PANAMJAS
Pan-American Journal of Aquatic Sciences

New species of *Protoopalina* (Opalinida) and first record of *Zelleriella hylaxena* (Opalinida) in tadpoles of *Boana pulchella* (Anura, Hylidae) from Argentina

MARÍA A. VILLEGAS OJEDA[1], SILVIA E. GUAGLIARDO[1,2] & RUBEN D. TANZOLA[1,2,*]

[1] *Laboratorio de Parasitología – Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670 (8000) Bahía Blanca, Argentina.*
[2] *Instituto de Ciencias Biológicas y Biomédicas del Sur, INBIOSUR/CONICET.*

*Corresponding author: rtanzola@uns.edu.ar

Abstract. Knowledge of unicellular eukaryotes that inhabit the digestive tract of anuran amphibians in Argentina is scarce and discontinued. In the present work, a new morphospecies of *Protoopalina* enterozoic in *Boana pulchella* tadpoles from Belisario stream, Ventania hydrographic basin, Buenos Aires province, is described. Its dimensions position *Protoopalina nana* n. sp. as one of the smallest of the known species to the present. No previous records are known of *Protoopalina* in Argentina. The geographic distribution of *Zelleriella hylaxena* is expanded and comparative data are provided.

Keywords: *Boana pulchella*, *Protoopalina*, unicellular enterozoic eukaryotes, *Zelleriella hylaxena*

Resumen: **Nueva especie de *Protoopalina* (Opalinida) y primer registro de *Zelleriella hylaxena* (Opalinidae) en larvas de *Boana pulchella* (Anura: Hylidae) de Argentina.** El conocimiento de los eucariotas unicelulares que habitan el tracto digestivo de los anfibios anuros en Argentina es escaso y discontinuado. En el presente trabajo se describe una nueva morfoespecie de *Protoopalina* enterozoica en *Boana pulchella* colectada en el arroyo el Belisario, de la cuenca hidrográfica de Ventania, provincia de Buenos Aires. Sus dimensiones posicionan a *Protoopalina nana* n. sp. como una de las más pequeñas especies conocidas hasta el presente. No se conocen registros previos de *Protoopalina* en Argentina. Se amplía la distribución geográfica y se aportan datos comparativos de *Zelleriella hylaxena*.

Palabras clave: *Boana pulchella*, eucariotas unicelulares enterozoicos, *Protoopalina*, *Zelleriella hylaxena*

Introduction

Previous records of unicelular eukaryotes, traditionally referred as “protozoans” or “protists” (Loker & Hofkin 2015), inhabiting the gut of anurans in Argentina are scarce and discontinued. Otamendi (1945) in his doctoral thesis on enterozoic protozoa of *Leptodactylus latrans* (cited as *Leptodactylus ocellatus* (L.)) and *Rhinella arenarum* (cited as *Bufo arenarum* Hensel) from surroundings of La Plata city, described and illustrated representatives of the genera *Zelleriella* Metcalf 1920, *Nyctotherus* Leidy 1849, *Trichomonas* Donné 1837, *Chilomastix* Alexeieff 1910, *Entamoeba* Casagrandi & Barbagallo, 1895 and *Endolimax* Kuenen & Swellengrebel 1917. Six decades later Cabagna Zenklusen *et al.* (2009) identified parasitic protozoa in five species of anurans from Entre Ríos and Santa Fe. These parasites were identified as *Zelleriella* sp., *Nyctotherus* sp., *Trichodina* sp. and *Trypanosoma* sp., the latter being a hemoparasite. To

date, the diversity of unicellular eukaryotes that interact with *Boana pulchella* (Duméril & Bibron 1841), particularly during their tadpole phase, remains unknown. Taking into account the aforementioned it has been considered relevant to provide novel information about the subject. The aim of this study is to describe and propose a new species of *Protoopalina* Metcalf 1918 and to report for the first time the presence of *Zelleriella hylaxena* Metcalf 1923 in Argentina.

Material and Methods

A total of 71 tadpoles of *B. pulchella*, of Gosner stages 25-41 (mode 28-30) were examined during the reproductive season of fall 2016 (N = 29) and spring 2017 (N = 42). The samples were collected with a 5 mm mesh handnet at the Belisario stream located in the southwest of the province of Buenos Aires, Argentina (38° 4´S, 61° 55´W). The amphibians were transported alive to the laboratory, prior to necropsy they were anesthetized with benzocaine and somatic measurements were taken. Body length (oral disc-cloaca distance) and maximum body length were measured with caliper. They were identified using the Kehr & Williams (1990) taxonomic key and the ontogenetic stage was determined according to Gosner (1960). The tadpoles were dissected by to the oro-anal ventral plane and using a stereoscopic microscope, the digestive tract and the coelomic cavity were examined. Enterozoic unicellular eukaryotes were identified by taking aliquots of intestinal content, observed by microscope fresh and fixed preparations (absolute methyl alcohol or 10% physiological formalin) and stained with Harris hematoxylin and Giemsa. Measurements were made with a 7X Shimaozu-Kalnew micrometer eyepiece using 40X and 100X objectives of an Olympus CH20 microscope on fresh and colored fixed preparations. For the measurements of the representative of the genus *Zeleriella*, the dimensions proposed by Galaviz-Silva & Jiménez-Guzmán (1986) were taken into account. Nomarski Interdifferential optical Microscopy (DIC) observations were made using a Leica Model TCS SP2 AOBS confocal laser microscope (CLSM) equipment (CCT Bahia Blanca CONICET). Photomicrographs were taken with a Mothic BA200 equipment fitted with the Motic Plus 2.0 photographic system. All measurements are given in µm and reported as the mean followed, in parentheses, by the standard deviation and range. The parasitic prevalence was calculated following Bush *et al.* (1997).

Results

Protoopalina nana n. sp. (Opalinidae: Protoopalininae)(Figures 1-2)

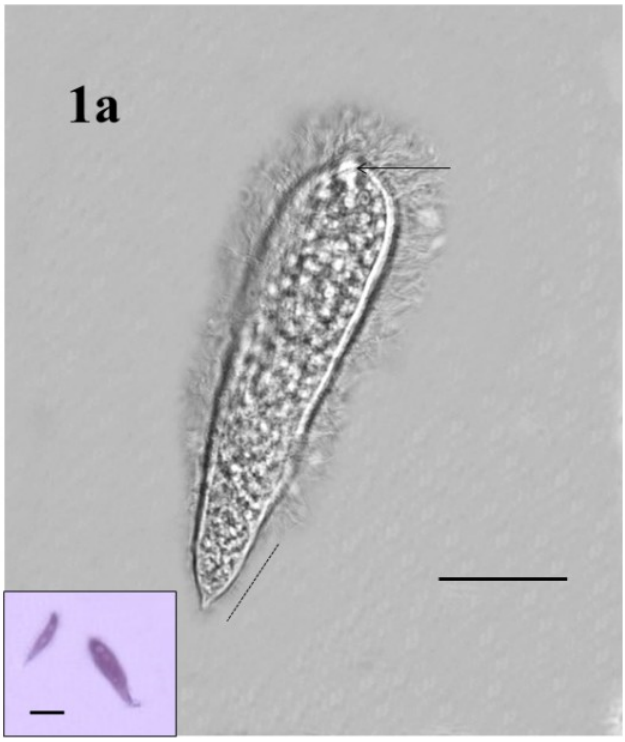
Location: rectum

Prevalence: 82.76% (autumn 2016), 100% (spring 2017)

Deposited specimens: Holotype (a marked slide) and paratype (a marked slide) deposited in the Collection of Invertebrates of Museo de La Plata, FCNyM-UNLP, with the Catallogue number MLP-Pr 102 and MLP-Pr 103, respectively.

Description (N= 30): Small flagellates with a cylindrical body, very elongated, covered with flagella 80-85%. Total length (L) 69.25 (4.79, 60.0-77.5) and maximum width (W) 7.0 (1.66, 5.0-10.0). Ratio L/W=9.89. Rounded frontal ends and pointed tail without flagella. *Falx* present little conspicuous in Giemsa-stained preparations (Fig.1). Flagella at the anterior end are longer and denser than those in the middle region. Kineties run slightly oblique to the longitudinal axis of the body and about 1.25 apart. The endoplasm is rich in spherules and granules of different sizes, more densely aggregated in the middle of the body. Two isometric nuclei of ellipsoidal contour, 7.0 (n=10) in diameter, in tandem on the widest region of the body. Several individuals with 4 and 5 nuclei of the same type and arrangement were observed (Fig. 2).

Remarks: The genus *Protoopalina* is the most common genus of the family Opalinidae Claus 1874 inhabiting amphibians (Li *et al.* 2014). It involves 79 species, mostly parasitic of anuran. Typically they present two nuclei with several nucleoli considered for a long time chromosomes in mitotic interphase (Chen 1948). However, in the same host, individuals with 4, 5 and up to 8 nuclei can be found as occurs in *P. pingi* Nie 1935 (n= 4) from *Rana plancyi* Lataste 1880 (China), *P. quadrinucleata* Metcalf 1923 (n= 4) from *Rana macrodon* Duméril & Bibron 1841 (Java), *P. axonucleata* Metcalf 1923 (n= 4, 6, 7 or 8) from *Bufo bufo asiaticus* (Stejneger 1907) (Korea) and in the present species (n= 2, rarely 4 o 5). *Protoopalina diplocarya* Metcalf 1923 was described from *Batrachyla leptopus* in Patagonia. It shows 2 monomorphic nuclei but is very large (217 µm in length). The dimensions of the specimens studied in *B. pulchella* are the smallest of most of the species described to date. Even the maximum recorded size is smaller than the lower sizes of most of their congeners. Likewise, a features that differentiates our specimens from the rest is the presence of a pointed posterior end totally devoid of cilia about 15 to 20% of the soma. For this reason,



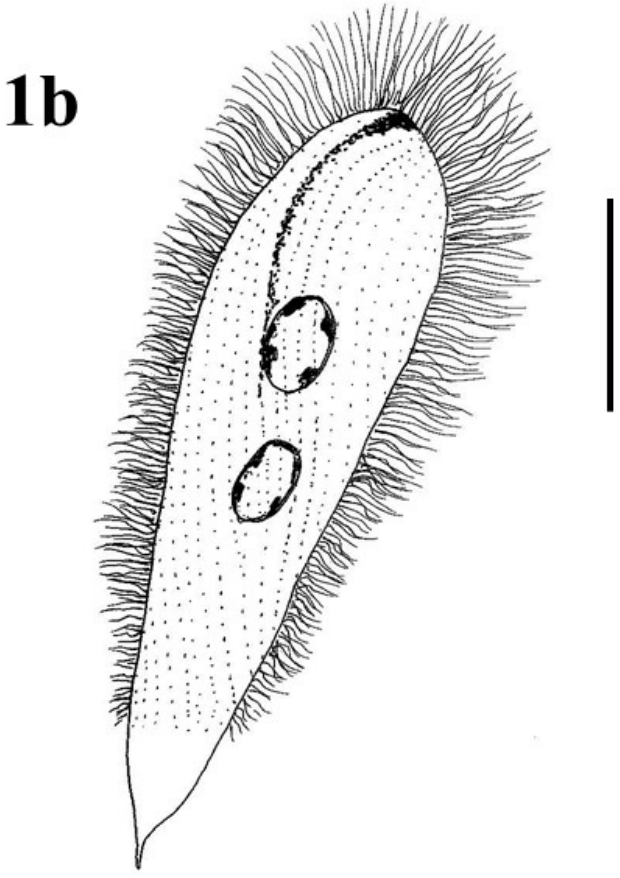


Figure 1. a. *Protoopalina nana* n. sp. *Falx* (arrow) and the caudal region without flagella (dotted line). Nomarski Interdifferential optical Microscopy. b. Schematic. Scale bar: 20 µm. Inbox: two binucleated individuals, Giemsa stain. Scale bar: 20 µm.

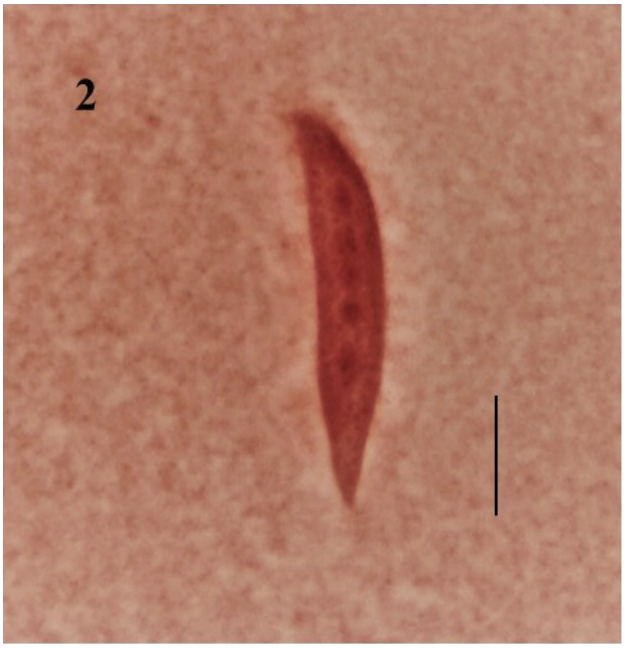


Figure 2. *Protoopalina nana* n. sp. with five monomorphic nuclei. Harris hematoxylin. Scale bar: 20 µm.

and because the taxonomic criteria of the consensus are eminently morphometric, it is proposed to consider the specimens as representatives of a new opalinid species and designate it as *Protoopalina nana* n. sp.

Zelleriella hylaxena Metcalf 1923 (Opalinidae: Protoopalininae)(Figure 3)
Location: rectum
Prevalence: 82.76% (autumn 2016), 100% (spring 2017)
Deposited specimens: Voucher (a slide) deposited in the Collection of Invertebrates of Museo de La Plata, FCNyM-UNLP, with the Catallogue number MLP-Pr 104.
Description (N= 7): Flattened and binucleated body with kineties run obliquely to the major axis. Ovoid or irregularly rounded contour with no defined frontal region. Body length 111.4 (14.6, 95-137.5) and maximum width 81 (12.6, 60-97.5). Two nuclei of slightly different circular contour, central and obliquely positioned with respect to the major axis of the soma. Nuclear diameter 16.07 (1.97, 15-20) independent of the total size of the opalinid; that is, large and small specimens were found uni or binucleated in which the nuclear dimensions remained in a constant range. Internuclear distance generally equivalent to a nuclear diameter or slightly greater (range 16-20). In nuclei at mitotic interphase, conspicuous accumulations of peripheral chromatin

can be seen, in numbers from 4 to 7. Uninucleated specimens were frequently found in which the nucleus becomes ellipsoidal (Fig. 3). Distance between flagellar rows approximately 1.2. Endoplasm densely loaded with granules and vacuoles of variable size.

Remarks: Metcalf (1923) described this opalinid in a unique *Boana pulchella* (cited as *Hyla pulchella*) specimen from the collection of the US National Museum that had been collected in Paraguay. The dimensions and body shape as well as the common host allow assigning the present specimens from *B. pulchella* to the species *Zelleriella hylaxena*. Metcalf (1923) recognized certain similarities between *Z. hylaxena* and some individuals of *Z. patagoniensis* Metcalf 1923, a parasite of *Pleuroderma bufonina* Bell 1843 (cited as *Paludicola bufonina*), collected in 1898 in the Strait of Magellan, Patagonia. However, both species can be differentiated by the structure of the nuclei as well as by the less pointed shape of the posterior end and the interflagellar distance (more than 2.0). Otamendi (1945) described in detail specimens of *Z. antillensis* (Metcalf 1914) in the cloaca of the common toad *Rhinella arenarum* from La Plata city. The measurements of his individuals are notably minor than those provided in the reference description of Metcalf (1923). Most of the Otamendi's specimens have a total length in the range of 120-130 and the nuclear diameter does not exceed 20, unlike *Z. antillensis* whose total length is over 180, and nuclei diameter 27.8. Given the overlapping habitat in Ventania hydrographic basin, further comparative studies are needed to rule out that the species parasitizing *B. pulchella* is the same that parasitizes *R. arenarum*. Other species described by Otamendi (1945) are *Z. brasiliensis* (Pinto, 1918) from the hindgut of *Leptodactylus latrans* and *R. arenarum* characterized by its pyriform shape and the presence of four macrochromosomes in the anaphasic nucleus, and *Z. carinii* Otamendi 1945, with circular shape and whose size exceedes 240 on average, grouped among the largest species in the genus. Cabagna-Zenklusen *et al.* (2009) identified as *Zelleriella* sp. small individuals (36-90 x 25-50) in the cloaca of tadpoles *Odontophrynus americanus* (Duméril & Bibron 1841) from Santa Fe and Entre Ríos provinces. Table I shows the *Zelleriella* species described in another Neotropical hosts. The present constitutes an expansion of the geographic range of *Z. hylaxena*.

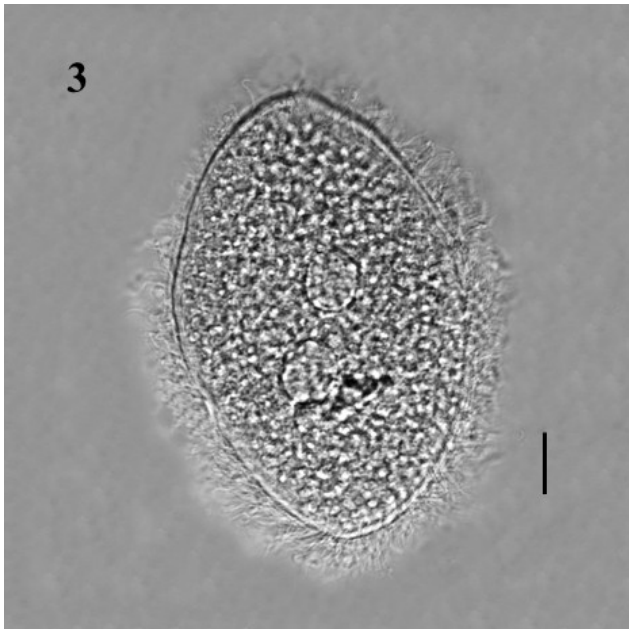


Figure 3. *Zelleriella hylaxena* Metcalf, 1923. Nomarski Interdifferential optical Microscopy. Scale bar: 20 µm.

Discussion

The family Opalinidae Claus 1874 includes flagellates that inhabit the hindgut or rectum of anuran amphibians, although few records are known in teleost fish and reptiles (Lom & Dyková 1992; Delvinquier & Patterson 1993; Grim & Clements 1996, Kostka 2016). Reptiles presumably acquired them by ingestion of an infected anuran (Kostka, 2016). They have the soma covered with flagella that resemble the cilia of the Ciliophora, but they lack citostoma, generally are bi or multinucleated with monomorphic nuclei, the subpelicular structure of microtubules arranged in parallel folds (that brings the "opalescent" aspect by which the group is named) and basal bodies of the undulipodia differ from ciliates (Kostka 2016). Peculiarities such as the nuclear structure (with chromatin condensed as macrochromosomes), mode of cell division (longitudinal inter-kinetial) and the biological cycle, motivated several authors to consider a separate paraflagellate phylum Opalinata Wenyon 1926 (Corliss, 1984). Molecular phylogeny demonstrated that opalinids belong to the lineage of Stramenopila or Heterokonts (Li *et al.* 2014). The meaning of their interaction with amphibians is controversial since some authors considered them endocomensals (Li *et al.* 2014), intestinal parasites (Bolek & Coggins 1998; Sulieman & Pengsakul 2015), symbionts (Schorr *et al.* 1990) and others simply enterozoic protozoa (Otamendi, 1945). They feed in a saprozoic way by endocitosis (Noirot-Timothee 1966, Li *et al.* 2017), or by an osmotrophic way (Li *et al.* 2014). To

Table I. Comparative morphometrical data between *Zelleriella* spp. from Neotropical hosts (all measurements in µm) (mean and range, in parentheses).

Species	*Zelleriella brasiliensis*	*Zelleriella brasiliensis*	*Zelleriella patagonensis*	*Zelleriella antillensis*	*Zelleriella carinii*	*Zelleriella hylaxena*	*Zelleriella hylaxena*
Hosts	*Leptodactylus latrans*	*L. latrans, Rhinella arenarum*	*Pleuroderma bufonina* Bell, 1843	*R. arenarum*	*R. arenarum*	*Hyla pulchella* (=*Boana pulchella*)	*Boana pulchella*
Location	La Plata (Arg.)	La Plata (Arg.)	Magellan Strait, Patagonia	La Plata (Arg.)	La Plata (Arg.)	Paraguay	Belisario stream, Ventania basin (Arg.)
References	Metcalf (1923)	Otamendi (1945)	Metcalf (1923)	Otamendi (1945)	Otamendi (1945)	Metcalf (1923)	Present study
Total length	130	105.4 (63-135)	152-170	122.9 (64-152)	243.7 (200-296)	111	111.4 (95-137.5)
Maximum width	82	64.9 (40.5-82)	91	81.2 (44-104)	185 (128-232)	65	81 (60-97.5)
Diameter of nuclei	15.2x10.9	14.2x11.1	15.6	16.5x15	20-32	15.2	16.07 (15-20)
Internuclear distance	Not stated	10	Not stated	Not stated	Not stated	Not stated	16-20
Interflagellar distance	1.9 to 2.7	Not stated	2.1 to 3.7	Not stated	Not stated	1.5-2.8	1.2

date five genera are known: *Opalina* Purkinje & Valentin, 1835, *Protoopalina*, *Cepedea*, Metcalf 1920, *Zelleriella* and *Protozelleriella* Delvinquier, Markus & Passmore 1991. Taxonomical criteria considered valid for the description of opalinids usually are based on morphology and morphometry, with species overlapping ranges or with data from a single individual (Li *et al.* 2014, Li *et al.* 2017). Molecular data are extremely sparse (Gentekaki & Chantangsi 2017). Opalinids exhibit a marked pleomorphism which makes difficult to accurately define a species, requiring detailed observation of a significant number of individuals rather than describing a single specimen. Analyzing the problem of the species in opalinids, with special reference to the genus *Protoopalina*, Sandon (1976) postulated that most of the supposed species in the group could be invalidated. Until new diagnostic methodologies are developed and validated, this scenario makes difficult to solve the taxonomy of the Opalinata. Recently, Gentekaki & Chantangsi (2017) have demonstrated the low degree of intra-morphotype genetic diversity, corresponding to that revealed by morphological features.

Finally, the present is the first report of the genus *Protoopalina* and of *Zelleriella hylaxena* in Argentina. This record is in addition to records of helminths such as *G. chabaudi*, Diplostomoidea gen. sp., *C. uruguayensis* and crustaceans like *Argulus ventanensis* for the same host in Argentina (Tanzola & Villegas Ojeda 2017, Villegas Ojeda & Tanzola 2019).

Acknowledgments

Authors are grateful to Lic. Edgardo Buzzi for his assistance with the Nomarski Interdifferential optical Microscopy (DIC) confocal laser microscope (CLSM) equipment (CCT Bahia Blanca- CONICET) and to an anonymous reviewer for their valuable criticism. This study was carried out with the aid of grant UNS-PGI 24/B238.

References

Bolek, M. G. & Coggins, J. R. 1998. Endoparasites of Cope's gray treefrog, *Hyla chrysoscelis* and western chorus frog, *Pseudacris t. triseriata*, from southeastern Wisconsin. **Journal of Helminthological Society of Washington**, 65: 212-218.

Bush, A. O., Lafferty, K. D., Lotz, J. M. & Shostak, A. W. 1997. Parasitology meets ecology on its own terms: Margolis *et al.* revisited. **Journal of Parasitology,** 83 (4): 575-583.

Cabagna Zenklusen, M. C., Lajmanovich, R. C., Peltzer, P. M., Attademo, A. M., Fiorenza Bianucci, G. S. & Bassó, A. 2009. Primeros registros de endoparásitos en cinco especies

de anfibios anuros del litoral argentino. **Cuadernos de Herpetología**, 23 (1): 33-40

Chen, T. T. 1948. Chromosomes in Opalinidae (Protozoa Ciliata) with special reference to their behavior, morphology individually, diploidy, haploidy, and association with nucleoli. **Journal of Morphology,** 83 281–359

Corliss, J. O. 1984. The Kingdoms Protista and its 45 Phyla. **BioSystems**, 33: 87-126.

Delvinquier, B. L. J. & Patterson, D. J. 1993. The opalines. In Kreier, J.P. & Baker, J.R. (Eds) Parasitic Protozoa. (2nd Edition). San Diego: Academic Press, 3, 247-325.

Galaviz-Silva, L. & Jiménez-Guzmán, F. 1986. *Zelleriella bayonai* n. Sp. and *Nyctotherus uscae* n. sp. (Protozoa) from *Leptodeira maculata* (Colubridae) of Guatemala, C.A. **Revista de Biología Tropical**, 34(2): 237-242

Gentekaki, E. & Chantangsi, Ch. 2017. Low level genetic diversity of Opalinid morphotypes from the digestive tract of *Hoplobatrachus rugulosus* (Batrachia, Amphibia) in Thailand. **Acta Protozoologica**, 56: 235-243.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. **Herpetologica,** 16: 183-190.

Grim, J. N. & Clements, K. D. 1996. Description of a new species of opalinid by light microscopy, SEM and TEM: *Protoopalina polykineta*, n. sp. from the intestines of the surgeonfish, *Acanthurus nigrofuscus*. **European Journal of Protistology**, 32: 81-89.

Kehr, A. I. & Williams, J. D. 1990. Larvas de anuros de la República Argentina. **Cuadernos de Herpetología**. Serie Monográfica 2.

Kostka, M. 2016. Opalinata. In: Archibald J. M. *et al.* (eds.). Handbook of the Protists. pp. 1-23. Springer International Publishing, Switzerland.

Li, C., Jin, X., Li, M., Wang, G., Zou, H., Li, W. & Wu, S. 2017. Light and transmission electron microscopy of *Cepedea longa* (Opalinidae) from *Fejervarya limnocharis*. **Parasite,** 24: 6.

Li, W., Wang, C., Huang, F., Li, M., Nilsen, F., Liu, H. & Xu, J. 2014. Redescription of *Protoopalina pingi* Nie, 1935 inhabiting the recta of *Hylarana guentheri* and *Pelophylax nigromaculatus* in China. **Parasite,** 21, 46.

Loker, E. S. & Hofkin, B. V. 2015. Parasitology: a conceptual approach. 560 pp. Garland Science, Taylor & Francis Group, New York.

Lom, J. & Dyková, I. 1992. Protozoan Parasites of Fishes. 315 pp. Elsevier, New York.

Metcalf, M. M. 1923. The opalinid ciliate infusorians. **Bulletin of the United States National Museum**, 120: 1–484.

Noirot-Timothee. C.1966. Presence simultanee de deux types de vesicules de micropinocytose chez *Cepedea dimidiata* (Protozoa, Opalinina). **Compte Rendus de l'Académie des Sciences**, 263: 1230- 1233.

Otamendi, J. C. 1945. Contribución al conocimiento de protozoarios enterozoicos de anfibios argentinos. Tesis del Museo de La Plata N°7. 71p.

Sandon, H. 1976. The species problem in the opalinids (Protozoa, Opalinata), with special reference to *Protoopalina*. **Transactions of the American Microscopical Society,** 95(3): 357-366.

Schorr, M. S., Altig, R. & Diehl, W. J. 1990. Populational changes of the enteric protozoans *Opalina* spp. and *Nyctotherus cordiformis* during the ontogeny of anuran tadpoles. **Journal of Protozoology**, 37(6): 479-481.

Sulieman, Y. & Pengsakul, T. 2015. Non-hemoparasitic protozoa of the subdesert toad, *Amietophrynus* (*Bufo*) *xeros* (Anura: Bufonidae). **International Journal of Fauna and Biological Studies**, 2(4): 89-92.

Received: September 2020
Accepted: December 2020
Published: December 2020