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CONTENTS

CAROL-CONSTANTIN PRUNESCU

(19th of December 1934, Turnu-Măgurele – 23th of September 2011, Bucharest)

He was born in Turnu-Măgurele, on 19th of December 1934. In 1951, CAROL-CONSTANTIN PRUNESCU graduated from "Sf. Haralambie" High-school in his native town, and after that he attended the Faculty of Biology of the University of Bucharest. He graduated from University with the State Exam (1955) and the Licence paper, "Parasitic insects to vertebrates", supervised by Professor Radu Codreanu.

Between 1955–1962, he carried on both scientific research and educational activities in different Research Stations (Timişul de Jos, Sinaia, Rucăr, Voila-Câmpina, Pantelimon) which belonged to the Faculty of Biology in Bucharest. In 1962, after a contest, he was appointed researcher within the Laboratory of Animal Morphology of "Traian Săvulescu" Institute of Biology of the Romanian Academy.

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In 1969, he presented at the University of Bucharest his PhD thesis "*Anatomia comparată şi evoluţia sistemului genital la Chilopode*" ["Comparative anatomy and the evolution of the genital system in chilopods"], supervised by Prof. Gh. Th. Dornescu.

Within the period 1962-2004 he continuously worked at the Institute of Biology Bucharest of the Romanian Academy, passing through all stages of scientific research. He was the director of the Institute of Biology (1990-1995), and during his directorship the Institute of Biology and the Institute of Biochemistry were reintegrated within the structure of the Romanian Academy. When he retired, he continued his scientific activity till the end of 2010.

Along his scientific activity, Dr. Carol-Constantin Prunescu approached numerous researching fields using the working methods specific to animal morphology research: microscopic anatomy, comparative anatomy, histology, ultrastructural studies.

In his very first years of activity, the main fields of interest were systematics, microscopic anatomy, issues of chilopod origin and evolution. In his PhD thesis, he published a series of papers dedicated to these items. Critical discussions of the data from specialized literature, corroborated with his own results in morphology of different representative types of the present orders of chilopods, led to the creation of the first phylogenetic tree of chilopods ("Contribution a l'étude de l'évolution des Chilopodes", Rev. Roum. Biol. – Zool., 10, 2: 89-102, 1965; "Considérations sur l'évolution de système génitale des Chilopodes", Bull. Mus. Natl. Hist. Nat. 2^{e} Série, T. 41, Suppl. No. 2: 108-111, 1969 (1970), Paris).

This phylogenetic tree was taken over by the international scientific community. It was cited and commented in: J.G.E. Lewis "The Biology of Centipedes", University Press, Cambridge, Oxford, New York, 1981, Chapter: "Relationships of the chilopod orders", pp. 418-424). Carol-Constantin Prunescu's studies continued and enriched along years with new remarks on some anatomical structures, interpreted on the basis of the ecological and ethological data, specific to different Chilopod orders ("Plesiomorphic and apomorphic characters states in the Class Chilopoda" in: Acta Myriapodologica**,** Geoffroy J.-J., Mauriès J.-P. & Nguyen Duy M (eds.), Mèm. Mus. Natn. Hist. Nat., 1996, 169: 299-306, Paris). Within last years, the old phylogenetic tree which presented a linear evolution was reanalyzed, and a well grounded and substantiated conclusion was reached, that is the chilopod evolution was simultaneous, in two distinct directions (Subclass Ovodispersa and Subclass Ovoconecta), starting from a common ancestor ("A new classification of the Class Chilopoda", paper presented at the 13th International Congress of Myriapodology, Bergen, Norway, 25th–29th of July 2005 and published as an abstract in Norw. J. Entomol., 53: 401-402, 2006; "Class Chilopoda: Evolution and Environment Adaptation" Rom. J. Biol. – Zool., 55 (2): 113-127,

2010). This paper was presented at the Anniversary Session of the Semicentenary of the Institute of Biology, Bucharest (29th of September – 1st of October 2010).

Dr. C.-C. Prunescu did not limit himself only to the study of the anatomical structures of the organisms within natural conditions. He was interested in the knowledge of the changes occurred in the organs and tissues of laboratory animals, submitted to some experimental models which reproduced human diseases (hemochromatosis, thalassemia, cirrhosis, alcoholic liver disease; bacterial infections (especially with *Mycobacterium*); parasites and lesions effects of some parasite species (*Plasmodium berghei, Dicrocoelium lanceatum (dendriticum), Aplectana acuminata, Schistosoma mansoni, Capillaria petruschewskii, Brandesia turgida, Soricimyxum fegati*); the lesional effects caused by the accumulation of some heavy metals (iron, lead) in the liver, kidney, lungs, testicles, or of different harmful substances (carbon tetrachloride, dimethylnitrosamina (DMN)) on the liver. He developed an original method to evaluate the effects of some non steroid anti-inflammatory drugs (AINS). He studied the pathogenesis of the liver fibrosis, degeneration of the liver sinusoids, liver capillarization, and the occurrence of liver cirrhosis in laboratory animals.

From the special studies on the iron loading experiments of the laboratory animals, it is worth mentioning papers such as: "Giant multinucleated iron-containing cells in the liver of polymaltosed iron injected rats" (Rev. Roum. Biochim., 25 (4**)**: 355-362, 1988); "Ferritin accumulation in nuclei of hepatocytes in polymaltosed iron injected mice" (Rev. Roum. Biochim., 28 (3-4**)**: 169-172, 1991); "Ultrastructure of fibrolamellar liver carcinoma occurred in an iron overloaded mouse", paper presented at the Annual Meeting European Iron Club (EIC), Louvain-La-Neuve, Belgium, 22-24, November 2001; "Bars bearing macrophages in the lungs of the iron overloading mice" (Clin. Lab. Haem, 17: 375, 1995).

Studies on the reactivity of the hamster liver submitted for long periods of time at noxious stimuli (iron, alcohol) led to the obtaining of some experimental patterns of human hemochromatosis, respectively, of human alcoholic liver disease (ALD): "The contribution of the iron overloaded hepatocytes to the synthesis and deposition of collagen *in vivo*", communication presented at EIC Meeting, Jaca, Spain, 11-14 September 1996; "The comparative study on the hepatic cirrhosis in the experimental iron loaded hamsters and the patients with primary and secondary haemochromatosis", communication presented at the International Symposium Iron in Biology and Medicine, Saint Malo, France, 16-20 June 1997; "Siderosomes and cellular lesions in alcoholic liver diseases (ALD)", communication presented at EIC Meeting, Vienna, Austria, 20-23 August 2003.

C.-Constantin Prunescu dedicated himself to some studies on the microscopic anatomy of the liver in different mammal species (cattle, deer, pigs, carnivores,

insectivores). He published papers which dealt with this field such as: C.-C. Prunescu, P. Prunescu, M. Krasińska, Z. Krasiński: "Liver histological structure in adult European bison *Bison bonasus* (Linnaeus, 1758)", Folia Morphol., 61 (3): 137-142, 2002; C-C. Prunescu, N. Şerban-Pârâu, J.H. Brock, D.M. Vaughan, P. Prunescu: "Liver and kidney structure and iron content in Romanian brown bears (*Ursus arctos)* before and after hibernation", Comparative Biochemistry and Physiology, Part A, 134: 21-26, 2003.

C.-C. Prunescu often demonstrated originality and competence in the achievement of some complex experiments, such as the demonstration with histological arguments of the biological cycle of the trematode *Dicrocoelium lanceatum (dendriticum),* a parasite worm which produces dicroceliosis in sheep. In this respect we mention the two papers signed by C.-C. Prunescu, P. Prunescu, V. Fromunda, D. Paraschivescu, S. Popescu: "Neurotropic stage of some cercariae of *Dicrocoelium lanceatum* in the intermediary host (*Helicella)* and complementary host (*Formica)*" and "Histopathological changes of the liver in hamsters in the experimental dicroceliosis", both papers published in Archiva Veterinaria, T. XIV, 1979, pg. 75-82, respectively, 83-89.

A remarkable scientific achievement was the first description of the myxosporidia *Soricimyxum fegati* (Myxozoa) in the liver of a terrestrial mammal: Prunescu C-C., P. Prunescu, Z. Pucek, J. Lom: "The first finding of myxosporean development from plasmodia to spores in terrestrial mammals: *Soricimyxum fegati* gen. et sp. n. (Myxozoa) from *Sorex araneus* (Soricomorpha)", Folia Parasitologica, 54: 159-164, 2007). This scientific novelty was presented and the 7th International Symposium dedicated to the parasites in fish (ISFP VII), Viterbo, Italy, 24-28 September 2007. The abstract of the paper was published by the same authors with the title: "Hepatic myxosporosis in a terrestrial mammal" in Parasitologia, 49, Suppl. 2: 166, 2007. *Soricimyxum fegati* is the first myxosporidia in which the myxosporean stage (development from plasmodia to spores) was observed in a terrestrial mammal. It was presumed that the entire development cycle (myxosporean and actinosporean stages) of this myxosporidian might be terrestrial, because it is known that *Sorex araneus*, the intermediary host, feeds exclusively on terrestrial organisms, including oligochaetes.

Within the last years, collaborating with Prof. R.R. Crichton and Prof. Roberta J. Ward, from the Biochemistry Unity of the University of Louvain La Neuve (Belgium), Carol-Constantin Prunescu remarked that the iron dextran, supplemented in cellular cultures of microglial cells, was found in these cells, as spicular iron, a rare depositing form, different from ferritin, not mentioned in the specialized literature, as yet (C.-C. Prunescu, P. Prunescu, P. Heushling, G. Dorrestein, F. Ledeque, R.R. Crichton, R.J. Ward: "Morphological and biochemical studies of microglia and alveolar macrophages iron loaded *in vivo* and

in vitro", First International Congress BioIron Soc., Prague, Czech Republic, 22-26 May 2005; C.-C. Prunescu, P. Prunescu, R.R. Crichton, R.J. Ward: "Spicular iron, a rare form of iron accumulation in living cells", paper presented at the 49th Annual Session of Scientific Communications of the Institute of Biology, Bucharest 2009 and in the "Report on the Annual Results" within the Romania-Belgium Francophone Inter-Governmental Cooperation (2004–2010). The purpose of this study was the understanding of the way of iron penetration in neurons, knowing that the oxidative stress generated by iron leads to the neurodegenerative disorders of the brain (Parkinson and Alzheimer Diseases).

As PhD guide, C.-C. Prunescu initiated training courses for his PhD students, in histological methods and the interpretation of the histological data. He was glad and proud when his students proved to be interested and passionate for their difficult and laborious work of histologists.

Carol-Constantin Prunescu was member of the International Society of Myriapodology (CIM) (Paris) since 1968, member of the European Iron Club (EIC) (London) since 1993, member of the International BioIron Society since 1997. He is the author of 88 scientific papers *in extenso*, 58 scientific papers published as abstracts, and co-worker to the drawing-up of three practical text books for the pupils of the sanitary high-schools or of those with biological profile (1978, 1981). The numerous results of his studies were the subject of many scientific communications presented in different symposia and international congresses.

In 1998, Carol-Constantin Prunescu got "Emanoil Teodorescu" Prize of the Romanian Academy, given for his papers on the evolution of the representatives of Chilopoda Class.

The approaching way, his studies and results reached along his entire career proved a great passion for knowledge and finding novelty, also supported by perseverance and hard work for achieving every goal of the experiments he made.

CAROL-CONSTANTIN PRUNESCU left us on 23rd of September 2011, discreet, as we knew him, leaving behind an indelible remembrance.

God rest his soul in peace!

PAULA PRUNESCU, PhD

A NEW *CYCLOSA* MENGE, 1866 (ARANEAE: ARANEIDAE) FROM INDIA

SOUVIK SEN[*], SUMANA SAHA[**], DINENDRA RAYCHAUDHURI[*]

Cyclosa bilobata n. sp. recorded from Mahananda Wildlife Sanctuary, West Bengal, India is described and illustrated.

Key words: Araneae, Araneidae, *Cyclosa bilobata* n. sp., West Bengal, India.

INTRODUCTION

Orb weavers (Araneidae) are globally represented by 3029 species under 168 genera (Platnick, 2012). These include 154 Indian species belonging to 29 genera (Sebastian & Peter, 2009). Fifteen out of the 175 species are known to compose the genus *Cyclosa* Menge 1866 of India (Simon, 1888, 1906; Thorell, 1881, 1892; Tikader, 1977, 1982; Biswas & Biswas, 1992, 2006; Gajbe, 2004; Saha & Raychaudhuri, 2004; Sen *et al*., 2009; Sebastian & Peter, 2009 and Platnick, 2012).

During our systematic survey (2007-2010) for the spiders of the eight reserve forests of Dooars and Darjeeling, West Bengal, India we came across with a *Cyclosa* species from Mahananda Wildlife Sanctuary. The species after critical examination is considered as new to science and accordingly described and illustrated.

The biotope. Mahananda Wildlife Sanctuary is situated in the terai region of Eastern Himalayas, on the west bank of river Teesta, lying between latitudes 26°55’33’’N & 26°47’54’’N and longitudes 88°33’31’’E & 88°23’36’’E. The notified area of the sanctuary is 129.04 sq. km.

Roughly 60% of the forest falls in the hilly region and remaining 40% in the plains. In the plains the forest subtypes include riverine to sal forests; khair, sissoo, simul, siris occur in the former while sal is the dominant species in the latter with admixture of lali, udal, bahera, asan, toon, etc. The lower hill forests extend to 800m and are of dry or wet mixed forests. Main species are panisaj, gamar, toon, dhobinut, choya bamboo, gokul, tejpat etc. Tree ferns and epiphytes are common.

Major fauna of the forest include gaur, leopard, tiger, elephant, spotted deer, sambar, common mongoose, wild pig, common hare, Assamese macaque, etc. A rich varied population of reptiles, birds, fishes and arthropods are found in the sanctuary (Anonymous 1996 a; Anonymous 1996 b; Anonymous, 2008).

MATERIAL AND METHODS

Orb weavers were collected and preserved following Tikader (1987) and Barrion & Litsinger (1995). The materials were studied using Stereo Zoom Binocular Microscope, model Zeiss SV-11. The measurements indicated in the text are in millimeters, made with an eye piece graticule. Leg measurements are shown as: total length (femur, patella, tibia, metatarsus, tarsus).

Abbreviations: CL = Cephalothorax length; CW = Cephalothorax width; AL = Abdominal length; AW = Abdominal width; TL = Total length; AME = Anterior median eyes; ALE = Anterior lateral eyes; PME = Posterior median eyes; PLE = Posterior lateral eyes; MWLS = Mahananda Wildlife Sanctuary; WB = West Bengal.

TAXONOMY

Family Araneidae Clerck, 1757
Subfamily Araneinae
Genus *Cyclosa* Menge 1866

Cyclosa bilobata n. sp.
(Figs. 1-9)

Type material

Holotype: ♀, Sukhna, MWLS, W.B., India, 13.X.2008, coll. S. Saha;
Paratype: 1 ♀, Sukhna, MWLS, W.B., India, 07.III.2009, coll. S. Sen.

Description

Female (Holotype):

CL-1.30, CW-1.17, AL-3.82, AW-1.91, TL-5.12.

Cephalothorax (Fig. 1) black, longer than wide, narrowing in front, posteriorly globose, cephalic region high, cervical furrows deeply distinct; thoracic region with 2 yellowish white, broad, lateral patch and pit like fovea. Eyes 8, pearly white, sub equal, laterals situated on moderately prominent tubercle, both rows recurved, ocular quad wider than long. Inter ocular distance: AME–AME = 0.17, ALE–AME = 0.13, ALE–ALE = 0.47, PME–PME = 0.06, PLE–PME = 0.17, PLE–PLE = 0.52, AME–PME = 0.08.

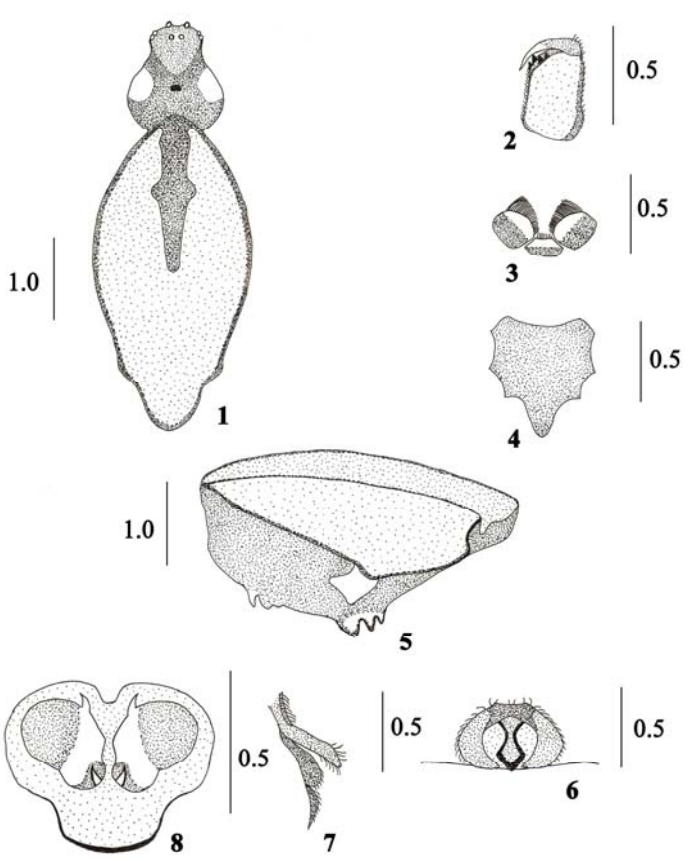
Clypeus black, height small.

Chelicerae (Fig. 2) yellowish brown, longer than wide, each margin with 2 teeth, fangs brown, strongly curved. Both maxillae and labium (Fig. 3) black with apices paler, scopulate, maxillae little wider than long, apically truncate, medially broad, labium wider than long.

Sternum (Fig. 4) black, cordate, anterior margin concave, posteriorly narrowed and acutely produced. Legs moderately long, clothed with hairs, segments with distinct transverse, brown bands, tarsal claw 3, toothed. Leg measurements: I 2.85 (0.53, 0.30, 0.93, 0.73, 0.33); II 3.02 (0.80, 0.40, 0.86, 0.66,

0.30); III 1.82 (0.50, 0.26, 0.53, 0.40, 0.13); IV 2.65 (0.60, 0.53, 0.66, 0.60, 0.26). Leg formula 2143.

Abdominal (Figs. 1-5) dorsum silvery, elongate, with 1 blunt, caudal hump and two lateral humps posteriorly, a midlongitudinal basal black band extending little beyond the middle, margins entirely black; venter blackish, with two narrow, lateral silvery bands extending from base to apex, spinnerets black, small, located medially.



Figs. 1–8. *Cyclosa bilobata* n. sp., Female (Holotype): 1. Cephalothorax and abdomen (dorsal view); 2. Chelicerae (ventral view); 3. Maxillae and labium (ventral view); 4. Sternum (ventral view); 5. Abdomen (lateral view); 6. Epigynum (ventral view); 7. Epigynum (lateral view); 8. Internal genitalia (dorsal view).

Epigynum – Internal genitalia (Figs. 6-8): Scape with a median constriction; spermatheca large, bilobed, atrium narrow, copulatory ducts short, copulatory openings close, fertilization ducts distinct.

Fig. 9. Habitus of *Cyclosa bilobata* n. sp. (female).

Distribution. India: West Bengal (known only from the type locality).

Etymology. The species name is derived from bilobed spermatheca.

Type deposition. Entomology Laboratory, Department of Zoology, University of Calcutta, registration no. EZC 0026 -12.

Remarks. The species shows a close affinity to *Cyclosa confraga* (Thorell, 1892), but can be separated by: i) medially constricted epigynal scape (scape absent but with a broad bulged structure in *C. confraga*); ii) large, bilobed spermatheca, narrow atrium, short copulatory ducts, closely placed copulatory openings (spermatheca never bilobed, small, circular, atrium wide, copulatory ducts long, with openings widely placed in *C. confraga*); iii) thoracic region with two yellowish white, broad, lateral bands (thoracic region devoid of any such band in *C. confraga*); iv) abdomen with a midlongitudinal, basal, black band (abdomen

without any band in *C. confraga*); v) each cheliceral margin with two teeth (each cheliceral margin with three teeth in *C. confraga*).

The species is therefore recognized as new to science.

Acknowledgements. We thank Department of Biotechnology, Govt. of India (BT/PR6391/NDB/51/078/ 2005 dated. 20.11.2006) for sponsoring the project and Directorate of Forests, Govt. of West Bengal and the Head, Department of Zoology, University of Calcutta for necessary support.

REFERENCES

ANONYMOUS, 1996 a, Wildlife conservation in West Bengal a decade at a glance. *Wild life Wing, Directorate of Forest, GOWB*, 55 pp.

ANONYMOUS, 1996 b, Survey of Flora and Fauna of Mahananda Wildlife Sanctuary. *Wild life Wing, Directorate of Forest, GOWB & National Environmet & Wildlife Society*, 34 pp.

ANONYMOUS, 2008, *West Bengal Forest Department.* Available at: http://www.westbengalforest.gov.in. (accessed on 12th January, 2012).

BARRION A.T., LITSINGER J. A., 1995, *Riceland spiders of South and Southeast Asia.* CAB International, UK & IRRI, Philippines, 716 pp.

BISWAS B.K., BISWAS K., 1992, *State Fauna Series 3: Fauna of West Bengal, Araneae: Spiders*, *part 3*. Zoological Survey of India: 357-500.

BISWAS B., BISWAS K., 2006, *Araneae: Spiders*. In: *Fauna of Arunachal Pradesh*, State Fauna Series. Zoological Survey of India, **13** (2): 491-518.

GAJBE P., 2004, *Spiders of Jabalpur, Madhya Pradesh (Arachnida: Araneae)*. Records of Zoological Survey India, Occasional Paper, **227**, 154 pp.

PLATNICK N.I., 2012, *The World Spider Catalog, version 12.5*. American Museum of Natural History. Available at: http://research.amnh.org/entomology/spiders/catalog/index.html. (accessed on 12th January, 2012).

SAHA S., RAYCHAUDHURI D., 2004, *A survey of spiders (Araneae: Araneidae) of Jaldapara Wildlife Sanctuary, West Bengal, with description of a new Zilla species*. Entomon, **29**: 245-252.

SEBASTIAN P.A., PETER K.V., 2009, *Spiders of India*. Universities Press (India) Pvt. Ltd., 614 pp.

SEN S., SAHA S., RAYCHAUDHURI D., 2009, *Typical Orb Weavers (Araneidae: Araneae) of Mahananda Wildlife Sanctuary, West Bengal*. Insect Environment, **15** (1): 30-31.

SIMON E., 1888, *Etudes sur le arachnides de l'Asie méridionale faisant partie des collections de l'Indian Museum (Calcutta). II. Arachnides recueillis aux îles Andaman par M.R.D. Oldham.* Journal of Asiatic Society Bengal, **57**: 282-287.

SIMON E., 1906, *Arachnides* (2e partie). In: *Voyage de M. Maurice Maindron dans l'Inde méridionale. 8e Mémoire*. Annals of Entomological Society of France, **75**: 279-314.

THORELL T., 1881, *Studi sui Ragni Malesi e Papuani. III. Ragni dell'Austro Malesia e del Capo York, conservati nel Museo civico di storia naturale di Genova*. Annali del Museo Civico di Storia Naturale di Genova, **17**, 727 pp.

THORELL T., 1892, *Novae species aranearum a Cel. Th. Workman in ins. Singapore collectae.* Bollettino della Società Entomologica Italiana, **24**: 209-252.

TIKADER B.K., 1977, *Studies on spider fauna of Andaman and Nicobar Islands, Indian Ocean*. Records of Zoological Survey of India, **72**: 153-212.

TIKADER B.K., 1982, *Family Araneidae (=Argiopidae), typical orbweavers: Fauna of India (Araneae)*. Zoological Survey of India, **2**: 1-293.

TIKADER B.K., 1987, *Hand book of Indian spiders*. Zoological Survey of India, 251 pp.

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DISTRIBUTION, ADULT POPULATION SIZE AND CONSERVATION ISSUES OF SOME MOOR FROG (*RANA ARVALIS*) AND COMMON FROG (*R. TEMPORARIA*) POPULATIONS IN THE GHEORGHENI BASIN, ROMANIA

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There are very few published data about amphibian population sizes in Romania, although these are essential for conservation. Our study aimed at filling this gap for the central-lower altitude parts of the Gheorgheni Basin (Eastern Carpathians) for two species, the moor frog (*Rana arvalis*) and the common frog (*Rana temporaria*). We made egg clump counts in 106 ponds on five sites and identified the largest known moor frog populations known so far in Romania. We counted close to 5000 moor frog and over 7000 common frog (*R. temporaria*) egg clumps. Maximum moor frog egg clump number in a pond was more than 1400. The total surface of the studied ponds is over 30 hectares representing a crucial factor for the maintenance of large amphibian populations. The most important threat to both frog species in the area is breeding habitat destruction through the filling of ponds with sawdust. The effect of filling is stronger for the moor frog. In the case of the common frog, collecting adults for frog legs during the breeding season and the regulation of streams are important threats. Urgent measures are needed to protect the breeding habitats, restore degraded or destroyed breeding habitats, and stop illegal frog-leg collection.

Key words: Common frog, egg clump counts, moor frog, periglacial ponds, pond filling.

INTRODUCTION

The moor frog is one of the rarest frog species of Romania, and Transylvania is its global southern distribution limit (Fuhn, 1960; Cogălniceanu *et al*., 2000). Although its status according to the IUCN Red List is Least Concern (Kuzmin *et al.*, 2009 b), in Romania it is considered endangered (Iftime, 2005) and critically endangered (Cogălniceanu & Venczel 1993; Sas *et al*., 2008 a, b). According to the national nature conservation law, it is a species of community interest that needs strict protection (Annex 4A, Ministerial Order 57/2007). However, practical measures for the conservation of this species in Romania are lacking. The common frog is also listed as Least Concern in the IUCN Red List (Kuzmin *et al*., 2009 a). In Romania it is listed in both Annex 4A and 4B of Ministerial Order 57/2007 (their collection requires the elaboration of management plans). This species is collected all over the Romanian Carpathians illegally – no permits are requested from the Environment Protection Agencies. Forestries and NGO-s fight against this

locally, but there is no national strategy for the management and conservation of this species.

Data about the size of populations are available only from the South-Eastern Carpathians and the North-Western Plain (Mara & Demeter, 2005; Demeter & Mara, 2006; Sas *et al.*, 2006; Demeter & Benkő, 2008; Sas *et al*., 2008 a). Iftime (2005) estimates a population size of 20.000 individuals for the whole country, however, the precise distribution and population sizes of this species are not known well enough at the country level, except that it has a fragmented distribution (Sas *et al.*, 2008).

The importance of the mountain basins of the Eastern Carpathians for the moor frog has been known for decades (Csata & Csata 1997; Fuhn, 1960). The largest moor frog population in Romania to date is known from the Ciuc Basin, with egg clump counts indicating the presence of between 800-1400 adult breeding females (Demeter & Benkő, 2006; Demeter, pers. obs. between 2005-2010).

In the Gheorgheni Basin, Eastern Carpathians, within the brown frog group Ghira *et al.* (2002) recorded *Rana temporaria* and *R. dalmatina* most frequently, while *R. arvalis* was recorded only in a few localities. This pattern is probably due to confusion between the three species (Demeter *et al*., 2006; Sas *et al.,* 2008 b; Gál, pers. com.). No population size estimates for this area are available on any amphibian species before the present study.

In the previous years we discovered important breeding sites in the Gheorgheni Basin. In this study we addressed the following questions:

1. What is the distribution and size of moor frog and common frog populations in the breeding sites near Joseni, Ditrau and Remetea villages?

2. How does pond size affect the number of egg clumps?

3. What is the effect of pond filling on the distribution and size of the populations of the two species?

MATERIAL AND METHODS

The study area

The Gheorgheni Basin (Fig. 1) is the northernmost of a chain of three tectonic mountain basins in the southern part of the Eastern Carpathians, having the highest altitude of the three (over 700 m a.s.l.). The climate of the basin is cold (mean annual temperature below 4 ^{o}C), thermal inversions are frequent in winter. There is a dense hydrographic network and wetlands are abundant, although a lot of the wetlands were damaged through drainage and river regulation works in the past four decades. In the 1990s huge deforestations happened in the mountains, and the wood was processed at a large number of private sawmills. The generated sawdust was treated as waste at that time, and it was often used to fill up ponds and other wetlands, to gain land for hay production or constructions.

We surveyed five sites with a high pond density located within three localities: Joseni, Ditrau and Remetea villages (Fig. 1). We define a pond site as a group of ponds on a geographically homogeneous area where the distance between nearest neighbours is generally less than 500 m.

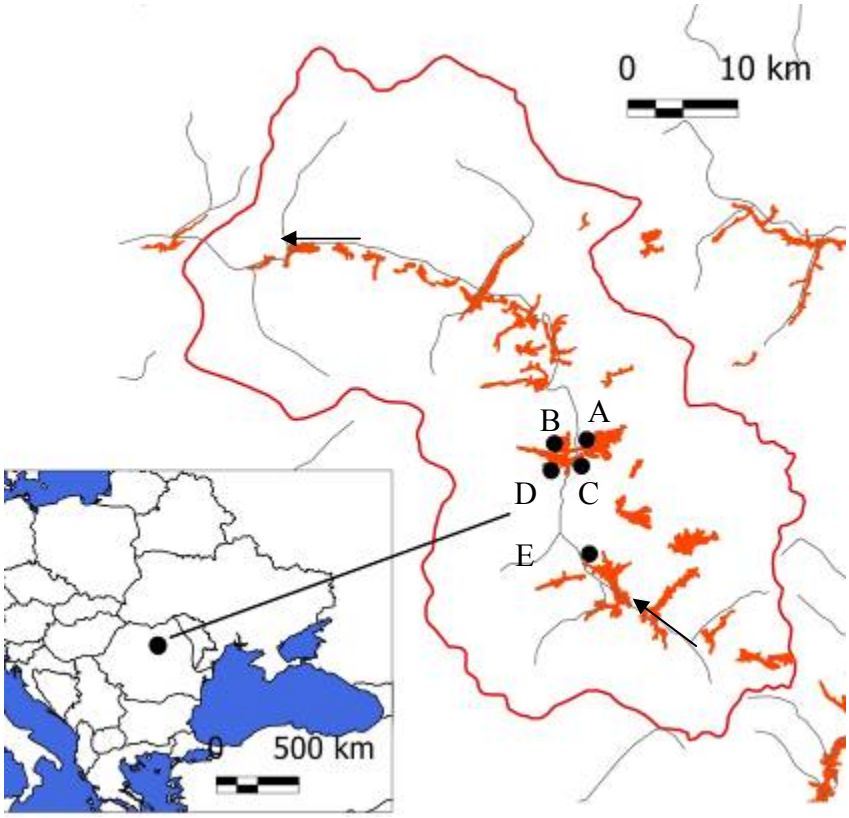

Fig. 1. The location of the study area in Eastern Europe and the location of the study sites (A-E) in the Gheorgheni Basin (closed line), showing hydrography (open lines) and settlements (areas). Arrows show direction of river flow.

Site A (25.4811°E, 46.8091°N, 709-738 m above sea level) is situated between Ditrau village, the railway and the Ditrau stream; we surveyed 53 ponds on this site. Site B (25.4424 °E, 46.8058 °N, 716-731 m asl) is situated on the western side of Remetea village; we surveyed 34 ponds here. Site C (25.4745 °E, 46.7872 °N, 711-717 m) is situated between the southeastern part of Remetea village and the railway; ten ponds were surveyed here. Site D (25.4377 °E, 46.7840 °N, 723-739 m) is situated southwest from Remetea village, five ponds and some streamside wetlands were surveyed here. Site E (25.4821 °E, 46.7155 °N, 727-

733 m) is situated north from Joseni village. Four isolated ponds were surveyed here (Fig. 1).

Table 1

Descriptive data of pond distance and size of the five studied sites

Sites (number of surveyed ponds)	Average distance between neighbours (min-max) (m)	Mean pond surface (min-max) (m^2)	Total pond surface (m^2)
A (53)	122.1 (45-504)	2445.3 (97-23056)	129602
B (34)	116.5 (56-500)	2134.9 (476-7998)	72586
C (10)	176.7 (87-242)	7158.8 (924-15889)	71558
D (5)	195.3 (38-340)	1723.8 (759-4680)	8619
E (4)	552.7 (321-756)	9299 (4333-15498)	37196
Total	–	**2986.8**	**319591**

We coded the degree to which a pond is filled up by sawdust on a 0-3 scale, where 0 represents ponds where no filling was observed, 1 represents ponds where a small amount was deposited, 2 represents ponds that have a large quantity of sawdust, and 3 represents ponds that are totally filled up.

Mapping of habitats and egg clump counts

We identified potential frog breeding habitats during field surveys and using aerial photographs from 2005 used by the Agricultural Payment Agency (APIA). This way we mapped more than 150 ponds, and we visited 105 in the first part of April 2010 (8, 11 and 17 April). One visit was made to each pond. The surveyed ponds are situated on alluvial fans on an undulating surface that indicates past landslides. Similar ponds in the Ciuc Basin were dated to between 8000 and 13000 years, and are hypothesized to have been formed in the early Holocene or late Pleistocene (Demeter *et al*., unpublished). Because of their specific location, we call them alluvial fan-ponds, and for their probable origin, we call them periglacial ponds.

We counted egg clumps of the common frog and the moor frog. Egg clump counts are frequently used in monitoring brown frog populations, since each female lays one clump, but the clumps of the species are often difficult to tell apart (Glandt, 2008). We have experience in monitoring egg clumps of the two species in the Ciuc Basin where they are also syntopic (Demeter & Benkő, 2008). We distinguish egg clumps of the two species based on: (1) the time of laying (common frog egg clumps are laid 1-3 weeks earlier, therefore the larvae are in a more advanced development stage); (2) size (egg clumps of moor frogs are generally smaller); (3) spatial arrangement of the egg clumps (moor frog egg clumps are more loosely laid, while common frog egg clumps are packed closely together) (Figs. 2-3).

During our first visit (8 April) the breeding of the common frog was over, and we observed calling moor frog males in several habitats. On the second surveying date (11 April) we did not observe breeding activity of any of the species. On the third surveying date (17 April) common frog larvae were already in an advanced phase of development, and one single moor frog male was observed, so we assume that the breeding of the moor frog was over too.

Fig. 2. Typical moor frog egg clumps. Egg clumps are smaller and more loosely laid than those of the common frog (Photo taken on 11 April 2010, pond nr. 28, site A).

Data analysis

As the data do not follow normal distribution, we used non-parametric tests of correlation and comparison. Correlation coefficients were calculated between the egg clump numbers per pond of the two species and between each species and pond surface area. The Mann-Whitney U-test was used to compare surface area and degree to which ponds are filled in ponds with or without each of the two species. Skewness was calculated for egg clump distribution per pond as a measure of the asymmetry of the distribution. Kurtosis was calculated as a measure of concentration of the distribution in a given range.

Fig. 3. Typical common frog egg clumps with a dead female on the bottom of the picture. Egg clumps are closer together than those of the moor frog (Photo taken on 8 April 2010, pond nr. 100, site E).

RESULTS

We estimated in total 7157 common frog and 4911 moor frog egg clumps. There were two sites (A, B) with over 1000 egg clumps in the case of both species. On sites B and E, the number of common frog egg clumps is 4-5 times the number of moor frog egg clump. On site A the number of common frog egg clumps is 30% larger than moor frog, and on site C the number of moor frog egg clumps is double than that of the common frog. Mean common frog egg clump counts per pond vary between 40 and 140 while mean moor frog egg clump counts have a larger variation towards the lower end of the scale, between 10 and 140 (Table 2).

Table 2

Summary data of egg clump counts and degree to which ponds are filled (column Filled, see Methods) in the studied sites

Common frog				Moor frog				
Site	**Total**	**Mean**	**Skewness/ Kurtosis**	**Total**	**Mean**	**Skewness/ Kurtosis**	**Filled**	**N**
A	4216	76.7	2.62 / 7.6	3244	59.0	6.82 / 48.83	0.7	55
B	1465	45.8	2.97 / 11.00	338	10.6	3.30 / 10.41	1.3	32

Table 2

(continued)

C	666	74.0	1.66 / 2.12	1279	142.1	2.18 / 4.72	0.8	9
D	275	55.0	2.23 / 4.97	50	10.0	0.87 / 0.11	1.5	5
E	535	133.8	2.00 / 4.00	121	30.3	1.97 / 3.90	2.1	4
Total	**7157**	**65.8**	–	**4911**	**44.9**	–	**1**	**105**

The number of ponds with no egg clumps is larger for the moor frog (38) than for the common frog (27). As a whole, there is a significant positive correlation between common frog and moor frog egg clump numbers ($r = 0.5$, $p < 0.0001$, $n = 105$) and between breeding pond surface and egg clump numbers of both species ($r = 0.61$, $p < 0.001$ for moor frog and $r = 0.4$, $p < 0.001$ for common frog, $n = 105$).

Mean common frog egg clump numbers per pond are larger (Table 2), but maximum value is much larger for the moor frog (1433 *vs*. 640). Egg clump counts and pond area are right skewed in all cases, most pronounced in the case of moor frog. The kurtosis of the moor frog egg clump counts is very high compared to that of common frog egg clump counts and pond area (Table 1). This implies that large moor frog egg clump counts are concentrated in fewer ponds than those of the common frog. This pattern can be observed in Figs. 2-3 as well.

We counted 3244 moor frog egg clumps on site A, of which almost half (1433) were in a single pond. The number of common frog egg clumps on this site was 4216. Pond filling happened in this area. Several ponds were filled in completely in the past two decades (Table 2). Common frog leg collection was observed in three ponds, a total number of 260 killed individuals were found on 8 and 11 April.

On site B average surface of ponds is similar to site A. However, common frog egg clump count is a little less than half the number of site A (1465) and moor frog egg clump count is just over one tenth of that (338). Half of the ponds on site B are largely or completely filled in with sawdust (Table 2).

On site C moor frog egg clump count was 1279 and common frog egg clump count was 666. Large quantities of sawdust are deposited on this site (Table 2).

On site D common frog egg clump count was 275, most of that number was found in one pond. Moor frog egg clump count was 50. Egg clumps of this species were recorded in shallow pools on a nearby wet meadow. A large amount of sawdust is deposited in the ponds of this site (Table 2).

One of the ponds on site E had a large number of common frog egg clumps (535) and fewer Moor frog egg clumps (121). The other ponds were strongly damaged or totally destroyed by filling with soil.

Fig. 4. The relative number of common frog egg clump counts (A) and moor frog egg clump counts (B) on site A. The relative abundance is indicated by the shades, darker shades represent more egg clumps). Arrows show hypothesized movement of frogs in spring. Crosses show frog killing. Arrows show direction of flow and probable spring migration directions of the common frog.

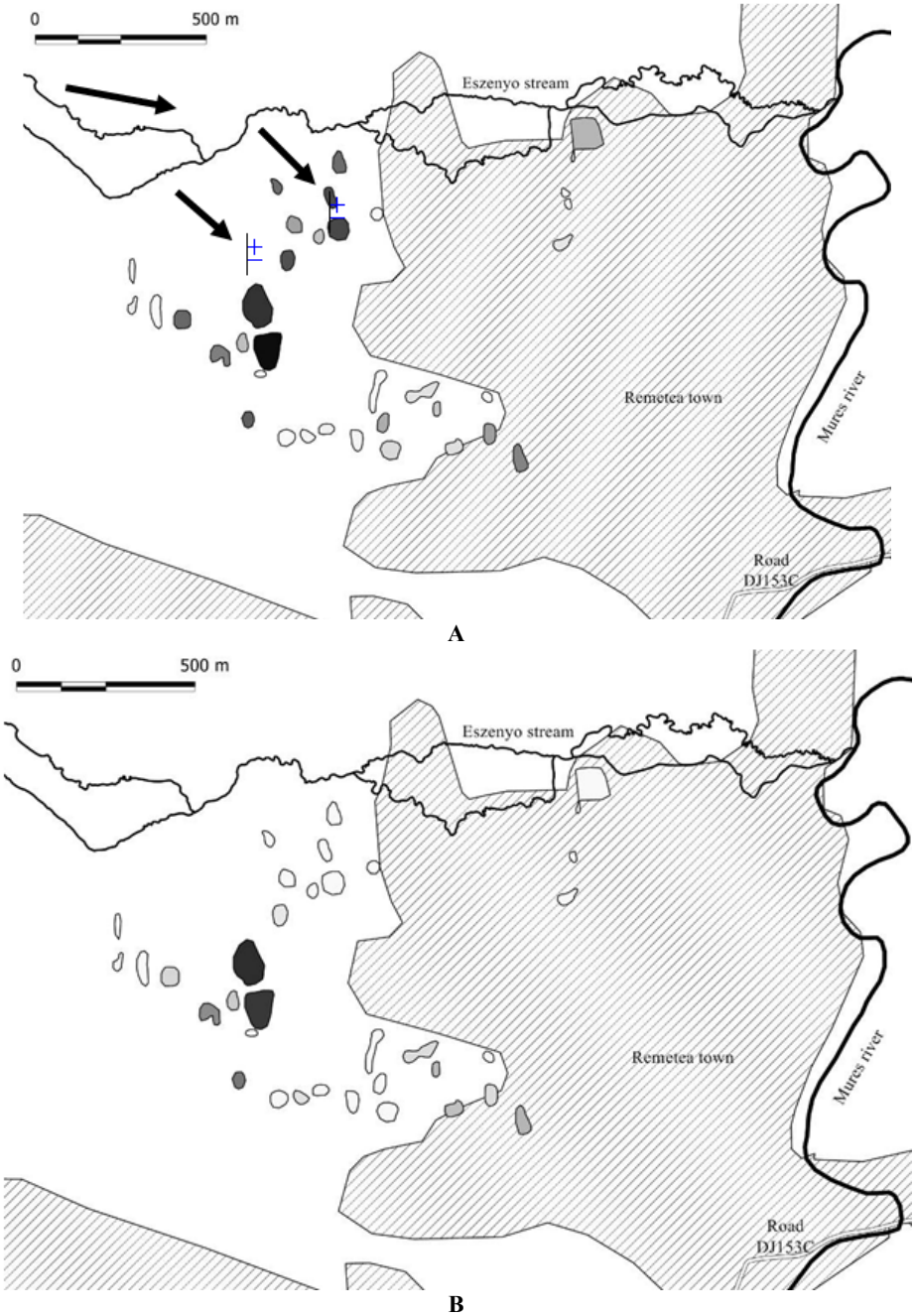

Fig. 5. The relative number of common frog egg clumps (A) and moor frog egg clumps (B) on site B. Arrows show direction of flow and probable spring migration directions of the common frog.

A

B

Fig. 6. The relative number of common frog egg clumps (A) and moor frog egg clumps (B) on site C.

DISCUSSION

Our data show that there are still large populations of common frog and moor frog in the studied areas of the Gheorgheni Basin. The estimated female adult moor frog population is the highest recorded in Romania so far. The recorded population size is strongly correlated in the case of both species with the availability and abundance of breeding habitats. The surroundings of Ditrau and Remetea village have a very high abundance of ponds of probable periglacial origin, associated with alluvial fans.

We surveyed about 60-70% of the available breeding habitats in the surroundings of Ditrau and Remetea village. Most of the breeding habitats that were not surveyed are found in the southern part of Ditrau village. This implies that the real size of the adult populations is higher than our estimations.

If we consider the frogs breeding on one site as one population, the one identified on site A is by far the largest moor frog population known so far in Romania. The second largest moor frog population known previously is in the Ciuc Basin with 1400 egg clump counts on one site, near Delnita village (Demeter & Benkő, 2007). On the site identified now, in one single pond 1433 moor frog egg clumps were counted. Site D, with an egg clump count of 1314, is also a very high number on the national level.

The distribution of moor frog egg clump counts has a very high kurtosis on site A (47.4% of egg clumps are found in one pond) and D (75.8% of egg clumps in two ponds). This also implies that these moor frog populations, although numerous, are very vulnerable to habitat destruction. The common frog egg clumps are more evenly distributed in the available breeding habitats.

Sites B, C and E show a high level of degradation, the cause of which is primarily filling with sawdust, and to a smaller extent, drainage or filling with other material. Our data show that the filling of habitats affected the moor frog to a higher degree than the common frog, possibly connected with a higher habitat specialization of the former species. No data are available about the population size before the filling, but based on the strong correlation between pond size and egg clump number we estimate that common frog population declined by 40% and the moor frog population declined by 80% in the studied sites.

Frog leg collection is an old practice in the area, but apparently it affects only the common frog. Killed frogs were observed on site A and B at six ponds. Although this frog is protected too, frog leg collection continues in Romania because of the lack of control by the authorities. In this case, it would be enough to guard 3-4 ponds for a week to remove this threat.

There is some debate on the national total population size and conservation status of the moor frog. Iftime (2005) estimated the national population size to 20000 individuals. Our data indicate that only on the studied area in the Gheorgheni Basin there are at least 8000 adults (assuming a 1:1 male to female sex ratio). Sas *et al.* (2007) found a sex ratio between 0.4:1 and 1.7:1, and as a whole sex ratio was 0.8:1, explained by higher female survival rate due to a more permanent habitat. If we include sub-adult individuals, the size of the whole population can easily reach 20000 in this site alone. At this point it is difficult to make estimations for the whole Gheorgheni Basin. As a comparison, in the Ciuc Basin, we estimate moor frog population size to about 4000 adults (Demeter unpublished). There are no population size data from the Braşov Basin, but based on habitat availability, population sizes may be similar to those in the Gheorgheni Basin.

The distribution of the egg clumps of the two species between ponds has different patterns. The moor frog has a higher kurtosis (large numbers detected in fewer ponds) than that of common frog. This is best observed on site A. The southeastern corner of this site is more used by the moor frog, while the northern part that is closer to the stream is used more by the common frog. We speculate that this can be explained by the difference in hibernation and spring movement of the two species. The common frog hibernates in streams and springs and a large percentage of the population arrives at the site through the stream (in this case, the Ditrau stream). Their movement is therefore from the stream towards the south, and many individuals stop to breed in the first ponds that they encounter. By contrast, the moor frog probably hibernates on the site, underground and/or in deep, boggy ponds, probably a large percentage of the population hibernates in the large bog-pond in the central-western part of the site.

Our data indicate that the distribution of the moor frog is highly fragmented in the studied area case too, although large populations are still present. Also, habitat destruction leads to a rapid decline of populations. A large percentage of the whole population is concentrated in a few breeding habitats. These habitats may play an important role in the terrestrial phase and hibernation of this species. Therefore it is crucial that they are strictly protected.

On the other hand, habitat restoration should be done in the case of at least some ponds. Sawdust can be relatively easily removed from the habitats. Special care should be taken when restoring such habitats not to make them too deep, because our observations suggest that deep ponds that have no emergent vegetation are not preferred by this species.

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REFERENCES

COGĂLNICEANU D., VENCZEL M., 1993, *Consideraţii privind ocrotirea şi conservarea populaţiilor de amfibieni şi reptile*. Ocrotirea Naturii şi a Mediului Înconjurător, **37** (2): 109-114.

COGĂLNICEANU D., AIOANEI F., BOGDAN M., 2000, *Amfibienii din România. Determinator*. Editura Ars Docendi, Bucureşti, 100 pp.

CSATA E., CSATA Z., 1997, *Răspândirea amfibienilor din partea centrală şi estică a Depresiunii Braşov*. Acta, **1**: 49-60.

DEMETER L., BENKŐ Z., 2008, *Note on a large moor frog population in Romania*. Zeitschrift für Feldherpetologie, Supplement, **13**: 355-358.

DEMETER L., HARTEL T., COGALNICEANU D., 2006, *Distribution and conservation status of amphibians in the Ciuc basin, Eastern Carpathians, Romania*. Zeitschrift für Feldherpetologie, Supplement, **10**: 217-224.

DEMETER L., MARA GY., 2006, *A mocsári béka (Rana arvalis) elterjedése és populációmérete a Csíki-medencében*. A Csíki Székely Múzeum Évkönyve, 2005: 439–450.

GLANDT D., 2008, *Methods of observation and population census of moor frogs (Rana arvalis) as a basis of conservation measures*. Zeitschrift für Feldherpetologie, **13**: 431-442.

FUHN I.-E., 1960, *Fauna R.P.R., Vol. XIV, Fascicula 1, Amphibia*. Editura Academiei R.P.R., Bucureşti.

GHIRA I., VENCZEL M., COVACIU-MARCOV S.-D., MARA GY., GHILE P., HARTEL T., TÖRÖK ZS., FARKAS L., RÁCZ T., FARKAS Z., BRAD T., 2002, *Mapping of Transylvanian herpetofauna*. Nymphaea, Folia Naturae Bihariae, **29**: 145–202.

IFTIME A., 2005, *Rana arvalis* Nilsson, 1842. In: BOTNARIUC N., TATOLE V. (Eds.), *Cartea Roşie a Vertebratelor din România.* Editura Academiei Române, Bucureşti, 260 pp.

KUZMIN S., ISHCHENKO V., TUNIYEV B., BEEBEE T., ANDREONE F., NYSTRÖM P., ANTHONY B., SCHMIDT B., OGRODOWCZYK A., OGIELSKA M., BOSCH J., MIAUD C., JON LOMAN, LOMAN J., COGALNICEANU D., KOVÁCS T., KISS I., 2009 a, *Rana temporaria*. In: IUCN 2011. IUCN Red List of Threatened Species. Version 2011.2. <www.iucnredlist.org>. Downloaded on 10 March 2012.

KUZMIN S., TARKHNISHVILI D., ISHCHENKO V., TUNIYEV B., BEEBEE T., ANTHONY B., SCHMIDT B., OGRODOWCZYK A., OGIELSKA M., BABIK W., VOGRIN M., LOMAN J., COGALNICEANU D., KOVÁCS T., KISS I., 2009 b, *Rana arvalis*. In: IUCN 2011. IUCN Red List of Threatened Species. Version 2011.2. <www.iucnredlist.org>. Downloaded on 10 March 2012.

MARA GY., DEMETER L., 2005, *Spatial distribution and habitat use of anurans in an area with mineral water springs in Lower Ciuc*. In: VELICIU S., LÁNYI S., LIEBE P. & MAKFALVI Z. (Eds.): *Second International Conference on the Mineral Waters of the Carpathian Basin:* 117-122.

SAS I., COVACIU-MARCOV S.D., DEMETER L., CICORT-LUCACIU A.S., STRUGARIU A. 2008 a, *Distribution and status of the Moor frog (Rana arvalis) in Romania*. Zeitschrift für Feldherpetologie, Supplement, **13**: 334-357.

SAS I., COVACIU-MARCOV S.D., KOVÁCS É.H., 2008 b, *Is the conservation of the moor frog problematic in Romania* ? Herpetologica Romanica, **2**: 57-60.

SAS I., COVACIU-MARCOV S.D., KOVÁCS É H., RADU N.R., TOTH A., POPA A., 2006*, The populations of Rana arvalis Nilss., 1842 from the Ier Valley (The Western Plain, Romania): present and future*. North-Western Journal of Zoology, **2** (1): 1-16.

SAS I., KOVÁCS É.-H., SZEIBEL N., RADU N.R., TÓTH A., FERENȚI S., 2007, *The populations of Rana arvalis Nills. 1842 from the Ier Valley (The Western Plain, Romania)*, Part II: *sex ratio and body size distribution of some population*s. Herpetologica Romanica, **1**: 38-44.

Ministerial Order 57/2007 regarding the functioning of nature reserves, protection of natural habitats, flora and fauna. Monitorul Oficial 442.

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ANIMAL SPECIES AND HABITATS PROTECTED IN "NATURA 2000" SITES CERNICA LAKE AND FOREST (ILFOV COUNTY, ROMANIA)

DOINA CIOACĂ

From Cernica area, a number of 151 species (119 birds, 10 fish, six invertebrates, five amphibians, five reptiles, three mamalians, three plants) are protected from Bern Convention, Bonn Convention, the Agreement on the Conservation of African-Eurasian Migratory Waterbirds (AEWA), National Law 49/2011. Among them, 12 bird species (*Gavia arctica*, *Nycticorax nycticorax*, *Aythya nyroca*, *Sterna hirundo*, *Coracias garrulus*, *Ficedula albicollis*, *Lanius collurio*, *Lanius minor*, *Phalacrocorax pygmeus*, *Dendrocopos syriacus*, *Larus ridibundus*, *Tyto alba*) have been selected to designate Natura 2000 ROSPA0122 Cernica Lake and Forest (under Birds Directive), and four fish species (*Aspius aspius*, *Cobitis taenia*, *Rhodeus sericeus amarus*, *Umbra krameri*), three amphibian species (*Bombina bombina*, *Bombina variegata*, *Triturus cristatus*) and three habitat types have been selected to designate Natura 2000 ROSCI0308 Cernica Lake and Forest (by Habitats Directive).

Key words: Natura 2000 site, Birds Directive, Habitats Directive, Cernica, Romania.

INTRODUCTION

The Natura 2000 network of protected natural areas was funded in 1992 by the European Commission with the aim to protect the most seriously threatened habitats and species in Europe. This network was created on the basis of two pieces of legislation: the *Habitats Directive* ("Council Directive 92/43/EEC on the Conservation of natural habitats and of wild fauna and flora") adopted in 1992 and the *Birds Directive* ("Council Directive 2009/147/EC on the conservation of wild birds", that replaces "Council Directive 79/409/EEC" of 1979).

Two types of areas are included in the Natura 2000 network: Special Protection Areas (SPAs), designated by the Member States under the Birds Directive, in areas with significant numbers of wild birds and their habitats and Special Areas of Conservation (SAC) designated by the Member States under the Habitats Directive, in rare, endangered or vulnerable natural habitats and species of plants or animals (other than birds). First, the Member State has to designate Sites of Community Interest (SCI), under the Habitats Directive, and then turn to the Special Areas of Conservation (SACs).

Natura 2000 is the largest network of protected areas in the world comprising nearly 26,000 sites and covering almost 18% of the total European Union terrestrial

environment as well as substantial marine areas. It is the cornerstone of EU biodiversity policy and has a key role to play in achieving the 2020 and 2050 EU biodiversity target and vision, aimed at halting and reversing the loss of biodiversity and ecosystem services.

The aim of the Natura 2000 Network is to protect vulnerable habitats and species across their natural range in Europe and ensure that they are restored to, or maintained at, a favourable conservation status.

The Natura 2000 sites do not exclude human presence or business activity, and must develop the activities that can be helpful to the protection of nature and the environment. Certain activities with a significant impact on the species or types of habitats for which the site has been designated must be restricted or stopped.

In order to designate Natura 2000 sites, each EU Member State must compile a list of the best wildlife areas containing the habitats and species listed in the Habitats and Birds Directives and submit to the European Commission, which develops an evaluation process and selects the areas that are of Community interest.

In Romania, under the first Emergency Government Ordinance no. 236/2000 on the regime of protected natural areas, conservation of natural habitats and wildlife, seven categories of protected natural areas were accepted as follows: National Park, Nature Park, Scientific Reserve, Natural Reserve, Natural Monument, Biosphere Reserve, Wetland of International Importance (Ramsar site).

After Romania became a member of the European Union, the EU Directives on Habitats and Birds were introduced into Romanian legislation under Emergency Government Ordinance no. 57 of 2007 on the regime of protected natural areas, conservation of wild fauna and flora, natural habitats, and later, with amendments, under Law No. 49/2011.

In Romania, with its highly valuable biodiversity, 530 Natura 2000 sites were registered, classified into 148 SPAs and 382 SCIs, many of them located in natural protected areas (national and natural parks, or other reservations). The number and diversity of Natura 2000 sites reflect peculiarities of the five bio-geographic regions on the territory of Romania (continental, alpine, pannonian, steppe and pontic).

In this work we present protected species and habitats found in the Natura 2000 Cernica sites of similar name (ROSPA0122 Cernica Lake and Forest and ROSCI0308 Cernica Lake and Forest).

MATERIAL AND METHODS

The process of obtaining special Natura 2000 status for Cernica area began in 2001 and ended in 2010.

The investigated zone was Cernica Lake and Forest and its surrounding areas, located in the jurisdiction of four localities (Cernica, Pantelimon, Brăneşti and Găneasa) from Ilfov County. Coordinates: Longitude 26°17'44"E: Latitude 44°26'36" N. Surface 3744 ha.

The investigated sites (Fig. 1) were: Cernica 1 (120 ha); Cernica 2 (250 ha); Cernica 3 (110 ha); Tânganu (150 ha); Pasărea (300-400 ha), with parts from Cernica Lake; Cernica, Pustnicu, Nisipiştea, Căldăraru forests (with *Quercus pedunculiflora*, *Q. pubescens*, *Tilia tomentosa*, *Carpinus betulus*, *Salix*, *Ulmus foliacea*, *Acer campestre*, *Corylus avelana*, *Fraxinus ornus*, *Crataegus monogyna*, *C. pentagyna*, *Cornus sanguinea*, *Prunus spinosa*, *Ligustrum vulgare*, *Rosa canina*); Pasărea river, movable or fixed reed formations; wetlands, agrosystems, ruderal zones (abandoned farming fields).

The type of habitats in Cernica area include: 80.00 % forests, 11.00 % water (lake and river), 3.00 % marshes, peats, 4.00 % agrosystems (especially cereal crops), 2.00 % grasslands.

The types of habitats from Cernica area are representing: 80.00 % in forests, 11.00 % water (lake and river), 3.00 % marshes, peats, 4.00 % agrosystems (especially cereal crops), and 2.00 % grasslands.

The collecting methods were adjusted to different animal categories. There were used also visual observations with the naked eye or with binoculars, bird counts in flight, on the ground or on the water, mammal counts and identification of certain bird and mammal sheltering, breeding, feeding or wintering ranges.

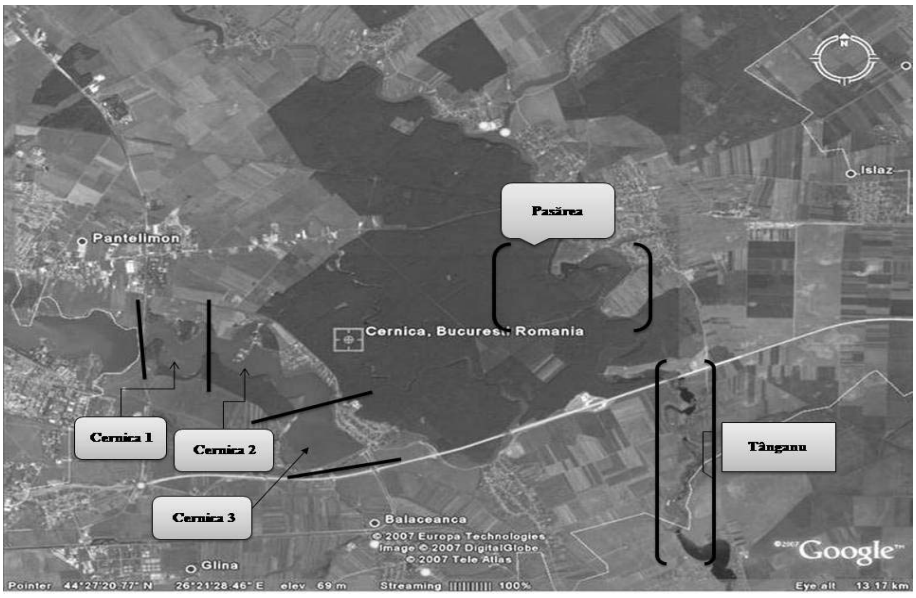

Fig. 1. The investigated sites in Cernica area (modified from www.earth.google.com).

RESULTS AND DISCUSSION

ARGUMENTS TO FIND THE NEED TO DESIGNATE NATURA 2000 SITE IN CERNICA AREA

1. It is a zone with rich diversity of species of wild animals, plants and habitats, many of them of Community interest and protected under the European Birds and Habitats Directives and other international Conventions and Agreements.

2. Cernica area is located on an important migration route of wild birds, most of which protected under the Birds Directive, the Bern Convention, Bonn Convention, Agreement on the Conservation of African-Eurasian Migratory Waterbirds or under National Law.

3. This zone offers rich and suitable food resources and favourable habitats for species of fauna protected under the Birds and Habitats Directives.

4. Many bird species, some of which rare and vulnerable, using Cernica zone for breeding, foraging, wintering or as migration stopover.

DIRECTION OF INVESTIGATIONS

1. Ecological systems diversity: ecosystems and habitats, particularly those listed in the Habitats Directive Annexes I and II, and habitats of protected species, especially those of wild birds.

2. Species diversity, particularly protected wild birds, listed in the Birds Directive, Annex I.

In Cernica zone we registered many wild species: invertebrates (141), amphibians (5), fish (23), reptiles (5), birds (122), mammals (11) and plants (185) (Botnariuc & Tatole, 2005; Cioacă, 2005, 2006; Dihoru & Negrean, 2009).

Invertebrates were represented by Lamellibranchia, Crustacea, Arachnida, Myriapoda and Insecta, some of them being protected under the Habitats Directive, Bern Convention and Law no 49/2011 (Table 1).

Table 1

Invertebrate species protected in Natura 2000 Cernica site

Species	**49/2011 Law**	**Habitat Directive**	**Convention**
Unio pictorum	–	–	Bern
Helix pomatia	–	Annex IV	Bern
Arion hortensis	–	Annex V	Bern
Astacus astacus	–	Annex V	Bern
Cerambyx cerdo	x	Annex II	Bern
Lucanus cervus	x	Annex II	Bern

Twenty two fish species, belonging to eight families (Cyprinidae, Clupeidae, Cobitidae, Esocidae, Percidae, Siluridae, Thymallidae and Umbridae), were

identified in Cernica Lake and Pasărea River of which 10 species are protected: five species protected under the Habitats Directive (Annex II), six under the Bern Convention and one species under Law no. 49/2011 (Table 2). Of these, four species (*Aspius aspius*, *Cobitis taenia*, *Rhodeus sericeus amarus* and *Umbra krameri*) were proposed and accepted for Natura 2000 site ROSCI0308 Cernica Lake and Forest (Natura 2000 Standard Form, 2011).

Table 2

Fish species protected in Natura 2000 Cernica site

Family	Species	49/2011 Law	Habitats Directive	Convention
Ciprinidae	*Astacus astacus*	x	Annex II	Bern
Ciprinidae	*Scardinius erythrophthalmus*	–	–	Bern
Ciprinidae	*Pelecus cultratus*	–	–	Bern
Ciprinidae	*Rhodeus sericeus amarus*	–	Annex II	–
Ciprinidae	*Chondrostoma* sp.	–	–	Bern
Cobitidae	*Misgurnus fosillis*	–	Annex II	–
Cobitidae	*Cobitis taenia*	–	Annex II	
Siluridae	*Siluris glanis*	–	–	Bern
Thymallidae	*Thymallus thymallus*	–	–	Bern
Umbridae	*Umbra krameri*	–	Annex II	

From amphibian species belonging to the Ranidae, Discoglossidae and Salamandridae families, all protected under the Bern Convention, three are also protected under the Habitats Directive (Annex II) and National Law (Table 3). *Bombina bombina*, *B. variegata* and *Triturus cristatus* were proposed and accepted for Natura 2000 site ROSCI0308 Cernica Lake and Forest (Natura 2000 Standard Form, 2011).

Table 3

Amphibian species protected in Natura 2000 Cernica site

Family	Species	49/2011 Law	Habitats Directive	Convention
Ranidae	*Rana ridibunda*	–	–	Bern
Ranidae	*Rana esculenta*	–	–	Bern
Discoglossidae	*Bombina bombina*	X	Annex II	Bern
Discoglossidae	*Bombina variegata*	X	Annex II	Bern
Salamandridae	*Triturus cristatus*	X	Annex II	Bern

Five reptile species, of the Emydidae, Lacertidae and Colubridae families are protected under the Bern Convention, and one of them is also protected by the Habitats Directive (Annex II) and four by the National Law (Table 4).

Table 4

Reptile species protected in Natura 2000 Cernica site

Family	Species	49/2011 Law	Habitats Directive	Convention
Emydidae	*Emys orbicularis*	X	Annex II	Bern
Lacertidae	*Lacerta viridis*	X	–	Bern
Lacertidae	*Lacerta agilis*	X	–	Bern
Colubridae	*Natrix natrix*	–	–	Bern
Colubridae	*Natrix tesselata*	X	–	Bern

A high level of richness was recorded for the Aves Class. From 122 identified bird species (belonging to 16 families), 119 species are legally protected: 27 species protected by the Birds Directive, Annex I, 80 species by the Bern Convention, 16 species by the Agreement on the Conservation of African-Eurasian Migratory Waterbirds (AEWA), 13 species by the Bonn Convention and 108 species by National Law.

Of the bird species, many are migratory, with seasonal migration (in Cernica zone some summer, some winter, others stop during passage), and some are non-migratory (resident or sedentary).

A number of 12 bird species were included in the Natura 2000 ROSPA0122 Cernica Lake and Forest Site, all protected by the Bern Convention, Bonn Convention, AEWA or National Law no. 49/2011. From these, 10 species are listed in Annex I of the Birds Directive (Table 5). In point of trophic categories, four of these bird species are dominantly ichtiophagous, while eight are insectivorous or with mixed regime (Bruun *et al*., 1999).

Of the 11 mammal species identified in Cernica area, eight species are protected under National Law, seven species by the Bern Convention and three species by the Habitats Directive, Annex II (Table 6).

Table 5

Bird species included in the Natura 2000 ROSPA0122 Cernica Lake and Forest Site

Family	Species	49/2011 Law	Bird Directive	Conventions	AEWA
Gaviidae	*Gavia arctica*	X	Annex I	Bern and Bonn	X
Ardeidae	*Nycticorax nycticorax*	X	Annex I	Bern	–
Anatidae	*Aythya nyroca*	X	Annex I	Bonn	X
Sternidae	*Sterna hirundo*	X	Annex I	Bern and Bonn	X
Coraciidae	*Coracias garrulus*	X	Annex I	Bern	–
Muscicapidae	*Ficedula albicollis*	X	Annex I	Bern	–

Table 5

(continued)

Family	Species	49/2011 Law	Bird Directive	Conventions	AEWA
Laniidae	*Lanius collurio*	X	Annex I	Bern	–
Laniidae	*Lanius minor*	X	Annex I	Bern	–
Phalacrocoracidae	*Phalacrocorax pygmeus*	X	Annex I	Bern and Bonn	X
Picidae	*Dendrocopos syriacus*	X	Annex I	Bern	–
Laridae	*Larus ridibundus*	X	–	–	–
Tytonidae	*Tyto alba*	X	–	Bern	–

Table 6

Protected mammalian species from Natura 2000 Cernica site

Family	Species	49/2011 Law	Habitats Directive	Conventions
Muridae	*Ondatra zibethica*	X	–	–
Mustelidae	*Putorius putorius*	X	–	Bern
Mustelidae	*Lutra lutra*	X	Annex II	Bern
Mustelidae	*Martes martes*	X	–	Bern
Mustelidae	*Mustela nivalis*	X	–	Bern
Mustelidae	*Meles meles*	X	–	Bern
Vespertilionidae	*Plecotus auritus*	X	Annex II	Bern
Vespertilionidae	*Myothis blythii*	X	Annex II	Bern

THE HABITATS PROPOSED TO DESIGNATED CERNICA NATURA 2000 SITE

1. Habitats for protected species: areas with fixed and mobile reed on Cernica Lake, zones with tall trees in Cernica and Căldăraru forests, zones with old hollow trees, wetlands and sandy shores.

2. Habitats that are of Community interest, protected by the Habitats Directive, Annex I and also on Natura 2000 Cernica SCI site (Doniță *et al*., 2005; Gafta & Mountford, 2008; Natura 2000 Standard Form, 2011): Balkan-Pannonian oak forests, with *Quercus petraea* and *Quercus cerris* (Natura 2000 code – 91M0); Dacian oak-hornbeam forests with *Quercus* and *Carpinus* species (Natura 2000 code – 91Y0) and Natural eutrophic lakes with *Magnopotamion* or *Hydrocharition*-type vegetation (Natura 2000 code – 3150).

CONCLUSIONS

In Cernica area, a number of 151 species (119 birds, 10 fish, six invertebrates, five amphibians, five reptiles, three mammalians, and three plants) are protected under the European Birds and Habitats Directives, the Bern Convention, Bonn Convention, the Agreement on the Conservation of African-Eurasian Migratory Waterbirds (AEWA) or National Law 49/2011.

Of the birds, 108 species are protected by Law 49/2011, 80 species by the Bern Convention, 13 species are protected by the Bonn Convention, 16 species by the AEWA, 27 species by the Birds Directive, Annex I). Of these, 12 species of birds (*Gavia arctica*, *Nycticorax nycticorax*, *Aythya nyroca*, *Sterna hirundo*, *Coracias garrulus*, *Ficedula albicollis*, *Lanius collurio*, *L. minor*, *Phalacrocorax pygmeus*, *Dendrocopos syriacus*, *Larus ridibundus*, *Tyto alba*), were the reason for designating the Natura 2000 ROSPA0122 Cernica Lake and Forest site and four fish (*Aspius aspius*, *Cobitis taenia*, *Rhodeus sericeus amarus* and *Umbra krameri*), three amphibians (*Bombina bombina*, *B. variegata* and *Triturus cristatus*), were selected to designate Natura 2000 ROSCI0308 Cernica Lake and Forest site.

As a result of investigations in Cernica zone during 2001–2010, some information was used in 2006 in proposing a Natura 2000 site as a Special Protection Area for birds (under the Birds Directive) by Doina Cioacă of the National Environmental Protection Agency, the proposal revised in 2010.

In 2011, Natura 2000 ROSPA0122 Cernica Lake and Forest was designated under Government Decision no. 971/2011 of 5 October 2011, published in The Official Journal of Romania no.715 of 11.10.2011.

At the same time, the other Natura 2000 ROSCI0308 Cernica Lake and Forest site (under Habitats Directive), proposed by Alexandru Iftime of the National Museum of Natural History Bucharest, was accepted under Order of the Minister of Environment and Forests no.2387/2011.

REFERENCES

BRUUN B., DELIN H., SVENSSON L., 1999, *Hamlyn Guide*: *Păsările din România şi Europa*, *determinator ilustrat*. Octopus Publishing Group Ltd, Londra, 54 pp.

BOTNARIUC N., TATOLE V. (Eds.), 2005, *Cartea Roşie a vertebratelor din România (Red Book of Vertebrates from Romania)*. Academia Română – Muzeul Național de Istorie Naturală "Grigore Antipa", Bucureşti, 260 pp.

CIOACĂ D., 2005, *The research study for diversity of flora and fauna to Cernica Lake*, Workshop on Environment / Biodiversity and Protected Areas, European Commission, TAIEX – Regional Technic Program (RTP) 11651, Cernica (Ilfov).

CIOACĂ D., 2006, *Măsuri de conservare pentru speciile de păsări de interes comunitar din România*, *dependente de zonele umede*. Publicație electronică a Agenției Naționale pentru Protecția Mediului, 63 pp. Available at: (http://www.scribd.com/doc/23569056/Masuri-Conservare-Pasari-Acvatice-Anexa-I-La-Dir-Pasari).

DIHORU G., NEGREAN G., 2009, *Cartea roşie a plantelor vasculare din România*. Editura Academiei Române, Bucureşti, 630 pp.
DONIŢĂ N., POPESCU A., PAUCĂ-COMĂNESCU M., MIHĂILESCU S., BIRIŞ I.-A., 2005, *Habitatele din România*, Editura Tehnică Silvică, Bucureşti, 496 pp.
GAFTA D., MOUNTFORD O. (coord.), 2008, *Manual de interpretare a habitatelor Natura 2000 din* România, Editura Risoprint, Cluj-Napoca, 101 pp.
* * *, Council Directive 79/409/EEC of 2 April 1979 on the conservation of wild birds (Directiva Consiliului Europei 79/409/EEC privind conservarea păsărilor sălbatice, adoptată la 2 aprilie 1979, cu modificările ulterioare).
* * *, Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora (Directiva Consiliului Europei 92/43/EEC referitoare la conservarea habitatelor naturale, a faunei şi florei sălbatice, adoptată la 21 mai 1992).
* * *, Legea nr. 13 din 11 martie 1993 pentru aderarea României la Convenţia privind conservarea vieţii sălbatice şi a habitatelor naturale din Europa, adoptată la Berna la 19 septembrie 1979.
* * *, Legea nr. 13 din 8 ianuarie 1998 pentru aderarea României la Convenţia privind conservarea speciilor migratoare de animale sălbatice, adoptată la Bonn la 23 iunie 1979.
* * *, Legea nr. 89 din 10 mai 2000 pentru ratificarea Acordului privind conservarea păsărilor de apă migratoare african-eurasiatice, adoptat la Haga la 16 iunie 1995.
* * *, Legea nr.49/2011 pentru aprobarea Ordonanţei de urgenţă a Guvernului nr. 57/2007 privind regimul ariilor naturale protejate, conservarea habitatelor naturale, a florei şi faunei sălbatice.
* * *, Raport privind starea mediului din România, 2001-2010, Ministerul Mediului şi Pădurilor, Available at: www.mmediu.ro.
* * *, Directive 2009/147/EC of the European Parliament and of the Council on the conservation of wild birds.
* * *, Order of the Minister of Environment and Forest no 2387/2011 to amend the order of the minister of environment and sustainable development. no. 1964/2007 establishing the regime of the protected areas as community interest sites as part of the European ecological network Natura 2000 in Romania.
* * *, Governmental Decision no. 971/ 2011 to amend and complete the Governmental Decision no. 1284/2007 regarding the declaration of the special protected areas for birds as part of the European ecological network Natura 2000 in Romania.
* * *, Formularele standard Natura 2000, Ministerul Mediului şi Pădurilor din România. Available at: http://www.mmediu.ro/beta/domenii/protectia-naturii-2/arii-naturale-protejate/.
* * *, http://ec.europa.eu/environment/nature/natura2000/.Natura 2000 network .

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MORPHOMETRY OF THREE TESTATE AMOEBAE OF *DIFFLUGIA* LECLERC, 1815 (AMOEBOZOA: ARCELLINIDA: DIFFLUGIIDAE) FROM BULGARIA

ROSITSA DAVIDOVA

Morphometric investigation of three new testate amoebae from the Bulgarian protozoan fauna – *Difflugia paulii* Ogden, 1983*, Difflugia minuta* var. *grandis* (Rampi, 1950) Gauthier-Lièvre and Thomas, 1958 and *Difflugia szczepanskii* Schönborn, 1965, collected from two freshwater basins in the North-eastern Bulgaria, is accomplished and compared to previous descriptions. The analysis of size frequency distribution is made. The coefficients of variation show that the test characteristic such as length, breadth and depth of shell, as well as diameter of aperture have low variability (CV 2.30 – 9.89%). The biometric comparison of closely related species – *Difflugia linearis*, *D. paulii* and *D. lacustris* is proposed. The ecological preferences for each testacean are reviewed.

Key words: biometry, *Difflugia*, Lake Durankulak, Golden Sands Nature Park, size frequency distribution.

INTRODUCTION

The genus *Difflugia* Leclerc, 1815 is one of widespread and the most abundant testacean genera. More than 300 species, varieties and forms of the genus have been established until now (Ogden, 1983). The representatives of the genus are typical inhabitants of the freshwater lakes, ponds, swamps and streams and relatively more rare in moss and soils. The genus *Difflugia* is also one of the most investigated and well-studied testate amoebae genera. Many authors give detailed descriptions of the morphological features, ecological preferences and geographical distribution of a large number of species (Rampi, 1950; Oye, 1953; Chardez, 1960; 1961; 1973; Gauthier-Lièvre & Thomas, 1958; Schönborn, 1966; Eckert & McGee-Russell, 1974; Ogden & Fairman, 1979; Ogden, 1979; 1980 a, b; 1983; 1988; Ogden & Hedley, 1980; Ogden & Živcovic, 1983; Casper & Schönborn, 1985; Lansac-Tôha *et al*., 2001; Bobrov & Mazei, 2004; Yang *et al.,* 2004; Yang & Shen, 2005; Lahr & Lopes, 2006; Nicholls, 2007; Todorov & Golemansky, 2007). However, some of species are difficult to be identified and distinguished from others due to the great diversity of shell shape and size as well as to the significant

variability that exists within the genus. The taxonomy of *Difflugia* genus is also inconvenient by the fact that the description of some species has been based on a few specimens, without detailed morphometry and microphotographs.

During our investigation on the testate amoebae in some freshwater basins in the North-eastern Bulgaria, we established three new testate amoebae in the Bulgarian protozoan fauna: *Difflugia paulii, Difflugia minuta* var. *grandis* and *Difflugia szczepanskii*. The aim of this study is to accomplish a morphometrical and ecological characterization of these new testate amoebae, comparing the results with data from literature.

MATERIAL AND METHODS

The material was collected in February, March and August 2010 from Durankulak Lake, and in July 2011 from Golden Sands Nature Park. Durankulak Lake is situated in the utmost North-eastern part of Bulgaria at 4-9 km from the Romanian border (28° 33' 43" E, 30° 40' 30" N). It is a shallow (maximum depth 4 m), eutrophic to hypertrophic lake, depending on the amount of biogenic elements and organic matter in its waters, covered with vast areas of vegetation, dominated mainly by *Phragmites australis*, *Typha angustifolia*, *Typha latifolia* and *Shoenoplectus triqueter*. The samples were collected from the benthos in the littoral zone of the lake. The Golden Sands Nature Park is located 17 km northeast of Varna. Its mean altitude is 110 m. The appearance of the park is determined by tree and shrub ecosystems, that occupy 90% of its area. The materials were collected from the benthos and hydrophyte vegetation (*Phragmites australis*) of a freshwater swamp from the park.

A half of each sample was fixed in 4% formaldehyde and was studied in a laboratory immediately after the collection; other half was kept alive for *in vivo* investigation.

The morphometric characterization of the species was made according to Schönborn *et al*. (1983).

The following parameters were calculated: x – arithmetic mean; M – median; SD – standard deviation; SE – standard error of the arithmetic mean; CV – coefficient of variation in %; Min, Max – minimum and maximum values; n – number of examined individuals.

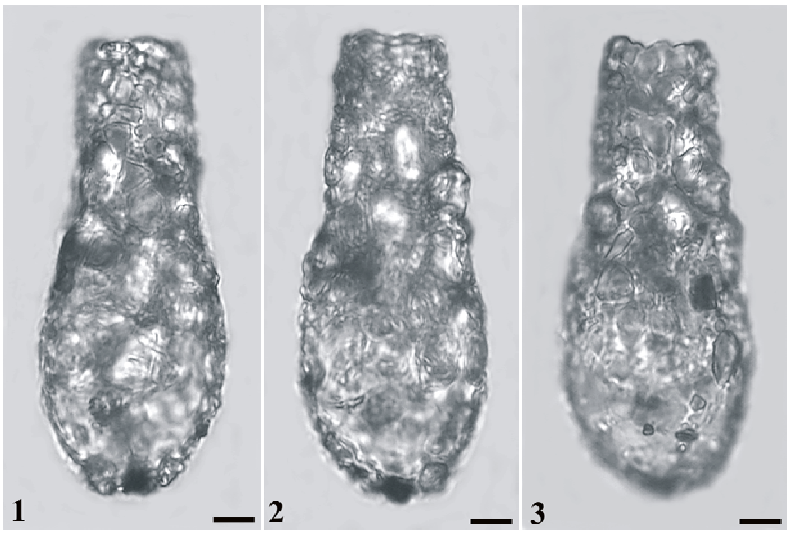
Shell size was measured under the light microscope at a magnification of 400 x. All measurements are in μm. The following abbreviations were used: L –length of shell, B-breadth of shell, D-depth of shell, Dm-diameter of shell, Da –diameter of aperture, B/L – ratio breadth of shell to length of shell and Da/L – ratio diameter of aperture to length of shell. Statistical analysis was carried out using software STATISTICA 6.

RESULTS AND DISCUSSION

***Difflugia paulii* Ogden, 1983** (Figs. 1–3).

Syn.: *Difflugia oblonga* var. *elongata* Oye, 1953

This species was described initially from Belgium as a variety of *D. oblonga* (Oye, 1953). According to this author the variety is "slender in comparison with *D. lacustris*" and has "the limited grains of quartz in the shell". Later Gauthier-Lièvre & Thomas (1958) found this variety in Africa. The authors agreed with the earlier description and indicated the following measurements: L = 130–142, B = 38–40, Da = 28–37. Ogden (1983) considered that this variety was the better to be treated as a distinct species and established *D. paulii*. According to Ogden (1983), *D. paulii* "appears to occupy a position mid-way between *D. linearis* and *D. lacustris*, which differed by its outline, elemental composition and pattering of the organic cement". Author noted the following measurements: L = 119–130, B = 48–54, Da = 19–23, B/L 0.40±0.01, Da/L 0.17±0.01.



Figs. 1–3. LM photographs of *Difflugia paulii* – lateral view of different specimens showing general test shape, outline and composition the shell (Scale bar: 10 μm).

The specimens found by us had shell morphology and dimensions in good agreement with the Ogden's description (1983). The morphometric characteristics of *D. paulii* show that the length and breadth of shell and diameter of aperture have normal variability (CV 3.52–7.35%) (Table 1). Size frequency distribution analysis indicates that the species is a size-polymorphic by the shell length (the measured

specimens have a shell length between 108–125 μm.) and a size-monomorphic species by the shell breadth (all specimens measured are in limits of 44–51 μm) (Fig. 4). Figure 4B shows that eighty percent of specimens have a shell breadth between 46 and 49 μm. The biometric comparison of three closely related species of *Difflugia* (*D. linearis*, *D. paulii* and *D. lacustris*) shows that they can be separated by their size and ratios B/L and Da/L (Fig. 5).

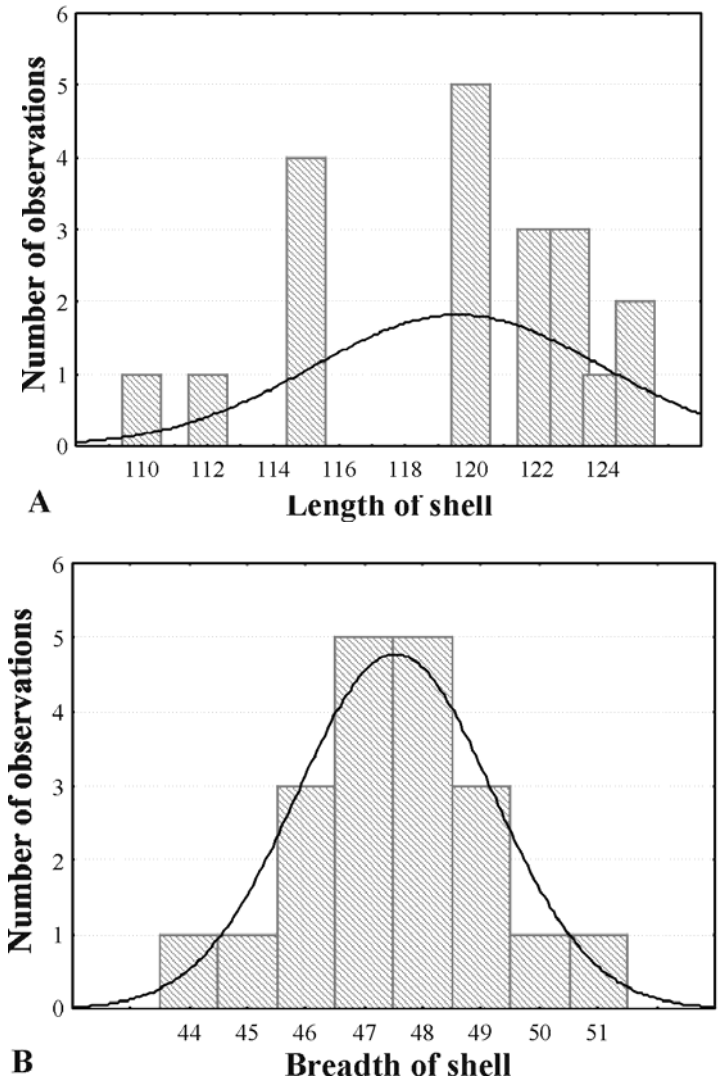


Fig. 4. Histograms showing the size frequency of the shell length (A) and shell breadth (B) of *D. paulii.*

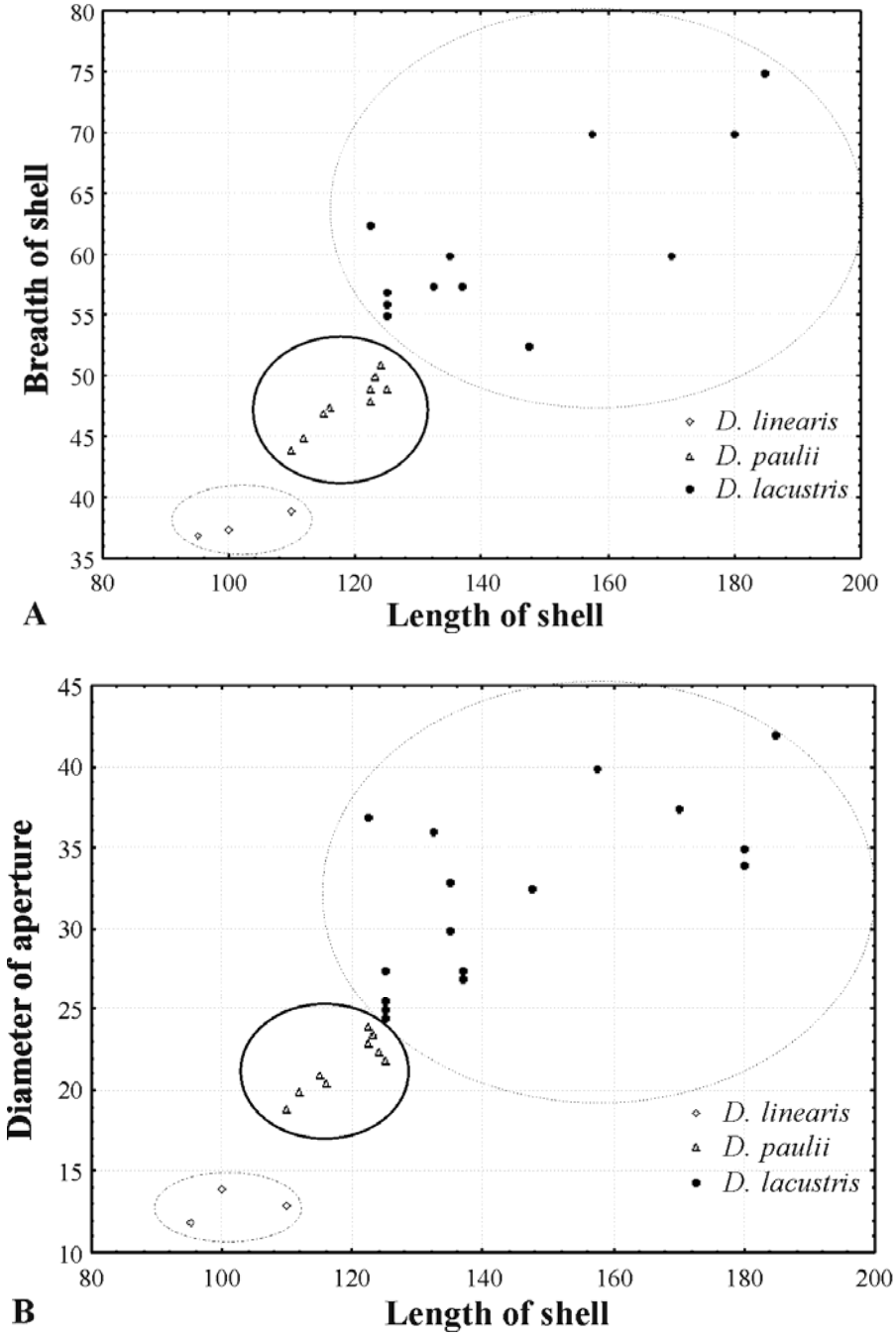


Fig. 5. Biometric comparison of the species *D. linearis*, *D. paulii* and *D. lacustris*.

Table 1

Biometric characterization of the investigated testacean species

Characters	x	M	SD	SE	CV	Min	Max	n
Difflugia paulii Length of shell (L)	119.5	120.0	4.39	0.98	3.66	110	125	20
Breadth of shell (B)	47.50	47.25	1.67	0.37	3.52	44	51	20
Diameter of aperture (Da)	21.23	21.00	1.56	0.35	7.35	18	24	20
B/L	0.40	0.39	0.01	0.002	2.50	0.38	0.41	20
Da/L	0.18	0.18	0.009	0.002	5.00	0.16	0.19	20
Difflugia minuta var. *grandis* Length of shell (L)	103.0	102.0	3.24	0.74	3.14	100	110	19
Breadth of shell (B)	99.42	100.0	2.29	0.53	2.30	97	105	19
Diameter of aperture (Da)	25.74	26.00	0.93	0.21	3.61	25	28	19
B/L	0.96	0.95	0.02	0.005	2.08	0.93	1.00	19
Da/L	0.25	0.25	0.005	0.001	1.60	0.24	0.26	19
Difflugia szczepanskii Length of shell (L)	54.20	55.00	2.83	0.57	5.22	50	60	25
Breadth of shell (B)	27.92	27.00	2.08	0.42	7.45	24	30	25
Depth of shell (D)	20.40	20.00	1.85	0.37	9.07	18	24	25
Diameter of aperture (Da)	9.20	10.00	0.91	0.10	9.89	8	10	25
B/L	0.51	0.52	0.02	0.004	3.92	0.47	0.54	25
D/L	0.38	0.40	0.03	0.006	7.89	0.34	0.41	25
Da/L	0.17	0.17	0.01	0.002	6.25	0.15	0.19	25

Oye (1953) and Gauthier-Lièvre & Thomas (1958) pointed out that this species occurred in small ponds. According to Ogden (1983), *D. paulii* inhabits the *Sphagnum* moss. Trappeniers *et al*. (1999) found it in samples of sediment of small lakes at Zackenberg, Northeast Greenland. Mazei (1999) reported that *D. paulii* occurred in flooded meadow near the lake in Pezenska District. In the present work the species was found in the benthic samples from the littoral zone in Durankulak Lake, in February and August. All these facts suggest that *D. paulii* is an inhabitant of small freshwater basins and *Sphagnum* moss.

***Difflugia minuta* var. *grandis* (Rampi, 1950) Gauthier-Lièvre and Thomas, 1958** (Figs. 6–9).

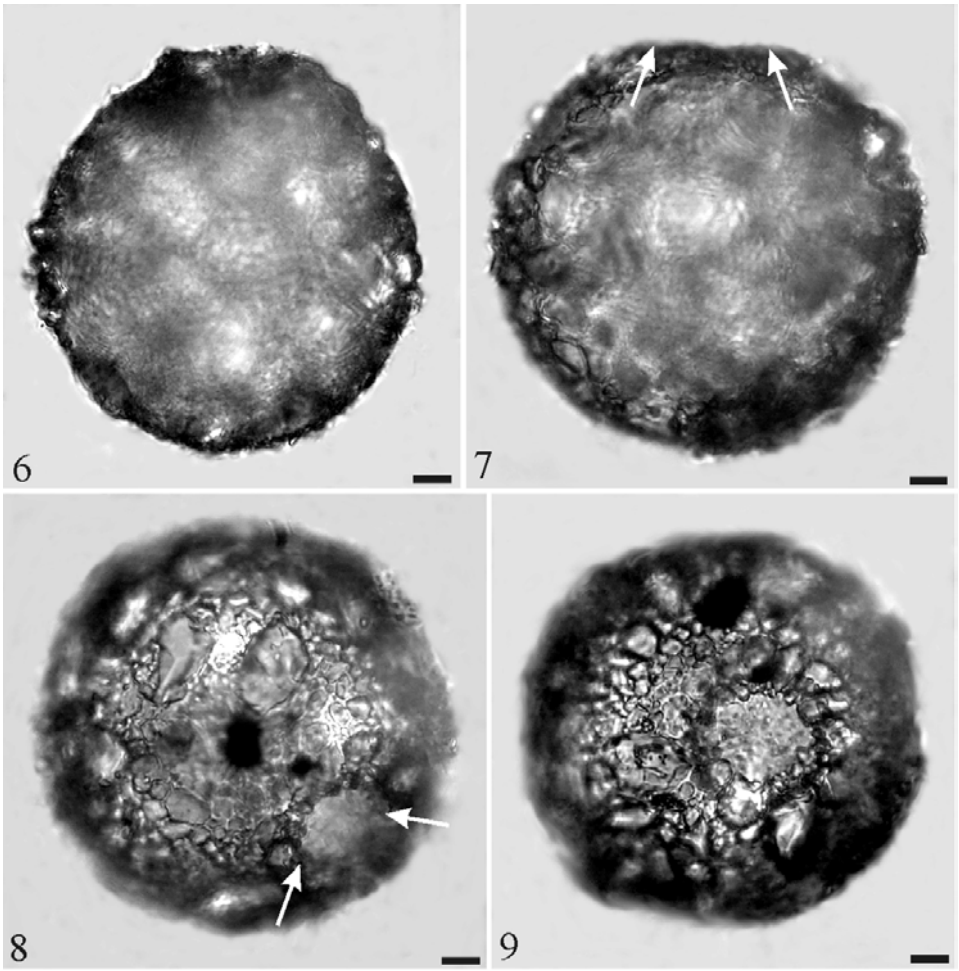
Gauthier-Lièvre & Thomas (1958) described *Difflugia minuta* var. *grandis* from Africa (Algeria) and remarked that this variety differed from *Difflugia minuta* Rampi, 1950 by its considerably (twice) larger shell dimensions. The authors indicate that it has a globular shell made mainly of large quartz particles and small aperture (about a quarter of shell breadth) and noted the following measurements: L = 100–130, B = 120–125, Da = 20–28.

By their morphometric characteristics the present specimens are in good agreement with the original description. Coefficients of variation of the measured

taxonomical characters are between 2.30–3.61% and show that shell parameters are moderately variable (Table 1). The analysis of the size frequency distribution indicates that *D. minuta* var. *grandis* is a size-monomorphic variety (Fig. 10).

About ninety percent of measured specimens of our population have a shell length 100–105 μm and a shell breadth 97–100 μm.

Data on its ecology find only in the original description, where Gauthier-Lièvre & Thomas (1958) states that it occurred in swamps. In the present study 19 specimens of this variety were found in benthic samples from the littoral zone of Durankulak Lake in March.



Figs. 6–9. LM photographs of *Difflugia minuta* var. *grandis*. 6, 7 – lateral view of different specimens showing general test form; 8 – oblique lateral view, showing composition of the shell and aperture (arrows); 9 – apertural view (Scale bar: 10 μm).

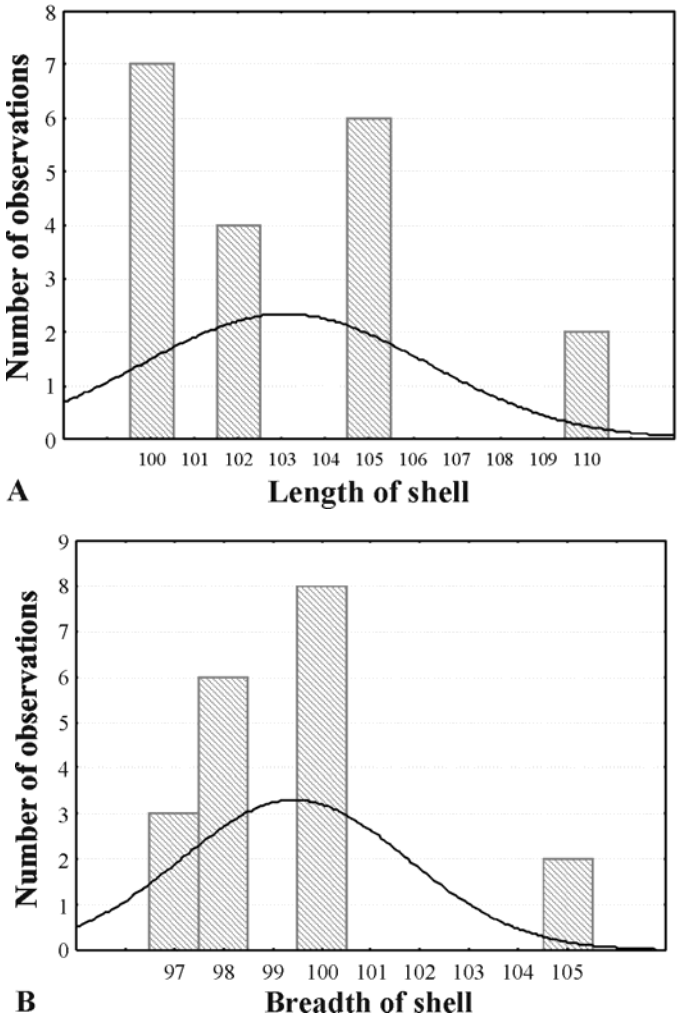


Fig. 10. Histograms showing the size frequency of the shell length (A) and shell breadth (B) of *D. minuta* var. *grandis.*

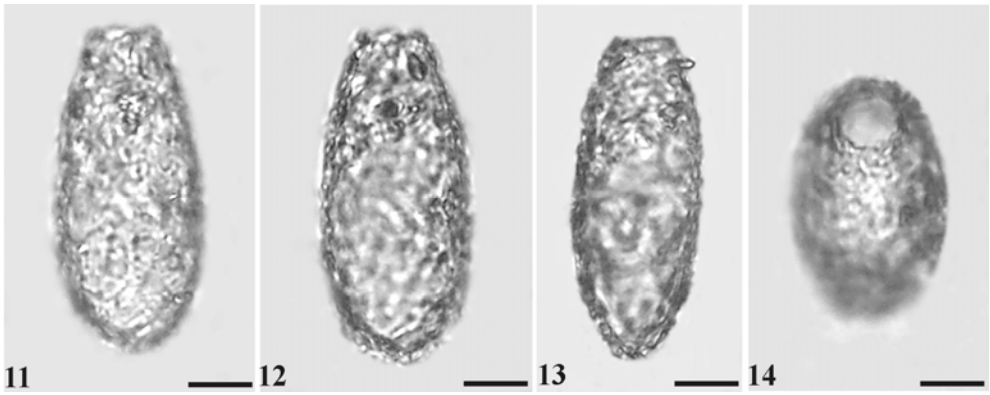
***Difflugia szczepanskii* Schönborn, 1965** (Figs. 11–14).

According to the original description of this species (Schönborn, 1965) the shell is oval and laterally flattened. The aperture is irregularly round. The shell is hyaline, covered with a meagre amount of particles. In the species description Schönborn (1965) indicate the following measurements: L = 60–80, B = 30–35, D = 20–27, but not reported data on the diameter of aperture.

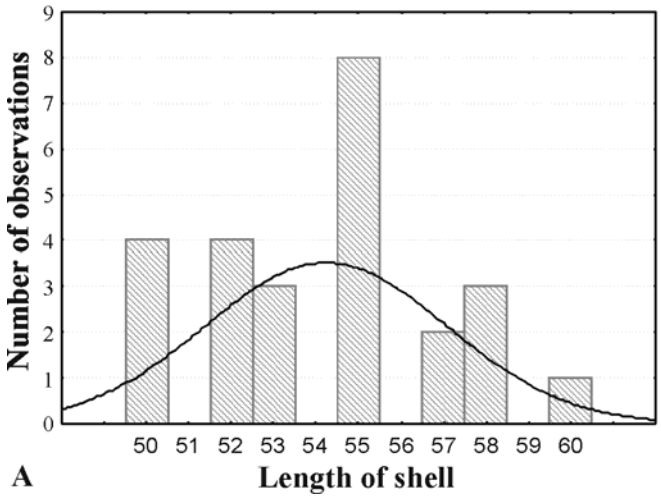
The coefficients of variation of all characters of specimens in our population are less than 10% and show that shell measurements are moderately variable. The specimens observed by us are in good agreement with the original description

concerning the form and structure of the shell, but established measurements are somewhat smaller. The size frequency distributions indicate that *D. szczepanskii* is a size-monomorphic species. Fig. 15 shows that the specimens of our population are characterized by small size range of the shell length (50–60 μm) and shell breadth (24–30 μm). 96% of measured specimens have a shell length 50–58 μm and 92 % of individuals have a shell breadth 26–30 μm.

In the present work the species was found in the benthic samples from the littoral zone in Durankulak Lake in February and March and in samples of benthal and of *Phragmites australis* of a swamp in Golden Sands Nature Park. So far the species has been reported only from fresh water samples from the Hancza Lake in Poland, from where it is described.



Figs. 11–14. LM photographs of *D. szczepanskii*. 11, 12 – broad lateral view of different specimens showing general test form; 13 – narrow lateral view; 14 – oblique lateral view, showing aperture (Scale bar: 10 μm).



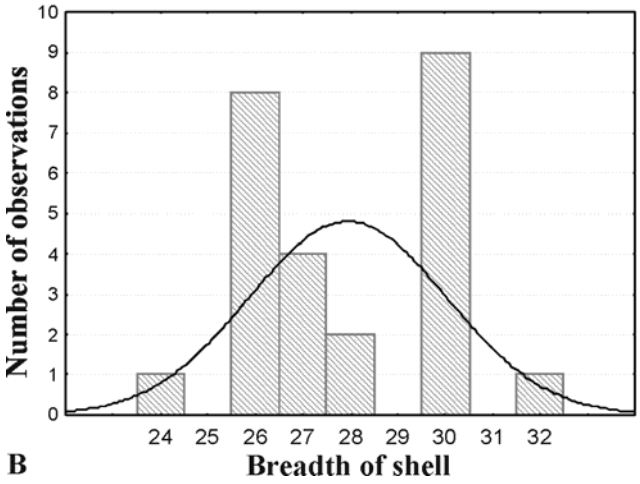


Fig. 15. Histograms showing the size frequency of the shell length (A) and shell breadth (B) of *D. szczepanskii.*

CONCLUSIONS

As a result of such research we obtain a clearer understanding for the importance of the morphological features of testate amoebae as well as for their variability. A detailed biometric analysis allows to outline the borders between studied species more clearly and successfully distinguishes closely related species.

Test morphology of *Difflugia paulii*, *Difflugia minuta* var. *grandis* and *Difflugia szczepanskii* from North-eastern Bulgaria are in good agreement with descriptions of these species from other parts of the world. Some differences were established only as regards the sizes of *Difflugia szczepanskii*. The specimens observed by us in Durankulak Lake and Golden Sands Nature Park are smaller than these described by Schönborn (1965) from the Hancza Lake. Evidently these species have a considerably wider range of test size.

The finding of *Difflugia paulii*, *Difflugia minuta* var. *grandis* and *Difflugia szczepanskii* in Bulgaria adds information about the geographical distribution and ecology of these species.

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REFERENCES

BOBROV A., MAZEI Y., 2004, *Morphological variability of testate amoebae (Rhizopoda: Testacealobosea: Testaceafilosea) in natural populations*. Acta Protozoologica, **43**: 133-146.

CASPER J., SCHÖNBORN W., 1985, *Difflugia limnetica (Levander) Penard (Protozoa: Testacea) as indicator organism of calcite precipitation in Lake Stechlin, GDR.*. Arch. Protistenk., **130**: 305-311.

CHARDEZ D., 1960, *Etudes sur deux Difflugia*. Hydrobiologia, **16**: 118-125.

CHARDEZ D., 1961, *Sur Difflugia acuminata Ehrenberg (Rhizopoda, Testacea)*. Bull. Inst. Agron. Stns Rech. Gembloux, **29**: 301-308.

CHARDEZ D., 1973, *Sur Difflugia ventricosa Deflandre, 1926 (Rhizopoda, Thecamoebien)*. Revue verviet. Hist. Nat., **30** (7-9): 57-60.

ECKERT B., S.MC GEE-RUSSELL, 1974, *Shell structure in Difflugia lobostoma observed by scanning and transmission electron microscopy*. Tissue Cell, **6**: 215-221.

GAUTHIER-LIÈVRE L., THOMAS R., 1958, *Les genres Difflugia, Pentagonia, Maghrebia et Hoogenraadia (Rhizopodes testaces) en Afrique*. Arch. Protistenk., **103**: 241-370.

LAHR D., LOPES S., 2006, *Morphology, biometry, ecology and biogeography of five species of Difflugia Leclerc, 1815 (Arcellinida: Difflugiidae) from Tiete River, Brazil.* Acta Protozoologica, **45**: 77-90.

LANSAC-TÔHA F., VELHO L., ZIMMERMANN-CALLEGARI M., BONECKER C., TAKAHASHI E., 2001, *On the occurrence of testate amoebae (Protozoa, Amoebozoa, Rhizopoda) in Brazilian inland waters. III. Family Difflugiidae: Genus Difflugia*. Acta Scientiarum Maringá, **23** (2): 305-321.

MAZEI Y., 1999, *Testacean fauna (Protista:Rhizopoda) in the Lakes from Pezenska District.* Proceedings: Ecology of animals and problems of regional environmental education. Saransk: 13-18 (in Russian).

NICHOLLS K., 2007, *Descriptions of Phryganella laurentiana n. sp. and Difflugia yorkui n. sp. two new species of testate amoebae from boreal forest wetlands in Ontario, Canada*. Acta Protozoologica, **46**: 65–72.

OGDEN C., 1979, *Comparative morphology of some pyriform species of Difflugia (Rhizopoda)*. Arch. Protistenk., **122**: 143-153.

OGDEN C., 1980 a, *Shell structure in some pyriform species of Difflugia (Rhizopoda)*. Arch. Protistenk., **123**: 455-470.

OGDEN C., 1980 b, *Notes on some Difflugiidae from Norfolk (Rhizopodea: Protozoa)*. Bull. Br. Mus. Nat. Hist. (Zool.), **39**: 125-138.

OGDEN C., 1983, *Observations on the systematics of the genus Difflugia in Britain.* Bull. Br. Mus. Nat. Hist. (Zool.), **44** (1): 1-73.

OGDEN C., 1988, *Morphology of the organic shell matrix of Difflugia (Rhizopoda) in culture, including modification by the addition of agglutinate particles.* Arch. Protistenk., **123**: 365-376.

OGDEN C., FAIRMAN S., 1979, *Further observations on piriform species of Difflugia (Rhizopodea)*. Arch. Protistenk., **122**: 372-381.

OGDEN C., HEDLEY R., 1980, *An atlas of freshwater testate amoebae*. Br. Mus. (Nat. Hist.), Oxford Univ. Press, London, 222 pp.

OGDEN C., ŽIVCOVIC A., 1983, *Morphological studies on some Difflugiidae from Yugoslavia (Rhizopoda, Protozoa)*. Bull. Br. Mus. Nat. Hist. (Zool.), **44** (6): 341-370.

OYE P. VAN, 1953, *Faune Rhizopodique de l'étang de Beernem*. Biol. Jaarb., **20**: 154-205.

RAMPI L., 1950, *Su alcuni Laghetti Alpini del Massiccio dell'Asbisso (Alpi Marittime)*. Bott. Pesca Pisci. Idrobiol., **26**: 207-224.

SCHÖNBORN W., 1965, *Die Sedimentbewohnenden Testaceen einiger Masurischer Seen.* Acta Protozoologica, **3** (27): 297-309.

SCHÖNBORN W., 1966, *Untersuchungen uber die Testaceen Schwedisch-Lapplands. Ein Beitrag zur Systematik und Ökologie der beschalten Rhizopoden*. Limnologica (Berlin), **4** (3): 517-559.

SCHÖNBORN W., FOISSNER W., MEISTERFELD R., 1983, *Licht- und rasterelektronen - mikroskopische Untersuchungen zur Schalen morphologie und Rassenbildung*

bodenbewohnender Testaceen (Protozoa: Rhizopoda) sowie Vorschläge zur biometrischen Charakterisierung von Testaceen-Schalen. Protistologica, **19**: 553-566.

TODOROV M., GOLEMANSKY V., 2007, *Morphological variability of Difflugia urceolata Carter, 1864 (Testacealobosia: Difflugiidae) and taxonomical status of its varieties D. urceolata var. olla Leidy, 1879, and D. urceolata var. sphaerica Playfair, 1917.* Acta Zoologica Bulgarica, **59** (1): 3-10.

TRAPPENIERS K., KERCKVOORDE A., CHARDEZ D., NIJS I., BEYENS L., 1999, *Ecology of testate amoebae communities from aquatic habitats in the Zackenberg area (Northeast Greenland).* Polar Biol., **22**: 271-278.

YANG J., BEYENS L., SHEN Y., FENG W., 2004, *Redescription of Difflugia tuberspinifera Hu, Shen, Gu et Gong, 1997 (Protozoa: Rhizopoda: Arcellinida: Difflugiidae) from China.* Acta Protozoologica, **43**: 281-289.

YANG J., SHEN Y., 2005, *Morphology, biometry and distribution of Difflugia biwae Kawamura,1918 (Protozoa: Rhizopoda)*. Acta Protozoologica, 44: 103-111.

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BILATERAL ASYMMETRY IN SOME MORPHOLOGICAL CHARACTERS OF *PARAPERCIS ALBOGUTTATA* (GÜNTHER, 1872) (FAMILY PINGUIPEDIDAE) COLLECTED FROM THE ARABIAN SEA COASTS OF OMAN

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Asymmetry analyses have been carried out for six bilateral characters of *Parapercis alboguttata* (Günther, 1872) (Pinguipedidae) collected from two localities, polluted and non-polluted, at the Arabian Sea coasts of Oman. In general, the asymmetry values of all characters studied were higher in the polluted than in the non-polluted one. The results showed that the level of asymmetry of the characters: preorbital length, postorbital length, and number of pectoral fin rays were higher than those of the rest of the characters studied in both areas. The number of pelvic fin rays showed the lowest asymmetry value. The possible cause of the asymmetry in this species has been discussed in relation to different pollutants and their presence in the area. In the polluted area, a weak trend of increase in the asymmetry values with the fish length was noticed in the number of lateral line scales. On the other hand, small individuals (< 120 mm) have shown a trend of increase in the asymmetry with the fish size of the preorbital length and number of pectoral fin rays. Similar trend was noticed in fishes collected from the non-polluted area with the difference that such trend is not confined to a certain fish length group.

Key words: Parapercis alboguttata, bilateral asymmetry, Arabian Sea, Sultanate of Oman.

INTRODUCTION

The differential development of a bilateral character between the sides of an organism is known as asymmetry (Van Vallen, 1962; Palmer & Strobeck, 1986; Leary & Allendrof, 1989).

Fluctuating asymmetry results when a trait present on both sides of the body does not undergo identical development. It is also known that fluctuating asymmetry represents a measure of developmental sensitivity to environmental stress (Møller & Pomiankowski, 1993; Jawad, 2001, 2003, 2004). Asymmetry usually increases under environmental stresses due to the failure of the homeostatic regulatory mechanism. These developmental effects might occur before the concentration of toxicants in the water or food reaches levels high enough to produce morbidity (Bengtsson & Hindberg, 1985).

Fluctuating asymmetry studies were never performed on the species in question, but there are few studies on record about the other fish species in Omani waters (Jawad *et al*., 2010; Al-Mamry *et al*., 2011 a, b). Therefore, the present

study is considered an addition to what previously published on morphological asymmetry in Omani fishes.

This work studied the bilateral asymmetry phenomenon in selected morphological characters of the pinguipedid species, *Parapercis alboguttata* collected from the Arabian Sea coasts of Oman and designed to determine: (1) if bilateral asymmetry occurs in the chosen characters; (2) the extent of asymmetries; (3) the direction of the asymmetrical development, as shown by one side tending to have a larger number of elements; (4) the possible usefulness of the information in future taxonomic studies of *Parapercis alboguttata*.

MATERIAL AND METHODS

According to several studies on the pollution in the Arabian Sea coasts of Oman (Badawy & Harthy, 1991; Ramamurthy, 1991; Sen Gupta *et al*., 1993), polluted (21º 09' 53.6'' N 59º 31' 13.06''E) and non-polluted areas (19º 52' 13.6'' N 59º 34' 20.36'' E) at the Arabian Sea coasts of Oman between latitudes 17° 28.86' N and 21° 54.85' N and longitudes 55° 17.14' E and 59° 45.82' E were chosen for this study (Fig. 1). One hundred and ninety two specimens, fish specimens of *Parapercis alboguttata* (Günther, 1872), were studied. The trawler, Al-Mustaquila ship was used in this study for the period 2006-2008. Six bilateral characters: pre and post orbital length, head length, number of pectoral fin rays, number of pelvic fin rays and number of lateral line scales were used to study the asymmetry level in the fish species in question. These characters are the most vulnerable to any changes in the environment (Bengtsson & Hindberg, 1985), and they are easy to evaluate.

Fig. 1. Map showing collecting site of *Parapercis alboguttata* (polluted and non-polluted area).

Table 1

Squared coefficient of asymmetry (CV^2_a) value and character means (X_{r+l}) of *Parapercis alboguttata*

Characters	Polluted area				Unpolluted area			
	CV^2_a	**N**	**Character mean**	**% of individuals with asymmetry**	**CV^2_a**	**N**	**Character mean**	**% of individuals with asymmetry**
Preorbital length	59.643	92	1.392	37.7	29.768	100	1.389	39.5
Postorbital length	39.642	92	1.882	70	19.543	100	1.877	75
Number of pelvic fin rays	4.385	92	5.012	1.11	0.356	100	5.043	1.2
Number of lateral line scales	6.813	92	59.850	72.53	1.769	100	59.796	75.5
Number of pectoral fin rays	34.702	92	16.395	39.56	16.969	100	16.388	40.8
Head length	12.395	92	3.958	69.57	4.543	100	3.976	75.5

The statistical analysis included calculating the squared coefficient of asymmetry variation (CV^2_a) for meristic and morphometric characters according to Valentine *et al*. (1973):

$$CV^2_a = (S_{r\text{-}l} \times 100/X_{r+l})^2,$$

where $S_{r\text{-}l}$ is the standard deviation of signed differences and X_{r+l} is the mean of the character, which is calculated by adding the absolute scores for both sides and dividing by the sample size. To obviate scaling problems associated with growth in morphometric characters, each measurement was divided by suitable general size measurements, *e.g.* head length was used as the standardizing measurement. Each of the morphometric characters was treated as such before obtaining the signed differences.

RESULTS

The results of asymmetry data analysis of the previously listed characters of *P. alboguttata* collected from both localities in the Arabian Sea coasts of Oman are shown in Table 1. In general, the asymmetry values of all characters studied were higher in the polluted than in the non-polluted one. In the polluted area, the results show higher values for the pre and postorbital length and number of pectoral fin rays. Among these, the asymmetry value of preorbital length was the highest. On the other hand, lowest asymmetry value is recorded for the number of pelvic fin

rays. Same pattern of distribution of asymmetry value was noticed in the non-polluted area.

Table 2

Squared coefficient of asymmetry and character means by size class of *Parapercis alboguttata*

Character	**CV^2_a**	**N**	**Character mean** X_{r+1}	**% of individuals with asymmetry**
Preorbital length				
80-90	126.4	6	0.98	83
91-100	571.4	13	1.07	61.5
101-110	131.99	39	1.2	66.6
111-120	51.27	8	1.31	66.6
121-130	0	1	1.5	0
131-140	20.82	5	1.64	60
141-150	7.8	7	1.77	57.14
151-160	11.7	6	2	50
161-170	12.4	6	2.11	66.6
171-180	0	1	2.3	0
Postorbital length				
80 - 90	98.708	6	1.383	50
91-100	50.248	13	1.519	84.6
101-110	52.57	39	1.72	67.5
111-120	55.19	8	1.85	83.3
121-130	0	1	2.1	100
131-140	11.46	5	2.21	60
141-150	32.69	7	2.32	83.33
151-160	9.23	6	2.45	80
161-170	18.81	6	2.37	57.14
171-180	0	1	2.55	0
Head Length				
80 - 90	126.42	6	3.07	33
91-100	6.57	13	3.29	76.9
101-110	6.44	39	3.63	66
111-120	14.62	8	3.83	66
121-130	0	1	4.2	0
131-140	1.88	5	4.61	80
141-150	2.98	7	4.83	100
151-160	5.32	6	5.26	83
161-170	1.73	6	5.23	50
171-180	0	1	5.65	100
Number of Pectoral rays				
80 - 90	9.66	6	16.08	50
91-100	27.26	13	16.54	53.8
101-110	42.59	39	16.25	89.6
111-120	58.31	6	16.33	66.6
121-130	0	1	16	0
131-140	20.57	5	16.5	66.6

141-150	18.15	7	16.43	57.1
151-160	19.23	6	17	66
161-170	17.46	6	16.75	50
171-180	0	1	16.5	100
Number of Pelvic fin rays				
80 - 90	0	6	5	0
91-100	0	13	5	0
101-110	0	39	5	0
111-120	53.7	8	5.08	16
121-130	0	1	5	0
131-140	0	5	5	0
141-150	0	7	5	0
151-160	0	6	5	0
161-170	0	6	5	0
171-180	0	1	5	0
Number of lateral line scales				
80-90	5.34	6	95.5	100
91-100	5.5	13	95.92	100
101-110	7.8	39	59.35	42.8
111-120	1.86	8	59.83	71.4
121-130	0	1	60.5	80
131-140	1.75	5	60.4	100
141-150	6.85	7	60.79	66
151-160	2.75	6	60.33	71.7
161-170	14.3	6	60.58	84.6
171-180	0	1	62.5	83

In the polluted area, the percentage of the individuals showing asymmetry in the number of pectoral fin rays was the highest among the percentages (72.53% of the total fish studied). The lowest percentage of individuals with asymmetry is recorded for the pelvic fin rays count (1.11% of the total fish studied). The same result obtained for the percentage of those two characters in the non-polluted area, but with lower values (39.5% and 1.2& for number of pectoral fin rays and pelvic fin rays respectively).

In both the polluted and non-polluted areas, the result of the asymmetry direction as whether individuals are left handed or right handed have shown that all the characters studied are senistral, where the left side showed higher value over the right side, except for pectoral fin ray count where shown to be dextral, where count on the right side is larger than that of the left side. Of specimens exhibiting pectoral fin ray asymmetry, 48.2% and 45.3 for polluted and non-polluted areas respectively, had larger right side counts. Of the specimens showing asymmetry in pre and postorbital length, pelvic fin ray count, head length and lateral line scale count, 68.5%, 79.5%, 55.7%, 48.9% and 46.7% are left sided respectively in the polluted area. Similar results were obtained in the non-polluted area but with slightly different values (67.5%, 75.5%, 50.7%, 45.9% and 45.7% for pre and

postorbital length, pelvic fin ray count, head length and lateral line scale count respectively.

Individuals of *Parapercis alboguttata* were grouped into length classes (Tables 2–3). A weak trend of increase in the asymmetry values was noticed in the number of lateral line scales also in the preorbital length and number of pectoral fin rays of small individuals less than 120 mm in the polluted area. Stronger trend is clear in the characters, the number of lateral line scales, the preorbital length and number of pectoral fin rays. Such trend is not restricted to certain fish length groups as in the polluted area.

Table 3

Squared coefficient of asymmetry and character means by size class of *Parapercis alboguttata* collected from the non-polluted area

Character	**CV^2_a**	**N**	**Character mean** X_{r+1}	**% of individuals with asymmetry**
Preorbital length				
80 - 90	1.4	6	0.98	83
91-100	1.9	13	1.07	61.5
101-110	2.2	40	1.2	66.6
111-120	2.4	10	1.31	66.6
121-130	2.4	1	1.5	0
131-140	2.6	8	1.64	60
141-150	2.8	9	1.77	57.14
151-160	3	6	2	50
161-170	3.2	6	2.11	66.6
171-180	3.7	1	2.3	0
Postorbital length				
80 - 90	3.708	6	1.383	50
91-100	0.248	13	1.519	84.6
101-110	2.57	40	1.72	67.5
111-120	5.19	10	1.85	83.3
121-130	0	1	2.1	100
131-140	1.46	8	2.21	60
141-150	2.69	9	2.32	83.33
151-160	3.23	6	2.45	80
161-170	1.81	6	2.37	57.14
171-180	0	1	2.55	0
Head Length				
80 - 90	6.42	6	3.07	33
91-100	6.57	13	3.29	76.9
101-110	6.44	40	3.63	66
111-120	4.62	10	3.83	66
121-130	0	1	4.2	0
131-140	1.88	8	4.61	80
141-150	2.98	9	4.83	100
151-160	5.32	6	5.26	83

161-170	1.73	6	5.23	50
171-180	0	1	5.65	100
Number of Pectoral rays				
80 - 90	2.66	6	16.08	50
91-100	2.98	13	16.54	53.8
101-110	3.59	40	16.25	89.6
111-120	3.67	10	16.33	66.6
121-130	3.89	1	16	0
131-140	4.0	8	16.5	66.6
141-150	4.15	9	16.43	57.1
151-160	4.23	6	17	66
161-170	4.46	6	16.75	50
171-180	4.78	1	16.5	100
Number of Pelvic fin rays				
80 - 90	0	6	5	0
91-100	0	13	5	0
101-110	0	40	5	0
111-120	3.7	10	5.08	16
121-130	0	1	5	0
131-140	0	8	5	0
141-150	0	9	5	0
151-160	0	6	5	0
161-170	0	6	5	0
171-180	0	1	5	0
Number of lateral line scales				
80-90	5.34	6	95.5	100
91-100	5.5	13	95.92	100
101-110	7.8	40	59.35	42.8
111-120	8.9	10	59.83	71.4
121-130	9.1	1	60.5	80
131-140	9.75	8	60.4	100
141-150	9.85	9	60.79	66
151-160	10.32	6	60.33	71.7
161-170	14.3	6	60.58	84.6
171-180	0	1	62.5	83

DISCUSSION

In the polluted area, the living habits of *Parapercis alboguttata* have shown a relationship between asymmetry and environmental stress in the form of water pollution such as heavy metals and hydrocarbon materials; and changes in other physical parameters. Indication for such conclusion has been obtained from the feeding behaviour and the high metabolic rate of this fish. Members of this species are usually gathered together and swim in school and move around water. Such pattern of movement exposes the individuals of *P. alboguttata* to the environmental stress mentioned above. On the other hand, greater energy is in need for such an

active fish to compensate for the energy loss during their development and towards manipulating changes in environmental factors. Other amount of energy is needed to be allocated for maintaining homeostasis (Mitton, 1994). Abnormal development may become evident if available energy is not sufficient to buffer the stress effects homeostasis (Mitton, 1994; Somarakis *et al*., 1997). Consequently, higher levels of asymmetry were resulted in *P. alboguttata*.

The asymmetry value showed some variation among the six morphological characters studied in *P. alboguttata*. There is a significant difference between the value of the asymmetry coefficient between the polluted and non-polluted areas studied ($P > 0.05$). Thus, it is possible to evaluate the level of asymmetry of those characters and to determine that they are higher than the average in the polluted area studied and conclude that there is a direct correlation between environmental stress such as pollution caused by hydrocarbon and heavy metals and asymmetry in this species.

Characters like pre and postorbital length and pectoral fin ray count showed higher asymmetry values than those of the remaining characters. Such high asymmetry values of these characters were also recorded in several freshwater and marine fish species. The agreement in the results of the asymmetry might indicate the vulnerability of those two characters to the immediate changes in the environment. On this particular issue, previous studies (Al-Hassan *et al*., 1990; Al-Hassan & Hassan, 1994; Jawad, 2001, 2003; Jawad *et al*., 2010; Al-Mamry *et al*., 2011a, b) have shown a direct correlation between environmental stresses due to pollution and asymmetry which can be applied in the case of the species in question.

The marine environment of the Sea of Oman is familiar with such environmental factors. The low fluctuating asymmetry values obtained for the other bilateral characters can be explained on the basis of that these characters are designated with high functional importance and highly canalized during ontogeny (Palmer & Strobeck, 1986; Møller & Pomiankowski, 1993).

The detrimental effect of asymmetry on the size of the fins which have functional importance is in fishes (Gonçalves *et al*., 2002). As they play an important role in locomotion, the pectoral fins have functional value and thus the efficiency of predator evasions probably depends on their functionality (Gonçalves *et al*., 2002). On the other hand, pectoral fins are of use during parental care as their advantage becomes clear in the process of egg oxygenation, and recently Künzler & Bakker (2000) have demonstrated that the area of the pectoral fins correlates with paternal quality in sticklebacks *Gasterosteus aculeatus* L. With the moderately high asymmetry value recorded for pectoral fin ray count of *P. alboguttata*, it is quite possible for such asymmetry to hinder the basic functions of the pectoral fin.

Effect of asymmetry on body proportions has been observed and discussed by several authors (Moodie & Reimchen, 1976; Reimchen, 1983; Bergstrom &

Reimchen, 2003). Among these effects is the effect of asymmetry on the orbital diameter, pre and postorbital length as it might increase or decrease the preorbital and postorbital areas in the fish skull. These changes can lead to differential enlargement which is restricted to the nasal pole of the eye. If such enlargement happens, retinal expansion will take place (Zygar *et al*., 1999). Moreover, by having more space in the naso-temporal region this might affect fish visual acuity (Cameron, 1995; Zygar *et al*., 1999).

The individuals of *P. alboguttata* appeared to be right handed for the pectoral fin ray count and left handed for the remaining five bilateral characters. Judging whether left handed or right handed individuals are the naturally successful individuals is not possible at this stage as such dextrality and senistrality in these characters might interfere with important biotic functions of the fish (Zygar *et al*., 1999).

Taxonomic and racial studies usually use data obtained from pectoral fin ray count and measurements of orbital diameter, pre and postorbital lengths. These characters are regularly used by taxonomists to set up the unique morphological features of the species in question. Any interchanging counts and differences in dimensions from left and right sides of *P. alboguttata* will introduce an additional source of variation to taxonomists who depend on these characters in separating this species or its populations. Bilateral asymmetry has shown to create problems to fish taxonomists (Parenti, 1986) and taxonomists of animal groups other than fish such as owl (Norberg, 1977).

Pollution of sea water and sediments by hydrocarbons, heavy metals, pesticides and organic matter are considered the main cause of environmental stress. This state of pollution is not unusual for the Arabian Sea coasts of Oman environment where different pollutants were reported to affect its water for at leas in the last twenty years (Ramamurthy, 1991; Badawy & Al-Harthy, 1991; Sen Gupta *et al*., 1993).

The environmental causes might be natural events, and several factors are known to produce nutritional deficiencies such as various pathogens and various population phenomena (Bengtsson & Hindberg, 1985), and it is highly possible that these factors may be in action in the Arabian Sea waters of Oman as they seem to be common in the aquatic environment.

Several authors have shown a relationship between the coefficient of asymmetry and fish length (Al-Hassan *et al*., 1990; Al-Hassan & Hassan, 1994; Al-Hassan & Shwafi, 1997; Jawad, 2001; Al-Mamry *et al*., 2011a, b) where there was a trend of increase in the asymmetry value with the increase in fish length. The body proportions and meristic characters studied were identical and gave zero value for the asymmetry coefficient in several length groups studied. This is because there is only one fish specimen in these groups and it happened to be identical for the chosen six morphological and meristic characters. For the pelvic fin rays, members of only one length group were non identical in respect of the

number of rays in their right and left sides. This means that the development of the pelvic fin rays is less vulnerable to the environmental factors.

The results also show a trend of increase with fish length in small individuals (>110 mm and < 120 mm) for preorbital length and number of pectoral fin rays. For the number of lateral line scales, there is a weak trend of increase with the increase of the fish length. This trend is probably the result of incomplete development; character means are always lowest in smaller size classes (Valentine *et al*., 1973). The same results were obtained by Valentine *et al*. (1973) in selected fish species collected from California, U.S.A. They suggested two possible hypotheses that may account for such a trend; these are the ontogenetic changes which are an increase in asymmetry with size (age) and the possible historical process which is a secular increase in asymmetry.

CONCLUSIONS

In the asymmetry study of *Parapercis alboguttata* (Günther, 1872) (Family: Pinguipedidae) collected from two localities, polluted and non-polluted, at the Arabian Sea coasts of Oman, the asymmetry values were higher in the polluted area than in the non-polluted one. Preorbital length, postorbital length and number of pectoral fin rays, the three characters with higher values for asymmetry coefficient in the polluted area, showed to have higher values in the non-polluted area too. On the other hand, the number of pelvic fin rays showed the lowest asymmetry value in both areas. Pollution with hydrocarbon and heavy metals is considered the possible cause of the asymmetry in this species. Trend of increase in the asymmetry values with the fish length was noticed in certain characters and in fish specimens from polluted and non-polluted areas with the difference that such trend is not restricted to certain fish length.

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REFERENCES

AL-HASSAN L.A.J., HASSAN S.S., 1994, *Asymmetry study in Mystus pelusius collected from Shatt al-Arab River, Basrah, Iraq*. Pakistan Journal of Zoology, **26**: 276-278.

AL-HASSAN L.A.J., SHWAFI N.A.A., 1997, *Asymmetry analysis in two marine teleost fishes collected from the Red Sea coast of Yemen.* Pakistan Journal of Zoology, **29**: 23-25.

AL-HASSAN L.A.J., AL-DOUBAIKEL A.Y., WAHAB N.K., AL-DAHAM N.K., 1990, *Asymmetry analysis in the catfish, Heteropneustes fossilis, collected from Shatt al-Arab River, Basrah, Iraq*. Rivesta di Idrobiologia, **29**: 775-780.

AL-MAMRY J.M., JAWAD L.A., AL-BIMANI S.M.H., AL-BUSAIDI H.K., AL- MARZOUQI

M.S., AL-HABSI S.H., 2011 a, *Asymmetry analysis study on Callionymus margaretae Regan, 1906 collected from the Arabian Sea coasts of Oman*. Ribarstvo, **69** (1): 3-9.

AL-MAMRY J., JAWAD L., AL-BIMANI S., AL-GHAFARI F., AL-MAMRY D., AL-MARZOUQI M., 2011 b, *Asymmetry in some morphological characters of Leiognathus equulus (Forsskål) (Leiognathidae) collec ted from the Sea of Oman*. Archive of Polish Fisheries, **19**: 51-55.

BADAWY M.I., AL-HARTHY F., 1991, *Hydrocarbons in seawater, sediment and oyster from the Omani coastal waters*. Bulletin of Environmental Contamination, **47**: 386-391.

BENGTSSON B.E., HINDBERG M., 1985, *Fish deformities and pollution in some Swedish waters*. Ambio, **14**: 32-35.

BERGSTROM C.A., REIMCHEN T.E., 2003, *Asymmetry in structural defences: insights into selective predation in the wild.* Evolution, **57**: 2128-2138.

CAMERON D.A., 1995, *Asymmetric retinal growth in the adult teleost green sunfish (Lepomis cyanellus)*. Visual Neurobiology, **12**: 95-102.

GONÇALVES D.M., SIMÕES P.C., CHUMBINHO A.C., CORREIA M.J., FAGUNDES T., OLIVEIRAI R.F., 2002, *Fluctuating asymmetries and reproductive success in the peacock blenny*. Journal of Fish Biology, **60**: 810-820.

JAWAD L.A., 2001, *Preliminary asymmetry analysis of some morphological characters of Tilapia zilli (Pisces: Cichlidae) collected from three localities in Libya*. Bolletino del Museo regionale de Science di naturale Torino, **18**: 251-257.

JAWAD L.A., 2003, *Asymmetry in some morphological characters of four sparid fishes from Benghazi, Libya.* Oceanological and Hydrobiological Studies, **32**: 83-88.

JAWAD L.A., 2004, *Asymmetry analysis in the mullet, Liza abu collected from Shatt al-Arab River, Basrah, Iraq.* Bolletino del Museo regionale de Science di naturale Torino, **21**: 145-150.

JAWAD L.A., AL-MAMRY J.M., AL-KHARUSI A.A., AL-HABSI S.H. , 2010, *Asymmetry in some morphological characters of the carangid species, Decapterus russelli collected from Lema coastal area, north of the Sea of Oman*. Oceanological and Hydrological Studies, **39** (2): 55-62.

KÜNZLER R., BAKKER T.C.M., 2000, *Pectoral fins and paternal quality in sticklebacks*. Proceedings of the Royal Society of London B, **267**: 999-1004.

LEARY A., ALLENDROF F.W., 1989, *Fluctuating asymmetry as an indicator of stress: implications for conservation biology*. Trend in Evolution, **4**: 214-217.

MITTON J.B., 1994, *Enzyme Heterozygosity, Metabolism, and Developmental Stability*: In: MARKOW T.A. (Ed.), *Developmental Instability and Evolutionary Implications*, Kluwer Academic Publishers, The Netherlands: 49-68.

MØLLER A.P., POMIANKOWSKI A., 1993, *Punctuated equilibria or gradual evolution: fluctuating asymmetry and variation in the rate of evolution*. Journal of Theoretical Biology, **161**: 359-367.

MOODIE G.E.E., REIMCHEN T.E., 1976, *Phenetic variation and habitat differences in Gasterosteus populations of the Queen Charlotte Islands*. Canadian Field Naturalist, **90**: 471-474.

NORBERG R.A., 1977, *Occurrence and independent evolution of bilateral ear asymmetry in owls and implications on owl taxonomy*. Philosophical Transaction of the Royal Society of London Series B, **280**: 375-408.

PALMER A.R., STROBECK C., 1986, *Fluctuating asymmetry: measurements, analysis and pattern*. Annual Review of Ecology and Systematics, **17**: 391-421.

PARENTI L.R., 1986, *Bilateral asymmetry in phallostethid fishes (Atherinomorpha) with description of a new species from Sarawak*. Proceedings of California Academy of Science, **44**: 225-236.

RAMAMURTHY V.D., 1991, *Effects of oil pollution on bio ecology and fisheries on certain enclosed coastal regions of Arabian Sea*. Marine Pollution Bulletin, **23**: 239-245.

REIMCHEN T.E., 1983, *Structural relationships between spines and lateral plates in three spine stickleback (Gasterosteus aculeatus)*. Evolution, **37**: 931–946.

SEN GUPTA R., RONDEKAR S.P., ALAGARSAMY R., 1993, *State of oil pollution in the northern Arabian Sea after the 1991 Gulf oil spill*. Marine Pollution Bulletin, **27**: 85-91.

SOMARAKIS S., KOSTIKAS I., TSIMENIDES N., 1997, *Fluctuating asymmetry in the otoliths of Lawa fish as an indicator of condition conceptual and methodological aspects*. Journal of Fish Biology, **51**: 30-38.

VALENTINE D.W., SOULE M.E., SAMOLLOW P., 1973, *Asymmetry in fishes: a possible statistical indicator of environmental stress*. Fisheries Bulletin, **71**: 357-370.

VAN VALLEN L., 1962, *A study of fluctuating asymmetry*. Evolution, **16**: 125-142.

ZYGAR C.A., RUSSELL M.J., FERNSALD D., 1999, *Nasotemporal asymmetry during teleost retinal growth: preserving an area of specialization.* Journal of Neurobiology, **41**: 435-442.

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EFFECT OF ARSENIC ON HAEMOLYMPH CELLS OF SHORT HORNED GRASSHOPPER

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The effects of dietary sodium arsenate on the hemocyte counts of *Gesonula punctifrons* (Stal, 1861) (Orthoptera: Acrididae) were studied. High levels of arsenic are present in the ground water in a large part of West Bengal, India. This water is used both for irrigation and drinking purpose. Being a toxic metal in the natural environments, arsenic may be taken up by the plants and transferred further up the food chain. Evaluation of the haemocyte counts showed a significant relation with the doses of sodium arsenate used in the experiment for the first time. Arsenic contamination in the grasshopper *Gesonula punctifrons* can alter its haemocyte counts, which might lead to impairment of the grasshopper ability to combat maladies.

Key words: grasshopper, arsenic, sodium arsenate, haemocyte counts.

INTRODUCTION

Ground water in a large part of West Bengal delta contains a high level of arsenic compounds. This arsenic contained ground water is used for irrigation leaving a risk of accumulation of this toxic element in the soil and the possible exposure of the food chain through plant intake and animal consumption (Imanul Haq & Naidu, 2005). A rapid population increase has led to increasing rice production and the resulting increase in the use of irrigation water has mainly been provided by shallow aquifers, which are particularly rich in arsenic compounds. In a terrestrial ecosystem acridid grasshopper occupied a significant position in food chain, representing 20-30% of arthropod biomass (Schmidt, 1986). Depending on the toxicity level of heavy metal and the tolerance of the exposed organism, this species spectrum of a contaminated ecosystem would be changed. Malakar *et al.* (2009) studied the effect of Hg on the development of *Oxya fuscovittata* and reported that survival, adult body weight and adult life span significantly decreased. Grasshoppers are ecologically significant because they serve as major food source for some species, especially spiders, reptiles, birds and small mammals. According to Belovsky (1993), grasshopper reduction might harm decline or threatened species that depend on those insects as food. Among different trophic levels commonly present in many fresh water and terrestrial ecosystems, insects occupy the critical middle links (Thompson, 1984).

Despite these important roles, very little is known about how pollutant affects insects, possible because of insect diversity, ubiquitous distribution and perceived

lack of importance in most anthromorphic activity (Tremblay *et al.*, 1998). Effects of heavy metals like Hg, Cd, Pb, Cu, Cr have been conducted on acridid grasshoppers in both field and laboratory conditions. But very few works have been done so far on the effect of arsenic compounds on the grasshoppers. Keeping this particular aspect in view, the present study is being done.

MATERIAL AND METHODS

Freshly collected adult female grasshoppers of almost equal size *Gesonula punctifrons* (Stal, 1861) were collected from a fallow land near Salt Lake City, Kolkata. Female grasshopper was selected because both sexes could equally accumulate the heavy metals in their bodies (Devkota & Schimdt, 2000). They were acclimatized in laboratory conditions for 7 days in insectariums. Ten of the acclimatized grasshoppers were caged in transparent plastic jars (5 litre capacity) containing moist and thoroughly washed builders-sand (3.0 cm thick) at the bottom. Conical flask (50 ml) containing water along with leaves of *Cynodon dactylon* Pers. was supplied as food (Nath & Rai, 2010).

For the control experiments, untreated adults were fed on leaves grown in distilled water. For contamination arsenic salt (Sodium arsenate) was dissolved in distilled water along with leaves of food plant (Schmidt & Ibrahim, 1994) and kept for 24 hours. Then the food was supplied to the adult female grasshoppers for seven days observation. Concentrations of 0.0125 $mg.l^{-1}$ and 0.025 $mg.l^{-1}$ arsenic salt water were tested respectively.

After seven days Blood films were prepared by taking a small drop of haemolymph on a slide by clipping of the hind leg of adult grasshopper. The drop was then drawn into a thin film by the edge of another slide and the film air-dried before staining (Jalali & Salehi, 2008). Leishman's stain was used following the standard method used in human blood film preparation (Swarup *et al*., 1986). Counting was done in narrow longitudinal strips of the blood film starting from the one end film to the other end avoiding the lateral edges. While counting, the numbers of the different types of leucocytes were observed. Counting was done in ten grasshoppers.

Data were subjected to statistical analyses and r and t values were calculated (Zar, 2009).

RESULTS

Exposure of *Gesonula punctifrons* to various doses of sodium arsenate results in disturbance in hematological parameters. Sublethal concentrations of sodium arsenate significantly decrease the plasmocyte (PL) count at 0.0125 $mg.l^{-1}$

(7.8 ± 0.58, $p < 0.05$) and 0.025 $mg.l^{-1}$ (6.7 ± 0.55, $p < 0.05$) dosed grasshopper (Table 1).

Table 1

Haemocyte counts in control and treated *Gesonula punctifrons* after 7 days of exposure to various concentrations of arsenic salt

Cells	Normal	DC	0.0125 $mg.l^{-1}$	DC	0.025 $mg.l^{-1}$	DC
Plasmocyte	24.3±1.63	20.87%	7.8±0.58 r = 0.97 t = 10.78*	6.50%	6.7±0.55 r = 0.97 t = 10.78*	6.60%
Granulocyte	17±1.78	14.78%	44.5±1.05 r = 0.96 t = 9.70*	36.59%	26.7±0.63 r = 0.98 t = 13.93*	25.47%
Spherulocyte	17.6±0.89	15.65%	29.1±0.61 r = 0.96 t = 9.70*	23.58%	22.8±0.65 r = 0.97 t = 10.78*	21.70%
Prohemocyte	14.8±0.95	13.04%	5.5±0.83 r = 0.98 t = 13.93*	4.88%	1.8±0.40 r = 0.98 t = 13.93*	1.89%
Podocyte	6.8±0.51	6.09%	0	0.00%	1.1 ±0.30 r = 0.94 t = 7.79*	0.94%
Coagulocyte	14.6±0.85	13.04%	22.3±0.62 r = 0.94 t = 7.79*	17.89%	29.6±0.55 r = 0.95 t = 8.61*	28.30%
Oenocytoid	11.6±1.20	10.43%	8.5±0.45 r = 0.96 t = 9.70*	7.32%	8±0.84 r = 0.96 t = 9.70*	7.55%
Reticulocyte	7±0.57	6.09%	3.6±0.41 r = 0.96 t = 9.70*	3.25%	7.5±0.52 r = 0.96 t = 9.70*	7.55%

*Experimental value is significantly different from control ($p < 0.05$).
Each value is mean of ten observations ±SE.

Podocyte (PO) count in non-exposed grasshopper was 6.8 ± 0.51, whereas it was absent in 0.0125 $mg.l^{-1}$ dosed grasshopper and present in negligible number in 0.025 $mg.l^{-1}$ dosed ones.

Granulocyte (GR) count was increased in 0.0125 $mg.l^{-1}$ dosed grasshopper (44.5 ± 1.05, $p < 0.05$; 36.59%) and decreased at 0.025 $mg.l^{-1}$ of sodium arsenate (26.7 ± 0.63, $p < 0.05$; 25.47%). Spherulocyte (SP) showed similar trend as in granulocyte count.

Prohemocyte (PR) and oenocytoid (OE) exhibited a decreasing trend with the increase of doses.

Coagulocyte (CO) and reticulocyte (RT) showed an increasing trend of cell count with increasing doses of sodium arsenate.

DISCUSSION

Phagocytosis usually takes place by plasmocytes. Non-pathogenic and weakly pathogenic organisms are dealt with effectively in this way (Chapman, 2004). The study revealed that plasmocyte count was decreased with increasing doses indicating the effect of sodium arsenate on plasmocyte production.

The study also revealed that podocyte count decreased with increasing doses of arsenic. Gupta and Sutherland (1966) suggested that podocyte is transferred from plasmocytes. When podocytes increase in differential counts, plasmocytes decrease (Rizki, 1962). The present study also revealed similar observations in arsenic dosed grasshopper indicating the effect of sodium arsenate on the production of plasmocytes.

With increasing amount of arsenic compounds in the food, prohemocyte count decreased. Prohemocyte is the stem cell, from which most other hemocyte types are formed. A decreasing trend of this cell count indicated a negative effect in the present study.

Granulocytes discharge their contents at the early part of defense response. The present study revealed that at low dose (0.0125 $mg.l^{-1}$) granulocytes out-numbered the normal count. Whereas, at high dose (0.025 $mg.l^{-1}$) the number again came low.

The present study represented high differential count of granulocytes at 0.0125 $mg.l^{-1}$ (36.59%, $r = 0.96$, $t = 9.70$) and 0.025 $mg.l^{-1}$ (25.47%, $r = 0.98$, $t = 13.93$) dosed grasshoppers. This indicates a response of granulocyte to sodium arsenate affected hemolymph.

Ratcliffe & Rowly (1979) reported that hemocyte which participated in phagocytosis can adsorb tracer substances by phagocytosis. The increase in the phagocytic hemocyte (PL, GR, and SP) counts also indicated a similar response in the presence of arsenic contamination. The perturbations in these haemocyte counts attributed to a defense reaction against toxicity of sodium arsenate through the stimulation of erythropoesis or may be due to the disturbances that occurred in both metabolic and haemopoietic activities of this grasshopper exposed to sublethal concentration as was observed in fish by Sasikala *et al*. (2011).

Due to continuous use of sodium arsenate contaminated water, it may accumulate in soils, decrease soil fertility, can be taken up by plants and thereby enter the food chain (Meharg & Rahman, 2003). Being herbaceous (primary consumer) and being preyed upon by other insectivorous vertebrates and arthropods, this arsenic compound affected grasshopper may play a significant role in accumulating and further transferring toxic metals to higher trophic levels in the

nature as was observed by Devkota & Schmidt (2000) in case of other heavy metals.

CONCLUSION

A significant relation was observed during evaluation of the haemocyte counts with the doses of sodium arsenate used in the experiment. Arsenic contamination in the grasshopper *Gesonula punctifrons* can alter its haemocyte counts, which might lead to impairment of the grasshopper ability to combat maladies.

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REFERENCES

BELOVSKY G.E., 1993, *Modeling avian foraging: implications for assessing the ecological effects of pesticides*. In: Kendall R.-J., Lacher T.E. Jr. (Eds), *Wildlife Toxicology and Population Modelling*: *Integrated studies of Agroecosystems*, pp.: 131-145, CRC Press, Inc., Boca Raton.

CHAPMAN R.F., 2004, *The Insects Structure and Function.* 4th Edn., Cambridge University Press, Cambridge, pp. 123-124.

DEVKOTA B., SCHIMDT G.H., 2000, *Life span and fecundity of Aiolopus thalassinus exposed to dietary heavy metals (Hg, Cd, Pb)*. Bollettino dl Zoologia Agraria e Bachicoltura Ser. II, **32** (2): 119-134.

GUPTA A.P., SUTHERLAND D.J., 1966, *In vitro transformations of the insect plasmatocyte in some insects*. Journal of Insect Physiology, **12**: 1369-1375.

IMANUL HAQ S.M., NAIDU R., 2005, *Arsenic in ground water and contamination of the food chain: Bangladesh scenario*. In: *National Arsenic Ground Water: Occurrence, Remediation and Management Balkema*, New York, pp. 95-101.

JALALI J., SALEHI R., 2008, *The Hemocyte types, differential and total count in Papilo demoleus L. (Lepidoptera: Papilionidae) during post embryonic development*. Munnis Entomology & Zoology, **3** (1): 199-216.

MALAKAR C., GANGULY A., HALDAR P., 2009, *Influence of Cadmium on Growth, Survival and Clutch size of a Common Indian Short Horned Grasshopper, Oxya fuscovittata*. American Eurasian Journal of Toxicological Science, **1** (1): 32-36.

MEHARG A.A., RAHMAN M.M., 2003, *Arsenic Contamination of Bangladesh Paddy field soils: implications for rice contribution to arsenic consumption*. Environmental Science and Technology, **37**: 229-234.

NATH S., RAI A., 2010, *Study of life table of Ceracris nigricornis laeta (Orthoptera: Acrididae) in laboratory condition*. Romanian Journal of Biology – Zoology, **55** (2): 159-165.

RATCLIFFE N.A., ROWLEY A.F., 1979, *Role of hemocytes in defense against biological agents*. In: A.P. Gupta (Ed.), *Insect Hemocytes*, pp. 331-414, Cambridge University Press, Cambridge.

RIZKI M.T.M., 1962, *Experimental analysis of hemocyte morphology in insects*. American Zoologist, **2**: 247-256.

SASIKALA G., PALANISAMY P., MALLIKARAJ D., BHUVANESHWARI N., NATARAJAN G. M., 2011, *Metasystox Induced Haematological Modulation in the South Indian*

Snakeheaded Chann striata. International Journal of Pharmaceutical & Biological Archives, **2** (2): 775-777.

SCHMIDT G.H., 1986, *Use of grasshoppers as test animals for the ecotoxicological evaluation of chemicals in the soil*. Agriculture Ecosystems and Environment, **16**: 175-188.

SCHMIDT G.H., IBRAHIM N.M.M., 1994, *Heavy metal content (Hg^+, Cd^+, Pb^+) in various body parts: Its impact on Cholinesterase activity and binding glycolprotein in grasshopper Aiolopus thalassinus adults*. Ecotoxicology & Environmental Safety, **29**: 148-164.

SWARUP N., ARORA S., PATHAK S.C., 1986, *Laboratory Techniques in Modern Biology*. Kalyani Publishers, New Delhi , pp. 191-192.

THOMPSON J.N., 1984, *Insect diversity and the trophic structure of communities*. In: Huffaker C.B., Rabb R.L. (Eds), *Ecological Entomology*, pp.: 591-606, John Wiley and Sons, New York.

TREMBLAY A., CLOUTIER L., LUCOTTE M., 1998, *Total mercury, methyl mercury fluxes via emerging insects in recently flooded hydroelectric reservoirs and a natural lake*. Science of the Total Environment, **219**: 209-221.

ZAR J.H., 2009, *Biostatistical Analysis*. 4th Edn. Pearson Education, New Delhi, India, pp. 377.

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THE FERRITIN STORAGE AND CEROID PLATES GENESIS IN KUPFFER CELLS FROM IRON OVERLOADED RATS

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This paper presents the ceroid genesis in the liver of iron overloaded rats. The ceroid plates were formed in the secondary lysosomes under the oxidative action of iron. The material submitted to the iron oxidative stress was furnished especially by the damaged membranes of the organelles of Kupffer and giant cells. This fact was illustrated by transmission electron microscopy (TEM) images. A succinct review on some recent experiments demonstrating the role of intra-lysosomal iron-catalysed peroxidation for the formation of the ceroid and lipofuscin pigments was also presented.

Key words: ceroid plates, ferritin, haemosiderin, iron-loading, Kupffer cells.

INTRODUCTION

A survey on the references with subject "ceroid" demonstrated a large interest about cellular pigments. A great number of papers were dedicated to cellular accumulations of ceroid pigment during pathological cases due to inborn errors of lipid metabolism. Other studies were related on the ceroid occurrence in the cytoplasm of various cellular types during different experimental models.

Ultrastructural observations on ceroid morphogenesis in Kupffer cells were realized after experimental intravenous injections of cod liver oil emulsion in rat (Kajihara *et al*., 1975 a, b.). Observations on the liver of an infant who received lipid nutrition by repeated intravenous inoculations revealed the occurrence of ceroid pigment in Kupffer cells (Van Haelst & Sengers, 1976).

Totović *et al*. (1980) realized an electron microscopic study on the Kupffer cells which were in the condition to realize an intensive erythrophagocytosis. The authors presented data on the endocytosis sequences, the relation of erythophagasomes with the primary lysosomes, the appearance of the oxidized and polymerized lipid from erythrocyte stroma and verified the identity between this product and ceroid pigment by the light microscopy and histochemical methods.

An experiment on the cultured human glial cells exposed to purified rat liver mitochondria aimed to know the origin of ceroid and lipofuscin pigments (Brunk, 1989). It was found that an incomplete degradation of mitochondria happened inside the secondary lysosomes and that the residual bodies contained iron.

Other experimental model aimed to simulate lipofuscinogenesis "in vitro" using cystein to stimulate the oxidative stress in the rat liver lysosomal-

mitochondrial fraction (Yin *et al*., 1995). The lipid membraneous components were submitted to peroxidation process with the formation of a residual material, similar to the material contained in the secondary lysosomes. The addition in the test-tube of an iron chelator (Desferal) led to complete inhibition of the peroxidation process, proving the main role of iron in the oxidative stress.

An experiment "in vivo" described the ceroid accumulation in the kidney and Kupffer cells of the rats, as the consequence of the lipid peroxidation using ferric nitriloacetate inoculations (Shimasaki *et al*., 2002).

Other research demonstrated that low density lipoprotein (LDL) endocytosed by mouse macrophages and human monocytes-derived macrophages presented an advanced oxidative process within the lysosomes. By confocal microscopy, the ceroid pigment was detected. This experiment represented the basis for the oxidized LDL hypothesis of atherosclerosis, which proposes that LDL undergoes oxidation in the interstitial fluid of the arterial wall (Wen and Leake, 2007).

Recently, an extensive review (Kurz *et al*., 2008) presented detailed molecular mechanisms developed for the ceroid and lipofuscin pigments genesis inside the secondary lysosomes.

It is interesting to mention a recent paper which studied the origin of brown pigments occurred in lipogranuloma of the canine liver in natural conditions (Isobe *et al*., 2008). Hepatic lipogranuloma were formed of Kupffer cells and/or macrophages presenting cytoplasmic brown pigments (haemosiderin and ceroid). Such structures were encountered relatively frequently in canine liver indifferently of sex, breed or age, the pathogenesis of this lesion remaining unclear.

The present paper aimed to describe the ceroid occurrence in the Kupffer cells and giant multinucleated cells of iron overloaded rats, as the direct effect of iron on the lipoprotein component of organelles membranes.

MATERIAL AND METHODS

Animals. 12 young adult male rats weighing 100±10 g received during a month, intra-peritoneally (ip) injections of Ferrum Hausmann, as iron III-hydroxide polymaltose complex, at intervals of three days, till the loading of 2 mg Fe/g body weight. The animals were sacrificed between 2-14 months from the beginning of the experiment. Controls were inoculated with saline and sacrificed at the same time with the animals of the experiment.

Histology. The liver samples were fixed in 8% formaldehyde in saline and processed according to standard technique, for the embedding in paraffin. 5 μm thick histological sections were stained with Hemalum-Eosin for structures observations. The Perls method made evident the presence of iron deposits of hemosiderin; Sudan B and Ziehl-acid fast methods made evident the presence of

ceroid pigment (Mureşan *et al.*, 1976). The autofluorescence of the ceroid pigment was observed in the UV light.

Electron microscopy. Small liver pieces were fixed in 2.5 % glutaraldehyde in sodium cacodylic acid salt buffer pH 7.2, for 24 h at 4 ºC. The samples were washed with cold buffer and then postfixed with 1.3 % Os O_4 in the same buffer, in the dark and at room temperature, for 2 h. The samples were dehydrated in ethyl alcohols of growing degrees concentrations and processed for the embedding in Epon 812, according to standard techniques. The ultrathin sections were contrasted with uranyl acetate and lead citrate. The ultrastructural observations were performed with TEM – Jeol 100.

RESULTS

Early during the iron treatment, the Kupffer cells and the hepatocytes became Perls-positive. The characteristic blue staining for the ferric iron presence was observed, in a decreasing gradient from the first to the third area of the hepatic acinus.

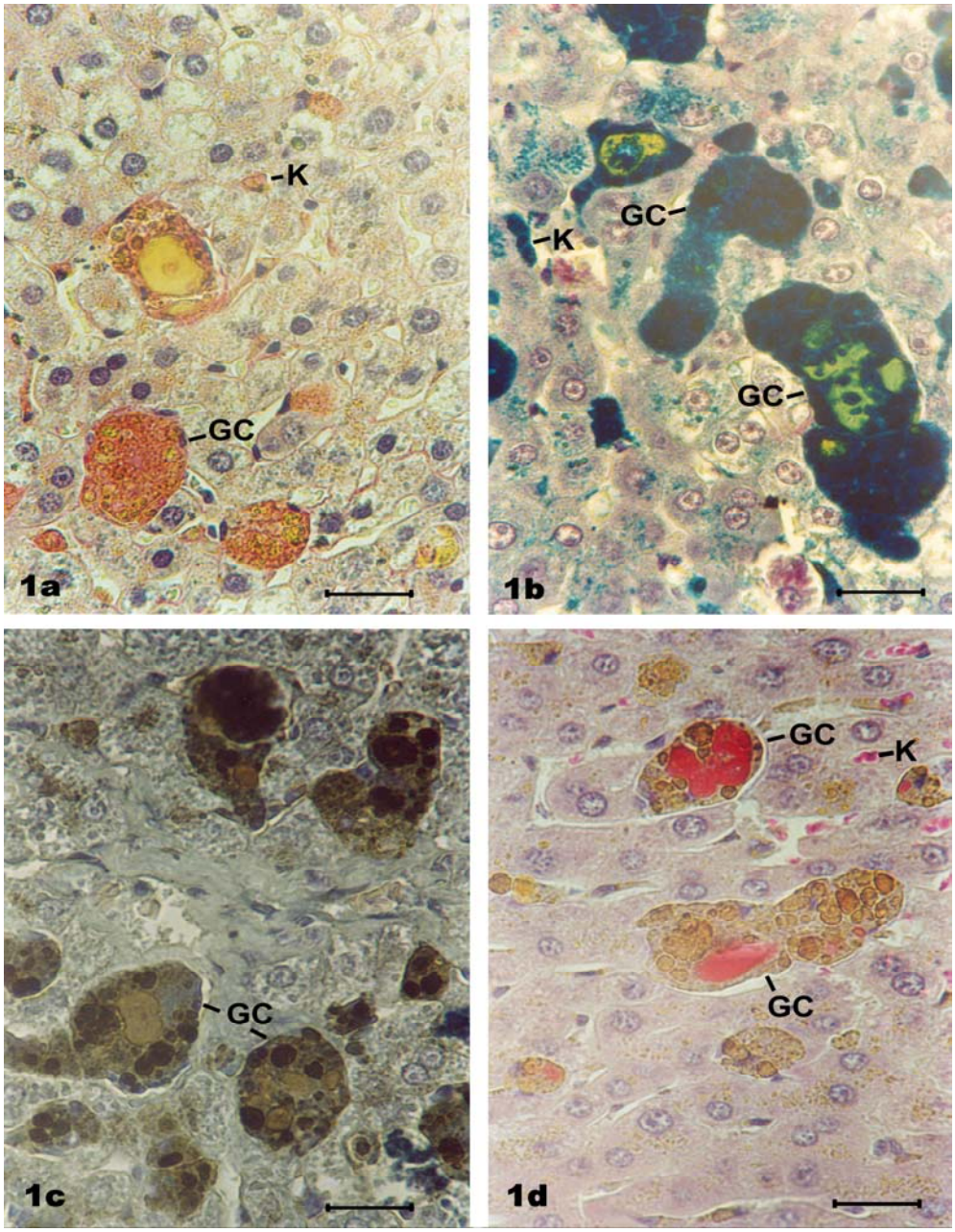
After the treatment ceasing, the most characteristic feature observed in the rat liver was the presence of numerous cellular aggregates (Fig. 1a) placed under Glisson capsule, in the spaces of the great portal veins and in the centrilobular zones. These cellular aggregates were Perls-positive (Fig. 1b) and presented a long persistency in time.

Some yellow round or ovoid structures (Fig. 1a), were made evident in the cytoplasm of Kupffer cells and/or the iron giant cells at 4-6 months from the ceasing of the iron treatment. These structures of 0.5-5 μm diameter were ceroid plates, presenting a variable degree of iron embedding (Fig. 1b). Specific cytochemical stainings to identify the ceroid have been applied (Table 1) (Figs. 1c,d). The ceroid presence was confirmed also by its golden-brown auto-fluorescence in the UV light.

Table 1

Cytochemical stainings used for ceroid identification

Specific cytochemical staining	Results (reaction colour)	Specific product	Illustration
Hemalum-Eosin	bright-yellow	ceroid	Fig. 1a
Perls method	green	ceroid embedded with ferritin	Fig. 1b
Sudan B	blue-black	ceroid	Fig. 1c
Ziehl-acid fast	bright-red	ceroid	Fig. 1d
UV light	golden-orange	ceroid	not shown



Figs. 1a –1d. Histological sections through the iron overloaded rat liver (Scale bar, 20 µm).
– 1a – Iron giant cells (GC) and Kupffer cells (K) with ceroid plates (CP). H-E.
– 1b – Ceroid plates were more or less ferritin loaded. Perls-H.
– 1c – Sudan black: ceroid plates were blue to black.
– 1d – Ziehl: ceroid plates were bright red.

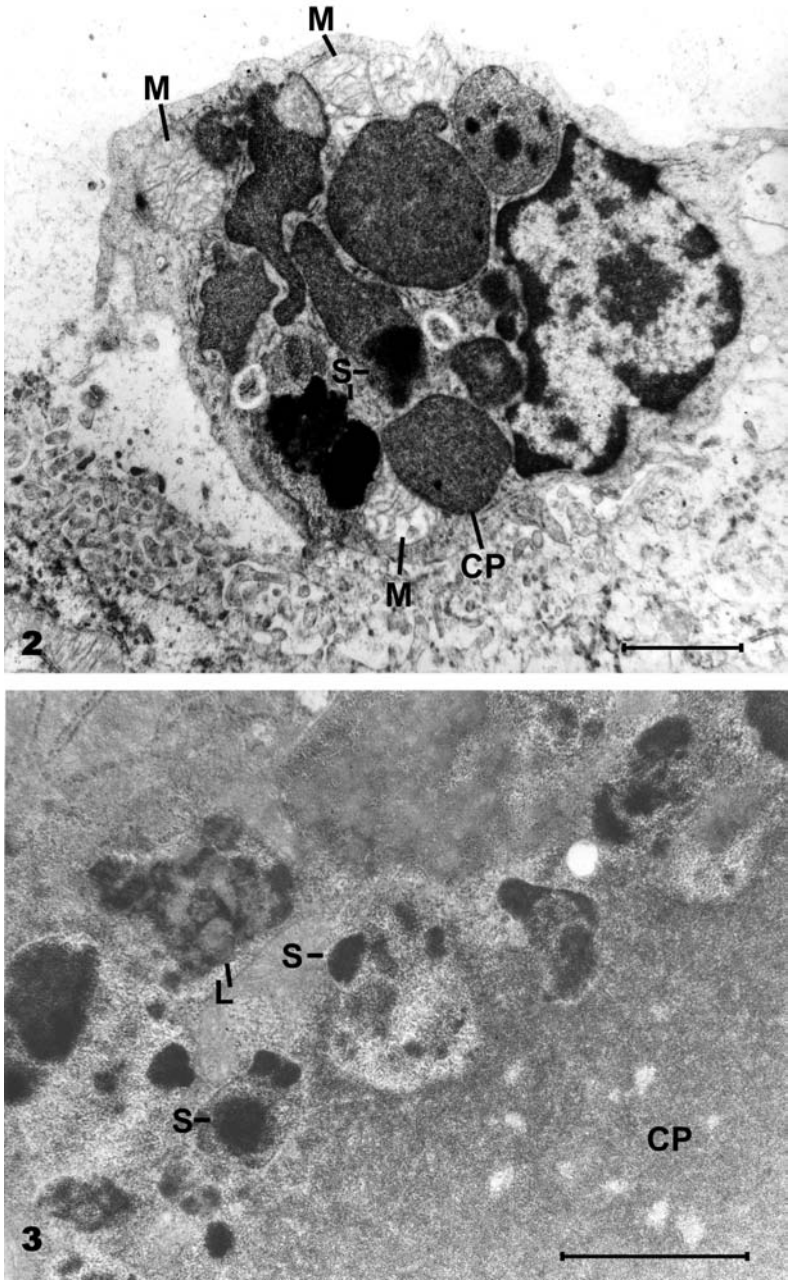
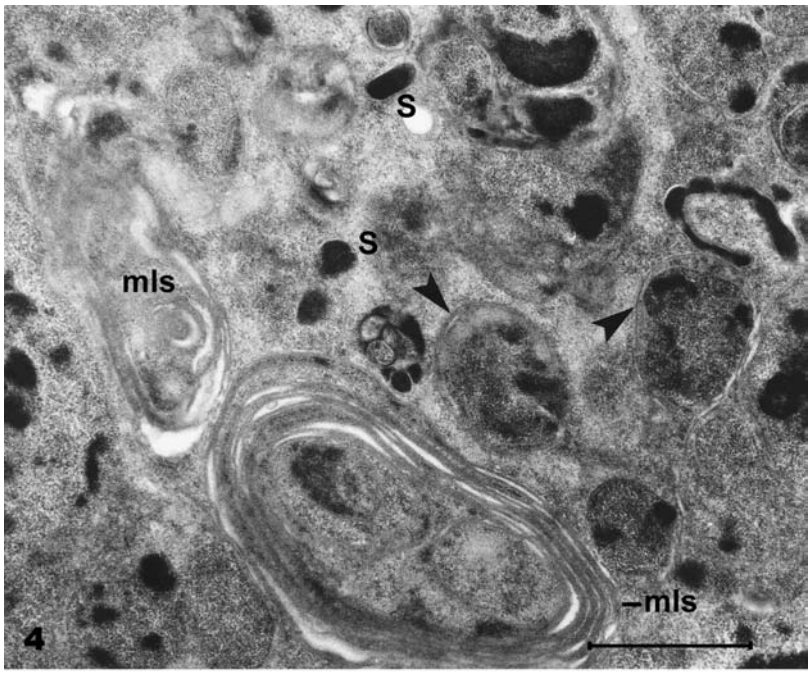


Fig. 2. Kupffer cell presenting electron dense siderosomes (S) and ceroid plates (CP) embedded with ferritin molecules. Mitochondria (M) were ferritin free (Scale bar, 1 μm).

Fig. 3. Iron giant cell (detail). A ceroid plate (CP) near many complex structures composed of lipids (L) and electron dense clumps of haemosiderin in siderosomes (S) (Scale bar, 1 μm).



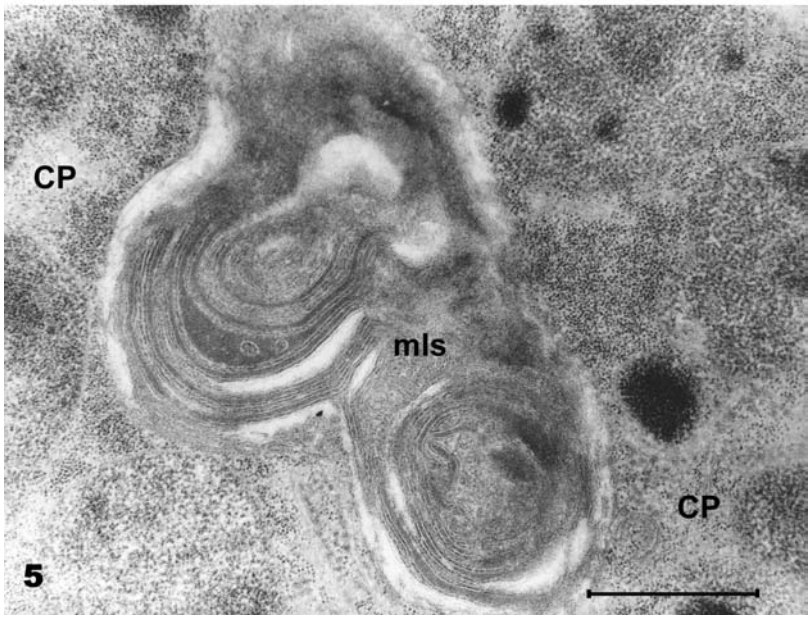


Fig. 4. Myelin-like structures (mls) and complex structures limited by a single membrane (arrow head) presenting ferritin molecules and electron dense haemosiderin in siderosomes (S) (Scale bar, 1 μm).
Fig. 5. Myelin-like structures (mls) near ceroid plates (CP) embedded with ferritin molecules in different densities (Scale bar, 0.5 μm).

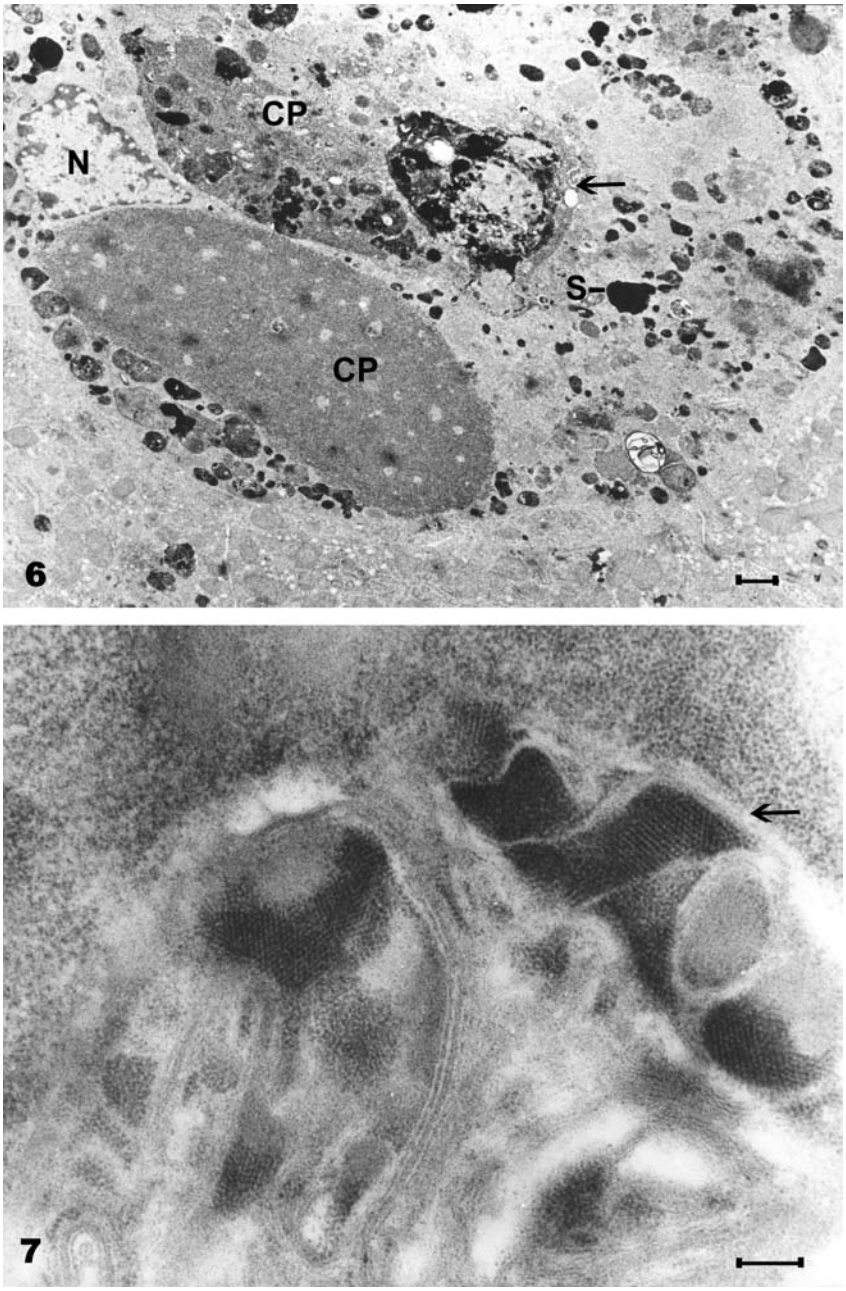


Fig. 6. Two ceroid plates in an iron giant cell (detail). One plate in formation (arrow) was loaded with electron dense siderosomes (S). Note a nucleus (N) of the giant cell (Scale bar, 1 μm).
Fig. 7. Ceroid plate (detail). Note the ferritin molecules organized in a paracrystalline arrangement (Scale bar, 0.1 μm).

Kupffer cells loaded with siderosomes and ceroid granules covered with ferritin molecules were frequent on the electron microscopic micrographs (Fig. 2).

The giant cells cytoplasm presented siderosomes heavy loaded with clamps of haemosiderin. Other siderosomes appeared associated with lipids in larger autophagolysosomes (Fig. 3). Sometimes, these secondary lysosomes appeared limited by a single membrane. The ceroid plates were observed near or inside autophagolysosomes containing myelin-like structures. These structures known also as "finger imprints" were multilayered packed damaged membranes. The lipid component of these membranes was osmiophilic and sometimes appeared covered with ferritin molecules (Figs. 4-5).

The ceroid plates presented characteristic circular or ellipsoidal profiles, devoid of any structure, which may be limited or not by a membrane (Figs. 2, 6). Some ceroid plates in course of formation were placed in close proximity with the siderosomes or secondary lysosomes containing damaged organelles (Fig. 6). The gradual transition from the intra-lysosomal damaged membraneous structures towards the ceroid plates may be observed (Figs. 3-6). The ceroid plates were often embedded with molecules of ferritin, forming areas of different densities (Fig. 5).

Generally, the ferritin molecules were randomly spread in the giant cells cytoplasm. Sometimes, ferritin clusters geometrically organized in paracrystalline structures may be observed in some zones of the compound siderosomes (Fig. 7).

DISCUSSION

During and after iron chronic loading, in the rat liver architecture many structural changes were observed. A morphological modification was the occurrence of the great cellular agglomerations placed in different zones of the liver. These Perls-positive cellular agglomerations were composed of the iron loaded Kupffer cells expelled from the sinusoidal endothelium and of giant cells appeared from the syncytialization of such Kupffer cells (Prunescu & Prunescu, 1988).

Inorganic iron presents a great oxidative potential for organs and tissues. At the same time, inorganic iron is an essential element for the existence of living cells. Therefore, during their evolution, the living cells have found the modalities to prevent the iron toxic effects. In animal organisms, the inorganic iron is combined with protective carrier proteins (transferrin and transferrin receptors), and with a protein (apoferritin) which makes the inorganic iron able to be stored in the cells as ferritin and haemosiderin (Crichton, 1991; Iancu, 1992).

The exogenous iron inoculated in great quantities as polymaltosed iron was stored in the liver and in other parenchymal organs, as ferritin and haemosiderin. In Kupffer cells and iron giant cells, the haemosiderin considered as a particularly iron-rich form of lipofuscin (Kurz *et al.*, 2008) was stored in siderosomes,

following the excess iron processing in the secondary lysosomes. The ferritin molecules were spread in the cytoplasm as diffuse clouds or in form of clusters lacked of membrane, forming more or less dense molecular agglomerations.

A contribution to the understanding of the iron pathogenesis was the discovery of the labile iron pool (LIP) which is an iron species lacked of protective proteins. It is a transitory iron form of very short life occurred in the cytoplasm. In the cytosol, it rapidly becomes associated with natural chelators (Crichton, 2001; Kurz *et al.*, 2008).

In the condition of iron overloading known in pathological cases or in experiments of iron loading, the labile iron pool presented in cytosol increased concentrations of about 10 times higher in comparison with the controls (Zanninelli *et al*., 2002). This iron becomes a real danger for the cellular integrity and homeostasis, because it has a great capacity to initiate the production of free radicals causing cellular damage (Crichton *et al*., 2002).

The oxidative stress mediated by the iron is implicated in the generation of reactive oxygen species (ROS) which attack the bio-molecules directly, with the enhancement on the lipid membranes peroxidation, DNA damage and protein oxidation (Crichton, 1991; Myiazaki *et al*., 2002).

During the present experiment, it is possible that the accumulation of a great quantity of labile iron in the cytosol of the iron overloaded Kupffer cells or iron giant cells, to lead to the oxidative reactions on the organelles membranes producing lesions of long duration or even permanently.

The multiplication of the secondary lysosomes containing myelin –like structures and the significant ferritin and haemosiderin accumulations represented aspects of a cycle in which the damaged cellular structures were associated with siderosomes and seemed to generate ceroid masses in a continuous growing.

The ceroid plates are the ultimate products from the lipoprotein membranes oxidation; the ceroid plates representing inert stores.

REFERENCES

BRUNK U.T., 1989, *On the origin of lipofuscin; the iron content of residual bodies and the relations of these organelles to the lysosomal vacuom. A study on cultured human glial cells*. Adv. Exp. Med. Biol., 266: 313-320.

CRICHTON R.R., 1991, Chpt. 11: *Iron and Oxidative Damage*, pp.: 190-213. In: *Inorganic Biochemistry of Iron Metabolism*, Ellis Horwood, Ltd., 1991, 263 pp.

CRICHTON R.R., 2001, *Inorganic Biochemistry of Iron Metabolism.(From Molecular Mechanisms to Clinical Consequences)*. Second Edition, John Wiley and Sons, Ltd., 2001, 319 pp.

CRICHTON R.R., WILMET S., LEGSSYER R., WARD R. J., 2002, *Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells*. J. Inorg. Biochem., **91** (1): 9-18.

Van HAELST U.J., SENBERG R.C., 1976, *Effects of parenteral nutrition with lipids on the mammalian liver. An electron microscopic study*. Virchows Arch. B. Cell Pathol, **22** (4): 323-332.

IANCU TH. C., 1992, *Ferritin and hemosiderine in pathological tissues*. Electron Microsc. Rev., **5**: 209-229.

ISOBE K., NAKAYAMA H., UETSUKA K., 2008, *Relation between lipogranuloma formation and fibrosis, and the origin of brown pigments in lipogranuloma of the canine liver*. Comparative Hepatology, **7** (5): 1-6. Available at: http://www.comparative-hepatology.com/content/7/1/5

KAJIHARA H., TOTOVIĆ V., GEDIGK P., 1975 a, *Ultrastructure and morphogenesis of ceroid pigment. I. Phagocytosis and formation of lipid containing after intravenous injection of unsaturated lipids* (in German). Virchows Arch. B. Cell Pathol., **19** (3): 221-237, (in German).

KAJIHARA H., TOTOVIĆ V., GEDIGK P., 1975 b, *Ultrastructure and morphogenesis of ceroid pigment.. II. Late changes of lysosomes in Kupffer cells of the rat after phagocytosis of unsaturated lipids.* Virchows Arch. B. Cell Pathol., **19** (3): 239-254.

KURZ T., TERMAN A., GUSTAFSSON B., BRUNK U.T., 2008, *Lysosomes in iron metabolism ageing and apoptosis*. Histochem Cell Biol, **129** (4): 389-406.

MYIAZAKI E., KATO J., KOBUNE M., OKUMURA K., SASAKI K., SHINTANI N., AROSIO P., NIITSU Y., 2002, *Denaturated H-ferritin subunit is a major constituent of hemosiderin in the liver of patients with iron overload*. Gut, **50** (3): 413-419.

MUREŞAN E., BOGDAN A.T., GABOREANU M., BABA A.I., 1976, *Normal and Pathological Histochemical Technic*s. Edit. Ceres, Bucharest, 416 pp. (in Romanian).

PRUNESCU C.-C., PRUNESCU P., 1988, *Giant multinucleated iron-containing cells in the liver of polymaltosed iron injected rats*. Rev. Roum. Biochim., **25** (4): 355-362.

SHIMASAKI H., SHIMIZU Y., DILIDAR D., UETA N., SHIGA J., 2002, *Ceroid accumulation in rat kidneys caused by uptake of ferric nitrilotriacetate*. Arch. Gerontol. Geriatr., **34** (3): 185-191.

TOTOVIĆ V., GEDIGK P., WANG T.N., PINO G., 1980, *Ultrastructural morphogenesis of lipids and iron containing pigments in Kupffer cells of the rat after erythrophagocytosis*. Virchows Arch. B Cell Pathol. Incl. Mol. Pathol., **34** (2): 123-141 (in German).

WEN Y., LEAK D.S., 2007, *Low density lipoprotein undergoes oxidation within lysosomes in cells*. Circ. Res., **100** (9): 1337-1343.

YIN D., YUAN X., BRUNK U.T., 1995, *Test tube simulated lipofuscinogenesis. Effect of oxidative stress on autophagocytic degradation*. Mec. Ageing Dev., **81** (1): 37-50.

ZANNINELLI G., LOREAL O., BRISSOT P., KONIJN A.M., SLOTKI Z.N., HIDER R.C., IOAV CABANTCHIK Z., 2002*, The labile iron pool of hepatocytes in chronic and acute iron overload and chelator induced iron.* J. Hepatol., **36** (1): 39-46.

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OXIDATIVE STRESS IN FRESHWATER BIVALVE OF INDIA EXPOSED TO AZADIRACHTIN BASED PESTICIDE

SUMAN MUKHERJEE*, MITALI RAY**, SAJAL RAY**

Lamellidens marginalis (Mollusca: Bivalvia: Eulamellibranchiata) is a freshwater edible mollusc distributed in the wetland of different districts of West Bengal. Natural habitat of the species is under risk of contamination by multineeem, a relatively newly introduced azadirachtin (limonoid) based pesticide in India. Haemocytes of *L. marginalis* are an immunoeffector cell of haemolymph which is capable of discrimination self and nonself surface, phagocytosis of foreign particles and production of cytotoxic molecules as an antimicrobial agent. Superoxide anion was estimated against 0.006, 0.03. 0.06 and 0.09 ppm of azadirachtin for different spans of exposure. Activities of superoxide dismutase and catalase were estimated for same concentration and span of exposure to study any possible impairment in antioxidant defence against toxic reactive oxygen species. Activity of superoxide anion was increased under all the concentrations of azadirachtin with a highest activity at 0.09 ppm for 7 days of exposure. Activity of catalase in haemocytes expressed a marked depletion against all concentrations and span of exposure to azadirachtin with a maximum depletion as 0.075±0.002 μM/mg protein/min for 0.09 ppm/7 days of exposure. Activity of superoxide dismutase was depleted against all concentrations and span of exposure to azadirachtin with a highest depletion as 1± 0.08 unit/mg protein/min for 0.09 ppm/ 7days of exposure against the control value of 16±0.4 unit/mg protein/min. Increment of activity of SOA and parallel decrease in the activities of SOD and catalase in the haemocytes appeared to be detrimental for survival of *L. marginalis* in the azadirachtin contaminated environment. Data is indicative of cellular oxidative stress in *L. marginalis* that may lead to decline population size in its natural habitat.

Key words: haemocyte, azadirachtin, reactive oxygen species, superoxide dismutase, catalase.

INTRODUCTION

The freshwater wetland systems of India support a rich biodiversity of molluscs. *Lamellidens marginalis* (Mollusca: Bivalvia: Eulamellibranchiata) is a freshwater filter feeding mollusc and a common dietary item of the rural mass of eastern India and a well recognised poultry feed. The species is harvested indiscriminately from the natural habitat. Occasionally, natural pearl is formed in the species which emphasises its commercial potentiality.

Moreover, *L. marginalis* is an important biological component of a freshwater ecosystem and an efficient bioaccumulator of toxin residues. Being a filter feeder, this species performs biological filtration of ambient water and keeps the water body biologically safe for other species. Azadirachtin is a natural

bioactive agent derived from the seeds of the neem tree *Azadirachtin indica*. Azadirachtin is a triterpenoid toxin and expresses insecticidal activity against a broad range of insect pests (Schmutterer & Singh, 2002). 'Multineem' (Multiplex India Pvt. Ltd.) is an azadirachtin based biopesticide which is being recently used by the Indian farmers for the purpose of protection of crops from pests. Insecticide applications to agricultural field may result in some of the product entering nearby fresh water bodies and it is therefore important to ensure that these insecticides do not post a risk of adverse effects on nontarget organisms like *L. marginalis*.

Small fresh water bodies are particularly susceptible to contamination by agricultural run off during monsoon and from inadvertent overspray. Haemocytes, the chief circulatory cells of blood of Bivalvia, play an important role in elicitation of immune response under the exposure of xenobiotics (Chen & Bayne, 1995).

Haemocytes are reported to be chief immuno-effector cells of the blood of bivalve which are capable of performing nonself surface adhesion, phagocytosis of particles, nodule formation and cytotoxicity (Sauve *et al*., 2002). On stimulation by foreign particles or humoral substances, haemocytes generate intracellular superoxide anion radical, a reactive defence molecule against intruding microorganisms (Vijayavel & Balasubramanian, 2006).

Strategy of oxygen radical mediated killing is based on the premises of toxicity evolved in high concentration of molecular oxygen and most of the oxygen derived molecules are extremely toxic for parasite, pathogen and self tissue (Galloway & Depledge, 2001). Aquatic organisms can serve as indicators for a variety of pollutant exposures related to biochemical oxidative stress. Thus, it provides sensitive biochemical marker for exposure and toxicity of xenobiotics (Regoli & Principato, 1995). Several reactive oxygen species occur as a result of normal oxygen metabolism, but can be produced in large quantities during toxicant-induced interactions, leading to intrahaemocyte oxidative stress (Vijayavel *et al*., 2009).The extent, to which such biological damage occurs, depends on the effectiveness of antioxidant defences to remove reactive oxygen species. Thus, oxyradical production ultimately poses a threat to the fitness and health of organisms (Rodriguez & Moullac, 2000).

In this study, superoxide anion radical was estimated as a representative of reactive oxygen species. Superoxide dismutase and catalase represent the main enzymatic defences against reactive oxygen species. For the study of xenobiotic induced impairment in relation to biochemical adaptive response, activities of antioxidant enzymes like superoxide dismutase and catalase were quantified in haemocytes. Chakraborty *et al.* (2012) reported toxicity of arsenic in the total haemocyte density and heart of *L. marginalis* and measured the toxin induced alteration of dimension of cardiac tissue by occular micrometer. Aggregation and chemical induced interference of haemocytes of *L. marginalis* was claimed as biomarker of aquatic pollution (Mukherjee *et al*., 2011). In a review Ray *et al*. (2011) highlighted the significance of pearl forming mussel as indicator of aquatic toxicity of diverse xenobiotics.

MATERIAL AND METHODS

Collection and treatment of animal. The adult healthy *L. marginalis* with shell size of 7-8 cm were manually collected from the selected wetlands of the district of South 24 Parganas of West Bengal. Prior to experimentation, animals were acclimatized for 15 days in the laboratory. During acclimatization, *L. marginalis* were maintained in aquaria with fresh supply of pond water with temperature of 29 °C ± 3 °C and the animals received uniform ration of illumination. During the course of acclimatization and experiment, the animals were fed with chopped *Hydrilla* sp. and common aquatic weeds (Raut, 1991). Aqueous solutions of Multineem (Multiplex, India Private Limited, Azadirachtin E.C. 0.03%) formulations were prepared in Borosilicate glass containers with azadirachtin concentrations of 0.006, 0.03, 0.06, and 0.09 ppm. The pH of the solution was maintained at 7.2. Each experimental set consisted of 10 animals of same shell length. Animals were exposed to a volume of 5 litre of pesticide solution for varied span of exposure, *i.e.* 1, 2, 3, 4, 7, 15 and 30 days along with control. The experiments were carried out in static water environment.

Nitrobluetetrazolium reduction assay (cytochemical localization)

Haemolymph was collected from the posterior adductor muscle at 4 ºC (Brousseau *et al*., 1999). A part of the freshly collected haemolymph was placed on ethanol cleaned coverslips and allowed to adhere for 30 mins at room temperature in a moist chamber. Haemocyte monolayers were incubated at room temperature for two hours with 1 mg/ml NBT solution (Anderson *et al*., 1992) following replacement by sterile snail saline. The coverslips were inverted, placed on slides and examined as wet mounts. Phase contrast image analyses of haemocyte monolayer were carried out microscopically fitted with camera.

Superoxide anion estimation. Superoxide anion productions by haemocytes were determined by a modified method of Bell & Smith (1993). The resulted deposition of formazan was estimated spectrophotometrically. Assay consisted of 1 ml of freshly collected haemolymph suspension (1×10^6 cells/ml) in a test tube and allowed to react with 1 ml of NBT solution (0.03 %) for 30 mins at 37 °C. The reaction was terminated by removing the NBT solution and addition of absolute methanol. After proper washings with 70 % methanol, the cells were treated with a solution of KOH (1 ml, 2 M) and DMSO (1 ml) to dissolve the cytoplasmic formazan. The optical density of the dissolved formazan was estimated spectrophotometerically (Cecil, Germany) at 630 nm. The superoxide anion generation was expressed as absorbance (optical density) at 630 nm/min/10^6 cells.

Preparation of haemocyte lysate. Animals were bled aseptically and the haemolymph was collected from posterior adductor muscle by a sterile syringe with needle (Brosseau *et al*., 1999). The bleeding and collection procedure was carried out at 4°C to prevent cell aggregation. Haemocytes were sedimented by centrifugation of haemolymph at 3000 rpm for 5 to 10 minutes and the cell density was adjusted with unit volume of sterile snail saline (SSS). The cell pellet was

resuspended thrice and the uniform cell density was adjusted with sterile snail saline (SSS). The pellet containing haemocytes were added with 1 ml of 0.1% TritonX-100 (SRL, India) for 15 minutes for cell lysis. The suspension was centrifuged at 3000 rpm for 5-10 minutes and the lysate was stored at –20°C.

Superoxide dismutase (sod, ec1.15.1.1). Activity of superoxide dismutase was determined in haemocyte lysate following the method of Krishnan *et al*. (2002). Assay involved reaction of cell suspension with Griess reagent followed by recording of absorbance at 560 nm by spectrophotometer (Cecil, Germany). One unit of enzyme activity is defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition. Activity of superoxide dismutase was expressed as unit of SOD/ mg of protein/min.

Catalase (ec1.11.1.6.). Catalase activity was estimated according to Prakash & Rao (1995). For test sample, the reaction mixture consists of 5 ml of hydrogen peroxide (6 mM) as substrate and 0.5 ml of enzyme (0.1% Triton-X lysed haemocyte). For standard, the reaction mixture consists of 5.5 ml of phosphate buffer (0.01 M). For both test and standard sample, the reaction was stopped by adding 1 ml of sulphuric acid (6 N) followed by 7 ml of potassium permanganate (0.01 N) after 3 min of reaction. The final reaction mixture was monitored at 480 nm for 30-60 sec after the stoppage of the reaction. The activity was expressed as µl of catalase/mg protein/min.

RESULTS

NBT reduction assay by haemocyte monolayer – a cytochemical localization. Superoxide anion production by the haemocytes was indicated by the intracellular deposition of blue formazan (reduced NBT). The haemocyte monolayer stained with NBT exhibited several positively stained cells, namely granulocytes, hyalinocytes and asterocytes. Granulocytes exhibited intense staining upon treatment with NBT which indicated a strong activity of superoxide anion (Fig. 1).

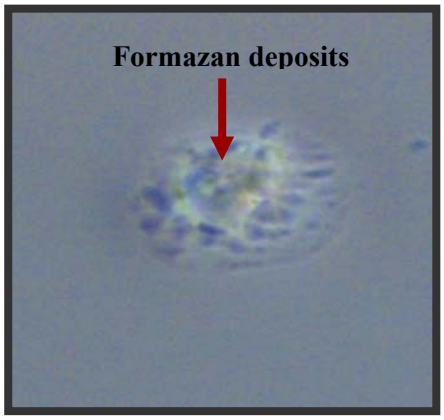

Fig. 1. Phase contrast microscopic image of granulocyte showing superoxide anion production (× 1000).

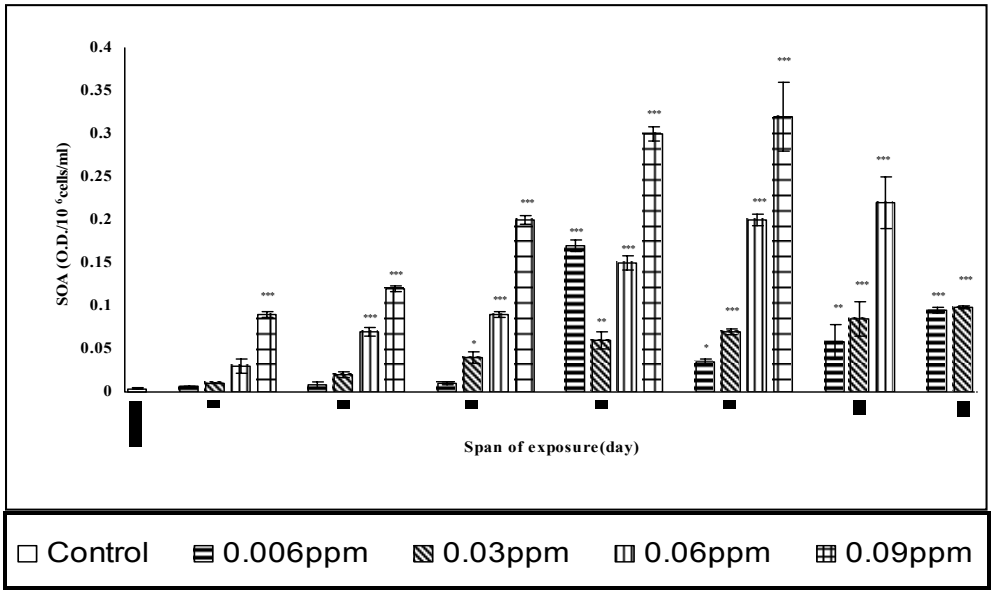

Fig. 2. Generation of superoxide anion in haemocyte of *L. marginalis* (7–8 cm shell length) exposed to azadirachtin *in vivo*. Data is represented as Mean ± S.D. Statistical significance is shown at $P < 0.05$*, $P < 0.01$**, $P < 0.001$*** (n = 5).

Quantitative NBT reduction. NBT reduction was found to be statistically uniform in animals collected from field during different seasons of the year. The control set showed an average activity of 0.01 O.D./10^6 cells/ml. Azadirachtin of 0.09 ppm exposure for 7 days showed a maximum elevation of activity of superoxide anion as 0.3 O.D./10^6 cells/ml. Superoxide anion production was higher in bivalves exposed to 0.09 ppm of pesticide compared to control for all span of exposure. Superoxide anion production expresses a dose dependent response (Fig. 2).

A marked and significant increase in intrahaemocyte superoxide anion production was detected against 0.006 ppm after 3 days of azadirachtin exposure up to a period of 30 days. A significant increase in the activity of superoxide anion was recorded during 2nd to 15th day of 0.06 ppm exposure of azadirachtin (Fig. 2).

Antioxidant activity. *L. marginalis* exposed to 0.09 ppm/7days of azadirachtin showed a maximum inhibition of intrahaemocytic SOD activity compared to that of control (Fig. 3). The intrahaemocyte SOD activity was significantly inhibited following 0.006 ppm of azadirachtin exposure after 3 days (Fig. 3). Azadirachtin exposure (0.06 ppm/15 days) significantly inhibited SOD activity compared with that of control. SOD activity was moderately inhibited by 0.03 ppm of azadirachtin exposure up to a period of 30 days (Fig. 3). Catalase has been used in aquatic organisms as a biomarker for monitoring environmental pollution and oxyradical induced damage.

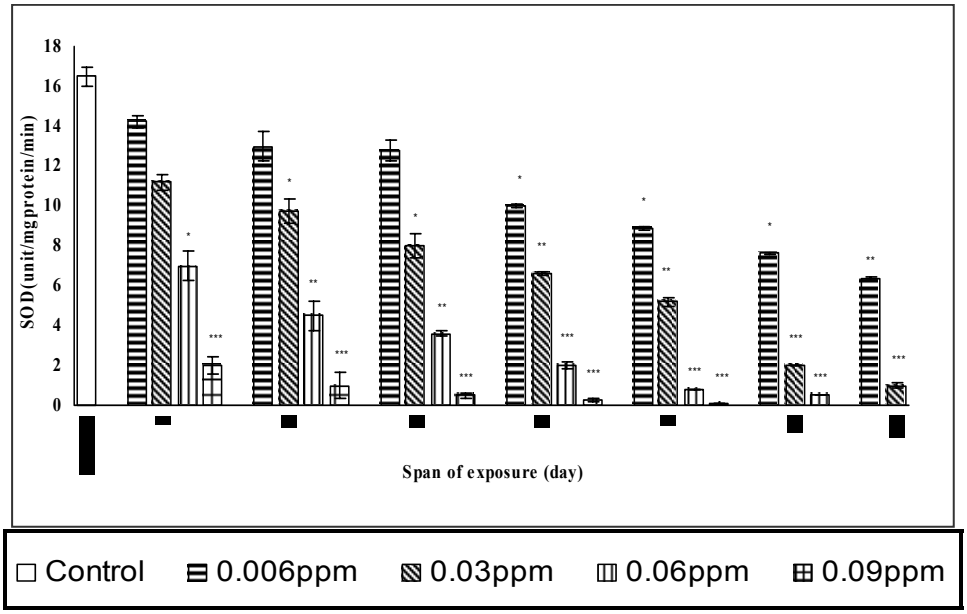

Fig. 3. Activity of superoxide dismutase of haemocyte of *L. marginalis* (7–8 cm shell length) exposed to azadirachtin *in vivo.* Data is represented as Mean ± S.D. Statistical significance is shown at $P < 0.05$*, $P < 0.01$**, $P < 0.001$*** (n = 5).

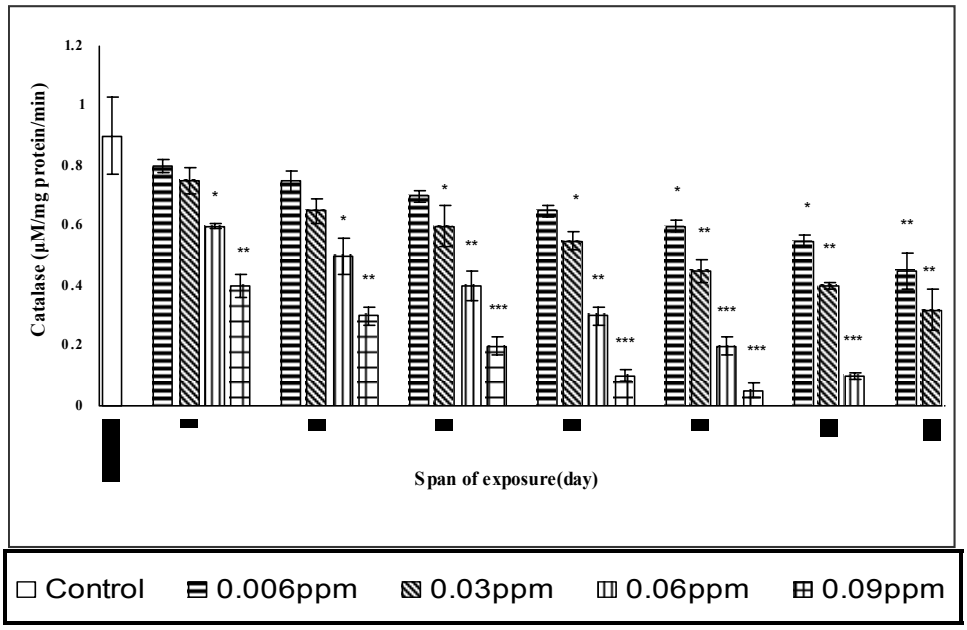

Fig. 4. Activity of catalase of haemocyte of *L. marginalis* (7–8 cm shell length) exposed to azadirachtin *in vivo*. Data is represented as Mean ± S.D. Statistical significance is shown at $P < 0.05$*, $P < 0.01$**, $P < 0.001$*** (n = 5).

Against all four selected concentrations of 0.006, 0.03, 0.06 and 0.09 ppm of azadirachtin, pattern of decrease of intrahaemocytic catalase activity expressed a dose dependent response against all spans of exposure (Fig. 4). *L. marginalis* exposed to 0.09 ppm/7days of azadirachtin showed a maximum inhibition of intrahaemocytic catalase activity as compared with control. Catalase activity was moderately inhibited in haemocyte by 0.006 ppm of azadirachtin up to 30 days of exposure (Fig. 4).

DISCUSSION

Invertebrates including bivalve molluscs are dependent on both oxygen dependent and oxygen independent modes of killing of intracellular pathogens (Munoz *et al*., 2000). In the biologically unsafe environment, the bivalves are capable of eliciting phagocytic response against invading pathogens and can generate superoxide anion as principal defence molecules (Nappi & Christensean, 2005). Stimulated haemocytes are reported to generate highly reactive oxygen species with effective microbicidal activity (Galloway & Depledge, 2001). The earliest product among highly reactive oxygen species is superoxide anion. However, reactive species is capable of resulting non target toxicity and thus affect the host tissue. Our studies revealed that upon exposure to nitrobluetetrazolium, the adherent mussel haemocytes become filled with diffused blue black formazan deposits and often showed a more intense positive reaction at the periphery. This result supports the idea of generation of cytotoxic oxygen radicals as general property of molluscan haemocytes (Fig. 1) as innate immunotoxicological response (Bogdan, 2001).

Ray *et al*. (2009) reported the toxicity of smoke in an amphobious gastropod mollusc of India. In *Pila globosa*, exposure to smoke resulted in fluctuation in the activities of NO, PO and SOA in the circulating haemocytes. Exposure to azadirachtin had increased the generation of superoxide anion for all four concentrations, which expressed a dose dependent response (Fig. 2). Azadirachtin contamination resulted in an induction of generation of reactive oxygen species in the haemocytes which is suggestive of increased capability of haemocytes for intracellular killing of pathogen. This response of haemocytes may be considered as cellular stress under the indefinite exposure of azadirachtin. Dose dependent response of haemocytes in generation of superoxide anion is suggestive of the parameter which may be considered as biomarker of toxicity. Superoxide anions are extremely toxic, powerful and hyperactive killing agents which are capable of creating damage of the cells and tissues of self.

Most reactive oxygen species are generated as superoxide anion having microbicidal activity and are rapidly dismutated by the action of superoxide

dismutase and catalase representing the main enzymatic defences against reactive oxygen species (Ordas *et al*., 2007). Azadirachtin exhibited an inhibitory effect on the production of both superoxide dismutase and catalase in haemocytes (Figs. 3, 4). However, Nadji *et al*. (2010) reported the dynamics in the activities of acetylcholine esterase and catalase in several tissues of bivalve mollusc (*Ruditapes decussatus*) under the exposure of malathion and observed increment in the activity of catalase. Inhibitory effect in the activity of acetylcholine esterase in the different species of musssel is in report (Moulton *et al*., 1996). Since xenobiotic induced production of reactive oxygen species varies significantly against environmental factors, necessary adjustments in antioxidant defences are required to maintain the steady state concentration of ROS for prevention and minimization of oxidative stress and cellular damage (Lesser, 2006) and is considered as a biochemical adaptive response. Phagocytic activity and generation of nitric oxide by the haemocytes of bivalve mollusc is established as biomarker of arsenic toxicity (Chakraborty *et al*., 2009). Phagocytosis of charcoal particulates by haemocytes of *L. marginalis* is reported as immunotoxicological marker of azadirachtin exposure (Mukherjee *et al*., 2011).

Contamination of the waterbodies by azadirachtin pesticide poses an environmental threat in *L. marginalis*. Many of the aquatic molluscs, including *L. marginalis*, are important bioresource. *L. marginalis* is a dietary source of human and bears ethnomedicinal value. Our data indicates a substantial level of cellular stress in haemocytes under azadirachtin exposure. Such a situation, if it remains uncontrolled, may lead to a gradual decline in the population of such an economically important species. Being a potential bioindicator, *L. marginalis* is considered to be an effective species in estimating the toxicity of azadirachtin and alike toxins of the aquatic ecosystem.

CONCLUSIONS

In this study, azadirachtin induced increase of intrahaemocytic generation of superoxide anion was recorded with simultaneous decrement of superoxide dismutase and catalase activity against increasing azadirachtin concentrations. In conclusion, present data demonstrate the sensitivity of antioxidant enzymes of *L. marginalis* as potential biomarker of toxicity.

Azadirachtin induced decrement of antioxidant enzymes is indicative to a possible onset of oxidative stress in the specimen distributed in water bodies contaminated with neem pesticide. Azadirachtin induced oxidative stress often alters the physiological status that may lead to decline of the population of *L. marginalis* in its natural habitat resulting in a gradual depletion of existing diversity of freshwater bioresource of India.

L. marginalis is a potential bioindicator and dynamics of the activities of SOA, SOD and catalase was indicative to be an effective biomarker of aquatic pollution. Biomarker research has been gaining a rapid significance in modern ecotoxicology. As such, the studied parameters bear a positive scope for estimation and analyses of toxicity of azadirachtin based agrotoxin.

REFERENCES

ANDERSON R.S., OLIVER L.M., BRUBACHER L.L., 1992, *Superoxide anion generation by Crassostrea virginica hemocytes by nitroblue tetrazolium reduction*. J. Invert. Pathol., **59**: 303-307.

BELL K.L., SMITH V.J., 1993, *In vitro super oxide production by hyaline cells of the shore crab, Carcinus maenas (L.)*. Dev. Comp. Immunol., **17**: 211-219.

BOGDAN C., 2001, *Nitrioxide and the immune response*. Nat. Immunol., **2** (10): 907-916.

BROUSSEAU P., PAYETTE Y., TRYPHONAS H., BLAKLEY B., BOERNAUS H., FLIPO D., FOURNIER M., 1999, *Manual of Immunological Methods*. CRC Press, Boca Raton, FL.

CHAKRABORTY S., RAY M., RAY S., 2009, *Evaluation of phagocytic activity and nitric oxide generation by molluscan haemocytes as biomarkers of inorganic arsenic exposure*. Biomarkers, **14** (8): 539-546.

CHAKRABORTY S., RAY M., RAY S., 2012, *Arsenic toxicity: A heart-breaking saga of a freshwater mollusc*. Tissue and Cell (in press).

CHEN J.H., BAYNE C.J., 1995, *Bivalve Mollusc Hemocyte Behaviours: Characterization of Hemocyte Aggregation and Adhesion and Their Inhibition in the California Mussel (Mytilus californiasus)*. Biol. Bull., **188**: 255-256.

GALLOWAY T.S., DEPLEDGE M.H., 2001, *Immunotoxicity in invertebrates: measurement and ecotoxicological relevance*. Ecotoxicol., **10**: 5-23.

KRISHNAN N., CHATTAPADHYAY J.K., CHAUDHURI A., 2002, *Superoxide dismutase activity in hemocytes and hemolymph of bombyx mori following bacterial infection*. Curr. Sci., **83** (3): 321-325.

LESSER M.P., 2006, *Oxidative stress in marine environment: biochemistry and physiological ecology*. Annu. Rev. Physiol., **68**: 253-278.

MOULTON C.A., FLEMING W.J., PURNELL C.E., 1996, *Effects of two cholinesterase inhibiting pesticides on freshwater mussels*. Environ. Toxicol. Chem., **15**: 131-137.

MUKHERJEE S., RAY M., RAY S., 2011, *Anticoagulant and carbohydrate induced interference of aggregation of mussel haemocyte under azadirachtin exposure*. In: LAUREN E. MCGEVIN (Eds.), *Mussels: Anatomy, Habitat and Environmental Impact"*, Chapter **20** (1). Nova publication, USA, pp.: 441-452.

MUKHERJEE S., RAY M., RAY S., 2011, *Phagocytosis of charcoal particulate as immunological marker of azadirachtin exposure*. In: V. GUPTA (Ed.), *Perspectives in Animal Ecology and Reproduction*, Chapter **5** (7), Daya publication, India, pp.: 55-67.

MUNOZ M., CEDENOR., RODRIGUEZ J., VAN DER KNAAP W.P.W., MIALHE E., BACHERE E., 2000, *Measurement of reactive oxygen intermediate production in hemocytes of the penaeid shrimp*, *Penaeus vannamei*. Aquacult., **191**: 89-107.

NADJI S., AMRANI A., MEBARKI R., KHEBBEB M., 2010, *Acetylcholinesterase and catalase activities in several tissues of a bivalve mollusc (Ruditapes decussatus) fished from Mellah lagoon (North East of Algeria) after malathion exposure*. Ann. Biol. Res., **1** (4):138-144.

NAPPI A.J., CHRISTENSEAN B.M., 2005, *Melanogenesis and associated cytotoxic reactions: application to insect innate immunity.* Insect. Biochem. Mol. Biol., **35**: 443-459.

ORDAS M.C., ALBAIGES J., BAYONA J.M., ORDAS A., FIGUERAS A., 2007, *Assessment of in vitro effect of the prestige fuel oil spill on the Mediterranean mussel immune system*. Arch. Environ. Contam. Toxicol., **52**: 200-206.

PRAKASH N.T., RAO K.S.J., 1995, *Modulations in antioxidant enzymes in different tissues of marine bivalve Perna viridis during heavy metal exposure*. Mol. Cell. Biochem., **146**: 107-113.

RAUT S.K., 1991, *Laboratory Rearing of Medically and Economically Important Molluscs*. In: M.S. JAIRAJPURI (Ed.), *Snails, Flukes and Man*, Zoological Survey of India Pub., pp. 79- 83.

RAY S., RAY M., BHUNIA N., 2009, *Toxicity of smoke in an Amphibian mollusc from India*. In: *Environmental and Regional Air Pollution,* Nova Science Publishers, Chapter **14**, pp. 1-16.

RAY S., RAY M., CHAKRABORTY S., MUKHERJEE S., 2011, *Immunotoxicity of environmental chemicals in the pearl forming mussel of India – a review*. In: LAUREN E. MCGEVIN (Eds.), *Mussels: Anatomy, Habitat and Environmental Impact*, Chapter **19** (1), Nova publication, USA. pp. 429-440.

REGOLI F., PRINCIPATO G., 1995, *Glutathione, glutathione-dependent and antioxidant enzymes in mussel, Mytilus galloprovincialis, exposed to metals under field and laboratory conditions*: *Implications for the use of biochemical biomarkers*. Aquat. Toxicol., **31**: 143-164.

RODRIGUEZ J., MOULLAC G.L., 2000, *State of the art of immunological tools and health control of penaeid shrimp*. Aquacult., **191**: 109-119.

SAUVE S., BROUSSEAU P., PELLERIN J., MORIN Y., SENECAL L., GOUDREAU P., FOURNIER M., 2002, *Phagocytic activity of marine and fresh water bivalves: in vitro exposure of hemocytes to met (Ag, Cd, Hg and Zn*). Aquat. Toxicol., **508** (3-4): 189-200.

SCHMUTTERER H., SINGH R.P., 2002, *List of insect pests susceptible to neem products*. In: MUMBAI SCHMUTTERER H. (Ed.), *The Neem Tree*: *Azadirachta indica A. Juss and other meliacious plants – sources of unique natural products for integrated pest management, medicine, industry and other purposes*, Second ed., Neem Foundation, India, pp.: 411–456.

VIJAYAVEL K., BALASUBRAMANIAN M.P., 2006, *Fluctuations of biochemical constituent and marker enzymes as a consequence of naphthalene toxicity in the edible estuarine crab, Scylla serrata*. Ecotoxicol. Environ. Saf., **63** (1): 141-147.

VIJAYAVEL K., GOPALAKRISHNAN S., THIAGARAJAN R., THILAGAM H., 2009, *Immunotoxic effects of nickel in the mud crab Scylla serrata.* Fish Shellfish Immunol., **26** (1): 133-139.

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SEMINAL VESICLE AS A TARGET FOR THE TOXIC MANIFESTATION OF CYCLOPHOSPHAMIDE: HISTOPATHOLOGICAL CHANGES AND SERUM TESTOSTERONE

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The goal of this study is to elucidate the effect of an anticancer/alkylating drug Cyclophosphamide (CPA) on seminal vesicle and on serum testosterone level in the rat model. White albino rats *Rattus norvegicus* were treated for 15 days with vehicle or CPA at doses 5mg and 15 mg/kg body weight given intraperitoneally. The behavioral changes were sluggishness, low appetite, withdrawn mood, hair fall all over the body and oral mucositis, alopecia bleeding from the cornea of the eye, etc. Body and seminal vesicle weights were significantly decreased ($P < 0.05$) in the treated groups. Cyclophosphamide treatment resulted into dose and duration dependent histopathological changes in the seminal vesicle as well as testosterone concentrations due to its antifertility and antiandrogenic properties which interfere with fertility of animal.

Key words: Cyclophosphamide, seminal vesicle, antifertility, antiandrogenic.

INTRODUCTION

The seminal vesicles are among the most important male accessory glands contributing to about 60% of the seminal plasma (Mann & Lutwak-Mann, 1981). Seminal vesicular secretion is rich in fructose, proteins, prostaglandins, complex carbohydrates and enzymes involved in the clotting of the ejaculate (Gonzales & Villena, 2001). It also provides nutrients for the spermatozoa and optimizes the conditions for transport, sperm motility, viability, elimination of non-viable spermatozoa from the uterus in both the male and female reproductive tracts (Agrawal & Vanha-Pertualla, 1987; Gonzales & Villena, 2001; Zubkova & Robaire, 2004; Troedsson *et al*., 2005). The potent immune-suppressive activities of the water-soluble fraction of the seminal vesicle fluid and its role in reproductive immunity have also been described (Rozeboom *et al*., 1999; Alghamdi *et al*., 2004). The seminal vesicle is an androgen dependent organ in terms of both structure and function (Gonzales, 1994; Almenara *et al*., 2000). The seminal vesicle possesses 5α-reductase activity, which converts testosterone to dihydrotestosterone, the active hormone. More recently it has been demonstrated that seminal vesicles contain LH/hCG receptors, thus making this accessory reproductive organ a potentional target of direct regulation by LH (Tao *et al*., 1998;

Nishino *et al*., 2004). Cyclophosphamide belonging to the class of Oxazaphosphorines is a bioactivated metabolite and alkylating agent that show cytostatic effects by forming covalent DNA adducts. The cytotoxicity of Cyclophosphamide is mediated by alkylation of DNA at the N7 position of guanine and the formation of DNA–DNA cross-links, DNA–protein cross-links, and single-strand breaks (Hemminki & Kallama, 1986; Crook *et al.*, 1986). Little is known about its toxic effect on accessory reproductive glands and testosterone level, therefore, the present study is undertaken to evaluate the correlation between testosterone and histopathological changes as these glands are androgen dependent.

MATERIAL AND METHODS

Drug

The anticancer drug Cyclophosphamide (Endoxan-N, CAS no. 50-18-0), with the chemical formula $C_7H_{15}Cl_2N_2O_2P$ and molecular weight, 261.086 g/mol, manufactured by Candila Healthcare Limited, Goa was used for the present experiments.

Experimental Animals

Wistar albino rats (*Rattus norvegicus*) with average body weight of 250–300 g were used for the experiments. Animals were maintained in the laboratory under an absolute hygienic condition as per the recommended procedures by fulfilling all the necessary ethical standards. They were housed in polypropylene box type cages, bedded with rice husk and kept at constant temperature 28±2°C and relative humidity (60±10%) with 12 h light: 12 h dark cycle. They were fed with pelleted diet (Hindusthan Liver Lt., India) and water *ad libitum*.

Treatment

Two doses of the drug, *i.e*. 5 and 15 mg/kg, were selected on body weight basis. CPA doses were prepared by dissolving the drug in saline and administered intraperitoneally to experimental animals (Table 1).

Table 1

Experimental protocol

Number of animals and sex	Treatment	Dose mg/kgBW	Route	Duration
6 males (Experimental)	CPA	5 mg	I.P.	15 days
6 males (Experimental)	CPA	15 mg	I.P.	15 days
6 males (Control)	Saline	Equal volume	I.P.	15 days

Abbreviations: I.P. = Intraperitoneal; BW = Body weight.

Histological assessment

The Animals were sacrificed using chloroform 24 hours after the last day of each experiment. Immediately the seminal vesicles were excised, weighed on an analytical balance and fixed in Bouin's fluid for 24 hrs and preserved in 70% alcohol. The tissues were dehydrated by passing through graded series of alcohol, cleared in xylol and after embedding in paraffin blocks were prepared and cut in numerous parallel 5 μm sections. For routine histological study the sections were stained with Ehrlich's haematoxylin and counter-stained with eosin. Measurements were taken with an occular micrometer wherever essential.

Enzyme-Linked Immunosorbent Assay for Measurement of Testosterone

For the determination of testosterone level in blood, rats were anesthetized by ether and 2 ml of blood was drawn by cardiac puncture with sterile syringe. The blood was allowed to clot at room temperature for half an hour. The clotted blood was then used for measurement of serum testosterone by ELISA (Delahunt & Hirsutism, 1993).

Statistical Analysis

To indicate individual variations in each corresponding region, the mean values and standard deviation (mean ± SD) were calculated from six animals. The statistical significance of differences for these values in different regions were assessed using 't-test' (Dalgaard, 2008). A significant level of $P < 0.05$ was accepted.

RESULTS

The behavioral changes revealed were sluggishness, low appetite, withdrawn mood, hair fall all over the body and oral mucositis etc., however, mortality rate was zero percent. A decrease in weight of body and seminal vesicle was notified in experimental groups in comparison to controls being significant for higher dose (Figs. 1, 2).

Evaluation of testosterone

The chronic low dose group showed insignificant decrease ($P < 0.1$) but the chronic high dose treatment resulted into significant decrease ($P < 0.001$) in serum testosterone concentration compared to control values (Fig. 3).

Histopathological Examination. Vehicle-treated controls

Seminal vesicle of the control rat was composed of tubular alveoli and the mucosa was thrown into an intricate system of folds lined by columnar epithelium containing a prominent basal nucleus overlaying the lamina propria. The lamina propria surrounding the epithelial cells was comprised of cellular connective tissue containing some smooth muscles rich in elastic fibres which appeared to be fairly thin and loose. The acini or alveoli were lined by muscularis consisting of inner circular and outer longitudinal layers of smooth muscles. Few basal cells, almost

rounded in shape and basal in position, were also observed between the columnar epithelial cells. Large numbers of dense secretory granules were visible in the apical cytoplasm. The lumen of acini was filled with the darkly stained secretory material (Figs. 5, 6).

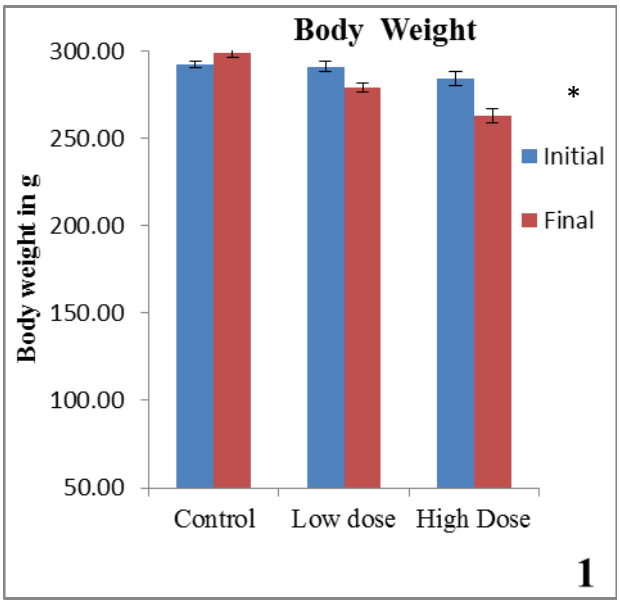


Fig. 1. Body weight changes over the course of 15 days treatment with Cyclophosphamide expressed as weight on the last day of treatment divided by weight on the first day of treatment (Control n = 6, Cyclophosphamide n = 6). Treated rats did not gain as much weight as the control rats. *P < 0.05.

Low dose treatment (5 mg/kg BW CPA for 15 days)

The changes undergone were remarkable increase in the fibro-muscular coat and an increase in the muscularis lining the secretory tubules causing reduction in the diameter of seminal vesicle acini (Fig. 4). There were streaks in the fibro-muscular tissue, suggestive of degeneration and blood capillaries exhibited hypermea. Most of the acini from the periphery as well as some from the central region showed degeneration due to sloughing off of mucosal folds into lumen imparting a shredded appearance to the acini. The degenerative changes also comprised intermingling of secretory epithelium and emptiness due to total loss of secretory activity at some portion of the gland. The regressive changes in some other acini were demarcated by restriction in the ramification of secretory mucosa which now appeared as finger-like projections or club-like projections. The pseudostratified appearance of the degenerative epithelium was due to irregular placement and pyknosis of the nuclei (Figs. 7, 8).

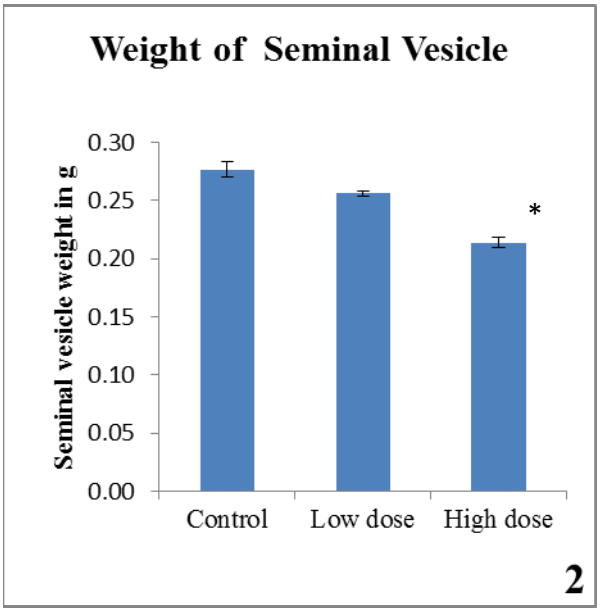


Fig. 2. Weight of seminal vesicle after 15 days treatment with vehicle or Cyclophosphamide (Control n = 6, Cyclophosphamide n = 6). There was a significant decrease in the weight of seminal vesicle of the Cyclophosphamide treated animals.*P < 0.001.

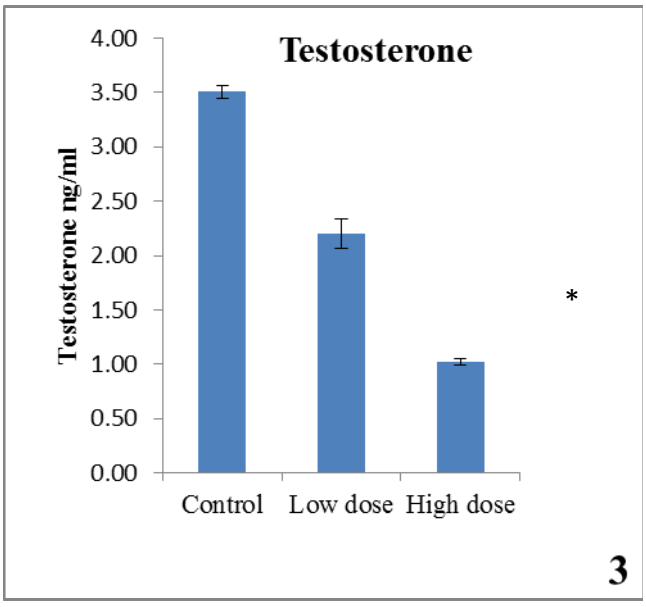


Fig. 3. Testosterone level changes over the course of 15 days treatment with Cyclophosphamide (Control n = 6, Cyclophosphamide n = 6). There was a significant decrease in level of testosterone in treated group when compared to vehicle. *P < 0.001.

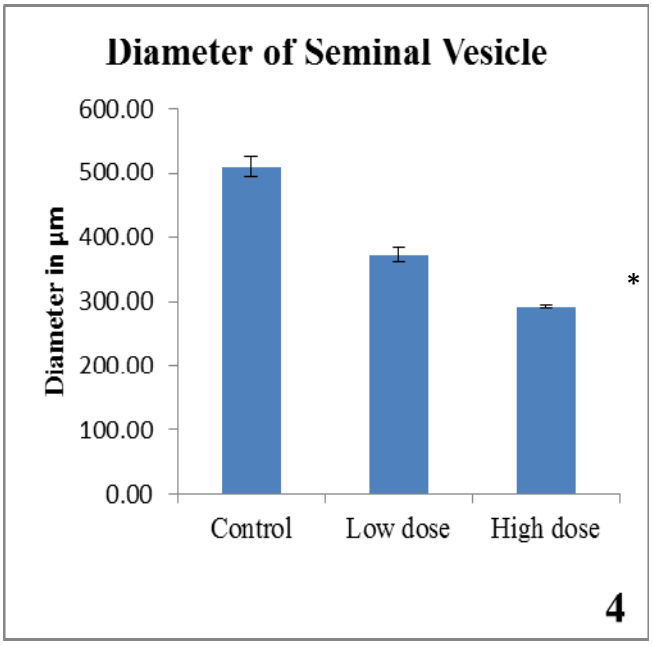


Fig. 4. Effect of 5mg and 15 mg/kg BW Cyclophosphamide chronic treatment for 15 days on diameter of seminal vesicle acini of rats (Control n = 6, Cyclophosphamide n = 6). There was a significant decrease in the diameter when compared with the vehicle. *P < 0.05.

Fig. 5. Few distended alveoli within the fibromuscular tissue from vehicle-treated control. Mucosa lining the alveolus is thrown into intricate system of folds (arrow) and lumen contains copious amount of secretion (arrow head). (× 100).

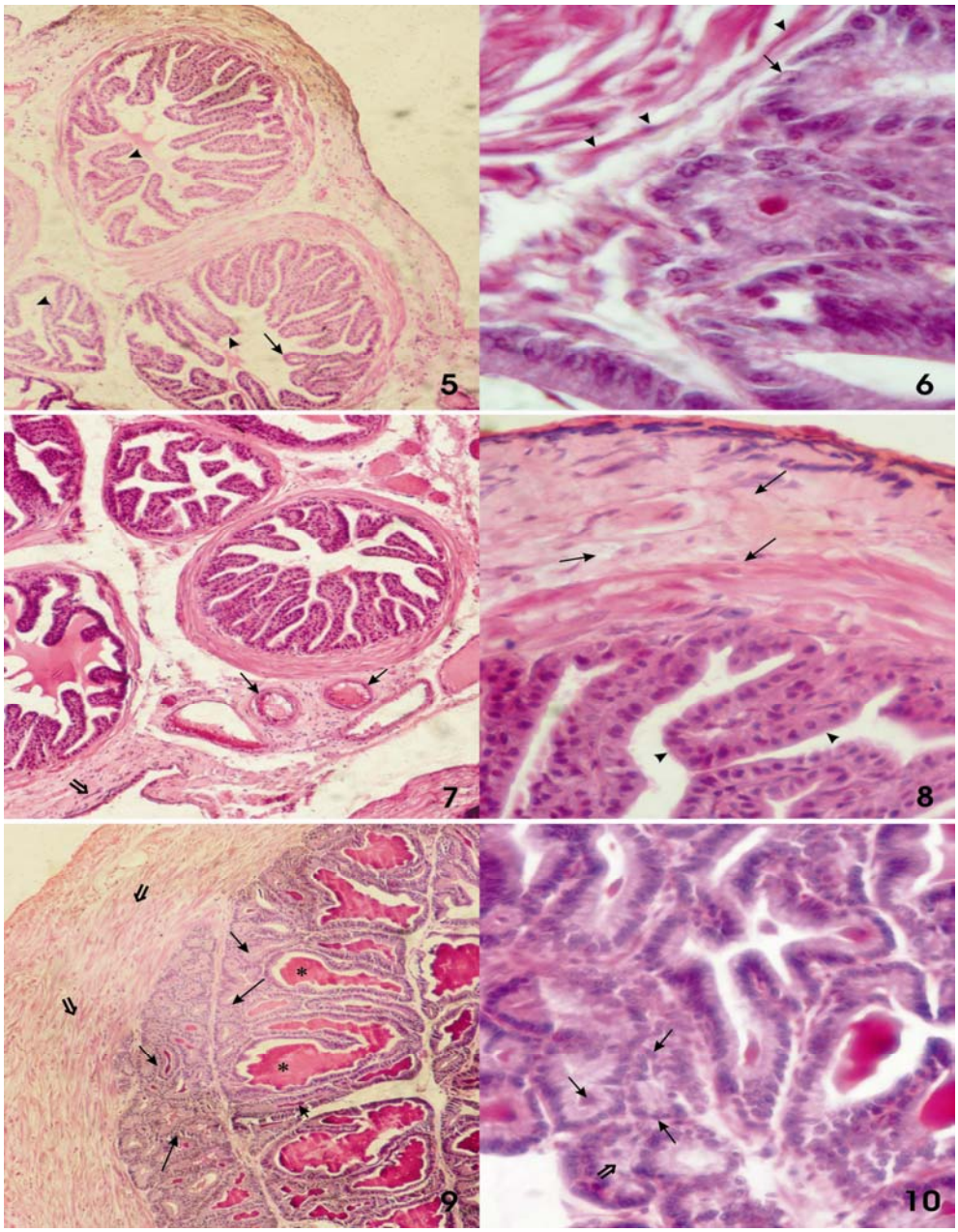
Fig. 6. A part of the secretory epithelium from vehicle-treated control which appears to be tall columnar containing a prominent basal nucleus. Few basal cells, almost rounded in shape and basal in position in between the columnar epithelial cells (arrow) are also seen. Large numbers of dense secretory granules are visible in the apical cytoplasm. The lamina propria surrounding the epithelial cells is comprised of cellular connective containing some smooth muscles rich in elastic fibres (arrow head). (× 400).

Fig. 7. Seminal vesicle from 5mg/kgBW/day for 15days. Note reduction and partial loss of secretory activity difference in size of secretory acini, irregularity in configuration due to an increase in the peripheral connective tissue (open arrow), and hyperemic blood capillaries (arrow). (× 100).

Fig. 8. Fig. 7 further magnified to show hypertrophy of muscularis (arrow) and reduction in the ramification of mucosa, the pathological changes in the mucosa are enlarged irregular, hyperchromatic nuclei (arrow head). (× 400).

Fig. 9. After administration of 15mg/kgBW Cyclophosphamide for 15 days there is an enormous increase in outer fibromuscular coat (open arrow), atrophy of peripheral secretory units (long arrow), remarkable shrinkage of acini leading to remarkable difference in size, some being smaller, bilaterally compressed without lumen, some with lumen but no secretion, some with little colloid (short arrow) but the larger one with coagulated dark secretion (asterisk arrow). (× 100).

Fig. 10. Secretory epithelium (high dose): Note the feathery appearance of mucosa due to extensive loss of cytoplasm. Also note loss of epithelium, loss of lumen in most of the acini (open arrow), secretion either being scanty or totally lost, displacement as well as loss of nuclei, sloughing off of epithelium from the basement membrane into the lumen (arrow). (× 400).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High dose treatment (15mg/kg BW CPA for 15 days)

The histologic features after chronic high dose treatment included noticeable reduction in the diameter of seminal vesicle acini (Fig. 4) due to enormous increase in the fibro-muscular coat as well as inter-tubular connective tissue. Almost all the tubules have lost their regular configuration due to extreme involution.

There was atrophy and degeneration of glandular epithelium with extensive vacuolation and loss of ramification, the nuclei were large, irregular (Figs. 9, 10). The nuclei of this degenerated epithelium appeared intermingled in the lumen due to detachment from the basement membrane with the dry flocculant secretion, however, most of the shrunk acini demonstrated presence of dark colloid.

DISCUSSION

In this study we investigated the toxicity of an antineoplastic agent Cyclophosphamide on seminal vesicle and serum testosterone level in albino rats. Seminal vesicular secretion is important for semen coagulation, sperm motility, stability of sperm chromatin and suppression of immune activity in female reproductive tract (Gonzales & Villena, 2001).

The accessory glands are morphologically and physiologically dependent on the production of the androgen and the circulating androgen which are in turn LH dependent (Lee *et al*., 1994; Nemeth *et al*., 1998; Sastry & Kashmiri, 2011; Sastry & Gupta, 2011). Cyclophosphamide manifested strong antiandrogenic effects, thereby causing reduction of most of the androgenic parameters due to androgen deprivation. It causes involution in the weight and size of the accessory glands due to reduction in the venous blood flow from the testis (Seaman *et al*., 2003). CPA being anti-androgenic causes decrease in serum testosterone (T) which results in decreased body weight, size and weight of androgen-dependent organs such as seminal vesicles (Bhasin *et al*., 1997; Delbes *et al*., 2009) and secretory activity of seminal vesicle (Higgins & Burchell, 1978; Fawell & Higgins, 1984; Zanato *et al*., 1994; Almenara *et al*., 2000). CPA is also a potent inhibitor of testicular 5α-hydroxysteroid oxidoreductase activity, binding itself to catalytic binding sites of the substrate like DHT (5α-dihydroxytestosterone) thus reducing androgen binding protein (ABP) production which would have helped in the maintenance of the accessories, since it is carrier of testosterone (Wong *et al*., 1978) or through hormone target cell interaction or the expression of seminal vesicle protein or by direct action on the metabolism of testosterone compartment of testis which are in the control of adenohypophysis.

The reduction or the shrinkage of the acini were in equivalence with the increase of fibro-muscular tissue defining stromal-epithelial interaction (Chan & Wong, 1992; Dünker & Kreiglstein, 2000; Blanchere *et al*., 2001). The above mentioned changes also point to androgen dependency of the gland since the male

accessory glands are highly dependent on androgenic hormones to maintain their normal structure and function and is also very sensitive to the level of circulatory androgen (Almenara *et al*., 2000; Nishino *et al*., 2004).

CONCLUSION

The Cyclophosphamide manifested a strong antiandrogenic effect, thereby causing reduction of most of the androgenic parameters due to androgen deprivation.

REFERENCES

AGRAWAL Y., VANHA PERTUALLA T., 1987, *Effect of secretory particles in bovine seminal vesicle secretion on sperm motility and acrosome reaction*. Journal of Reproductive Fertility, **79**: 409-419.

ALGHAMDI A.S., FOSTER D.N., TROEDSSON M.H.T., 2004, *Equine seminal plasma reduces sperm binding to polymorphonuclear neutrophils (PMNs) and improves the fertility of fresh semen inseminated into inflamed uteri.* Reproduction, **125**: 593-600.

ALMENARA A., ESCALANTE G., GAZZO E., GONZALES G.F., 2000, *Transillumination to evaluate spermatogenesis: Effect of testosterone enanthate in adult male rats*. Archives of Andrology, **46**: 21-28.

BHASIN S., STORER T.W., BERMAN N., YARASHESKI K.E., CLEVENGER B., PHILLIPS J., LEE W.P., BUNNELL T.J., CASABURI R., 1997, *Testosterone replacement increases fat free mass and muscle rise in hypogonadal men.* Journal of Clinical Endocrinology and Metabolism, **82** (2): 407-413.

BLANCHERE M., MESTAYER C., SAUNIER E., BROSHUIS M., MOWSZOWICZ I., 2001, *Transforming growth factor β in the human prostate: its role in stromal–epithelial interactions in non-cancerous cell culture*. Prostate, **46**: 311-318.

CHAN L., WONG Y.C., 1992, *Cytochemical localisation and characterisation of the proteoglycans (glycosaminoglycans) in the epithelial-stromal interface of seminal vesicle of guinea pig*. Journal of Anatomy, **180** (1): 41-56.

CROOK T.R., SOUHAMI R.L., MCLEAN A.E., 1986, *Cytotoxicity, DNA cross-linking, and single strand breaks induced by activated Cyclophosphamide and acrolein in human leukemia cells*. Cancer Research, **46**: 5029–5034.

DELAHUNT J., HIRSUTISM W., 1993, *Practical therapeutic guidelines.* Drugs, **45** (2): 223-231.

DELBES G., CHAN D., PAKARINEN P., TRASLER J.M., HALES B.F., ROBAIRE B., 2009, *Impact of the chemotherapy cocktail used to treat testicular cancer on the gene expression profile of germ cells from male brown-Norway rats*. Biology of Reproduction, **80**: 320-327.

DALGAARD P., 2008, *Introductory statistics with R*, 2nd Edition. Springer Verlag, 364 pp.

DÜNKER N., KREIGLSTEIN K., 2000, *Targeted mutations of transforming growth factor-beta genes reveal important roles in mouse development and adult homeostasis*. European Journal of Biochemistry, **267**: 6982-6988.

FAWELL S.E., HIGGINS S.J., 1984, *Androgen regulation of specific mRNAs, endoplasmic reticulum and Golgi-system.* Molecular and Cellular Endocrinology, **37**: 15-27.

GONZALES G.F., 1994, *Test for androgen activity at the male reproductive tract in infertile men*. Archives of Andrology, **32**: 235-242.

GONZALES G., VILLENA A., 2001, *True corrected seminal fructose level: a better marker of the function of seminal vesicles in infertile men*. International Journal Andrology, **24** (5): 255-260.

HEMMINKI K., KALLAMA S., 1986, *Reactions of nitrogen mustards with DNA*. IARC Scientific Publication, **78**: 55-70.

HIGGINS S.J., BURCHELL J.M., 1978, *Effects of testosterone on messenger ribonucleic acid and protein synthesis in rat seminal vesicle*. Biochemical Journal, **174**: 543-551.

LEE C., GOOLSBY C.L., SENSIBAR J.A., 1994, *Cell cycle kinetics in rat prostatic epithelia: Nuclear migration during G2 phase*. Journal of Urology, **152**: 2294-2299.

MANN T., LUTWAK-MANN C., 1981, *Male reproductive function and semen*. Springer-Verlag, Berlin, 495 pp.

NEMETH J.A., ZELNER D.J., LANG S., LEE C., 1998, *Keratinocyte growth factor in the rat ventral prostate: androgen–independent expression*. Journal of Endocrinology, **156**: 115-125.

NISHINO T., WEDEL T., SCHMITT O., BUHLMEYER K., SCHONFELDER M., HIRTREITER C., THORS, 2004, *Androgen dependent morphology of prostate and seminal vesicle in the Hershberger Assay: Evaluation of immunohistochemical and morphometric parameters.* Annals of Anatomy Anatomischer Anzeiger, **189**: 247-253.

ROZEBOOM K.J., TROEDSSON M.H., MOLITOR T.W., CRABO B.G., 1999, *The effect of spermatozoa and seminal plasma on leukocyte migration into the uterus of gilts.* Journal of Animal Science, **77**: 2201-2206.

SASTRY M.S., KASHMIRI Z.N., 2011, *Toxic study of an oncolytic Drug Cyclophosphamide on the Accessory Reproductive Glands of Male Squirrel Funambulus pennanti (Wroughton): Histological Approach*. Asian Journal of Experimental Biological Science, **2** (1): 119-126.

SASTRY M.S., GUPTA S.S., 2011, *Male reproductive toxicity of DMPA on seminal vesicle of Indian palm squirrel, Funambulus pennanti*. International Journal of Science and Nature, **2** (4): 764-768.

SEAMAN F., SAWHNEY P., GIAMMONA C.J., RICHBURG, J.H., 2003, *Cisplatin-induced pulse of germ cell apoptosis precedes long-term elevated apoptotic rates in C57/BL16 mouse testis*. Apoptosis, **8**: 101-108.

TAO Y.X., LEI Z.M., RAO C.V., 1998, *Seminal vesicles are novel sites of luteinizing hormone/human chorionic gonadotropin receptor gene expression*. Journal of Andrology, **19**: 343-347.

TROEDSSON M.H., DESVOUSGES A., ALGHAMDI A.S., DAHMS B., DOW C.A., HAYNA J., VALESCO R., COLLAHAN P.T., MACPHERSON M.L., POZOR M., BUHI W.C., 2005, *Components in seminal plasma regulating sperm transport and elimination.* Animal Reproduction Science, **89** (1-4): 171-186.

WONG P.Y.D., AU C.L., NGAI H.K., 1978, *Electrolyte and water transport in rat epididymis, its possible role in sperm maturation.* International Journal of Andrology (Suppl.), **2**: 608-628.

ZANATO V.F., MARTINS M.P., ANSELMO-FRANCI J.A., PETENUSCI S.O., LAMANO-CARVALHO T.L., 1994, *Sexual development of male Wistar rats*. Brazilian Journal of Medical and Biological Research, **27**: 1273-1280.

ZUBKOVA E.V., ROBAIRE B., 2004, *Effect of Glutathione Depletion on Antioxidant Enzyme in the Epididymis, Seminal Vesicles and Liver and on Spermatozoa Motility in the Ageing Brown Norway Rat*. Biology of Reproduction, **71**: 1002-1008.

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