the black proteus are in dire need of protection if they are to survive in their own natural habitat.

Key words: Proteus anguinus parkelj, discovery, distribution, phylogeny, morphology, threats

IZVLEČEK

Zgodovina in biologija črnega močerila (*Proteus anguinus parkelj* Sket & Arntzen 1994; Amphibia: Proteidae): pregledni članek

Črni močeril *Proteus anguinus parkelj* Sket & Arntzen 1994 je temno pigmentirana podvrsta človeške ribice *P. anguinus* Laurenti 1786. Ni troglomorf, ima razvite oči, vendar je troglobiont. Je endemit belokranjskega krasa v JV Sloveniji in verjetno spominja na površinskega prednika. Zato je zelo informativen pri raziskavah adaptacij na življenje v podzemlju. Ker je bil odkrit šele leta 1986, je o njegovi biologiji znanega relativno malo. Namen tega preglednega članka je povzeti objavljene podatke o okoliščinah njegovega odkritja, geografski razširjenosti, filogeniji, taksonomiji in biologiji, vključno z okoljskimi grožnjami, varstvenim statusom in ukrepi za zagotovljanje njegovega preživetja. Zaradi izredno omejene razširjenosti je črni proteus še posebej ranljiva rasa. Ogroža ga onesnaževanje okolja, kot tudi različni potencialni patogeni, ki ogrožajo dvoživke po vsem svetu. Črni proteus potrebuje zaščito in varstveni status, če ga želimo ohraniti v njegovem naravnem habitatu.

Ključne besede: Proteus anguinus parkelj, odkritje, razširjenost, filogenija, morfologija, ogroženost

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INTRODUCTION

The purpose of this review paper is to summarize what we know from studies of the mysterious "black proteus", *Proteus anguinus parkelj* Sket et Arntzen 1994, endemic to underground waters of the karst of Bela krajina in southeast Slovenia. The researches were mainly conducted by two research groups at the Department of Biology of the Biotechnical Faculty, University of Ljubljana, with their collaborators from different institutions, including graduate students involved in the research work over many years. The group for Zoology and Speleobiology is focused on taxonomy, evolution and biogeography of *Proteus*. The group for Functional Morphology of Vertebrates is focused on the functional morphological adaptations of *Proteus* to its habitat in the context of vulnerability to toxic chemical and organic pollution with the long-term goal of understanding the factors that can affect its survival in its natural environment. The private Cave Laboratory Tular has also contributed to studies of proteus, and has also made numerous contributions to public outreach and dissemination of the information.

BACKGROUND

The species *Proteus anguinus* Laurenti 1786 was first mentioned by Valvasor in 1689, although he did not recognize its taxonomic affiliation, let alone its biological uniqueness and scientific importance. The species (also known as *človeška ribica*, proteus, the European blind cave salamander, or german *Olm*, among other common names) was first formally described by Laurenti (1786) based on a specimen that was thought to be from the karst lake Cerkniško jezero, in present-day Slovenia, although some researchers are doubtful about the exact locality (Sket 2007). *Proteus anguinus* was the first troglobiont ever to be scientifically described and is also one of the largest ones.

The taxonomical and biogeographical history of the species is highly problematic. Neither the exact location of the type specimen nor the identity of the author who described it are known for certain, and information about its basic biology and reproduction and even its taxonomic identity are still unclear, although most evidence places it with the North American genus *Necturus* in the Family Proteidae (Kezer et al. 1965; Sessions 1980; Trontelj & Gorički 2003). The putative species has been taxonomically split (Fitzinger 1850) and then fused again, and might be split once again into at least six species based on current molecular and biogeographical information (Gorički 2004, 2006; Gorički et al. 2006; Gorički & Trontelj 2006; Trontelj et al. 2009, 2017). Its current geographic distribution is thought to extend through an area characterized as holodinaric, from easternmost Italy through southern Slovenia, Croatia and along the eastern border of Hercegovina and Dalmacija (Sket 1997). Its presence in Montenegro is questionable. Some populations may be reproductively isolated.

Note that the names 'white proteus', 'white subspecies' or '*Proteus anguinus anguinus*' denote here any population of depigmented protei, presenting just momentarily and formally a subspecies. In fact, it is an assembly of morphologically and ecologically very similar populations which are genetically distinct and may present even several independent species.

DISCOVERY OF THE »BLACK PROTEUS«

Historically, the only known populations of *P. anguinus* over the entire known range of the species were depigmented, cave-adapted »white proteuses« with regressed eyes and other troglomorphic characteristics (Sket 2017) (Fig. 1). Occasional reports have been made of darkly pigmented specimens of *P. anguinus* (Aljančič et al. 1986, Aljančič 1988), either from natural populations or captive animals, but these were all troglomorphic white proteus individuals that, at least in the case of the captive animals, had been exposed to light for some time. The first dark pigmented, non-troglomorphic specimens of the »black proteus« (designated as the subspecies *P. a. parkelj*), complete with functional eyes (Fig. 1), were found in October 1986 (nearly 300 years after the white *Proteus* was first mentioned by Valvasor) by researchers of the Inštitut za raziskovanje krasa (from Postojna), during a pumping experiment for water supply of the

Dobličica spring near Črnomelj, in Bela krajina, SE Slovenia (Aljančič et al. 1986; Mihevc 1987; Sket & Arntzen 1994; Aljančič 2017). Following the discovery of the first specimens of the black proteus, searching in surface waters of Dobličica spring gave no positive results, even after a thorough inspection via scuba diving of the 40 m wide and 10 m deep limnocrene spring of Dobličica (Sket 1993c). But gradually, over time, short-term visits and observation of nearby Jelševnik spring resulted in the collection of about 15 individuals. Occasionally, after heavy rains, the animals can be observed penetrating from underground to approach the surface through a thin turf, covering the cracked rock that is characteristic of its type locality (Fig. 2A). The currently known geographic distribution of *P. a. parkelj* is highly restricted in SE Slovenia, southwest to west of Črnomelj where it is found only at spring outlets in an area that is less than 5 km^2 in size (Sket & Arntzen 1994; Gorički et al. 2017; Hudoklin & Aljančič 2017). Adjacent populations of *P. a. anguinus* (the white proteus) are located all around this area.

The first specimen of *P. a. parkelj* that was morphologically analyzed was a female with a total body length of 187 mm and body mass of 13.5 g and with immature ovaries. Detailed reports of the anatomical peculiarities of *P. a. parkelj* were first published by Istenič (1987) and later by Sket (1993b, c). All subsequent samples of *P. a. parkelj* exhibited a remarkable uniformity in external morphology, which allowed the formal description of the black proteus as a new taxon. The morphological description included detailed osteological data and was supplemented by allozyme analysis (Sket & Arntzen 1994). Superficially, many of the morphological characters of the black proteus appear to be plesiomorphies. In addition to dark pig-

Figure 1: Proteus anguinus Laurenti, the ‚first cave animal', described in 1786 from Slovenia as distributed along the almost whole Dinaric karst. Proteus anguinus parkelj Sket & Arntzen, a non-troglomorphic subspecies of P. anguinus, found 1986 and described 1994 as a surprising discovery. It is a troglobiont, but not adaptively morphologically transformed as a ‚fully' cave-adapted troglomorph. It is endemic to several kilometres in the SE corner of Slovenia. Scale bar: 2 cm. Photos: D. Dalessi, Speleological laboratory at Department of Biology, BF, University of Ljubljana.

Slika 1: Proteus anguinus Laurenti, ‚prva jamska žival'. Opisana 1786 iz Slovenije, razširjena je vzdolž skoraj vsega Dinarskega krasa. Proteus anguinus parkelj Sket & Arntzen 1994, ne troglomorfna rasa in veliko odkritje iz leta 1986. Je endemit le nekaj kilometrov velikega območja v JV kotu Slovenije. Je troglobiont, vendar ni adaptivno morfološko preoblikovan. Merilo: 2 cm. Fotografiji: D. Dalessi, Speleološki laboratorij na Oddelku za biologijo, BF, Univerza v Ljubljani.

mentation and fully developed (and presumably functional) eyes, the black proteus lacks conspicuous snout elongation, has differently shaped skull bones, lower teeth number, shorter legs, shorter tail, and a longer trunk with a higher number of trunk vertebrae compared with the white proteus. The differences between the two subspecies were later confirmed by detailed analysis of the skull and whole skeleton by micro-CT scanning (Centrih-Genov 2011; Ivanović et al. 2013).

The existence of a population of non-troglomorphic *Proteus* inhabiting the underground waters in the low karst of Bela krajina is an important biological puzzle. First, it suggests lower selection pressure for troglomorphy in this area, or possibly selection against troglomorphism. This conclusion is supported by the existence in the same area of the only Dinaric cave shrimp *Troglocaris* race that has non-troglomorphic traits such as pigmented eye rudiments (*Troglocaris anophthalmus ocellata,* Jugovic et al. 2012; Sket 1993a). Some epigean and even terrestrial animals (Diplopoda, Chilopoda) drift out of the springs (Sket 1993c, d), which indicates a relatively good food supply and weaker selection pressure compared to the »typical« cave environments inhabited by populations of white proteus.

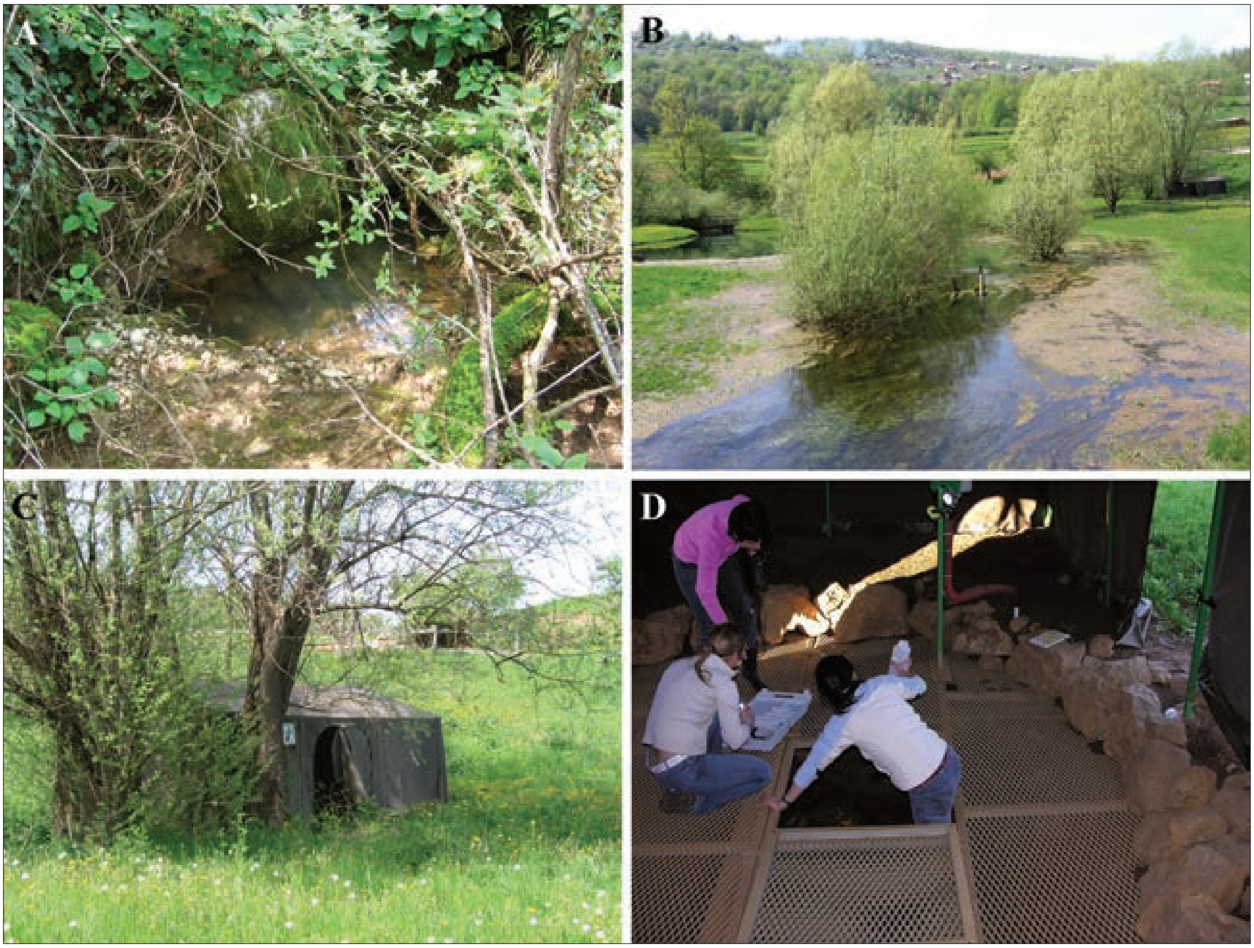

Figure 2: The Jelševnik locality of Proteus anguinus parkelj Sket & Arntzen 1994 in Bela krajina. A. The native typical appearance of the spring. B. The flooded meadow during springtime. C & D. The spring, covered by a tent for in situ observation of animals. Photos: B. Bulog in April 2006.
Slika 2: Lokaliteta črnega močerila Proteus anguinus parkelj Sket & Arntzen 1994 na Jelševniku v Beli krajini. A. Izgled nativnega izvira. B. Poplavljen travnik spomladi. C & D. Izvir, pokrit s šotorom, za lažje opazovanje živali in situ. Fotografije: B. Bulog v aprilu 2006.

MORPHOLOGICAL DESCRIPTION

The black proteus (Fig. 1) can be simply defined as a darkly pigmented and short-snouted proteus with externally visible eyes, a head with more angular and convex lateral sides, a longer trunk, shorter legs, and a shorter tail.

Specimens of adult *P. a. parkelj* mostly range from 200-280 mm in total length (exceptionally, larger specimens have been found, up to 360 mm total length). Its coloration is always close to black with some paler parts and a violet or brownish hue (Fig. 1); a large quantity of dark pigments is found in the outer layers of the dermis (Istenič 1987; Bulog 1994). The eyes are proportionally only slightly smaller than in the North American *Necturus maculosus* Rafinesque 1818, an

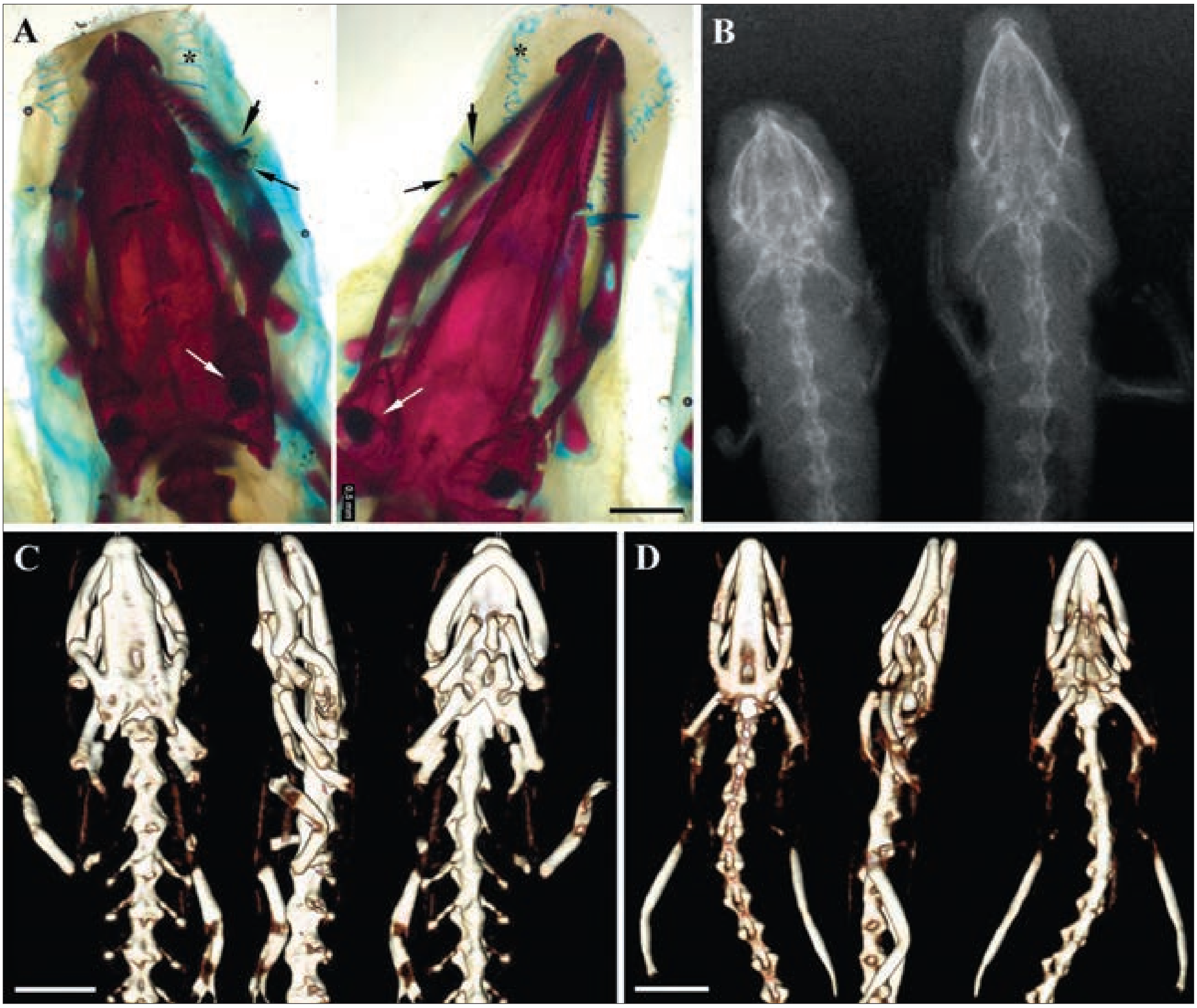

Figure 3: The skull morphology of Proteus anguinus parkelj Sket & Arntzen 1994 (left on photos) and P. a. anguinus Laurenti 1786 (right on photos). A. Clearing and staining technique for bone (red) and cartilage (blue). Asterisk – cartilages of nasal cavity, long arrow - eye, short arrow - antorbital cartilage, white arrow – otic capsule. B. X-ray technique. C & D. Computed micro-tomography from dorsal, lateral and ventral side. Scale bar: 5 mm. Photos: L. Bizjak Mali, 2015 (A), B. Sket, 1992 (B) and T. Centrih-Genov, 2011 (C&D).
Slika 3: Morfologija lobanje Proteus anguinus parkelj Sket & Arntzen 1994 (levo na slikah) in P. a. anguinus Laurenti 1786 (desno na slikah). A. Tehnika presvetlitve tkiv in barvanja za kostno (rdeče) in hrustančno (modro) tkivo. Zvezdica – hrustanci v nosni votlini, dolga puščica - oko, kratka puščica – antorbitalni hrustanec, bela puščica – slušna kapsula. B. Rentgenski posnetek. C & D. Računalniška mikro-tomografija s hrbtne, bočne in trebušne strani. Merilce: 5 mm. Fotografije: L. Bizjak Mali, 2015 (A), B. Sket, 1992 (B) in T. Centrih-Genov, 2011 (C&D).

epigean but permanently aquatic and paedomorphic species. The eyes of the black proteus are clearly visible, with a transparent cornea but lack eyelids (which is typical for other paedomorphic salamanders). The three pairs of gills are feathered with bright red filaments (when alive), and with a partly blackish cover and pale stems. The head is relatively shorter, but broader and with more massive head musculature than in *P. a. anguinus*. The skull morphology has been studied in detail after clearing and staining of whole animals where bones were stained and the remaining tissue made transparent (»cleared«) so that the bones can be easily seen (Sket & Arntzen 1994; Centrih-Genov 2011), with X-ray (Sket & Arntzen 1994) and by computed microtomography (micro-CT) (Centrih-Genov 2011) (Fig. 3A-D) and geometric morphometrics with *Necturus* as an outgroup (Ivanović et al. 2013). All of these studies showed that the skull is similar overall to that of *P. a. anguinus,* but with different proportions of bones; it is shorter and more similar to *Necturus* than to the white proteus. These differences include a head that is not duck-bill shaped (like it is in the white proteus) and with a lower number of teeth. This is especially pronounced on the dentale with only 16 to 19 teeth (while there are 23 to 33 in *P. a. anguinus*). The black proteus' skull is also wider at the jaw articulation point, its snout is wider with laterally extended, larger premaxillae, and shorter vomers which are positionend further apart. The position of the jaw articulation and the shape of buccal cavity are also different (Ivanović et al. 2013). The maxilla bones of the upper jaw are missing which is characteristic of the whole family Proteidae. Compared to *P. a. anguinus*, the black proteus also has a larger number of trunk vertebrae and myomeres. Both legs of the black proteus are remarkably shorter, but with the same reduced number of digits: three on the forelimb and two on the hind limb, a unique feature in *Proteus* among amphibians. The Wolterstorff index (an indicator of body shape) is significantly lower than in *P. a. anguinus* (WI = 10.7 ± 0.57 in *P. a. parkelj* vs. 17.0 ± 1.5 for different populations of *P. a. anguinus*) (Sket & Arntzen 1994). The black proteus also has a substantially shorter tail with a lower number of vertebrae.

CYTOGENETICS AND GENOMICS

A cytogenetic analysis of three populations of *Proteus*, including the black proteus and two populations of the white proteus, showed that the karyotype of *P. a. parkelj* is identical to that of *P. a. anguinus* with 19 pairs (2n = 38) of mainly bi-armed chromosomes with a nucleolus organizer region at the telomere of chromosome pair 12 revealed by AgNOR banding (Fig. 4) (Sessions et al. 2016). Unlike its closest relative *Necturus*, which has pronounced heteromorphic XY sex chromosomes (Sessions 1980; Sessions & Wiley 1985; Sessions et al. 2016), both subspecies of *Proteus* lack heteromorphic sex chromosomes (Sessions et al. 2016). However, C-banding of the chromosomes revealed a prominent concentration of alternating bands of C-band heterochromatin in the telomeric/subtelomeric region of the long arm of both homologues of the largest chromosome 1 in both subspecies of *Proteus*. No other C-band heterochromatin was detectable in the chromosomes of *Proteus*, and even the centromeres were unstained. The conspicuous cluster of alternating light and dark C-bands in chromosome pair 1 exactly matches the pattern of alternating C-bands in the *Necturus* Y chromosome (Fig. 4) and was interpreted to be an X-Y translocation that has become fixed as a homologous pair in both subspecies of *Proteus* (Sessions et al. 2016).

The published mean haploid genome size (C-value) of *P. a. anguinus* is 49 pg (approximately 47.9 Gb of DNA) which is relatively large in comparison with most other amphibians but is only about half as large as the genome sizes of *Necturus* species (Gregory 2016). Nevertheless, the *Proteus* genome is about 16 times the size of the human genome. Current research suggests that the large genomes seen in salamanders are due to the proliferation of transposable elements ("selfish DNA") and accumulation of various kinds of repetitive DNA (Sessions 2008; Sun & Mueller 2014). Preliminary comparative research of genome size of *P. a. parkelj* and *P. a. anguinus* with Feulgen microdensitometry of erythrocyte nuclei (Bizjak Mali, Vokač & Kladnik unpublished) showed that the C-value of *P. a. parkelj* appears to be slightly lower than in *P. a. anguinus*, but these differences are not statistically significant (p value = 0.908). Further study on a much larger sample of individuals for analyzing the genome size of different populations of protei is in progress.

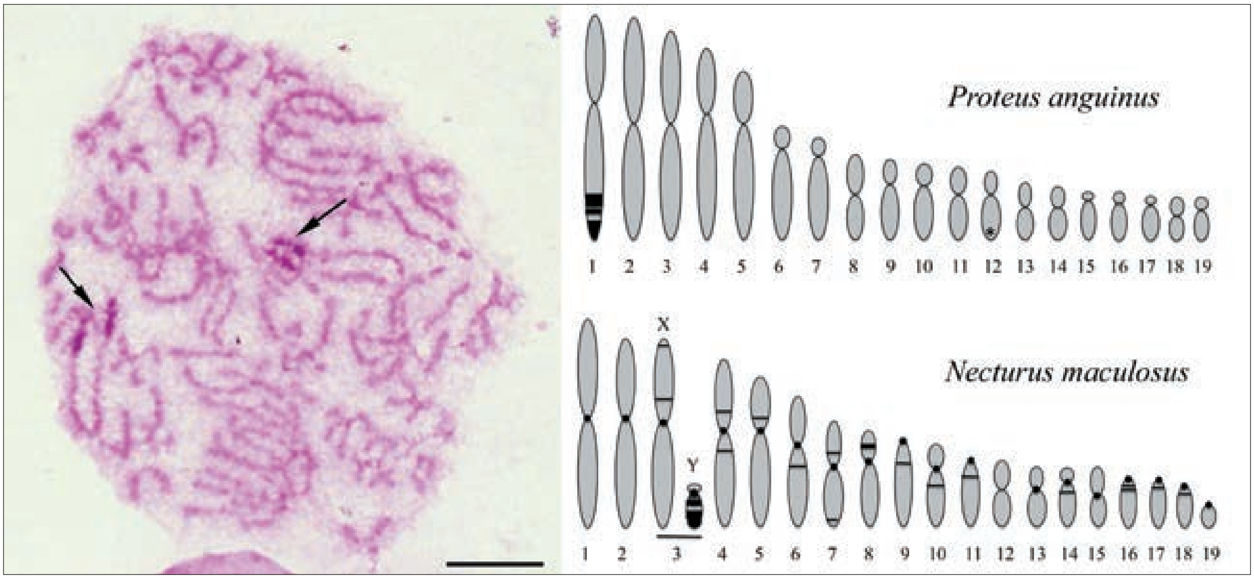

Figure 4: C-banded chromosome spread of Proteus anguinus parkelj Sket & Arntzen 1994 (left) with visible heterochromatic bands on chromosome 1 (arrows), and idiograms (right) of P. anguinus (both subspecies) and Necturus maculosus. Scale bar: 10 µm. Modified from Sessions et al. 2016.
Slika 4: Razmaz C-proganih kromosomov črnega močerila Proteus anguinus parkelj Sket & Arntzen 1994 (levo) z vidnim heterokromatinskim vzorcem na kromosomu 1 (puščica) in idiogram (desno) od P. anguinus (obe podvrsti) and Necturus maculosus. Merilce: 10 µm. Prirejeno po Sessions et al. 2016.

DISTRIBUTION AND HABITAT

The biogeographic distribution of *Proteus anguinus* is holodinaric, with north-western localities close to the Soča/Isonzo estuary and south-eastern ones in SE Hercegovina (Fig. 5) (Sket 1997; Sket et al. 2004). The data from Montenegro (Ćurčić et al. 2008; Gorički et al. 2017) are not reliable. The known locality of the black proteus is in SE Slovenia WSW of Črnomelj (Fig. 5), in some springs positioned within an area of less than 10 km^2 , although the actual habitat of the black proteus might be under the massif of Poljanska gora which encompasses 55 km^2 at most (Sket & Arntzen 1994). Unfortunately, there are no cave entrances in the area that are accessible for searching. Most specimens of the black proteus were found on the surface immediately at the springs, evidently brought from underground after rains. In the year 2004 the black specimen was observed for the first time in its real habitat by divers at a depth of 15 to 20 meters when they dived into the siphon of Dobličica spring (Mihajlovski 2012; Hudoklin & Aljančič 2017). The only locality where the black proteus can be routinely observed *in situ* is the Jelševnik spring where researchers from the Department of Biology at the University of Ljubljana set up a »research station« (Fig. 2B-D) (Bulog & Bizjak Mali 2014). The location is covered by a tent for perpetual observation with infrared camera.

The method for specific eDNA (environmental DNA) detection allows a more detailed investigation of the distribution of the black proteus. This methodology revealed five additional sites (beside the four previously known sites) that may be inhabited by the black proteus (Stanković et al. 2016; Gorički et al. 2016, 2017). Three of these springs lie outside the limits of its previously known range and represent an extension of its presumed range north-eastward, along with the general direction of the flow of the Dobličica River. Populations of *P. a. parkelj* and *P. a. anguinus* appear to be generally allopatric, however at one locality (in the spring Šprajcarjev Zdenec), the eDNA of both taxa was found together (Gorički et al. 2017). While these data do not prove that the two populations are really syntopic, they do highlight the potential for contact through an existing intermittent hydrogeological connection between the above-mentioned springs occupied by the black proteus, and a nearby Otovski Breg which is occupied by the white proteus (Gorički et al. 2017). Even though the black and white protei in these two populations show a low degree of DNA sequence

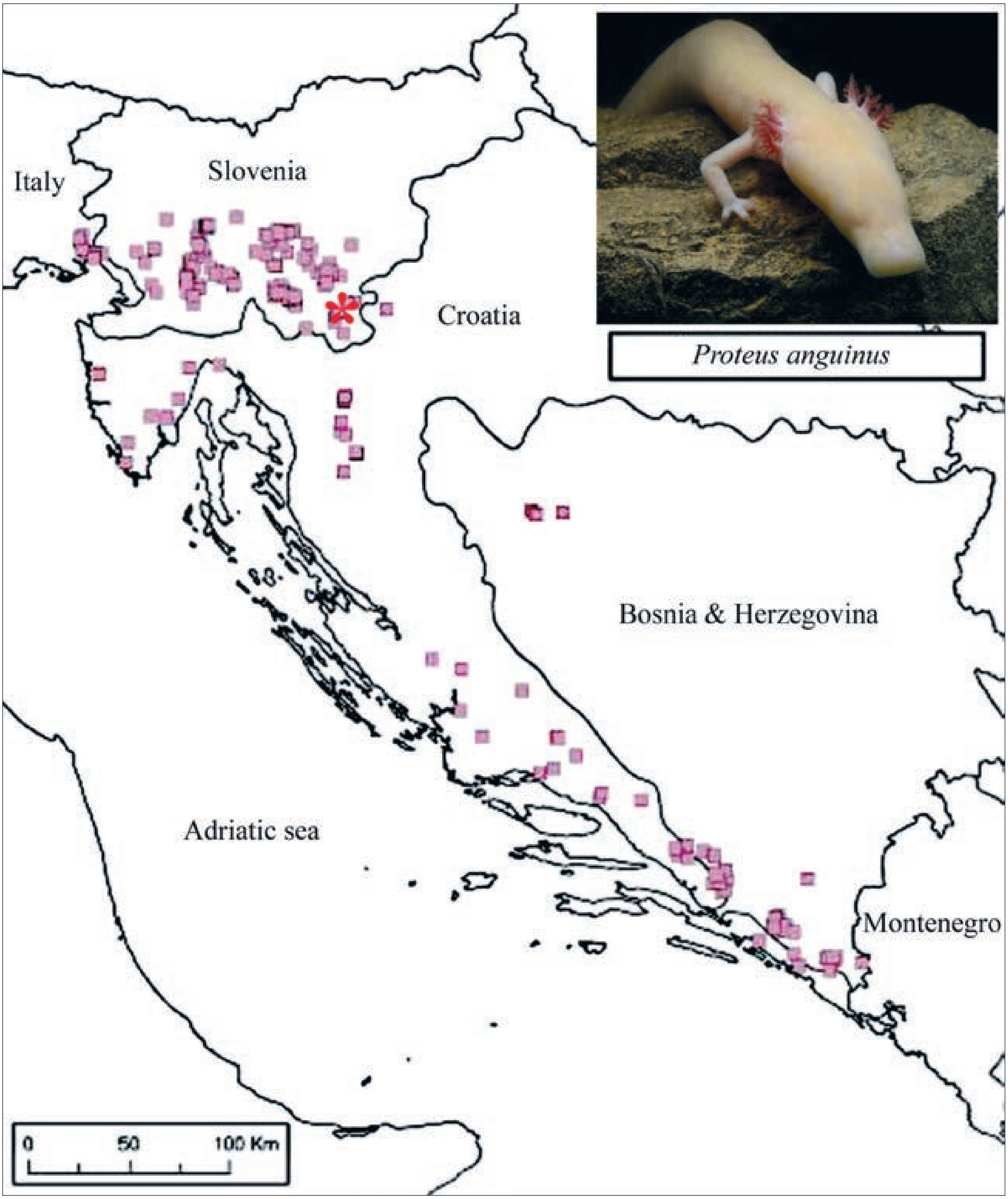

Figure 5: Distribution of Proteus anguinus Laurenti 1786 in its entire natural range. Proteus anguinus parkelj Sket & Arntzen 1994 is known only from SE Slovenia WSW of Črnomelj (asterisk). Map: B. Sket.
Slika 5: Razširjenost Proteus anguinus Laurenti 1786 v njegovem celotnem naravnem območju. P. anguinus parkelj Sket & Arntzen 1994 je poznan samo iz SV Slovenije ZSZ od Črnomlja (zvezdica). Zemljevid: B. Sket.

divergence in their mitochondrial control regions (Gorički 2006; Gorički & Trontelj 2006; Trontelj et al. 2009), none of the comparative studies to date have detected any signs of their interbreeding, e.g. haplotype sharing or intermediate morphology (Gorički et al. 2017). Thus, if the black and white protei do come into contact with each other, there is no evidence for reproductive interaction between them.

PHYLOGENY & TAXONOMY

The morphological description of the black proteus as a new taxon was accompanied by biochemical allozyme study of over 40 loci by Sket & Arntzen (1994). A more detailed phylogenetic study using mitochondrial DNA (Gorički 2004, 2006; Gorički et al. 2006; Gorički & Trontelj 2006; Trontelj et al. 2009) presented evidence that the black proteus shows closest affinity to a population of white proteus among the SE Slovenian group of populations. It seems reasonable that the black proteus are populations that experienced selection favoring either the retention or the re-acquisition of non-troglomorphic traits (e.g. body proportion, functional eyes and dark pigmentation).

The proposed taxonomy of the black proteus by Sket & Arntzen (1994) was rejected by Grillitsch & Tiedemann (1994) who claimed that it falls within the clinal variation of *Proteus anguinus* and does not deserve a taxonomical (i.e. subspecies) status. However, Arntzen & Sket (1996) used the Wolterstorff index, a morphometric tool that has been used for defining the body proportions in newts, to demonstrate clear and consistent differences between *P. a. parkelj* and three populations of the white proteus *P. a. anguinus*, making the black subspecies diagnosable, at least morphologically. In particular, the black proteus exhibits some characters, including head shape, eyes, and pigmentation, that are clearly outside the range of the white proteus. Furthermore, Arntzen & Sket (1997) presented a morphometric analysis of a number of populations and found *P. a. anguinus* and *P. a. parkelj* to be morphologically distinct. Multivariate morphometric analysis produces very clear differences and Mantel permutation tests show no firm evidence for clinal variation within the species that would encompass these differences. Thus the claim that the black population falls within the clinal variation of *P. anguinus* is not tenable, at least on purely morphological grounds.

The black proteus presents us with an interesting paradox. On the one hand, morphological data show it to be clearly distinct from any white proteus in a large number of important characters including head shape (especially lack of an elongated snout), eye development and function, pigmentation, limb length, trunk length, number of vertebrae, etc. (Arntzen & Sket 1996, 1997). On the other hand, biochemical, molecular, and cytogenetic studies (Sket & Arntzen 1994; Gorički 2004, 2006; Gorički et al. 2006; Gorički & Trontelj 2006; Trontelj et al. 2009; Sessions et al. 2016) do not support the recognition of these subspecies, the molecular genetic data revealing that both black and white protei are nested within a southeastern Slovenian clade of *P. anguinus*. In other words, the black proteus is more closely related to a geographically adjacent population of white proteus than that population of white proteus is to other populations of white proteus. One possible explanation for these differences is that the black proteus, though troglobiotic with a subterranean habitat, represents an ancestral condition that lacks most of the striking troglomorphic characters seen in populations of the white proteus. In other words, the black proteus, which occupies a relatively young and low type of karst (Paušič & Čarni 2012), represents a population that is in the early stages of troglobiotic adaptation. An alternative interpretation is suggested by phylogenetic analysis of molecular data indicating that the black proteus is genetically more closely related to adjacent populations of white proteus than either are to any other populations of *Proteus* (Gorički et al. 2006). These data suggest that, instead of representing an ancestral form that never evolved a troglomorphic phenotype, the black proteus was derived from within a population of white proteus that was exposed to a different set of selective pressures that did not favor extreme troglomorphism resulting in the evolutionary reversal of troglomorphic traits (Ivanović et al. 2013, Sessions et al. 2015). Thus, the non-troglomorphic characteristics of *P. a. parkelj* would be homoplasies rather than plesiomorphies (Sessions et al. 2015). A close relationship between white and black protei is also supported by recent cytogenetic information (Sessions et al. 2016). Thus, either i) the troglomorphic form has evolved several times independently, or ii) the black form evolved once from a white ancestor by reversing troglomorphic traits. As both Ivanović et al. (2013) and Sessions et al. (2015) have pointed out, the second scenario is formally far more parsimonious on phylogenetic grounds. The research on the developmental genetics of cave adaptations in the Mexican Blind Cave Fish, *Astyanax mexica-*

nus De Filippi 1853 (Jeffery 2005), offers a possible evolutionary developmental mechanism, involving a genetic switch, by which such a reversal could have occurred in *Proteus* (Sessions et al. 2015). Testing this idea will require access to embryonic material of both subspecies of *Proteus*.

FUNCTIONAL MORPHOLOGY

Skin morphology and bacteriome

Proteus is a paedomorphic salamander and the skin of the adults of both subspecies of *Proteus* thus retains many larval characteristics of amphibian skin as well as showing some adult characteristics. The thin, non-keratinized epidermis with outermost *stratum mucosum*, numerous unicellular glands (Leydig cells) and

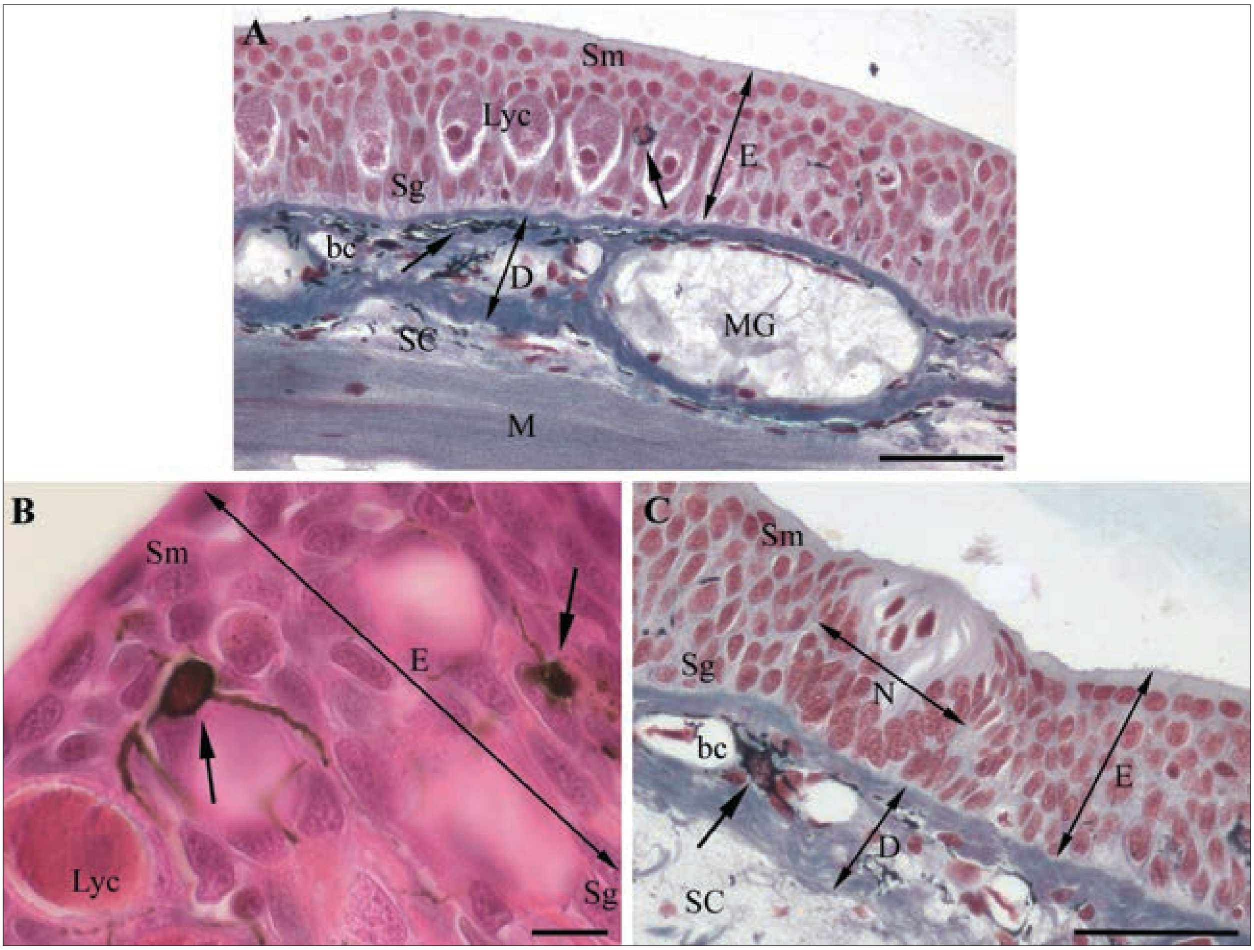

Figure 6: The skin histology of Proteus anguinus parkelj Sket & Arntzen 1994. A. The skin is thin and non-keratinized with numerous epidermal Leydig cells (Lyc), dermal multicellular mucous glands (MG) and stelate melanophores (arrows) in the epidermis (E) and dermis (D). B. The epidermal melanophores (arrows) with projections. C. The mechanoreceptive neuromast (N) in the epidermis. bc – blood capillary, M- skeletal muscles, SC – subcutis, Sg – stratum germinativum, Sm – stratum mucosum. A & C. Silver nitrate - Pollak staining. B. H & E staining. Scale bar: 100µm (A & C) and 20µm (B). Photos: L. Bizjak Mali.
Slika 6: Histologija kože črnega močerila Proteus anguinus parkelj Sket & Arntzen 1994. A. Koža je tanka in neporoženela s številnimi Leydigovimi celicami (Lyc) v vrhnjici, z večceličnimi mukoznimi žlezami (MG) v usnjici in zvezdastimi melanoforami v vrhnjici (E) in usnjici (D). B. Melanofore z odrastki v vrhnjici (puščice). C. Mehanoreceptorni nevromast v vrhnjici. bc – krvna kapilara, M- skeletne mišice, SC – podkožje, Sg – stratum germinativum, Sm – stratum mucosum. A & C. Barvanje srebrov nitrat - Pollak. B. Barvanje H & E. Merilce: 100µm (A & C) and 50µm (B). Fotografije: L. Bizjak Mali.

mechanoreceptive neuromasts of the lateral line system (Fig. 6A-C) (Bulog 1991; Kos 1992; Kos & Bulog 1993) are unique features of the larval epidermis that are also seen in other paedomorphic salamanders. On the other hand, the numerous multicellular alveolar mucous glands in the dermis (Fig. 6A) are characteristic of adult amphibian skin and provide an important protective mucus layer over the non-keratinized surface. A comparative analysis of skin of white and black protei showed that the dark pigmented skin in the black protei has fewer larval characteristics and shows some structural characteristics of metamorphosed forms such as more numerous multicellular mucous glands in dermis (Bulog 1991), an increased number of layers of epidermal cells, where the surface epidermal cells tend to be more flattened and the basement lamella is thicker (Kos 1992; Kos & Bulog 1993). In general, the skin of black protei is thicker with numerous processes of stellate melanophores concentrated mainly in the outer part of the dermis under the basement lamella (Fig. 6A, C) (Bulog 1991; Kos 1992; Kos & Bulog 1993). Melanophores are also present in the epidermis (Fig. 6B). Even though the processes of melanophores are more numerous than in white protei, it is not known if the darker skin is due to the higher number of melanophores in the skin or more melanosomes (pigment granules) in the cytoplasm of the processes of pigment cells.

Interestingly, immunolabelling of the skin with anti-opsin antibody was positive in cells of the basal layer of the epidermis (*stratum germinativum*) of both subspecies of *Proteus* indicating that the skin is photosensitive (Kos et al. 2001). Indeed, the animals show strong photophobic reaction when their skin is illuminated (unpublished observation). Further studies are required to clarify the significance of this non-specific staining and to identify the molecules and cellular structures in the skin that bind this antibody.

Recently, a comparative metagenomic analysis of the skin bacteriomes from natural population of *Proteus* showed that all of them, with the exception of *P. a. parkelj*, exhibited considerably lower bacterial diversity compared to water samples from their habitats (Kostanjšek et al. 2017; Prodan 2018; Kostanjšek et al. 2019). The bacteriomes in these populations of white proteus individuals included similar proportions of mostly known taxa of environmental microbes: Alphaproteobacteria, Betaproteobacteria, and Actinobacteria. In stark contrast to the white protei, the bacteriome of the black subspecies was dominated by enterobacteria and aeromonads possibly originating from sewage and farming pollution (Kostanjšek et al. 2017, 2019). These data are alarming because the skin microbiome is an essential part of the innate immune system in amphibians (see in McCoy & Peralta 2018), and changes to the skin microbiome can lead to increased vulnerability to infection by potential pathogens and consequently to population decline. Indeed, besides playing a critical role in a wide range of physiological processes (e.g. regulating fluid balance, ion transport, and respiration), amphibian skin also plays a critical role in regulating the animal's health by producing antioxidants and antimicrobial peptides, and by harboring diverse, symbiotic microbial communities that protect against foreign and potentially harmful pathogens (see in McCoy & Peralta 2018). These symbiotic skin bacteria are adapted to persist in the presence of antimicrobial mucosal peptides and can inhibit pathogen colonization and infection of the skin. The black *Proteus* may be the first case of such replacement of the endemic skin bacteriome of an amphibian due to human agricultural activity. Future research will be needed to understand the long-term threat of this phenomenon to the vulnerable populations of *P. a. parkelj*.

Perception in the dark

The eye

Adult black protei have small but totally normal eyes, although they are paedomorphic and lack eyelids, which is typical for larval amphibians (Fig. 7A). The eyes have all the important parts (transparent cornea, lens, retina and optical nerve) of the optical-neural pathway leading to the central nervous system (Bulog 1992, 1993; Kos 2000) (Fig. 7B). The diameter of *P. a. parkelj* eyes (730 μm) is much larger then in *P. a. anguinus* (450 – 500 μm) (Kos 2000) but well below the size of surface dwelling (epigean) salamanders. The lens has a diameter of about 200 μm in comparison to *P. a. anguinus* where the lens is markedly reduced or even totally missing (Kos et al. 2001). Compared to the regressed retinal morphology of the white proteus, detailed morphological analyses revealed that the black proteus eye has a normal amphibian retinal structure (Bulog 1992; Kos & Bulog 1996a; Kos 2000; Kos et al. 2001). Individual retinal layers are discernible and photoreceptor cells exhibit recognizable inner and outer segments (Fig. 7C) (Bulog 1992; Kos 2000; Kos et al. 2001). Retinal immuno-cytochemistry with various anti-opsin antibodies (Kos 2000; Kos et al. 2001; Bulog et al. 2002) confirmed the light sensitive visual pigments (the first important member of the phototransduction cascade in vertebrate photoreceptor cells) in the photoreceptors of *P. a. parkelj* (as well as in the variable rudimentary eye of *P. a. anguinus*) that indicates a light sensitivity of the eyes. This finding correlates with electrophysiological investigations that revealed that retinal photoreceptor cells of *P. a. angui-*

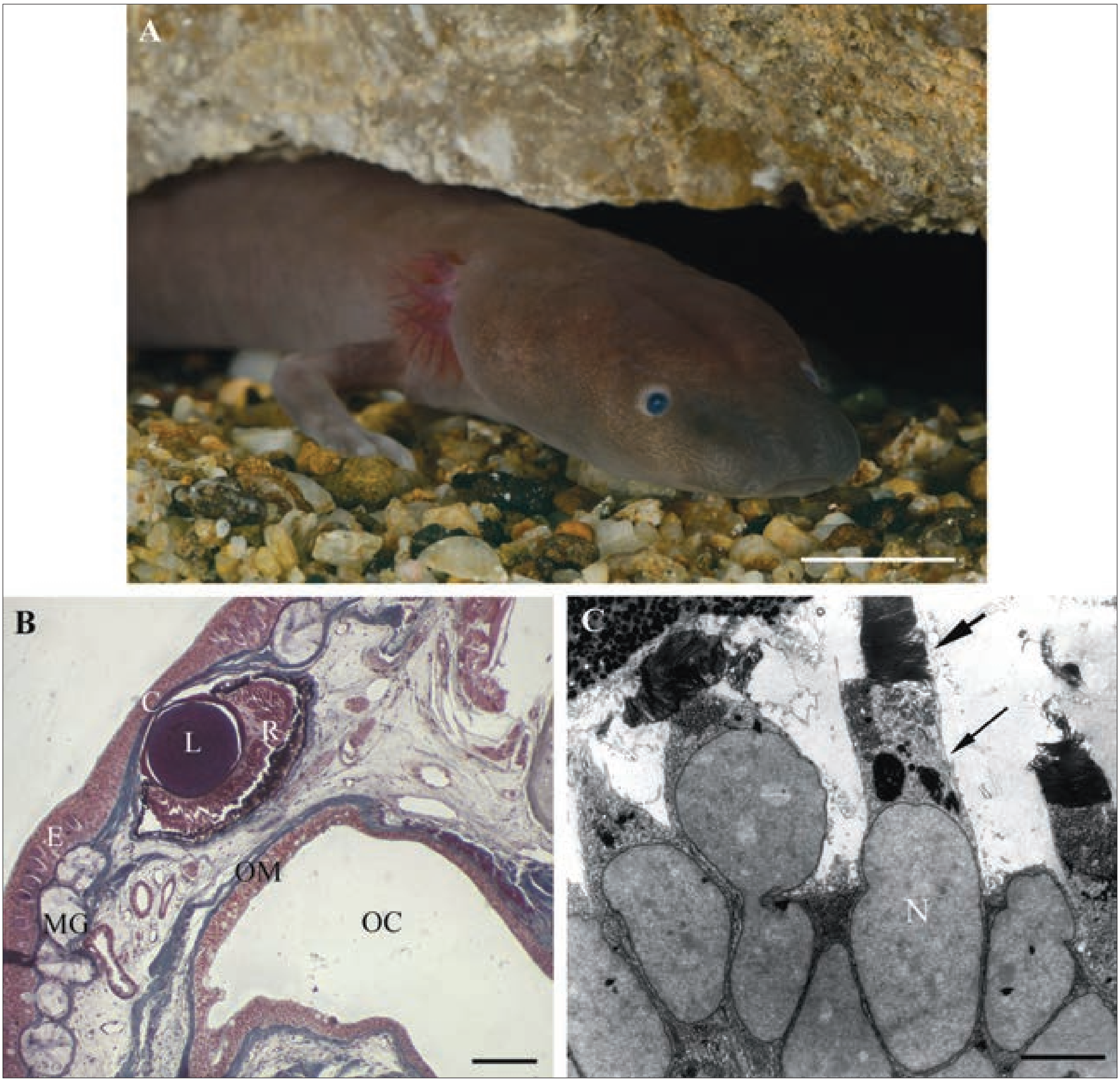

Figure 7: A. The head of Proteus anguinus parkelj Sket & Arntzen 1994 with its typical »larval« eyes and visible mechanoreceptive neuromasts on the snout. B. Cross section through the head with eyeball. C – cornea, E – epidermis, MG – multicelullar mucous gland in dermis, L – lens, R – retina with pigment epithelium, OC – oral cavity, OM – oral mucosa. Silver nitrate - Pollak staining. C. Electron-microscopy micrograph of retina photoreceptors. N – nucleus of photoreceptor cell, thick arrow – outer segment, thin arrow – inner segment. Scale bar: 1 cm (A), 200µm (B) and 10 µm (C). Photos: D. Dallesi (A), L. Bizjak Mali (B) and M. Kos (C).

Slika 7: A. Glava črnega močerila Proteus anguinus parkelj Sket & Arntzen 1994 z očmi značilnimi za ličinke in vidnimi mehanoreceptornimi nevromasti na gobcu. B. Prečni prerez glave v predelu oči. C – roženica, E – epidermis, MG – večcelična mukozna žleza v dermidi, L – leča, R – mrežnica s pigmentnim epitelom, OC – ustna votlina, OM – ustna sluznica. C. Posnetek fotoreceptorjev mrežnice narejen s presevnim elektronskim mikroskopom. N – jedro fotoreceptorne celice, debela puščica – zunanji segment, tanka puščica – notranji segment. Merilce: 1 cm (A), 200µm (B) in 10 µm (C). Fotografije: D. Dallesi (A), L. Bizjak Mali (B) in M. Kos (C).

nus are still responsive to light even though the retina itself is rudimentary (Gogala et al. 1965 cit. in Kos et al. 2001; Žener 1973 cit. in Kos et al. 2001). In comparison with other amphibians which have two types of rods (green and red) and three types of color-specific cones (red-, blue- and UV sensitive cones) (cit. in Kos et al. 2001), the retina of both subspecies of *Proteus* contains »red« or principal rods, red-sensitive cones but that the black proteus differs from white protei in having a third photoreceptor type, which might represent blue- or UV-sensitive cones (Kos 2000; Kos et al. 2001). The number of photoreceptor cells in both is relatively low and the cones predominate. The latter is quite surprising given that the retina of most animals that are nocturnal or live underground or in turbid conditions (e.g *Necturus*, the closest relative of *Proteus*, the larval Ozark cave salamander, *Eurycea spelaea* Stejneger 1892 (syn. *Typhlotriton s.*), and the Mexican cave fish *Astyanax*) contains mostly rods which operate in dim light (cit. in Kos et al. 2001). It appears that during the regressive evolution of the eyes of *Proteus*, rods degenerated sooner than cones (Kos et al. 2001).

Pineal organ

The pineal organ of lower vertebrates is a photosensitive neuroendocrine gland in the roof of diencephalon part of brain, controlling circadian rhythms, gonadal development, metamorphosis and, body pigmentation. Photosensory cells in the pineal organ of vertebrates are structurally and functionally homologous to retinal photoreceptors having well-developed outer segments with visual pigments similar to those in the retina. The pineal organ in both subspecies of *Proteus* is reduced in size and can be found only with serial semithin sectioning of the brain (Kos 1998; Kos et al. 2001). Detailed ultrastructural studies (Kos & Bulog 1996b, 2000; Kos 1998; Kos et al. 2001) revealed structural degeneration of the outer segments of pineal photoreceptors in both subspecies, composed of irregular membranes and vesicular aggregations instead of a stack of membrane disks known for normal photoreceptors. The synaptic ribbons with clear synaptic vesicles in soma and processes of pineal photoreceptors of *Proteus* indicate the involvement of pineal cells in the transmission of light stimuli. The possibility of retained light sensitivity is supported by the finding of immunopositive red-sensitive visual pigments in the pineal photoreceptors of both subspecies (Kos et al. 2001). Supposedly, the atrophy of the outer segments of photoreceptors does not lead to the loss of the photoreceptive function of pineal in *Proteus*. The pineal also seems to have a secretory activity as evidenced by the ultrastructure of the inner segment of pineal photoreceptors (Kos 1998; Kos & Bulog 2000).

Hearing abilities

Inner ear morphology and sound detection is of particular importance in overall mechanoreception and acoustic orientation in the underground aquatic environment inhabited by *Proteus*. Like the other aquatic urodeles, *Proteus* has the ability to register sound sources via water medium and vibrations over the substrate with a specialized and complex inner ear. The mouth cavity (which is frequently filled with air that is not used in breathing activity) and lungs, together with the inner ear, act as underwater sound pressure transducers (Bulog 1990). The ability of sound pressure detection in *Proteus* is probably enhanced with the tight anatomical junction between the ceiling of the gas filled oral cavity and the oval window of inner ear (Bulog 1998; Bulog & Schlegel 2000; Schlegel et al. 2009). A three-dimensional model of the inner ear made from serial semithin sections of the otic region in both subspecies of *Proteus* enabled a comparative analysis and reconstruction of sensory epithelia and the perilymphatic system (Konec 2009; Konec & Bulog 2010) (Fig. 8A-C). This study showed that membranous labyrinth of *P. a. parkelj* is slightly shorter and less flattened, but structurally mostly identical to *P. a. anguinus.* All seven sensory epithelia (Fig. 8B-C) previously described in *P. a. anguinus* (Istenič & Bulog 1976; Bulog 1995), are present in the membranous labyrinth of *P. a. parkelj* (Konec 2009; Konec & Bulog 2010). The only difference found is in the size of the saccular macula and crysta externa, the first one is larger in *P. a. anguinus* and former is larger in *P. a. parkelj.* The saccular macula of *P. a. anguinus* has also a very complex structure with a large otoconial mass according to the utricular and lagenar; it is assumed that it plays a role in detecting the direction of the sound source, in other words, it is a specific adaptation to life in an underground, aquatic habitat (Bulog & Schlegel 2000).

The sound detection ability in both subspecies of *Proteus* is in the frequency range from 10 to 15000 Hz with the greatest sensitivity reached at 1500 Hz for *P. a. anguinus* and 2000 Hz for *P. a. parkelj* (Bulog & Schlegel 2000; Schlegel et al. 2009). This range of audible frequencies provides the unique ability to detect sound waves underwater and surpasses all amphibians and most fish that have been studied. The frequencies below 50 Hz are most probably detected at the expense of the mechanosensitive neuromasts of lateral line sensory system. It is assumed that inner ear of *Proteus* also plays an important role in the detection of sound waves created by the rising water level during the rainy seasons that enables the animal a timely retreat into the deeper regions of their aquatic habitat before they are thrown out onto the surface (Bulog & Bizjak Mali 2014). Further research is needed in order to confirm and explain this phenomenon.

Mechano- and electroreceptors of the lateral-line sensory system

Proteus can detect its prey in total darkness over some distance using its excellent sense of smell (olfactoreceptors) as well as mechanoreceptive neuromasts and electroreceptive ampullary organs of the lateral line sen-

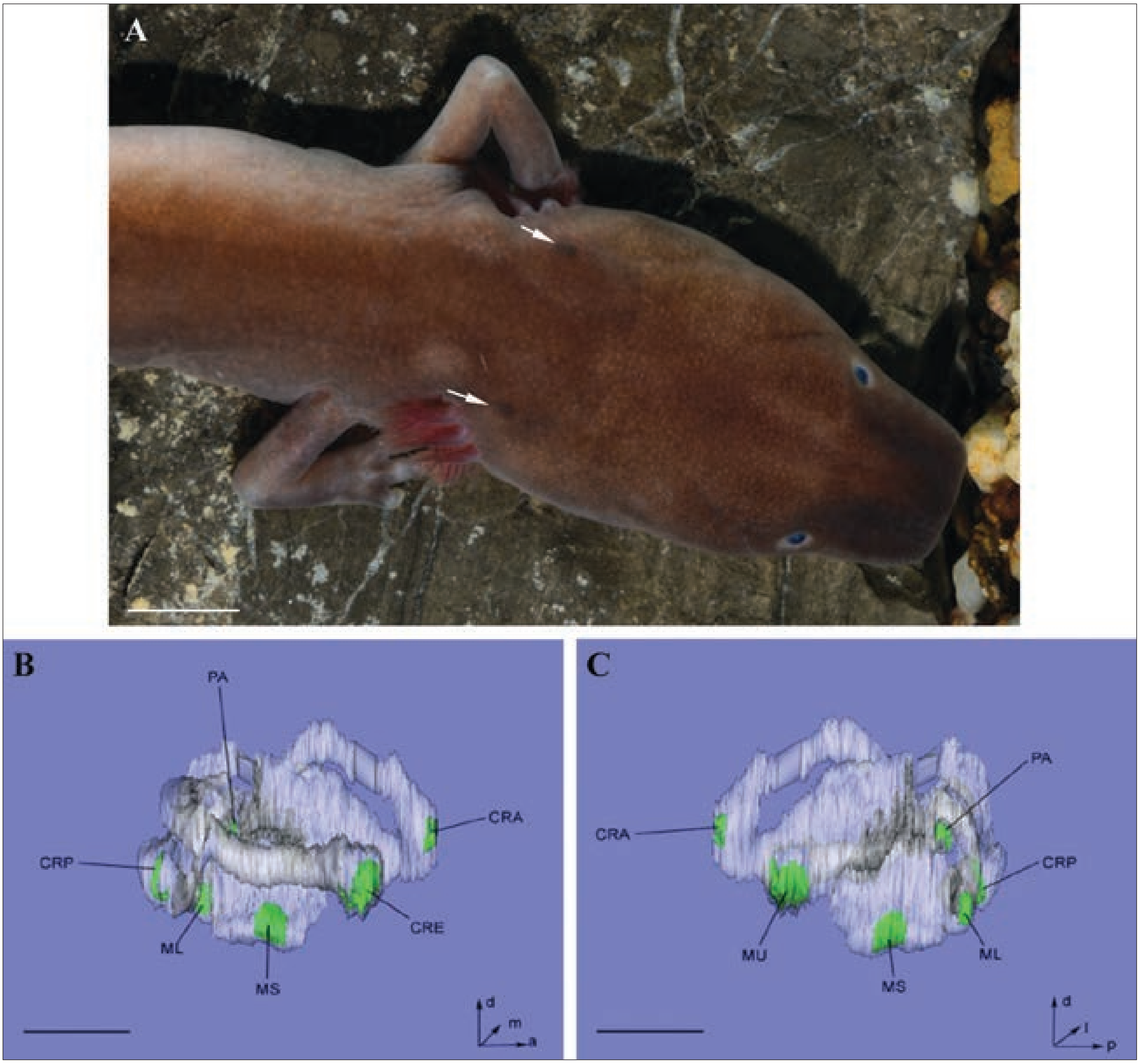

Figure 8: Head of Proteus anguinus parkelj Sket & Arntzen 1994 with visible otic region (arrow) (A) and 3-D model of inner ear from lateral (B) and medial view (C). The sensory epithelia are labeled in green. CRA- crysta anterior, CRP - crysta posterior, CRE – crysta externa, ML – macula lagenae, MS – macula sacculi, MU – macula utriculi, PA – papillae amphibiorum. Scale bar: 5 mm (A) and 1 mm (B & C). Photos: D. Dallesi (A) and M. Konec, 2009 (B & C).
Slika 8: Glava črnega močerila Proteus anguinus parkelj Sket & Arntzen 1994 z vidno slušno regijo (puščica) (A) in 3-D model notranjega ušesa z lateralne (B) in mediane strani (C). Senzorični epiteli so označeni z zeleno. CRA- anteriorna krista, CRP – posteriorna krista, CRE – zunanja krista, ML – makula lagene, MS – makula sakula, MU – makula utrikula, PA – papilla amphibiorum. Merilce: 5 mm (A) in 1 mm (B & C). Fotografije: D. Dallesi (A) in M. Konec, 2009 (B & C).

sory system (overview in Bulog, 1994). These sensory organs of lateral line system lie in the epidermis of the skin (Figs. 6C, 7A) and, while the ampullary organs are present only on the head, the neuromasts are distri-

Figure 9: The post-hatched larva of Proteus anguinus parkelj Sket & Arntzen 1994 with visible hind limb bud (arrow). Fixed specimen from archive collection at the Department of Biology, BF, University of Ljubljana. Scale bar: 5 mm (A) and 2 mm (B). Photos: L. Bizjak Mali.

Slika 9: Izležena ličinka črnega močerila Proteus anguinus parkelj Sket & Arntzen 1994 z vidnim brstom zadnje okončine (puščica). Fiksiran osebek iz arhivske zbirke Oddelka za biologijo, BF, Univerza v Ljubljani. Merilce: 5 mm (A) in 2 mm (B). Fotografiji: L. Bizjak Mali.

buted also along both sides of body (Istenič & Bulog 1984; Istenič 1986; Bizjak Mali 1990; Fric 2017). The sensory organs of the lateral line system are present in aquatic amphibian larvae and adult paedomorphic salamanders that retain an aquatic lifestyle (Webb 2014). The mechanosensory neuromasts enable the animals to detect water disturbances caused by currents, the movements and sounds of nearby animals, and a variety of other sources. They function as a "distant touch" sensory system important to locate objects in their environment and also play an active role in localization and orientation. With electrosensory ampullary organs the animals sense the weak bioelectrical fields generated by other animals (due to the activity of their nerves and muscles) and use it to locate them or avoid them. Behavioral studies have demonstrated that the white subspecies can detect weak electrical fields all the way to a value of 0.1 mV / cm, while the *P. a. parkelj* are slightly less sensitive (Schlegel & Bulog 1997). These mechano and electroreceptive sense organs, including a specialized and very complex inner ear, obviously play an important role in *Proteus* in orientation, search for prey, intraspecies communication, and mutual recognition in underground habitats.

Behavioral studies suggest that *Proteus* (both subspecies) is also able to detect and orient itself to magnetic fields (Aljančič & Bulog 1999; Schlegel et al. 2009). For these studies the animals were in round containers surrounded by large coils for manipulating the magnetic field. A computer-assistant program and infrared camera was used to record the direction faced by the animals at different times in the experimental arena.

What we know about the reproductive biology of the black proteus?

We understand very little about the reproductive biology of the black proteus even though captive black protei laid eggs in the Tular Cave laboratory in Kranj, Slovenia in the years 2007, 2013, and 2015 (where seven individuals have been kept in captivity since 2002) (Aljančič, personal communication). Unfortunately, none of the eggs developed successfully, and some of them were eaten by conspecifics sharing the same tank. The egg laying process (in October 2015) was recorded with an infrared camera (Jaksetič 2015) but there are no further published data for the size of the egg clutches or female behavior during laying. One captive female *P. a. parkelj* (body length 250 mm) laid eggs after one year and half in captivity in the Speleological laboratory at the Department of Biology of Biotechnical faculty, University of Ljubljana (B. Bulog, personal communication). The female laid more than 30 eggs but she ate most of them and the remaining eggs failed to develop because they were most probably not fertilized. Since this female was kept in her own, separate aquarium, the eggs laying can obviously happen in the absence of a male.

An early post-hatched black proteus larva was found at Jelševnik spring (Sket & Arntzen 1994), confirming that the black proteus, like the white proteus, is oviparous. This post-hatched larva was 21 mm in length and was at Briegleb's developmental stage 21 (Briegleb 1962) with almost three digits on the short front limbs and hind limb buds at a pre-digit stage (Fig. 9A-B). The intestinal wall was still swollen with yolk and the larva was not yet able to move around in an organized way. The eye diameter (0.4 mm) was 8% of the head length and the embryo was pigmented densely grey dorsally, fading on the sides to a lighter venter.

Studies of the gonads morphology and gametogenesis in *Proteus* have been performed to gain better base-line knowledge of their reproductive biology, with potential applications in conservation issues as well as in the management of their reproduction in captivity. Studies of detailed morphology of the ovaries, including descriptions of the developmental stages of the oocytes, was mostly performed on the white subspecies of *Proteus* (Talaber 2008; Bizjak Mali & Bulog 2010, 2011; Žibert 2010; Bizjak Mali et al. 2010, 2013). These studies showed that, at least in *P. a. anguinus*, ovarian maturation is not seasonal, although it is positively correlated with body length and mass (Bizjak Mali et al. 2010, 2013). However, a recent analysis of testes morphology of preserved adult individuals of *Proteus* (with total body lengths between 200 to 360 mm) from different populations, including both *P. a. anguinus* and *P. a. parkelj*, showed that body size is a poor predictor of reproductive/maturity state in males of *Proteus* (Kovačič 2013; Bizjak Mali et al. 2015, 2016; Bizjak Mali & Bulog 2016; Bizjak Mali 2017). The shape and meiotic condition of the testes are highly variable and totally independent of the length (age) of the animal. Even more, the two different morphological types of testes (multilobed and broad single-lobed testis) that were found indicate a possible intraspecies variability of morphological types of testes. The multilobed testis was found only in *P. a. parkelj* and broad single-lobed testis was found only in *P. a. anguinus.* Another interesting discovery is also that, contrary to *Proteus* females, meiotic activity in *Proteus* males appears to be seasonal (Bizjak Mali 2017) suggesting that gametogenesis in male and female *Proteus* is only loosely synchronous. These issues underline how incompletely we still understand reproduction in this enigmatic animal.

The digestive system and accessory glands

The basic anatomical and histological structure of the digestive tract of *Proteus* is similar to other amphibians and it is divided into a short esophagus, a slightly widened muscular stomach, a short duodenum, a medium long small intestine that is more or less coiled depending on the size of the animal, and a short, slightly wider large intestine that opens into the cloaca (Fig. 10A-B). A preliminary analysis of the intestinal microbiota from the fecal samples of both subspecies of *Proteus* by molecular typing method (T-RFLP) showed that there was a difference in microbiota between the white proteus from Planina cave and black proteus and no clear resemblance of either to the gut microbiota of the axolotl (*Ambystoma mexicanum*) (Borštnar Vasle 2016; Avguštin et al. unpublished). Cloning and sequencing of the amplified PCR products revealed that the majority of the sequences from *P. a. anguinus* belonged to an unclassified group from the family Peptostreptococcaceae of the Gram-positive phylum Firmicutes, whereas the majority of the sequences from the *P. a. parkelj* belonged to the family Clostridiaceae from the same phylum and to the Gram negative members of the genus *Parabacteroides* from the phylum Bacteroidetes. Further studies on this topic are in progress.

The spleen and liver morphology in both proteus subspecies were investigated and compared with their closest surface-dwelling relative, *Necturus maculosus*. The spleen is a major lymphoid organ in the immune response of all vertebrates and, in fish and amphibians, it also has hematopoietic and hemocateretic functions (destruction of red blood cells and "recycling of haemoglobin"), in addition to phagocytosis, storage, and release of erythrocytes (Alvares 1990). The liver is the central organ for metabolic activity in vertebrates and is also an important storage depot for many raw materials of digestion (sugars, fats, vitamins, metal ions) (Hadley 1985).

The spleen of both subspecies of *Proteus* is diffuse, with the lymphoid tissue (white pulp) evenly distributed with the red pulp (Fig. 11 A-B) (Mrak 2007). Most of the spleen is red pulp, consisting of cell cords (lymphocytes, erythrocytes, hemocytoblasts and erythroblasts) and numerous sinusoids full of erythrocytes. The spleen of both subspecies of *Proteus* also has pigmented melanomacrophages similar to macrophages with phagocytic activity described for ectothermic animals, including amphibians (Augis 1980).

Figure 10: A. Proteus anguinus parkelj Sket & Arntzen 1994 with open posterior part of the body cavity. A higher magnification view (B) of the visible posterior part of liver (l) with gall bladder (gb), stomach (st), small (si) and large intestine (li), tiny, immature testes (arrows), kidney (k) with urinary (Wolffian) ducts, urinary bladder (asterisk), and cloaca (c). Scale bar: 1cm. Photos: L. Bizjak Mali.

Slika 10: A. Črni močeril Proteus anguinus parkelj Sket & Arntzen 1994 z vidnim odprtim posteriornim delom telesne votline. Pod večjo povečavo (B) je viden posteriorni del jeter (l) z žolčnikom (gb), želodec (st), tanko (si) in debelo (li) črevo, drobceni nezreli testisi (puščici), ledvica (k) s stranskima sečevodoma, sečni mehur (zvezdica) in stok ali kloaka (c). Merilce: 1cm. Fotografije: L. Bizjak Mali.

The liver of both subspecies of *Proteus* occupies more than half of the length of body cavity (Fig. 10A-B) and represents the 5.35 (±1.68) % of the body mass. The hepatocytes are large and arranged in laminae that are two or more cells thick, separating adjacent sinusoids (Fig. 11C-D). One of the most outstanding features of the liver in *Proteus* is a remarkable accumulation of energy–rich storage material (e.g. lipid droplets and glycogen) in the hepatocytes (Fig. 11C) (Bulog et al. 2000; Bizjak Mali et al. 2001; Lužnik 2004) that are used to meet metabolic needs during food deprivation (Bizjak Mali et al. 2013) and reflect adaptation to stress situations in its environment, such as sporadic and discontinuous food supplies and/or hypoxia. The second outstanding feature is the numerous pigment cells which are grouped in large clusters among the hepatocytes (Bizjak Mali et al. 1999, 2001; Prelovšek et al. 2005, 2008), and contain haemosiderin (iron-storage complex), melanin, and lipofuscin (lipid-containing residues of lysosomal digestion) (Fig. 11C-D). In comparison with *Necturus,* the pigment cells are more numerous and form larger clusters, and are also structurally more heterogeneous and contain a larger amount of haemosiderin. It seems that the liver pigment cells are an important site of iron accumulation in *Proteus*. Furthermore, synthesis of melanin was confirmed in the liver pigment cells of *Proteus* (Prelovšek & Bulog 2003). Melanin can absorb and neutralize free radicals, cations, and other potentially toxic agents derived from degradation of phagocytosed cellular material (Zuasti et al. 1989). It is assumed that the pigment cells of this extracutaneous system in *Proteus* has an important role in supporting the antioxidant system of reactive oxygen species (Bizjak Mali, unpublished) in defense against free radicals that are harmful to cells, protecting the liver from the negative effects.

Behavior

Virtually almost nothing is known about behavior of the black proteus except for the following report by prof. Tine Valentinčič (Sket et al. 2014; Trontelj et al. 2017), who showed that black protei were so aggressive towards white ones in the common aquarium that the experiment was discontinued and the animals had to be separated. In terms of behavioral patterns in their natural environment, we know that black protei come regularly to surface water bodies in the evening, where it is also possible to see them early in the morning (Aljančič, Bulog, Hudoklin, Mlinar, Župančič, pers. comm.). There is no evidence that this behavior is associated with feeding patterns, even after one month of continuous observation (Bulog, pers. comm.). Similar is the behavior of the white protei.

THREATS AND CONSERVATION STATUS & ACTIONS

The water resources in the karst underground are extremely vulnerable to all kinds of pollution and contamination with hazardous organic and inorganic compounds. Any pollution of the karst surface may affect the quality of water in its underground. The self-cleaning capacities of the karst underground are poor (Sket & Velkovrh 1981; Novak 1995; Sket 1996). Among the most serious chemical pollutants are pesticides, polychlorinated biphenyls (PCBs), and heavy metals which persist in the environment, being slowly, if at all, degraded by natural processes (Bulog et al. 2002).

Most of pollution in karst areas is due to unsustainable anthropogenic activities including intensive agriculture and industry, unregulated communal infrastructure and industrial landfills. *Proteus anguinus parkelj* is particularly endangered due to its limited distribution, which might be estimated at even less than 3 km^2 (Gorički et al. 2017), as well as its specific biology, such as permanent aquatic life history, its thin, non-keratinized skin, and its longevity and consequent potential bio-accumulation of pollutants. The chronic exposure to contaminants can cause the weakening of the immune system of amphibians, and consequently increased susceptibility to parasitic infections and other pathogens (Hayes et al. 2010). Many pollutants in the environment act as endocrine disrupters, leading to hormonal balance disorders and a negative impact on the reproductive system and reproduction. The main threats for *Proteus* in Bela Krajina are the overuse of pesticides and nitrogen fertilizers, and, after 2009, the free distribution of biogas slurry (Bulog 2007, 2012; Hudoklin 2011; Bulog & Bizjak Mali 2014; Bizjak Mali & Bulog 2016; Năpăruş-Aljančič et al. 2017). Furthermore, only 700 m away from the black proteus locality at Jelševnik springs, foundry sand with heavy metals was dumped into a doline between the years 1989 and 1993 (Bulog et al. 2002; Hudoklin 2011). The groundwater was loaded with a high content of aromatic hydrocarbons, phenols, and iron (Novak 1995; Hudoklin 2011). The accumulation of zinc and arsenic in both habitat and tissues of *P.*

a. parkelj has been shown (Bulog et al. 2000, 2002). The sand from this landfill was drained into the spring and because of its coarseness the thin skin of the protei was injured and subsequent lethal infections with pathogenic water mold *Saprolegnia* sp. developed (Kogej 1996), resulting in the death of some of the protei (Bulog personal; Hudoklin 2011).

Human sewage represents another potential problem because local villages have no centralized sewage system, and the waste water is decanting from septic tanks directly into the karst underground. The presence of proteus individuals in the low karst plain indicates its higher exposure to these sewage and agricultural pollutants. Increased levels of nitrates and phosphates have been detected in the Jelševnik springs (Bulog 2007, 2009, 2011, 2012, 2015; reinterpreted by: Hudoklin 2011; Bulog & Bizjak Mali 2014; Bizjak Mali & Bulog 2016). The values of nitrates were significantly increased in 2010 and 2011 when a biogas plant at Lokve in Črnomelj began to operate and especially in the spring reached values higher than 15 mg/L of NO_3 (in some nearby localities the levels reached

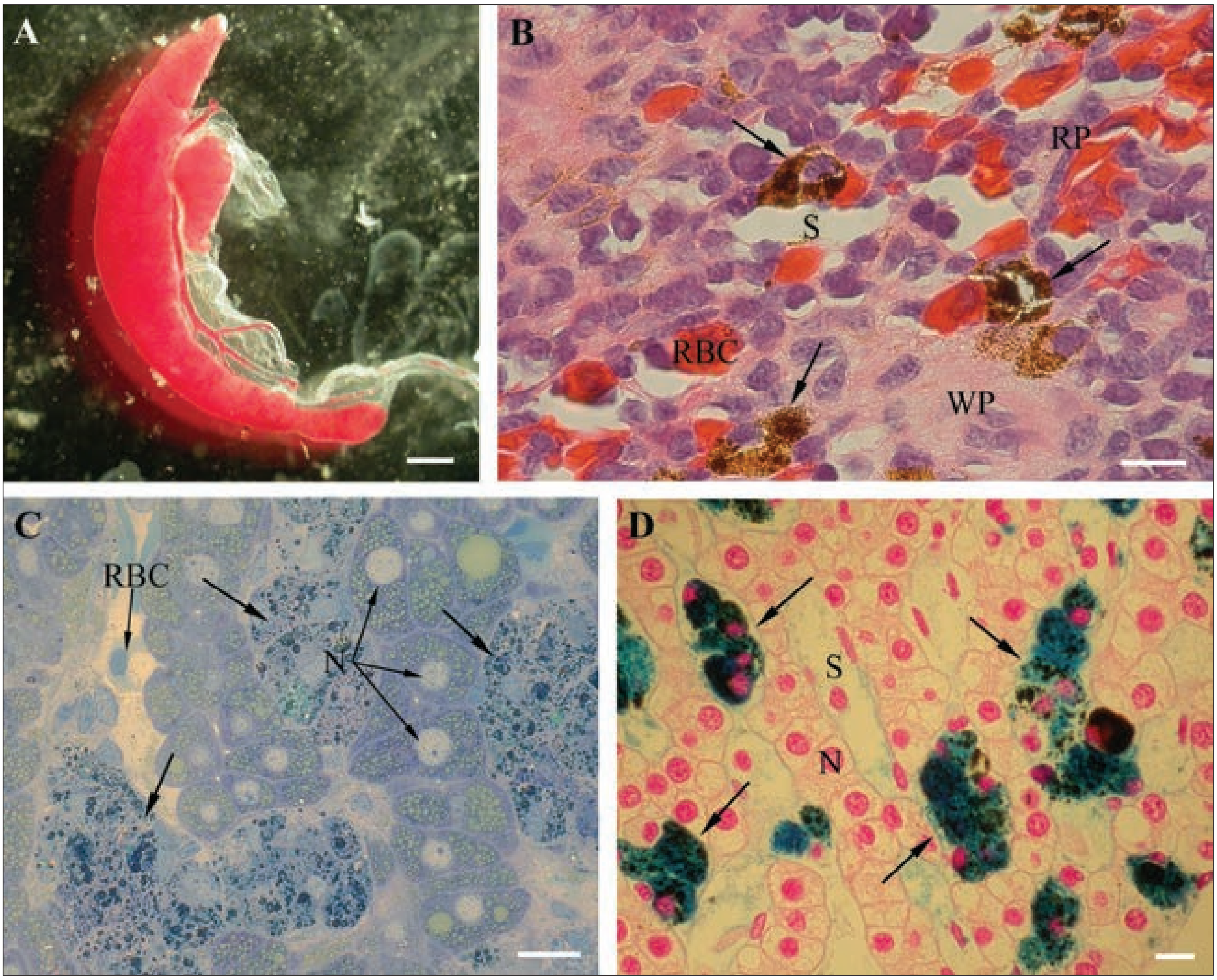

Figure 11: Spleen (A & B) and liver (C & D) of Proteus anguinus parkelj Sket & Arntzen 1994. arrows – clusters of pigment cells, N – nuclei of hepatocytes, S – sinusoid, RBC – red blood cell in sinusoid, RP – red pulp, WP – white pulp. B. H&E staining. C. Azur II - Methylen blue staining, semithin section. Lipid droplets in hepatocytes are green. D. Perls histochemical staining for hemosiderin (blue). Scale bar: 1 mm (A), 20 µm (B, C & D). Photos: P. Mrak, 2007 (A & B) and M. Lužnik, 2004 (C & D).
Slika 11: Vranica (A & B) in jetra (C & D) črnega močerila Proteus anguinus parkelj Sket & Arntzen 1994. Puščice – skupki pigmentnih celic, N – jedra hepatocitov, S – sinusoid, RBC – rdeča krvnička v sinusoidu, RP – rdeča pulpa, WP – bela pulpa. B. Barvanje H&E. C. Barvanje Azur II - metilensko modro, poltanka rezina. Lipidne kaplje v hepatocitih so zelene. D. Histokemijsko barvanje po Perlsu za hemosiderin (modro). Merilce: 1 mm (A), 20 µm (B, C & D). Fotografije: P. Mrak, 2007 (A & B) in M. Lužnik, 2004 (C & D).

even up to 20 mg/L), probably as a consequence of intensive manuring and pouring of slurry onto agricultural surfaces, and the consequent leaching of nitrates into underground water. Natural background concentration of nitrate in groundwater in temperate regions range from trace amounts to 3 mg/L (in Slovenia the levels of nitrate in groundwater are evaluated on average 3.81 mg NO_3/L; Mezga 2014); concentrations above 3 mg/L reflect anthropogenic contamination (see in Rouse et al. 1999).

The pollution trends with nitrates and orthophosphates at *P. a. parkelj* localities were assessed using the IDW (Inverse Distance Weight) interpolation tool from ArcGIS 10.3.1 (Năpăruş-Aljančič et al. 2017) and published data from springs Dobličica and Jelševnik in the years 1987 and 2014. This pilot GIS analysis revealed a growing trend for both of these parameters implicating intensified use of fertilizer in agriculture and unregulated sewage disposal in human settlements of this area. The study also pointed out the necessity for the implementation of a monitoring scheme for the black proteus and its habitat, and also exposed the importance of GIS analysis as an indispensable tool for future conservation measures for protection of the endangered black proteus.

Increasing nitrate levels in surface and ground water are of global concern to amphibians, causing death and developmental anomalies in amphibians and impact other animals in aquatic ecosystems. The field data suggest that nitrogen fertilizers may be contributing (along with pesticides) to the decline of amphibian populations in agricultural areas (review in Camargo et al. 2005). Laboratory studies have shown that the toxicity of nitrate compounds to amphibians increases with exposure times (review in Camargo et al. 2005). A nitrate concentration of 10 mg NO_3-N/L (USA federal maximum level of nitrogen for drinking water) can adversely affect freshwater invertebrates, fishes and amphibians, at least during long-term exposures (Camargo et al. 2005). Safe levels below this nitrate concentration are recommended to protect sensitive freshwater animals from nitrate pollution. Furthermore, a maximum level of 2 mg NO_3-N/L would be appropriate for protecting the most sensitive freshwater species (Camargo et al. 2005). Nitrates have been shown to cause methaemoglobinaemia (cit. in Hamer et al. 2004), in which the symbiotic microbiota in the intestine convert nitrates to nitrites, which is absorbed and then oxidizes iron in hemoglobin to form methaemoglobin that is unable to bind oxygen (Huey & Beitinger 1980; Rouse et al. 1999).

The groundwater quality standard in Europe for the protection of human health for drinking water for nitrates is a concentration of up to 50 mg NO_3/L (Fontelles & Pekkarinen 2006). However, water quality criteria for nitrate for the protection of wildlife do not exist. At the request of the RS Nature protection agency, the National Laboratory for Health, Environment and Food in Maribor, Slovenia, performed a study for the assessment of risk that nitrates pose to the groundwater ecosystem in the project area of LIFE Kočevsko (Kolar 2017). They propose several risk mitigation measures that should apply in the Karst region in order to reduce the risk of nitrate for proteus. One of the measures listed is the implementation of the threshold value of 9.2 mg NO_3/L (which is equivalent to 2.1 mg NO_3-N /L) in groundwater as an environmental quality standard for proteus habitats.

In Slovenia, *Proteus* has been protected by national legislation since 1922 and is currently classified as 'Vulnerable' by the International Union for Conservation of Nature (IUCN) red list of threatened species (Arntzen et al. 2009) because of its fragmented and limited distribution and declining population. In 1982 *Proteus* was placed on a list of rare and endangered species in Slovenia. After joining the European Union, Slovenia had to establish mechanisms for protection of the species included in the EU Habitats Directive. *Proteus anguinus* is included in the Slovenian Red List of endangered species (Sket 1992). The caves inhabited by protei were also included in the Slovenian part of the Natura 2000 network (Hudoklin 2014).

The black proteus (*P. a. parkelj*) does not have any special classification, but it is protected within the species *Proteus anguinus*. However, it is in need of a particular protection, as its distribution range is so limited. This makes it the most vulnerable and threatened population of *Proteus* to environmental pollution and to various potential pathogens that are of global concern to amphibians. The Council for the Protection of the Environment of the Slovenian Academy of Science and Arts (Kranjc 2018) formulated the standpoints to support the actions of scientists and non-government organizations (e.g. the civil society Društvo Proteus in the region of Bela krajina, the Cave laboratory Tular) (Aljančič et al. 2017) for special protection of *P. a. parkelj*. Some protection measures in force should to be made stricter. An application to the state ministries of environment and of agriculture was made for the rapid preparation of a provisional law for particularly strong protection measures for the black proteus area. These should be replaced by well-considered permanent legislation. *»Black proteus and its habitat in Bela krajina are unique, so the state, the local community and local inhabitants must take special responsibility for it*!« (Kranjc 2018).

During the rainy season and flooding, proteus individuals are regularly washed out from their subterranean habitat. Unfortunately, current laws and policies concerning the protection of *Proteus* limit the use of such specimens for scientific purpose. With the permission of the ARSO the "rescue" and return of washed-out individuals of *Proteus* to their original natural habitat is currently performed by the Tular Cave laboratory (SOS Proteus – Refuge for protei) in collaboration with a veterinarian (Zatočišče za zaščitene prostoživeče živali Golob d.o.o) (Aljančič & Aljančič 2015). The return of rescued proteus to cave habitats, however, is potentially dangerous, especially if they are housed in captivity for any length of time, due to emerging pathogens that are of global concern to amphibians and can be inadvertently spread by human contact. Therefore, we recommend that microbial analyses should be a part of routine procedure before the animals are released. Even more importantly, non-destructive samples for genetic analyses should be obtained from such animals to avoid inadvertent contamination of genetically divergent populations. An unfortunate example is the rescue of three black protei washed out to the surface at Kanižarica (Ivanovič 2012), which was at the same time a newly found locality. These protei were then released into another locality at Jelševnik spring, because it was the only accessible location; this was done without any regard for possible contamination or accidental mixing of different gene pools. For these reasons we strongly discourage attempts to return stranded protei to their natural habitat after human contact. Instead, these animals should be kept in captivity in a proper laboratory setting where they can be utilized for much needed scientific research on this important animal.

PUBLICITY AND POPULAR SCIENCE

From the time it was first discovered, *Proteus anguinus* (*človeška ribica*, the »human fish«), in the form of the strange-looking troglomorphic white proteus, has long attracted the interest of the public. It is of iconic significance for the country of Slovenia and a proteus used to be featured on a Slovenian coin. In the first years of discovery of the black proteus, newspapers and popular scientific journals in Slovenia frequently presented short news stories on this strange creature, sustaining a certain amount of interest on the need for environmental protection of this species and in general (see list of »other references«: Istenič & Bulog 1986; Sket 1992; Hodalič 1993; Bulog 2005, 2007; Ušaj 2005; Pavlovič 2006; Ivanovič 2012; Kukman 2012; Aljančič et al. 2015; Bezek-Jakše 2015; Dolžan 2015; Krese 2015; Marušič 2015, 2016; Cvjetkovič 2016; Červek 2016; Maljevec et al. 2016; Žiberna 2016; Levstik 2017a, b; Rajšer 2017; Stankovič 2017; Vlašič 2017; Žmahar 2017; Mezinec 2018a, b). Among particularly interesting news was the laying of black proteus eggs in the Tular Cave laboratory in Kranj (Jaksetič 2015). Various interviews and documentary films about the black proteus and its environmental problematic have been recorded over the years, including the famous Nick Baker's documentary (see list of »other references«: Mlinar 1999, 2018a, b; Bulog et al. 2003; Kutin 2005; Baker et al. 2007; Mihelčič & Bizjak, 2008; Jecič 2015; Aljaničič et al. 2016; Bizjak Mali et al. 2016; Šuštarič et al. 2017). A permanent exhibition was set up at Jelševnik by the Department of Biology, BF, University of Ljubljana (Žnidaršič 2002, Bulog & Bizjak Mali 2014) in the house of the Župančič family who owns the land where the black proteus localities are found and who has played an important role in the public awareness and conservation of this subspecies. A sculpture of the black proteus made by the academic sculptor Boštjan Kavčič was placed near the Jelševnik locality in October 2018 (Fig. 12) to raise awareness on the vulnerability and threats to this remarkable animal (Bezek-Jakše 2017). In 2018 a special postage stamp was issued at the 250th anniversary of the formal description of *Proteus anguinus*. It was supplemented by a bulletin with some illustrated data on *Proteus*, includ-

Figure 12: A sculpure of Proteus anginus parkelj Sket & Arntzen 1994 by the academic sculptor B. Kavčič (2018) at the Jelševnik locality to raise awareness on this remarkable, vulnerable and threatened animal. Photo: L. Bizjak Mali.
Slika 12: Skulptura akademskega slikarja B. Kavčiča (2018) črnega močerila Proteus anguinus parkelj Sket & Arntzen 1994 na Jelševniku, ki opozarja na to izjemno, ranljivo in ogroženo žival. Fotografija: L. Bizjak Mali.

ing *P. a. parkelj*. A descriptive article of the black proteus by Sket & Arntzen 1994 was used in a popular educational article (Sket 1993c) to illustrate the scientific process and writing; in one column a popularly re-shaped scientific paper was presented, in the other were presented principles of scientific writing. The black subspecies of *Proteus* is presented in detail in several scientific encyclopedias (e. g. Bulog 2004; Durand 2005; Gorički et al. 2012; Sket 2012), in a monograph (Aljančič et al. 1993), in natural science books (Parzefall et al. 1999; Pobolšaj 2003; Bulog & Bizjak Mali 2014), textbooks (Sket & Dermastia 2013), and on verified webpages e.g. Wikipedia (Bulog & Polajnar 2011), AmphibiaWeb (Bulog & Van Der Meijden 1999), and IUCN (Arntzen et al. 2009).

Despite this attention, the black proteus is still not nearly as familiar among ordinary citizens, and even among biologists, as the iconic white one. But its discovery, with its swarthy skin, beady eyes, and bright red gills, still seems a little shocking, as reflected by its

Figure 13: Proteus anguinus parkelj Sket & Arntzen 1994 in all its glory. Photos: D. Dalessi, 2012, Speleological laboratory at Department of Biology, BF, University of Ljubljana.
Slika 13: Črni močeril Proteus anguinus parkelj Sket & Arntzen 1994 v vsem svojem veličastju. Fotografije: D. Dalessi, 2012, Speleološki laboratorij na Oddelku za biologijo, BF, Univerza v Ljubljani.

taxonomic name »parkelj«, the Slovenian name for Krampus, a terrifying mythological black demon who goes from house to house during the Christmas season, frightening naughty children. It seems ironic that the proteus named after such an »evil« creature is itself so vulnerable due to human-made pollution in its natural habitat in Bela krajina. The true evil (and even national shame) is the fact that the skin of living individuals of the black proteus has become so contaminated with fecal bacteria from human agriculture and sewage that they might not be able to survive in their own natural habitat.

POVZETEK

Namen tega preglednega članka je bil, zbrati sklepe vseh dosedanjih raziskav o črni rasi in podvrsti močerila (*Proteus anguinus parkelj* Sket et Arntzen 1994), ki je endemit podzemeljskih voda belokranjskega krasa in jugovzhodne Slovenije.

Prikazali smo glavna področja raziskav na črnem močerilu, ki so potekale predvsem na Oddelku za biologijo Biotehniške fakultete, Univerze v Ljubljani. Sodelovali so tudi raziskovalci z drugih ustanov, pa tudi dodiplomski in podiplomski študentje. Skupina za zoologijo in speleobiologijo pokriva široko področje od taksonomije, evolucije in biogeografije podzemeljskih habitatov in organizmov, do splošne biodiverzitete. V skupini za funkcionalno morfologijo vretenčarjev pa se ukvarjajo predvsem s funkcionalno morfološkim prilagajanjem proteja na njegov habitat, v kontekstu ranljivosti od strupenega kemičnega in organskega onesnaženja, z daljnosežnim ciljem razumevanja dejavnikov, ki lahko prizadenejo močerilovo preživetje v njegovem naravnem okolju. Pomemben doprinos predstavljajo tudi raziskave jamskega laboratorija Tular s prispevki za ozaveščanje javnosti o naravovarstveni problematiki.

Prvi črni osebek močerila so ujeli raziskovalci Inštituta za raziskovanje krasa (iz Postojne) oktobra 1986 ob poskusnem črpanju podzemeljske vode na izviru Dobličice pri Črnomlju v Beli krajini (Aljančič in sod. 1986; Mihevc 1986; Sket & Arntzen 1994). Prvo poročilo o anatomskih značilnostih je podala Lili Istenič 1987, pozneje Boris Sket (1993 b, c), novi takson je bil že zelo temeljito znanstveno opisan in poimenovan kot *Proteus anguinus parkelj* Sket et Arntzen leta 1994 na osnovi zunanje morfologije, osteološke slike in biokemijske alocimske analize.

Podrobnejše filogenetske študije po kontrolni regiji mitohondrijske DNK so uvrstile črno raso v jugovzhodno slovensko skupino populacij (Gorički 2006; Gorički & Trontelj 2006; Trontelj et al. 2009). Kljub opravljeni filogenetski študiji ni povsem jasno, katera rasa je filogenetsko in evolucijsko prvobitnejša.

P. a. parkelj potrebuje strogo varstvo, saj je njegova razširjenost skrajno omejena (izračunan areal je le nekaj kvadratnih kilometrov (Gorički et al. 2017), nekaj bioloških posebnosti pa dela njegove populacije najbolj ranljive in ogrožene pripadnike proteja; ogroža ga tako onesnaženje okolja, kot tudi potencialni patogeni, ki ogrožajo dvoživke po vsem svetu.

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