Toute commande sera adressée à:
EDITURA ACADEMIEI ROMÂNE, Calea 13 Septembrie nr. 13, Sector 5, P.O. Box 5–42, Bucureşti, România, Tel. 40-21-411 9008, Tel./Fax. 40-21-410 3983, 40-21-410 3448; e-mail: edacad@ear.ro
RODIPET S.A., Piaţa Presei Libere nr. 1, Sector 1, P.O. Box 35-37, Bucureşti, România, Tel. 40-21-222 4126, Fax 40-21-224 0558.
ORION PRESS IMPEX 2000 S.R.L., P.O. Box 77-19, Bucureşti, Sector 5, România, Tel. 40-21-301 8786, Fax 40-21-335 0296.

Les manuscrits ainsi que toute correspondance seront envoyés à la rédaction; les livres et les publications proposés en échange seront adressés à: INSTITUTUL DE BIOLOGIE, Splaiul Independenţei 296, P.O. Box 56-53, 060031, Bucureşti.

REVUE ROUMAINE DE BIOLOGIE
SÉRIE DE BIOLOGIE ANIMALE
Calea Victoriei 125
010071, Bucureşti, România
Tél. 650 76 80

REVUE ROUMAINE DE BIOLOGIE

SÉRIE DE BIOLOGIE ANIMALE

TOME 47, N^{os} 1–2 janvier-décembre, 2002

SOMMAIRE

REV. ROUM. BIOL.–BIOL. ANIM., TOME 47, N^{os} 1–2, P. 1–128, BUCAREST, 2002

CONTRIBUTION TO THE KNOWLEDGE OF OPILIONID FAUNA (ARACHNIDA: OPILIONES) FROM TIMIŞ DISTRICT

ANDA BĂBĂLEAN

The present paper reports the presence of 16 epigeic Opilionid species in a very little studied region from Romania: Timiş district. From the 16 species, only one – *Opilio parietinus* (De Geer) was mentioned in Timiş Plain. The presence of *Nemastoma transsylvanicum* Gruber et Martens in Răchita extends the species areal in Romania. Taking the areal into consideration the share of different Opilionid species met within the collecting made in Timiş is given.

1. INTRODUCTION

Because of the lack of systematic collecting actions only incomplete data concerning the Opilionid fauna from Banat can be found in the specialized literature.

In the last years some valuable information were brought by:

1) Ştefania Avram, whom studies were focused more on cavernous Opilionids (4),

2) Dan Dumitrescu- especially for Caraş-Severin district (10).

2. MATERIALS AND METHODS

I have collected Opilionids from the following stations: Răchita; Făget (Dealul-Înalt), Românești (Izvorul lui Miron Monastery-Dealul Cărării); Nădrag, Lugoj Forest, Rudna, Green Forest (Pădurea Verde)-Timişoara; Buziaş (Buziaş Park); Grabați, Lovrin. The stations are in plain, hill and mountain areas as shown in Table 1 and Fig. 1.

I have studied especially the forest leafage fauna during June 1999 – August 2001. Opilionids have been collected mostly in Barber traps ½ filled with 10 % formaldehyde.

Table 1

Collecting station	Altitude	Geographical site
Răchita Făget (Dealul Înalt)	200 m 311 m	N-W part of Poiana Ruscă Mountains
Românești (Dealul Cărării)	346 m	N of Poiana Ruscă Mountains on the bank of Bega-Uricani River

REV. ROUM. BIOL. – BIOL. ANIM., TOME 47, N^{os} 1–2, P. 3–8, BUCAREST, 2002

Table 1 (continued)

Collecting station	Altitude	Geographical site
Nădrag	710 m	W-S-W of Poiana Ruscă Mountains
Lugoj Forest	242 m	Lugoj Plain in N of Silagiu and Sacoş Hills
Rudna	70 m	Timiş Plain -bank of Timiş River
Green Forest-Timişoara	90 m	Timiş Plain
Buziaş-Park	128 m	N of Buziaş Hills
Grabaţi	85 m	N of Old Canal in Timiş Plain
Lovrin	90 m	N of Timiş Plain, W of Arad and Vinga Plains

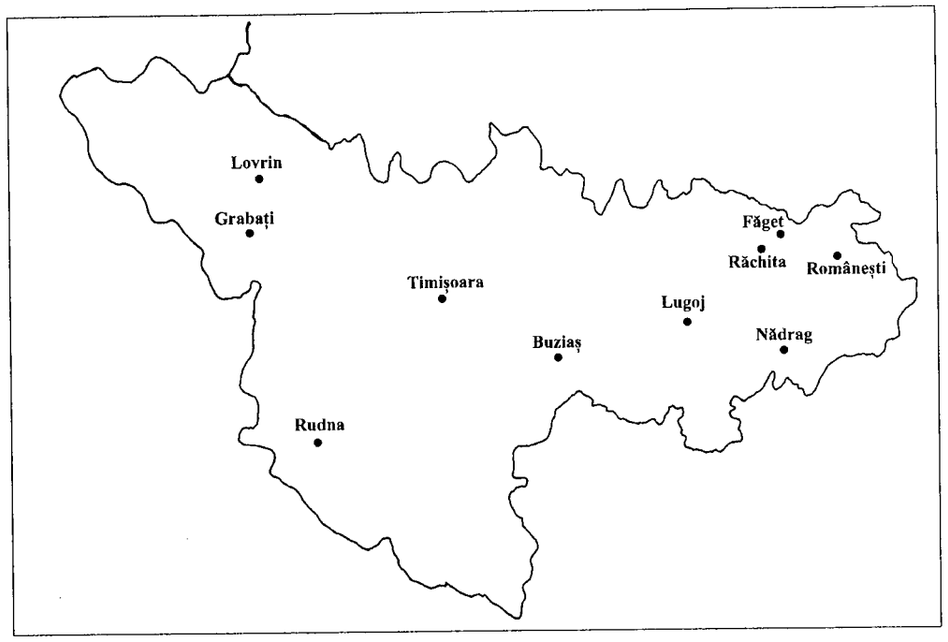

Fig. 1 – Map of Timiş district with collecting station.

3. RESULTS AND DISCUSSION

Further on, the list of the identified species is presented, set in order according to Professor dr. J. Martens – "Spinnentiere, Arachnida Weberknechte, Opiliones", Veb Gustav Fischer Verlag Jena, 1978 (13).

Subord. Laniatores

Suprafam. Travunoidea

Fam. Erebomastridae

1. Holoscotolemon jaqueti (Corti, 1905)

Subord. Palpatores

Suprafam. Troguloidea

Fam. Nemastomatidae

2. Nemastoma transsylvanicum Gruber et Martens, 1968
3. Carinostoma elegans (Soerensen, 1894)
4. Mitostoma chrysomelas (Hermann, 1804)

Fam. Dicranolasmatidae

5. Dicranolasma scabrum (Herbst, 1799)

Fam. Trogulidae

6. Trogulus tricarinatus (Linnaeus, 1767)
7. Trogulus nepaeformis (Scopoli, 1763)

Suprafam. Phalangioidea

Fam. Phalangiidae

8. Phalangium opilio Linnaeus, 1761
9. Opilio parietinus (De Geer, 1778)
10. Platybunus pinetorum (C. L. Koch, 1839)
11. Rilaena triangularis (Herbst, 1799)
12. Zacheus crista (Brullé, 1832)
13. Egaenus convexus (C. L. Koch, 1835)

Subfam. Oligolophinae

14. Oligolophus tridens (C. L. Koch, 1836)
15. Lacinius sp.

Subfam. Leiobuninae

16. Leiobunum rupestre (Herbst, 1799)

A synoptic table (Table 2) including collecting stations, collecting time, zoogeografic distribution and the number of individuals for all species is given.

Table 2

Species	Collecting stations	Collecting time	Nr. of ind.	Areal
1	2	3	4	5
Holoscotolemon jaqueti	Făget	18.05.2001	1	S-E-European, Carpathian, dinaric
Nemastoma transsylvanicum	Răchita	18.05-1.07.2001 1.07-27.08.2001	3 19	Endemic in Romania
Carinostoma elegans	Lugoj Făget	19.05.2001 16.05.2001	1	S-E-European, Carpathian
Mitostoma chrysomelas	Răchita Nădrag	18.05-1.07.2001 1.07-27.08.2001 4.07.2001	2 10 4	European-Atlantic

Table 2 (continued)

1	2	3	4	5
Dicranolasma scabrum	Făget Românești Lugoj Răchita	18.05-1.07.2001 1.07-27.08.2001 3.07-29.08.2001 19.05-31.08.2001 18.05-1.07.2001 1.07-27.08.2001	17 13 1 3 10 5	European, Carpathian, dinaric
Trogulus tricarinatus	Buziaş	20.05-2.07.2001	1	Subatlantic and continental
Trogulus nepaeformis	Buziaş Românești Făget Răchita Pădurea Verde	20.05-2.07.2001 3.07-29.08.2001 18.05-1.07.2001 1.07-27.08.2001 1.07-27.08.2001 12.05-27.06.2001	2 1 3 3 3 16	Antlantic and continental
Phalangium opilio	Răchita Grabaţi	18.05-1.07.2001 18-20.07 1999	3 37	Palearctic
Opilio parietinus	Grabaţi	8.11.2000	15	Palearctic
Platybunus pinetorum	Făget	18.05-1.07.2001	3	Montane, alpine, carpathian
Rilaena triangularis	Pădurea Verde Buziaş	21.05-27.06.2001 20.05-2.07.2001 2.07-27.08.2001	21 12 3	Atlantic and continental
Zacheus crista	Făget Rudna Pădurea Verde Răchita Buziaş	18.05-1.07.2001 1.07-27.08.2001 12.05-28.06.2001 12.05-27.06.2001 27.06-22.08.2001 18.05-1.07.2001 1.07-27.08.2001 2.07-28.08.2001	2 2 12 5 25 1 2 3	Ponto-mediterranean
Egaenus convexus	Făget Românești Lugoj Rudna Pădurea Verde Răchita Buziaş Lovrin	18.05-1.07.2001 1.07-27.08.2001 3.07-29.08.2001 19.05.2001 12.05-28.06.2001 12.05-27.06.2001 27.06-22.08.2001 18.05-1.07.2001 1.07-27.08.2001 20.05-2.07.2001 17.06.2001	3 2 15 1 10 10 10 6 7 6 4	S-European
Oligolophus tridens	Răchita Românești Nădrag Pădurea Verde	1.07-27.08.2001 3.07-29.08.2001 4.07.2001 27.06-22.08.2001	21 1 8 4	Atlantic and continental

Table 2 (continued)

1	2	3	4	5
Lacinius sp.	Răchita	18.05-1.07.2001	2 larva	
Leiobunum rupestre	Nădrag	30.08.2001 4.07.2001	3 ad. 6 juv	Alpine, Carpathian-secondary subatlantic

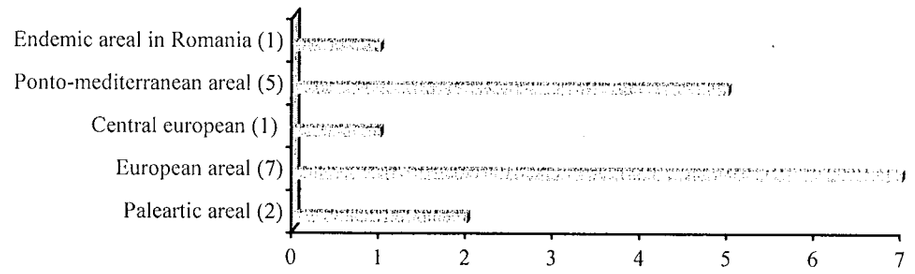

Fig. 2 – Diagrama with the share of different Opilionid species collected in Timiş district.

Considering the areal, the 16 species can be separated into 5 groups (4, 13, 14):

1) Palearctic areal-2 species: Phalangium opilio, Opilio parietinus
2) European (atlantic and subatlantic) areal-7 species: Trogulus nepaeformis, Trogulus tricarinatus, Dicranolasma scabrum, Leiobunum rupestre, Rilaena triangularis, Mitostoma chrysomelas, Platybunus pinetorum.
3) Central european-1 species: Ologolophus tridens
4) Ponto-mediteranean (balcanic)-5 species: Holoscotolemon jaqueti, Carinostoma alagans, Egaenus convexus, Zacheus crista, Lacinius sp.
5) Endemic areal (in Romania)-1 species: Nemastoma transsylvanicum.

Taking this caracter into consideration, the share of different Opilionid species met within the collecting made in Timiş is given into the Fig. 2 (number of species in brackets). According to the local general climate (moderate continental climate with oceanic and submediteranean influences) the most represented groups are number 2-seven species and 4-five species.

Acknowledgements. For literature, loan of comparative material, help in identification and confirming the identification of some species I thank the following persons and institutions: Acad. Dr. Petre Bănărescu; Dr. Dan Dumitrescu; Dr. Jürgen Gruber–Naturhistorisches Museum Wien; Natural History Museum "Grigore Antipa" Bucharest.

REFERENCES

1. Avram, Şt., Un nou opilionid: *Platybunus cîrdei* n.sp. Stud. Cerc. Biol., Ser. Zool., **9**, 6:481–486, 1964.
2. Avram, Şt., Laniatores cavernicoles de Roumanie. Revision des genres Scotolemops Rwr., Brigestus Rwr. Et Bukowina Rwr. (Opiliones, Phalangodidae). Annls. Speleol., 23, 1968a.
3. Avram, Şt., Contribuţie la cunoaşterea speciilor genului Platybunus din România (Opiliones, Phalangiidae): *Platybunus juvarae* n.sp., *P. decui* n.sp., *P. jeporum* n.sp. Lucr. Inst. Speol. «Emil Racoviţă».**8**: 163–185, 1968b.
4. Avram, Şt., Dumitrescu, D., Contribuţii la cunoaşterea răspândirii geografice şi a ecologiei opilionidelor cavernicole, endogee şi epigee din România. Lucr. Inst. Speol. «Emil Racoviţă», **8**: 99–145, 1969.
5. Avram, Şt., Quelques especes nouvelles ou connues du genre Trogulus Latr. (Opiliones). Trav. Inst. Speol. «Emile Racovitza», **10**, 245–271, 1971.
6. Bănărescu, P., Boşcaiu, N., *Biogeografie*, Editura Ştiinţifică, Bucureşti, 1973.
7. Dumitrescu, D., Sur Platybunus decui Avram, 1968 (Arach., Phalangiidae). Trav. Mus. Hist. Nat. «Grigore Antipa», **8**, 2. Le centenaire Grigore Antipa, 187–1967: 1021–1027, 1968.
8. Dumitrescu, D., Opilionide din Dobrogea. Comun. Zool. Soc. Şti. Biol., Rep. Social. Rom.,: 315–326, 1970.
9. Dumitrescu, D., Contributions à l'étude de la morphologie des organes génitaux externes chez quelques Opiliond de la famille Phalangiidae de Roumanie. Trav. Mus. Hist. Nat. «Grigore Antipa», **10**: 79–90, 1979.
10. Dumitrescu, D., Contributions à la connaissance de la faune d'Opilions (Arachnida) de Roumanie. Trav. Mus. Hist. Nat. «Grigore Antipa», **12**: 69–83, 1972.
11. Dumitrescu, D., Contribuţie la cunoaşterea faunei de Opilionide (Arachnida) din Masivul Ciucaş şi de pe valea superioară a Teleajenului. Comun. Ref., Muz. Şti. Nat., Ploieşti, 101–109, 1973.
12. Gruber, J., Martens, J., Morphologie, Systematik und Okologie der Gattung Nemastoma C. L. Koch (s. str.) (Opiliones, Nemastomatidae). Senckenbergiana biol., **49**, 137–172, 1968.
13. Martens,J., Spinnentiere, Arachnida. Weberknechte, Opiliones. In: Die Tierwelt Deutschlands, **64**: 1–464, 1978.
14. Weiss, I., Die Weberknechtfauna Siebenburgens (Arachnida: Opiliones), Stapfia, **45**, 259–280, 1996.

Received 9 December, 2002.

Faculty of Biology
University of Craiova
13 Al. I. Cuza, Craiova

RARE AND ENDANGERED FISHES IN THE DRAINAGE AREA OF THE MIDDLE AND LOWER DANUBE BASIN

PETRU M. BĂNĂRESCU

Six fish and one lamprey are endemic to the middle/lower Danube basin, two other species are shared only with the upper Danube. Some of them are abundant in their small range, but must be considered vulnerable. Some running water species are still widely distributed and locally abundant, but must be considered vulnerable because their habitat is endangered. Endangered standing water species are *Umbra krameri* and especially *Carassius carassius*.

Key words: endangered fish and lampreys, middle and lower Danube basin.

1. INTRODUCTION

The drainage area of the Middle and Lower Danube has the richest fish fauna in Europe, consisting of three lampreys, six sturgeons and 81 native species and well differentiated subspecies of bony fishes, eight of which (and one lamprey) are endemic, while two bony fishes and one lamprey are endemic to the entire Danube basin and six other species center in the Danube basin.

2. MATERIAL AND METHODS

The data included in the paper are the results of many years investigations of the fish fauna of Romania, eastern Hungary and la Transcarpathian Ukraine.

3. RESULTS

Many of these species are threatened, strongly threatened or vulnerable. Several categories can be distinguished among them.

Two sturgeons are probably extinct: the migratory Atlanto – Mediterranean *Acipenser sturio* and the sedentary Aralo – Caspo – Pontic *A. nudiventris*.

Three species and one subspecies, endemic to the middle/lower Danube area have quite restricted ranges (1, 4, 5):

– the almost extinct *Romanichthys valsanicola* that is dealt with in another paper (2);

REV. ROUM. BIOL. – BIOL. ANIM., TOME 47, N^{os} 1–2, P. 9–19, BUCAREST, 2002

– *Scardinius racovitzai*, endemic to a small thermal pond in western Romania (Fig. 1);

– *Telestes polylepis*, endemic to a few tributaries of Sava river in Croatia (Fig. 1);

– an undescribed subspecies of *Leuciscus borysthenicus* confined to the shallow lake Comana and its tributary brook in south central Romania (Fig. 1).

S. racovitzai and *L. borysthenicus* ssp. retained their abundance and are presently not endangered, but because of their very small ranges must be considered vulnerable (1). The present status of *T. polylepis* is not yet known.

Endemic with wider ranges are:

- the predatory lamprey *Endontomyzon danfordi*, confined to the mountain tributaries of Tisa River and some rivers in the Banat (Fig. 2); it underwent a rather strong numerical decline, must be considered endangered.
- the loach *Cobitis elongata*, distributed in many southern tributaries of the Middle and Lower Danube from Slovenia to Bulgaria and in a single northern tributary, the Nera in Romania (Fig. 1); it is abundant in the latter river (which flows through a natural park) and in some Serbian rivers, being presently not threatened; because of its small range it must be included in the vulnerable category;
- another loach, *Sabanejewia romanica*, distributed in many rivers in south-central Romania (some in the drainage area of the Middle, others in that of the Lower Danube (Fig. 3), locally abundant, again a vulnerable species (1, 5);
- the gudgeon *Gobio kessleri antipai*, ranging in the main channel of the Danube, from the confluence of the River Argeş down streams, and in the arms of the Danube Delta (Fig. 1); its present status is not known.

Two of the species shared only with the Upper Danube drainage area deserve mention:

- the non-predatory lamprey *Endontomyzon vladykovi*, reported only from quite few localities in Slovakia, western Hungary, Serbia and Romania (rivers Timis and Bega in the Banat, extinct from tributaries of the Olt) and everywhere rare (Fig. 4);
- the roach *Rutilus pigus*, present in small number in southern Slovakia, western Hungary, Croatia and at the limits between Hungary, Ukraine and Romania (4) (Fig. 5).

Both species must be considered endangered in the Middle Danube area, but the latter is safe in the Upper Danube drainage.

Many non-endemic species have a restricted distribution in the Middle/Loweer Danube drainage area, but most of them can not be considered threatened or vulnerable, e.g. the eastern sculpin *Cottus poecilopus* (1, 3), confined

to the northern tributaries of the Middle Danube, of the Tisa, Siret and Prut (Fig. 5), since it inhabits the uppermost stretches of rivers, where hydro tehnical construction can not be built, *Leuciscus borysthenicus borysthenicus* and many gobies, one *Pungitius* and one *Syngnathus* of marine Ponto – Caspian origin, present in the Danube Delta and Lower Danube, many of which are expansive species.

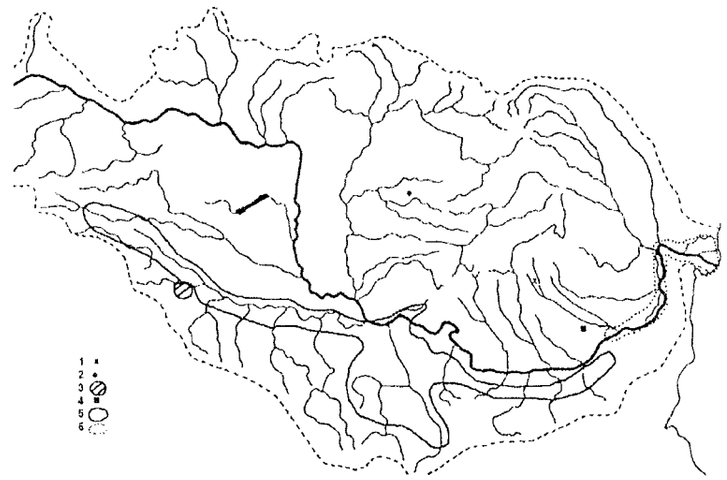

Fig. 1 – Distribution of six fishes endemic to the middle and lower Danube basin with restricted ranges. 1. *Romanichthys valsanicola*; 2. *Scardinius racovitzai*; 3. *Telestes polyepis*; 4. *Leuciscus borysthenicus ssp*; 5. *Cobitis elongata*; 6. *Gobio kessleri antipai.*

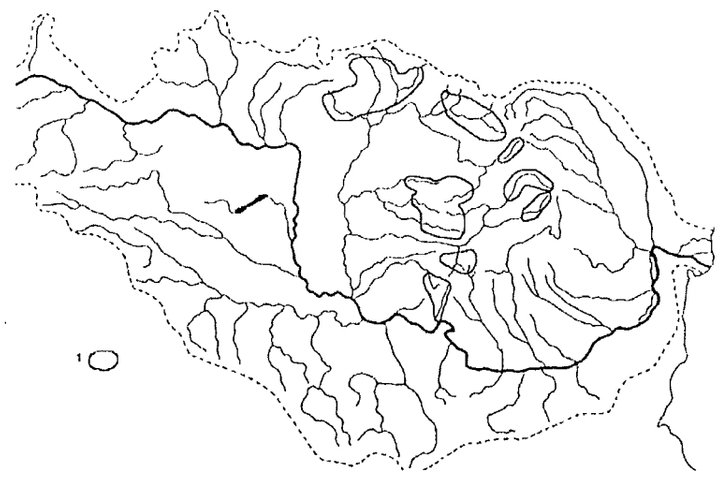

Fig. 2 – Distribution of the predatory lamprey *Eudontomyzon danfordi.*

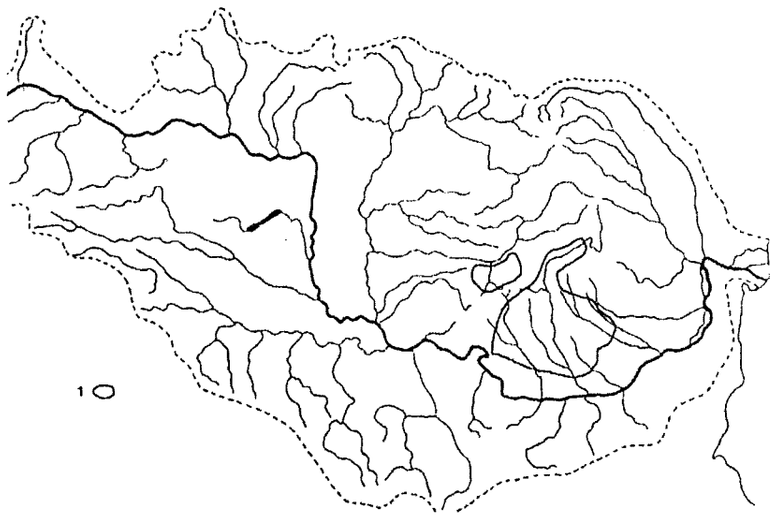

Fig. 3 – Distribution of *Sabanejewia romanica*.

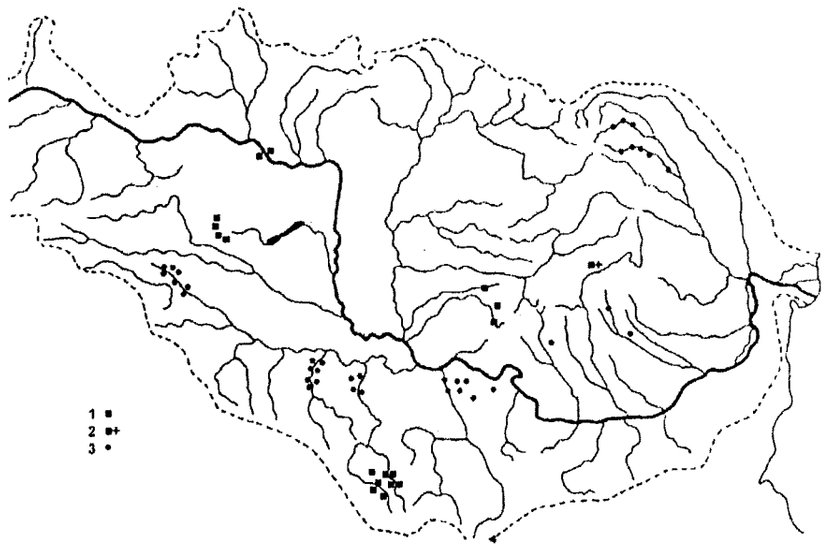

Fig. 4 – Distribution of two non-predatory lamprey in the basin of the middle and lower Danube
1. *Eudontomyzon vladykovi*, present; 2. *E. vladykovi*, extinct; 3. *E. mariae*.

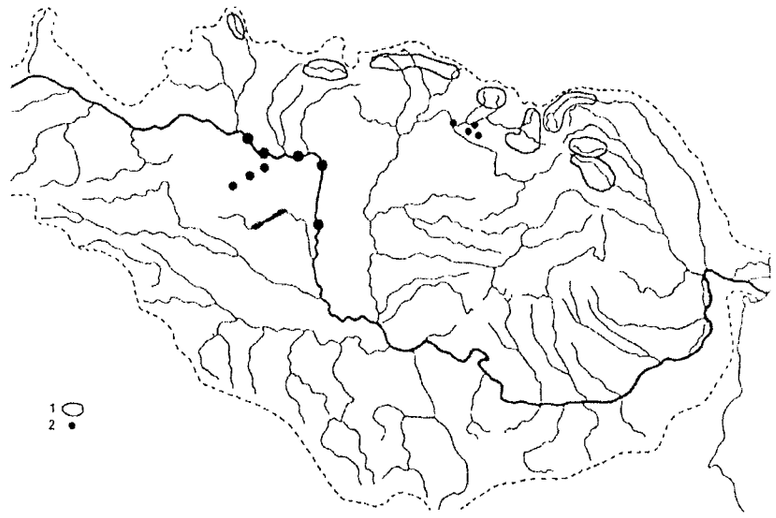

Fig. 5 – Distribution of *Cottus poecilopus* (1) and *Rutilus pigus* (2) in the basin of the middle and lower Danube.

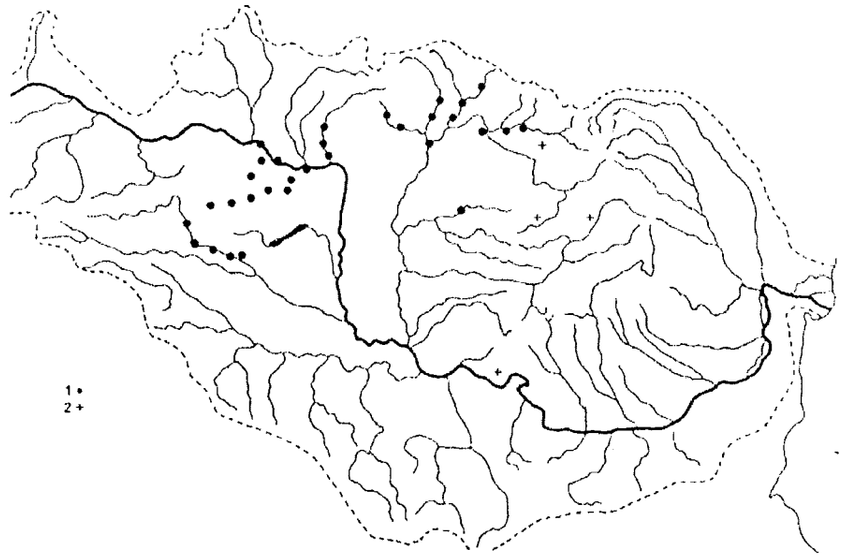

Fig. 6 – Distribution of *Leuciscus leuciscus* in the basin of the middle and lower Danube
1. resent; 2. extinct.

On the contrary, *Telestes souffia*, disjuntly distributed in the Middle Danube area (upper Drava and Sava on one hand, upper Tisa and tributaries on the other

hand) is vulnerable, its habitat (submontane reaches of large rivers) is potentially endangered by hydro tehnical constructions.

Endangered are also:

- the non-predatory lamprey *Endontomyzon mariae*, distributed in the montane, submontane and hilly areas of the tributaries of the Lower Danube (Fig. 4) (larvae occasionally found also in lowlands) and in the basins of more eastern rivers (Nistru Dnieper etc.), which became extinct from many rivers and is rare in others;
- the three migratory sturgeons of Ponto – Caspian origin: *Huso huso*, *Acipenser gueldenstaedti*, in a lesser measure *A. stellatus*. Because of the construction of the Iron-Gates dam lake, their best spawning places disappeared and they can no longer migrate up streams. Over fishing, mainly illegal, has also determined a numerical decline;
- a large sized bleak, *Chalcalburnus chalcoides mento* was present (but not numerous) in the Upper Danube area, in the Danube at the Iron-Gates stretch and in the Lower Danube (including many shallow lakes of its floodplain but not of the Delta). It became extinct from the Iron-Gates area and has no more been found in the floodplains of the Danube, being found recently only in the Danube near Galaţi (this species is endangered);
- a pike-perch species, *Stizostedion volgense*, has probably become extinct from the Lower Danube area, survives in many localities in Hungary and Slovakia (where it is considered not very rare: Barus & Oliva, 1995); it became extinct also from several rivers in the Middle Danube catchment of Romania, surviving in Crişul Negru river. The species must be considered endangered in Romania, vulnerable in Hungary and Slovakia;
- the dace, *Leuciscus leuciscus*, has a wide Euro – Siberian range. It is safe in the drainage area of the Upper Danube, but more rare in that of the Middle Danube. It was recorded from the Lower Danube drainage of southern and eastern Romania (Antipa, 1909), but not in Bulgaria (Drensky, 1951). In the Middle Danube drainage it is present, in small number, in Czekia, Slovakia, Croatia, western and north-eastern Hungary, was also present in several rivers in the Romanian area of the Middle Danube, becoming extinct in some of them (e.g., Someşul Mic); it survives in the Crişul Repede River (Fig. 6). The species must be considered threatened;
- the salmonids *Hucho hucho* and *Thymallus thymallus* have undergone numerical decline.

Both are protected by law and have been repopulated in some stretches of rivers.

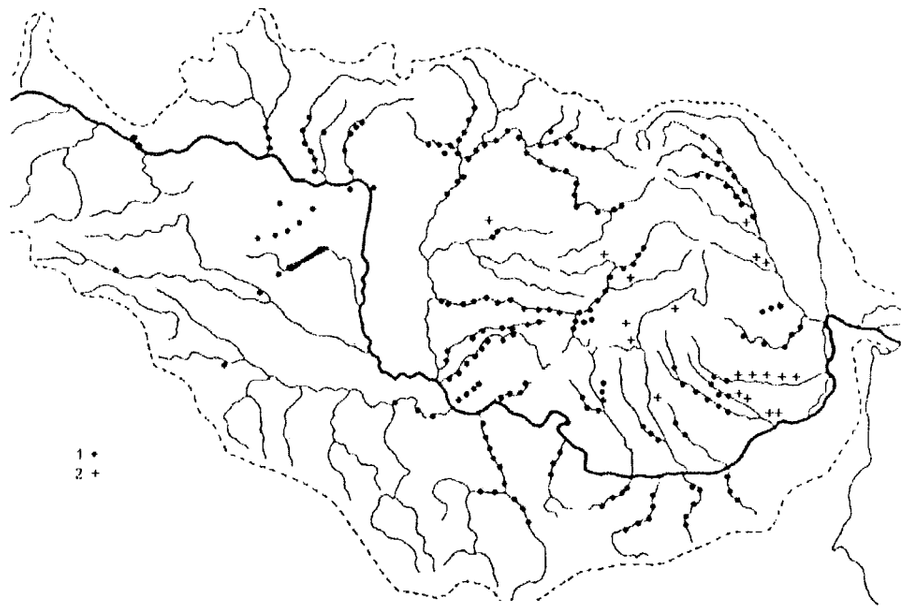

Fig. 7 – Distribution of Gobio kessleri in the Danube basin
1. resent; 2. extinct.

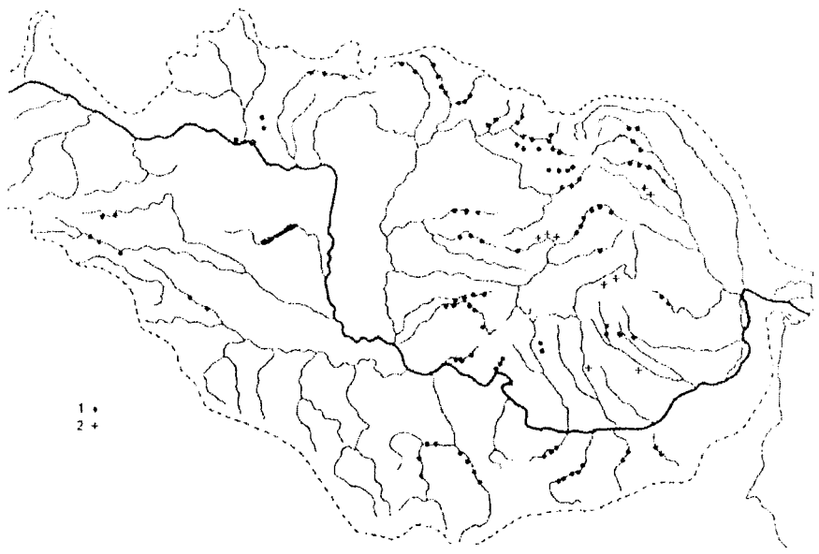

Fig. 8 – Distribution of *Gobio uranoscopus* in the basin of the middle and lower Danube
1. resent; 2. extinct.

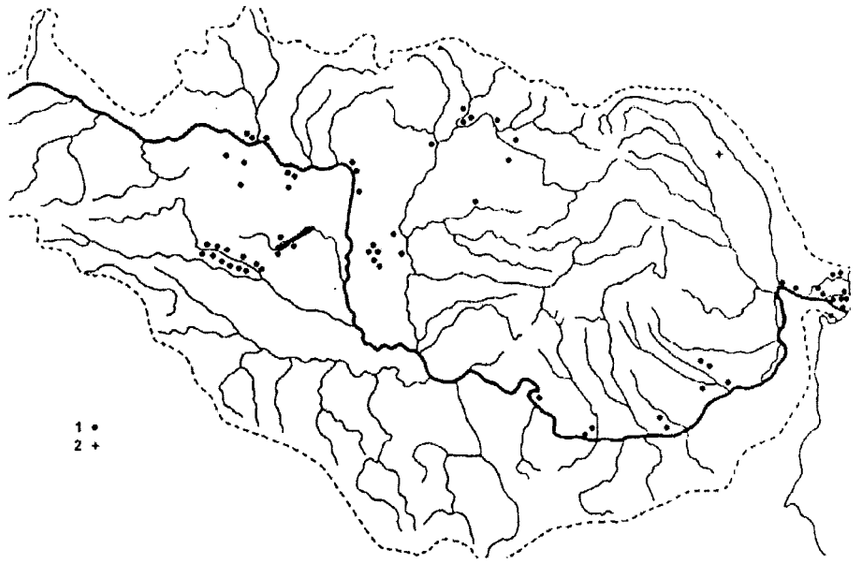

Fig. 9 – Distribution of *Umbra krameri* in the Danube basin
1. resent; 2. extinct.

A special category comprises the rather widely distributed species which are, at least locally, still abundant, but whose habitat is endangered by future economical activities, mainly damming of rivers. Most of these are confined to running water, either in submontane areas or in lowlands. To this category belong:

- four percids: *Gymnocephalus baloni*, *G. schraetser*, *Zingel zingel* and *Z. streber*, all inhabitants of lowland river, the latter also living in submontane rivers; it became extinct in many rivers, very rare in others, retained its abundance in few other rivers (in Romania only in Crişul Negru and in Nera). The three former must be considered vulnerable, Z. streber endangered;
- the loach *Sabanejewia aurata* that is especially interesting because of its strong geographical variability (four accepted subspecies connected by intergrading populations; it is more vulnerable than its congener *S. romantica*, since it lives in not too small rivers while the latter inhabits also brooks and rivulets of no hydroenergical interest.
- the sedentary sturgeon *Acipenser ruthenus*, which underwent a strong numerical decline and must be considered endangered (1);
- the gudgeons *Gobio uranoscopus*, inhabitant of rapidly flowing stretches of submontane rivers (Fig. 8) and *G. kessleri* (Fig. 7), which lives in moderately running sandy rivers, mainly in lowlands. Both

species became extinct from strongly polluted or dammed stretches of rivers but retained their abundance in non-modified stretches *G. kessleri* is especially abundant in many rivers, living in large swarms, sometime comprising hundreds of specimens. Both species are vulnerable.

Two species living in stagnant waters are also threatened by modification of the habitat:

- the European mud minnow, *Umbra krameri* lives in marginal areas of lakes and especially in isolated ponds and pools with much vegetation and in slowly flowing muddy rivulets throughout the lowlands in the drainage area of the Middle and Lower Danube (and also of the nearby river Nistru or Dniester) (Fig. 9). It is still quite abundant in many localities, but its habitat is endangered through the draining of ponds and rivulets. The species is rather vulnerable than endangered.
- the crucian carp, *Carassius carassius*, until a few decades ago quite abundant in the Middle and Lower Danube drainage area (it was the most abundant species in many lakes of the Danube Delta), has undergone a spectacular and unexpected numerical decline, becoming extinct from many water bodies and very rare in others. This decline has two causes:
  1) the eutrophisation of water which determined almost disappearance of the submerse aquatic vegetation;
  2) the competition from the apparently exotic *C.auratus gibelio*. The crucian carp still survives, even in high number, in some isolated dystrophic pools, especially in hilly areas. It must be considered either as endangered or as vulnerable.

Contrary to the endangered or vulnerable fish species, other are "expansive", enlarged their ranges and became more numerous, e.g. *Perca fluviatilis*, *Carassius auratus gibelio*, *Leuciscus cephalus*, *Gobio gobio*, *Alburnus alburnus*, in a certain measure also *Gobio albipinnatus*. Expansive are also most gobies of brackish water Ponto – Caspian origin, especially *Neogobius fluviatilis*, but also *N. kessleri*, *N. gymnotrachelus*, *N. melanostomus*, *Bentophilus stellatus*: only *N. syrman* underwent a numerical decline, while *N. eurycephalus* (until recently reported as *N. cephalarges*), *Proterorhinus marmoratus* and *Knipowitschia caucasia* retained their ranges and abundance. Very rare (present only in the arms of the Danube Delta and very small number) is *Bentophiloides brauneri*.

4. PROTECTION MEASURE

Many of the above listed species are officially "protected by law". But what does that practically mean? People usually considered that protection means

interdiction of fishing. Actually interdiction or limitation of fishing is an efficient measure only for large-sized, late maturing species: sturgeons, *Hucho*, *Thymallus*. But most endangered and vulnerable fish species are small, maturate in the second or third year and have no commercial value. If an ichtyologist collects one or a few specimens of *Umbra krameri* for study in Hungary or Slovakia is punished; but who prevents the draining of the pond where the species lives, this draining determining the death of hundreds or thousands of *Umbra* specimens? Few people realize how abundant are most of the endangered small-sized fish species (with few exceptions, above all *Romanichtys valsanicola*) when the habitat is not affected. One can collect without danger tens of *Cobitis elongata* in the river Nera, of *Umbra krameri* in the rivulet Gurbanu (tributary of the lake Comana), of *Gobio uranoscopus* in some rivers, many hundreds of *Gobio kessleri* in the rivers Timis, Somes or Crişul Negru. The only efficient measure to protect these species is to protect their habitat.

5. ADDITIONAL REMARKS

Several red books or red lists include erroneous data. E.g. *Gobio albipinnatus* is listed (also included in the Berna Convention) as endangered. Actually it has a wide range, from Germany to the upper Volga and the Volga Delta, in many places quite abundant and it is extending its range. On the contrary, *Carassius carassius* is nowhere mentioned as endangered; actually it became very rare, at least in Slovakia, Hungary and Romania.

The protection status of the same species can be different in the same country or river basins; e.g., *Leuciscus leuciscus* is safe in the Upper Danube drainage, vulnerable in Hungary and Slovakia (Middle Danube drainage), endangered in western Romania (again in the Middle Danube drainage), extinct in eastern and southern Romania (Lower Danube drainage), absent in northern Bulgaria (southern part of the Lower Danube drainage area).

The term "rare", recommended by I.U.C.N. is quite ambigous since it includes two quite distinct categories of species: widely distributed ones which are everywhere rare, and species with restricted ranges, but quite abundant in their range, hence not endangered, unless their habitat is subject to drastic modifications.

6. CONCLUSIONS

The protection of the habitats can be realized by:

- interdiction of the construction of dam lakes, of installation of industries which endangered the water quality, of capture of water on some rivers, e.g., the Nera (the only river in Romania where *Cobitis elongata* lives (and in great quantity) and in which vulnerable fish species, *Gobio uranoscopus, G. kessleri*, *Sabanejewia aurata*, *Zingel streber* are numerous, and the brook Gurbanu (where *Umbra krameri* and the undescribed *Leuciscus borysthenicus* subspecies are abundant);
- interdiction of constructing dam lakes on long stretches of other rivers;
- preservation of rather great number of ponds inhabited by *Umbra krameri* and *Carassius carassius*; for the latter species it is necessary to prevent the introduction of *C. auratus gibelio*.

REFERENCES

1. Bănărescu P, The present-day conservation status of the Freshwater fish fauna of Romania. Ocrot.nat.med.inconj., **38** (1): 5–20, 1994.
2. Bănărescu P, *Romanichtys valsanicola, a highly theatrened species from the Danube River Basin in Romania*. In: Collares-Pereira, M., I. Cowx & M. Coelho (eds.), *Conservation of Freshwater Fishes: Options for the Future*. Blackwell Sc., London, 2000, p. 174–177.
3. Barus, V. & O. Oliva (eds), *Fauna CR a SR Mihulovici a ryby*, 2 vols., Nakladatel. Akad.Věd České rep., 623 and 698, Praha, 1995.
4. Harka, A., Halaink Természet és Körn, *Tanár. Egyesülete*, Budapest, 176 pp, 1997.
5. Maitland, P.D., *Conservation of threatened freswater in Europe*. Council of Europe Press, Strasbourg, 76 p, 1991.

Received November 2002.

Institute of Biology, 296 Spl. Independenței, 060031, București
e-mail: petre.banarescu@ibiol.ro

COMPARISON STUDY OF ENCEPHALON IN *OPSARIICHTHYS PACHYCEPHALUS, ZACCO PLATYPUS* AND *ZACCO TEMMNICKII* (PISCES, TELEOSTEI, CYPRINIDAE)

CARMEN BĂLESCU

The research carried on external morphology of encephalon, in 3 species of cyprinids: *Opsariichthys pachycephalus, Zacco platypus* and *Z. temmnickii*, show few differences, regarding the aspect of encephalon vesicles, depending on body length, environment and life style. By taking in account the development of vagal and facial lobes at the myelencephalon level, cyprinids species studied are in the group I, a group with vagal and facial lobes medium developed. By morphological criterion, the encephalon of analyzed species belongs to the group of basis brains of cyprinids type (generalised). Varability of encephalon divisions and subdivisions is low and medium at the studied species being influenced by environmental factors and fish size and they have normal parameters.

1. INTRODUCTION

Cyprinids species *Opsariichthys pachycephalus, Zacco platypus* and *Z. temmnickii*, that belong to Danioninae subfamily, have a similar exterior morphology. They are characterised by: elongated and side compressed body; lateral line is slanting and placed closer to the abdomen; superior mouth is lacking barbels. They swim in midwater and towards surface. These species have origin in fresh-waters of South-Eastern Asia.

In reference literature, I did fiind litťle data on encephalon structure in genera *Opsariichthys* and *Zacco*. Itto, in a paper in 1978, reminded the fact that encephalon sructure was studied in species *Zacco platypus*, but he did not mention any kind of data about that. Howes (1980), describes very summarily the encephalon in *Zacco platypus;* of the genera *Opsariichthys*, he studied the encephalon in the *Opsariichthys uncirostris* and *O. barbatus* species. Attempts on Internet in order to find out other information on the brain of the mentioned species failed.

That is the reason I present by comparison the specific features of 5 encephalon vesicles, in 3 cyprinids: *Opsariichthys pachycephalus, Zacco platypus* and *Z. temmnickii*, closely related to the environment and to the way of life.

REV. ROUM. BIOL. – BIOL. ANIM., TOME 47, N^{os} 1–2, P. 21–30, BUCAREST, 2002

2. MATERIAL AND METHODS

Species that were beforehand preserved in alcohol were obtained from the personal collection of Acad. Dr. Doc. Petru Bănărescu to whom I give sincere acknowledgements. I have studied few individuals (one in *Opsariichthys pachycephalus*, two in *Zacco temmnickii*, three in *Zacco platypus*), but they were relevant to follow up by comparison the variability of varions vesicles, in the 3 species of cyprinids.

The encephalon of the individuals studied, has been extracted out of the skull by dissection and put in a tray with water, to prevent dehydration. In order to observe the details of exterior morphology of encephalon, viewing was made through a binocular magnifying glass. Drawings were made at clear chamber. In each individual studied, measurements were made for viewing the following: body length (from nose to caudal basis) and the main divisions and subdivisions of encephalon, that were carried out in areas with maximum dimensions (olfactory bulbs, olfactory tracts, cerebral hemispheres, lateral lobes of hypothalamus, optic lobes, corpus cerebelli, valvula cerebelli, myelencephalon). The weight was measured with mechanical scales while encephalon weight was measured with analytical scales. Data were processed by using mathematical statistics methods (Ceapoiu, 1968). The following mathematical statistical indices were used: arithmetical average ($\bar{x}$), variance (S^2), standard aberration of arithmetical average ($S_{\bar{x}}$) and variability coefficient (S%), upon which an estimation of encephalon divisions and subdivisions variability can be made.

3. RESULTS AND DISCUSSIONS

CHARACTERIZATION OF ENCEPHALON

The observations on telencephalon have led to pointing out the following characteristics: In species analized, on the surface of the two halfs of telencephalon (cerebral hemispheres), sulci are distinguished, that delimit tubercles. In *Opsariichthys pachicephalus*, the sulci and tubercles can be hardly distinguished; more obvious is latero-dorsalis sulcus that separates lateral tuberculus from the medial one, which is smaller, and posterior sulcus, under which tuberculus posterior, which is larger, is distinguished; sulcus latero-dorsalis is forking in sulcus antero-lateralis and antero-medialis, dimly shaped out, that delimit tuberculus anterior, which is barely shaped out; by leaning the telencephalon in front, it is obseved tuberculus postremum, which is separated from the posterior one, through sulcus postero-postremum; laterally to tuberculus postremum it is distinguished tuberculus latero-postremum; the two tubercle are separated by the sulcus having the same name (latero-postremum); by leaning the telencephalon side wards, it is remarked tuberculus pars ultima tuberculi lateralis, which is small, triangular, placed also in posterior part of lateral tuberculus, towards the exterior side (Fig. 1. a.). In *Zacco platypus* (Fig. 1.b., c.), sulci and

tubercles are maintained similarly as in *Opsariichthys pachicephalus*; sulci are weakly visible and tubercles are not prominent. In *Zacco temmnickii* (Fig. 1.d.), sulci are much more evident, as compared with the other 2 species; it is observed very clearly the sulcus posterior, which delimits tuberculus posterior, which is large; sulcus latero-dorsalis, which is also evident, delimits tuberculus lateralis, which is proeminent, by tuberculus medialis; tuberculus posterior is separated from tuberculus postremum by sulcus postero-postremum; tuberculus postremum is visible by leaning the telencephalon forward; laterally to tuberculus postremum, it is distinguished sulcus latero-postremum, which delimits a small tuberculus towards exterior, which is named tuberculus latero-postremum; this is more visible by leaning the telencephalon on one side. In all three species, olfactory bulbs are oval and are linked to olfactory lobes, that are assimilated in cerebral hemispheres, by relatively long olfactory tracts. The length of olfactory tracts is in direct proportion to body size for example: in *Z. platypus*, at the individual with larger size body (10.07cm.), olfactory tract is 3.2 mm. and at the individual with smaller body size (7 cm.), olfactory tract length is 2.1 mm. (Fig. 1. b., c.). In the species *Opsariichthys pachicephalus*, in comparison with the species *Opsariichthys uncirostris*, *O. barbatus* (Howes, 1980), I haven'not come across the following: - the obvious elongation of the two cerabral hemispheres; -the separation of the telencephalon from the mesencephalon by long posterior trabeculae. The form of the telencephalon in species *Z. platypus*, does not differ from the same species studied by Howes (1980).

Concerning the diencephalon, hypothalamus lobes are very evident and they are placed on ventral side. Medial lobe, included among lateral lobes, has rostral area rounded in *Opsariichthys pachycephalus* (Fig. 1. a.), and straight one in *Zacco platypus and Z. temmnickii* (Fig. 1. b., d). It was interesting to see a sulcus transversalis present in *Z. platypus*, on the surface of lobus medialis, at its caudal side; this sulcus transversalis is evident and it separates the 2 halves of lobus medialis into 2 unequal parts (Fig. 1. b., c.). In *Opsariichthys pachycephalus* and *Z. temmnickii* (Fig. 1. a., d.), the two halves of lobus medialis are departed between them at their caudal side, a level in which one can distinguish an elongated, diamond-like formation, which I assign to saccus vascularis. The separating distance is more pronounced in *Opsariichthys pachycephalus* than in *Z. temmnickii*. In *Z. platypus*, following the lobus medialis it is remarked the formation coresponding to saccus vascularis, but it is very small (Fig. 1. b., c.). Lateral lobes with reniform shape, are close to each other caudally and have three sulci on their dorsal surface: longitudinal, medial and mammillary. Corresponding tuberculi are not visible. The shape of hypothalamus lobes taken together, is almost spherical in *Opsariichthys pachycephalus* and two individuals in *Z platypus* (Fig. 1. a., c.), is a rounded hexagone in the 3-rd individual of *Z. platypus* (Fig. 1. b.), and is a trapezium in *Z. temmnickii* (Fig. 1. d.).

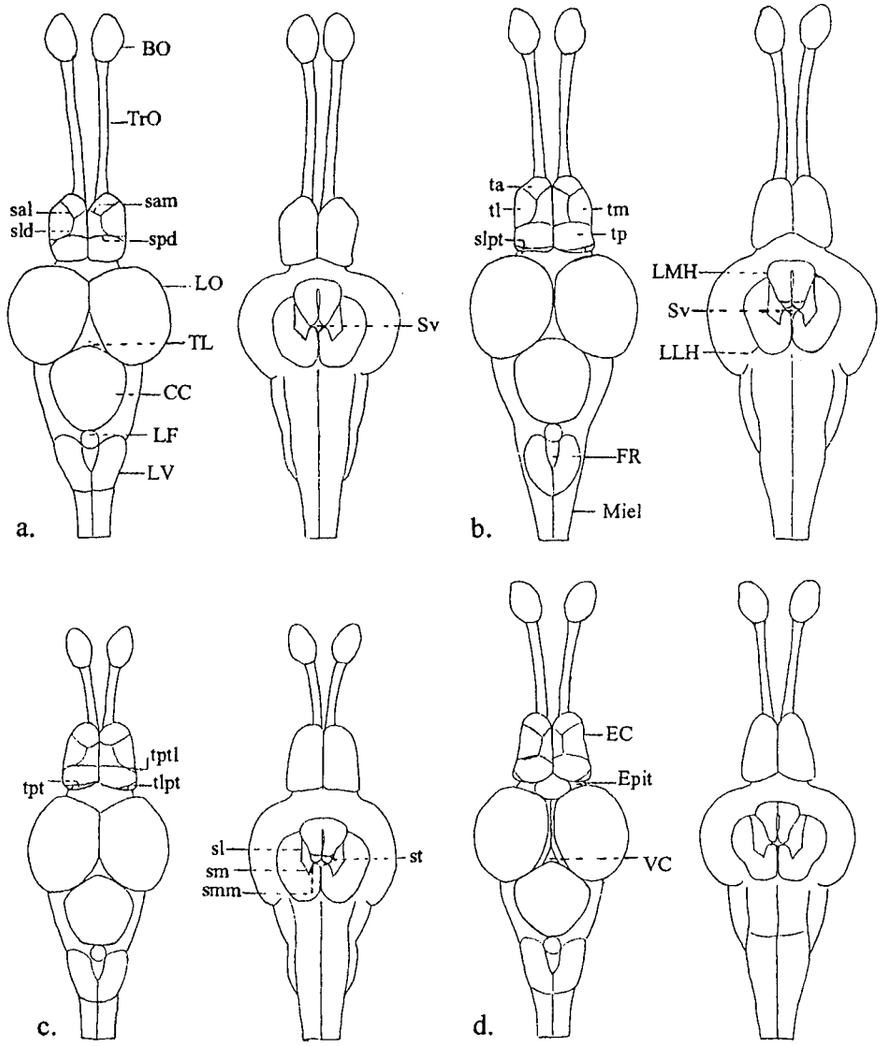

Fig. 1 – Dorsal (left) and ventral (right) view of encephalon in: a.- *Opsariichthys pachycephalus*; b., c. - *Zacco platypus*; d. - *Z. temmnickii*. BO=bulbus olfactorius; TrO=tractus olfactorius; EC = cerebral hemisphere ; Epit=epitalamus; LLH=lobus lateralis hipotalamic; LMH=lobus medianus hipotalamic; Sv=saccus vascularis; LO=lobus opticum; TL=torus longitudinalis; VC= valvula cerebelli; CC = corpus cerebelli; Miel = mielencephalon; LF=lobus facialis; LV=lobus vagus; FR = fossa rhomboidea; sld=sulcus (s.) latero-dorsalis; sal=s. antero-lateralis; sam=s. antero-medianus; spd=s. postero-dorsalis; slpt= s. latero-postremum; sl = s. longitudinal; sm=s. median; smm=s. mamilar; st=s. transversal; ta=tuberculus (t.) anteriorus; tl=t. lateralis; tm=t. medianus; tp= t. posteriorus; tptl=t. pars ultima tuberculi lateralis; tpt=t. postremum; tlpt=t. latero-postremum. Scale - 4,9:1 (a., b., c.); 5,15:1 (d.)

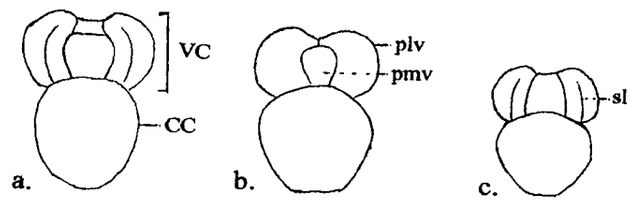

Fig. 2 – Valvulla cerebelli (VC) in: a. -*Opsariichthys pachycephalus*; b. - *Zacco platypus*; c. - *Z. temmnickii*. CC-corpus cerebelli; pmv-median side of valvulla cerebelli; plv-lateral side of valvula cerebelli; sl-sulcus longitudinalis. Scale - 5,5:1(a.); 5:1 (b.); 6:1(c.)

Regarding the mesencephalon, optic lobes are large and rounded. In the individual with smaller body of *Z. platypus*, the optic lobes seem flattened and elongated, being a likely consequence of skull pressing upon encephalon. In *Opsariichthys pachycephalus* and two individuals of *Z. platypus*, the optic lobes join on medial line and on front side and they are a little departed on back side. Departing is more evident in *Opsariichthys pachycephalus* (Fig. 1. a.), than in *Z. platypus* (Fig. 1. c.). At this level the torus longitudinal is distinguished. In an individual of *Z. platypus* and *Z. temmnickii* (Fig. 1. b, d.), optic lobes are departed between themselves on their entire medial length, more caudally than rostrally, a level in which the torus longitudinalis are seen on their entire medial length this is more evident in *Z. temmnickii* than in *Z. platypus*. Caudally and medially, in *Z. temmnickii* the valvula cerebelis is very litlle distinguished (Fig. 1. b.).

Among metencephalon components, I analyzed the corpus cerebelli and valvula cerebelli. In all analyzed species, the corpus cerebelli is smaller than the optic lobe. The shape of corpus cerebelli is different, being in correlation to the skull shape. Pentagonal shape with rounded sides is found in *Opsariichthys pachycephalus* (Fig. 1. a.) and *Z. platypus* (Fig. 1. b., c.), the shape of rounded rhomb in *Z. temmnickii* (Fig. 1. d.). Metencephalon development is related to mobility degree. It is well known that species that swim in midwater and at the surface, have a larger corpus cerebelli. The species of both genera are medium and surface species, but they have a smaller corpus cerebelli. Nevertheless they remove and orientate pretty well in Asiatic rivers. In this respect, the other formations play an important role, such as: valvula cerebelli, eminentia granularis, lateral line. Valvula cerebelli, a component part of metencephalon, is of cyprinid type (Franz, 1912). In *Opsariichthys pachycephalus*, valvula cerebelli is slightly exceerding the width of corpus cerebelli (Fig. 2. a.). In *Z. platypus* and *Z. temmnickii*, valvula cerebelli and corpus cerebelli have almost the same width value (Fig. 2. b., c.). In *Z. platypus*, lateral parts of valvula cerebelli are closed to each other in their anterior side, so that they exceed the medial portion at thip level (Fig. 2. b.). In *Opsariichthys pachycephalus* and *Z. temmnickii* (Fig. 2. a., c.), lateral portions are

departed to each other at anterior side. On each lateral lobes surface we have noticed a longitudinal sulcus, except in the individual of *Z. platypus*, with small body length. Medial portion in *Opsariichthys pachycephalus* has a trapesius shape with lesser basis placed caudaly (Fig. 2. a.); in *Z. platypus* it has a egg shape (Fig. 2. b.); in *Z. temmnickii* it has a bell shape (Fig. 2. c.). From the shape point of view, the valvula cerebelli shape is varions among species. I think this variation is given by the size of mesencephalon ventricle.

Myelencephalon through its divisions is in close correlation to feeding mode (Evans M. E., 1931; Evans H. E., 1952; Miller and Evans, 1965; Davis and Miller, 1967, etc). Facial lobe is very small, spherical, visible, being not necessary to depart the corpus cerebelli; laterally-posteriorly it is flanked by vagal lobes, which are hardly visible and placed on one side and the other of rhomboidal fossa. Rhomboidal fossa has a triangle aspect, with its basis placed previous (Fig. 1. a-d.). Depending on facial lobe and vagal lobes development, species are classified in group I, a group characterized by less developed facial lobe and vagal lobes. Classification was made by taking into account the classification made by Bănărescu (1949). Acoustical tubercles have a large surface. There is not a clear delimitation between anterior and posterior parts, they being at the same level; they are not prominent. Anterior parts are flattened, only in the medial part, as a result of corpus cerebelli pressing them. Acoustical tubercles are not placed on drawing, being very hardly visible.

Taking into account recent data in the reference literature (Brandstätter and Kotrschal, 1990; Kotrschal and collab., 1991, 1992, 1998), from a morphological point of view, species are classified in group of brains of generalised type-cyprinid primary brain. This kind of brain is characterised by well-developed visual lobes, but a medium size of octavolateralis lobes and taste lobes. Analyzed species are living in midwater and detecting the food by sight.

INTERPRETATION OF ALLOMETRICAL DATA

The three species have been characterised also based on statistical interpretation, of relative size of brain divisions and subdivisions with respect to length. Ratio was made at total length of brain, which includes small olfactory bulbs and tracts.

We can see in Table 1, where the data regarding the average of relative values of the length of brain divisions and subdivisions in species studied is presented, that the highest value is found at the myelencephalon level: 28,1954 % in *Zacco temmnickii*, 27,5923 % in *Z. platypus*. Myelencephalon remains a basis component, at its level are the important nervous centers (taste, acoustical,

movement coordination ones). Optic lobes are on the second place after the mylencephalon, recording high values: 22,807 % in *Zacco temmnickii*, 22,2097 % la *Z. platypus*. Optic lobes have a special role in these fish lives; with their help, the species are easily detecting food. I haven't taken into discussion *Opsariichthys pachycephalus*, since I had a single individual, I have written down only direct values of divisions and subdivisions length, in ratio to encephalon length.

Table 1

Values of statistical indices in *Opsariichthys pachycephalus* (O.p.), *Zacco platypus* (Z.p.) and *Zacco temmnickii* (Z.t.). $\bar{x}$ -average of relative values of encephalon divisions and subdivisions length; $S_{\bar{x}}$ = standard aberration of arithmetical averge; S%=variability coefficient. In the species specified with star, only values of encephalon divisions and subdivisions are written, in relation to encephalon length

Species	Indices	BO/En	TrO/En	EC/En	LH/En	LO/En	CC/En	VC/En	Miel/En
*Op	$\bar{x}$	9,1503	26,1437	15,0326	18,3006	20,2614	15,6862	13,0718	22,2222
Z.p.	$\bar{x}\pm S_{\bar{x}}$	9,6483 ± 0,5038	22,2021 ± 2,27	17,5311 ± 1,0327	19,7001 ± 0,8572	22,2097 ± 1,161	15,4516 ± 0,7828	12,8886 ± 0,2131	27,5923 ± 0,1976
	S%	9,0440	17,7086	10,203	7,537	9,0537	8,7751	1,0567	1,2405
Z.t.	$\bar{x}\pm S_{\bar{x}}$	10,818 ± 0,2066	20,4051 ± 1,5211	18,1704 ± 0,6202	20,0918 ± 0,3839	22,807 ± 0,7088	17,3767 ± 0,0590	11,9673 ± 0,0314	28,1954 ± 0,2657
	S%	2,7008	10,5499	4,8276	2,7025	4,3955	0,4807	2,25	1,3328

After myelencephalon and optic lobes, I have seen high values also at the level of lower lobes of hypothalamus, where we found values of 20,0918 % in *Zacco temmnickii* and 19,7001% in *Z. platypus*. This aspect shows the important role of lower hypothalamic lobes in adjusting the vegetative functions and feeding behaviour. I haven't discussed olfactory tracts (whose values are high), since they are linking elements (considered as secondary olfactive nerves), between olfactory bulbs and olfactory lobes of cerebral hemispheres.

Regarding the variability of encephalon divisions and subdivisions, in table 1., I found the following facts: in *Zacco temmnickii*, variability coefficient has values between 0–10%, variability being low on most divisions, except the olfactory tracts, where S% is 10,5499%, in his case variability is medium. For *Z. platypus*, variability coefficient is between 10–20%, for only 2 divisions: cerebral hemisphere (where S% is 10,2030%) and olfactory tracts (where S% is 17,7086%), for the rest of them variability is low for divisions, variability coefficient being between 0–10%.

Table 2

Absolute values of body weight, brain weight, values of brain weight on body weight, as well as body size of individuals studied in *Opsariichthys pachycephalus, Zacco platypus* and *Z. temmnickii*

No.	Species	No. of individuals	Body size (mm)	Body weight (g)	Brain weight (g)	Brain weight / Body weight (%)
1.	***Opsariichthys pachycephalus***	1	8.7	12	0.0620	0.5166
2.	***Z. platypus***	1.	11.47	20	0.0838	0.4190
		2.	10.07	12	0.0602	0.5016
		3.	7	4	0.0422	1.0550
3.	***Z. temmnickii***	1.	7.34	7	0.0464	0.6628
		2.	7.13	6	0.0402	0.67

Table 3

Values of statistical indices in *Zacco platypus* and *Z. temmnickii* $\bar{x}$ =arithmetical average of relative value of brain weight; S $\bar{x}$ = standard aberation of arithmetical average; S% = variability coefficient

No.	Species	$\bar{x} \pm S_{\bar{x}}$	S%
1.	***Z. platypus***	0.6585 ± 0.1629	42.87
2.	***Z. temmnickii***	0.6664 ± 0.0560	13.602

As regards absolute weight of the brain, I found out that it increases in direct proportion to the body length, from the individuals with small body to the individuals with large body and absolute weight increases in reverse proportion to body size; it decreases from individuals with small body to individuals with large body (Table 2). Variability coefficient is 13.602 % in *Zacco temmnickii* and 2.87% in *Z. platypus*, variability of brain weight being medium in *Z. temmnickii* and it is very high in *Z. platypus* (Table 3).

4. CONCLUSIONS

In species studied: *Opsariichthys pachycephalus*, *Zacco platypus* and *Z. temmnickii* it is observed slight interspecific differences as regards the brain shape, depending on body length, life style and life environment.

Cerebral hemispheres have dorsal surface furrowed by weak sulci, that are delimiting slightly visible tubercles. They are more evident in *Zacco temmnickii*.

In caudal part of hypothalamic medial lobe, between its two halves, one can see a diencephalic formation which. I think is the saccus vasclararis. If in

Opsariichthys pachycephalus this formation is more evident, in *Zacco platypus* it is very small.

The optic lobes are large and they have 2 aspects, both intraspecific and interspecific. The distance betwen optic lobes is influenced by the development of valvula cerebelli.

Depending on the degree of vagal lobes development and facial lobe development, *Opsariichthys pachycephalus*, *Zacco platypus* and *Z. temmnickii* are classified in group I, a group characterized by weakly developed vagal lobes and facial lobe.

By the morphological criterion, of the encephalon one can see that species analyzed would belong to the group of primary brain of cyprinid type, a group characterized by well developed visual lobes, in detriment of octavolateralis and gustative lobes, which are small. In these species, that have mouth lacking barbels and they live in midwater, the eyes have an important role in identifying the food.

Relative weight of encephalon is oriented in the sense of a reversely proportional increase to body weight and body length, respectively. The low number of individuals does not allow me to draw a certain conclusion on medium and very high variability of relative brain weight.

The variability of brain divisions and subdivisions is low and medium, being influenced by environmental factors and fish divisions and they have normal parameters.

REFERENCES

1. Bănărescu P. 1949. *Contribuţiuni la studiul encefalului la teleosteeni în legătură cu felul de viaţă şi filogenie*. Teză de doctorat. 188 pp.
2. Brandstätter R. & K. Kotrschal. 1990. *Brain growth patterns in our European cyprinid fish species **(Cyprinidae,Teleostei)**, roach **(Rutilus rutilus)**, bream **(Abramis brama,**)common carp **(Cyprinus carpio)**, and sabre carp (**Pelecus cultrâtus)***. Brain. Behav. Evol., **35:** 195–211.
3. Ceapoiu N. 1968. *Metode statistice aplicate în experienţe de cîmp şi biologice*. Edit. Agro-Silvică. Bucureşti.
4. Davis B. J. and Miller R. J. 1967. *Brain patterns in Minnows of the genus **Hybopsis** in relation to feeding habits and habitat*. Copeia **1:** 1–39.
5. Evans H. E. 1952. *The correlation of brain pattern and feeding habits in four cyprinid fishes*. J. Comp. Neurol. **97**, nr. *1*:133–142.
6. Evans H. M. 1931. A comparative study of the brains in British Cyprinoids in relation to their habits of feeding with special reference to the anatomy of themedulla oblongata. Proc. Roy. Soc.Lond. **108**, 233–257.
7. Howes G. J. 1980. The anatomy, phylogeny and classification of bariliine cyprinid fishes. Bull. Br. Mus. nat. Hist. (Zool.). London, **37** (3): 129–198.
8. Ito H. 1978. A catalogue of histological preparations of the teleost brains. Med. J. Osaka Univ., **28:** 219–228.

9. Kotrschal K., R. Brandstätter, A. Gomahr, H. Junger, M. Palzenbeger and M. Zaunreiter. 1992, *Brain and sensory systems*. In Winfield I. J. and Nelson J. S., eds. *Cyprinid Fishes, Systematics, Biology and Exploitation*. London: Chapman and Hall, p.285–331.
10. Kotrschal K. and Palzenberger M. 1992, *Neuroecology of cyprinids: comparative, quantitative reveals diverse brain patterns*. Env. Biol. of Fishes Dordrecht: Kluwer Academic Publishers. **33:** 135–152.
11. Kotrschal K., M. J. Van Staaden and R. Huber. 1998. *Fish brains: evolution and environmental relationships*. Reviews in Fish Biology and Fisheries **88:** 377–408.
12. Miller R. J. & H. E. Evans. 1965. *External morphology of the brain and lips in Catostomid fishes*. Copeia **1:** 467–487.

Received 16 December, 2002.

Craiova University,
Department of Vertebrate Zoology

TAXONOMIE ET NOMENCLATURE ACTUALISÉES CONCERNANT LES ESPÈCES DES SCARABÉOÏDÉS (INSECTA: COLEOPTERA: *SCARABAEOIDEA*) EN ROUMANIE (À L'EXCLUSION DE LA FAM. DES LUCANIDAE)

CORNELIA CHIMIŞLIU

There is presented a checklist of the Romanian Scarabeidae fauna (excepting the species of the Lucanidae family) using literature data from 1891 until now. This paper completes and updates the taxonomy and the nomenclature of the Scarabeidae species presented by Panin (1955, 1957). The phyletic classification of the Scarabeoidea superfamily taxons follows the new taxonomical system proposed for the Romanian Scarabeidae fauna (5).

1. INTRODUCTION

La taxonomie ainsi que la nomenclature des coléoptères scarabéoïdés de la faune de Roumanie ont résté les mêmes depuis des années 1955 et 1957, quand S. Panin a adopté le système taxonomique et la nomenclature pour ces coléoptères.

Les recherches scientifiques effectuées ultérieurement par des spécialistes du monde ont déterminé des changements dans la taxonomie et dans la nomenclature des scarabéoïdés.

Pour la taxonomie de la superfamille *Scarabaeoidea* de la faune de Roumanie on a proposé un nouveau système taxonomique (5).

Dans ce travail l'auteur présente la liste complète des espèces des scarabéoïdés signalées dans la faune des coléoptères de Roumanie depuis l'année 1891 jusqu'à présent, en actualisant en même temps la taxonomie et la nomenclature des espèces.

2. MATERIEL ET METHODES

Pour l'élaboration de la liste complète des espèces, l'auteur a utilisé les dates publiées par Panin (17, 18) et par des autres spécialistes qui ont signalé des

REV. ROUM. BIOL. – BIOL. ANIM., TOME 47, N^{os} 1–2, P. 31–41, BUCAREST, 2002

espèces nouvelles pour la faune de la Roumanie, qui ne sont pas compris dans l'ouvrage de Panin (9, 10, 11, 12, 13, 16, 17, 21).

Pour l'actualisation de la taxonomie et de la nomenclature des taxons, l'ouvrage de référence a été celui de Mary Liz Jameson & B. C. Ratcliff (15), qui ont utilisés aussi et d'autres ouvrages (J. Baraud, 1989, 1995, 1990; 3, 6, 7, 14). Pour la sous-famille j'ai utilisé comme l'ouvrage de référence celui d'Adam (1).

La structure actuelle de la superfamille *Scarabaeoidea* en concordance avec le nouveau système taxonomique (4) est rendue dans le Tableau 1.

Tableau 1

Familles	Sous-familles	Tribus	No. gen.	No. sbgen.	No. esp. et sous-espèces
GLARESIDAE			1	-	1
TROGIDAE			1	-	4
GEOTRUPIDAE	***BOLBOCERATINAE***	*BOLBOCERATINI*	2	-	2
	GEOTRUPINAE	*GEOTRUPINI*	3	-	5
	LETHRINAE	*LETHRINI*	1	-	1
OCHODAEIDAE	***OCHODAEINAE***		2	-	3
HYBOSORIDAE	***HYBOSORINAE***		1	-	1
GLAPHYRIDAE	***GLAPHYRINAE***		1	-	1
SCARABAEIDAE	***APHODIINAE***	*AEGIALINI*	1	-	1
		APHODIINI	5	40	80
		PSAMOBIINI	4	-	7
	SCARABAEINAE	*SCARABAEINI*	1	1	2
		GYMNOPLEURINI	1	-	3
		SISYPHINI	1	-	1
		COPRINI	1	-	2
		ONITINI	2	-	2
		ONITICELLINI	1	-	2
		ONTHOPHAGINI	3	2	31
	MELOLONTHINAE	*HOPLIINI*	1	2	7
		SERICINI	3	2	7
		MELOLONTHINI	7	6	18
	RUTELINAE	*RUTELINI*	7	5	20
	DYNASTINAE	*ORYCTINI*	1	-	1
		PENTODONTINI	1	-	3
	ORPHNINAE	*ORPHNINI*	1	-	1
	CETONIINAE	*CETONIINI*	9	-	15
	TRICHIINAE	*TRICHIINI*	3	-	6
	VALGINAE	*VALGINI*	1	-	1
7	14	23	66	58	228

SCARABAEOIDEA (LAICHARTING, 178)

GLARESIDAE SEMENOV & MEDVEDEV, 1932
(après **BARAUD J, 1995**)
Glaresis ERICHSON, 1848
rufa ERICHSON, 1848

TROGIDAE PÉRINGUEY 1901
(après **JAMESON, 2002)**
Trox FABRICIUS, 1775
hispidus hispidus (PONTOPIDAN, 1763)
hispidus (PONTOPIDAN, 1763)
perrisi FAIRMAIRE, 1868
sabulosus (LINNAEUS, 1758)
scaber (LINNAEUS, 1767)

GEOTRUPIDAE ERICHSON, 1847
(après **ENDRÖDI, 1985)**
BOLBOCERATINAE
BOLBOCERINI CASTELNAU, 1840
Bolbelasmus BOUCOMONT, 1904
unicornis (SCHRANK, 1789)
Odontaeus KLUG, 1843
armiger (SCOPOLI, 1772)

GEOTRUPINAE ERICHSON, 1847
GEOTRUPINI (ERICHSON, 1847)
Anoplotrupes JEKEL, 1866
stercorosus (SCRIBA, 1791)
Geotrupes (A.) stercorosus SCRIBA, 1791
Geotrupes LATREILLE, 1796
(Geotrupes) JEKEL, 1865 (1866)
mutator (MARSHAM, 1802)
spiniger (MARSHAM, 1802)
stercorarius (LINNAEUS, 1758)
Trypocopris MOTSCHULSKY, 1845
vernalis vernalis (LINNAEUS, 1758)
Geotrupes (T.) vernalis LINNÉ, 1758

LETHRINAE
MULSANT & REY, 1871
LETHRINI WINKLER, 1929
Lethrus SCOPLOLI, 1768
(Lethrus) SEMENOV, 1894
apterus (LAXMANN, 1770)

OCHODAEIDAE
MULSANT & REY, 1871
(après **CARLSON, 2002**)
OCHODAEINAE ARRENS, 1904
Codocera ESCHSCHOLTZ, 1839
ferrugineum (ESCHSCHOLTZ, 1821)
ferruginea ESCHSCHOLTZ, 1821
Ochodaeus SERVILLE, 1831
chrysomeloides (SCHRANK, 1781)
alleonis FAIRMAIRE, 1884

HYBOSORIDAE ERICHSON, 1847
(après **JAMESON, 2002)**
HYBOSORINAE PÉRINGUEY 1901
Hybosorus MAC-LEAY, 1861
illigeri REICHE, 1853

GLAPHIRIDAE MACLEAY, 1819
GLAPHYRINAE BEDEL, 1889
(après **BARAUD, 1990)**
Pygopleurus MOTSCHULSKY, 1859
vulpes (FABRICIUS, 1781)
Amphicoma (P) vulpes FABR., 1781)

SCARABAEIDAE REITTER, 1892
APHODIINAE MULSANT, 1842
(après **DELLACASA, 1987)**
AEGIALINI
Aegialia LATREILLE, 1807
(Dimalia) MULSANT 1842
sabuleti (PANZER, 1797)

APHODIINI SCHMIDT, 1922
Aphodius ILLIGER, 1798
(Acanthobodilus) DELLACASA, 1983
immundus CREUTZER, 1799
(Bodilus) immundus CREUTZER, 1799
(Acrossus) MULSANT, 1842
depressus (KUGELANN, 1792)
luridus (FABRICIUS, 1775)
rufipes (LINNAEUS, 1758)
(Agrilinus) MULSANT, 1870

ater (GEER 1774)
constans DUFTSCHMIDT, 1805
(Agrilinus) ***constans*** DUFT., 1805
rufus MOLLER , 1782
(Bodilus) rufus MOLLER , 1782
sordidus (FABRICIUS, 1775)
(Bodilus) sordidus FABRICIUS, 1792
convexus (ERICHSON, 1848)
vittatus mundus REITTER , 1893
(Alocoderus) SCHMIDT, 1913
hydrochaeris (FABRICIUS, 1798)
hydrochoeris FABRICIUS, 1798
(Amidorus) MULSANT, 1870
obscurus obscurus (FABRICIUS, 1792)
obscurus (FABRICIUS, 1792)
(Ammoecius) MULSANT, 1842
brevis (ERICHSON, 1798)
(Aphodius) MULSANT, 1871
foetens (Fabricius, 1787)
aestivalis STEPHENS, 1839
conjugatus (PANZER, 1795)
fimetarius (LINNAEUS, 17658)
foetidus (HERBST, 1783
(Agrilinus) putridus HERBST, 1789
scybalarius FABRICIUS, 1781
sulcatus FABRICIUS, 1792
(Biralus) MULSANT, 1870
satellitus (HERBST, 1789)
(Bodilus) Mulsant, 1870
ictericus LAICHARTING, 1781
nitidulus FABRICIUS, 1792
ictericus ghardimaouensis BALTHASAR, 1929
ghardimaouensis BALTH., 1929
lugens CREUTZER, 1799
punctipennis ERICHSON, 1848
(Calamosternus) LINNAEUS 1767
granarius (LINNAEUS, 1767)
(Chilothorax) MOTSCHULSKI, 1859
(Volinus) MULSANT, 1870
conspurcatus LINNAEUS 1758
distinctus (MÜLLER, 1776)
melanostictus SCHMIDT, 1840
pictus STURM, 1805
sticticus (PANZER, 1798)
tessulatus PAYKUL,1798
(Colobopterus) MULSANT, 1842
erraticus LINNAEUS 1758
(Coprimorphus) MULSANT, 1842
scrutator (HERBST, 1789)
(Colobopterus) scrutator (HERBST, 1789)
(Erytus) MULSANT, 1870
stepicola BALTHASAR, 1941
(Esymus) MULSANT, 1870
merdarius (FABRICIUS, 1775)
suturinigra SCHMIDT , 1922
pusillus (HERBST, 1789)
(Orodalus) pusillus HERBST, 1789
(Eudolus) MULSANT & REY, 1870
quadriguttatus (HERBST, 1783)
(Emadus) quadriguttatus HERBST, 1789
(Euorodalus) DELLACASA, 1983
coenosus (PANZER, 1798)
(Orodalus) tristis ZENKER, 1801
cittelorum SEMENOV & MEDVEDEV, 1928
(*Phalacronotus*) *cittelorum* SEMENOV & MEDVEDEV, 1928
paracoenosus BALTHASAR & HRUBANT, 1960
(Phalacronotus) paracoenosus BALTHASAR & HRUBANT, 1960
(Eupleurus) MULSANT, 1842
subterraneus (LINNAEUS, 1758)
(Colobopterus) subterraneus L., 1758
(Labarrus) MULSANT, 1870
lividus (OLIVIER, 1789)
(Nialus) lividus OLIVIER, 1789
(*Limarus*) MULSANT, 1870
maculatus STURM, 1800
(Liothorax) MOTSCHULSKI, 1859
kraatzi HAROLD, 1868
(Nialus) kraatzi HAROLD, 1868
niger (PANZER, 1797)
(Nialus) niger PANZER, 1795
plagiatus (LINNAEUS, 1767)
(Nialus) plagiatus LINNÉ, 1767
(Loraphodius) REITTER, 1892

suarius FALDERMAN, 1835
(Melaphodius) REITTER, 1892
circumcinctus W. SCHMIDT, 1840
(Melinopterus) circumcinctus SCHMIDT, 1840
(Melinopterus) MULSANT, 1842
consputus CREUTZER, 1799
prodromus (BRAHM, 1790)
pubescens STURM,1800
sphacelatus (PANZER, 1798)
(Neagolius)
(Agolius) MULSANT, 1871
mixtus VILLA, 1833
montanus ERICHSON, 1848
(Nialus) MULSANT, 1870
varians DUFTSCHMIDT, 1805
(Nimbus) MULSANT, 1870
contaminatus (HERBST, 1783)
obliteratus PANZER, 1823
(Nobius) MULSANT, 1870
serotinus (PANZER, 1799)
(Orodaliscus) REITTER, 1900
rotundangulus REITTER,1900
(Oromus) MULSANT, 1870
alpinus (Scopoli, 1763)
(Otophorus) MULSANT, 1842
haemmorhoidalis (LINNAEUS 1758)
(Teuchestes) haemmorhoidalis LINNAEUS 1758
(Paramoecius) SEIDLITZ, 1891
corvinus ERICHSON, 1848
(Oromus) corvinus ERICHSON, 1848
gibbus GERMAR, 1817
(Phalacronothus) MOTSCHULSKI, 1859
biguttatus GERMAR, 1824
(Emadus) biguttatus GERMAR, 1824
quadrimaculatus (LINNAEUS, 1761)
(Emadus) quadrimaculatus L., 1761
(Plagigonus) MULSANT, 1842
arenarius OLIVIER, 1789
(Planolinus) MULSANT & REY, 1870
borealis GYLLENHAL, 1827
(Agrilinus) borealis GYLLENHAL, 1827
(Pseudacrossus) REITTER, 1892
przewalskyi REITTER, 1887
thermicola STURM, 1800
(Amidorus) thermicola STURM, 1800
(Sigorus) REITTER, 1892
porcus (FABRICIUS, 1792)
(Amidorus) porcus FABRICIUS, 1792
(Subrinus) MULSANT & REY, 1870
sturmi HAROLD, 1870
(Nialus) sturmi HAROLD, 1870
(Teuchestes) MULSANT, 1842
fossor (LINNAEUS, 1758)
(Trichonotulus) BEDEL, 1911
scropha (FABRICIUS, 1787)
(Euheptaulacus) DELLACASA, 1983
carinatus (GERMAR, 1824)
(HEPTAULACUS) CARINATUS GERM., 1824
SUS HERBST, 1783
(HEPTAULACUS)
SUS HERBST, 1783
VILLOSUS (GYLLENHAL, 1806)
(HEPTAULACUS)
VILLOSUS GYLL., 1827
Mothon SEMENOV & MEDVEDEV, 1927
sarmaticus SEMENOV & MEDVEDEV, 1927
Paracoptochirus BALTHASAR, 1963
singularis HAROLD, 1868
Oxyomus ESCHSCHOLTZ, 1839
sylvestris (SCOPOLI, 1763)

PSAMMOBIINI SCHMIDT, 1922
Diastictus MULSANT, 1842
tibialis FABRICIUS, 1789
vulneratus STURM, 1805
Pleurophorus MULSANT, 1842
caesus (CREUTZER, 1796)
Psammobius HEER, 1841
basalis MULSANT & REY, 1869
laevipennis COSTA, 184
sulcicollis ILLIGER, 1802
Rhyssemus MULSANT, 1842
germanus *(LINNAEUS, 1767)*

SCARABAEINAE
(după **BALTHASAR, 1964)**
SCARABAEINI WINKLER, 1929

Scarabaeus LINNAEUS, 1758
(Scarabaeus) LINNAEUS, 1758
pius ILLIGER, 1803
affinis BRULLÉ, 1832

GYMNOPLEURINI
(LACORDAIRE, 1856)
Gymnopleurus ILLIGER, 1803
goffroy (FUESSLIN, 1775)
mopsus (PALLAS, 1781)
sturmi MAC-LEAY, 1821

SISYPHYNI MULSANT, 1842
Sisyphus LATREILLE, 1807
schaefferi schaefferi (LINNAEUS, 1758)
schaefferi (LINNÉ, 1758)

COPRINI REITTER, 1892
Copris GEOFFROY, 1762
hispanus (LINNAEUS, 1764)
lunaris (LINNAEUS, 1758)

ONITINI BEDEL, 1892
Chironitis LANSBERGE, 1875
furcifer (ROSSI, 1792)
Onitis FABRICIUS, 1798
damoetas OLIVIER, 178
ONITICELLINI D'ORBIGNY, 1916
Euoniticellus JENSSEN, 1953
Oniticellus D'ORBIGNY, 1916
fulvus (GOEZE, 1777)
pallipes (FABRICIUS, 1781)
ONTHOPHAGINI
LACORDAIRE, 1856
Caccobius THOMSON, 1859
(Caccobius) JEKEL, 1872
histerioides (MÉNÉTRIÉS, 1832)
schreberi (LINNAEUS, 1767)
Euonthophagus BALTHASAR, 1959
amyntas (OLIVIER, 1789)
Onthophagus (s.str.) amyntas (OLIVIER, 1789)
gibossus (SCRIBA, 1790)
Onthophagus (s.str.) gibossus (SCRIBA, 1790)
Onthophagus LATREILLE, 1807
(*Onthophagus*) LATREILLE, 1807
coenobita (HERBST, 1783)
fissicornis STEVEN, 1809
fracticornis (PREYSSLER, 1790)
furcatus (FABRICIUS, 1781)
furcicornis REITTER, 1892
Onthophagus (s.str.) parmatus REITTER, 1893
gibbulus (PALLAS, 1781)
grossepunctatus REITTER, 1905
illyricus (SCOPOLI, 1763)
joannae GOLJAN, 1953
kindermanni HAROLD, 1871
lemur (FABRICIUS, 1781)
lucidus (STURM, 1800)
marginalis GEBLER, 1817
nuchicornis (LINNAEUS, 1758)
opacicollis D'ORBIGNY, 1897
ovatus (LINNAEUS, 1767)
ponticus HAROLD, 1871
ruficapillus BRULLÉ, 1832
semicornis (PANZER, 1798)
similis (SCRIBA, 1790)
taurus (SCHREBER, 1759)
vacca (LINNAEUS, 1767)
verticicornis (LAICHARTING, 1781)
vitulus (FABRICIUS, 1776)
MELOLONTHINAE REITTER, 1902
(après **JAMESON & RATCLIFF, 2002)**
HOPLIINI (LATREILLE, 1829)
Hoplia ILLIGER, 1803
(*Decamera*) MULSANT 1842
philanthus philanthus FEESSLY, 1775
philanthus FEESSLY, 1775
praticola DUFTSCHMIDT, 1805
(*Hoplia*) REITTER 1903
argentea (PODA, 1761)
farinosa LINNAEUS, 1761
dilutipes REITTER, 1890
graminicola *(FABRICIUS, 1792)*
hungarica *BURMEISTER, 1844*
parvula *KRYNICKY, 1832*
SERICINI WINKLER, 1929

Maladera MULSANT, 1842
holosericea (SCOPOLI, 1772)
Omaloplia SCHONHERR, 1817
Homaloplia STEPHENS, 1829
(Acarina) BARAUD, 1965
spiraea (PALLAS, 1773)
Homaloplia spiraea PALLAS, 1773
(Omaloplia) SCHÖNHERR, 1817
alternata KÜSTER, 1849
Homaloplia alternata KÜSTER, 1849
erythroptera FRIVALDSZKZY, 1835
Homaloplia erythroptera FRIV., 1835
marginata FUESSLY, 1775
Homaloplia marginata FUESSLY, 1775
ruricola (FABRICIUS, 1775)
Homaloplia ruricola (FABRICIUS, 1775)
Serica MAC-LEAY, 1861
brunea (LINNAEUS, 1758)

MELOLONTHINI MAC- LEAY, 1819
Amphimallon BERTHOLD, 1827
(*Amphimallon*) REITTER ,1902
altaicum (MANNERHEIME, 1825)
altaicus (MANNERHEIME, 1825)
assimile (HERBST, 1790)
assimilis HERBST, 1790
caucasicum (GYLLENHAL, 1817)
caucasicus GYLLENHAL, 1817
ruficorne (FABRICIUS, 1775)
ruficornis FABRICIUS, 1775
solstitiale solstitiale (LINNAEUS, 1758)
solstitialis LINNÉ, 1758
Anoxia CASTELNAU, 1832
(*Anoxia*) MEDVEDEV, 1951
pillosa (FABRICIUS, 1792)
villosa villosa FABRICIUS, 1781
(*Protanoxia*) MEDVEDEV, 1951
orientalis (KRYNICKY, 1832)
Melolontha FABRICIUS, 1775
(Melolontha) REITTER, 1877
hippocastani FABRICIUS, 1801
melolontha (LINNAEUS, 1758)
pectoralis MEGERLE VON MUEHLEFELD, 1812
Miltotrogus REITTER 1902
aequinoctialis (HERBST, 1790)
Rhizotrogus(Miltotrogus) aequinoctialis HERBST, 1790
pilicollis (GYLLENHAL, 1817)
Rhizotrogus(Miltotrogus) pilicollis GYLLENHAL, 1817
tauricus (BLANCHARD, 1850)
Rhizotrogus(Miltotrogus) tauricus BLANCHARD, 1850
vernus (GERMAR, 1823)
Rhizotrogus(Miltotrogus) vernus GERMAR, 1823
Polyphylla HARRIS, 1842
(*Polyphylla*) MEDVEDEV, 1951
fullo (LINNAEUS, 1758)
Rhizotrogus BERTHOLD, 1827
(*Rhizotrogus*) REITTER, 1903
aestivus (OLIVIER, 1789)
Monotropus ERICHSON, 1843
nordmanni BLANCHARD, 1832

RUTELINAE REITTER (1903)
RUTELINI MEDVEDEV, 1949
Anisoplia SERVILLE, 1825
(*Anisoplia*) MEDVEDEV, 1949
agricola PODA, 1761
bromicola (GERMAR, 1817)
(Lasioplia) bromicola (GERMAR, 1817
deserticola FISCHER, 1824
(Ammanisoplia) deserticola FISCHER, 1824
dispar ERICHSON, 1847
flavipennis BRULLÉ, 1832
lata lata ERICHSON, 1847
tempestiva ERICHSON, 1847
zwicki FISCHER, 1824
(*Autanisoplia*) MEDVEDEV, 1949
austriaca HERBST, 1783
(*Lasioplia*) MEDVEDEV, 1949
aprica ERICHSON, 1847
Anomala SAMOUELLE, 1819
(*Anomala*) REITTER, 1903
dubia (SCOPOLI, 1763)
solida ERICHSON, 1847
oblonga FABRICIUS, 1756

vitis (FABRICIUS, 1755)
(Psammoscaphaeus) MOTSHSULSKY, 1853
errans (FABRICIUS, 1755)
Blitopertha REITTER, 1903
lineata (FABRICIUS, 1798)
Chaetopteroplia MEDVEDEV, 1949
segetum (HERBST, 1783)
Exomala
Trichopertha REITTER, 1903
hirtella BRULLÉ, 1832
Mimela KIRBY, 1823
Rhombonyx HOPE, 1837
aurata (FABRICIUS, 1801)
Phyllopertha STPHENS, 1837
horticola (LINNAEUS, 1758)

DYNASTINAE KOLBE, 1897
(après ENDRÖDI, 1985)
ORYCTINI MULSANT, 1842
Oryctes ILLIGER, 1803
nasicornis LINNÉ 1758

PENTODONTINI MULSANT, 1842
Pentodon HOPE, 1837
bidens bidens (PALLAS, 1771)
bidens PALLAS, 1771
bidens sulcifrons KÜSTER, 1789
sulcifrons KÜSTER, 1789
idiota idiota HERBST, 1789
idiota HERBST, 1789

ORPHNINAE PÉRINGUEY, 1901
(après **JAMESON, 2002)**
***Chaetonyx* Schaum, 1862**
robustus robustus SCHAUM, 1862
robustus SCHAUM, 1862

CETONIINAE DISTANT,
1900-1911
(après **ADAM,** 1994 şi
KRAL &VITNER, 1996)
CETONIINI SEILITZ, 1875
Aethiessa BURMEISTER, 1842
floralis (FABRICIUS, 1787)
Cetonia FABRICIUS, 1787
aurata aurata (LINNAEUS, 1761)
Oxythyrea MULSANT, 1842
cinctella SCHAUM, 1841
FUNESTA (PODA,1761)
Cetonischema REITTER ,1898
speciosisima (SCOPOLI, 1786)
aeruginosa (DRURY, 1770)
Eupotosia MIKSIC, 1954
affinis affinis (ANDERSCH, 1797)
Potosia affinis ANDERSCH, 1797
Liocola THOMSON, 1859
marmorata (FABRICIUS, 1792)
lugubris (HERBST, 1783
Netocia COSTA, 1852
fieberi (KRAATZ, 1880)
hungarica hungarica (HERBST, 1790)
hungarica HERBST, 1790
vidua (GORY & PERCHERON, 1833)
Potosia MULSANT, 1842
cuprea cuprea FABRICIUS, 1775
cuprea metallica (HERBST, 1782)
cuprea obscura (ANDERSCH, 1797)
Tropinota MULSANT, 1842
hirta (PODA, 1761)
Epicometis hirta PODA. 1761
squalida (SCOPOLI, 1783)

TRICHIINAE KOLBE, 1897
TRICHIINI LA CONTE, 1861
Gnorimus SERVILLE, 1825
nobilis (LINNAEUS, 1758)
Octopunctatus
(LINNAEUS, 1758)
osmoderma SERVILLE, 1825
eremita (SCOPOLI, 1763)
trichius FABRICIUS, 1775
fasciatus LINNAEUS, 1758
sexualis BEDEL, 1906
zonatus GERMAR, 1817

VALGINAE MULSANT, 1842
VALGINI MULSANT, 1842
Valgus *SCRIBA, 1857*
haemipterus (LINNAEUS, 1758)

DES ESPÈCES SIGNALÉES PAR D'AUTRES AUTEURS

► K. PETRI (1912) - ***Hoplia (s. str.) brunnescens*** REITTER,1903; ***Aphodius (Liothorax) plagiatus*** LINNÉ 1767;

► O. MARCU (1928) - ***Heptaulacus carinatus*** GERMAR, 1824;

► A. HORION (1957) - ***Aphodius (Liotorax) kraatzi*** HAROLD, 1868;

► M. IENIŞTEA (1957) - ***Aphodius (Loraphodius) suarius*** FALDERMAN, 1835; ***Trox perrisi*** FAIRMAIRE, 1868; ***Hoplia (Hoplia) parvula*** KRYNICKY, 1832;

► M. IENIŞTEA (1975) - **Onthophagus (Onthophagus) ponticus** HAROLD, 1871; **Onthophagus (Onthophagus) joannae** GOLJAN, 1953; ***Onthophagus (Onthophagus) grossepunctatus*** REITTER, 1905; ***Onthophagus (Onthophagus) opacicollis*** D' ORBIGNY, 1916; ***Onthophagus (Onthophagus) similis*** SCRIBA, 1790; ***Aphodius (Agrilinus) convexus*** ERICHSON 1848; ***Aphodius (Bodilus) ghardimaouensis*** BALTHASAR, 1929; ***Mothon sarmaticus*** SEMENOV & MEDVEDEV, 1927; ***Paracoptochirus singularis*** HAROLD, 1868.

►M. IENIŞTEA (1982) - ***Aphodius (Erytus) stepicola*** BALTHASAR, 1941; ***Aphodius (Eurodalus) paracoenosus*** BALTHASAR & HRUBANT, 1960; ***Aphodius (Eurodalus) cittelorum*** SEMENOV& MEDVEDEV, 1928; ***Aphodius (Melinopterus) pubescens*** STURM, 1800; ***Aphodius (Pseudacrossus) przewalskyi*** REITTER, 1887; ***Aphodius* (Orodaliscus) *rotundangulus*** REITTER, 1900; ***Aphodius (Esymus) suturinigra*** SCHMIDT, 1922; ***Aphodius (Agrilinus) vittatus mundus*** REITTER, 1893.

► ŞT. NEGRU & ATENA ROŞCA (1967) - ***Chironitis furcifer*** (ROSSI, 1792); ***Onthophagus (Onthophagus) furcicornis*** REITTER, 1892; ***Aphodius (Agrilinus) scybalarius*** FABRICIUS, 1781; ***Pentodon sulcifrons*** KÜSTER, 1789.

► A. RUICĂNESCU (1993) - ***Copris hispanus*** LINNAEUS, 1764.

DES ESPÈCES POSSIBLES DANS LA FAUNE DE ROUMANIE
(S. PANIN, 1957)

► ***Chironitis hungaricus*** HERBST, 1789; ***Aphodius (Amidorus) tomentosus*** MÜLLER, 1776 ***Aphodius (Esymus) pyreti*** PENEKE, 1911; ***Heptaulacus porcellus*** FRIVALDSZKZY, 1879; ***Hoplia (s. str.) brunnipes*** BONELLI, 1807; ***Hoplia (s. str.) subnuda*** REITTER, 1903; ***Hoplia (s. str.) brunnescens*** REITTER, 1903.

DES ESPÈCES RAYÉES DE LA FAUNE DE ROUMANIE

► ***Scarabaeus sacer*** L.,. ***Onthophagus (s. str.) nigellus*** ILLIG., ***Onthophagus (s. str.) atricapillus*** D' ORBIG,.***Onthophagus (s.str.) maki*** ILLIG., ***Geotrupes (Trypocopris) alpinus*** HAGEN., ***Lethrus (s. str.) macrognatus*** FAIRM., ***Rhizotrogus (s. str.) cicatricosus*** MULS., ***Rhizotrogus (s. str.) maculicollis*** VILLA, ***Pentodon punctatus*** VILL., ***Potosia (Netocia) oblonga*** GORY, ***Potosia (s. str.) bessarabica*** PANIN.

3. CONCLUSIONS

Dans la faune des coléoptères de Roumanie ont été signalées jusqu'à présent 228 espèces et sous-espèces des *scarabéoïdés* (à l'exception des espèces de la famille *Lucanidae*), encadrées dans 58 sous-genres, 66 genres, 23 tribus, 14 sous-familles, 7 familles. Ont été rayées 11 espèces signalées par erreur dans la faune de notre pays.

La taxonomie et la nomenclature des scarabaeoidés sont à présent actualisées environ un demi-siècle après.

BIBLIOGRAPHIE

1. Adam L., 1994, *A check-list of the Hungarian Scarabaeoidea with the description of ten new taxa (Coleoptera)*. Folia Ent. Hungarica Rovartani Közlemények. **LV**:5 –17.
2. Balthasar V., *Monographie der Scarabaeidae und Aphodiidae der palearktischen und orientalischen Region. Coleoptera: Lamellicornia.* **I.** 1. *Scarabaeinae (Pinotini, Coprini).* Verlag der Tschechoslowakischen Akademie der Wisenschaften: 390, Praga, 1963.
3. Balthasar V., 1963, *Monographie der Scarabaeidae und Aphodiidae der palearktischen und orientalischen Region. Coleoptera: Lamellicornia.* **I.** *2. Coprinae (Onitini, Oniticellini, Onthophagini).* Verlag Tschechoslowak. Akad. Wisensch, Praga: 627.
4. Carlson D.C., 2002, *Glaphyridae* MACLEAY, 1819, *Glaphyrid Scarab Beetles.* www-museum-unl.edu/research/entomology/Guide/scarabaeoidea/scarabaeoideaK.
5. Chimişliu Cornelia & Paşol P., *Contribuţii la cunoaşterea istoricului taxonomiei scarabaeoideelor (Insecta: Coleoptera: Scarabaeoidea).* Oltenia. Stud şi com. Şt. Nat. **XVIII**. Museul Olteniei Craiova: "in press", 2002
6. Dellacasa M., *Contribution to a word-wide catalogue of Aegialidae, Aphodiidae, Aulocnemidae, Termitotrogidae* (*Coleopt. Scarabaeoidea).* Mem. Soc. Entom. Ital. **66:**1–455, 1987.
7. Endrödi S., *The Dynastinae of the world.* Dr. W. Junk Publisher.: 800, London, 1985.
8. Fleck E., *Die Coleopteren rümaniens.* Bul. Soc. Sci. **XIII**, *3–4:* 135 – 153,Bucureşti, 1904.
9. Horion A., *Lamellicornia, in: Verzeichnis der kafer mitteleuropäs. 2* Abteilung. Alfred Kernen Verlag.: 358 – 373, Stuttgart, 1951.
10. Ieniştea M., *Specii noi de coleoptere pentru fauna R.P.R.* Bul. Şt. Secţia Biol. şi Şt. Agr. (Seria Zoologie). **IX** (2): 155 – 164, Bucureşti, 1957.
11. Ieniştea M., *Die Onthophagiden rümaniens (Coleoptera, Insecta).* Trav. Mus. d'Hist. Nat. "Gr. Antipa " **XVI**: 139–163, Bucureşti, 1975b.
12. Ieniştea M., *Bemekenswerten neue Aphodiiden aus der Fauna Rümaniens (Coleoptera).* Trav. Mus. d'Hist. Nat. "Gr. Antipa" **XXIV**: 113–123, Bucureşti, 1982.
13. Jameson Mary Liz., *Scarabaeidae Subfamily Rutelinae* MACLEAY, 1819 - *Shining Leaf Chafers.*www-museuunl.edu/research/entomology/Guide/scarabaeoidea/ scarabaeoidea K, 2002.
14. Jameson Mary Liz & Ratcliff B. C., *Scarabaeoidaea.* www.au.poznan.pl/wydzial/ ogrodniczy/ ke/ scarab. htm#aphodiinae, 2002.
15. Kral D. & Vitner J., Coleoptera Scarabaeoidea. In: Rozkosny R. & Vanhara J. (eds). Terrestrial invertebrates of the Palava Biosphere Reserve of UNESCO , **III.** Folia Fa. Sci. Nat. Uni. Masaryk, Brun, Biol, 94: 419–431.
16. Marcu O., *Contribuţii la cunoaşterea coleopterelor Olteniei.* Arh. Olteniei. **VII.** 39–40: 473–487, Craiova, 1928.

17. Negru Şt. & Roşca A., *Ord. Coleoptera, in: L` entomofaune des forêts du sud de la Dobroudja.* Trav. Mus. d'Hist. Nat. "Gr. Antipa" **VIII**: 119 – 145, Buc., 1967.
18. Panin S., *Fam. Scarabaeidae I.* Faune R.P.R. **10** (3). Ed. Acad. Române, 121,Bucureşti, 1955.
19. Panin S., *Fam. Scarabaeidae II.* Faune R.P.R. **10** (4). Ed. Acad. Române, 315, Bucureşti, 1957.
20. Petri K., *Siebenbürgens Käferfauna.* Sieb. Ver fus Naturwiss zu Hermannstadt: 351–362, 1912.
21. Ruicănescu A., *Copris hispanus (L.) (Coleoptera-Scarabaeidae) specie nouă în fauna României.* Stud. şi serc. de biol. S Biol. Umană. **45** (1), Ed. Acad. Române, 4–6, Bucureşti, 1993.

Reçu le 13 November 2002.

Muzeul Olteniei Craiova – Ştiinţele Naturii
Strada Popa Şapcă nr. 8
1100 Craiova

THE KARYOTYPE CHARACTERIZATION IN *CARASSIUS AURATUS GIBELIO* (BLOCH) MALES AND FEMALES OF UNISEXUAL AND BISEXUAL POPULATIONS

LILIANA GREGORIAN*, ATENA SCRIPCARIU**

The analysis of the relationships between the sexes in *C. a. gibelio* revealed that the two sexes appear in certain populations considered bisexual; but near boundaries of the spreading area (Far East-Europe) the male proportion decreases. Thus some populations became unisexual, as a result of prevalent gynogenetic way of reproduction. The aim of investigations was to identify any differences at the chromosomal level to justify this type of population dimorphism. The cytogenetic was made on two morphotypes ("old form" and "new form") which set up unisexual and bisexual populations in natural habitat. The cytogenetic characterisation of some bisexual *C. a. gibelio* populations showed the existence of dimorphism between males 2n (2x) = 98 ± 2 and females. The study of female karyotypes did not revealed notable differences at the chromosomal level two morphotypes.

1. INTRODUCTION

The *Carassius auratus* subspecies determination passes beyond the classic systematic boundaries, as it is a peculiar phenomenon among vertebrates. *Carassius auratus* has a rare speciation process, for which only a few adequate explanations or certain classification criteria exist. Anyway, a correlation between unisexuality and polyploidy was evidenced, the gynogenetic reproduction being accompanied by triploidy or even tetraploidy.

Berg showed in 1932, that a crucian form, closer to Eastern Asian crucian and initially named *Cyprinus gibelio*, appeared in the European part of former Soviet Union and in some countries of Europe. This form was further described as subspecies *Carassius auratus gibelio* (Bloch). This subspecies, autochthon in Europe was firstly mentioned in our country by Borcea (1935) who characterized it in comparison with *Carassius carassius* from Romania.

Many authors agree that *Carassius auratus gibelio* subspecies was introduced in Europe by Mongols, many years ago, from China. Thus, *C. a. gibelio,* derived from Eastern Asia, is present nowadays in European and Central Asian waters. As simpatric race, *C. a. gibelio* represents a taxon with an evolution unclearly understood by taxonomists, ecologists and geneticians (1), (8).

REV. ROUM. BIOL. – BIOL. ANIM., TOME 47, N^{os} 1–2, P. 43–52, BUCAREST, 2002

In natural habitats of *C. a. gibelio* subspecies, an unbalanced sex ratio exists, namely, a female excess in some populations, caused by gynogenetic reproduction.

The unisexual populations observed in several aquatic basins of our country and of other geographical zones, present triploid chromosome numbers, ranging from 2n=140 to 2n=160. Sometimes, they have even tetraploid forms in Far East. The unisexual *C. a. gibelio* populations with about 160 chromosomes are named "triploids" comparatively to those comprising 2n = 100, consisting in functionally diploidized tetraploids.

The unisexual populations, consisting mostly of females, were found in several hydrographical basins of Romania. The unisexual *C. a. gibelio* populations of some geographical areas present a triploid chromosome number as was established by several cytogenetic studies (4), (9).

The researches performed at Fish Culture Center in Nucet, resulted in identification of two morphotypes, conventionally named "old form" (o.f.) and "new form" (n.f.). The "old form" includes generally, unisexual populations of triploid females, considered to have an earlier origin (caused by a reproduction seeming strictly gynogenetic). The "new form" includes bisexual populations in which we also observed a chromosomal numerical dimorphism, the females being triploids and males diploids.

2. MATERIAL AND METHODS

3–4 years old adult males and females specimens of *C. a. gibelio,* both "old form" and "new form" were obtained from Nucet Fish Culture Center.

Anterior kidney preparations were used to visualize the metaphase chromosomes. The flame dried squashes were stained by Giemsa technique. The karyotype characteristics were established after criteria stated by Levan and col. (1964).

3. RESULTS AND DISCUSSION

As scarce researches on *C. a. gibelio* population genotype were done in our country, we considered interesting to start a systematic study in order to characterize by genetical point of view the stocks existent in fish ponds, as well as to efficiently utilize them in amelioration.

The results regarding the chromosomal complement in *C. a. gibelio* vary from one to another author. Thus, researchers like Makino (1941, 1966), Post (1965), Ojima (1967), (1997), Chiareli (1969), Kobayasi & col. (1971), Muramato (1975), Raicu & col. (1981) showed a variation of 2n between 94-104 chromosomes, in this subspecies.

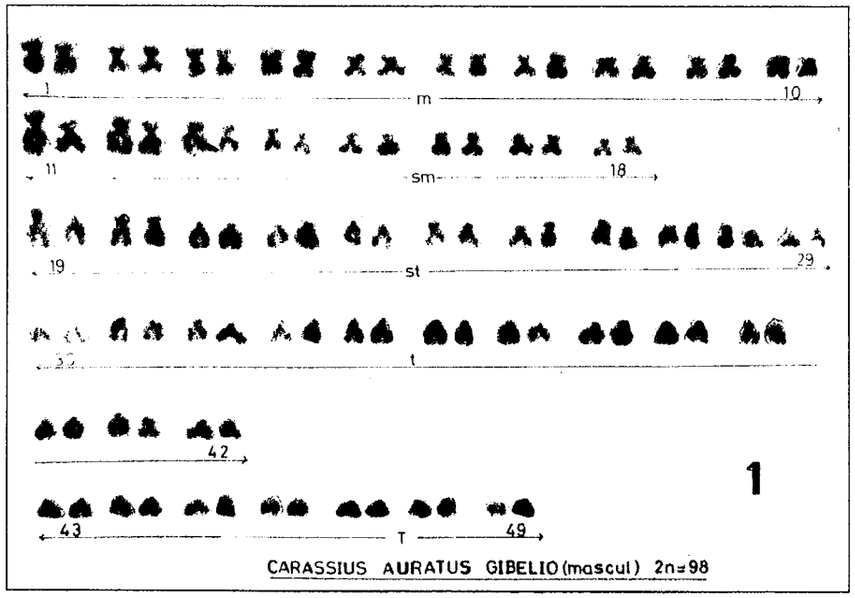

Fig. 1 – *C. a. gibelio* males karyotype with 2n = 98 somatic chromosomes.

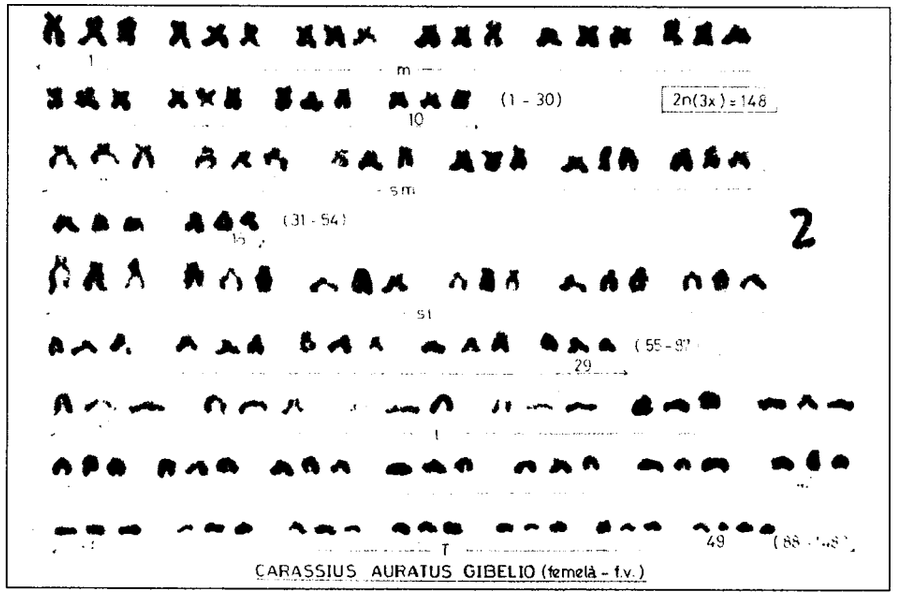

Fig. 2 – *C. a. gibelio* ("old form") females karyotype with 2n = 148 somatic chromosomes.

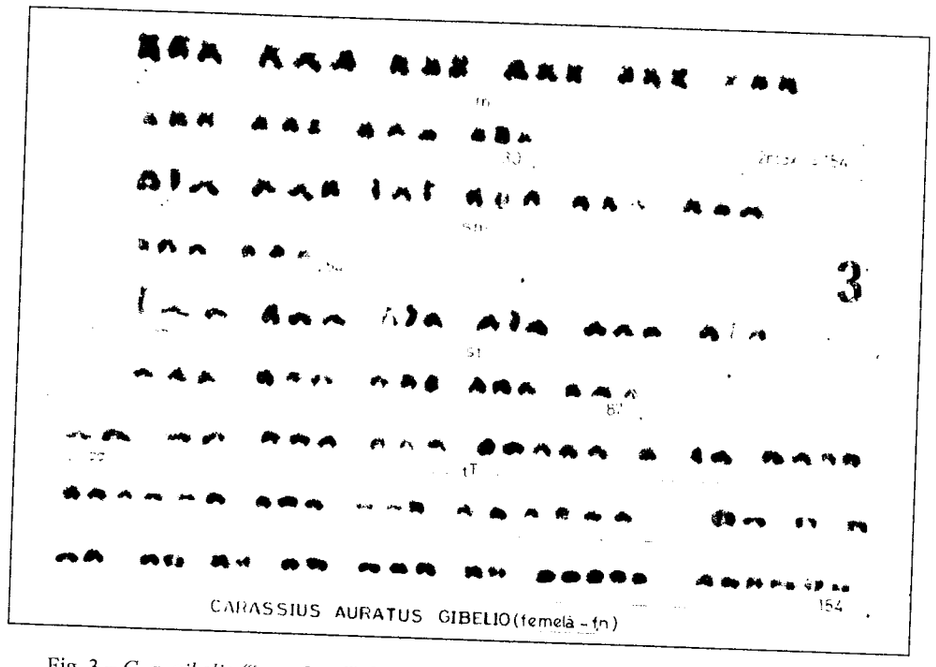

Fig. 3 – *C. a. gibelio* ("new form") females kariotype with 2n = 154 somatic chromosomes.

Our study regarding the cytogenetic analysis of some females and males of *C. a. gibelio* populations established that counting differences in complement, and also different ploidy levels exist between the two sexes. The males had 2n ≅ 100 somatic chromosomes, therefore were diploids (2x), while females had between 146–162 chromosomes, which indicate their triploidy (3x).

The chromosome numbers of 2n = 94–100 could be found in male *C. a. gibelio* karyotype. The karyotype in Figure 1 comprises 2n = 98 chromosomes, grouped in 4 morpholog groups, based on measurements, shown in Table 1.

Table 1

Biometrical measurements in *C. a. gibelio* males (μ)

Nr crt	Long arm (l)	Schort arm (s)	Total length (c)	Relative length (c)	Arm ratio (γ)	Centromeric coeff. (i)	Chromosome type
1	1.90	1.46	3.36	27.31	1.30	43.45	M 3.36÷1.96 $\bar{X}$m=2.57
2	1.82	1.32	3.14	25.52	1.38	42.04	
3	1.68	1.17	2.85	23.17	1.44	41.05	
4	1.57	1.08	2.65	21.54	1.45	40.75	
5	1.53	1.01	2.54	20.65	1.51	39.76	
6	1.54	0.97	2.51	20.40	1.59	38.65	
7	1.48	0.90	2.38	19.34	1.64	37.82	
8	1.40	0.84	2.24	18.21	1.67	37.50	
9	1.27	0.77	2.04	16.58	1.65	37.75	
10	1.23	0.73	1.96	15.93	1.68	37.24	
11	2.85	1.58	4.43	36.01	1.80	35.67	Sm 4.43÷2.04 $\bar{X}$sm=2.94
12	2.53	1.20	3.73	30.32	2.11	32.17	
13	2.34	1.10	3.44	27.96	2.13	31.98	
14	1.81	0.92	2.73	22.19	1.97	33.70	
15	1.86	0.81	2.67	21.70	2.30	30.34	
16	1.57	0.69	2.26	18.37	2.28	30.53	
17	1.56	0.66	2.22	18.04	2.36	29.73	
18	1.46	0.58	2.04	16.58	2.52	28.43	
19	3.39	1.08	4.47	36.33	3.14	24.16	St 4.47÷2.17 $\bar{X}$st=2.84μ
20	2.69	0.82	3.51	28.53	3.28	23.36	
21	2.28	0.64	2.92	23.73	3.56	21.92	
22	2.24	0.64	2.88	23.41	3.50	22.22	
23	2.22	0.62	2.84	23.08	3.58	21.83	
24	2.03	0.59	2.62	21.30	3.44	22.52	
25	1.96	0.57	2.53	20.56	3.44	22.53	
26	1.95	0.54	2.49	20.24	3.61	21.69	
27	1.94	0.53	2.47	20.08	3.66	21.46	
28	1.87	0.51	2.38	19.34	3.67	21.43	
29	1.75	0.42	2.17	17.64	4.17	19.35	
30	2.25	0.32	2.57	20.89	7.03	12.45	

31	2.23	0.31	2.54	20.65	7.19	12.20	t,
32	2.22	0.28	2.50	20.32	7.93	11.20	
33	2.22	0.25	2.47	20.08	8.88	10.12	
34	2.19	0.28	2.47	20.08	7.82	11.34	
35	2.17	0.24	2.41	19.59	9.04	9.96	2.57÷1.33
36	2.16	0.25	2.41	19.59	8.64	10.37	
37	2.15	0.23	2.38	19.34	9.35	9.66	$\bar{X}tT=2.13\mu$
38	2.09	0.22	2.31	18.78	9.50	9.52	
39	2.08	0.22	2.30	18.69	9.45	9.57	
40	2.07	0.21	2.28	18.53	9.86	9.21	
41	2.00	0.22	2.22	18.04	9.09	9.91	
42	1.81	0.19	2.00	16.26	9.53	9.50	
43	1.84	0.16	2.00	16.26	11.50	8.00	T
44	1.84	0.13	1.97	16.01	14.15	6.60	
45	1.75	0.09	1.84	14.96	19.44	4.89	
46	1.46	0.09	1.55	12.60	16.22	5.81	
47	1.46	0.06	1.52	12.35	24.33	3.95	
48	1.43	0.06	1.49	12.11	23.83	4.03	
49	1.30	0.03	1.33	10.81	43.33	2.26	

The measurements were done at a 3150 fold microscope magnifying power.

The genetical analyses were performed in *C. a. gibelio* females preparations on methaphases with a chromosome number of 148 (o.f.) and 154 (n.f.).

The chromosome arrangement in order of sizes and the effectued measurements showed that the karyotype consisted in groups of 3 chromosomes, two of which having almost identic dimensions (Figs. 2 and 3, Tabs 2 and 3).

Table 2

Biometrical measurements in ***C.a.gibelio*** females*(f.v.)* (μ)

Nr crt	Long arm (l)	Short arm (s)	Total length (c)	Relative length (c)	Arm ratio (γ)	Centromeric coeff (i)	Chromosome type	
1.	2.25	1.56	3.81	3.56	1.44	40.94	1	$\bar{X}m=2.49\mu$
2.	1.90	1.62	3.38		1.17	46.02		
3.	1.81	1.55	3.36		1.17	46.13		
4.	1.68	1.30	2.98	2.87	1.29	43.62	2	
5.	1.56	1.30	2.86		1.20	45.45		
6.	1.56	1.20	2.80		1.30	43.48		
7.	1.58	0.90	2.48	2.54	1.76	36.29	3	
8.	1.52	1.05	2.57		1.45	40.86		
9.	1.55	1.02	2.57		1.52	39.69		
10.	1.52	1.02	2.54	2.53	1.49	40.16	4	
11.	1.55	0.98	2.53		1.58	38.74		
12.	1.56	0.95	2.51		1.64	37.85		
13.	1.52	0.95	2.50		1.60	38.46		

14.	1.49	0.95	2.44	2.45	1.57	38.93	5	
15.	1.46	0.98	2.44		1.49	40.16		
16.	1.27	1.17	2.44	2.36	1.09	47.95	6	
17.	1.30	1.02	2.32		1.27	43.97		
18.	1.30	1.02	2.32		1.27	43.97		
19.	1.33	0.95	2.28	2.27	1.40	41.67	7	
20.	1.30	0.98	2.28		1.33	42.98		
21.	1.27	0.98	2.25		1.30	43.56		
22.	1.27	0.95	2.22	2.20	1.34	42.79	8	
23.	1.24	0.95	2.19		1.31	43.38		
24.	1.21	0.98	2.19		1.23	44.75		
25.	1.17	0.96	2.13	2.08	1.22	45.07	9	
26.	1.11	0.95	2.06		1.17	46.12		
27.	1.11	0.95	2.06		1.17	46.12		
28.	1.21	0.79	2.00	1.99	1.53	39.50	10	
29.	1.11	0.89	2.00		1.25	44.50		
30.	1.18	0.79	1.97		1.49	40.10		
31.	2.22	1.21	3.43	3.33	1.83	35.28	1	$\bar{X}sm=2.43\mu$
32.	2.22	1.14	3.36		1.95	33.93		
33.	2.06	1.14	3.20		1.81	35.63		
34.	2.06	1.02	3.08	3.00	2.02	33.12	2	
35.	2.00	0.98	2.98		2.04	32.89		
36.	2.03	0.92	2.95		2.21	31.19		
37.	1.68	0.76	2.44	2.43	2.21	31.15	3	
38.	1.68	0.76	2.44		2.21	31.15		
39.	1.68	0.73	2.41		2.30	30.29		
40.	1.58	0.79	2.37	2.36	2.00	33.33	4	
41.	1.52	0.83	2.35		1.83	35.32		
42.	1.49	0.86	2.35		1.73	36.60		
43.	1.52	0.79	2.31	2.28	1.92	34.20	5	
44.	1.49	0.83	2.32		1.80	35.78		
45.	1.46	0.76	2.22		1.92	34.23		
46.	1.43	0.76	2.19	2.15	1.88	34.70	6	
47.	1.39	0.79	2.18		1.76	36.24		
48.	1.36	0.73	2.09		1.86	34.93		
49.	1.32	0.73	2.05	2.04	1.81	35.61	7	
50.	1.30	0.73	2.03		1.78	35.96		
51.	1.30	0.73	2.03		1.78	35.96		
52.	1.27	0.63	1.90	1.88	2.02	33.16	8	
53.	1.24	0.63	1.87		1.97	33.69		
54.	1.21	0.66	1.87		1.83	35.29		
55.	4.13	1.30	5.43	4.73	3.18	23.94	1	St –
56.	3.49	1.15	4.64		3.03	24.78		
57.	3.17	0.96	4.13		3.30	23.24		
58.	2.67	0.60	3.27	3.14	4.45	18.35	2	
59.	2.57	0.67	3.24		3.84	20.68		
60.	2.26	0.66	2.92		3.42	22.60		

61.	2.22	0.63	2.85	2.82	3.52	22.11	3	$\bar{X}$ st = 2.59 μ
62.	2.16	0.69	2.85		3.13	24.21		
63.	2.08	0.67	2.75		3.10	24.36		
64.	2.06	0.57	2.63	2.58	3.61	21.67	4	
65.	1.92	0.64	2.56		3.00	25.00		
66.	1.94	0.60	2.54		3.23	23.62		
67.	1.91	0.60	2.5	2.50	3.18	23.90	5	
68.	1.91	0.60	2.5		3.18	23.90		
69.	1.90	0.57	2.47		3.33	23.08		
70.	1.94	0.47	2.41	2.40	4.13	19.50	6	
71.	1.90	0.51	2.41		3.73	21.16		
72.	1.84	0.54	2.38		3.41	22.69		
73.	1.78	0.54	2.32	2.30	3.30	23.28	7	
74.	1.75	0.57	2.32		3.07	24.57		
75.	1.75	0.51	2.26		3.43	22.57		
76.	1.75	0.50	2.25	2.23	3.50	22.22	8	
77.	1.74	0.51	2.25		3.41	22.67		
78.	1.68	0.51	2.19		3.29	23.29		
79.	1.68	0.48	2.16	2.11	3.50	22.22	9	
80.	1.59	0.50	2.09		3.18	23.92		
81.	1.58	0.51	2.09		3.10	24.40		
82.	1.65	0.41	2.06	2.01	4.02	19.90	10	
83.	1.59	0.47	2.06		3.38	22.82		
84.	1.55	0.35	1.90		4.43	18.42		
85.	1.49	0.26	1.75	1.72	5.73	14.86	11	
86.	1.46	0.29	1.75		5.03	16.57		
87.	1.27	0.38	1.65		3.34	23.03		
88.	2.51	0.34	2.85	2.80	7.38	11.93	1	t $\bar{X}$tΓ=1.89μ
89.	2.50	0.35	2.85		7.14	12.28		
90.	2.39	0.31	2.70		7.71	11.48		
91.	2.30	0.31	2.61	2.59	7.42	11.88	2	
92.	2.28	0.32	2.60		7.13	12.31		
93.	2.26	0.30	2.56		7.53	11.72		
94.	2.23	0.31	2.54	2.52	7.19	12.20	3	
95.	2.23	0.28	2.51		7.96	11.16		
96.	2.23	0.28	2.51		7.96	11.16		
97.	2.21	0.26	2.47	2.43	8.50	10.53	4	
98.	2.17	0.24	2.4		9.04	9.96		
99.	2.16	0.25	2.4		8.64	10.37		
100.	2.15	0.23	2.38	2.34	9.35	9.66	5	
101.	2.10	0.22	2.32		9.55	9.48		
102.	2.09	0.22	2.31		9.50	9.52		
103.	2.07	0.21	2.28	2.26	9.86	9.21	6	
104.	2.04	0.21	2.25		9.71	9.33		
105.	2.03	0.22	2.25		9.23	9.78		
106.	2.00	0.22	2.22	2.21	9.09	9.91	7	
107.	2.00	0.22	2.22		9.09	9.91		

108.	2.00	0.20	2.20		10.00	9.09	
109.	1.99	0.21	2.20	2.19	9.48	9.55	8
110.	1.98	0.21	2.19		9.43	9.59	
111.	1.98	0.21	2.19		9.43	9.59	
112.	1.96	0.20	2.16	2.15	9.80	9.26	9
113.	1.96	0.20	2.16		9.80	9.26	
114.	1.93	0.21	2.14		9.19	9.81	
115.	1.87	0.22	2.09	2.08	8.50	10.53	10
116.	1.86	0.23	2.09		8.09	11.00	
117.	1.84	0.23	2.07		8.00	11.11	
118.	1.86	0.20	2.06	2.05	9.30	9.71	11
119.	1.85	0.21	2.06		8.81	10.19	
120.	1.84	0.19	2.03		9.68	9.36	
121.	1.72	0.18	1.90	1.80	9.56	9.47	12
122.	1.58	0.17	1.78		9.29	9.71	
123.	1.58	0.17	1.78		9.29	9.71	
124.	1.49	0.16	1.65	1.55	9.31	9.70	13
125.	1.35	0.15	1.50		9.00	10.00	
126.	1.34	0.16	1.50		8.38	10.67	
127.	1.34	0.13	1.47	1.46	10.31	8.84	1
128.	1.33	0.12	1.45		11.08	8.28	
129.	1.33	0.12	1.45		11.08	8.28	
130.	1.29	0.10	1.39	1.38	12.90	7.19	2
131.	1.29	0.10	1.39		12.90	7.19	
132.	1.26	0.11	1.37		11.45	8.03	
133.	1.23	0.12	1.35	1.34	10.25	8.89	3
134.	1.23	0.12	1.35		10.25	8.89	
135.	1.20	0.13	1.33		9.23	9.77	
136.	1.18	0.09	1.27	1.26	13.11	7.09	4
137.	1.18	0.09	1.27		13.11	7.09	
138.	1.16	0.09	1.25		12.89	7.20	
139.	1.14	0.09	1.23	1.22	12.67	7.32	5
140.	1.14	0.09	1.23		12.67	7.32	
141.	1.12	0.08	1.20		14.00	6.67	
142.	1.13	0.07	1.20	1.18	16.14	5.83	6
143.	1.11	0.06	1.17		18.50	5.13	
144.	1.11	0.06	1.17		18.50	5.13	
145.	1.11	0.05	1.16	1.14	22.20	4.31	7
146.	1.08	0.05	1.13		21.60	4.42	
147.	1.08	0.05	1.13		21.60	4.42	
148.	1.07	0.05	1.12		21.40	4.46	

T

The measurements were done at a 3150 fold microscope magnifying power.

The morphologous groups were the same as in male karyotype case (but 3x replaced the chromosome pairs), while chromosome dimensions differ, but in

narrow limits. Each group included approximately a three fold number of chromosomes, as the calculations revealed.

The biometrical measurements were done in same way at "n.f.". In table 3 is presented a general data view.

Table 3

Biometrical measurements in ***C.a.gibelio*** females *(n.f.)* (μ)

Nr. crt.	Nr. of triplet chromosoms		Relativ length by triplet l÷s (c)	Arm ratio by triplet (γ) l/s	Chromosome type	
1	10	1÷10 (1÷30)	4.06÷1.81	1.15÷1.62	M	$\bar{X}m = 2.68\mu$
2	8	11÷18 (31÷54)	2.99÷2.02	1.77÷2.80	Sm	$\bar{X}sm = 2.43\mu$
3	11	19÷29 (55÷87)	3.71÷1.87	3.08÷3.98	St	$\bar{X}st = 2.63\mu$
4	13	30÷42 (88÷126+1)	2.42÷1.28	7.45÷9.71	T	$\bar{X}t, T = 1.73\mu$
5	9	43÷51 (128÷154)	2.10÷1.27	10.60÷20.17	T	

The measurements were done at a 2362 fold microscope magnifying power.

The biometrical data in Tables 2 and 3 evidence the fact that in both "n.f." and "o.f.", about 96–100 chromosomes present pairs of identic dimensions (sometimes four chromosomes in t group). We attempted to complete the 3x of these triploid kariotypes with the rest of about 50 chromosomes (a haploid set), but they hadn't identical dimensions to other two chromosomes. This fact suggest that they belong to a haploid chromosomal set added by hybridogenesis. This observation determines us to consider that the genetic structure of these females was allotriploid in both cases.

From the comparison of *C. auratus* and *C. a. gibelio* populations in Europe, it could be state that few morphological differences exist between them, though their 2n karyotypes are different, at least regarding the chromosome classification. The unisexual 3n forms differ much more among them; it is unclearly if this variability could be interpreted only by a systematic point of view. Cherfas showed in 1966, that in the case of *C. a. auratus* populations which differ as regards chromosome number and morphology probably appears, an opinion confirmed by Penaz & col. (1979).

Our studies about cytogenetic characterization of some differentiated forms of *C. a. gibelio* agree with the above mentioned. We consider that this represents an intra- and interpopulational chromosomal polymorphism which may constitute a premise in the simpatric speciation.

Taking into account the allotriploid nature, especially of *C.a.gibelio* females, the karyotype analyses, carried out by diverse authors in different populations and generations, may result in various results and therefore the respective studies seem to be not very valid.

It is interesting to note that the studied specimens generally presented constant phenotypes, in spite of karyotype differences. On the other hand, marked karyotype differences didn't appear at different morphotypes as those discussed in this paper (o.f. and n.f.). As consequence, we suggest that both our previous observations are based on a gene determinism: a basic "gene portion" which is preponderantly expressed, while a major part of the genetical material is expressed only in particular conditions, leading to a relative variability of unisexual populations.

4. CONCLUSIONS

✓ The genetic analysis of different specimens of *Carassius auratus gibelio* populations resulted in identification of a chromosomal dimorphism between males (which presented 2n(2x)=100) and females (2n(2x) =148 ÷142);

✓ The karyotype characterisation in females of *C. a. gibelio,* "old form"and "new form", didn't show marked differences between the two morphotypes;

✓ The chromosome biometrical parameters evidenced the existence of a complement in formula: 2n+n (two identical genomes + a heterologous genome) so both "old form" and "new form" were allotriploid forms;

✓ It is assumed that mechanisms modifying meiosis exist at the origin of allo-triploid females, such as automixis and apomixis, or the restraint of secondary polar body. The oocytes, which undergo these abnormalities, can subsequently generate triploid organisms (auto- or allotripoid), by gynogenesis or hybridogenesis. (17)

REFERENCES

1. Bănărescu, P., 1960, Einige Fragen zur Herkunft und Verbreitung der Süsswasserfischfauna der europäisch-mediterranenen Unterregion. Arch. f. Hydrobiol. **57**: 16-134.
2. Berg, L. S., 1932, Uber *Carassius carassius* und C. gibelio. Zool. Anz, **98**: 15÷18.
3. Borcea, I., 1935, Etude sur la gibele (*Carassius gibelio* Bloch). Ann. Sci. Univ Jassy, **21**: 526÷536.
4. Cherfas, N. B., 1966, Natural triploidy in females of the unisexual form of the goldfish (*Carassius auratus gibelio* Bloch). Genetica, 16÷24.
5. Chiarelli, B., 1969, *The karyotype of some teleostean fish obtained by tissue culture in vitro.* Experientia, **25**: 426.
6. Kobayasi, H., 1971, A cytological study on gynogenesis of the triploid ginbuno (*Carassius auratus langsdorfii*). Zool. Mag., **80**: 316÷322.
7. Levan, A., Fredga, K., Sandberg, A.A., 1964, Nomenclature for centromeric position on chromosomes. Hereditas, **52**: 201÷220.
8. Libovarski, J., 1962, Zur palaeoborealen Verbreitung der Gattung *Carassius*. Zool. Yh. Syst. **90**: 197÷210.
9. Lieder, U., 1967, Polyploidisierungsversuche bei Fischen mittels Temperaturschoch und Colchizinbehnadlung. Z. Fisch. N. F. **12**: 247÷257.
10. Makino, S., 1941, A karyological study of gold-fish of Japan. Cytologia, **12**: 96÷111.

11. Muramato, Y., 1975, A note on triploidy of the fauna (Cyprinidae, Pisces). Proc. Japan. Acad., 51.
12. Ojima, Y., Hitotsumachi, S., 1967, Cytogenetic studies in lower vertebrates IV. A note on the chromosomes of the carp (*Cyprinus carpio*) in comparison with these of the funa and the goldfish (*Carassius auratus*). Japan. J. of Genetics, **42**, *3*, p. 163÷167.
13. OJIMA, Y., ASANO, N., 1997, A cytological evidence for gynogenetic development of the ginbuna (Carassius auratus longstorfii). Proc. Japan Acad., 53(4): 138÷142.
14. Penaz, M., Rob, P., Prokes, 1979, Cytological analysis gynogenesis and early development of *Carassius auratus gibelio*. Acta Sc. Nat. Brno, **13**/7, 1÷33.
15. Post, A., 1965, Verleichende Untersuchungen der Chromosomenzahlen bei Susswasser-teleosteen. Z. für Zool. Syst. and Evol. Forsch., **3**, 47–93.
16. Raicu, P., Taisescu, E., Bănărescu, P., 1981, *Carassius carassius* and *Carassius auratus*, a pair of diploid and tetraploid representative species (Pisces, Cyprinidae). Cytologia, **46**: 233÷240.
17. ZANG, F., OSHIRO, T., TAKASHIMA, F., 1999, Fertility of triploid back-cross progeny (gengoroubuna Carassius auratus cuvieri E x carp Cyprinus carpio Γ)F1 E x carp gengoroubuna Γ. Japan J. Ichthyol., 39(3): 229÷233.

Received May 16, 2003.

*Romanian Academy
Calea Victoriei nr. 125
**Institute of Biology
Spl. Independenței nr.126, P.O.Box.56-53,
Bucharest 79651, Romania

IMMUNOCYTOCHEMICAL LOCALIZATION OF ACTIN AND α-TUBULIN IN PREVITELLOGENIC OOCYTES OF *Carassius auratus gibelio*

OTILIA ZĂRNESCU

The organization of cytoplasmic actin and α-tubulin during previtellogenic phase of crucian carp, *Carassius auratus gibelio*, was examined by immunoperoxidase technique.

Actin has been seen in the cortical cytoplasm and microvilli in all previtellogenic oocytes. In addition, a network of actin cables appears in the perinuclear cytoplasm surrounding the germinal vesicle.

α-tubulin was present throughout cytoplasm in oogonia (32 μm). During stage I (oocyte diameters from 41 to 58 μm) microtubules were concentrated in the perinuclear cytoplasm and often observed in association with perinuclear mitochondrial aggregates that correspond to Balbiani's body. In the early previtellogenic oocytes with diameters of 92-116 μm the perinuclear network of microtubules begins to dispersate throughout the cytoplasm. At the end of early previtellogenic phase of oogenesis (183 μm) a dense network of α-tubulin fills the entire cytoplasm. The onset of cortical alveoli formation coincided with an apparent reduction of cytoplasmic microtubules, although some of these oocytes show a subplasmalemal concentration of α-tubulin. Finally, in the oocytes greater than 392 μm cytoplasmic α-tubulin disappeared from most areas of cytoplasm except for the perinuclear region where occur a punctate pattern.

1. INTRODUCTION

Studies mainly using oocytes of *Xenopus laevis*, but including oocytes from other species have shown that fish and amphibian oocytes contain all major classes of cytoskeletal elements found in animal cells, including microtubules (3, 4, 7, 8, 11, 17), actin microfilaments (1, 13, 14) and intermediate filaments (2, 5, 9, 10, 15).

Despite rapid progress in studies of fish oocyte very little is known about the organization of the cytoskeletal components such as microtubules and microfilaments and their possible role in the previtellogenic phase of oogenesis.

In the present study the organization of actin and α-tubulin in previtellogenic oocytes of crucian carp was examined using immunoperoxidase technique.

REV. ROUM. BIOL. – BIOL. ANIM., TOME 47, N^{os} 1–2, P. 53–57, BUCAREST, 2002

2. MATERIALS AND METHODS

Fragments of *Carassius auratus gibelio* ovaries were fixed in 3% glutaraldehyde in 0.1 M phosphate buffered saline, pH-7.4 (PBS), with 1% dimethylsulphoxide (DMSO), overnight at 4 °C. After washing in PBS, samples were transferred in 2% glycine in PBS, to quench the free aldehyde groups, dehydrated and embedded in paraffin.

Slides were given several 10-min rinses in 0.1 M PBS and sequentially incubated in methanol:H_2O_2 (9:1) to remove endogenous peroxidase (30 min), PBS plus 10% normal rabbit serum to remove non-specific background staining (1h), mouse monoclonal anti-α-tubulin, primary antibody (Sigma), diluted 1:1000, or mouse monoclonal anti-actin primary antibody (Sigma), diluted 1:100 (overnight, at 4 °C), rabbit anti-mouse IgG peroxidase conjugate (Sigma), diluted 1:200 (1 h, at room temperature). Each antibodies incubation step was followed by four 5 min rinses in PBS. To visualize the primary antibody binding sites, the slides were incubated for 5–15 min in a solution of 3,3'-diaminobenzidine (0.05%) and 0.015% hydrogen peroxide, dissolved in 0.1 M PBS.

3. RESULTS

The previtellogenic ovary of crucian carp contains oogonia and ovarian follicles in different stages of growth, which oocyte diameters varies between 41–400 μm.

The pattern of actin and α-tubulin in the oocytes of crucian carp varied both temporally and spatially during oogenesis.

Actin has been seen in all previtellogenic oocytes in the cortical cytoplasm (Fig. 1) and microvilli. In addition, a "three-dimensional" network of actin cables appears in the perinuclear cytoplasm surrounding the germinal vesicle (nucleus) (Fig. 2).

α-tubulin was present throughout cytoplasm in oogonia (32 μm) (Fig. 3). During stage I (oocyte diameters from 41 to 58 μm) microtubules were concentrated in the perinuclear cytoplasm (Fig. 4) and often observed in association with perinuclear mitochondrial aggregates that correspond to Balbiani's body (Fig. 5).

In the early previtellogenic oocytes with diameters of 92–116 μm the perinuclear network of microtubules begins to dispersate throughout the cytoplasm (Fig. 6). At the end of early previtellogenic phase of oogenesis (183 μm) a dense network of α-tubulin fills the entire cytoplasm (Fig. 7).

The onset of cortical alveoli formation coincided with an apparent reduction of cytoplasmic α-tubulin although some of these oocytes show a subplasmalemal concentration of microtubules (Fig. 8).

Finally, in the oocytes greater than 392 μm α-tubulin disappeared from most areas of cytoplasm except for the perinuclear region where a punctate pattern occurs (Fig. 9).

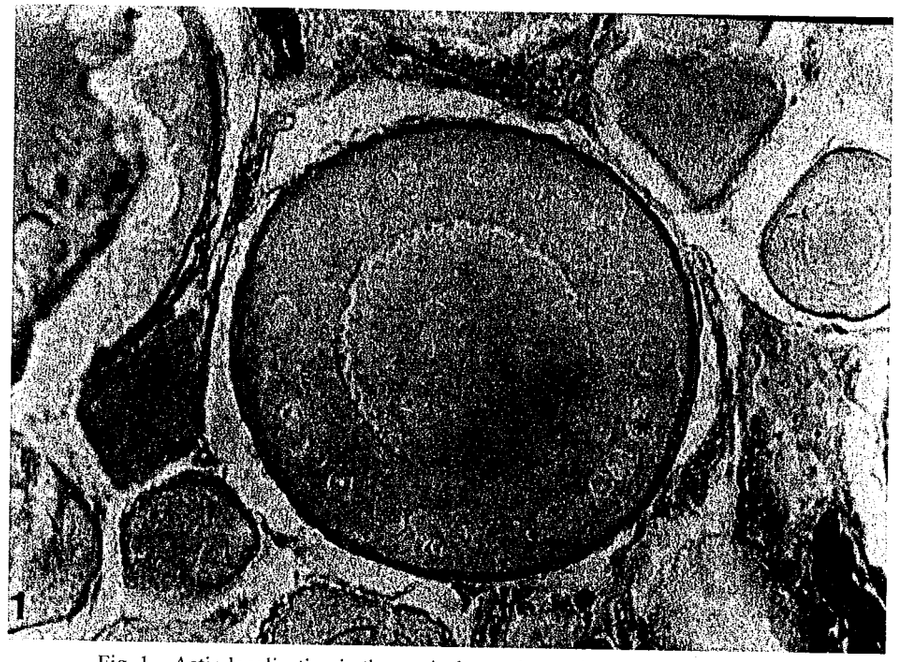

Fig. 1 – Actin localization in the cortical cytoplasm of previtellogenic oocyte. * – cortical alveoli. × 1225.

Fig. 2 – Immunolabelling of actin was associated with oocyte microvilli which were passing through the pores of zona radiata (zr). × 1960.

Fig. 3 – Network of actin cables that surrounding the germinal vesicle. × 1960.

Fig. 4 – Nests of oogonia (arrow) showing the intense cytoplasmic immunostaining of α-tubulin. × 1960.

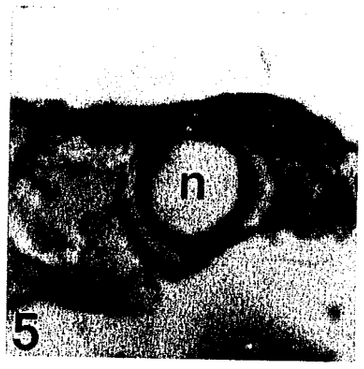

Fig. 5 – In the early previtellogenic oocytes α-tubulin was concentrated in the perinuclear cytoplasm. n-nucleus. × 1225.

Fig. 6 – α-tubulin associated with Balbiani body (arrow).

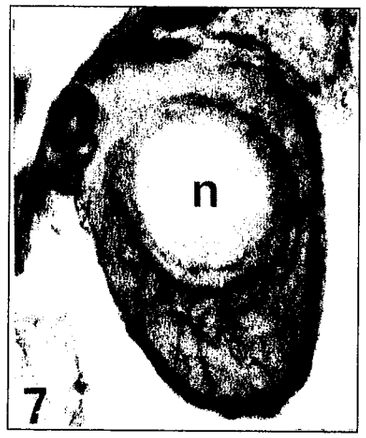

Fig. 7 – In the previtellogenic oocytes with diameters of 92–116 μm the perinuclear network of microtubules beginning to dispersate throughout the cytoplasm. n-nucleus. × 1960.

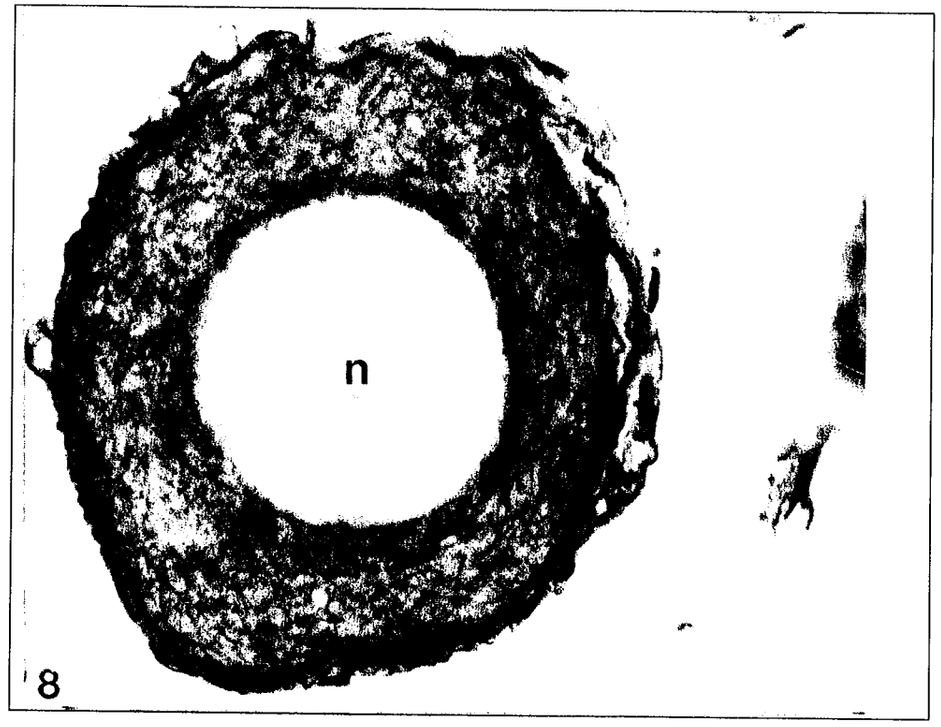

Fig. 8 – Previtellogenic follicle with an diameter of 183 μm shows a dense network of α-tubulin throughout the cytoplasm. n-nucleus. × 1960.

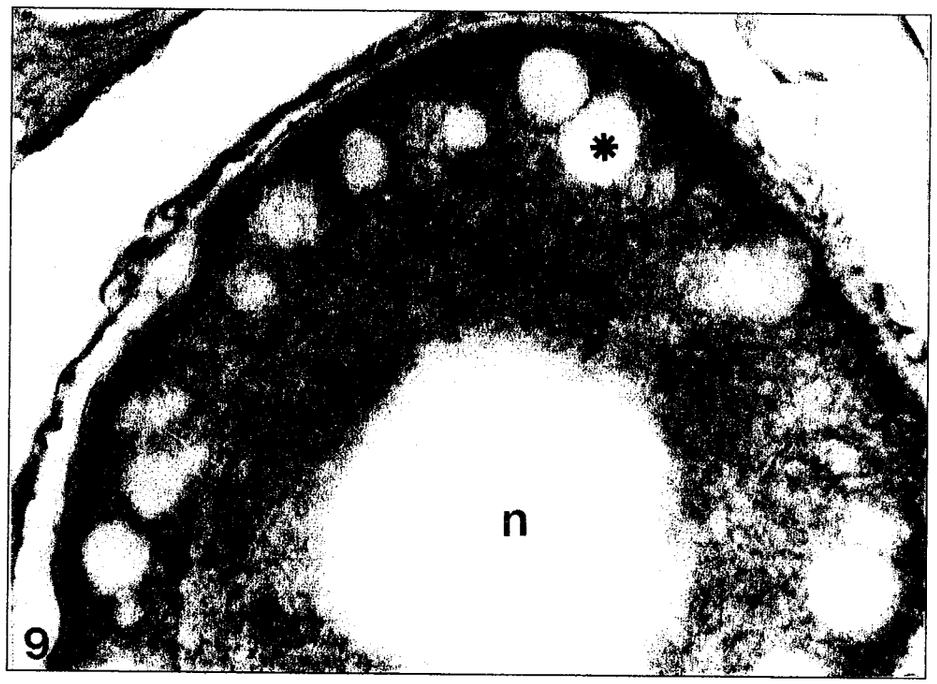

Fig. 9 – In the previtellogenic follicle with cortical alveoli (*) α-tubulin was detected in cytoplasm and more concentrated in the oocyte cortex (arrow). n – nucleus. × 1960.

4. DISCUSSION

Although anti-actin and anti-tubulin immunostaining have been demonstrated in oocytes of many animal species this is the first report that describes the distribution of these cytoscheletal proteins in the previtellogenic oocytes of fish.

The distribution and organization of microfilaments and microtubules have been investigated with conventional electron microscopy and fluorescence imunocytochemistry, mainly using oocytes of African frog *Xenopus laevis* (1, 3, 4, 7, 8, 11, 13, 14). From this reason the results of this study will be compare with those carried out on amphibians.

In the crucian carp previtellogenic oocytes, actin has been seen in the cortical cytoplasm, microvilli and as a network of cables.

In the unfertilized eggs of zebrafish the F-actin network was restricted to the plasma membrane. Nonfilamentous actin was localized to the cytoplasm housing the cortical granules and other organelles of the cortex (6).

Subplasmalemmal actin networks have been proposed to act as a barrier to the movement and docking of secretory granules at the plasma membrane (12, 16). In zebrafish eggs, cortical granules are immobilized within a cytoskeletal framework and their movement to the plasma membrane would appear to be restricted by the F-actin meshwork (6).

Confocal immunofluorescence microscopy of *Xenopus laevis* oocytes has revealed numerous cables in the cytoplasm immediately surrounding the germinal vesicle (13). The network of actin cables suggests that actin might play an important role in maintaining the morphology and positions of the large oocyte nucleus.

The actin network that surrounded the germinal vesicle in crucian carp oocytes is similar to the planar network of anastomosing keratin filaments from *Xenopus* oocytes (4, 9). Because the previous studies were demonstrated a close interaction between actin filaments and intermediate filaments, the structure observed in this study may be an intermediate filament network associated with actin.

At fish, previous studies that using monoclonal antibody against α-tubulin were made only in fully grown oocytes of goldfish, *Carassius auratus* and in zygotes and early embryo of medaka, *Oryzias latipes* (6, 8).

Throughout the previtellogenic phase the dense array of microtubules observed in the oogonia and very small oocytes of crucian carp was replaced in the early previtellogenic oocytes by the complex network surrounding the nucleus. Immunocytochemical studies on the distribution of microtubules in oocytes of *Xenopus laevis* indicated that this transition is accompanied by two significant changes in the organization of cytoplasmic microtubules: (a) dispersal of

microtubules throughout the oocyte cytoplasm and (b) inactivation of the maternal microtubule-organizing center, or centrosome (4).

At *Xenopus laevis* staining of previtellogenic oocytes with antibodies against components of the cytoskeleton showed that the mitochondrial cloud and its breakdown product react strongly with anti-tubulin antibodies (3). These aspects are similar to those observed in this study.

In conclusion, distribution of actin and α-tubulin previtellogenic oocytes of *Carassius auratus gibelio* shows a similar pattern with those observed by other authors at amphibians.

REFERENCES

1. Franke W.,W., Rathke P.C., Seib E., Trendelenburg M.F., *Distribution and mode of arrangement of microfilamentous structures and actin in the cortex of the amphibian oocytes.* Cytobiologie **14**, 111–130 (1976).
2. Franz J.K., Gall L., Williams M.A., Picheral B., Franke W.W., *Intermediate-sized filaments in a germ cell: expression of cytokeratins in oocytes and eggs of the frog Xenopus.* Proc. Natl. Acad. Sci. USA **80**, 6254–6258 (1983).
3. Gard D.L. *Organization, nucleation, and acetylation of microtubules in Xenopus laevis oocytes: a study by confocal immunofluorescence microscopy.* Dev. Biol. **143**, 346–362 (1991).
4. Gard D.L., *Confocal microscopy and 3-D reconstruction of the cytoskeleton of Xenopus oocytes.* Microsc. Res. Tech. **44**, 388–414 (1999).
5. Godsave S.F., Wylie C.C., Lane E.B., Anderton B.H., *Intermediate filaments in the Xenopus oocyte: the appearance and distribution of cytokeratin-containing filaments.* J. Embryol. Exp. Morphol. **83**, 157–167 (1984).
6. Hart N. H., Fluck R. A., *Cytoskeleton in teleost eggs and early embryos: Contributions to cytoarchitecture . and motile events.* In: *Cytoskeletal mechanisms during animal development* (D. V. Capco, Ed.), 343–381 (1995).
7. Huchon D., Jessus C., Thibier C., Ozon R., *The mitochondrial cloud of Xenopus oocytes: the source of germinal granule material.* Dev. Biol. **154**, 415–420 (1988).
8. Jiang J-Q., Yamashita M., Yoshikuni M., Fukada S., Nagahama Y., *Organization of cytoplasmic microtubules during maturation of goldfish oocytes.* Zool. Sci. **13**, 899–907 (1996).
9. Klymkowsky M.W., *Intermediate filament organization, reorganization, and function in the clawed frog Xenopus.* In: Cytoskeletal mechanisms during animal development (D. V. Capco, Ed.), 455–486 (1995).
10. Klymkowsky M.W., Maynell L.A., Nislow C., *Cytokeratin phosphorylation, cytokeratin filament severing, and the solubilization of the maternal mRNA Vg1.* J. Cell Biol. **114**, 787–797 (1991).
11. Palecek J., Habrova V., Nedvidek J., Romanovsky A., *Dynamics of tubulin structures in Xenopus laevis oogenesis.* J. Embryol. Exp. Morphol. **87**, 75–86 (1985).
12. Perrin D., Moller K., Hanke K., Soling H. D., *cAMP and Ca^{2+}-mediated secretion in parotid acinar cells is associated with reversible changes in the organization of the cytoskeleton.* J. Cell Biol. **116**, 127–134 (1992).

13. Roeder A.D., Gard D.L., *Confocal microscopy of F-actin distribution in Xenopus oocytes.* Zygote **2**, 111–124 (1994).
14. Ryabova L.V., Vassetzky S.G., *Contractile proteins and non-erythroid spectrin in oogenesis of Xenopus laevis oocyte and its visualization in deep layers of ooplasm (in russian).* Ontogenez **24**, 55–61 (1992).
15. Torpey N.P., Heasman J., Wylie C.C., *Distinct distribution of vimentin and cytokeratin in Xenopus oocytes and early embryos.* J. Cell Sci. **101**, 151–160 (1992).
16. Vitale M. L., Rodriguez Del Costillo A., Tchakarov L., Trifaro J. M., *Cortical filamentous actin disassembly and scinderin redistribution during chromaffin cell stimulation precede exocytosis, a phenomenon not exhibited by gelsolin.* J. Cell Biol. **113**, 1057–1067 (1991).
17. Zarnescu O., *Immunocytochemical localisation of α-tubulin in oocytes of Rana ridibunda.* Rev. Roum. Biol.-Biol. Anim. **46**, 79–83 (2001).

Received April 19, 2002.

Faculty of Biology
Bucharest, Spl. Independentei 91–95
E-mail: otilia@dbio.ro

FINE STRUCTURE OF THE LIVER IN HAMSTER CHRONICALLY INTOXICATED WITH CARBON TETRACHLORIDE

C-C. PRUNESCU, PAULA PRUNESCU

This paper describes the ultrastructure of the cellular lesions persisted in the hamster liver after the ceasing of the CCl_4 chronic treatment. The damage, death and disappearance of the hepatic sinusoid endothelial cells and also different types of hepatocyte injuries were presented. Hepatic cirrhosis features like the extension of the necrotic areas, biliary proliferations, inflammation and fibrogenesis were discussed. The generalized hepatic sinusoidal injury seemed to represent the essential condition of these processes.

1. INTRODUCTION

The effects of carbon tetrachloride (CCl_4) experimental administration to laboratory animals made the object of numerous papers. From the multitude of these papers, there were relatively few ultrastructural studies, especially dedicated to the cell, which synthesize the collagen fibers (9, 18) or other components of extracellular matrix (7, 17, 18). Some papers considered the parenchymal cells at the origin of the collagen fibers of the liver (3, 8). Other studies dealt with the hepatic microvascular system (10, 20).

The present paper aimed to study the hamster liver reactivity in condition of chronic CCl_4 intoxication, using observation in the electron microscopy by transmission (TEM).

The early cellular lesions, occuring in the hepatocytes or in the cells of the hepatic sinusoidal lining during the CCl_4 intoxication, will contribute to the desorganization of the hepatic lobular structure. This tissular reaction was observed later during the experiment. The understanding of the relationship between the cellular and tissular level of the damage, following the chronic action of the toxic, was the objective of this research.

During CCl_4 chronic intoxication, the first injury supported by hepatic parenchyma with essential effects on its metabolic functions, was probably related to the alteration and even the disappearance of the hepatic sinusoidal lining.

2. MATERIAL AND METHODS

The chronic CCl_4 treatment design was adapted by Stenger (19).

Animals. 14 male, young Syrian golden hamsters of 95±5 g, body weigh.

REV. ROUM. BIOL. – BIOL. ANIM., TOME 47, N^{os} 1–2, P. 59–64, BUCAREST, 2002

Inoculation. Injectable solution of 10% carbon tetrachloride (CCl_4) in alimentary oil.

Dose. 0.3 ml injectable solution/100 g body weight.

Treatment. A number of 14 subcutaneously (sc.) inoculations were performed. The toxic was weekly administered. Hamsters were sacrificed 5, 30 and 60 days after the last inoculation.

Control group. 10 young male hamsters weighing 95±5 g, were sc. inoculated with 0.3 ml/dose alimentary oil. The treatment and killing were performed similarly with the experimental group.

Material processing. Small samples were collected from different zones of the liver soon after the sacrification and fixed in 2.5% glutaraldehyde solution in cacodylate buffer 0.1 M, pH 7.2 at 4° C, for 24 h. After washings in PBS at 4° C, blocks of dimensions of about 2/2/2 mm were realized. The fixation in 1.33% osmium tetroxid solution in the same buffer, for 2 h, at the room temperature was performed. The blocks were washed, dehydrated and finally impregnated and embedded in Epon 812. The blocks were cut with an ultramicrotome Tesla.The ultrathin sections were double contrasted with uranyl acetate and lead citrate. The observation was realized with ME Zeiss and JEM 7.

Samples of liver were collected in all the moments of the sacrification and fixed in 20% formaldehyde solution in saline (v/v). After the routine histological techniques for paraffin embedding, 5μm thick sections were stained with hemalum – eosin (H-E), Picro-Sirius red – hemalum (PSR) and silver impregnation Gömöri.

3. RESULTS

The research on the ultrastructure of the hepatic cellular lesions was realized after 5, 30 and 60 days from the ceasing of the chronic CCl_4 treatment.

Macroscopically, the liver presented the modified aspect than the normal. On the surface of the organ there were observed several ascitic and/or hemorrhagic vesicles sustained by fibrous strands. The remaining of the hepatic tissue presented a granulous aspect.

The histological study of the liver revealed an aspect of randomly mixed processes of necrosis and regeneration (14). At 5 days after the ending of the treatment, the hepatic lobules were replaced in a great extent by the regeneration nodules, separated by connective strands. At 30 and/or 60 days from the ceasing of the treatment, the persistence of the internodular connective septa was noted. The hepatocytes with steatosis and necrotic nuclei were observed on the areas of the regenerative nodules. The hepatic sinusoids were difficult to identify. The hepatic inflammation with great masses of cellular infiltrations were frequently in the periportal areas or spread in the hepatic territory. Specific staining for collagen demonstrated the high degree of the liver fibrosis.

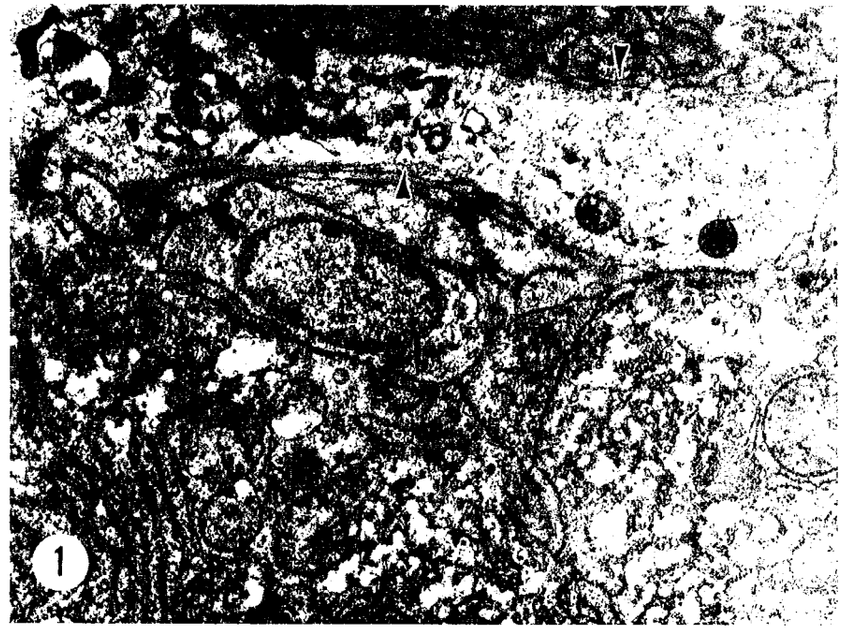

Fig. 1 – Hepatic sinusoid lacked of endothelial cells (arrow heads). In the lumen there were cellular electrondense remnants as mitochondria, peroxidated cytoplasmic membranes. A lymphocyte (L) was in extravascular migration. × 16,000.

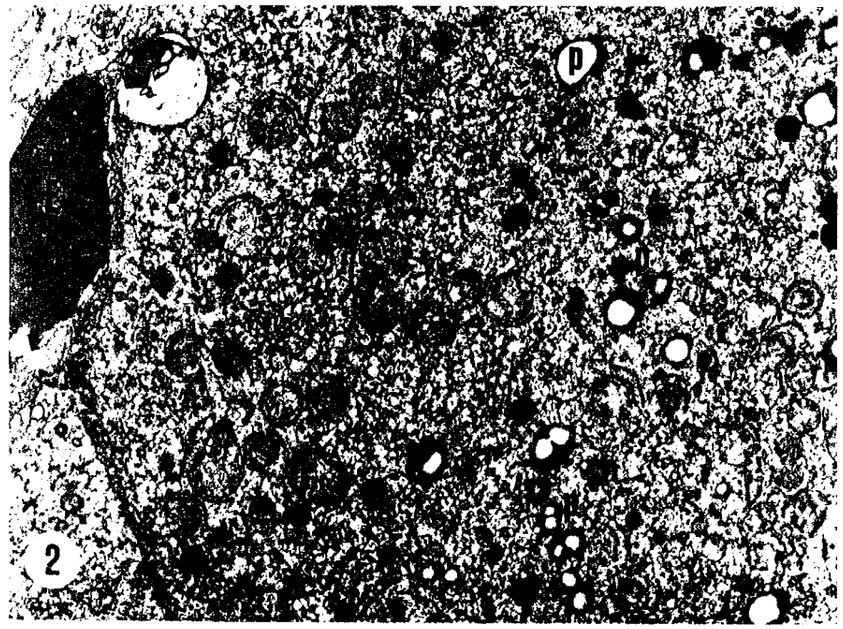

Fig. 2 – Erythrocyte (E) arrested in a hepatic sinusoidal space. Peroxidated lipids (p) and fragmented rough endoplasmic reticulum were noted in an atrophic hepatocyte. × 6,000.

Fig. 3 – Collagenous fascicles (C) at the hepatocyte vascular pole. An erythrocyte (E) marked the place of the damaged hepatic sinusoid. × 6,000.

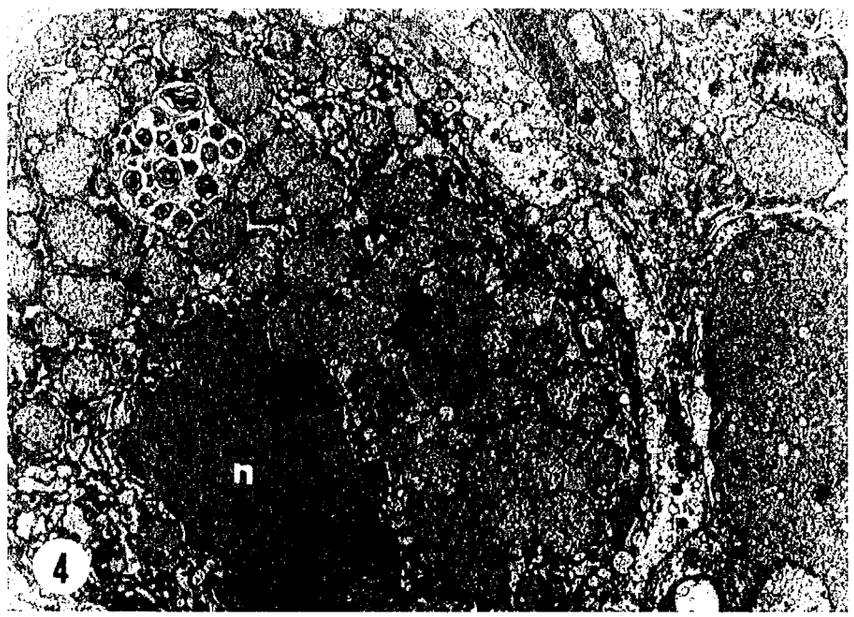

Fig. 4 – Prenecrotic hepatocyte with a typical necrotic nucleus (n), electrondense mitochondria, autolysed zone (Z). × 6,000.

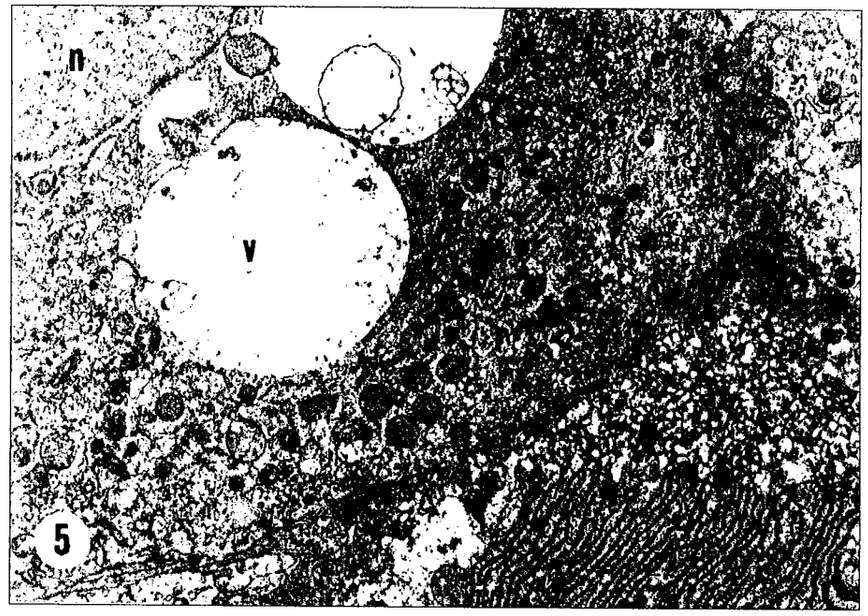

Fig. 5 – Hepatocyte with great lipidic vacuoles (v) near the nucleus (n). × 5,100.

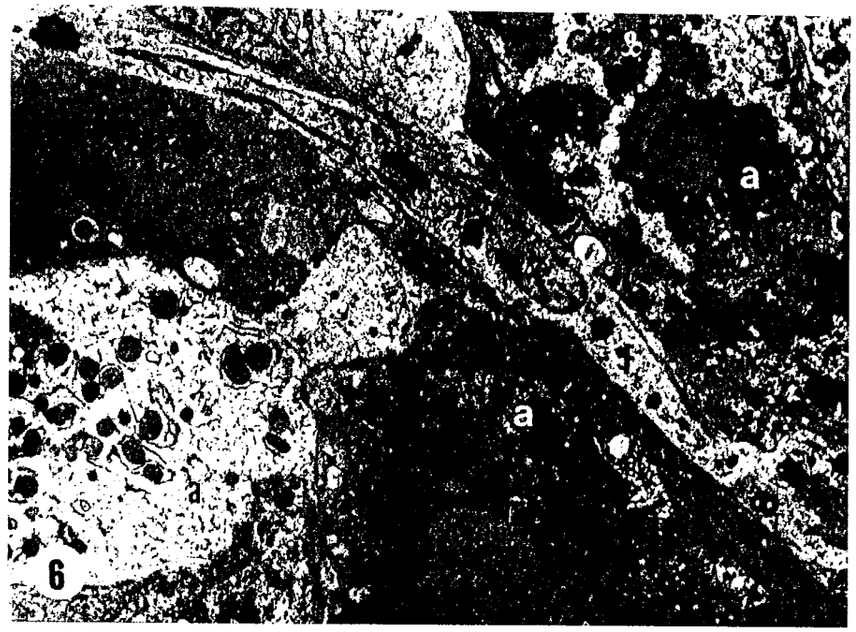

Fig. 6 – Atrophic hepatocytes (a) and an elongated cell of connective type (F). × 3,000.

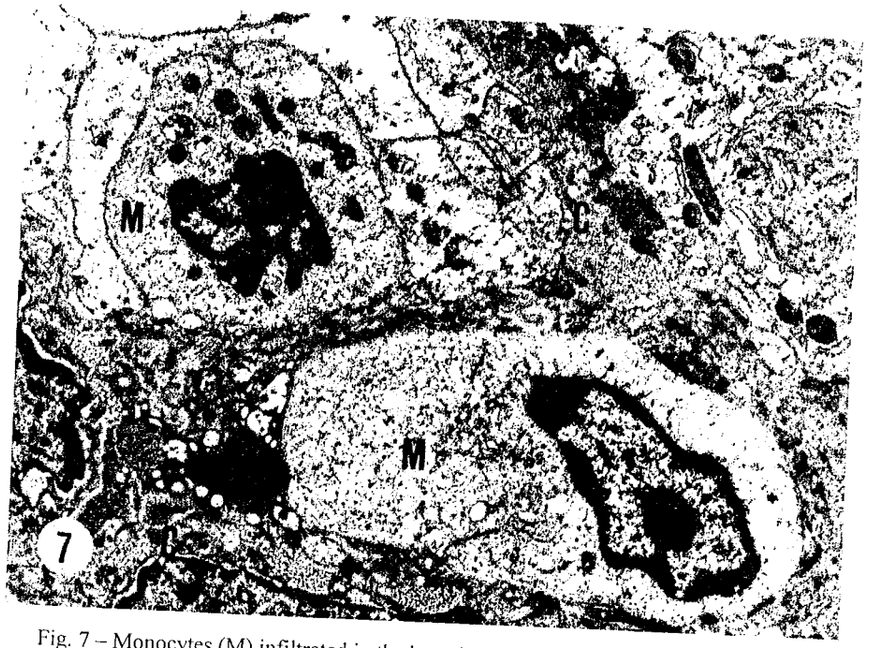

Fig. 7 – Monocytes (M) infiltrated in the hepatic cord. C: collagen fascicles. × 5,100.

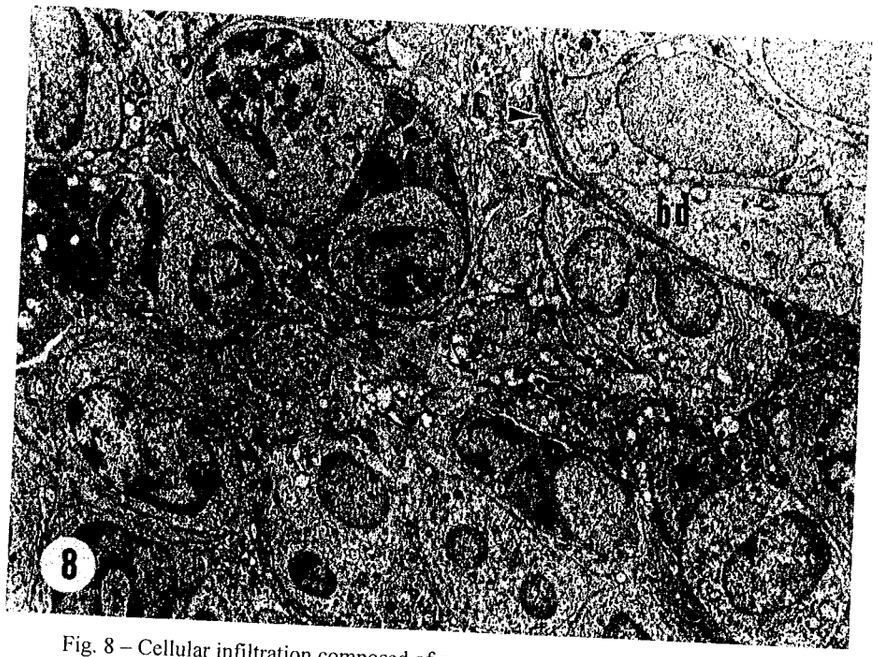

Fig. 8 – Cellular infiltration composed of monocytes and neutrophil polymorphs. A biliary duct (bd) presented a thick basal membrane (arrow head). × 3,000.

At the different moments of sacrification, the ultrastructural observations presented the coexistence of necrosis, inflammation, hepatic regeneration and collagen fascicles proliferation.

The hepatic sinusoids cells presented heavy lesions. Frequently, was observed the endothelial cells necrosis. Electrondense cellular remnants in the sinusoidal lumens were observed (Fig. 1). In some cases necrosed sinusoids lacked of the endothelial cells were identified only by the presence of the erythrocytes arrested between atrophic or necrotic hepatocytes (Fig. 2) or even surrounded by masses of collagen (Fig. 3).

The hepatocytes presented different types of organelles alterations. There were mitochondria with dense matrix (Fig. 1), accumulations of peroxidated lipids (Fig. 2, 3), poor developed rough and/or smooth endoplasmic reticulum (Fig. 4).

Different categories of the damaged hepatocytes were distinguished:

– Steatotic hepatocytes with accumulations of lipid vacuoles of different sizes; some were in a juxtanuclear position (Fig. 5);

– Atrophic hepatocytes detached from the hepatobiliary tree, retracted to a sphaerical form and surrounded by large intercellular spaces (Fig. 6);

– Necrotic hepatocytes with different cellular lesions, the most important being a typical necrotic nucleus. These hepatocytes might be identified as Councilman bodies (Fig. 4).

Connective cells with elongated cellular and nuclear form were observed near the atrophic hepatocytes. Often, in their cytoplasm were noted granules of the peroxidated lipids (Fig. 6).

Lymphocytes and/or monocytes migrated from the blood flow to the necrotic parenchyma (Fig. 7). Great accumulations of leukocytes (neutrophils and eosinophils) and mononuclears were also frequently observed in the periportal areas (Fig. 8).

In the extended periportal areas, a neoformation process of biliary ducts with thick basal membrane was registered (Fig. 8).

The fibrogenesis was present in hamster liver during the chronic CCl_4 treatment and two months after. Hepatic fibrosis implied the synthesis and deposition of the collagen fibers perihepatocytary, in the Disse spaces. Intralobullary were observed great collagen fascicles (Fig. 3). New formed collagen fibers appeared especially in the regions with atrophic hepatocytes, where the capillaries and biliary ducts proliferation was observed.

4. DISCUSSION

Carbon tetrachloride was a hepatic poison experimentally used to reproduce the model of human hepatic cirrhosis, in laboratory animals. (10, 13, 14, 20). In

spite of this interest, there were few papers, which conducted the research till the hepatic cirrhosis occurence.

This paper presented the cellular reactivity of the hamster liver for two months after the ceasing of CCl_4 treatment. The chronic treatment had a duration of three months and two weeks.

It was interesting that in hamster, at one week, at one month and at two months after the ceasing of the treatment, the hepatic cellular and tissular damage were similar with the period when the toxin was yet administrated (14).

The late intoxication effects seemed to be due to a long lasting injury supported by the liver circulatory system. The vascular alterations of the liver were produced at the several levels.

The hepatic sinusoid was the first affected by the treatment. Endothelial cells alterations were observed. Frequently in the lumens of damaged sinusoids were only remnants of endothelial cells.

At the moment of the research, endothelial cell necrosis was generalised and hepatic sinusoidal deendothelialization was observed. A hepatic sinusoid lacked of its lining cells ceased to be functional: this meant that the blood circulation was stopped in the respective vascular segment. The consequence was the interruption of the nutrients and oxygen passage from the blood to the Disse space and to the hepatocytes.

Using dimethylnitrosamine intoxication, Fujiwara et al. (6) demonstrated massive hepatic necrosis following the destruction of the endothelial cells of the sinusoidal lining.

The endothelial cells sieve plates modifications which had the effects on the passage of different molecules types and chylomicrons toward the vascular pole of the hepatocytes were studied (5, 22).

Sinusoidal capillarization represented an important step in the liver pathogenesis (1, 2, 4, 15). The capillarization of the sinusoids was manifested when the endothelial cells lost the sieve plates and began to synthesize and depose a basal membrane, perisinusoidally.

The degradation of the hepatic microcirculatory system was completed by the true arterialization (11, 21). This meant that in a zone, which suffered a metabolic alteration were found only true capillaries from portal arterioles and there were never observed sinusoids or active hepatocytes.

The hepatic sinusoid necrosis (12, 20) was often ignored, probably because it was the earliest sign of the lasting hepatic alteration. In hamsters after CCl_4 treatment, this process was a very extensive and persistent injury.

The evidence of the hepatic sinusoidal necrosis was presented in this paper.

The alteration of the hepatic sinusoids led to the occurrence of the porto-hepatic shunts vessels which must deliver the high portal pressure. By the shunt vessels, the blood was directed on the shortest way from the terminal portal vein to

the terminal hepatic vein. Sometimes the shunt was realized through one or more sinusoids with less damaged walls.

In the areas lacked of the sinusoids, the hepatic parenchyma was necrosed and the cellular infiltration was installed. Alternatively, if the hepatic portal arterioles sprouted and the capillaries replaced the sinusoids from the necrosed hepatic parenchyma, the fibrogenesis was installed.

The hepatic necrosis, biliary ducts proliferation and fibrogenesis were three processes in a continuous intercondition, even two months after the ceasing of chronic CCl_4 administration.

Acknowledgements. This paper was supported by Grant MEC-CNCSIS Nr. 5193/1999-2001 and by Institute of Biology Bucharest.

REFERENCES

1. Le Bail B., P. Bioulac-Sage, R. Senuita, A. Quinton, J. Saric, C. Balabaud (1990), J. Electron Microscopy Technique, **14**: 257–282.
2. Bioulac-Sage P., B. Le Bail, J. Carles, P. Bernard, G. Janvier, C. Brechenmachen, J. Saric, C. Balabaud (1992) in: Mollecular Aspects of Cirrhosis, B. Clément, A. Guillouzo eds., Colloque INSERM, J. Libbey Euro text Ltd., vol. **216**, 91–101.
3. Chojkier M., K.D. Lyche, M. Filip (1988), Hepatology, **8** (4): 808–814.
4. Dubuisson L., Boussarie L., C. A. Bedin, C. Balabaud, P. Bioulac-Sage (1995), Hepatology, 21: 805–814.
5. Fraser R., G. W. T. Rogers, L. E. Sutton, B. R. Dobbs (1995) in: Cells of the Hepatic Sinusoid, Kupffer Cell Foundation, E. Wisse, D. L. Knook, K. Wake eds., Vol. **5**, 263–266.
6. Fujiwara K., Ogata I., Mochida S., Hirata K., Tomiya T., Yamada S., Ohta Y. (1991) in Cells of the Hepatic Sinusoid, E. Wisse, D. L. Knouck and R. S. McCuskey eds., Kupffer Cell Foundation vol. **3**, 428–432.
7. Geerts A., J. M. Lazou, P. de Blesser, E. Wisse (1991), Hepatology, 13(6), 1193–1202.
8. Martinez-Hernandez A. (1985), Lab. Inverst., **53**(2), 166–186.
9. McGee J., Patrick R. (1972), Lab. Invest., **26**, 429–440.
10. Onori P., S. Morini, A. Franchito, R.Sferra, D. Alvaro, E. Gaudio (2000), J. Hepatol., **33**(4), 555–563.
11. Phillips M. J., P. S. Latham, S. Poucell (1987): Electron Microscopy of Human Liver Diseases (Chap. 2), in: Diseases of the Liver, L. Schiff, E. R. Schiff, 6-th. Ed., J. B. Lippincott Co., 47–76.
12. Prunescu C-C., P. Prunescu, H. K. Koerten, H. van der Meulen (1999) "*The hepatic sinusoid destruction precedes the appearance of the porto-hepatic shunts in experimental iron loaded hamsters*, in: World Congress on Iron Metabolism BioIron'99, Sorrento (Italy), 23-28 May 1999, Abstr. Book, 335.
13. Prunescu C-C., P. Prunescu (2000) *Evolution of lesions induced in rat liver by chronic carbon tetrachloride intoxication*, Proc. Inst. Biol., **3**, 491–497.
14. Prunescu C-C., P. Prunescu (2002) *Tissue lesions induced in the hamster liver by chronic carbon tetrachloride treatment*, Proc. Inst. Biol., vol. **IV**, 497–507.
15. Schaffner f., H. Popper (1963), Gastroenterology, **44**,239–242.
16. Shiratori Y., A. Geerts, T. Ichida, T. Kawase, E. Wisse (1986), J. Hepatol., 3, 294–303.

17. Shiratori Y. T. Ichida, A. Geerts, E. Wisse (1987), Digestive Diseases and Sciences, **32**(11), 1281–1289.
18. Stenger R. J. (1965), Exp. Molec. Pathol., **4**, 357–369.
19. Stenger r. j. (1966), J. Ultrastruct. Res., **14**, 240–253.
20. Vollmar B., S. Siegmund, M. D. Menger (1998), Hepatology, **27**, 1544–1553.
21. Wanless I. R. (1995), Hepatology, **22**(3), 983–993.
22. Wisse E., R. B. de Zanger, R. Charles, P. van der Smissen, R. S. McCuskey (1985), Hepatology, **5**(4), 683–692.

Received April 15, 2002.

Institute of Biology,
296 Spl. Independenţei,
P.P. Box 56–53, Bucharest, 060031
Romania

HEPATIC FIBROSIS IN HAMSTERS AFTER TMH-FERROCENE CONTAINING DIET

PAULA PRUNESCU, C-C. PRUNESCU

Syrian golden hamsters received a diet enriched with 0.5 % TMH-ferrocene for a period of 30 days. Immediately after the ceasing of the treatment, the hepatocytes were homogenously loaded with Perls-positive material. In time, the hepatocytes iron loading discharged gradually. The Kupffer cells overloaded with siderosomes detached from the sinusoid walls. These cells concentrated in the subcapsular zones or in the portal spaces. Often, they syncytialized forming iron multinucleated giant cells. Near the heavy iron loaded cell agglomerations, around the portal zones, fibroblast like cells were present. These cells were activated to synthesize and depose important quantities of collagen fibres. The central veins were fibrosed. The portal cirrhosis occurred in about half of the number of experimental hamsters.

1. INTRODUCTION

Human primary (idiopathic) iron overloading leads always to hepatic cirrhosis. The same is valid for the human diseases due to secondary iron loading (1, 2, 3, 4, 5).

The methods using TMH-ferrocene diet for obtaining the iron loading of the laboratory animals similarly to the iron overloading in the human idiopathic haemochromatosis, maintain a constant interest (6, 7, 8, 9, 10, 11).

The feeding of the rats, mice and even minipigs with a diet enriched with TMH-ferrocene led to massive iron loading of the liver and to a fibrosis restricted around the great agglomerations of iron loaded macrophages (7, 12, 13). The generalized fibrosis of the liver was not obtained. Also, the presence of the cirrhotic nodules and the setting of the portal and the central vein fibrosis like in the pattern of the pigmentary cirrhosis (14) was not observed.

The obtaining of a hepatic fibrosis similar to pigmentary cirrhosis of the human idiopathic hemochromatosis was realised until now only in experiments using *Grahamella gerbil* (15, 16). This species attained a cirrhotic status following iron treatment associated with the natural exposure to enterotoxic *Escherichia coli*.

In this paper was presented the hamster liver fibrosis obtained by iron overloading following the TMH-ferrocene diet.

REV. ROUM. BIOL. – BIOL. ANIM., TOME 47, N^{os} 1–2, P. 65–70, BUCAREST, 2002

2. MATERIALS AND METHODS

12 Syrian golden hamsters male, of 80–100 g weight, received for a period of 30 days, TMH-ferrrocene containing diet with the concentration of 0.5 % 3,5,5–trimethylhexanoyl-ferrocene. After the ceasing of the treatment, hamsters continued the conventional feeding. The effects of the iron loading on the liver were observed during the following six months.

Control male Syrian golden hamsters of 80–100 g body weight, fed with conventional feeding were sacrificed in the same time with the experimental individuals.

The animals were sacrificed by exsanguinations from the carotid under ether anaesthesia. The livers were processed for the light and electron microscopy.

Samples of liver were fixed in 4 % formaldehyde in saline, processed by routine histological techniques and embedded in paraffin. 5 μm thin sections were stained with Perls-Hemalum Mayer for the ferric ions (Fe^{+++}), silver impregnation Gömöri and trichrome stain of Krutzay for the collagen fibres (17).

Thin liver fragments were fixed in 2.5 % glutaraldehyde in cacodylate buffer pH 7.2 at 4° C, postfixed in 1.3 % Osmium tetraoxid, in the same buffer for 1 h, in the dark, at room temperature. After washing and dehydrations, the pieces were embedded in Epon 812. Ultrathin sections were contrasted with uranyl acetate and lead citrate. Observations were made using TEM Zeiss.

3. RESULTS

Immediately after TMH-ferrocene administration, the histological sections showed a moderately and relative uniformly Perls-positive thin granules loading of the hepatocytes (Fig. 1). 1–3 months after the treatment, the hepatocytes placed at the periphery of the hepatic lobules remained still charged with Perls-positive material. With the lapse of time, the liver of experimental animals presented an unequal iron loading.

Iron overloaded Kupffer cells detached from the sinusoid walls appeared concentrated in the portal spaces or inside the hepatic lobule (Fig. 2). The iron loaded Kupffer cells syncyalized and formed giant multinucleated cells (Fig. 3). The morphology of the iron giant cell is particular. The nuclei were placed at the peripheral position, beneath the plasma membrane (Fig. 3 and 7). The siderosomes were agglomerated more centrally, behind the nuclei disposition. Frequently, the right centre of the giant cell remained with a poor iron loading. (Fig. 3).

Usually, near the central veins, isolated iron loaded Kupffer cells and/or small Kupffer cells agglomerations were seen (Fig. 2 and 4).

As more Perls-positive material was concentrated on different structures of the hepatic lobules, more collagenous deposition was observed around that area.

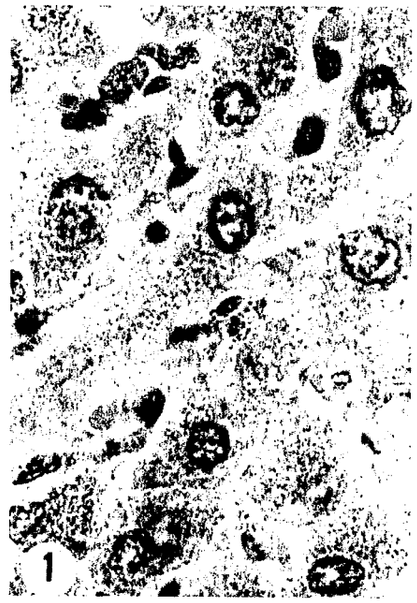

Fig. 1 – Hepatocytes loaded with Perls positive granules. Perls-Hemalum, × 1,000.

Fig. 2 – Massive accumulation of iron multinucleated giant cells in a portal zone; portal vein (pv). Biliary ducts (arrow) were observed between siderotic nodules. Hepatic central vein (hv) with thickened walls (double arrow). Trichrome Krutzay-Perls-Hemalum, × 200.

Fig. 3 – Iron multinucleated giant cell (arrow). Perls-Hemalum, × 500.

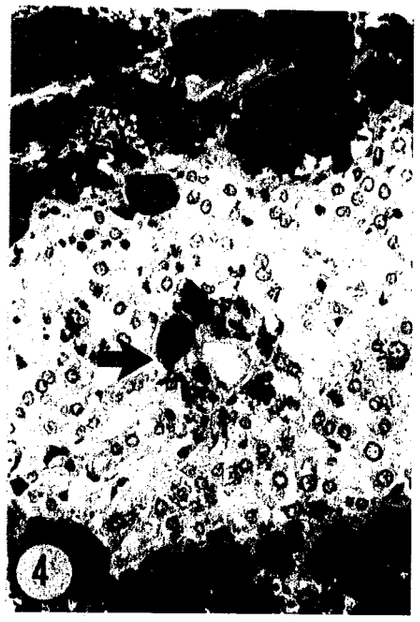

Fig. 4 – Iron loaded Kupffer cells and small iron multinucleated cells around the central vein with thickened wall (arrow). Trichrome Krutzay-Perls-Hemalum, × 200.

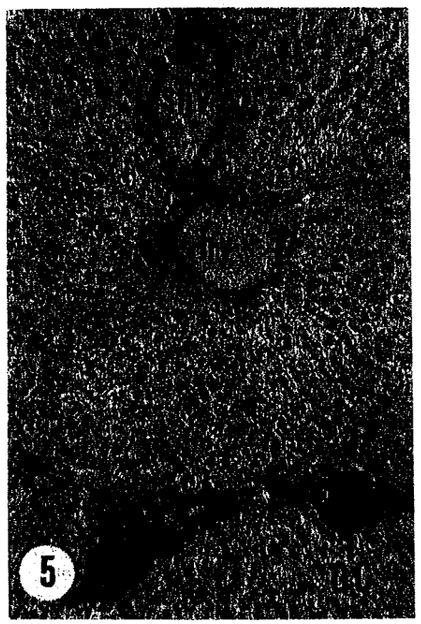

Fig. 5 – Central hepatic vein (hv) with thickened wall and shunt vessel (shv). A distinct giant cell (GC) in the portal area.
Trichrome Krutzay-Perls-Hemalum, × 80.

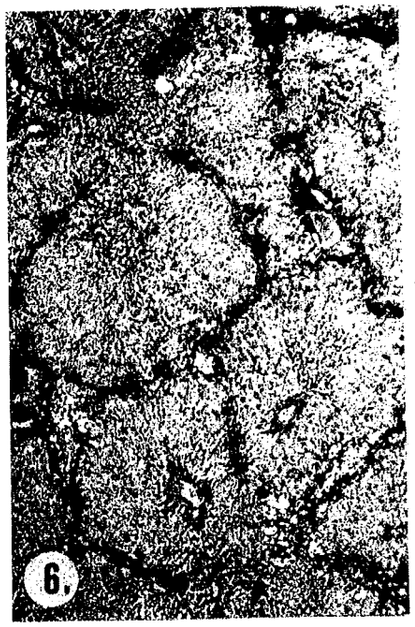

Fig. 6 – Cirrhotic nodules. The portal areas were bound together by collagenous strands.
Trichrome Krutzay-Perls-Hemalum, × 25.

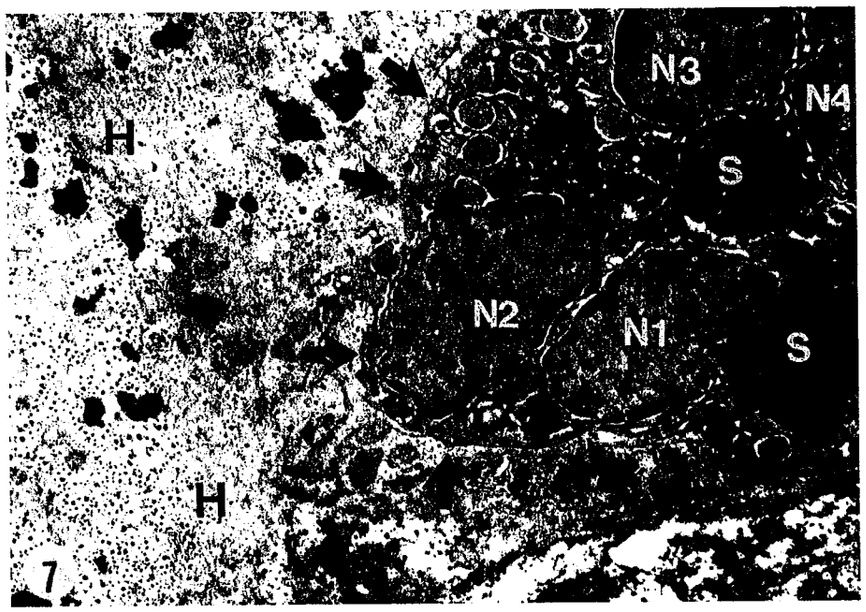

Fig. 7 – Peripheral zone of the iron giant multinucleated cell (arrows).N_1, N_2 N_3, N_4: giant cell nuclei; S: siderosomes, H: hepatocyte. × 5,300.

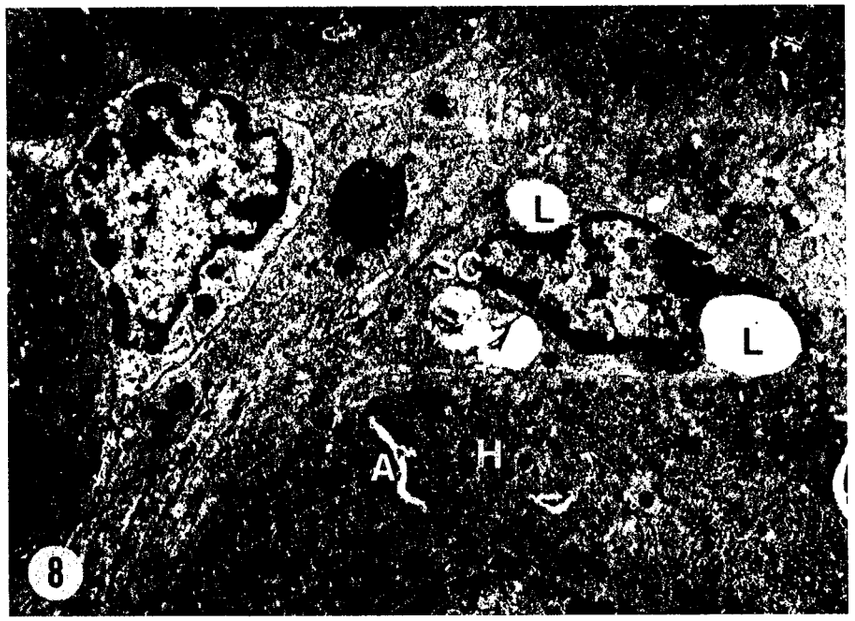

Fig. 8 – Perisinusoidal stellate cell (SC); L: lipid vacuole; H: hepatocyte; A: artefact. × 6,800.

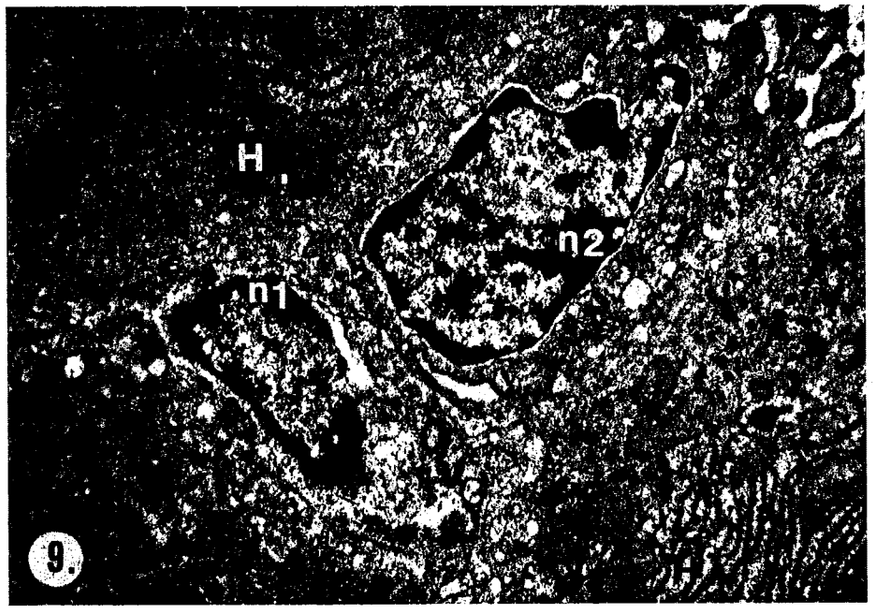

Fig. 9 – Fibroblast like cell with two nuclei (n_1, n_2) and swollen rough endoplasmic reticulum. H: hepatocyte. × 11,700.

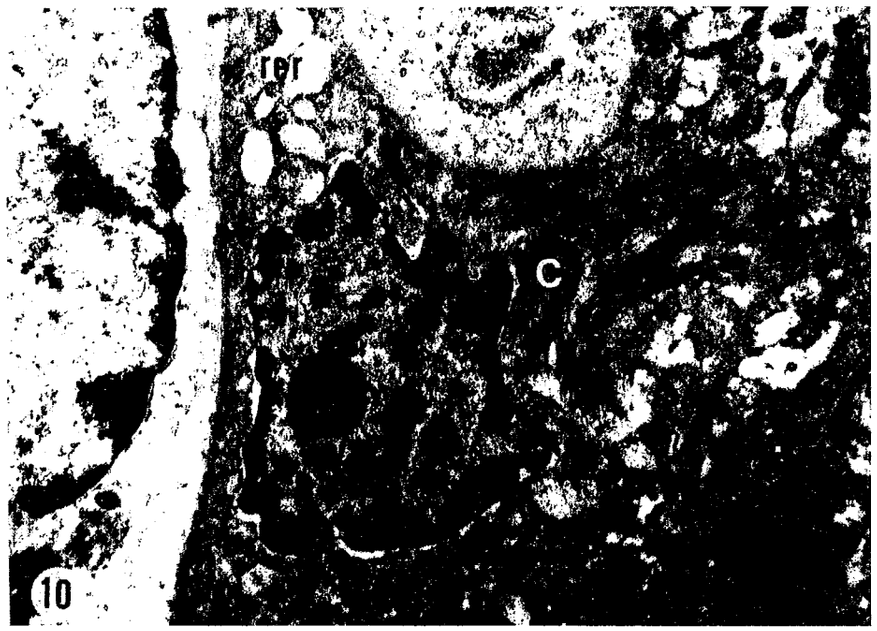

Fig. 10 – Fibroblast like cell with swollen rough endoplasmic reticulum (rer) and a collagen fascicle (C). × 13,600.

Around the central veins fibroblasts like cells actively deposed significant amounts of collagen fibres. Consequently, the central veins appeared dilated with thick collagenous walls, devoid of elasticity (Fig. 2 and 4). Sometimes a sinusoid in the vicinity presenting a greater diameter could become a shunt vessel taking over a part of the portal blood and opening in the fibrosed central vein (Fig. 5).

A similar process was observed in the peripheral zones of the hepatic lobule. Near the perisinusoidal stellate cells marked by great lipid vesicles (Fig. 8), the fibroblast like cells, with a remarkable development of the endoplasmic reticulum (Fig. 9) were observed. They synthesized and deposed filaments of collagen (Fig. 10).

The installed fibrosis in the hamster liver was observe towards the final period of six months established for the study.

The portal area surrounded by iron loaded Kupffer cells and iron multinucleated giant cells showed significant modifications. Portal vein walls were fibrosed. The great cellular agglomerations in the neighbourhood of the portal spaces were surrounded by fibres revealed by silver impregnation. The collagenous strands bound different portal areas and sometimes they joined the collagen fascicles from the central vein zones. The collagenous strands marked hepatic micronodules (Fig. 6) displaying the histological picture of constituted hepatic cirrhosis.

The bile ducts proliferated in the portal spaces showing numerous tortuous profiles, which penetrated between iron Perls-positive masses (Fig. 2).

Polymorphs, monocytes and plasmocytes were infiltrated in the close vicinity of the fibrosed portal spaces.

The connective cells of the capsule of Glisson and the fibroblast like cells of the subcapsular zones were activated, contributing to the thickening of the capsule Glisson. Also, the capsule was the target of the passive displacements of numerous iron overloaded Kupffer and/or giant multinucleated cells.

4. DISCUSSION

The attempts to model the liver lesions characteristic for the human idiopathic hemochromatosis failed firstly because the impossibility to obtain the hepatic cirrhosis following the iron loading in experimental rodents (7, 8, 18, 19).

Long standing iron-loading experiments with dogs (20) or baboons (21) had very limited positive results.

The hepatic fibrosis in *Grahamella gerbil* obtained after the iron parenteral administration was simultaneous with a natural continuous penetration of the gut bacterial endotoxin lipopolysaccharide in the liver. This endotoxin induced liver

haemorrhages and generated important inflammatory reactions. Finally, gerbils presented pigmentary cirrhosis (15).

The reciprocal condition was demonstrated by Ramm and col. (1999), namely the pathogen-free gerbils did not develop bridging fibrosis or cirrhosis after hepatic iron overloading (22).

Hamsters, which received moderate amounts of iron by the alimentary way during TMH-ferrocene treatment, presented hepatic fibrosis.

The collagen deposits of iron-loaded liver were always constituted near the Kupffer and giant multinucleated cells heavy charged with siderosomes.

In the controls, which were never fed with TMH-ferrocene, the histological sections of the liver did not present the spontaneous iron loading of the liver, or the spontaneous liver fibrosis.

Immediately after the ceasing of TMH-ferrocene administration, the hepatocytes were homogenously iron charged, like in experiments of other authors (10, 11). During the following months, a moving of the Perls-positive material from the hepatocytes was registered.

The iron overloaded Kupffer cells presented the tendency to move and concentrate in the subcapsular zones or in the portal spaces. This phenomenon was similar to that one observed in parenterally iron overloaded hamsters (23) or rats (24). This dynamics represented one of the liver own way to eliminate the iron excess or any other foreign material (25).

The iron concentrations determined the activation of the perisinusoidal stellate cells (16). The evidence of the perisinusoidal stellate cells activation to express mRNA type I collagen, to transform in myofibroblasts and to synthesize and depose collagen or other extracellular matrix proteins has been reported in the literature (26, 27, 28, 29).

An interesting feature of fibrosis in the hamster liver, as a response to the TMH-ferrocene feeding, was the weak reactivity of the hepatic stellate cells situated in zones lacked of iron loading.

As a matter of fact, in the human idiopathic haemochromatosis, the collagenous fascicles occurred in all Disse spaces (2, 3, 4).

Perhaps, the reactivity failure of the hepatic stellate cells observed in this experiment, might be bound to the episodic manner of iron administration. An iron-overloading model by TMH-ferrocene enriched diet, in which the iron daily intake will be reduced but continuous, could give an answer to this question.

The positive reactivity of the Syrian golden hamster liver to the iron overloading propose this species as an interesting model for the study of the hepatic fibrogenesis initiated by perisinusoidal stellate cells. Also, it could be an useful model for the pharmacological studies on the iron-chelating capacity of some medicaments.

Hamsters with advanced portal cirrhosis died during the experiment. This fact suggested the possibility to use hamsters with advanced cirrhosis for establish the efficacy of the essential drugs.

Acknowledgements. We thank Dr. Dr. P. Nielsen for his present of food enriched with TMH-ferrocene used as diet in this experiment and for the revision of the manuscript.

REFERENCES

1. Anthony P.P., Ishak K.G., Poulse H. E., Scheuer P. J., Sobin L. H. (1977), Bull. W. H. O., **55** (4): 521–540.
2. Arthur M. J. P. (1995), Kupffer Cell Foundation, Cells of Hepatic Sinusoid, Vol. **5**, E. Wisse, D. L. Knook, K. Wake eds., 372–376.
3. Brissot P., Campion J. P. Guillouzo A., Allain H., Messmer M., Simon M., Ferrand B., Bourel M. (1983), Digestive Diseases and Sci., **28** (7): 616–624.
4. Cabble E. E., Connor J. R., Isom H. C. (1998), Am. J. Pathol., **152** (3): 781–792.
5. Cabble E. E., Isom H. C. (1999), Drug. Metab. Dispos., **27** (2): 255-260.
6. Carthew Ph., Edwards R. E., Smith A. G., Cox T. M. (1992), in: Molecular and Cell Biology of Liver Fibrogenesis edited by A. M. Gressner and G. Ramadori Kluwer Academic Publishers, 316–319.
7. Carthew Ph., Edwards R. E., Smith A. G., Dorman B., Francis J. E. (1991), Hepatology, **13**: 534–539.
8. Crichton R., (2001), Inorganic Biochemistry of Iron Metabolism, J. Wiley Ltd, Second Edition, 223–235.
9. Cox T. M. (1990), Blood Revs., **4**: 75–87.
10. Düllmann J., Wulfhekel U., Nielsen P., Heinrich H. C. (1992), Acta Anat., 1992, **143**: 96–108.
11. Enzan H., Iwamura S., Himeno H., Saibara T., Onishi S., Yamamoto Y., Hara H. (1995), Kupffer Cell Foundation, Cells of the Hepatic Sinusoid, Vol. **5**, E. Wisse, D. L. Knook, K. Wake eds., 422–423.
12. Gressner A. M. (1991), Eur. J. Clin. Chem. Clin. Biochem., **29**: 293–311.
13. Iancu Th., Landing B. H., Neustein B. (1977), Path. Ann. vol. 12, S. C. Sommers ed. New York Appleton Century Crofts, 171–200.
14. Iancu Th., Shiloh H. (1988), in: Haemochromatosis edited by L. R. Weintraub, C. Q. Edwards, M. Krikker, Ann. N. Y. Acad. Sci., **526**, 169–178.
15. Lisboa P. E. (1971), Gut, **12**: 363–368.
16. Mureşan E., Gaboreanu M., Bogdan A. T., Baba A. I. (1974), Tehnici de Histologie Normală şi Patologică, Edit. Ceres, Bucureşti, 239–240.
17. Nielsen P., Heinelt S. Düllmann J. (1993), Comp. Biochem. Physiol., C., **106** (2): 429–436.
18. Nielsen P., Heinrich H. C. (1993), Biochem. Pharmac., **45** (2): 385–391.
19. Nielsen P., Zimmermann I. Fisher R., Dresow B., Düllmann J., Gabbe E. E. (1993) (abstr.), European Iron Club Meeting London (abstr.).
20. Phillips M. J., P. S. Latham, S. Poucell (1987), in Diseases of the Liver, L.Schiff and E. Schiff eds., J. B. Lippincott Co., 47–76.
21. Pietrangelo A., Gualdi R., Casalgrandi G., Geerts A., De Blesser P., Montosi G., Ventura E. (1994), Hepatology, **19** (3): 714–721.
22. Powell L. W., (1987), *The Pathogenesis of Cirrhosis associated with Iron Overload* in: Falk Symposium 44, edited by J. L. Boyer and L. Bianchi, MTP Press Ltd, 147–153.

23. Prunescu C-C., Prunescu P., (1988): "Giant multinucleated iron containing cells in the liver of polymaltosated iron injected rats", Rev. Roum. Biochim., **25** (4), 355–362.
24. Prunescu P., Prunescu C-C. (1975): "Pigment bearing multinucleated cells in the liver of malarial rodents during recovery", Rev. Roum. Biol. (Zool), **20** (2), 107–111.
25. Prunescu P., Prunescu C-C., (2000): "Early cellular and tissular lesions induced by iron overloading of hamster liver", Proc. Inst. Biol., Vol. **3**, 449–506.
26. Ramm G. A., R. S. Britton, E. M. Brunt, R. O'Neill, B. R. Bacon (1999), BioIron'99 Sorrento Naples Italy, Congress Book, Abstr. 327.
27. Richter G. W. (1974), Am. J. Pathol., **74** (3): 481–506.
28. Scheiber-Mojdehkar B., Zimmermann I., Dresow b., Goldenberg H. (1999), J. Hepatol., **31** (1): 61–70.
29. Stremmel W., Riedel H. D. Niederau C., Strohmeyer G. (1993), **23**, 321–329.

Received April 15, 2002.

Institute of Biology, Bucharest
296, Spl. Independenței,
P.O. Box 56–53, Bucharest, 060031
Romania

NEUROPEPTIDE TYROSINE (NPY-)-IMMUNOREACTIVE NEURAL AND ENDOCRINE ELEMENTS IN THE AMPHIBIAN GASTROENTERIC TRACT

LUMINIȚA UNGUREANU*, IOANA TRANDABURU**, T. TRANDABURU**

The gastrointestinal tract of four amphibians species (newts and frogs) was investigated immunohistochemically for the occurrence and topographic distribution of neuropeptide tyrosine (NPY) by using the avidin-biotin-peroxidase complex (ABC-peroxidase) procedure. A relative poor NPY innervation consisting of nerve fibers in stomach and of nerve fibers and ganglion perikarya in the entire gut was seen in newts. In addition to the neural structures, the large intestine of these urodeles displayed several immunostained endocrine cells. As compared to newts, the endocrine cells were the sole immunolabelled elements encountered in the frog gastroenteric tract. The above findings are discussed in connection with those previously reported in other taxa of vertebrates.

1. INTRODUCTION

The neuropeptide Y family of peptides consists of neuropeptide Y (NPY), which is expressed in the central and peripheral nervous systems but also in the gastro-entero-pancreatic (GEP) endocrine system, and peptide YY (PYY) and pancreatic polypeptide (PP), which are considered gut endocrine peptides [26]. All three peptides are 36 aminoacids long and act on G-protein – coupled receptors [16, 18, 26]. According to the current concept [5, 11, 26, 28], NPY is one of the most conserved peptide during evolution, PYY is more variable, particularly in mammals, whereas PP, probably arosed as a copy of PYY in an early tetrapod ancestor, may be the most rapidly evolving endocrine peptide.

The NPY-family peptides have many and diverse effects including vasoconstriction [19, 29], stimulation of food intake [13, 19], regulation of circadian rythms [28] and release of pituitary sex hormones [13]. To the above effects, the inhibitory actions on gastrointestinal motility, secretion and blood flow should be added [22].

Although the topographic distribution of NPY immunoreactive neural and/or endocrine elements has been demonstrated in the GEP organs of various mammalian species [1, 11, 12, 22, 23, 24, 25] including the human one [35], their occurrence, particularly in the gastrointestinal tract of poikilotherm vertebrates, was only sporadically investigated.[4, 10, 32]. Moreover, according to our information, there are only two reports regarding the distribution of NPY

immunopositive structures in the amphibian gut [30, 31]. This study was therefore designed to enlarge the present knowledge about the occurrence and topographic distribution patterns of this neuropeptide in the amphibian gastroenteric tract by carrying out comparative immunohistochemical investigations in several newt and frog species. It could be also provides a further evidence for the good conservation of NPY during phylogeny.

2. MATERIALS AND METHOD

ANIMALS

Adult specimens of amphibians, of both sexes, collected in spring-time in the neighbourhood of Craiova city were kept unfed in fresh-water aquaria for 1–3 days. The species and the number of specimens employed are listed bellow:

Urodela newts: *Triturus vulgaris* (5 specimens)
Triturus cristatus (4 specimens)
Anura (frogs): *Rana esculenta* (6 specimens)
Rana temporaria (4 specimens)

TISSUE PREPARATION

All animals were anesthetized with chloroform and the entire gastrointestinal tracts were removed and divided into defferent regions: stomach (upper and lower), small intestine (duodenum, middle and distal) and large intestine. The tissue fragments were fixed for 24–36 h in Bouin's fluid, dehydrated through a graded ethanol series, cleared with toluene and parrafin-embedded. Sections of 5–6 μm-thickness, prepared on a sledge microtome, were mounted on poly-L-lysine (Sigma, USA) – coated slides.

PRIMARY ANTIBODY

The primary antiserum – polyclonal rabbit anti-synthetic porcine NPY- has been purchased from Chemicon, Temecula, Ca., USA.

IMMUNOHISTOCHEMICAL PROTOCOL

The deparaffinized, rehydrated sections were immunostained according to the avidin-biotin-peroxidase complex (ABC-peroxidase) procedure [21]. It includes successive exposures of the sections at room temperature to 1) the adequately diluted (1:700) primary antiserum overnight, 2) the biotinylated goat anti-rabbit IgG (Sigma, USA) diluted 1:100 for 60 min., and 3) the preformed complex of extravidin-peroxidase (Sigma, USA) diluted 1:100 for 60 min.

The antigen-antibody binding sites were revealed by a 3-4 min. immersion of the sections at room temperature in a solution of 0.5 mM DAB (3,3'-diaminobenzidine tetrahydrochloride – Serva Feinbiochem. GmbH., FRG) prepared in 0.05 M Tris-HCl buffer (pH 7.6), to which 0.05 % hydrogen peroxide had been added just before use. 0.01 M PBS (phosphate buffer saline) (pH 7.4) containing 0.3% Triton X-100 was used as diluent for every step of the procedure and as rinsing solution between the steps. Subsequently, the sections were dehydrated in ethanol, cleared with xylene and mounted in Entellan (E. Merck, FRG).

SPECIFICITY CONTROLS

Pertinent tests to prevent procedural non-specificity were performed as recommended in the literature [6, 7, 17].

The specificity of the primary antibody was tested by its preadsorption (24h at 4°C) with varying quantities (6-128 μg/ml working dilutions) of the corresponding antigen (swine synthetic NPY – Biotrend Chem. GmbH., FRG) and of the structurally related antigens (human synthetic PP – Biotrend Chem. GmbH., FRG; human synthetic PYY – Sigma-Aldrich Chem. GmbH., FRG). Preadsorption of the primary antibody with its corresponding antigen at 6 μg/ml antibody concentration completely abolished the staining, while preadsorption with the related antigens, even at concentrations up to 128 μg/ml antibody did not affect the immunoreaction.

3. RESULTS

The antiserum used revealed both neural (nerve cell bodies and fibers) and endocrine elements in the gastrointestinal tract of newts and only those endocrine in that of frogs. The density of immunolabelled elements varied considerably along the gut and between the taxa (Urodela and Anura) studied and was roughly the same among the individuals of one species (Table 1). Moreover, the staining intensity showed variations in the different gut segments depending on the nature (neural or endocrine) of immunoreactive structures.

Newts. Except for the sparse weavy profiles of the nerve fibers scattered in submucosa and lamina propria, the stomach was devoid of any other NPY immunopositive element.

The entire small intestine showed also a poor NPY-innervation made up of nerve fibers and ganglion cell bodies (Figs. 1a-d; 2a,b). Immunostained fine nerve profiles occured mainly distributed in the deep myenteric plexus but also in the superficial one, in submucosa and even in mucosa running parallel with the villi long axis (Figs. 1a,d; 2a,b). Despite of the scarce NPY immunopositive nerve

fibers identified in the superficial myenteric plexus, it was the sole layer of the enteric wall displaying immunolabelled perikarya (Figs. 1b,c).

The density and distribution pattern of immunoreactive neural elements in the large intestine were roughly similar to those recorded in the remainder gut. At this level, has been, however, detected a moderate amount of NPY containing endocrine cells disseminated throughout the villi epithelium, most of them showing the morphological features of the "open" cell variety (Figs. 2c,d).

Table 1

The occurrence and relative density of NPY-immunoreactive structures along the gastrointestinal tract of amphibians studied (1:700 dilution of antiserum)

Gut segments	Immunoreactive elements	Newts	Frogs
Stomach			
– Upper	En/P/Nf	–/–/+	–/–/–
– Lower	En/P/Nf	–/–/++	+/–/–
Small intestine			
– Duodenum	En/P/Nf	–/+/++	++/–/–
– Middle	En/P/Nf	–/+/++	++++/–/–
– Distal	En/P/Nf	–/±/++	++++/–/–
Large intestine	En/P/Nf	++/±/++	+++/–/–

Abbreviations: En = endocrine cells; P = perikarya; Nf = nerve fibers
Symbols (number of immunoreactive structures/gut cross section):
++++, >16; +++, 10–16; ++, 5–9; +, 1–4; ±, occasional presence; –, none

Frogs. The endocrine cells, as the sole NPY immunopositive elements, appeared scattered in the mucosa epithelium along the gastrointestinal tract, reaching the highest density in the middle and distal small intestine. They were absent in the upper stomach and only a few in its lower region, where they occurred located at the base of the pyloric glands (Fig. 3a).

Both immunoreactive cell populations of the "open" and "closed" varieties were encountered along the entire gut (Figs. 3b-e). In the duodenum prevailed the enterocytes of the "open" variety, in the remainder small intestine the amounts of "open" and "closed" cell varieties occurred fairly balanced, whereas in the large intestine the number of "closed" type enterocytes exceeded that of the "open" one. Irrespective of their morphological features, the immunostained endocrine cells appeared scattered throughout the villi epithelium.

4. DISCUSSION

The available information regarding the distribution of NPY immunoreactivity in the gastrointestinal tract of poikilotherm vertebrates and

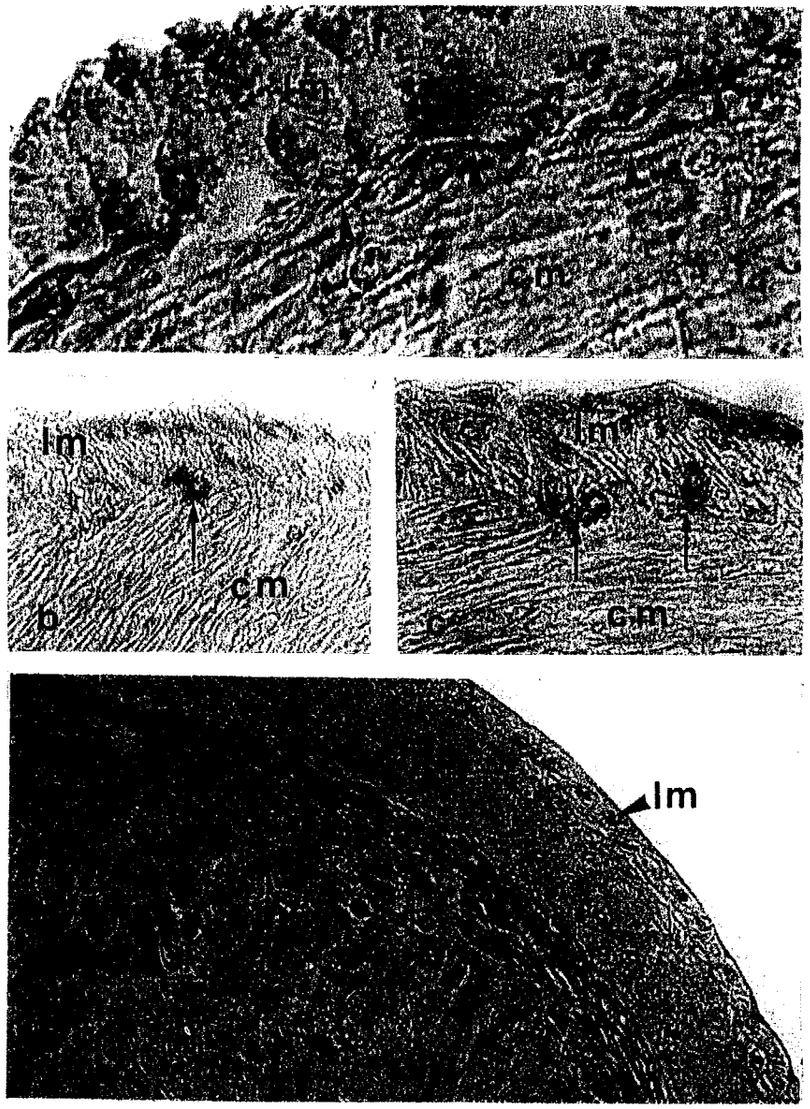

Fig. 1 – NPY immunoreactive nervous elements in the gut of newts.
a. – loose network of immunopositive nerve fibers (arrowheads) located in the superficial myenteric plexus of the duodenum of the newt, *Triturus cristatus* ; b, c. – immunolabelled ganglion cells (arrows) localized in the superficial myenteric plexus of the distal (b) and middle (c) small intestine of the newts, *Triturus vulgaris* (b) and *Triturus cristatus* (c);
d. – cross section through the large intestine of the newt, *Triturus vulgaris* showing strongly immunostained nerve fibers (small arrowheads) in the deep myenteric plexus.
lm = longitudinal muscle layer; cm = circular muscle layer; sm = submucosa; a. – ×1090; b. – × 560; c. – × 740; d. – × 480.

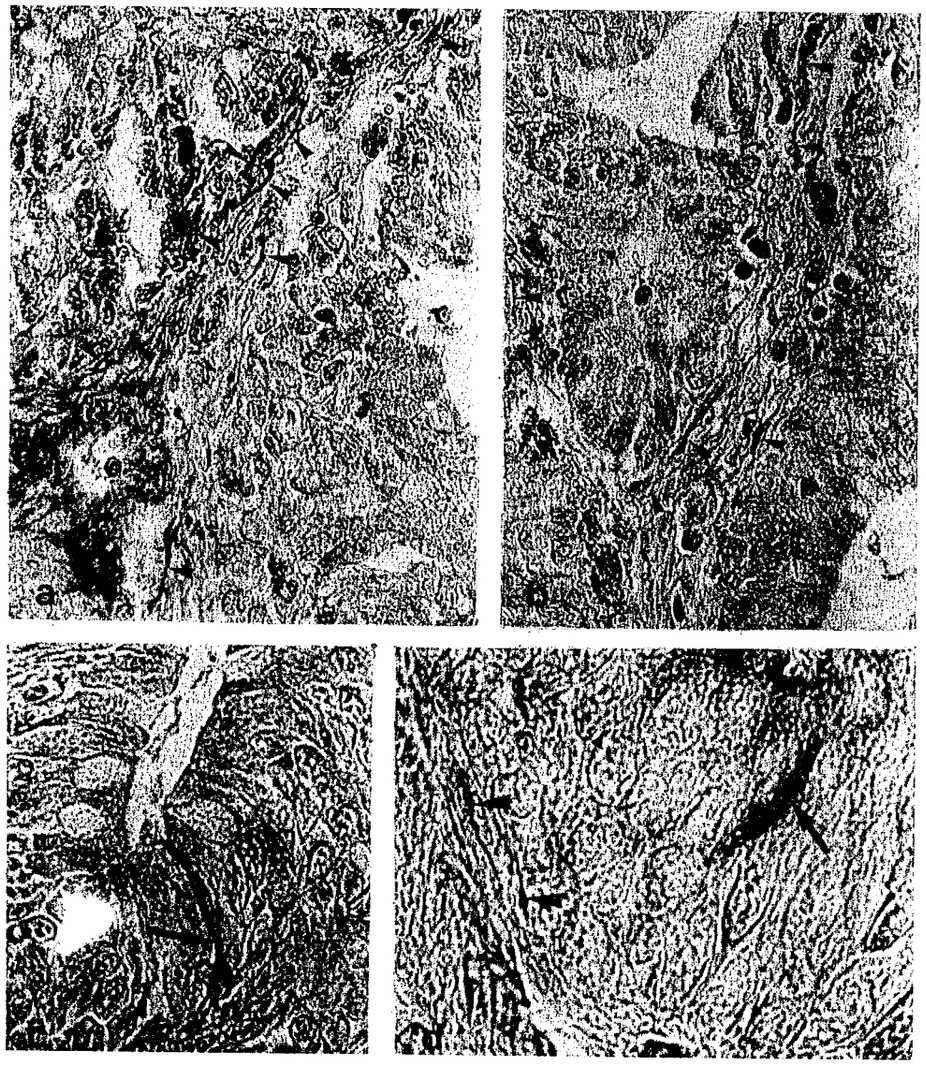

Fig. 2 – NPY immunopositive neural (a, b) and endocrine (c, d) elements in the newt gut. a, b. – immunostained slender nerve profiles (small arrowheads) in the proximal (a) and middle (b) small intestine mucosa of the newt, *Triturus vulgaris* showing parallel arrangements with the villi long axes; c,d. – immunolabelled endocrine cells (arrows) of the "open" variety and nerve profiles (arrowheads) detected in the large intestine of the newts, *Triturus cristatus* (c) and *Triturus vulgaris* (d). a, b. – × 990; c, – × 640; d. – × 1090.

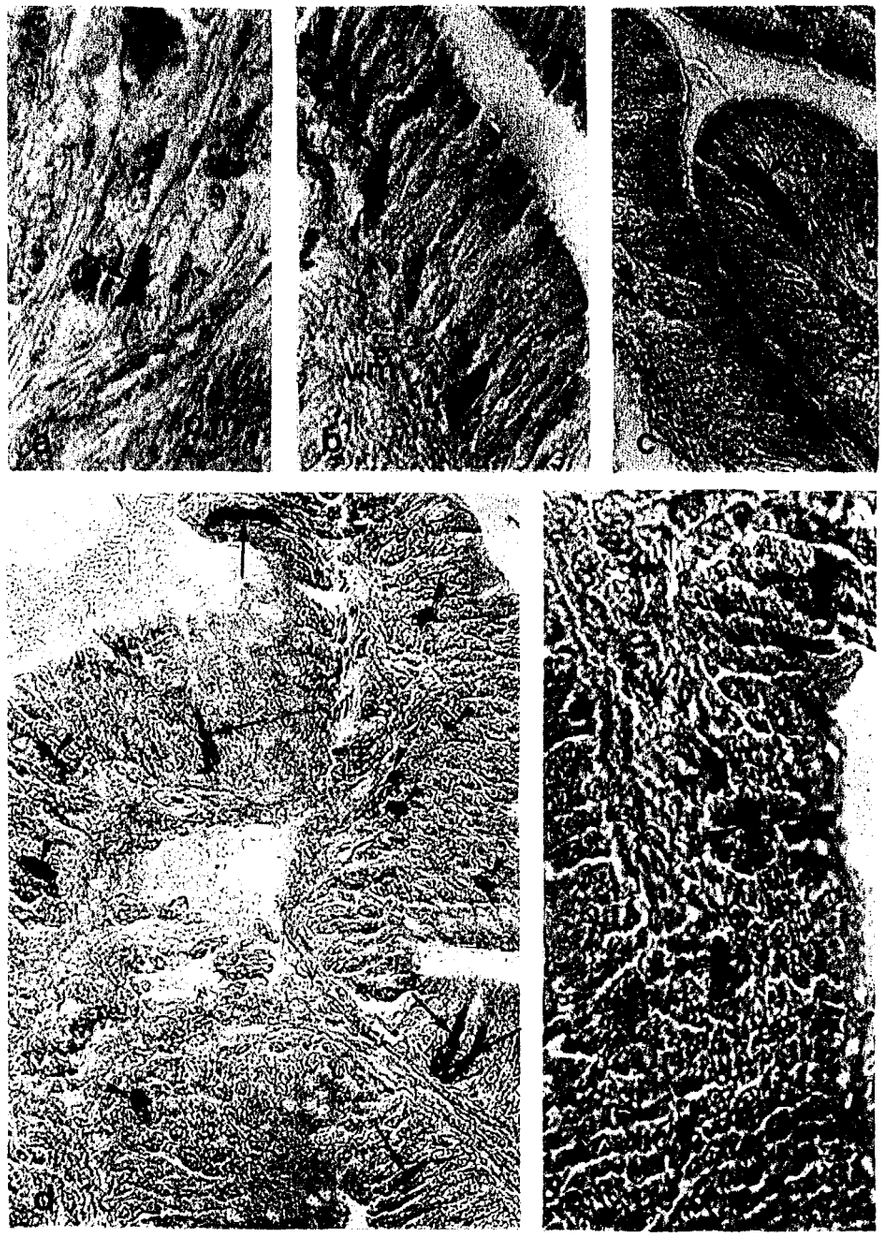

Fig. 3 – NPY containing endocrine cells in the frog gastroenteric tract. a. – an immunolabelled endocrine cell at the base of a pyloric gland in the stomach of a *Rana esculenta* specimen; b,c. – immunostained enterocytes (arrows) of the "open" variety located in the duodenal epithelium of the frogs, *Rana esculenta* (b) and *Rana temporaria* (c); d. – immunopositive enterocytes of the "open" (arrows) and "closed' (small arrowheads) varieties scattered in the mucosal epithelium of the middle small intestine of the frog, *Rana esculenta;* e. – densely immunostained endocrine cells of the "closed" variety (arrowheads) detected in the large intestine of the frog, *Rana temporaria*. om = oblique musculature; vm = vilus mucosa; a. – × 800; b,c. – × 740; d. – × 500; e. – × 900.

mammals appears somehow contradictory. Thus, more reports [2, 12, 31] have revealed the storage of this neuropeptide exclusively in the neural structures (perikarya and/or nerve fibers), while others [4, 8, 9, 22, 24, 33] both in neural and endocrine enteric elements. In this context, our results demonstrating immunopositive enterocytes, perikarya and nerve fibers in the newt gut undoubtedly support the dual nervous and endocrine distribution of NPY.

With strict reference to the NPY containing ganglion cells, their distribution in one or both nervous plexuses (superficial and deep) of the intestinal wall has been previously reported in dogfish [10], turtle [15, 32] and rat [3, 22, 34], but not in toad [31], frog [30] and the majority of investigated mammals [12, 23, 24, 25]. In our opinion, the presence, even sporadical in the newt gut of immunolabelled perikarya could express the more potent inhibitory actions of NPY on the organ motility, secretion and blood flow.

The results obtained in frogs showing the distribution of NPY immunoreactivity exclusively in the enteroendocrine cells deserve particular attention.

They disagree with those reported previously in toad [31] and also in the same frog species [*Rana temporaria* – 5], in which have been detected only NPY immunopositive nerve fibers. These contradictory findings could be assigned, at least partly, to the different purenesses or to the differences, even minor, between the primary structure of the antibody employed by us and that isolated by McKay et al.(1992) from the frog brain extracts. An indirect prove supporting the first explanation could be considered the much more widespread neuronal expression of the NPY related PYY in lower vertebrates described by several authors [26, 27].

The present study has revealed two distinct distribution patterns of NPY immunoreactive enteroendocrine cells in newts and in frogs, respectively. In comparision with the relative high number of such cells recorded throughout along the frog gut, their sparse occurrence in newt, restricted only to the large intestine, could – as has been postulated for other neuropeptides [20] – be ascribed to the functional pecularities of this organ segment controlling its own motility prevalent humorally.

*Acknowledgements.*This investigation was supported by a Humboldt grant (Bonn, FRG) assigned to T.T. in 2000 and was partly carried out in the Institute of Anatomy II, J.W.Goethe University (Frankfurt am Main, FRG). The skilful technical assistance of Mr. S. Leslie is gratefully acknowledged by T.T.

REFERENCES

1. Allen J. M., Hughes J., Bloom S.R.(1987) Presence, distribution, and pharmacological effects of neuropeptide Y in mammalian gastrointestinal tract. Dig.Dis.Sci. **32**: 506–512.
2. Branchek T.A., Gershon M.D. (1989) Time-course of expression of neuropeptide Y, calcitonin gene-related peptide and NADPH diaphorase activity in neurons of the developing murine

bowel and the appearance of 5-hydroxitryptamine in mucosal enterochromaffin cells. J.Comp.Neurol. **285**: 262–273.

3. Buchan A.M., Baimbridge K.G. (1988) Distribution and co-localization of calbindin D28k with VIP and neuropeptide Y but not somatostatin, galanin and substance P in the enteric nervous system of the rat. Peptides **9**: 333–338.
4. Burkhardt-Holm P., Holmgren S. (1989) A comparative study of neuropeptides in the intestine of two stomachless teleosts (*Poecilia reticulata, Leuciscus idus melanotus*) under conditions of feeding and starvation. Cell Tissue Res. **255**: 245–254.
5. Cereda-Reverter J.M., Martinez-Rodriguez G., Zanuy S., Carillo M., Larhammar D. (2000) Molecular evolution of the neuropeptide Y (NPY) family of peptides: cloning of three NPY-related peptides from the sea bass (*Dicentrachus labrax*). Regul.Peptides **95**: 25–34.
6. Cetin Y., Aunis D., Bader M.F., Galinda E., Jörns A., Bargsten G., Grube D. (1993) Chromostatin, a chromogranin A-derived peptide, is present in human pancreatic (β) cells. Proc.Natl.Acad.Sci.USA **90**: 2360–2364.
7. Cetin Y., Müller-Köppel L., Aunis D., Bader M.F., Grube D. (1989) Chromogranin A (CgA) in the gastro-entero-pancreatic (GEP) endocrine system. II CgA in mammalian entero-endocrine cells. Histochemistry **92**: 265–275.
8. Cheung R., Andrews P.C., Plisetskaya E.M., Youson J.H. (1991) Immunoreactivity to peptides belonging to the pancreatic polypeptide family (NPY, aPY, PP, PYY) and to glucagon-like peptide in the endocrine pancreas and anterior intestine of adult lampreys, *Petromyzon marinus*: an immunohistochemical study. Gen.Comp.Endocrinol. **81**: 51–63.
9. Chiba A., Honma Y. (1995) Occurrence of neuropeptide Y (NPY)-like-immunoreactive cells in the vitellointestinal duct and yolk sac of two species of elasmobranchs. Zool.Sci. **12**: 611–614.
10. Chiba A., Honma Y., Oka S. (1995) Ontogenetic development of neuropeptide Y-like-immunoreactive cells in the gastroenteropancreatic endocrine system of the dogfish. Cell Tissue Res. **282**: 33–40.
11. Ding W.G., Kimura H., Fujimura M., Fujimiya M. (1997) Neuropeptide Y and peptide YY immunoreactivities in the pancreas of various vertebrates. Peptides **18**:1523–1529.
12. Domeneghini C., Massoletti P., Arrighi S. (1997) Localization of regulatory peptides in the gastrointestinal tract of the striped dolphin, *Stenella coeruleoalba* (Mammalia: Cetacea). An immunohistochemical study. Eur.J.Histochem. **41**: 285–300.
13. Dumont Y., Martel J.-C., Fourmier A., St-Pierre S., Quirion R. (1992) Neuropeptide Y and neuropeptide Y receptor subtypes in brain and peripheral tissues. Prog.Neurobiol. **38**: 125–167.
14. Ekblad E., Alm P., Sundler F. (1994) Distribution, origin and projections of nitric oxide synthase-containing neurons in gut and pancreas. Neurosci. **63**: 233–248.
15. Gabriel R., Timmermans J.P., De Groodt-Lasseel M.H., Scheuermann D.W. (1990) High density of neuropeptide Y-innervation in the myenteric plexus of the turtle midgut. An immunocytochemical study. Acta Biol.Hung. **41**: 283–288.
16. Gehlert D.R. (1994) Subtypes of receptors for neuropeptide Y: implications for the targeting of therapeutics. Life Sci. **55**: 551–562.
17. Grube D., Aunis D., Bader M.F., Jörns A., Yoshie S. (1986) Chromogranin A (CgA) in the gastro-entero-pancreatic (GEP) endocrine system. I CgA in the mammalian endocrine pancreas. Histochemistry **85**: 441–452.
18. Grundemar L., Håkanson R. (1994) Neuropeptide Y effector systems : perspectives for drug development. Trends Pharmacol.Sci. **15**: 153–159.
19. Haselwood L.R. (1990) Pancreatic polypeptide (PP) and its relevant relatives. In : Progress in Comparative Endocrinology (Epple A., Scanes C.G., Stetson HM.eds). Viley-Liss Inc., New York, Toronto, Singapore, **342**: 250–256.
20. Holmgren S., Jensen J., Lönsson A.-C., Lundin K., Nilsson S. (1985) Neuropeptides in the gastrointestinal canal of *Necturus maculosus*. Distribution and effects on motility. Cell Tissue Res. **241**: 565–580.

21. Hsu S.M., Raine L., Fanger H. (1981) Use of avidin-biotin-peroxidase techniques. A comparision between ABC and unlabelled antibody (PAP) procedures. J. Histochem. Cytochem. **29**: 577–580.
22. Jackerott M., Larsson L.I. (1997) Immunocytochemical localization of the NPY/PYY Y1 receptor in enteric neurons, endothelial cells and endocrine-like cells of the rat intestinal tract. J. Histochem. Cytochem. **45**: 1643–1652.
23. Keast J.R. (1993) Innervation of the monotreme gastrointestinal tract: a study of peptide and catecholamine distribution. J.Comp.Neurol. **334**: 228–240.
24. Keast J.R., Furness J.B., Costa M. (1987) Distribution of peptide-containing neurons and endocrine cells in the rabbit gastrointestinal tract, with particular reference to the mucosa. Cell Tissue Res. **248**: 565–577.
25. Koide M., Kitamura N., Yamada J., Yamashita T. (1990) Immunohistochemical study of peptide-containing nerves in the gastrointestinal tract of the Japanese field vole, *Microtus montebelli*. Anat.Histol.Embryol. **19**: 109–119.
26. Larhammar D. (1996) Evolution of neuropeptide Y, peptide YY, and pancreatic polypeptide (PP). Regul.Peptides, **62**: 1–11.
27. Larhammar D., Blomqvist A.G., Sodenberg C. (1993) Evolution of neuropeptide Y and its related peptides. Comp.Biochem.Physiol. C **106**: 743–752.
28. Larhammar D., Söderberg Ch., Lundell I. (1998) Evolution of neuropeptide Y family and its receptors. Trends in Comparative Endocrinol.Neurobiol. (Vaudry H., Tonon M.C., Roubos E.W., Loof A.eds.) Ann.NY Acad.Sci. **839**: 35–41.
29. Lundberg J.M., Tatemoto K. (1982) Pancreatic polypeptide family (APP,BPP,NPY and PYY) in relation to sympathetic vasoconstriction. Acta Physiol.Scand. **116**: 393–412.
30. McKay D.M., Shaw C., Halton D.W., Thim L., Buchanan K.D. (1992). *The primary structure and tissue distribution of an amphibian neuropeptide* Y. Regul.Peptides **37**: 143–153.
31. Murphy S., Campbell G (1993) An immunohistochemical study of the innervation of the large intestine of the toad (*Bufo marinus*). Cell Tissue Res. **274**: 115–125.
32. Scheuermann D., Gabriel R., Timmermans J.P., Adriaensen D., De Groodt-Lasseel M.H. (1991) The innervation of the gastrointestinal tract of a chelonian reptile, *Pseudemys scripta elegans*. II. Distribution of neuropeptides in the myenteric plexus. Histochemistry **95**: 403–411.
33. Van Noorden S. (1990) Gut hormones in cyclostomes. Fish Physiol. Biochem. **8**: 399–408.
34. Wang Y.N., Mc Donald J.K., Wyatt R.J. (1987) Immunocytochemical localization of neuropeptide Y-like immunoreactivity in adrenergic and non-adrenergic neurons of the rat gastrointestinal tract. Peptides **8**: 145–151.
35. Wattchow D.A., Furness J.B., Costa M. (1988) Distribution and coexistence of peptides in nerve fibers of the external muscle of the human gastrointestinal tract. Gastroenterology **95**: 32–41.

Received May, 2002.

*Craiova University
Faculty of Biology
Al.I. Cuza 13
1100 – Craiova
Romania
** Institute of Biology
Splaiul Independenței 296
P.O. Box 56–63
060031 – Bucharest
Romania

NEUROPEPTIDE TYROSINE (NPY) IMMUNOREACTIVITY IN THE GASTROINTESTINAL TRACT OF SEVERAL REPTILES

IOANA TRANDABURU*, T. TRANDABURU*, LUMINIȚA UNGUREANU**

The occurrence and topographic distribution of neuropeptide tyrosine (NPY) have been investigated immunohistochemically in the gastrointestinal tract of turtles, lizards and snakes by using the avidin-biotin-peroxidase procedure. The stomach of all reptiles displayed exclusively immunostained neural structures (perikarya and/or nerve fibers), whereas the intestine both neural and endocrine elements. Their numerical distribution varied widely among the taxa under study and along the gastroenteric tract and only slightly among the individuals of the same species. Two distribution patterns of NPY-containing enteroendocrine cells – specific for turtles and for lizards and snakes, respectively – have been identified. The localizations and numerical distribution pecularities of NPY-immunopositive elements in the reptilian gastrointestinal tract are discussed in connection with the findings in other taxa of vertebrates.

1. INTRODUCTION

Neuropeptide tyrosine (NPY) and peptide tyrosine tyrosine (PYY) are 36-aminoacid peptides characterized by a high degree of sequence conservation during phylogeny [24]. They form a family of neuroendocrine peptides that in tetrapods also includes pancreatic polypeptidic (PP) and in certain fish species a pancreatic polypeptide tyrosine (PY) [25].

Although the physiological effects of NPY are not entirely known yet, it appears involved in central and peripheral vasoconstriction [27], stimulation of the heart contractions [26], feeding and obesity [12], enhancement of the neuronal excitability and memory retention [16], regulation of circadian rhythms [25] and release of pituitary sex hormones [12]. According to several authors [15, 17], the above effects seem to be mediated by G protein-coupled receptors.

Originally isolated from the porcine brain [30], the presence of NPY was subsequently demonstrated in neuronal and/or endocrine elements of various organs, including those of the gastro-entero-pancreatic (GEP) system [8, 13, 28]. At gastrointestinal level, NPY and PYY are considered to mediate inhibitory actions on motility, secretion and blood flow [19].

Despite the important position of reptiles in phylogeny, there are still only two studies on the presence and distribution of NPY immunoreactivity in the

REV. ROUM. BIOL. – BIOL. ANIM., TOME 47, N^{os} 1–2, P. 79–86, BUCAREST, 2002

gastrointestinal tract and both are confined to a single chelonian species [14, 29]. Considering the above, the present comparative investigation carried out in turtles, lizards and snakes was designed to improve our knowledge about the occurrence and distribution profiles of NPY immunoreactive structures in different segments of the reptilian gut. Another aim was to provide additional evidence for the phylogenetic perenniallity of this neuropeptide.

2. MATERIALS AND METHODS

ANIMALS

Adult specimens of reptiles, captured in spring-time (April-May) from the surroundings of Bucharest, were kept unfed in fresh water aquaria (turtles) and in terraria (lizards, snakes) for 2–3 days. The species and the number of specimens (in brackets) employed are listed bellow:

Chelonia turtles: *Emys orbicularis* (4)
Squamata lizards: *Lacerta viridis* (4)
Lacerta agilis (2)
snakes: *Natrix natrix* (3)

TISSUE PREPARATION

All the animals were killed under chloroform anesthesia and the entire gastrointestinal tracts (from cardia to cloaca) were removed. In order to get comparable materials, the gastrointestinal tracts of the individuals of each species were measured with a ruler and equal segments (1–1.5 cm long fragments depending on species) of stomach (middle and lower regions) and intestine (proximal, middle and end regions) were cut from corresponding sites. The excised fragments of both organs were immersed in Bouin's fluid for 36 h, dehydrated through a graded ethanol series, cleared with toluene and embedded in paraffin. Serial sections of 6 μm-thickness, prepared on a sledge microtome, were mounted on poly-L-lysine (Sigma, USA)-coated slides.

PRIMARY ANTIBODY

The primary antiserum – polyclonal rabbit anti-synthetic porcine NPY – has been purchased from Chemicon, Temecula, Ca.,USA.

IMMUNOHISTOCHEMICAL PROTOCOL

The deparaffinized, rehydrated sections were treated according to the avidin-biotin-peroxidase (ABC-peroxidase) procedure [18]. Briefly, it includes three

consecutive incubations of the sections at room temperature with 1) the appropiately diluted (1:800) primary antiserum overnight, 2) the biotin conjugated goat anti-rabbit IgG (Sigma, USA) diluted 1:100 for 60 min, and 3) the performed complex of extravidin-peroxidase (Sigma, USA) diluted 1:100 for 6o min. 0.01 M phosphate buffer saline (PBS) (pH 7.4) containing 0.3% Triton X-100 was used as diluent for every step of the procedure and as rinsing solution between the steps.

The antigen-antibody binding sites were vizualized by a 3–4 min. immersion of the sections in a solution of 0.05% DAB (3,3'-diaminobenzidine tetrahydrochloride – Serva Feinbiochem. GmbH., FRG) prepared in 0.05M Tris-HCL buffer (pH-7.6), to which 0.05% hydrogen peroxide was added just before use. The sections were finally dehydrated in ethanol, cleared in xylene and mounted in Entellan (E. Merck, FRG).

SPECIFICITY CONTROLS

Procedure-dependent nonspecificities were excluded by running the following controls:

– use of ascending dilutions of the primary antiserum;
– omission of a single step in the immunohistochemical protocol;
–addition of poly-L-lysine (MW 388; 2 mg/ml) to the primary antiserum;
– use of high molar rinsing solution (0.5M PBS) between the steps of the immunohistochemical procedure (to exclude the binding of antibodies by no-immunological mechanisms).

The specificity of the primary antiserum was tested by its preadsorption (24h at 4°C) with the corresponding antigen (swine synthetic NPY – Biotrend Chem.GmbH., FRG) and with heterologous, but structurally related antigens (human synthetic PP – Biotrend Chem. GmbH., FRG; human synthetic PYY – Sigma-Aldrich Chem. GmbH., FRG). The antiserum could be preadsorbed only by its corresponding antigen even at concentration of 6 μg/ml (working dilution of antiserum); preadsorption of the antiserum with heterologous antigens (up to 128 μg/ml) had no effect on immunostaining.

3. RESULTS

The antiserum used revealed variable amounts of immunoreactive endocrine cells and neural structures (perikarya and/or nerve fibers) in the reptilian gastrointestinal tract. Their occurrence and density varied widely both along the gut and among the taxa under study and only slightly among the individuals of the same species (Table 1).

Turtles. As compared to the organ of the other reptiles studied, the stomach of turtles displayed by far the most numerous neural elements immunoreactive for

NPY. The labelled elements included strongly stained nerve cell bodies mainly localized in the deep myenteric plexus (Meissner) and more rarely in the superficial one (Auerbach), as well as a moderate number of nerve fibers within both plexuses and around the blood vessels penetrating the submucosa and even the villi mucosa (Figs. 1a,b). No nerve fiber or terminal was seen in the depth of longitudinal or circular muscle layers.

Table 1

Occurrence and relative density of NPY- immunoreactive structures along the gastrointestinal tracts of investgated reptiles (1:800 dilution of antiserum)

Gut segments	Immunoreactive elements	Turtles	Lizards	Snakes
Stomach				
– Middlle	Ec/P/Nf	–/+/++	–/–/+	–/–/+
– Lower	Ec/P/Nf	–/+++/+++	–/–/++	–/±/+++
Small intestine				
– Proximal	Ec/P/Nf	++++/++/++	++/±/+	+/++/++
– Distal	Ec/P/Nf	++/+/++	+++/±/+	++/++/++
Large intestine	Ec/P/Nf	+/–/–	++++/–/±	+++/++/+

Abbreviations: Ec = enterocytes; P = perikarya; Nf = nerve fibers
Symbols (amount of each category of immunoreactive structures /gut ross section : ++++, >12; +++, 8–12; ++, 4–7; +, 1–3; ±, occasional presence; –, none.

Apart from nervous structures, endocrine cells of both "open" and "closed" varieties were encountered throughout the intestinal epitelium (Fig. 1c). Their amount, however, drastically decreased from proximal to the end intestinal region, where most of them were of the "closed" variety. A similar decreasing trend along the small intestine was recorded also for NPY- containing nervous elements, which seemed to be missing in the large intestine.

Lizards. Sparse nerve fibers located in the depth of circular musculature were the sole NPY immunoreactive structures encountered in the lizard stomach (Fig. 2a). Besides them, immunostained endocrine elements showing the morphological features of the "open" and "closed" cell varieties appeared scattered along the entire intestine, the highest density being, however, recorded in the large one (Figs. 2b–d). The cells of the "open" variety prevailed in the small intestine and were mainly localized in the lower half of the villi epithelium (Fig. 2b). Contrary, the "closed" cell population exceeded the "open" one in the large intestine and both cell populations were disseminated throughout the villi epithelium (Fig. 2d).

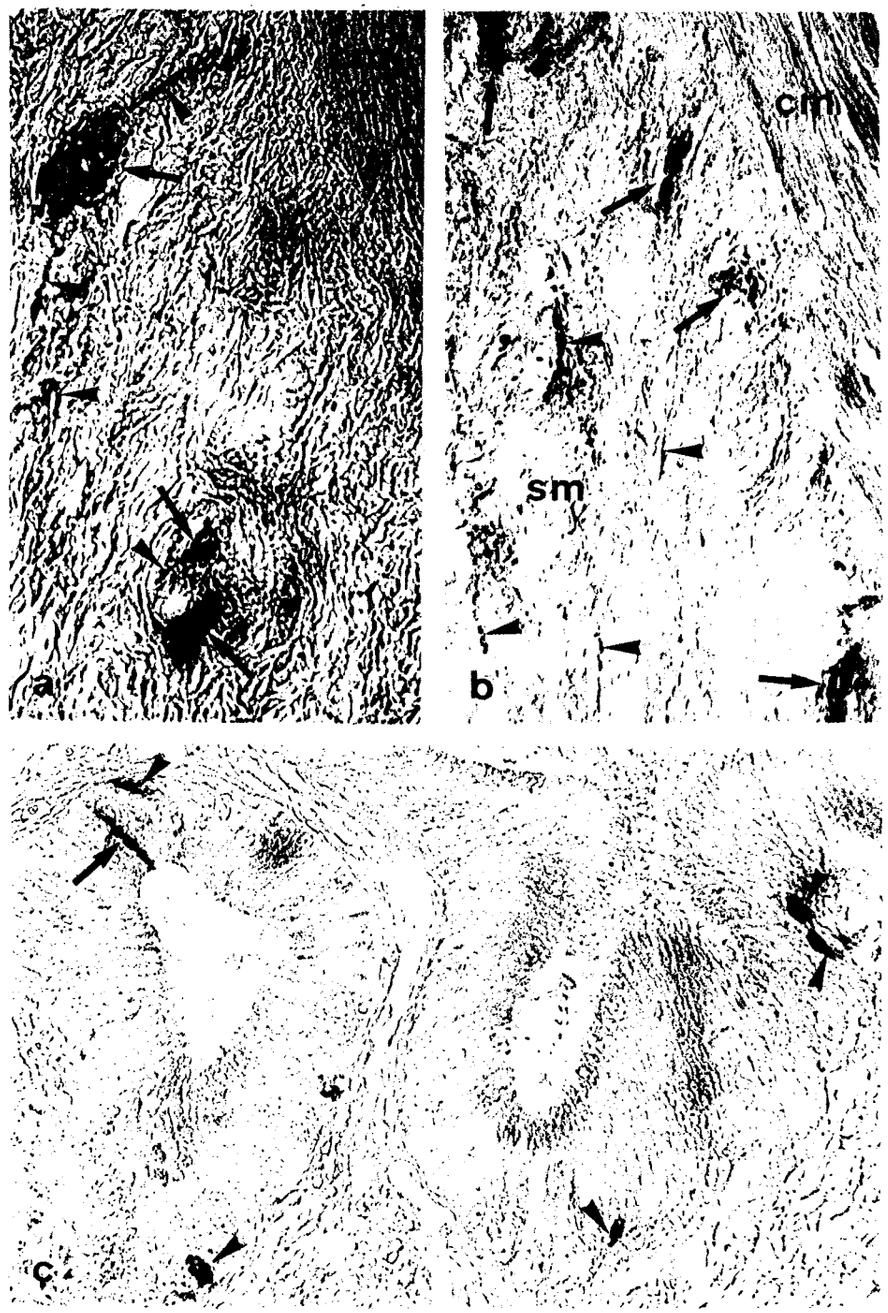

Fig. 1 – NPY immunoreactive neural (a,b) and endocrine (c) structures located in the lower stomach (a, b) and distal small intestine (c) of fresh water turtle. a.– strongly immunolabelled ganglion cell bodies (arrows) and nerve fibers (arrowheads) detected in the gastric submucous plexus; b. – bundles of densely stained nerve fibers (arrows) localized in the deep myenteric plexus and several single fibers (arrowheads) spread in the gastric submucosa (sm); c. -- several immunostained endocrine cells of the "closed" variety (arrowheads) and one of the "open" variety (arrow) scattered in the mucosa epithelium of the distal small intestine. cm = circular muscle layer; a,c. – × 690; b -- × 180.

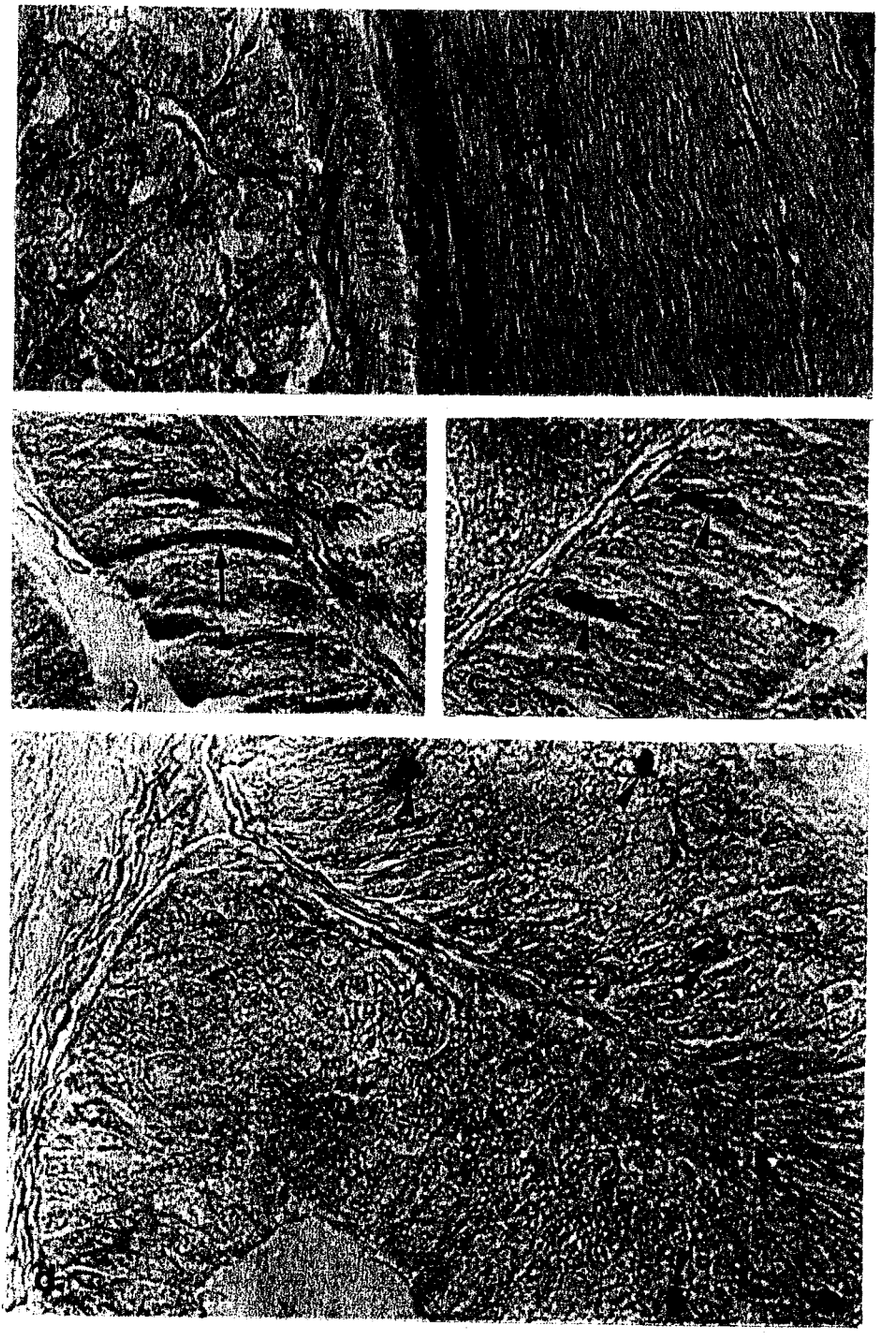

Fig. 2 – NPY immunoreactivity in neural (a) and endocrine elements (b-d) of the lizard gastrointestinal tract. a. – several immunostained nerve fibers (arrowheads) running in parallels in the gastric circular musculature. Note also the presence of few short nerve profiles (small arrowheads) in submucosa (sm) and the lack of immunoreaction in the deep myenteric plexus (dmp); b. – immunostained enteroendocrine cell (arrow) of the "open" variety located in the proximal small intestine; c,d. – neuropeptide containing cells of the "closed" variety observed in the distal small (c) and large (d) intestine. sm = submucosa; a-c. – × 690; d. – × 830.

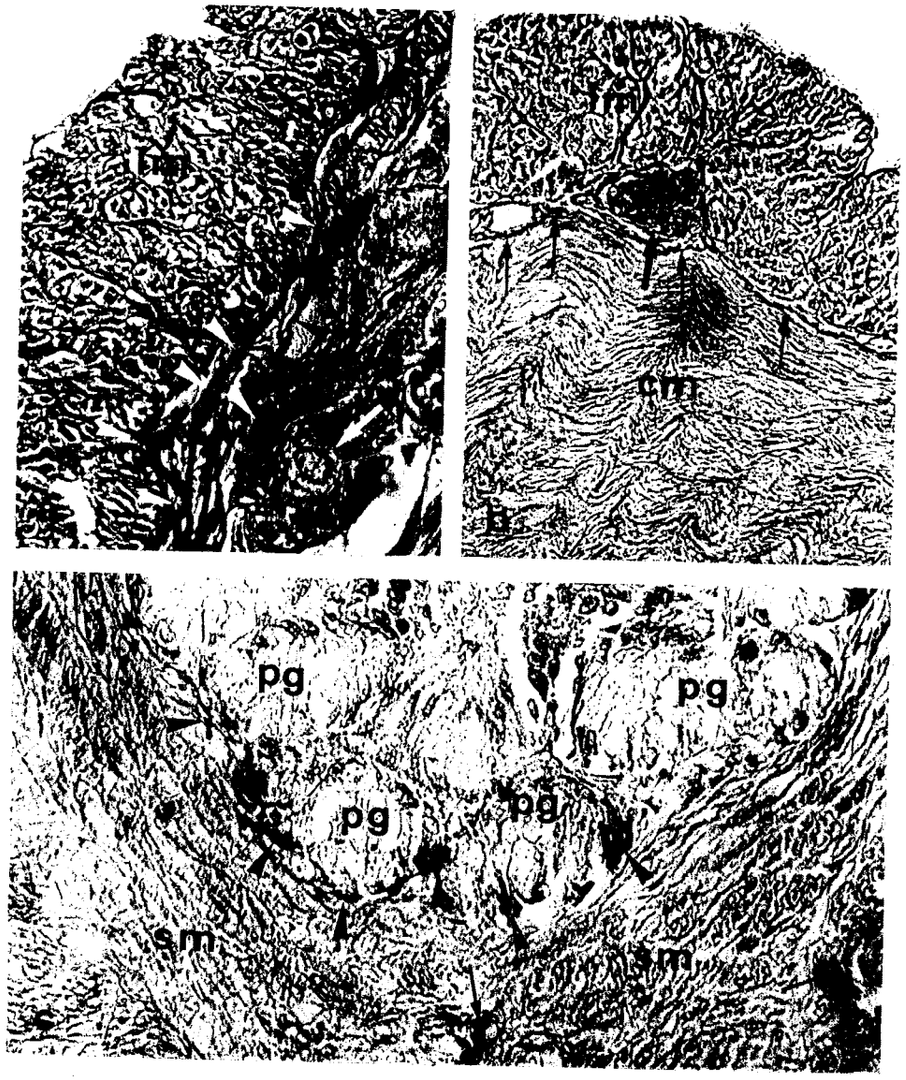

Fig. 3 – The distribution of NPY containing neural elements in the snake lower stomach. a. – detail of the superficial myenteric plexus showing dense network of strongly immunoreactive nerve fibers (arrowheads) and an unstained perikaryon (arrow); b. – immunostained ganglion cell body (arrow) and few thin nerve fibers (thin arrows) located in the superficial myenteric plexus; c. – immunolabelled short nerve profiles detected at the base of the pyloric glands (arrowheads) and in submucosa (sm) (arrows). lm = longitudinal muscle layer; cm = circular muscle layer; pg = pyloric glands; a,c. – × 690; b. – × 830.

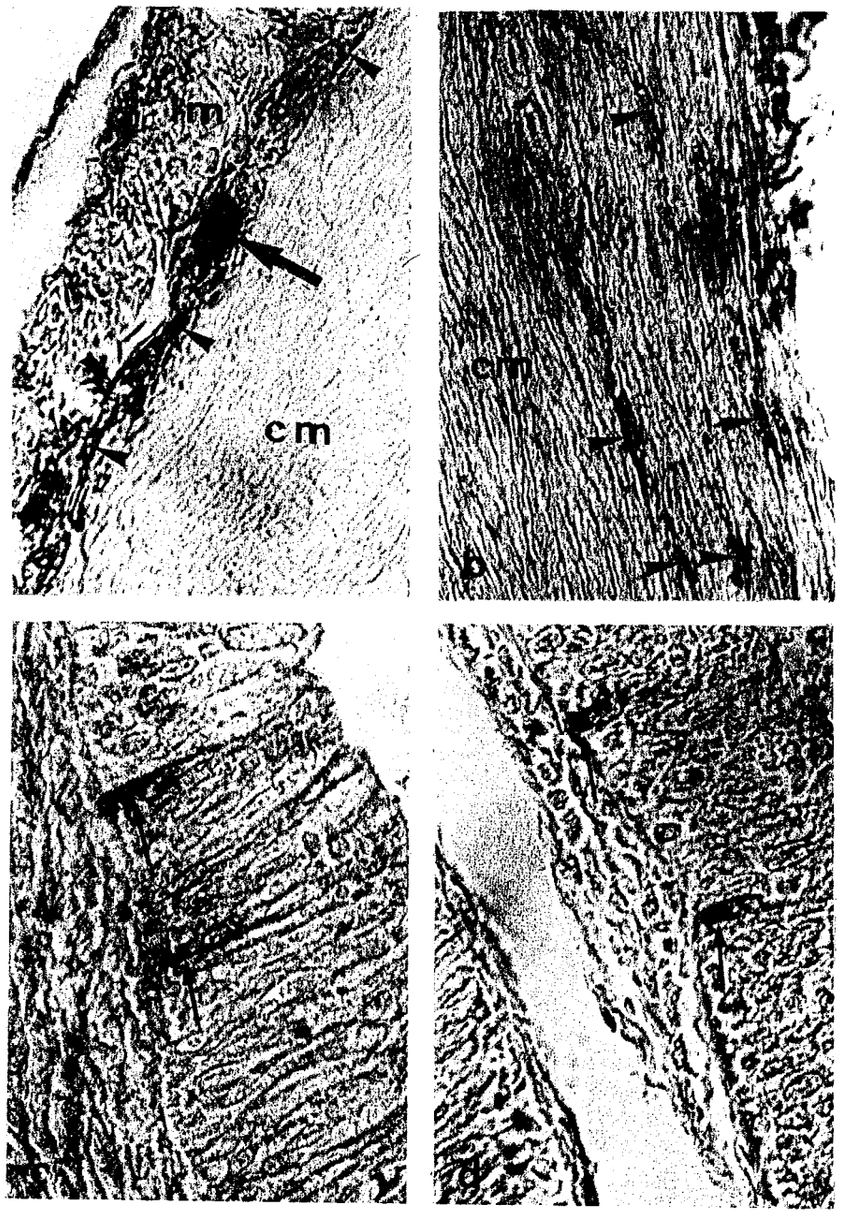

Fig. 4 – NPY immunoreactive nervous (a,b) and endocrine (c,d) structures in the snake gut. a. – a densely immunostained perikaryon (arrow) and several nerve fibers (arrowheads) found in the distal small intestine; b. – immunoreactive nerve fibers (arrowheads) located in the depth of circular musculature of the proximal small intestine; c. – two immunostained enterocytes (arrows) of the "open" variety detected in the distal small intestine; d. – two immunoreactive enterocytes (arrows) of the "closed" variety observed in the large intestine. lm = longitudinal musculature; cm = circular musculature; a. – × 880; b,c. – × 960; d. – × 990.

With the antiserum used both superficial and deep myenteric plexuses of the lizard small intestine displayed only occasionally perikarya and also a few nerve fibers immunostained for NPY. Like in turtle, the lizard large intestine appeared devoid of immunoreactive ganglion cells.

Snakes. In the lower stomach of these reptiles the immunoreaction revealed short weavy nerve fibers distributed in both myenteric plexuses, around the blood vessels of submucosa and also outlyining the base of the pyloric glands. Except for the strongly immunostained nerve fibers and several neurons detected in the superficial nervous plexus, no immunoreactive endocrine element was observed in the gastric organ (Figs. 3a–c).

NPY immunopositive ganglion cells, nerve fibers and endocrine cells were identified along the entire intestine (Figs. 4a–d). Most perikarya were seen in the superficial myenteric plexus, whereas the nerve fibers and terminals occurred scattered in both myenteric plexuses, submucosa and only seldom in the circular musculature (Figs. 4a,b). In spite of the relative uniform distribution of perikarya in the different gut segments, the amount of immunoreactive nerve fibers was obviously lower in the large intestine. NPY-containing enterocytes, most of them of the "closed" variety, appeared disseminated throughout the villi epithelium, showing the highest density in the large intestine (Figs. 4c,d).

4. DISCUSSION

So far as we know, the present study represents the first comparative approach to the occurrence and topographic distribution of NPY immunoreactivity in the reptilian stomach and intestine and one of the few carried out in the alimentary tract of lower vertebrates [1, 5, 7].

Generally, our results demonstrating the presence of both neural and enteroendocrine elements containing NPY in the reptiles studied were similar to those previously reported in cyclostomes [6,31], elasmobranchs [7, 8], teleosts [5] and even in certain mammals [19, 22]. On the other hand, they disagree with those recorded in toad [28], turtle [14, 29] and most mammalian species [2, 3, 11, 21] including the human one [33], showing the storage of this neuropeptide exclusively in the enteric nervous system (ganglion neurons and/or nerve fibers). The results appear contradictory the more so as in pancreas – organ originated from the gut wall – the NPY immunoreactive endocrine cells are a common presence in vertebrates series [8, 9, 10, 20]. An explanation could be ascribed to the different pureness degrees of antibody employed by other authors, taking into account the molecular structure of the antigen partly identical with those of the other members (PP, PYY, PY) of NPY family.

With the antiserum used the immunostained endocrine cells were absent throughout the stomach of investigated reptiles. On the hand, it revealed two opposite patterns of the distribution of such cells along the intestine from pylorus to cloaca. The first profile, showing decreasing amounts of immunoreactive enterocytes was seen in turtles, whereas the second one, characterized by their numerical increasing towards the hindgut, seemed to be valid for lizards and snakes.

The topographic distribution of NPY immunopositive neural structures in the gastrointestinal tract of the reptiles under study showed many similar features to those described in fishes [1, 5, 8] anurans [28] and mammals [2, 21, 23, 32]. With strict reference to turtles we did not, however, as Scheuermann et al. (1991) have reported in the stomach of *Pseudemys scripta elegans*, find immunostained ganglion neurons mainly localized in the superficial myenteric plexus. According to our observations in *Emys orbicularis*, the bulk of NPY immunostained perikarya was, like in the mammalian gastric organ [3, 4, 32], found in the deep myenteric plexus. In addition, unlike the findings in *Pseudemys scripta*, [14, 29] both longitudinal and circular muscle layers of the Emys midgut were obviously less innervated. Finally, the lack of immunoreactive perikarya in the large intestine of the turtle under study represented another different finding from that reported in *Pseudemys scripta* [29].

Among the reptiles studied, the snakes displayed the most abundant NPY innervation. Although like in turtles the density of nerve fibers decreased from the pyloric region of the stomach towards the cloaca, the amount of immunostained perikarya occurred uniformly distributed, at least along the intestine. As compared to snakes and even to turtles, the lizards showed only scarce nerve fibers and occasionally immunostained ganglion neurons along the entire gut.

Acknowledgements. This research was supported by a grant from the Alexander von Humboldt Foundation (2000) to T.T. It was partly carried out in the Institute of Anatomy II, University of Frankfurt am Main, FRG. The skilful technical assistance of Mr. S.Leslie is gratefully acknowledged by T.T.

REFERENCES

1. Bjenning C., Holmgren S. (1988) Neuropeptides in the fish gut. An immunohistochemical study of evolutionary patterns. Histochemistry **88**: 155–163.
2. Bottcher G., Ekblad E., Ekman R., Håkanson R., Sundler F. (1993) Peptide YY: a neuropeptide in the gut. Immunocytochemical and immunochemical evidence. Neuroscience **55**: 281–290.
3. Branchek T.A., Gershon M.D. (1989) Time-course of expression of neuropeptide Y, calcitonin gene-related peptide and NADPH diaphorase activity in neurons of the developing murine bowel and the appearance of 5-hydroxitryptamine in mucosal enterochromaffin cells. J.Comp.Neurol. **285**: 262–273.
4. Buchan A.M., Baimbridge K.G. (1988) Distribution and co-localization of calbindin D28k with VIP and neuropeptide Y but not somatostatin, galanin and substance P in the enteric nervous system of the rat. Peptides **9**: 333–338.

5. Burkhardt-Holm P., Holmgren S. (1989) A comparative study of neuropeptides in the intestine of two stomachless teleosts (*Poecilia reticulata*, *Leuciscus idus melanotus*) under conditions of feeding and starvation. Cell Tissue Res. **255**: 245–254.
6. Cheung R., Andrews P.C., Plisetskaya E.M., Youson J.H. (1991) Immunoreactivity to peptides belonging to the pancreatic polypeptide family (NPY, aPY, PP, PYY) and to glucagon-like peptide in the endocrine pancreas and anterior intestine of adult lampreys, *Petromyzon marinus*: an immunohistochemical study. Gen.Comp.Endocrinol. **81**: 51–63.
7. Chiba A., Honma Y. (1995) Occurrence of neuropeptide Y (NPY)-like-immunoreactive cells in the vitellointestinal duct and yolk sac of two species of elasmobranchs. Zool.Sci. **12**: 611–614.
8. Chiba A., Honma Y., Oka S. (1995) Ontogenetic development of neuropeptide Y-like-immunoreactive cells in the gastroenteropancreatic endocrine system of the dogfish. Cell Tissue Res. **282**: 33–40.
9. Del Rosa A., Putti R. (1985) The endocrine pancreas of lacertids: an immunocytochemical study of the genera Pediopłanis and Meroles. Eur.J.Histochem. **39**: 47–58.
10. Ding W.G., Kimura H., Fujimura M., Fujimiya M. (1997) Neuropeptide Y and peptide YY immunoreactivities in the pancreas of various vertebrates. Peptides **18**: 1523–1529.
11. Domeneghini C., Massoletti P., Arrighi S. (1997) Localization of regulatory peptides in the gastrointestinal tract of the striped dolphin, *Stenella coeruleoalba* (Mammalia: Cetacea). An immunohistochemical study. Eur. J. Histochem. **41**: 285–300.
12. Dumont Y., Martel J.-C., Fourmier A., St-Pierre S., Quirion R. (1992) Neuropeptide Y and neuropeptide Y receptor subtypes in brain and peripheral tissues. Prog. Neurobiol. **38**: 125–167.
13. Ekblad E., Alm P., Sundler F. (1994) Distribution, origin and projections of nitric oxide synthase-containing neurons in gut and pancreas. Neurosci. **63**: 233–248.
14. Gabriel R., Timmermans J.P., De Groodt-Lasseel M.H., Scheuermann D.W. (1990) High density of neuropeptide Y-innervation in the myenteric plexus of the turtle midgut. An immunocytochemical study. Acta Biol. Hung. **41**: 283–288.
15. Gehlert D.R. (1994) Subtypes of receptors for neuropeptide Y: implications for the targeting of therapeutics. Life Sci. **55**: 551–562.
16. Gehlert D.R. (1998) Multiple receptors for the pancreatic polypeptide (PP-fold) family: physiological implications. Proc.Soc.Exp. Biol. Med. **18**: 7–22.
17. Grundemar L., Håkanson R. (1994) Neuropeptide Y effector systems : perspectives for drug development. Trends Pharmacol.Sci. **15**: 153–159.
18. Hsu S.M., Raine L., Fanger H. (1981) Use of avidin-biotin-peroxidase techniques. A comparision between ABC and unlabelled antibody (PAP) procedures. J. Histochem. Cytochem. **29**: 577–580.
19. Jackerott M., Larsson L.I. (1997) Immunocytochemical localization of the NPY/PYY Y1 receptor in enteric neurons, endothelial cells and endocrine-like cells of the rat intestinal tract. J. Histochem. Cytochem. **45**: 1643–1652.
20. Kawakami T., Kusakabe T., Takenaka T. (1995) Distribution of immunoreactive neuropeptides in the pancreas of the bullfrog, *Rana catesbiana*, demonstrated by immunofluorescence. Cell Tissue Res. **280**: 307–311.
21. Keast J.R. (1993) Innervation of the monotreme gastrointestinal tract : a study of peptide and catecholamine distribution. J. Comp. Neurol. **334**: 228–240.
22. Keast J.R., Furness J.B., Costa M. (1987) Distribution of peptide-containing neurons and endocrine cells in the rabbit gastrointestinal tract, with particular reference to the mucosa. Cell Tissue Res. **248**: 565–577.

23. Koide M., Kitamura N., Yamada J., Yamashita T. (1990) Immunohistochemical study of peptide-containing nerves in the gastrointestinal tract of the Japanese field vole, *Microtus montebelli*. Anat. Histol. Embryol. **19**: 109–119.
24. Larhammar D. (1996) Evolution of neuropeptide Y, peptide YY, and pancreatic polypeptide (PP). Regul. Peptides, **62**: 1–11.
25. Larhammar D., Söderberg Ch., Lundell I. (1998) Evolution of neuropeptide Y family and its receptors. Trends in Comparative Endocrinol.Neurobiol. (Vaudry H., Tonon M.C., Roubos E.W., Loof A.eds.) Ann. NY Acad. Sci. **839**: 35–41.
26. Lundberg J.M., Hua X.Y., Franco-Cereda A. (1984) Effect of neuropeptide Y (NPY) on mechanical activity and neurotransmission in the heart, vas deference and urinary bladder of the guinea-pig. Acta Physiol. Scand. **121**: 325–333.
27. Lundberg J.M., Tatemoto K. (1982) Pancreatic polypeptide family (APP,BPP,NPY and PYY) in relation to sympathetic vasoconstriction. Acta Physiol. Scand. **116**: 393–412.
28. Murphy S., Campbell G (1993) An immunohistochemical study of the innervation of the large intestine of the toad (*Bufo marinus*). Cell Tissue Res. **274**: 115–125.
29. Scheuermann D., Gabriel R., Timmermans J.P., Adriaensen D., De Groodt-Lasseel M.H. (1991) The innervation of the gastrointestinal tract of a chelonian reptile, *Pseudemys scripta elegans*. II. Distribution of neuropeptides in the myenteric plexus. Histochemistry **95**: 403–411.
30. Tatemoto K. (1982) Neuropeptide Y: complete aminoacid sequence of the brain peptide. Proc. Natl. Acad. Sci. USA **79**: 5485–5496.
31. Van Noorden S. (1990) Gut hormones in cyclostomes. Fish Physiol. Biochem. **8**: 399–408.
32. Wang Y.N., Mc Donald J.K., Wyatt R.J. (1987) Immunocytochemical localization of neuropeptide Y-like immunoreactivity in adrenergic and non-adrenergic neurons of the rat gastrointestinal tract. Peptides **8**: 145–151.
33. Wattchow D.A., Furness J.B., Costa M. (1988) Distribution and coexistence of peptides in nerve fibers of the external muscle of the human gastrointestinal tract. Gastroenterology **95**: 32–41.

Received April, 2002.

* *Institute of Biology*
Splaiul Independentei 296
P.O. Box 56–63
060031 – Bucharest, Romania

** *Craiova University*
Faculty of Biology
Al.I. Cuza 13
1100 – Craiova, Romania

EFFECTS OF HEAVY WATER ON ULTRASTRUCTURAL AND FUNCTIONAL STATUS OF HEP 2 AND CHO CELLS LYSOSOMES

WANDA BUZGARIU*, OTILIA ZĂRNESCU**, MARIA CALOIANU*, ANIȘOARA CÎMPEAN**, GH. TIȚESCU***, I. ȘTEFĂNESCU***

The heavy water effects on the ultrastructure and function of Hep 2 and CHO lysosomal cell compartment were investigated using electron microscopy and enzymatic studies. The cell viability, measured by neutral red uptake assay and the total protein content determination, has shown a dose dependent decrease in cell growth for both studied cell types. The electron microscopy study has revealed a progressive increase in number and size of lysosomes and autophagosomes after 96 h exposure to different deuterium concentrations media in a dose dependent manner.
The enzymatic determination in the lysosomal pellet revealed an increased acid phosphatase activity in both cell types (15% and 33% for Hep 2, respectively 24% and 52% for CHO) exposed to media with high (65%, 90%) D_2O content.

1. INTRODUCTION

The D/H ratio in nature is around 1:6600 and the deuterium concentrations vary in limited range not exceeding 150 ppm. The considerable differences between H and D atomic masses have a significant impact in the chemical reactions involving molecules containing the two isotopes, which occur in biological systems. The heavy water, (water in which D replaced the H) has been widely studied upon various biological systems: microorganisms (1, 2), plants (3, 4) and animals (5, 6, 7).

Heavy water is less toxic to microorganisms, which, after a period of adaptation, succeeded in growing and developing even in 99.98% D_2O (8). Previous studies have shown a linear relationship between the toxicity induced in mammals and the administered dose: increased concentrations of D_2O (over 50%) are toxic, while a concentration up to 30% (ingested as drinking water) allowed the long term survival of laboratory animals (9). High level of deuteration has led to acute neurological symptoms, liver hyperplasia, anemia and finally even death (9, 10).

D_2O ingestion in high dose determined the impairment of hematopoiesis, and a decreased formation of platelets, neutrophils, and especially lymphocytes in the mouse (11). In this latter case a 29% concentration stimulates the animal resistance to lethal doses of gamma irradiation (12).

REV. ROUM. BIOL. – BIOL. ANIM., TOME 47, N^os 1–2, P. 87–96, BUCAREST, 2002

One of the intensely studied effects concerns the inhibition of mitosis (13, 14), which is due partly to the action of D_2O on tubulin polymerization, and on its effects on microtubule organizing centers and other structures governing the formation of the mitotic spindle (13).

Among the biochemical and biophysical effects exerted by heavy water on membrane function, the most important are the membrane depolarization and Ca^{+2} channels activation, Na^+, K^+ ATP-ase inhibition and interference with $Cl^-HCO_3^-$ exchange in hepatic cells (15, 16).

Some studies suggested the pharmacological and clinical effects of high D_2O concentrations: the experiments performed on rat aortae segments by Vasdev *et al.* (17, 18) have shown that *in vitro* heavy water normalizes the high uptake of Ca^{+2} induced by KCl through Ca^{+2} voltage–operated channels. The anti-hypertensive effect determined by D_2O lay on the basis of a patent obtained by Liepins (19) for the use of heavy water in the treatment of hypertension of human subjects.

The antitumoral effect exerted by D_2O upon the neoplasic brain cells was studied by Uemura et al. (20). They concluded that the heavy water-mediated cytotoxicity on malignant astrocytoma cells involves the induction of apoptosis and cell accumulation during the G_2/M phase. The reported results cannot be extrapolated, the D_2O induced effects being dependent on cell type (21).

The present investigation attempts to evaluate the effects of various deuterium concentrations on cell proliferation and some ultrastructural and biochemical features of lysosomes, which are important in intracellular protein degradation.

2. MATERIALS AND METHODS

Cell culture. Hep 2 cell line (derived from a human larynx carcinoma) and CHO cell line (derived from Chinese hamster ovary) was routinely cultured as a monolayer in MEM respectively RPMI 1640 supplemented with 10% fetal calf serum, 0.14% sodium bicarbonate, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere and subcultured twice a week. I.N.T.C.I. Râmnicu Vâlcea (Romania), on the basis of cooperation agreement has supplied the heavy water (99.98%). The tested culture media obtained by dissolving the powdered MEM and RPMI 1640 in water with different deuterium concentrations (150 ppm, 25%, 50%, 75%, 99.98%) and were sterilized by filtration.

Cell viability tests. Cells were seeded in 24-well plates at a density of 3×10^4 cells/well for Hep and CHO. After 24 hours, the medium was removed and fresh medium with different D_2O final content (150 ppm, 20%, 40%, 65%, 90%) was added in quadruplicate. A cell number to optical density standard curve was established for each cell type.

At different times (0h, 24h, 48h, 72h, 96h), the neutral red uptake assay was performed (22): the medium was discarded and the plates were washed and incubated with neutral red solution (50 μg /ml) at 37°C for 3 hours. After the removal of the dye, the plates were gently washed for 5 minutes (?) with 0.5% formaldehyde, 1% $CaCl_2$) and then incubated with a solvent solution (50% ethyl alcohol, 1% acetic acid) for 20 minutes at room temperature. The measurement of absorbance was performed by a UV-VIS spectrophotometer (Jasco, model V-530) at 540 nm.

The same plates used for the neutral red uptake assay were washed twice with phosphate buffered saline salt solution and the Bradford method (23) for protein content was performed, after solubilization of the cells in 0.5N NaOH for 3 hours at 60°C.

Transmission electron microscopy. Hep 2 and CHO cells were seeded in medium with 150 ppm deuterium (naturally occurring concentration). After the lag period (24 hours), the medium was changed with deuterated medium (finale concentrations after serum adding 20%, 40%, 65%, 90%) and the cells were left to grow for 4 days. At the end of experiment, the medium was removed and the monolayer was prefixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4, postfixed for 10 minutes with 1% OsO_4 in the same buffer, serial dehydrated with ethanol and propylene oxide and included in Epon 812. The probes were cut at ultramicrotome and the grids stained with lead citrate and uranyl acetate, were visualised at a Philips EM 208 S electron microscope.

Subcellular fractionation. After 4 days of culture in deuterated and normal media in 80 mm Petri dishes, the cells were washed with protease inhibitors (1 mM PMSF, 5 mM benzamidine, 1 μg/ml pepstatin A), 1 mM EDTA, 0.25 mM $MgCl_2$ in phosphate buffer saline 0.1M, *p*H 7.4, harvested in 0.25M sucrose inTris-HCl 10 mM *p*H 7.4 with the same inhibitors cocktail, EDTA and $MgCl_2$, sonicated at 50% of the power in 5 pulse of 10 seconds and centrifuged for 10 minutes at 1 000 g, 4°C, for removing the nuclei and intact cells. The supernatants were centrifuged at 12 000 g, 30 minutes, at 4°C and the deposited lysosomes and other organelles like mitochondria, peroxisomes were resuspended in 10 mM Tris-HCl with inhibitors and processed for the estimation of enzymatic activity. The protein content was estimated with the Bradford method (23).

Estimation of acid phosphatase activity. The enzymatic activity of lysosomal markers (acid phosphatase) was determined by measuring P_I (phosphate) release following the hydrolysis of β-glycerol phosphate. The final incubation medium contains 0.05 M β-glycerol phosphate, 0.01% Triton X-100 in 0.05 M acetate buffer, *p*H 5. The reaction was stopped after 30 min by adding 30% trichloracetic acid, and the released phosphate was determined by using the

reaction with ammonium molybdate in acidic medium and the reducing agent ascorbic acid. The absorbance was read at 820 nm. The specific activity was expressed as nmol of P_I released/hour/mg protein.

3. RESULTS

In the present study, the cytotoxic and cytostatic activity of heavy water was assessed using the human cancer cell line (Hep 2) and the Chinese hamster ovary cell line (CHO). The cell viability was measured using two assays: neutral red uptake and total protein content.

The increase in cell number over 96 hours of growth for both cell types showed a clear dose-dependent inhibition in the neutral red uptake assay (Fig. 1).

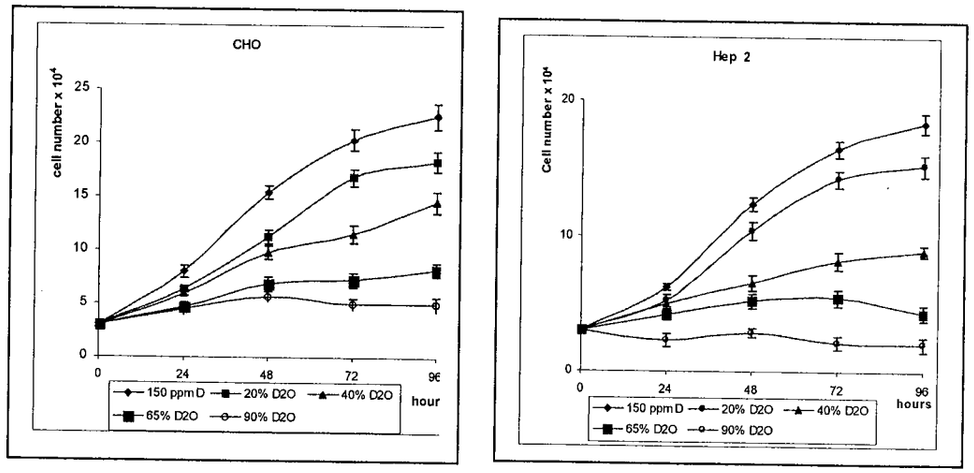

Fig. 1 – *In vitro* growth curves of Hep 2 and CHO cells in the presence of D_2O at the indicated concentrations. The cell numbers were obtained using the neutral red uptake assay (mean ± SD). The differences are significant at $p < 0.05$ (paired *t*-test).

In the case of cells exposure at medium with 20% D_2O, it was observed a proliferation decrease by 17% for Hep 2 and 18% for CHO. Growth inhibition of approximately 50% (Hep 2) and 35 % (CHO) was noted in culture with 40% deuterated medium. The inhibitory action of heavy water was more extended for the medium with 65% D_2O (77% for Hep 2 and 64% for CHO). A concentration of 90%, D_2O was cytotoxic both for the CHO and Hep 2 cells.

The total protein content determinations have shown a similar decrease in cell proliferation (Fig. 2) for both studied cell types. The cell viability was measured as total protein content and expressed as percentage of protein of the exposed cells with respect to those of the untreated ones. A slight modification of

the rate of surviving cells measured with the total protein content method in comparison with the neutral red uptake assay can be observed after the treatment with heavy water.

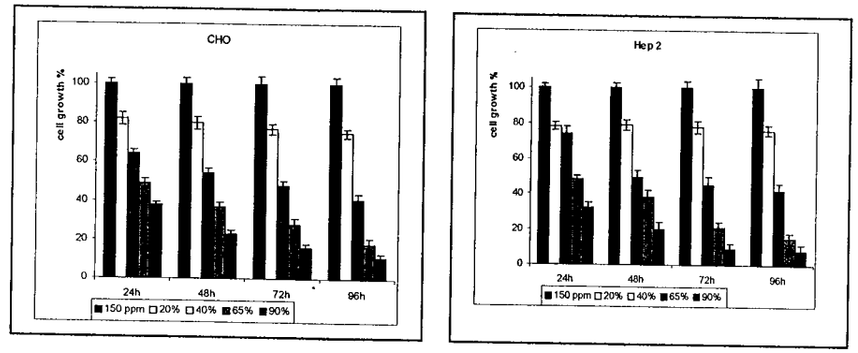

Fig. 2 – Effect of different D_2O concentrations (20%, 40%, 65%, 90%) on Hep 2 and CHO cells viability determined by total protein content assay. Data are presented as the percentage of surviving cells relative to the control (150 ppm D). The values are mean of quadruplicate determination ± SD. ($p < 0.05$ in paired *t*-test).

In the case of Hep 2 cells exposed 4 days to media with different deuterium contents, the ultrastructural studies shown a progressive increase in number and size of lysosomes and autophagosomes compared with the control (Figs. 3, 4). Ultrastructurally, many autophagic secondary lysosomes (of uneven electron density), with the remains of organelles or with myelin figures were observed (Fig. 4).

Fig. 3 – Electron microscope image of Hep 2 cells cultured in medium with naturally deuterium content (150 ppm)(TEM × 30 000).

The CHO cells exposed for 4 days to various deuterated media (as have been presented) contain also a significantly greater number of lysosomes, autophagic

vacuoles or multivesicular body than cells maintained under normal conditions for the same period of time (Figs. 5, 6).

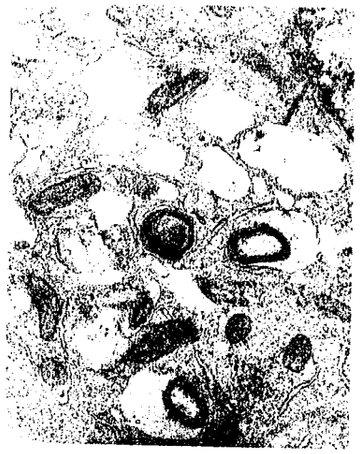

Fig. 4 – Ultrastructural image of CHO cell exposed to medium with 65% D_2O. (TEM × 26 000).

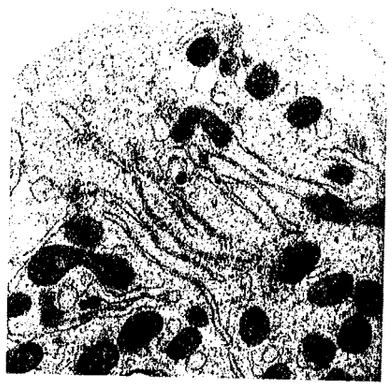

Fig. 5 – CHO cell grew in RPMI 1640 with 150 ppm deuterium. (TEM × 20 000).

The enzymatic determination in the lysosomal pellet revealed an increased acid phosphatase activity in both cell types exposed to media with high (65%, 90%) heavy water content (Fig. 7). In Hep 2 cells the activity was increased by 15% and 33% for cells treated with 65% and 90% D_2O media respectively. In the case of CHO cell line, this increase was more substantially: 24% for 65% D_2O and 52% for 90% D_2O.

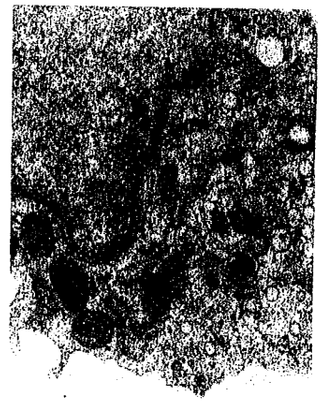

Fig. 6 – Increasing number of lysosomes, autophagic vacuoles and multivesicular body in Hep 2 cells exposed to medium with 65% D_2O. (TEM × 20 000).

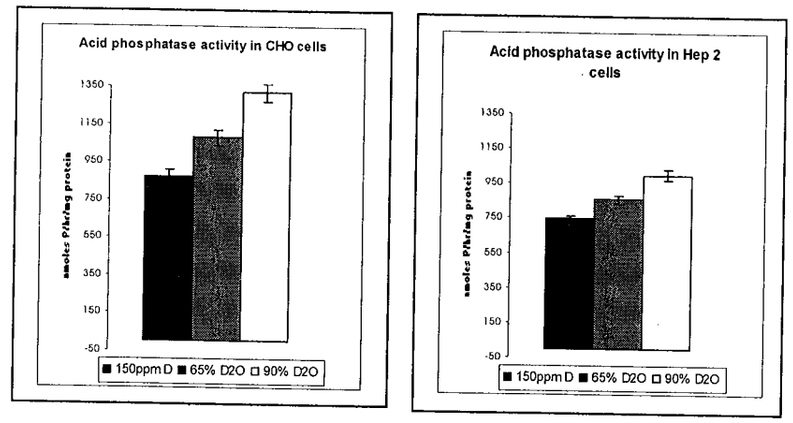

Fig. 7 – Effect of heavy water content (65%, 95%) on acid phosphatase activity in Hep 2 and CHO cells. Results are shown as the mean ± SD of 4 experiments. Statistical analysis was done by paired *t*-test. Differences were considered significant at $p < 0.05$.

4. DISCUSSION

In the present study, we investigated the heavy water effects on the ultrastructure and function of Hep 2 and CHO lysosomal cells compartment.

The viability of tested cells was measured using 2 different assays on the same cultured cells. By the method developed by Borenfreund (22), the neutral red accumulates in the viable cells lysosomes. A caveat of this assay is that agents such as polyols (24) and weak bases (25) trigger lysosomes hypertrophy and consequently an overestimation of the cell viabillity can occur. The total protein

content showed a slight difference in the percentage of cell growth inhibition due to heavy water exposure. In conclusion, the neutral red uptake assay has led to a slight underestimation of cytotoxicity.

Dose dependent damage was observed in both studied cell lines, which showed slight differing sensitivities to heavy water. A different sensitivity to D_2O was observed in other cell types such as: Hep G2 hepatic, Panc-1 pancreatic, Kato-3 gastric and Colo 205 colonic cancer cell lines (21), 3T3 fibroblast and RSVM (murine malignant astrocytoma) cell line, which could also be interpreted that D_2O is more toxic to malignant than normal animal cells (10).

The lysososmal compartment is critical for the intracellular protein degradation. In the case of cell exposure to chemicals or other stress factors (26), the affected or modified organelles such as mitochondria are removed by autophagocytosis. The electron microscopy study has revealed an increase in the volume fraction of lysosomal/autophagic vacuole compartment of Hep 2 and CHO cells, determined by the accumulation of degraded material in cells.

The presence of multivesicular bodies is a consequence of the degrading function of lysosomal compartment, due to the accumulation of undegraded material resulting from the decreased rate of lysosomal protein degradation in the cells exposed to heavy water, which may cause the overload and expansion of the lysosomal compartment. Another aspect that may be involved here is an imbalance between the degrading lysosomal processes and the massive and fast degradation of subcellular structure upon the heavy water action with the whole system destabilization.

The detection of an increased acid phosphatase activity, an enzymatic marker for the lysosomes, may be due to a lysosomal compartment extension in these cells, as has been suggested in the case of increased lysosomal enzymes activity of irradiated cells (27). On the other hand, the "solvent isotope effect" determined by the incorporated D_2O in cells could contribute to the enhancement or the decrease of enzyme activities. This is in agreement with the results obtained in the studies of acid phophatase from deuterated algae (8), when the activity of this enzyme increased by 30% in comparison with the algae growing in normal deuterated medium. However, in the case of acid phosphatase from rat testis, after 6 weeks of D_2O (30%) ingestion, the activity has decreased by 50%, but these changes have been determined by the destruction of germinal testis cells (28). Other studies upon the deuterium effects on metabolic pathways concern the photosynthetic metabolism (29) of carbon in fully deuterated algae and the glycolysis in rat hepatocytes (30). The results have shown a significant decrease in the enzymatic activity of the enzymes involved in the photosynthetic pathway (29) and an increase in lactate production by 350% (30).

The heavy water action on living systems can be discussed from two points of view (31):

– primary and secondary isotopic substitution (the substitution of H with D in biological molecules that contributes to the increasing of C-D bonds strength (32) and to changes in the conformational stability of proteins);

– solvent effect (D_2O acts as a general solvent instead of H_2O in which cellular processes take place).

It is difficult to distinguish between solvent and deuterium isotope effects. Both of them contributes to the chemical reaction rate changes and consequently to alteration of normal status function of cellular mechanisms.

Acknowledgements. The authors would like to thank Luciana Ciotec for her excellent technical assistance.

REFERENCES

1. De Giovanni R., *The effects of deuterium oxide on certain microorganisms,* Ann.N.Y.Acad.Sci. **84**, 644–647 (1960).
2. Crespi H.L., Conrad S.M., Uphaus R.A., Katz J.J., *Cultivation of microorganisms in heavy water,* Ann.N.Y.Acad.Sci. **84**, 648–666 (1960).
3. Blake M.I., Crane F.A., Uphaus R.A., Katz J.J., *The effect of deuterium oxide on the growth of peppermint (Mentha piperita),* J. Pharm. Sci. **53**, 79–86 (1964).
4. Sacchi G.A., Cocucci M., *Effects of deuterium oxide on growth, proton extrusion, potassium influx and in vitro plasma membrane activities in maize root segments*, Plant Physiol., **100**, 1962–1971 (1992).
5. Hughes A.M., Benett E.L., Calvin M., *Further studies on sterility produced in male mice by deuterium oxide*, Ann.N.Y.Acad.Sci. **84**, 763–769 (1960).
6. Thompson T.J., *Physiological effecst of D_2O mammals*, Ann.N.Y.Acad.Sci. **84**, 736–745 (1960).
7. Vasdev S., Prabkaran V., Sampson C.A., *Deuterium oxide normalizes blood pressure and vascular calcium uptake in Dahl salt-sensitive hypertensive rats,* Hypertension **15**, 183–191 (1990).
8. Unno K., Ando I., Hagima N., Yokogaki S., Koike C., Okada S., *Growth delay and intracellular changes in Chlorella ellipsoidea C-27 as a result of deuteration,* Plant Cell Physiol. **33**, 963–969 (1992).
9. Thomson J.F., *Biological effects of deuterium*. Pergamon Press, Macmillan, New York (1963).
10. Kushner D.J., Baker A., Dunstall T.G., *Pharmacological uses and perspectives of heavy water and deuterated compounds*, Can. J. Physiol. Pharmacol. **77**, 79–88 (1999).
11. Adams W.H., Adams D.G., *Effects of deuteration on hematopoiesis in the mouse*, J. Pharmacol. Exp. Ther. **244**, 633–639, (1988).
12. Laissue J.A., Altermatt H.J., Bally E., Gebbers J.O., *Protection of mice from whole body gamma irradiation by deuteration of drinking water: hematologic findings*, Exp. Hematol. **2**, 177–189 (1987).
13. Lamprecht J., Schroeter D., Paweletz N., *Mitosis arrested by deuterium oxide. Light microscopic, immunofluorescence and ultrastructural characterization*, Eur. J. Cell Biol. **51**, 303–312 (1990).
14. Schroeter D., Lamprecht J., Eckardt R., Futterman G., Paweletz N., *Deuterium oxide (heavy water) arrests cell cycle of PtK_2 cells during interphase*, European J. of Cell Biology **58**, 365–370 (1992).

15. Andjus P.R., Kataev A.A., Alexandrov A.A., Vucelic D., Berestovsky G.N., *D_2O-induced ion channel activation in Characeae at low ionic strength*, J. Membr. Biol. **142**, 43–53 (1994).
16. Andjus P.R., Vucelic D., *D_2O-induced cell excitation*, J. Membr. Biol. **115**, 123–127 (1990).
17. Vasdev S., Gupta I.P., Sampson C.A., Longerich L., Parai S., *Deuterium oxide normalizes blood pressure and elevated cytosolic calcium in rats with ethanol-induced hypertension*, Can J. Cardiol., **9**, 802–808 (1993).
18. Vasdev S., Prabhakaran V.M., Whelan M., Ford C.A., Longerich L., Parai S., *Fructose-induced hypertension, hyperglyceridemia and eleveted cytosolic calcium in rats: prevention by deuterium oxide*, Artery **21**, 124–132 (1994).
19. Liepins A., PATENT US 5223269, (1993).
20. Uemura T., Moritake K., Akiyama Y., Kimura Y., Shingu T., Yamasaki T., *Experimental validation of deuterium oxide-mediated antitumoral activity as it relates to apoptosis in murine malignant astrocytoma*, J. of Neurosurgery **96**, 900–908 (2002).
21. Takeda H., Nio Y., Omori H., Uegaki K., Hirahara N., Sasaki S., Tamura K., Ohtani H., *Mechanisms of cytotoxic effects of heavy water (deuterium oxide: D_2O) on cancer cells*, Anticancer Drugs **9**, 715–25 (1998).
22. Borenfreund E., Puerner J.A., *Toxicity determined in vitro by morphological alterations and neutral red absorption*, Toxicology Letters, **24** 119–124 (1985).
23. Bradford M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*, Anal.Biochem. **72**, 248–254 (1976).
24. Olivier P., Testard P., Marzin D., Abott D., *Effect of high polyol concentrations on the neutral red absoption assay and tetrazolium-MTT test of rat hepatocytes in primary culture*, Toxicology in Vitro **9**, 133–138, (1995).
25. Ohkuma S., Poole B., *Cytoplasmic vacuolation of mouse peritoneal macrophages and the uptake into lysosomes of weakly basic substances*, J. of Cell Biol **90**, 656–664 (1995).
26. Ohkuma S., Poole B., *Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents*, Proc. Natl. Acad.Sci. USA **75**, 3327–3331, (1978).
27. Somosy Z., Takacs A., Bognar G., Kovacs J., Koteles G.J., *X-irradiation induced disorganization of cytoskeletal filaments and cell contacts in HT29 cells*, Scanning Microscopy **9**, 763–772, (1996).
28. Kanwar K.C., Verma R., *Oral D_2O administration and enzymatic changes in rat testis*, Acta biol.med.germ. **35**, 577–581, (1976).
29. Shimba S., Unno K., Okada S., *Characteristics of the photosynthetic metabolism of carbon in deuterated Chlorella ellipsoidea C-27*, Plant cell Physiol. **34**, 255–262, (1993).
30. Wals P.A., Katz J., *The effect of D_2O on glycolysis by rat hepatocytes*, Int. J. Biochem. **25**, 1561–1564, (1993).
31. Katz J.J., *Chemical and Biological studies with deuterium*, 39th Annual Priestly Lecture, Pennsylvania State University, University Park, Pa. 1–110, (1965).
32. Thomas A.F., *Deuterium labeling in organic chemistry*, Appleton-Century Crofts, New York, (1971).

Received August 15, 2002.

*National Institute of Biological Sciences Research
Bucharest, Spl. Independentei 296
E-mail: wanda@dbio.ro
**Bucharest University, Faculty of Biology,
Spl. Independentei 91–95
***I.N.T.C.I. Ramnicu Valcea

TURBELLARIA SPREADING WITHIN IRON GATES AREA EXISTING IN BENTHIC AND PHYTOPHILE FAUNA

*MARIA NĂSTĂSESCU, ** VIRGINIA POPESCU-MARINESCU

The work presents a review on turbellaria in the Iron Gates area, based both on data of the literature and on our own, unpublished data. The analyzed material enabled us to state that most of benthic species are concentrated within the higher area of the Iron Gates (i.e. Iron Gates I damlake), where they also reach the highest densities. Among them, the main taxa are *Palaeodendrocoelum romanodanubialis, Otoplana antipai, Oligochaerus limnophyllus,* all of them being Ponto-Caspian elements. The favourite substrata for the respective species are the stony, muddy and stony-muddy ones. *Dugesia tigrina,* usually a phytophilic one, was found both among the zoobenthic components and those on the vegetation. *D. tigrina* as well as *D. gonocephala* habitate, the former, usually and the latter exclusively on the macrophyte vegetation in the Iron Gates II damlake.

1. INTRODUCTION

The work represents a synthesis of data regarding turbellaria occurring in the Danube within the Iron Gates area both prior and after the creation of the two damlakes in the area until 2002.

We have used data from the specialty literature, as well as personal, unpublished materials.

In carrying out this work, the main difficulty consisted in the lack of some uniform methods in the researches performed during the period of time analyzed by us. In this respect, the quantitative methods were not always used for sample collection, only certain sections belonging to the perimeter of the existing Iron Gates damlakes were included, or in certain years of study, only the river riparian, the shallow section, was considered. All these inconsistencies concerning the investigations made difficult for us the interpretation of results and their integration in the present study.

Nevertheless, making use of a rich bibliographic material and a number of personal observations, most of them in the field, but in the laboratory as well, we present this work as a synthesis of the researches carried out during a long period of the time which enable us to point out some important conclusions on systematicity, ecology and geographic spreading of the representative species.

REV. ROUM. BIOL. – BIOL. ANIM., TOME 47, N^{os} 1–2, P. 97–105, BUCAREST, 2002

2. RESULTS AND DISCUSSIONS

Turbellaria material presented in the work includes: A. benthic fauna components and B. components of the fauna on the submersed macrophyte vegetation.

On the other hand, during the Iron Gates area evolution there are two periods of time and namely, before the formation of Iron Gates I damlake and after the creation of the two accumulations in this area.

A. BENTHIC FAUNA TURBELLARIA

Among the early data on the studies begun by M. Bacescu during 1942 – 1943 regarding the upstream stretch of the Romanian Danube, some of them were published in 1944 under the suggestive title "Traces of marine fauna in the waters of the Danube at Cazane and Iron Gates", the other, such as "Remarks on the benthic fauna in the Danube gorge", in 1948 (2). It is worth to be mentioned the fact that in both works the author outlines the occurrence of Ponto – Caspian elements at Iron Gates and at Cazane gorge. Among them, he enumerates the *Microstomum* genus, present near Vârciorova (Kralina Stena and downstream from Prigrada) at 50 m depth and at 5 m/s flow velocity. Also, at Cazane (Svinița and Greben) he found a species of a genus, probably similar to *Paradendrocoelum*. On the respective turbellaria material, R.Codreanu describes *Palaaedendrocoelum romanodanubialis*, a new genus and a new species, triclada set up on a stony and rocky substratum (4).

Later, in 1958 in the lithorheophilic assemblage, between rkm 1042 –943, both on river medial, at the 70 m depth, and on river riparian at 3–4 m depths *P. romanodanubialis* was found, sometimes in a great number, in this area, by Ec. Popescu and El. Prunescu – Arion and from their data we show Table 1 (10).

Table 1

Numerical density of triclada present in benthic fauna in the Danube, within the Iron Gates area in 1958

Station	Date	Numerical density		Water depth (m)	Substratum nature
		Ind./ sample	%		
1	2	3	4	5	6
Danube rkm 1005	28. VIII	299	0.91	3.80	Stony
	28. VIII	58	0.07	8.00	Stony
Danube rkm 969	28. VIII	39	1.06	15–20	Stony
	28. VIII	548	1.23	70	Stony
Danube rkm 966	28. VIII	4	0.16	45	Stony
Danube rkm 957	20. VI	3	0.55	8–10	Stony
Danube rkm 957	17. VII	4	0.47	9	Stony
Danube rkm 957	26. IX	3	0.38	8–10	Stony

Table 1 (continued)

1	2	3	4	5	6
Danube rkm 957	21. X	3	0.59	7–10	Stony
Danube rkm 957	19. XI	7	0.64	4–8	Stony
Danube rkm 957	19. XI	4	2.00	8–9	Stony
Danube rkm 957	15. XII	435	2.16	4–8	Stony
Danube rkm 957	15. XII	21	3.37	7	Sandy

Following R. Codreanu's determinations, in the material of 1958 at rkm 957 (Orsova, where the two above mentioned authors carried out monthly investigations), *P. romanodanubialis* in most samples recorded 2–18 individuals. But, in December the species reached 76 individuals on river medial and 69 on the intermediate left riparian one. Usually the higher frequency of this species was in the intermediate area between the Danube left bank and the river medial.

Also, prior to the creation of the first damlake on the Romanian stretch of the Danube, V. Popescu-Marinescu in the Monograph of the Iron Gates area (3) in 1968, between rkm 1 007 (in the Danube, at the Eliseva river mouth) and at rkm 960 (in the Danube, at the Ieşelnița river mouth) mentions the same species, in shallow water areas (1–2 m), on a stony-sandy substratum, in a smaller number of individuals (Table 2).

Table 2

Numerical density of turbellaria present in benthic fauna in the Danube, within the Iron Gates area in 1968

Station	Date	Numerical density		Water depth (m)	Substratum nature
		Ind./m^2	%		
Danube Elişeva rkm.1007	4. VII.1968	17	0.25	1–2	Stony, sandy
Danube Mraconia rkm. 967	3.VII.1968	6	0.21	1–2	Stony, sandy
Danube Ieşelnita rkm. 960	2.VII.1968	8	0.44	1–2	Stony, sandy
	25. X. 1968	9	0.85	1–2	Stony, sandy

After the Danube was bloked at the Iron Gates, in the second year of the damlake existence (1972), Cure et al. mention at rkm 967 (in the Danube at the Mraconia river mouth), on the rocky riparian, on a mosaicked substratum (stony-sandy-muddy) *P. romanodanubialis,* as well as a new species belonging to *Macrostomum* genus (5).

During 1972–1986, V. Popescu-Marinescu studies (11–15) refer to the presence of *P. romanodanubialis* both in the Iron Gates I damlake (rkms 967, 954) and in the Iron Gates II damlake (rkm 931) (Table 3).

The maximal density of the respective triclada during this period of time (1972–1986) was of 1 122 ind/m^2 at rkm 967 on the accumulation medial. The

same author found the most abundant species among turbellaria in the two damlakes (rkm 1072.4-866; Baziaş-Ostrovul Mare), *P. romanodanubialis* during the years 1995–1996 (16), (Table 4).

Recent investigations we carried out in 2002 reveals that from the total amount of turbellariates (Table 5) *P. romanodanubialis* is also dominant (Table 6).

Table 3

Numerical density of turbellaria present in benthic fauna in Danube, within the Iron Gates area (annual mean values of transverse profile) during 1972–1986

Profile	ind./m²					ind %				
	Years:					Years:				
	1972,	1973,	1981,	1982,	1986	1972,	1973,	1981,	1982,	1986
Iron Gates I damlake Mraconia rkm 967	33	41	144	153	612	0.61	0.16	0.09	0.15	0.75
Cerna rkm 954	During 1972–1982 no samples were collected 85					During 1972–1982 no samples were collected 0.14				
Iron Gates II damlake Downstream Turnu Severin rkm 931	During 1972–1982 no samples were collected 102					During 1972–1982 no samples were collected 0.40				

Table 4

Numerical density of turbellaria present in benthic fauna from Iron Gates I and II damlake during 1995–1996

Station	Date (month, year)	Numerical density		Water depth (m)	Substratum nature
		Ind./m²	%		
Iron Gates I damlake					
Moldova Veche rkm 1044,5					
• Left riparian	VI 1995	50	0.15	0.90	Muddy
Baziaş rkm 1072,4					
• Left riparian	V 1996	32200	7.38	10.20	Muddy
• Intermediate	V 1996	2300	0.33	15.50	Muddy,sandy, stony
Iron Gates II damlake					
Ostrovul Corbului rkm 911					
• Intermediate	VI 1995	50	0.03	14.80	Sandy
• Intermediate	VI 1996	200	0.03	9.65	Stony
Tigănaş rkm 879					
• Medial	VI 1995	500	1.08	11.80	Stony
• Medial	VI 1996	26	0.12	10.90	Stony
Ostrovul Mare rkm 866					
• Medial	VI 1995	300	1.29	20.00	Stony, sandy, muddy
• Intermediate	VI 1996	100	1.37	14.10	Stony

Intermediate = between left riparian and medial lake

The studied section extends throughout the length of Iron Gates I damlake I between rkm 1072.4–942, the investigations being carried out both at the lake left riparian and at the middle, as well as in the intermediate area between them. The substratum on which the triclada were set was a stony, muddy or a mosaicked one. We outline the fact that as the Table 6 shows, *P. romanodanubialis* was not found below rkm 967 in the Iron Gates I damlake, but it reappeared in the samples from rkm 911 in the Iron Gates II damlake (Ostrovul Corbului) on a stony substratum. The depths of the two lakes where the triclada were found ranged between 8.4 and 20 m.

Regarding the numerical density of *P. romanodanubialis* in 2002, this ranges from 8 ind./m² to 14 318 ind/m² in Iron Gates I damlake.

After a period of time of almost 20 years since *P. romanodanubialis* has been found in the Danube, at the Iron Gates, other two turbellaria, *Oligochaerus limnophyllus*, were pointed out by Ax and Dorges and *Otoplana antipai* by H. An der Lan, in the Danube waters, in various areas.

In the Romanian stretch of the river, *O.antipai* was firstly mentioned also by H. An der Lan at Iron Gates in large amounts, at depths ranging between 1 and 70 m (1).

Table 5

Numerical density of turbellaria present in benthic fauna from Iron Gates I and II damlake in 2002

Station	Date (month)	Numerical density		Water depth (m)	Substratum nature
		Ind./m²	%		
Iron Gates I damlake					
Baziaş rkm 1072,4					
• Intermediate	VI	22500	1.65	9.50	Muddy
• Intermediate	IX	25	0.19	8.90	Muddy
Ostrov rkm 1062					
• Mediale	VI	900	1.59	8.40	Stony, muddy
• Mediale	IX	125	0.73	9.06	Stony, little muddy
Moldova Veche rkm 1048,7					
• Mediale	VI	125	0.75	17.40	Stony, little muddy
• Mediale	IX	125	1.02	19.50	Stony
Cozla rkm 1012,3					
• Intermediate	VI	8	1.78	11.30	Stony
• Left riparian	IX	50	2.17	9,00	Stony
Dubova bay rkm 970					
• Middle	IX	25	0.21	15.00	Muddy
Mraconia rkm 967					
• Mediale	IX	25	0.14	20.00	Stony, sandy
Iron Gates II damlake					
Ostrovul Corbului rkm 911					
• Mediale	IX	25	0.23	15.46	Stony

Intermediate = between left riparian and medial lake

In "Fauna. Monograph study"(17) *Oligochaerus limnophyllus* is mentioned as being collected in small number in the Danube, in April 1958, October 1969 and July 1972, co-existing with *O. antipai* in the Iron Gates area. The same *O. limnophyllus* is also mentioned by V. Mack-Firă in the Danube gorges (6).

In our studies carried out in 2002 we found *O. limnophyllus* with a density of 2 046 ind/m^2 in the Iron Gates damlake at rkm 1 072.4 in the intermediate area, at a depth of 9–5 m on a muddy substratum (Table 6).

O. antipai was also mentioned in the Iron Gates area, between rkms 1007 and 960 in the unblocked Danube, in shallow water (2–3 m) on the riparian stones (V. Popescu-Marinescu, the Monograph of the Iron Gates) on a material from 1968 summer (3). On the same species, V. Mack-Firă (6) says that it is massively represented in the Cataracts' area of the Iron Gates, co-existing with *P. romanodanubialis* and *O. limnophyllus*. But in "Fauna Monograph Study" *O. antipai* is mentioned in the Iron Gates area at 1–70 m depths, this Danubian endemicity being collected in April 1958, October 1968 and July 1972 (17).

Table 6

Spreading of turbellaria from benthic and phytophilic fauna from Iron Gates I and II in 2002

Taxonomic group	Numerical density Ind./m^2	Station	Date	Water depth (m)	Substratum nature
Order Acoela		**Iron Gates I damlake**			
Oligochaerus limnophyllus	2046/m^2	rkm 1072.4-Intermediate	6.VI	9.50	Muddy
Order Proseriata	6136/m^2	**Iron Gates I damlake**			
Otoplana antipai	12/m^2	rkm 1072.4-Intermediate	6.VI	9.50	Muddy
Otoplana antipai	450/m^2	rkm 1072.4-Intermediate	10.IX	8.90	Sandy, stony
Otoplana antipai	62/m^2	rkm 1062-Mediale	6.VI	8.40	Stony, muddy
Otoplana antipai	50/m^2	rkm 1048.7-Mediale	11.IX	19.50	Stony
Otoplana antipai		rkm 1012.3-Intermediate	11.IX	9.00	Stony
Order Tricladida		**Iron Gates I damlake**			
Palaeodendrocoelum romanodanubialis	14318/m^2	rkm 1072.4-Intermediate	6.VI	9.50	Muddy
Palaeodendrocoelum romanodanubialis	13/m^2	rkm 1072.4-Intermediate	10.IX	8.90	Sandy,stony
Palaeodendrocoelum romanodanubialis	450/m^2	rkm 1062-Mediale	6.VI	8.40	Stony, muddy
Palaeodendrocoelum romanodanubialis	125/m^2	rkm 1048.7-Mediale	6.VI	17.40	Stony, muddy
Palaeodendrocoelum romanodanubialis	63/m^2	rkm 1048.7-Mediale	11.IX	19.50	Stony
Palaeodendrocoelum romanodanubialis	8/m^2	rkm 1012.5-Riparian	6.VI	11.30	Stony
Palaeodendrocoelum romanodanubialis	25/m^2	rkm 970-Middle bay Dubova	12.IX	15.00	Muddy
Palaeodendrocoelum romanodanubialis	25/m^2	rkm 967-Mediale	13.IX	20.00	Stony, sandy
Palaeodendrocoelum romanodanubialis	25/m^2	rkm 911-Mediale	15.IX	15.46	Stony

Table 6 (continued)

Taxonomic group	Numerical density Ind./m^2	Station	Date	Water depth (m)	Substratum nature
		Iron Gates I damlake			
Dugesia lugubris	63/m^2	rkm 1062-Mediale	11.IX	19.50	Muddy
Dugesia gonocephala	1/sample	rkm 970-Riparian	12.IX	3-4	Macrophytic
		Iron Gates II damlake			
Dugesia gonocephala	1/sample	rkm 879-Riparian	16.IX	6	Macrophytic
Dugesia gonocephala	4/sample	rkm 875-Riparian	17.IX	6	Macrophytic
Dugesia gonocephala	1/sample	rkm 866-Riparian	17.IX	6	Macrophytic
		Iron Gates II damlake			
Dugesia tigrina	10/sample	rkm 920-Riparian	15.IX	4	Macrophytic
Dugesia tigrina	1/sample	rkm 911-Riparian	15.IX	4	Macrophytic
Dugesia tigrina	6/sample	rkm 875-Riparian	17.IX	6	Macrophytic

During 1972–1973 and 1981–1982 *O. antipai* was found at rkm 967 on the accumulation medial. In 1981–1982 it recorded a maximal density of 10 ind./m^2 and 10% frequency (11; 12).

In 2002, we determined *O. antipai* only in the higher area of the Iron Gates I damlake between rkms 1072.4–1012.3 from the stony or muddy substratum, at depths ranging between 8.40 and 19.50 m on the lake medial and intermediate. Numerical density varies between 50 ind/m^2 and 6 136 ind/m^2. The highest values were recorded between rkms 1 072.4 and 1 062 (the lake tail) (Table 6).

Besides the mentioned turbellaria, other species determined by us in the accumulation benthos in the lower Danube are *Planaria torva* and *Dugesia tigrina*. *Planaria torva* had a small number of specimens, being spread in the both damlakes, on medial, on a mosaicked substratum, at rkm 967 in 1981, and at rkms 931 and 878 in 1985–1986. We found *Dugesia tigrina* more numerous, but with a lower frequency, on the medial of the Iron Gates I damlake, only at rkm 967, on a substratum rather stony, in 1982.

Also, in the benthic fauna, in 2002, in the higher area of the Iron Gates I accumulation (rkm 1062) we found *Dugesia lugubris* triclada. The number of individuals reached 62/m^2 on the lake medial at 19 m depth, on a muddy substratum.

Prior to the Danube blockage in the Romanian stretch, between rkm 1 042 and 1 043, in the stony substratum assemblage, *Polycelis nigra* and *P. felina* were also mentioned (in synonimy with *P. cornuta*). Other references in bibliography are from various sources (7; 9). The first of the mentioned species was reported also in 1966–1967 in the marshes at rkms 961 (Ieşelnita), 1 015 (Cozla) and 1 034 (in Alibeg river). After the creation of the two accumulations in the Romanian stretch of the Danube, even in 2002, these turbellaria have been no more found in the area.

B. PHYTOPHILIC FAUNA TURBELLARIA

Phytophilic fauna in the Iron Gates area was very little studied, the main cause being the lack of the macrophyte vegetation, particularly prior the creation of the accumulations. The only investigations concerning the turbellaria on the submersed plants in the two damlakes are those we carried out in 2002.

The determined species are *Dugesia gonocephala* (juvenils) and *D. tigrina*. Most identification (Table 6) were in the Iron Gates II damlake, *i.e.* where in the riparian area (left side bank), between rkms 920 and 866, the depths were of 3–6 m. Fauna sampling from the macrophyte vegetation by quantitative methods being more unwieldy, our samples are qualitative ones.

D. gonocephala was also reported in the benthos of some inner rivers such as Dubova and Alibeg at confluence with the Danube (3; 8).

In other sections of the Danube, particularly at the border of channels, brooks and lower Danube branches, where rich vegetation develops, *D. tigrina* was found by M. Năstasescu (9).

3. CONCLUSIONS

1. Most benthic turbellaria are concentrated in the higher area of the Iron Gates I damlake, where they reach the highest densities.

2. The favourite substratum is the stony or the muddy one for *Oligochaerus limnophyllus, Otoplana antipai* and *Palaeodendrocoelum romanodanubialis*.

3. Regarding *Dugesia gonocephala* and *D. tigrina* we mention: the former was found only on the macrophyte vegetation, preponderantly in the Iron Gates II damlake, and the latter, both on the macrophyte vegetation in the same aquatic basin and in benthic fauna in the Iron Gates I damlake.

4. We outline the fact that *Oligochaerus limnopkyllus*, *Otoplana antipai* and *Palaeodendrocoelum romanodanubialis* are Ponto-Caspian, rheophilic elements.

REFERENCES

1. An der Lau H., *Zwei neue tiergeographisch bedeutsame Turbellarien-Funde in der Donau*, Arch. Hydrobiol. Suppl. Donauforschung, **27**. *1* (4) (1964).
2. Băcescu M, *Quelques observations sur la faune benthonique du défilé roumain du Danube: son importance zoogeographique et pratique; la description d'une espace nouvelle de Mermithidae, Pseudomermis cazanica n.sp.*, Ann. Sci. Univ., Jassy, **31** (1948).
3. Th. Busnita, Gh. Brezeanu, M. Oltean, Virginia Popescu-Marinescu, Elena Prunescu-Arion, *Monografia zonei Porţilor de Fier, Studiul hidrobiologic al Dunarii si al afluentilor sai*, 85–174, Ed. Acad. RS.R, (1970).
4. Radu Codreanu, *Sur un nouveau Triclade oculé du Défilé du Danube: Palaeodendrocoelum romanodanubialis n.g.,n.sp.*, Bul.biol. France et Belgique, **LXXXIII**, *3*, 284–287, Paris, (1949).

5. Cure Victoria, Popescu-Marinescu Virginia, Schneider Adrian, *Dinamica zoobentosului in lacul de acuumulare Portile de Fier in cel de-al doilea an de la formare. – 1972*, Trav. St., "Stejarul ", Limnologie 1974–1975, 141–155, (1975).
6. Mack-Firă Valeria, *Turbelariate din Romania (Archoophora, Prolecithophora, Prosesiata, Rhabdocoela, Lecithoepitheliata), studiu sistematic, ecologic si zoogeografic*, Teza de doctorat, 62–401 (1970).
7. Maria Năstasescu, *Despre trei specii ale genului Polycelis Ehrenberg 1831 (Tricladide-Paludicola) prezente în apele Romaniei*, St. cerc. biol., Seria biol. anim., **28**, *1*, 3–8, Bucuresti, (1970).
8. Maria Năstasescu, *The Study of Dugesia gonocephala Dugis 1830 (Tricladida-Paludicola) from romanian waters*, Ann. Univ. Buc., biol. anim., XXII, 25–36, (1973).
9. Maria Năstasescu, *Triclade din România, studiu morfologic, sistematic, ecologic şi zoogeografic*. Teza de doctorat, Tipo. Univ. Bucureşti, 103–174 (1974).
10. Popescu Ecaterina, Prunescu–Arion Elena, *Contribuţii la studiul faunei bentonice din Dunăre, în regiunea cataractelor (km 1042–955)*, St. cerc. biol., Seria biol. anim., **13**, *2*, Bucureşti (1961).
11. Popescu-Marinescu Virginia, *Etappen der Zoobenthos–Entwicklung der Donau, im Gebiet des "Eisernen Tors ", Abschnitt Mraconia*, Rev.roum. biol., Biol.anim. **31**, *1*, 73–80, Bucarest, (1986).
12. V. Marinescu (Popescu-Marinescu), *Die Strucktur der Benthos-Zoozönosen bei Mraconia (Stausee Eisernes Tor I) in den Jahren 1981–1982*, 26.Arbeitstagung der IAD, Passau/Deutschaland, 1987, Wissenschaftliche Kurzreferate, 147–151, (1987).
13. V. Marinescu (Popescu-Marinescu), *Die Entwicklung der Benthos- Zoozönose in der Donau in Bereich des Stausees Eisernes Tor II*, 24. Arbeistagung der IAD, Passau /Deutschland 1987, Wissenschaftliche Kurzreferate, 152–155, (1987).
14. Virginia Popescu-Marinescu şi Ion Diaconu, *Structura zoocenozelor bentonice din lacul de baraj Porţile de Fier I (Profilele Mraconia, Cerna, Bahna) in anul 1986*, St. cerc. biol., Seria biol. anim., **41**, *1*, 71–76, Bucureşti, (1989).
15. Popescu-Marinescu Virginia, *Strucutura zoocenozelor bentonice din Dunăre, în sectorul românesc, în perioada 1971–1986*, Hidrobiologia, **20**, 111–134, Bucureşti (1992).
16. Popescu-Marinescu V., Oaie G., *Angaben uber die Abundanz und Biomasse der benthischen Wirbellosen in den Stauseen Eisernes Tor I und II*, 31. Konferenz der IAD, Baja-Ungarn, 1996, Wissenschaftliche Referate, 261–266, (1996).
17. * * * Fauna, Grupul de cercetari complexe."Porţile de Fier"Serie monografică, Ed. Acad. R.S.R, 20–22, (1975).

Received September 5, 2002.

* *Faculty of Biology*
Splaiul Independenţei, 93–95
Bucharest

** *National Institute of Research and Development for Biological Science*
Splaiul Independenţei, 296
Bucharest

IXODIDAE SPECIES IN DOMESTIC MAMMALS IN ROMANIA

IRINA TEODORESCU*, ELENA POPA**

During 1984–1995, a total of 5,733 domestic mamals (ovins, bovines, equides) were investigated. The research was done in 152 localities of 34 districts of Romania. Six Ixodidae species (*Ixodes ricinus*, *Boophilus calcaratus*, *Dermacentor reticulatus*, *Hyalomma plumbeum*, *Haemaphysalis punctata*, *Rhipicephalus bursa*) belonging to *Metastigmata* order were identified. The species frequence and dominance, the infestation intensity, the host specificity, host body distribution, the polyparasitism cases and the vectorial role were also determined.

1. INTRODUCTION

Ixodida (ticks) includes two families: *Ixodidae* (hard ticks) and *Argasidae* (soft ticks), and has a cosmopolitan distribution. Both sexes are bloodsucking ectoparasites of mammals, birds, reptiles and amphibians and are able of bitting humans (1, 2, 3, 4, 5, 6, 7).

Parasitic ticks are dangerous by producing mechanical injury at their bites, local itching, formation of pruritic nodules or granulomas, by venom injecting that produces paralysis (sometimes fatal). They also might be vectors for human and animal diseases. Their vectorial importance was discovered in 1893, when Smith and Kilbourne established that *Boophilus annulatus* transmited in cattle the "Texas fever", produced by *Babesia bigemina*. The ticks, as vectors, are the most important for domestic animal diseases and after mosquitoes, for human diseases.

Aethiological agents of tick borne diseases, which affect man are viruses, bacteria and protozoa. The main tick borne viruses are *Flavivirus* (which produce Powassan encephalitis, Russian spring-summer encephalitis, Louping-ill virus, Omsk hemorrhagic fever, Kyasanur forest disease), *Nairovirus* (Crimean-Congo hemorrhagic fever), *Orbivirus* (Colorado tick fever). The main tick borne rickettsial diseases are: Q. fever (*Coxiella burnetii*), Butoneous fever (*Rickettsia conorii*), Rocky Mountain spotted fever (*Rickettsia rickettsii*), North Asian tick typhus (*Rickettsia syberica*), Spotted fever rickettsiae (*Rickettsia akari*), Scrub typhus rickettsia (*Rickettsia tsutsugamushi*), Queensland tick typhus (*Rickettsia australis*). The tick species are vectors for borreliosis and spirochetal diseases: Lyme disease (*Borrelia burgdorferi*), Relapsing fever (*Borrelia* species) and Tularemia (*Francisella tularensis*). Protozoan diseases transmitted by ticks are babesiosis produced by *Babesia* species.

In this work the authors present, based on investigation made upon 5,733 mammal individuals, their results regarding six *Ixodidae* species.

REV. ROUM. BIOL. – BIOL. ANIM., TOME 47, N^os 1–2, P. 107–115, BUCAREST, 2002

2. MATERIAL AND METHODS

In order to know the Ixodidae situation in Romania, a study was carried out on a great number of domestic mammals, during 1984–1995. The researches were done in 152 localities of 34 districts and implied the checking of randomly selected hosts (ovines, bovines, equides).

The visual host examination, the parasite counting, collecting and identified were made in order to establish the ixodids species, their frequency and dominance, infestation intensity, host specificity and distribution, polyparasitism cases, vectorial role.

3. RESULTS AND DISCUSSIONS

Six haematophagous species (*Ixodes ricinus*, *Boophilus calcaratus*, *Dermacentor reticulatus*, *Hyalomma plumbeum*, *Haemaphysalis punctata*, *Rhipicephalus bursa*) from order *Metastigmata*, family *Ixodidae* were identified, the hosts being ovines, bovines and equides (Table 1).

Table 1

Mammalian hosts of *Ixodidae* species

No.	Ixodidae species	HOSTS		
		Bovines	Equides	Ovines
1.	*Boophilus calcaratus*	+	-	-
2.	*Dermacentor reticulatus*	+	+	+
3.	*Ixodes ricinus*	+	+	+
4.	*Haemaphysalis punctata*	+	+	-
5.	*Hyalomma plumbeum*	+	+	+
6.	*Rhipicephalus bursa*	-	+	+

Generally, the ixodids don't show host specificity (euryxenous). Thus, *I. ricinus*, *D. reticulatus* and sometimes *H. plumbeum* were idendified on ovins, bovins, equides, while *R. bursa*, usually on ovins but sometimes on equides. *B. calcaratus* and *H. punctata* were identified only in bovines, but their specificity against host was due to the absence of other species hosts in proximity. *Boophilus* species have a **monophasic life cycle** (with only one host species), *Rhipicephalus bursa* has a **diphasic life cycle** (with two host species), other species have **triphasic life cycle** (with three host species).

Intensity of infestation had seasonal and annual variations. A high level of infestation was registered in *I. ricinus*, with 100–150 parasites on the same host animal. Most investigated hosts presented moderated and low infestation level. The *H. plumbeum* infestations were moderate (about 50 individual parasites on the same host), when those of *D. reticulatus* and *H. punctata* were under 5–10 individuals on one animal host. A high level of infestation was registered during

the summer. A low infestation level was registered on January-February months, in years with mild winters.

The tick dwelling on host body was correlated with the infestation degree: in low one, the ectoparasites were placed especially on ears and on inguinal zone, but in high one, they were distributed on the whole body.

The mechanical and toxic bite actions registered: an inflammatory reaction of the perivascular tissue, with local hyperemia, oedema, hemorrhage; the wound may become necrotic or secondarily infected.

A vectorial role of the ticks was found in bovines, for *Babesia bovis* and *Anaplasma marginale* parasites.

As regards the **numerical abundance,** *I. ricinus* species was dominant in collected material throught the whole interval. Of 21,553 collected and analised ixodide individuals, *I. ricinus* represented 80.40%, *D. reticulatus* 12,74%, but other species only 6.86% together, among which *Boophilus* and *Haemophisalis,* each under 0.30% (Table 2).

As regards the species **frequency** in the investigated zones, *I. ricinus* was present in all 34 districts (constant species with 100% frequency). *D. reticulatus* had a value of 35.29 % and other species very low ones.

Polyparasitism was registered in some hosts, *I. ricinus* and *D. reticulatus* always present.

The female/male ratio in the collected material, was in the female favour, in all species: 4/1 in *B. calcaratus* and *H. punctata*, 3/1 in *I. ricinus* and *D. reticulatus*, 2/1 in *R. bursa* and *H. plumbeum*.

Favourable environmental conditions include abundant vegetation, moisture, and numerous animal hosts.

The parasitism effects observed in the parasitised hosts included restlessness, pruritis, scratching, local oedema and, in severe infestations, weight losses, milk production reduction, zones of hair loss.

Distribution: unlike the acarina species producing diseases, which are distributed relatively even on the country surface, in the case of ixodids, higher frequency values were revealed in Tulcea district, especially of *I. ricinus*, as well as the exclusively presence of *B. calcaratus* (Table 2).

Investigations were made in Tulcea district, where five ixodide species were identified, *H. punctata* being absent. The dominant species was *I. ricinus*, with an abundance of 72.58%. Values two times increased against country average had *R. bursa* and *H. plumbeum*. The highest annual values of *I. ricinus* species were registered in 1984 (above 92%), 1985 and 1986 (100%). In 1987, the relative abundance values of *I. ricinus* species decreased around 63%, as the numerical abundance increased in *D. reticulatus* (above 8%) and *H. plumbeum* (above 5%). Ixodidae species number varied in time, in Tulcea district, being four in 1987 (*B. calcaratus* missed) and three in 1988 (*B. calcaratus* and *R. bursa* species missed).

Ticks control used: hosts and habitats spraying (infested grounds, floors and walls in animal shelters and houses) with acaricides or repellent substances (containing diethyltoluamide).

Table 2

Ixodidae species distribution in Romania (1984–1995)

No.	Districts	Localities	Number of infested hosts	Ixodidae number	*Ixodes ricinus*	*Dermacentor reticulatus*	*Rhypicephalus bursa*	*Boophylus calcaratus*	*Hyalomma plumbeum*	*Haemophysalis punctata*
1	2	3	4	5	6	7	8	9	10	11
1	Alba	Alba Iulia	60	185	145	40	-	-	-	-
		Cenade	7	38	38	-	-	-	-	-
		Cetatea de Balta	7	41	41	-	-	-	-	-
		Rodesti	77	188	168	20	-	-	-	-
		Ramet- Panov	7	38	38	-	-	-	-	-
		Sebes	35	119	104	15	-	-	-	-
		Iglun	19	74	63	11	-	-	-	-
		Monarade	27	98	83	15	-	-	-	-
		Tatarlana	25	84	69	15	-	-	-	-
2	Arad	Arad	150	550	550	-	-	-	-	-
		Petris	145	324	324	-	-	-	-	-
		Savarsin	21	68	68	-	-	-	-	-
3	Arges	Paduresti	24	96	96	-	-	-	-	-
		Stalpeni	15	48	48	-	-	-	-	-
4	Bihor	Oradea	25	78	78	-	-	-	-	-
		Balc	81	73	73	-	-	-	-	-
		Bistrita	29	114	114	-	-	-	-	-
5	Bistrita Nasaud	Chirales	10	68	68	-	-	-	-	-
		Lechinta	18	54	42	12	-	-	-	-
6	Botosani	Radauti	40	125	125	-	-	-	-	-
		Catamarasti	10	41	41	-	-	-	-	-
	2	3	4	5	6	7	8	9	10	11
7	Braila	Urleasca	10	57	57	-	-	-	-	-
		Padina	50	165	165	-	-	-	-	-
		Buzau	20	119	119	-	-	-	-	-
8	Buzau	Beceni	60	206	186	20	-	-	-	-
		Berca	50	180	150	30	-	-	-	-
		Cernatesti	68	155	143	12	-	-	-	-
		Naidas	14	72	56	16	-	-	-	-
9	Caras Severin	Pojejana	21	93	74	19	-	-	-	-
		Slatina Nero	30	122	92	30	-	-	-	-
		Caras Severin	42	228	150	78	-	-	-	-
		Modelu	24	112	112	-	-	-	-	-
		Unirea	20	47	47	-	-	-	-	-
10	Calarasi	Borcea	20	81	81	-	-	-	-	-
		Beresti	18	54	54	-	-	-	-	-
		Hanu Conachi	28	93	93	-	-	-	-	-
		Calarasi	10	40	40	-	-	-	-	-
		Rasuceni	57	202	202	-	-	-	-	-
		Chinteni	40	114	114	-	-	-	-	-
11	Cluj	Iara	23	96	96	-	-	-	-	-
		Cacova	14	74	74	-	-	-	-	-
		Turca	18	81	81	-	-	-	-	-
		Santeia	22	140	-	140	-	-	-	-
		Medgidia	49	236	236	-	-	-	-	-
12	Constanta	Mihai Viteazu	20	161	161	-	-	-	-	-
		Palas	75	255	255	-	-	-	-	-
		Sf. Gheorghe	55	197	197	-	-	-	-	-
13	Covasna	Tg. Secuiesc	124	268	-	268	-	-	-	-
		Gherlinta	34	154	-	154	-	-	-	-
		Baraolt	20	89	-	89	-	-	-	-
	2	3	4	5	6	7	8	9	10	11
14	Dolj	Podari	2	15	15	-	-	-	-	-
		Bailesti	3	16	16	-	-	-	-	-
		Poiana	33	95	78	17	-	-	-	-

15	Dambovita	Crevedia	4	21	21	-	-	-	-	-
		Doicesti	50	214	214	-	-	-	-	-
		Tecuci	8	42	42	-	-	-	-	-
16	Galati	Beresti	170	287	287	-	-	-	-	-
		Namoloasa	40	122	122	-	-	-	-	-
		Cosmesti	80	190	190	-	-	-	-	-
		Gradinari	10	51	51	-	-	-	-	-
17	Giurgiu	Ogrezeni	14	50	50	-	-	-	-	-
		Giurgiu	30	110	110	-	-	-	-	-
		Talpasesti	5	29	29	-	-	-	-	-
18	Gorj	Targu Jiu	89	289	238	25	-	-	26	-
		Borascu	70	148	148	-	-	-	-	-
		Grivilesti	72	160	160	-	-	-	-	-
19	Harghita	Miercurea Ciuc	30	70	70	-	-	-	-	-
		Feliceni	20	63	63	-	-	-	-	-
		Hunedoara	40	103	83	20	-	-	-	-
		Gioagiu	26	92	68	24	-	-	-	-
		Martinesti	20	74	54	20	-	-	-	-
		Romas	34	118	92	26	-	-	-	-
		Brad	20	90	80	10	-	-	-	-
		Crisor	55	183	148	35	-	-	-	-
		Petrila	15	53	38	15	-	-	-	-
		Tomesti	17	55	42	13	-	-	-	-
		Petrosani	10	75	54	21	-	-	-	-
		Ghelari	23	61	34	27	-	-	-	-
	2	3	4	5	6	7	8	9	10	11
		Calan	25	85	60	25	-	-	-	-
		Britea	11	37	28	9	-	-	-	-
20	Hunedoara	Hateg	6	22	18	4	-	-	-	-
		Haran	47	109	96	13	-	-	-	-
		Soimus	18	42	30	12	-	-	-	-

		Bronis	11	40	21	19	-	-	-	-
		Chilia	10	25	15	10	-	-	-	-
		Zom	25	60	45	15	-	-	-	-
		Raul de Mori	10	47	27	20	-	-	-	-
		Salasul de Sus	14	37	31	6	-	-	-	-
		Vulcan	7	21	18	3	-	-	-	-
		Lupeni	10	25	15	10	-	-	-	-
		Vesel	37	93	70	23	-	-	-	-
		Dobra	18	41	29	12	-	-	-	-
		Labugin	11	59	40	19	-	-	-	-
		Densus	47	112	89	23	-	-	-	-
		Sarmisegetusa	39	115	94	21	-	-	-	-
21	Iasi	Dimitresti	7	56	56	-	-	-	-	-
22	Ialomita	Stelnica	67	214	214	-	-	-	-	-
		Movila	9	29	29	-	-	-	-	-
		Targu Mures	35	124	109	-	-	-	15	-
		Reghin	25	60	-	6	-	-	-	-
23	Mures	Brancovenesti	50	160	-	50	110	-	-	-
		Socolul de Padure	50	150	-	110	40	-	-	-
		Idicel	63	183	-	183	-	-	-	-
		Cernesti	26	84	84	-	-	-	-	-
24	Mehedinti	Bosonci	25	40	40	-	-	-	-	-
		Orsova	10	30	-	-	-	-	-	30
	2	3	4	5	6	7	8	9	10	11
		Salcea	12	117	117	-	-	-	-	-
25	Olt	Valeni Olt	80	297	297	-	-	-	-	-
		Mandra	60	211	211	-	-	-	-	-
26	Prahova	Ploiesti	30	81	81	-	-	-	-	-
		Livada	5	58	58	-	-	-	-	-

27	Satu Mare	Moftinul Mic	50	116	116	-	-	-	-	-
		Cehal	100	110	110	-	-	-	-	-
		Satu Mare	23	119	69	50	-	-	-	-
		Preutesti	5	75	75	-	-	-	-	-
		Ipotesti	20	31	31	-	-	-	-	-
		Suceava	69	180	180	-	-	-	-	-
28	Suceava	Iacobeni	20	63	63	-	-	-	-	-
		Breaza	30	81	81	-	-	-	-	-
		Fundu Moldovei	30	79	79	-	-	-	-	-
		Carun	20	48	48	-	-	-	-	-
29	Timis	Jomu Mare	30	70	70	-	-	-	-	-
30	Tulcea	25 localitati	1186	7517	5455	821	540	62	639	-
31	Vaslui	Husi- Rasesti	145	424	424	-	-	-	-	-
		Ivanesti	40	112	112	-	-	-	-	-
32	Valcea	Rm. Valcea	20	61	61	-	-	-	-	-
33	Vrancea	Suraia	50	124	124	-	-	-	-	-
34	S.A.I.	Chitila	137	208	208	-	-	-	-	-
Total 34 districts		**152 localities**	**5733**	**21553**	**17336**	**2755**	**690**	**62**	**680**	**30**
					80.40 %	**12.74%**	**3.20%**	**0.28%**	**3.25%**	**0.13%**

4. CONCLUSIONS

- Six haematophagous species (*Ixodes ricinus, Boophilus calcaratus, Dermacentor reticulatus, Hyalomma plumbeum, Haemaphysalis punctata, Rhipicephalus bursa*) were identified during 1984–1995, in 152 localities of 34 Romania districts, on 5,733 domestic mammals (ovines, bovines, equids).
- The female/male ratio in the collected material was in favor of the female, in all species.
- As regards numerical abundance and frequency, *I. ricinus* was dominant and constant, throught whole interval.
- *I. ricinus* showed the highest infestation intensity.
- The tick vectorial role was evidenced in bovines, for *Babesia bovis* and *Anaplasma marginale* protozoan parasites.
- From the registered polyparasitism *I. ricinus* and *D. reticulatus* were always present.
- In Tulcea district, five ixodide species were identified (*H. punctata* being missed), dominant and constant beying *I. ricinus*.

REFERENCES

1. Acha N. P, Szyfres B., *Zoonoses et maladies transmissibles communes à l'homme et aux animaux*, Office International des epizooties, Paris, 1989.
2. Brumpt E., *Precis de Parasitologie*, Paris, Masson et Cie. (1949).
3. Cosoroaba I., *Acarologie veterinara*, Bucuresti, Editura Ceres (1994).
4. Knudsen B. A, Slooff R., *Vector-borne disease problems in rapid urbanization: new approaches in vector control*, Bulletin OMS, **70**, *1*, 1–6 (1992).
5. Knudsen B. A, Slooff R., *Problemes dus aux maladies à transmission vectorielle et urbanisation accélérée: nouvelles approches de la lutte antivectorielle*, Bulletin OMS, **70**, *2*, 165–171 (1992).
6. Markell K. E., Marietta, Voge M. A., David T. J., *Medical parasitology*, 7-th Edition, Philadelphia, London, Toronto, Montreal, Sydney, Tokyo, W. B. Saunders Company (1992).
7. Neva A. F., Brown W. H., *Basic clinical parasitology*, Norwalk, Connecticut, Appleton & Lange (1995).

Received January 27, 2003.

*Faculty of Biology,
Spl. Independentei 91–95, Bucharest
e-mail: iteodorescu@ bio.bio.unibuc.ro
**Institute of Diagnosis and Animal Health,
Bucharest, Romania
Dr. Staicovici 63,
e-mail: popa.elena@idah.ro

BIOLOGICAL CONTROL OF COLORADO POTATO BEETLE IN ROMANIA BY INUNDATIVE RELEASES OF *PODISUS MACULIVENTRIS* SAY (*HETEROPTERA*: *PENTATOMIDAE*)

T. MANOLE, MARIA IAMANDEI*, IRINA TEODORESCU**

The possibility of using various natural enemies in the biological control of pests is now a necessity more than ever. The use of biocontrol agents implies the availability of adequate rearing techniques enabling large-scale production at low costs. Mass production and periodic colonization of predators or parasitoids species is one of strategy to increase the effectiveness of the biological control.
Our researches establish for the first time in Romania that the species *Podisus maculiventris* Say could be a valuable agent for the biological control of of *L. decemlineata* in the field conditions. *P. maculiventris* was reared under controlled conditions in the small biostation at Research Institute for Plant Protection (RIPP)-Bucharest. The technical line and ensemble of facilities was previously described (Manole T., 1993). After mass rearing tests, based of its economic growing in industrial facilities, the four instar larvae *M. domestica* host we recommend for predatory feeding. The obtained results establish the moment and rate of release in field and effectiveness of predatory rate against Colorado potato beetle in four regions of Romania.

1. INTRODUCTION

Biological control by mass rearing of beneficial insects will require increasing attention in the agricultural context in which it is applied. Many authors considered that biological control offers real potential as a major tactic for maximization in a strategy of integrated control of crops pests (6, 9, 4, 5, 7). Researches reffer to mass rearing of different species of *Asopinae*, but the field trials in large plots are rare (3, 1, 2, 5, 8).

Since 1990 *Podisus maculiventris* Say (*Heteroptera*: *Pentatomidae*), an efficient predator for *Leptinotarsa decemlineata* Say, has currently mass reared in Romania.

2. MATERIALS AND METHODS

TECHNOLOGY OF MASS REARING

Podisus maculiventris were reared in controlled condition at 25 ± 2°C, 60 ± 10 % RH and a photophase of 12 hours on *Musca domestica* larvae.

REV. ROUM. BIOL. – BIOL. ANIM., TOME 47, N^os 1–2, P. 117–121, BUCAREST, 2002

APPLICATION IN THE FIELD

The field experiments were carried out in the four region of Romania, at the Research Institute for Plant Protection, Bucharest and at Institute for Culture and Production of Potato from Braşov, Danube Delta Research and Design Institute from Tulcea and Plant Protection Inspection Service from Focşani.

The field plots had the surface of 500 m^2 and the biological material transport was made in recipients of 1 000 cm^3 capacity. The water and the food were assured. Correlated with predator stage each recipient contains 100–300 specimens. The releases were made by setting the insects down in the spreading points with the help of special brushes and a pleated carton paper.

EXPERIMENTAL VARIANTS

Variant 1

Moment of release: apparition of eggs on 50% of the potato plants. Rate of release: 40,000 specimens/ha. Number of releases: 1. Stage of predator: larvae L_2 instar.

Variant 2

Moment of release: apparition of first larvae. Rate of release: 70,000 specimens/ha. Number of releases: 1. Stage of predator: larvae L_2 instar.

Variant 3

Moment of release: the warning for chemical treatment. Rate of release: 140,000 specimens/ha. Number of releases: 1. Stage of predator: larvae L_2 instar.

Variant 4: standard control.

Chemical treatment with DECIS 2.5 EC. Dose: 0.5 l/ha.

All variants had 4 replicates plots, each with the surface of 500 m^2.

Observations and measurements registered: total density and mean relative density of pest larvae expressed by specimens/plant; specimens/ha; mortality of pest larvae induced by predatory insect at 24, 48 hours and 10 days after the release; pest larvae mortality induced by chemical treatment.

3. RESULTS AND DISCUSSION

A. In the **Muntenia region**, the climate is typical temperate with lightly excessive shades. The spring is generally short, with clearly daily thermal contrasts and between months (6–7°C between March and April). The means values of temperature swing between 5 and 17°C and the mean precipitations level 150 mm. The summer is hot (means values between 20–23°C) with few rains and maximum of typical tropical days (t° between 35–40°C). The researches were organized in 1998, in two localities: Bucureşti and Ştefăneşti. The values of mortality induced by the *P. maculiventris* were significantly higher than the case of the variant treated with deltametrin (Table 1).

B. In the **Moldova region**, the climate is alike those of the south regions with the mention that the continental influences are more felt than in the rest of country. In our investigations, in 1997, in Focsani locality, the mortality of pest larvae was higher (between 89.13 % 100 %) in comparison with chemical treatment (85.70 %) (Table 3).

C. In the **Transilvania region** the climatic conditions in the Brasov district are different, because is placed into a plain surrounded by Carpathian Mountains.

Table 1

The efficiency of biological control of species *Leptinotarsa decemlineata* Say using the predator *Podisus maculiventris* Say

Variant	Rate of release (specimens/ha)	Mean host density (specimens/ha)	Mortality induced by predator P. maculiventris after:		
			24 hours %	48 hours %	10 days %
BUCHAREST – 1998					
V_1	40.000	388.200	48.01	38.16	94.44
V_2	70.000	433.760	1.40	13.98	95.83
V_3	140.000	883.680	9.57	33.44	98.24
V_4	Decis 2.5 EC 0.5 l/ha	806.400	Mortality induced by deltametrin		
			96.83	-	71.83
ŞTEFĂNEŞTI – 1997					
V_1	40.000	346.500	71.4	95.2	100.0
V_2	70.000	445.500	67.9	90.1	90.1
V_3	140.000	452.000	29.0	92.7	100.0
V_4	Decis 2,5 EC 0,5 l/ha	429,000	Mortality induced by deltametrin		
			89.7	-	70.9
FOCŞANI – 1997					
V_1	40,000	137,500	52.0	96.0	100.0
V_2	70,000	165,000	40.0	96.6	96.6
V_3	140,000	253,000	52.1	73.9	89.1
V_4	Decis 2,5 EC 0,5 l/ha	209,000	Mortality induced by deltametrin		
			92.1	-	85.7
BRAŞOV – 1997					
V_1	40,000	286,000	28.8	57.6	100.0
V_2	70.000	220.000	57.5	70.0	97.5
V_3	140.000	214.500	41.0	61.5	97.4
V_4	Decis 2.5 EC 0.5 l/ha	269.500	Mortality induced by deltametrin		
			81.6	-	79.5
BRAŞOV – 1998					
V_1	40.000	317.000	33.33	60.48	96.60
V_2	70.000	460.000	12.86	61.29	98.95
V_3	140.000	629.000	35.58	66.40	87.26
V_4	Decis 2.5 EC 0.5 l/ha	580.625	Mortality induced by deltametrin		
			84.61	-	72.80

Table 1 (continued)

UZLINA – 1997					
V_1	40.000	72.400	11.6	24.0	74.3
V_2	70.000	116.800	13.7	22.9	87.4
V_3	140.000	130.800	34.2	55.1	100.0
V_4	Decis 2.5 EC 0.5 l/ha	122.540	Mortality induced by deltametrin		
			94.5	-	87.2
UZLINA – 1998					
V_1	40.000	265.200	3.16	12.82	96.60
V_2	70.000	452.600	6.09	14.18	96.28
V_3	140.000	466.200	3.64	16.34	94.64
V_4	Decis 2.5 EC 0.5 l/ha	367.800	Mortality induced by deltametrin		
			73.35	-	71.45

Winters are relatively mildly, springs are relatively short and coldish and summers are warm but damped. This climate is very unfavourable for the development of Colorado potato beetle, but is favourable from the predatory and his predatory activity is highest at the lowest densities of pest. The activities were organized in 1997–1998 and the same high values registered in all the releases with *P. maculiventris* (Tables 4 and 5).

In the **Dobrogea region**, the climate is the driest in Romania (132.7–189.8 mm annual average precipitation), the sunniest days (150–160 days/year, over 2 500 hours annual at Sfântu Gheorghe on the Black Sea coast). In these conditions the effectiveness of predatory action, in Uzlina locality, in 1997–1998, was significantly higher than chemical treatment (Tables 6 and 7).

4. CONCLUSIONS

The species *P. maculiventris* is able to protect the potato crops against the attack of Colorado potato beetle over the entire period of vegetation in the various pedoclimatic conditions of Romania.

The reasons for L_2 instar larvae considered the best stage for release were: start feeding immediately after release; could be very easy manipulated and conveyed; are less movable and the migration is made progressively searching the food, the spreading in the crop is slow, according to the food consumption.

The most efficient level of release, strongly correlated with the biology of Colorado potato beetle was 40,000 specimens per hectare, released correlated with about 50 % eggs laying on the potato plants.

REFERENCES

1. DECLERCQ, P., DEGHEELE, D., *Laboratory measurement of predation by Podisus maculiventris Say and P. sagitta* (*Hemiptera*: *Pentatomidae*) *on beet armyworm* (*Lepidoptera*: *Noctuidae*), Journal of Economic Entomology, 87: 6–83 (1994).
2. DECLERCQ, P., DEGHEELE, D., Effects of mating status on body weight, oviposition, egg load, and predation in the predatory stinkbug *Podisus maculiventris* (*Heteroptera*: *Pentatomidae*), Annals of the Entomological Society of America, 90:121–127 (1997).
3. DRUMMOND, F., A., JAMES, R., L., CASAGRANDE, R.,A., FAUBERT, H., *Development and survival of Podisus maculiventris* Say (*Hemiptera*: *Pentatomidae*), *a predator of the Colorado potato beetle* (*Coleoptera*: *Chrysomelidae*), Environmental Entomology 13:1283–1286 (1984).
4. HOUGH-GOLDSTEIN, J., A., HEIMPEL, G., E., BECHMANN, H.,E., MASON, C., E., *Arthropod natural enemies of the Colorado potato*, Crop Protection, nr. 5, 12:324–334 (1993).
5. HOUGH-GOLDSTEIN, J., A., COX, J., ARMSTRONG, A., *Podisus maculiventris* (*Hemiptera*: *Pentatomidae*) *predation on ladybird beetles* (*Coleoptera*: *Coccinelidae*), The Florida Entomologist, 79: 64–68 (1996).
6. HUFFAKER, C., B., *Biological control in the management of pests*, Agro-Ecosystems, 2:15–31 (1975).
7. MANOLE, T., *Mass rearing of Podisus maculiventris Say under controlled environment conditions* (I), Anals of Res., Instit., Pl., Prot., 24:101–110 (1993).
8. WIEDENMANN, R., N., O' NEIL, R., J., *Searching strategy of the predator Podisus maculiventris* Say (*Heteroptera*: *Pentatomidae*). Environmental Entomology, 21:1–9 (1992).
9. WILLIAMS, D., W., LEPPLA, N., C., *The future of augmentation of beneficial arthropods*, USDA Report, 1–23 (1992).

Received January 27, 2003.

*Research Institute for Plant Protection, Bucharest
**Faculty of Biology, Bucharest

ACARIDAE SPECIES CAUSING ACARIOSIS IN DOMESTIC MAMMALS AND BIRDS IN ROMANIA

ELENA POPA*, IRINA TEODORESCU**

During 1984–1995 over 6,000 domestic mammals (ovins, bovines, equids, suides, canides, felides, leporines) and birds (gallinaceae) were investigated. The researches carried out in 152 localities from 35 districts of Romania. A number of 8 acaridae species (*Sarcoptes scabiei* var. ***ovis***, *bovis*, *equi*, *suis*, *Notoedres cati* var. *cuniculi*, *Cnemidocoptes laevis* and *Cnemidocoptes mutans*, *Otodectes cynotis*, *Psoroptes equi* var *cuniculi*, *Chorioptes bovis*, *Demodex canis*), belonging to *Astigmata* and *Prostigmata* orders were identified. These species caused acariosis diseases (sarcoptosis, notoedrosis, psoroptosis, chorioptosis, otodectosis, cnemidocoptosis and demodicosis).

1. INTRODUCTION

The underclass *Acarida* (*Acari*) includes *Metastigmata* (*Ixodida*), *Mesostigmata* (*Gamasida*), *Prostigmata* (*Trombidida*) and *Astigmata* (*Acarida*) orders. Within the order *Prostigmata* (*Trombidida*), the *Demodecidae* family includes species parasite in canides, suides, bovines, equids, ovins, man. In the order *Astigmata* (*Acarida*) some families (*Sarcoptidae*, *Psoroptidae*, *Cnemidocoptidae*) are parasitic and cause dermatitis both in human and animals (1, 2, 3, 4, 5, 6, 7).

In this study based on an investigation made upon over 6,000 mammals and bird species, the authors present their results regarding 8 parasite species belonging to *Prostigmata* (*Demodecidae* family) and *Astigmata* (*Sarcoptidae*, *Psoroptidae*, *Cnemidocoptidae* families) orders, which caused acarosis.

2. MATERIAL AND METHODS

The study was carried out during 1984–1995. The researches effectued in 152 localities of 35 districts implied the checking of randomly selected hosts of different groups (ovins, bovines, equids, suines, canines, felines, leporines, gallinaceae). The diagnosis was established through the identification of aetiological agent (guided by clinical evidences), phaner examination, derma scraping.

The observations made on 6,240 domestic animals (6,216 mammals and 24 birds).

REV. ROUM. BIOL. – BIOL. ANIM., TOME 47, N^{os} 1–2, P. 123–128, BUCAREST, 2002

3. RESULTS AND DISCUSSIONS

The quantitative and qualitative analyses of the obtained material, lead to the identification of 8 acaridae species belonging to *Astigmata* and *Prostigmata* orders (Table 1).

The produced diseases were sarcoptosis, notoedrosis, psoroptosis, chorioptosis, otodectosis, cnemidocoptosis and demodecosis.

Seven species belonged to order *Astigmata*, families *Sarcoptidae*, *Psoroptidae*, *Cnemidocoptidae* and only a single species to order *Prostigmata*, family *Demodecidae* were identified.

Table 1

Main acarids species causing acariosis

ORDER/ FAMILY	Genus	Species	Variety	Disease
Astigmata				
Sarcoptidae	*Notoedres*	*Notoedres cati*	*cuniculi*	notoedrosis
		Notoedres cati		notoedrosis
	Sarcoptes	*Sarcoptes scabiei*	*bovis*	sarcoptosis scabies
		Sarcoptes scabiei	*equi*	sarcoptosis scabies
		Sarcoptes scabiei	*ovis*	sarcoptosis scabies
		Sarcoptes scabiei	*suis*	sarcoptosis scabies
Cnemidocoptidae	*Cnemidocoptes*	*Cnemidocoptes laevis*		cnemidocoptosis
		Cnemidocoptes mutans		cnemidocoptosis
Psoroptidae	*Psoroptes*	*Psoroptes equi*	*cuniculi*	psoroptosis
		Psoroptes equi	*equi*	psoroptosis
	Chorioptes	*Chorioptes bovis*	*bovis*	corioptosis
		Chorioptes bovis	*equi*	corioptosis
	Otodectes	*Otodectes cynotis*	*canis*	otodectosis
Prostigmata				
Demodecidae	*Demodex*	*Demodex canis*		demodecosis

The family *Sarcoptidae* was represented by species of genera *Sarcoptes* and *Notoedres*.

Four varieties of *Sarcoptes scabiei* species (*ovis*, *bovis*, *equi*, *suis*), were present in sheep, cow, horse and pig. The highest percent of the parasitized animals was found in pigs (10 %) (Table 2).

Notoedres cati was present in rabbits through *cuniculi* variety, but the rabbit notoedrosis incidence was low.

The family *Psoroptidae* was represented by three species of genera *Psoroptes*, *Chorioptes* (each with two varieties) and *Otodectes*.

Psoroptes was represented by *equi* variety in horses and by *cuniculi* variety in rabbits. The last indicated an increased psoroptosis incidence in r bes.

Two varieties of *Chorioptes bovis* species (*bovis* and *equi*) parasitized boars and horses, and an increased incidence was registered in the case of *bovis* variety in calves.

Table 2

Acarian parasitism degree of mammal and bird hosts

Hosts	Number of investigated hosts	Parazitated hosts		PARASITES	
		Number	%	Species number	Genus
Ovins	1789	48	2.68	1	*Sarcoptes*
Bovins	3164	97	3.06	2	*Sarcoptes, Chorioptes*
Equids	935	9	0.96	3	*Sarcoptes, Chorioptes, Psoroptes*
Suines	50	50	100.00	1	*Sarcoptes*
Canidae and felidae	4	4	100.00	3	*Otodectes, Notoedres, Demodex*
Leporidae	275	275	100.00	2	*Notoedres, Psoroptes*
Total Mammals	**6216**	**483**	**7.77**	**6**	
Galiinacea	24	24	100.00	2	*Cnemidocoptes*
Total birds	**24**	**24**	**100.00**	**2**	
Total general	**6246**	**507**	**8.12**	**8**	

Otodectes cynotis was represented in dogs by *canis* variety, with a low incidence.

The family *Cnemidocoptidae* was represented by species *Cnemidocoptes laevis* and *C. mutans*, discovered in gallinaceae. Cnemidocoptosis was characterised by thin crusts, less adherent, with loss of feathers and open lesions as a result of pruritis, in the case of *C. laevis* and thick crusts, adherent on feet, caused by *C. mutans*.

The single species within the order *Prostigmata* was *Demodex canis*, discovered on two dogs. The parasite is placed in hair folicles and sebaceous glands, in periocular areas, on cheeks and ears, in dorsal neck areas. The induced cutaneous modifications were oedema, papulovesicles, crusts, and hair losses.

As regard the distribution of infested animals (Table 3), the most acarioses were found out in Bucharest area and Ilfov district.

A great number of infested animals were discovered in Alba (159) and Prahova (102) districts, but the diseases were caused only by two acarids species.

Psoroptes equi var. *cuniculi* and *Chorioptes bovis* var. *bovis* were present on hosts from four districts, while *Demodex canis* and *Otodectes cynotis* var. *canis*, *Notoedres cati* var. *cuniculi* and *Sarcoptes scabiei* var. *equi* were found only in Bucharest area.

The analysis of the material collected on the 507 identified hosts enabled us to determine the values of the **species frequency** of acarids and of the most infested hosts.

The highest frequency values had *Psoroptes equi*, present in 56.93% of investigated hosts, *Sarcoptes scabiei* found in 27.33% ones and *Corioptes bovis* in 14.28% ones. The lowest frequency values had *Otodectes cynotis*, *Notoedres cati*, *Cnemidocoptes mutans*, *C. laevis* and *Demodex canis* (between 0.41 and 0.62%). *Psoroptes equi* represents a constant species through its high frequency values.

The **infestation intensity** was higher in horses and rabbits, caused by *Psoroptes equi* var. *equi* and in pigs infested with *Sarcoptes scabiei* var. *suis*. Low levels of infestation with *Otodectes cynotis* var *canis* in dogs, with *Notoedres cati* in cats, and with *Notoedres cati* var. *cuniculi* in rabbits were found.

Polyparasitism was identified in horses (with *Sarcoptes scabiei* var. *equi*, *Psoroptes equi* var. *equi* and *Chorioptes bovis* var. *equi*), bovines (with *Sarcoptes scabiei* var. *bovis*, *Chorioptes bovis* var. *bovis*), dogs (with *Otodectes cynotis* var. *canis* and *Demodex canis*), rabbits (with *Notoedres cati* var. *cuniculi* and *Psoroptes equi* var *cuniculi*) and in galinaceae (with *Cnemidocoptes laevis* and *C. mutans*). A variety of *Sarcoptes scabiei* (*equi*, *ovis*, *bovis*) was identified in the case of the other examined host species.

The parasitism local effects were due especially to the mechanical and spoliation actions of parasites, while the general effects were caused by the toxins and by the feeding alteration (affecting the animal weights, milk production a. o.).

Sarcoptidae and *Psoroptidae* species burrow into host skin and cause irritation; the scratching often causes supplementary injuries or leads to secondary infection.

Table 3

Sarcoptidae, *Psoroptidae* and *Demodecidae* distribution in Romania (1984–1995)

District	Locality	Host	No. infested animals	Parasites
1	2	3	4	5
Bucureşti	Bucureşti	suides	10	*Sarcoptes scabiei var. suis*

Table 3 (continued)

1	2	3	4	5
		equids	2	*Sarcoptes scabiei var. equi*
		leporides	9	*Psoroptes equi var. cuniculi*
		equids	1	*Psoroptes equi var. equi*
		canides	2	*Otodectes cynotis var. canis*
		canides	2	*Demodex canis*
		leporides	3	*Notoedres cati var. cuniculi*
		equids	2	*Chorioptes bovis var. equi*
		bovines	3	*Chorioptes bovis var. bovis*
Alba	Alba Iulia	leporides	21	*Psoroptes equi var. cuniculi*
	Sebeş	leporides	54	*Psoroptes equi var. cuniculi*
	Rodeşti	leporides	68	*Psoroptes equi var. cuniculi*
	Alba Iulia	bovines	4	*Chorioptes bovis var. bovis*
	Sebeş	bovines	12	*Chorioptes bovis var. bovis*
Constanţa	Palas	ovines	26	*Sarcoptes scabiei var. ovis*
Dâmboviţa	Crevedia	gallinacee	9	*Cnemidocoptes mutans*
	Crevedia	gallinacee	4	*Cnemidocoptes laevis*
Giurgiu	Giurgiu	bovines	15	*Sarcoptes scabiei var. bovis*
	Ogrezeni	equids	2	*Psoroptes equi var. equi*
	Grădinari	equids	2	*Chorioptes bovis var. equi*
Neamţ	Piatra Neamţ	leporides	48	*Psoroptes equi var. cuniculi*
Prahova	Ploieşti	leporides	72	*Psoroptes equi var. cuniculi*
	Ploieşti	bovines	30	*Chorioptes bovis var. bovis*
S.A.I.	Chitila	suides	40	*Sarcoptes scabiei var. suis*
	Chitila	bovines	17	*Sarcoptes scabiei var. bovis*
	Buftea	galinacee	8	*Cnemidocoptes mutans*
	Buftea	galinacee	3	*Cnemidocoptes laevis*
Sibiu	Sibiu	bovines	16	*Chorioptes bovis var. bovis*
Suceava	Preuţeşti	ovines	22	*Sarcoptes scabiei var. ovis*
Total 10 districts	**15 localities**		**507**	

4. CONCLUSIONS

– Eight species belonging to *Astigmata* (*Sarcoptes scabiei* var. *ovis*, *bovis*, *equi*, *suis*, *Notoedres cati* var. *cuniculi*, *Cnemidocoptes laevis* and *Cnemidocoptes mutans*, *Otodectes cynotis*, *Psoroptes equi* var *cuniculi*, *Chorioptes bovis*), and *Prostigmata* (*Demodex canis*), orders were identified.

– These species caused various acarosis diseases (sarcoptosis, notoedrosis, cnemidocoptosis, otodectosis, psoroptosis, chorioptosis and demodicosis).

– Parasitism degree is high (100 %) on suides, leporines, canides, felides and gallinaceae.

– The highest frequency values had *Psoroptes equi*, *Sarcoptes scabiei* and *Chorioptes bovis*.

– The lowest frequency values had *Otodectes cynotis*, *Notoedres cati*, *Cnemidocoptes mutans*, *C. laevis* and *Demodex canis*.

– *Psoroptes equi* represents a constant species.

– The infestation intensity was higher in horses and rabbits, caused by *Psoroptes equi* var. *equi* and in pigs infested with *Sarcoptes scabiei* var. *suis*.

– The polyparasitism was identified in horses, bovines, dogs, rabbits and birds.

REFERENCES

1. Acha N. P, Szyfres B., *Zoonoses et maladies transmissibles communes à l'homme et aux animaux*, Office International des epizooties, Paris, 1989.
2. Brumpt E., *Précis de Parasitologie*, Paris, Masson et Cic., 1949.
3. Cosoroaba I., *Acarologie veterinară*, Bucuresti, Editura Ceres, 1994.
4. Knudsen B. A, Slooff R., *Vector-borne disease problems in rapid urbanization: new approaches in vector control*, Bulletin OMS, **70**, *1*, 1–6, 1992.
5. Knudsen B. A, Slooff R., *Problèmes dus aux maladies à transmission vectorielle et urbanisation accelerée: nouvelles approches de la lutte antivectorielle*, Bulletin OMS, **70**, *2*, 165–171, 1992.
6. Markell K. E., Marietta, Voge M. A., David T. J., *Medical parasitology*, 7-th Edition, Philadelphia, London, Toronto, Montreal, Sydney, Tokyo, W. B. Saunders Company, 1992.
7. Neva A. F., Brown W. H. *Basic Clinical Parasitology*, Norwalk, Connecticut, Appleton & Lange, 1995.

Received January 27, 2003.

*Institute of Diagnosis and Animal Health, Bucharest, Romania
Dr. Staicovici 63,
E-mail: popa.elena@idah.ro
**Faculty of Biology,
Spl. Independenţei 91–95, Bucharest
E-mail: iteodorescu@ bio.bio.unibuc.ro

GRAFICA XX
TIPOGRAFIE

AVIS AUX COLLABORATEURS

La «Revue roumaine de biologie – Série de biologie animale» publie des articles originaux d'un haut niveau scientifique de tous les domaines de la biologie animale: taxonomie, morphologie, physiologie, génétique, écologie, etc. Les sommaires des revues sont complétés par d'autres rubriques, comme: 1. La vie scientifique, qui traite des manifestations scientifiques du domaine de la biologie (symposiums, conférences, etc.); 2. Comptes rendus des plus récentes parutions dans la littérature.

Les auteurs sont priés de présenter leurs articles en double exemplaire, imprimés de préférence sur une imprimante laser et espacés à double interligne. Le contenu des articles sera introduit sur des disquettes dans un langage connu, préférablement Word 6.0. La composition et la mise en vedette seront faites selon l'usage de la revue: caractères de 11/13 points pour le texte, de 12/14 points pour le titre de l'article et de 9/11 pour les annexes (tableaux, bibliographie, explication des figures, notes, etc.) et le résumé en anglais de 10 lignes au maximum, qui sera placé au début de l'article. Il est obligatoire de spécifier sur les disquettes le nom des fichiers ainsi que le programme utilisé.

Le matériel graphique sera envoyé sur disquette, scanné, avec les mêmes spécifications. En l'absence d'un scanner, le matériel graphique sera exécuté en encre de Chine sur papier calque.

Les tableaux et les illustrations seront numérotés en chiffres arabes dans l'ordre de l'apparition. Les titres des revues seront abrégés conformément aux usages internationaux.

Les textes ne doivent pas dépasser 10 pages (y compris les tableaux, la bibliographie et l'explication des figures).

La responsabilité pour le contenu des articles revient exclusivement aux auteurs.