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REVUE ROUMAINE DE BIOLOGIE

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SOMMAIRE

SUR LES GRÉGARINES (PROTOZOA, SPOROZOA) D'INSECTES PLÉCOPTÈRES, À PROPOS DE DEUX ESPÈCES PARASITES CHEZ DES LARVES RHÉOPHILES DES CARPATES MÉRIDIONALES

DOINA BĂLCESCU-CODREANU

The development and the completed diagnosis of two species of Gregarines, *Ancyrophora codreanui* Bălcescu-Codreanu, 1973 and *A. obtusa* Bălcescu-Codreanu 1973, found as intestinal parasites in Plecopteran larvae (Subord. Systellognatha) from mountain streams in Southern Carpathians (Romania) are presented. There are discussed the taxonomic value of the epimerite morphology in the *Ancyrophora* genus and the systematic status of Gregarines parasites of Plecoptera, with reference to some polyparasitic states.

Les Grégarines des Plécoptères sont peu étudiées et n'ont fait pas l'objet d'un inventaire systématique. Chez des hôtes appartenant à diverses familles ont été mentionnées deux espèces du genre *Pileocephalus* Aimé Schneider, 1875: la première, *P. nemurae* (Foerster, 1938) Stein, 1960, décrite chez des larves de *Leuctra* sp. et *Nemoura* sp. de Silésie (9) et retrouvée chez des larves de Chloroperlidae de Karélie (le lac Syam) (10, 18), ainsi que chez des larves de *Perlodes* sp. en Allemagne (10); la deuxième, *P. chinensis* Aimé Schneider, 1875 connue des larves des Trichoptères (11), a été citée chez des larves d'*Isoperla oxylepis* en Pyrénées-Orientales (8). Chez des larves d'*Arcynopterix compacta* et *Isoperla minima* (Carpates méridionales, Roumanie) nous avons distingué deux espèces nouvelles, rapportées au genre *Ancyrophora* Léger, 1892, respectivement *A. codreanui* Bălcescu-Codreanu, 1973 et *A. obtusa* Bălcescu-Codreanu, 1973, brièvement décrites auparavant (1). Ultérieurement, dans le même genre on a trouvé une autre espèce, *A. cornuta* Baudoin et Mouthon, 1976 chez des larves de *Dinocras megacephala* en France (6).

Ce travail présente la phase végétative du développement des deux espèces d'*Ancyrophora* que nous avons établi (1), en complétant leurs diagnoses. Ces faits nous conduisent parallèlement à revoir les Grégarines parasites des Plécoptères, ainsi que la valeur taxonomique de la morphologie des épimérites, à propos des espèces du genre *Ancyrophora*.

MATÉRIEL ET MÉTHODES

Les larves rhéophiles des Plécoptères proviennent du ruisseau Valea Cerbului, afluent droit de la Prahova, Massif des Bucegi, à 800 m. d'altitude; température de l'eau de 5–11°C. On a prélevé des échantillons répétés pendant plusieurs

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années (les mois d'avril – octobre) et quelques centaines de larves récoltées dans plusieures stations au long de la vallée ont été prospectées. Dans les récoltes de printemps prédomine l'espèce *Isoperla minima*, accompagnée d'*Arcynopterix compacta*, *Brachyptera seticornis* et *Perlodes microcephala*, tandis que dans les matériels d'automne prédomine *Arcynopterix compacta*, associée avec *Perlodes microcephala*.[1]

En disséquant des intestins des larves nous avons trouvé divers stades de développement des grégarines, observées sur le vivant ou fixées aux vapeurs osmiques (mesures, dessins à la chambre claire, microphotographies en contraste de phase). Sur des fragments des intestins parasités, fixés au Bouin alcoolique, on a pratiqué des coupes histologiques (colorations hématoxyline ferrique Heidenhain-érythrosine-orange G ou Azan et réaction APS.

RÉSULTATS

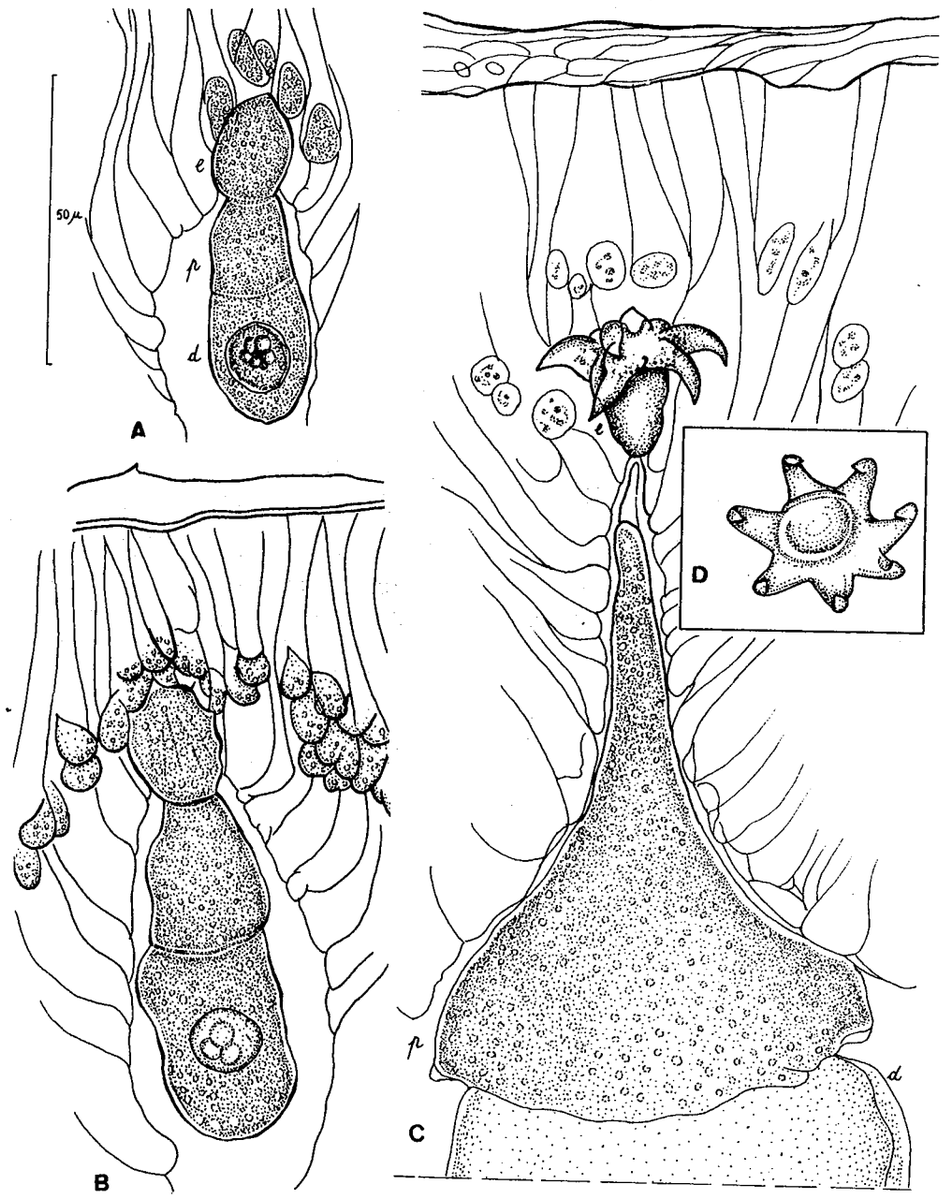
Chez les larves d'*Arcynopterix compacta* et *Isoperla minima* nous avons constaté des infestations grégariniennes à un pourcentage élevé (55–80 %) et en grand nombre, localisées dans le mésenteron, surtout dans les caecums digestifs d'un aspect crayeux. L'intestin postérieur ne contient que des stades de préenkystement et des kystes.

1. LA PHASE TROPHIQUE DU CYCLE DE DÉVELOPPEMENT ET LA DIFFÉRENTIATION DE L'ÉPIMÉRITE

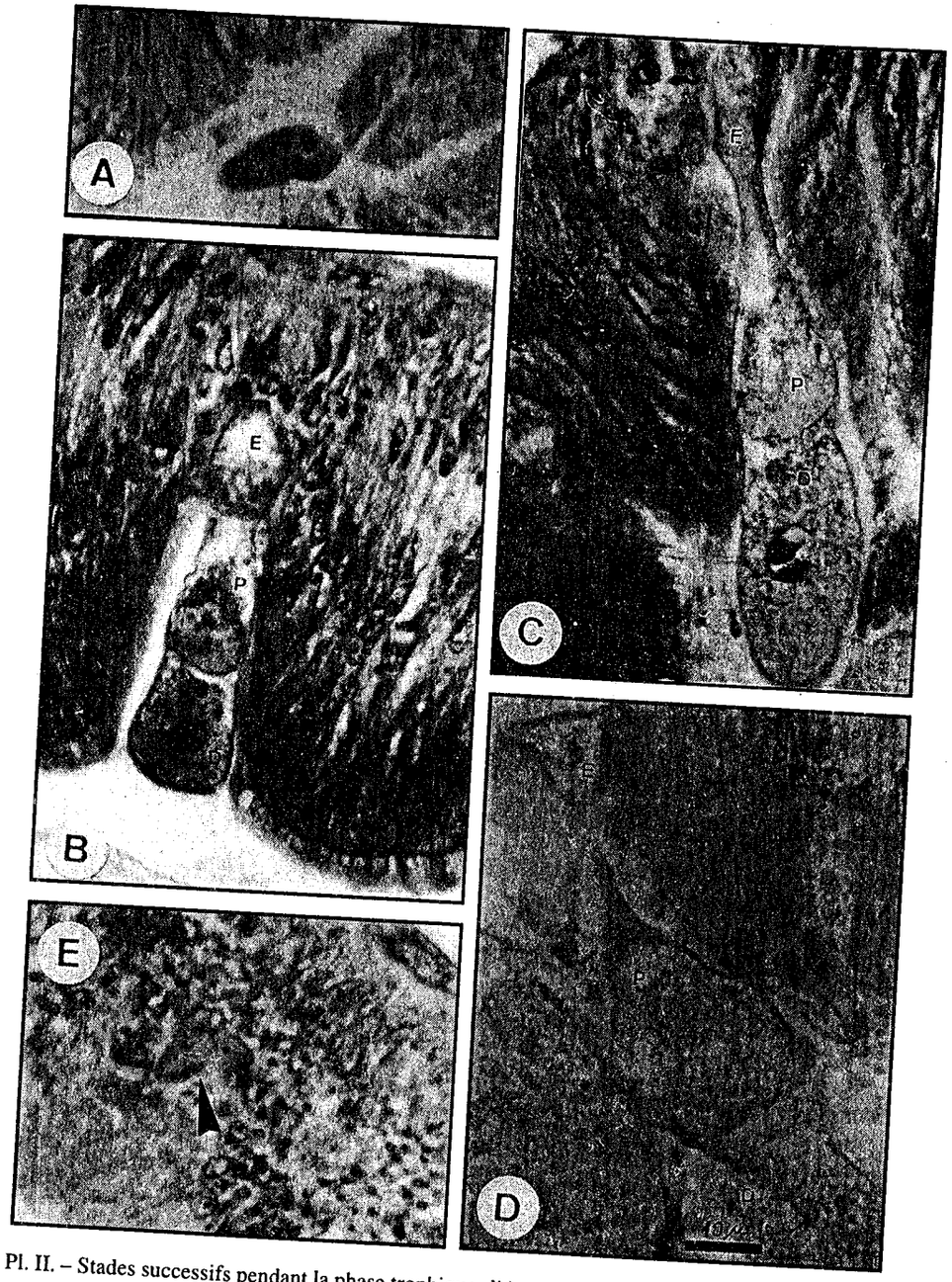
D'après la conformation des trophozoïtes, nous avons distingué 2 espèces, en infestations singulières ou mixtes. Celles-ci ont été atribuées au genre *Ancyrophora* Léger, 1892 du fait de leur type très caractéristique de l'épimérite, dont l'évolution est suivie sur le vivant (en dilacérant très attentivement les cellules intestinales auxquelles ils sont attachés) et surtout sur les coupes histologiques en série, pendant leur phase de croissance végétative, similaire chez les deux espèces et peu étudiée pour ce genre.

On n'a pas trouvé de stades intracellulaires. De très petits trophozoïtes, dont le corp cellulaire n'est pas cloisonné (Pl. II, A) s'attachent à l'épithélium digestif. Ils s'allongent en se découpant en 3 segments: l'antérieur, qui sert à la fixation est constitué par un col prolongeant le protomérite et terminé en avant par un renflement sous la forme d'une massue lisse, s'enfonçant dans la cellule intestinale-hôte. Chez de jeunes céphalins d'une taille de 40–50 µm, cette région représente un tiers ou la moitié du corps de la grégarine (Pl. I, A; Pl. II, B). Ultérieurement, l'épimérite

[1] Nous tenons à remercier Mr. le Prof. Béla Kis de l'Université de Cluj qui nous a si aimablement aidé dans ces déterminations.



Pl. I. – Différentiation de l'épimérite pendant la phase trophique du développement chez *Ancyrophora codreanui*. **A**–jeune céphalin tricystidé attaché à l'épithélium intestinal; **B**–céphalin pendant la croissance de sa partie extracellulaire; **C**–céphalin adulte, ayant l'épimérite intracellulaire complètement différentié; **D**–couronne de crochets épiméritiques, vue transversale (e–épimérite; p–protomérite; d–deutomérite). (Coupes histologiques, dessins à la chambre claire, échelle commune.)



Pl. II. – Stades successifs pendant la phase trophique *d'Ancyrophora obtusa*. **A**–trophozoïte initial au cours de son attachement à l'épithelium intestinal; **B**–très jeune céphalin ayant la tête épiméritique intracellulaire d'une taille maximale; **C**–jeune céphalin pendant la croissance de sa portion extracellulaire; **D**–différentiation complète de l'épimérite; **E**–épimérite intracellulaire (flèche) détaché du reste du céphalin (E–épimérite; P–protomérite; D–deutomérite). (Coupes histologiques, photos échelle commune).

et son long diamérite ne vont presque pas augmenter, alors que se sont le protomérite et surtout le deutomérite qui subiront un accroissement considérable (Pl. I, B; Pl. II, C).

Le protomérite en forme de cloche haute, présente au début du développement un rhytme plus intense de croissance, atteignant bientôt la taille maximum et sa forme définitive. Dans la phase suivante, c'est le deutomérite qui s'accroît jusqu'à sa taille maximale, prenant la forme caractéristique pour chacune de deux espèces (Pl. I, C; Pl. II, D). L'épimérite inclus entièrement dans la cellule-hôte, n'acquiert que tardivement sa complète différentiation au terme de la croissance des céphalins (taille maximum de 200–250 µm), attachés au paroi intestinale. Il est constitué d'une tête ovoïde, en massue (25×15 µm) qui porte dans sa partie supérieure une couronne étoilée de 6–8 appendices forts, acuminés, relativement courts et recourbés arrière. L'appareil épimeritique s'attache au protomérite par un col diaméritique qui diffère par sa longueur chez les deux espèces, ainsi que les détails des appendices (Pl. I, C–D; Pl. II, D–E).

Les céphalins âgés se détachent de l'épithélium intestinal par une rupture entre le protomérite et l'épimérite (Pl. II, D), qui reste en place dans la cellule-hôte, apparent sur les coupes (Pl. II, E-flèche). Les sporadins ainsi libérés dans la cavité digestive ont leur extrémité antérieure réduite à une vésicule cicatricielle, ou, le plus souvent, à un simple mucron (Pl. III, 1, 4, 5; Pl. IV, 5, 6). Ils restent continuellement solitaires, ne s'accouplant en syzygies tardives, frontales qu'avant l'enkystement. Les gamonts (Pl. III, 6; Pl. IV, 7) ont un aspect massif (180×145 µm), avec de gros noyaux (20 µm de diamètre). Les gamontokystes sphériques, d'un diamètre de 120–200 µm, passent dans l'intestin moyen.

2. LES DIAGNOSES DES ESPÈCES ÉTUDIÉES

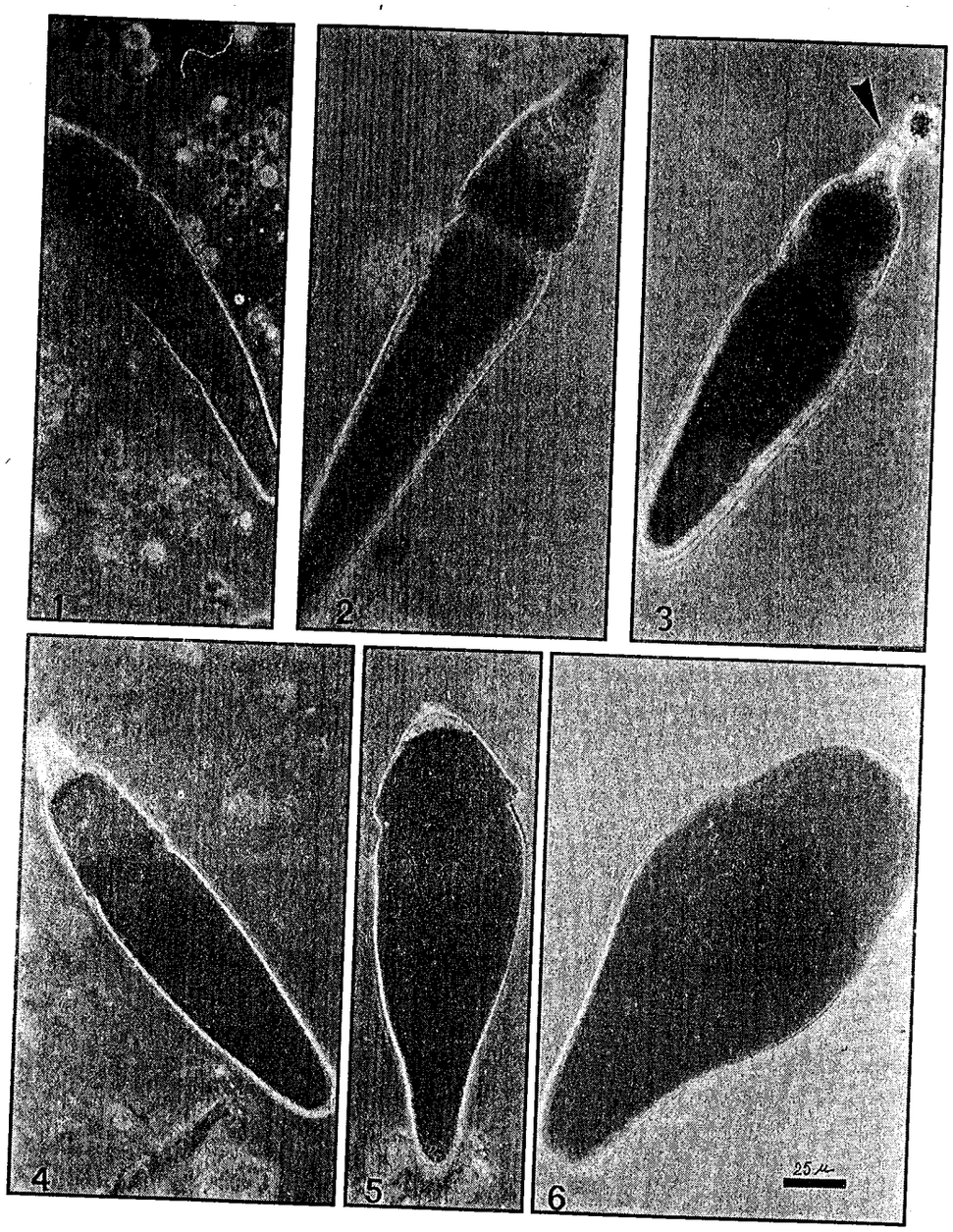
Ancyrophora codreanui Bălcescu-Codreanu, 1973[2]

(Fig. 1; Pl. III)

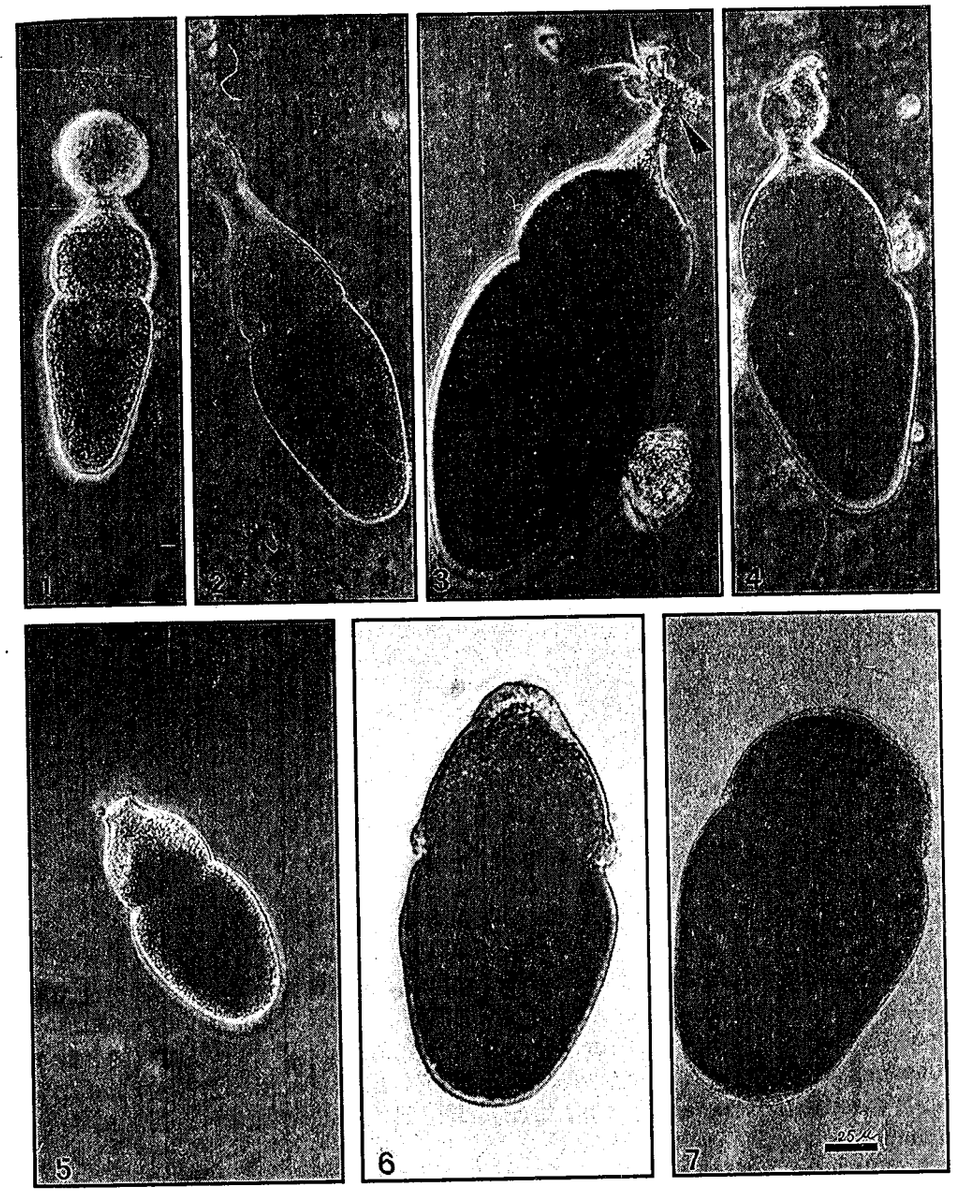
Les trophozoïtes d'une allure svelte, avec le deutomérite acuminé à l'extrémité caudale.

Les plus jeunes céphalins sont étroits, aigus, d'un aspect cunéiforme et mesurent 40–50 µm de long sur 24 µm de largeur maximum. Les céphalins plus âgés, dont la taille atteint 240–250 µm sur 50 µm de large, ont une forme générale allongée; ils sont acuminés à l'extrémité postérieure et exécutent de très vifs mouvements, surtout des contractions latérales (Fig. 11, A; Pl. III, 1–4). L'épimérite a la forme d'une massue d'env. 25 µm d'hauteur, pourvue d'une couronne apicale de 6–8 crochets, courts et pointus, recourbés en arrière (Pl. I, C–D; Pl. III, 3). Il

[2] Respectueusement dédiée à Radu Codreanu (1904–1987), réputé biologiste, membre de l'Académie Roumaine, pour ses contributions dans le domaine de la protistologie.



Pl. III. – *Ancyrophora codreanui* Bălcescu-Codreanu, 1973. 1–4 – différents aspects des céphalins détachés de l'épithélium intestinal; 5–6 – sporadins. («In toto», fixation osmique, photos échelle commune.)



Pl. IV. – *Ancyrophora obtusa* Bălcescu-Codreanu, 1973. 1–5 – différents aspects des céphalins détachés de l'épithélium intestinal; 6–7 – sporadins âgés. («In toto», fixation osmique, photos échelle commune.)

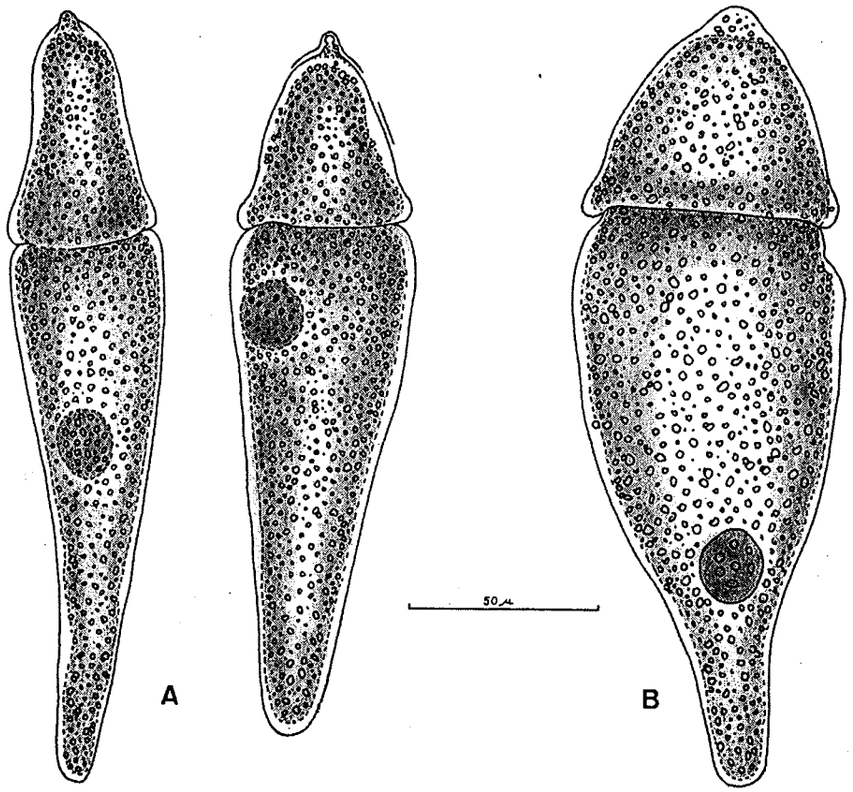


Fig. 1. – *Ancyrophora codreanui* Bălcescu-Codreanu, 1973. A–céphalins libérés dans la lumière intestinale (taille maximale); B–sporadin avant l'accouplement en syzygie. («In toto», fixation osmique, dessins à la chambre claire.)

s'attache au protomérite par un long (15 μm) col diaméritique hyalin. Le protomérite d'aspect conique-haut, a son rebord caudal élargi, entourant comme un épaississement la partie supérieure du deutomérite. Le rapport du protomérite à la longueur totale est de 1:4. Le deutomérite allongé est éfilé dans son tiers caudal, où le cytoplasme a un aspect raréfié, contenant le noyau arrondi (12–15 μm de diamètre).

Les sporadins conservent la même forme générale que les céphalins (Fig. 1, B; Pl. III, 5–6).

Les kystes sphériques, d'un diamètre de 125–135 μm.

Les indices morphométriques de l'espèce (sporadins solitaires):

TL	LP	LD	LP:TL	WP	WD	WP:WD	N
200μm	49μm	150μm	1:4	35μm	41μm	1:1.1	13μm

L'espèce a été trouvée dans des infestations singulières chez des larves d'*Arcynopterix compacta* (Mac Lachlan, 1872) recoltées au mois de septembre–octobre; le pourcentage d'infestation de 80%. Dans la même hôte, mais dans des récoltes de printemps (avril–juin) cette espèce peut coexister jusqu'à 55 % pourcentage d'infestation avec *Ancyrophora obtusa*.

Ancyrophora obtusa Bălcescu-Codreanu, 1973
(Fig. 2; Pl. IV)

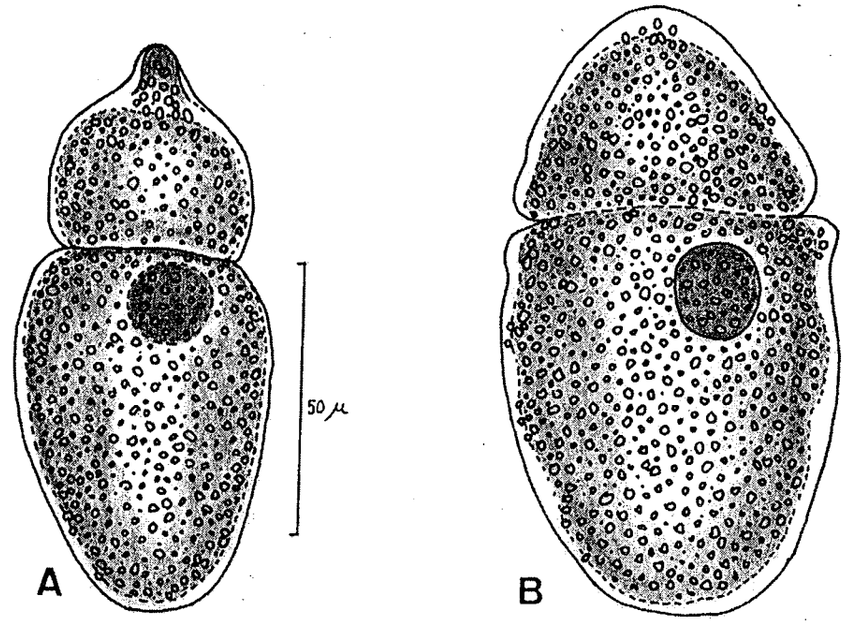


Fig. 2. – *Ancyrophora obtusa* Bălcescu-Codreanu, 1973. A–céphalin libéré dans la lumière intestinale; B–sporadin (tailles moyennes). («In toto», fixation osmique, dessins à la chambre claire.)

Les trophozoïtes ont l'aspect général volumineux, avec le deutomérite rebondi, à l'extrémité postérieure arrondie.

Les plus jeunes céphalins, d'une longueur de 40–50 μm, en forme d'ovale-arrondie, ont la largeur maximale de 40 μm. Les céphalins adultes ont une forme générale ovale, robuste, dilatée vers le milieu et arrondie au pôle caudal (Fig. 2, A; Pl. IV, 1–5); ils peuvent atteindre 225 μm de long sur 100 μm de large. L'épimérite a une tête ovoïde (20×15 μm) munie à l'extrémité apicale de 5–7 prolongements courts pétaliformes disposés en étoile, avec les pointes récurrentes (Pl. II, D–E). Il se rattache au protomérite par un con diaméritique. Le protomérite, en forme d'une voûte, a la marge postérieure rétrécie au-dessus du deutomérite,

qui le déborde. Le rapport du protomérite à la longueur totale est de 1:3. Le deutomérite a une forme ovale, rebondie, avec le bord caudal arrondi; le noyau est situé au-dessous du septum proto-deutomériticque.

Les sporadins conservent le même aspect général (Fig. 2. B; Pl. IV, 6–7).

Les kystes sphériques mesurent 200 µm de diamètre.

Les indices morphométriques de l'espèce (sporadins solitaires):

TL	LP	LD	LP:TL	WP	WD	WP:WD	N
210µm	72µm	138µm	1:3	82µm	105µm	1:1.2	15µm

On a trouvé cette espèce dans des infestations singulières chez des larves d'*Isoperla minima* Illies, 1953 dans des récoltes de printemps (avril–juin), le pourcentage d'infestation étant d'env. 75%. Pendant la même saison, on la trouve souvent chez des larves d'*Arcynopterix compacta* en association avec *Ancyrophora codreanui* (55% pourcentage d'infestation).

DISCUSSION ET CONCLUSIONS

1. LA VALEUR TAXONOMIQUE DES ÉPIMÉRITES CHEZ LE GENRE *ANCYROPHORA*

Les épimérites d'Eugrégarines tricystidées sont très variables et leur morphologie constitue un des critères taxonomiques essentiels, notamment au niveau générique (12, 17, 19). C'est ainsi le cas du genre *Ancyrophora* Léger, 1892 dont le type très particulier de l'épimérite, constitué par «une tête garnie d'**appendices flexibles ou rigides et recourbés en crochets**», auquel s'ajoutent les caractères du sporocyste (biconique, épineux, avec des aigrettes de soies polaires et une couronne de six soies équatoriales) sont utilisés par son auteur pour le définir (15).

Chez les plus de 15 espèces, atribuées ultérieurement à ce genre (1, 2, 4, 5, 6, 11, 13), on a constaté une large diversité dans la forme de la «tête» et des appendices épiméritiques, dont la disposition n'est pas précisée dans la description initiale de Léger (1892). Ainsi, Tuzet et coll. (19) ont limité le genre *Ancyrophora* à des épimérites constitués par «une tête garnie d'**appendices flexibles ou rigides, recourbés ou non en crochets, insérés en étoile** autour de cette tête». En transférant les espèces *A. cervicornis*, *A. puytoraci* et *A. tuzetae*, ainsi que les espèces *Cometoides licini* et *C. wellmeri* dans le genre *Ramicephalus* Obata, 1953, ils ont complété la diagnose initiale de ce dernier genre: épimérites avec «une tête garnie d'**appendices flexibles ou peu ou pas rigides, insérés sans ordre apparent** sur la surface de cette tête… donnant à l'épimérite un **aspect hirsute**». Donc, c'est le mode d'insertion des appendices autour de la «tête» épimériticque qui sépare les genres *Ancyrophora* et *Ramicephalus*, dont les sporocystes sont assez semblables.

D'autre part, Baudoin (2, 4) souligne l'originalité d'*Ancyrophora*, notamment par la **grande variabilité morphologique d'un type d'épimérite** à travers les différentes espèces du genre, c'est-à-dire «un épimérite **globuleux ou discoïde**, sur lequel s'insèrent des **appendices en forme de tentacules ou de crochets**, qui se disposent sur **une couronne apicale ou sur 2 couronnes ou bien encore sur toute la surface sphérique** de l'épimérite. Leur forme est généralement **simple**, mais chez quelques espèces, on observe une **tendance à la bipartition**». Cet auteur propose même une clef ayant comme principal élément la forme de la tête et des appendices et leur disposition, pour distinguer les 8 espèces considérées dans ce genre, qu'il sépare du genre *Rhizionella* Baudoin, 1971, caractérisé par «l'épimérite en bouton porteur de longs appendices filamenteux ascendants».

La morphologie de l'épimérite chez *Ancyrophora codreanui* et *A. obtusa*, dont les descriptions nous venons de compléter dans ce travail, confirme leur appartenance au genre *Ancyrophora*, même «dans le sens restreint» de Tuzet et coll. (19). Mais, à propos de nos deux espèces, si on compte la forme ovoïde, d'une massue, de leurs têtes, il faudrait élargir en plus la définition des épimérites d'*Ancyrophora* proposée par Baudoin (4), c'est-à-dire «tête globuleuse ou discoïde». Cet auteur même indiquait pour l'espèce *A.cornuta* (6) encore une autre forme, respectivement «d'**un mamélon**».

Les espèces *A. codreanui* et *A. obtusa* ont leurs épimérites assez semblables, mais elles diffèrent nettement par l'allure du corps, surtout par la forme de leurs deutomérites, ainsi que des protomérites, et par leurs indices biométriques.

Peut-être qu'il faut penser, d'une part, à l'hiérarchie des critères taxonomiques dans la systématique des Grégarines Acanthosporinae, et d'autre part, aux limites des variations morphologiques de ces caractères, au moins dans le cadre du genre et de l'espèce. Ainsi, l'établissement des limites de la variabilité morphologique d'un type d'épimérite et des soies des sporocystes pourrait soutenir la révision taxonomique des genres étroitement apparentés, comme *Ancyrophora*, *Cometoides*, *Ramicephalus*, *Rhizionella*.

2. LE STATUT SYSTÉMATIQUE DES GRÉGARINES PARASITES DES PLÉCOPTÈRES

Initialement, Foerster (1938) a établi chez des larves de Plécoptères l'espèce *Gregarina nemurae*; Stein (1960) la transfère dans le genre *Pileocephalus*, en considérant que Foerster n'a pas bien regardé les épimérites. En accord avec Stein, Geus (1969) décrit *P. nemurae* chez des larves de *Perlodes*. D'autre part, Desportes (1963), pensant aussi que Foerster n'a pas observé que des sporadins, maintient le genre *Pileocephalus*, mais elle n'accepte pas l'espèce *nemurae*; d'après les figures de Stein (18), elle considère qu'il s'agit de *P. chinensis*, commune chez les Tri-

choptères, espèce qu'elle-même la retrouva chez *Isoperla*. Il est vrai, que ces auteurs (8, 11, 18) n'ont pas revu le matériel-type de Foerster, retrouvant «son» espèce chez d'autres genres-hôtes de Plécoptères et dans des régions différentes d'Europe. Malheureusement, aucun auteur n'a vu les sporocystes de cette grégarine des Plécoptères.

Pour la première fois, nous avons établi (1) la présence du genre *Ancyrophora* chez les Plécoptères, par les espèces *A. codreanui* Bălcescu-Codreanu, 1973 et *A. obtusa* Bălcescu-Codreanu, 1973; plus tard, Baudoin et Mouthon (1976) ajoutent une nouvelle espèce, *A. cornuta*. Ainsi, le genre *Ancyrophora* reste inféodé, comme toutes les Acanthosporinae, aux Insectes carnassiers surtout aquatiques (12) tels: larves de Plécoptères et de Trichoptères, larves ou imagos de Coléoptères (Fam. Caraboidae, Dytiscidae, Silphidae, Staphylinidae, Hydrophilidae) et larves d'Odonates (13).

Les observations de longue durée, que nous avons fait sur un très riche matériel concernant nos espèces d'*Ancyrophora*, ont relevé la difficulté de mettre en évidence chez ce genre l'appareil épiméritique complètement différentié. La plupart des stades vivants obtenus en disséquant les intestins des larves parasitées ont l'aspect des sporadins, d'une longueur totale de 50–250 µm, portant à leur extrémité apicale une proéminence conique hyaline (3–5 µm d'hauteur) (Fig. 1 A; Fig. 2 A), provenant probablement par le déchirement du diamérite pendant les manœuvres de dissection (Pl. III, 3; Pl. IV, 3–5). De même, à cause de la fixation solide de ce type morphologique d'épimérites, il peut arriver que les céphalins accrochent leurs cellules-hôtes en se détachant de l'épithélium intestinal (Pl. III, 2; Pl. IV, 1–2). Tels débris qu'entourent l'épimérite peuvent masquer sa vraie forme, en lui donnant, aussi bien que le réliquat du diamérite ou la vésicule cicatricielle, l'apparence d'un «bonnet chinois», suggérant facilement une fausse appartenance au genre *Pileocephalus* (Pl. III, 2, 4; Pl. IV, 2–4). Il y a des figures d'autres auteurs, qui pourraient aussi soutenir cette possibilité; (8)–Fig. 14 B, p. 359 et (12)–Fig. 507 C, p. 653 pour *P. chinensis*; (11)–Fig. 223, p. 386 pour *P. nemurae* et même pour *Ancyrophora puytoraci*, (2)–Pl. XIV, Fig. 3, p. 77.

Comme nous l'avons déjà signalé (1), c'est pourquoi les données des auteurs sont à réviser, non seulement à propos des Grégarines des Plécoptères, mais aussi concernant la présence de ce genre chez les Trichoptères. L'altération rapide des appendices épiméritiques ou son aspect mutilé peuvent empêcher la détermination taxonomique correcte du genre *Ancyrophora* et c'est pourquoi Geus (11) transfère aussi *Gregarina similis* Foerster, 1938 dans ce genre. Rappelons que c'est le même motif de la fragilité des épimérites qui a conduit Baudoin (4) à séparer son nouveau genre *Rhizionella* du genre *Ancyrophora*.

Quant à la signification de la diversité morphologique des épimérites, elle reste encore confuse (17). Ayant pu mettre en évidence chez nos espèces (sur le vivant et sur les coupes histologiques) la différentiation tardive, à la fin de la phase de croissance, d'une couronne de 2–8 appendices acuminés, caractérisant le genre

Ancyrophora, ces crochets forts et recourbés dans l'épithelium intestinal suggèrent un rôle d'encrage solide de l'épimérite, pour les céphalins arrivés à leur taille maximale assez importante (200–250 µm); ceux-ci vont se détacher de leurs cellules-hôtes juste avant leur accouplement et l'enkystement.

Ces trophozoïtes restant pendant toute leur phase végétative attachés et abrités dans les nombreuses criptes des caecums intestinaux, dont les lumières très étroites sont bien agglomérées, pour que les grégarines exécutent de vifs mouvements, ils nous semble un peu difficile de mettre en corrélation stricte ce type de fixation avec le régime carnassier des hôtes, en ce qui concerne l'agilité des larves de Plécoptères, comme le pensent Baudoin et ses colaborateurs (2, 3, 5, 6). Ces auteurs (6) opposent la présence d'*A. cornuta*, liée au régime carnivore des larves de la Fam. des Perlidae (dont fait partie son hôte *Dinocras megacephala*) aux espèces du genre *Pileocephalus* (*P. nemurae* et *P. chinensis*) qu'ils considèrent propres aux larves herbivores. Mais, ce n'est pas tellement exact, car, à part les genres-hôtes *Nemoura* et *Leuctra* (Plecoptera, sous-ordre Holognatha – phytophages), *P. nemurae* a été retrouvée chez des larves de la Fam. Chloroperlidae (10, 18) et Fam. Perlodidae (g. *Perlodes*) (11); *P. chinensis* a été aussi cité chez *Isoperla* de la même famille (8). Aussi que les Perlidae, les deux dernières familles appartiennent au sous-ordre Systellognatha des Plecoptera (14), dont les larves sont surtout carnivores, ou phytozoophages.

Nous-mêmes, nous avons trouvé *Ancyrophora obtusa*, spécifique chez *Arcynopterix compacta* et *Isoperla minima* (Fam. Perlodidae), rarement (au-dessous de 10% pourcentage d'infestation et très faible pouvoir d'invasion) chez *Brachyptera seticornis*, un autre Holognathe phytophage.

Comme nous l'avons déjà signalé (1, 7), les deux espèces que nous venons de décrire, peuvent coexister chez une même-hôte. Les états polyparasitaires sont assez peu connus chez des invertebrés; nous les avons rencontré surtout chez des crustacés Amphipodes et Phyllopodes, ou chez des larves d'insectes Ephéméroptères et Lépidoptères (7); Baudoin (1967, 1971) et Desportes (1963) citent des infestations grégariniennes simultanées chez des larves de Trichoptères et de Coléoptères. Telles infestations mixtes arrivent fréquemment dans des populations isolées ou dans les biocénoses simples, dans des biotopes homogènes et limités, assez restrictifs, comme c'est le cas des habitats rhéophiles, ou coexistent des larves de Plécoptères, Ephéméroptères, Trichoptères et Gammarides. Le polyparasitisme est favorisé par une faible résistence des hôtes et par l'influence réciproque des parasites, aussi que par des facteurs du milieu ambiant, notamment par le biais de la nourriture (5, 8). C'est aussi par l'apport de la nourriture qu'on peut expliquer le taux réduit d'infestation (au-dessous de 20%) avec *Ancyrophora codreanui*, qu'on trouve dans des récoltes de printemps et d'automne chez des larves de *Perlodes microcephala*, ou avec *A. obtusa* chez *Brachyptera seticornis*, dans des récoltes de printemps; pour ces espèces de plécopteres-hôtes on les considère comme des infestations accidentales, «de biotope».

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IN VIVO AND *IN VITRO* STUDY OF CHONDROITIN SULPHATE UPTAKE AND DISTRIBUTION IN OOCYTES OF CRUCIAN CARP *Carassius auratus gibelio*

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Chondroitin sulphate (Mr~ 32,000) isolated from bovine tracheal cartilage labelled with fluorescein isothiocyanate (FITC) was used to study the uptake and distribution into developing oocytes of crucian carp, *Carassius auratus gibelio*.
Fluorescein-labelled chondroitin sulphate accumulated *in vivo* and *in vitro* in pre and vitellogenic follicles.
In the previtellogenic follicles accumulation was observed in all the cytoplasm except for cortical alveoli. In these oocytes that internalized chondroitin sulphate, fluorescence was detected in the nucleoplasm and nucleoli.
In the vitellogenic follicles fluorescent chondroitin sulphate accumulates in vesicles among cortical alveoli and yolk platelets.

Vitellogenesis is the principal event responsible for the enormous growth of oocytes in many teleosts and may account far as much as 95% of the final egg size (40). This is the period of ovarian development when extraovarian proteins are sequestered, processed and packaged into oocytes.

Vitellogenin provides most of the amino acids, lipid and calcium for the growing oocyte, but there are an array of other substances that although present in far lower quantities are equally necessary for life, such as vitamins, hormones, many metals (required for enzyme activity). Some of these substances may pass into the oocyte attached to vitellogenin (VTG), others adventiously in the fluid phase during the receptor-mediated uptake of VTG, but most of these substances probably enter quite specifically bound to distinct, specific receptors (40).

Numerous studies on other tissues have suggested that glycosaminoglycans (GAG_S) composition of the extracellular matrix plays an important role in controlling cell migration, proliferation and differentiation during development (17, 38). A large number of embryonic cells that are not precursors of cartilage cells synthesize sulphated GAG_S including chondroitin sulphate during early stage of development (21, 22, 24, 27). Furthermore, in *Rana pipiens* following injections of embryos with ^{35}S-sulphate, incorporation can be detected in unfertilized and fertilized eggs along the periphery of yolk platelets (20).

Biochemical analysis of isolated GAGs showed that the principal component in early metamorphosis larvae of bonefish was a type of keratan sulphate while in advanced larvae the predominant component was a type of chondroitin sulphate and it is proposed that GAGs breakdown may be directly related to larval water loss during this period (30).

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The function of GAGs is an important example of polysaccharide diversity and specificity with high impact in development (8). Hyaluronan, chondroitin sulphate and heparan sulphate have all been reported to be associated with chromatin in nuclei (9, 14, 37). In addition glycosaminoglycans are internalized and processed by alternate intracellular pathways which generate GAGs fragments.These fragments have been shown to accumulate in nuclei (10, 19, 44). It is possible that GAGs fragments could regulate cell growth at a nuclear location (19).

The present communication describes a morphological study on endocytosis of chondroitin sulphate in developing oocytes of crucian carp, *Carassius auratus gibelio.*

MATERIALS AND METHODS

ANIMALS

The experiments were carried out on crucian carp, *Carassius auratus gibelio*, obtained from the Fisheries Research Farm Nucet. The specimens were acclimatized to laboratory conditions for two or more weeks, at room temperature, in glass aquaria containing tap water.

CONJUGATION OF CHONDROITIN SULPHATE WITH FLUORESCEIN

The chondroitin sulphate (50 mg/ml) dissolved in 0.01 M carbonate/bicarbonate buffer, pH-9.2 containing 0.15 M NaCl was incubated with fluorescein isothyocyanate (15 mg/ml) at 4°C, overnight. To remove unreacted dye the solution of CS-FITC was filtered through filter paper and dialysed at 4°C against sodium phosphate buffers 0.2 M, pH-7.2, overnight. The nondiffusible material was then purified by gel filtration on a Sephadex G-50 column and eluted with phosphate buffer 0.2 M, pH-7.2. The fractions were concentrated by evaporation to a final concentration of 1.5 mg/ml uronic acids.

UPTAKE OF CS-FITC IN VIVO

Each fish was injected intraperitoneally (I.P) with 1 ml of CS-FITC. Animals were kept in aquaria at 20°C and sacrificed at 24 hours after a single injection.

UPTAKE OF CS-FITC IN VITRO

The ovarian follicles from female crucian carp were dissected and maintained in culture medium for 1 hour before the incubation had been started. After this period follicles were examined under a dissecting microscope and used in the

subsequent cultures only those maintaining the appearance of freshly dissected follicles. The ovarian follicles were cultured in Leibovitz (L-15) complete growth medium (Sigma, USA). Both Penicillin G (250 U.I/ml) and streptomycin sulphate (250 µg/ml) were added. The medium was adjusted to pH-7.8 by using 1 N HCl and then sterile filtered by using 0.22 µm filters (Sartorius AG, Germany). Individual ovarian follicles or lamella (containing 10-15 follicles) were incubated in 1 ml culture medium with 10% fetal calf serum (Gibco, USA) and 50 µg/ml CS-FITC in flat-bottom 24 well polystyrene culture plates with lids (Corning, New York). After 1 and 4 hours incubation follicles were washed three times in L-15 containing bovine serum albumin (BSA, Merck, Germany).

HISTOLOGICAL PROCEDURES

Small pieces of tissue dissected from ovary of fish injected I.P with CS-FITC and ovarian follicles cultured in medium with CS-FITC were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH-7.2), for 4 hours at room temperature. After fixation samples were rinsed with 0.1 M cacodylate buffer, dehydrated in ethanol and embedded in paraffin. The sections were examined in a Zeiss fluorescence microscope.

BIOCHEMICAL CHARACTERIZATION OF CHONDROITIN SULPHATE

The following investigations of chondroitin sulphate were performed:
- uronic acids according to Bitter and Muir's method (3);
- hexosamines according to Elson-Morgan's method (7);
- total nitrogen according to Kjeldahl's method (6);
- total sulphate estimated by the gravimetric method (29);
- molecular weight according to the viscosimetric method (31).

RESULTS

To determine the cellular site of uptake, CS-FITC was injected I.P and dissolved in culture medium. Sections of the ovary and cultured follicles were examined in the fluorescence microscope.

Chondroitin sulphate consists of alternating D-glucuronic acid and N-acethyl D-galactosamine units and the most common sites of sulphation are the 4 and/or 6 position of the N-acetyl D-galactosamine unit (26).

Table 1 shows the biochemical characteristics of chondroitin sulphate isolated by us from bovine tracheal cartilage and used for endocytosis experiments.

The ovary of crucian carp used in vivo and in vitro experiments characterize by the presence of previtellogenic and vitellogenic follicles. The follicle consists

of an oocyte surrounded by a sheath of follicle (or granulosa) cells, a vascularized thecal layer containing capillaries within a connective tissue meshwork and a thin surface epithelium.

Table 1

Analytical determination for chondroitin-sulphate extracted from bovine tracheal cartilage

Determination	Chondroitin-sulphate
Uronic acids*	25
Hexosamines*	18.1
Total nitrogen*	5.3
Sulphate*	9.6
Average mol.wt.*	32,000

* *The values are related to % of weight of dry substance.*

UPTAKE OF CS-FITC IN VIVO

In the previtellogenic follicles the fluorescence appears in all cytoplasm except cortical alveoli (Fig. 1A). In these follicles there are a cortical and perinuclear ring which exhibit a higher fluorescence. In the vitellogenic follicles the chondroitin sulfate-FITC was observed in the endosomes that appear as very bright fluorescent spots (vesicles) among cortical alveoli and yolk platelets (Fig. 1B). Apart from these localizations a weak fluorescence was identified in the nucleoplasm and the nucleoli of previtellogenic follicles (Fig. 1C).

UPTAKE OF CS-FITC IN VITRO

After 1 hour of incubation in a medium containing CS-FITC the fluorescence was observed in follicle cells around the previtellogenic oocyte with cortical alveoli (Fig. 2A). Uptake of fluorescent ligand was significantly higher at 4 hours of incubation when the fluorescence was localized in the cortical cytoplasm of previtellogenic (Fig. 2B) and vitellogenic follicles. Moreover, follicle cells detached from oocytes exhibit an intense fluorescence (Fig. 2C).

DISCUSSION

It has generally been assumed that the oocyte is a storage depot for many of the macromolecular components required by the developing embryo, for its metabolic and nutritional needs as well as for rapid cellular proliferation.

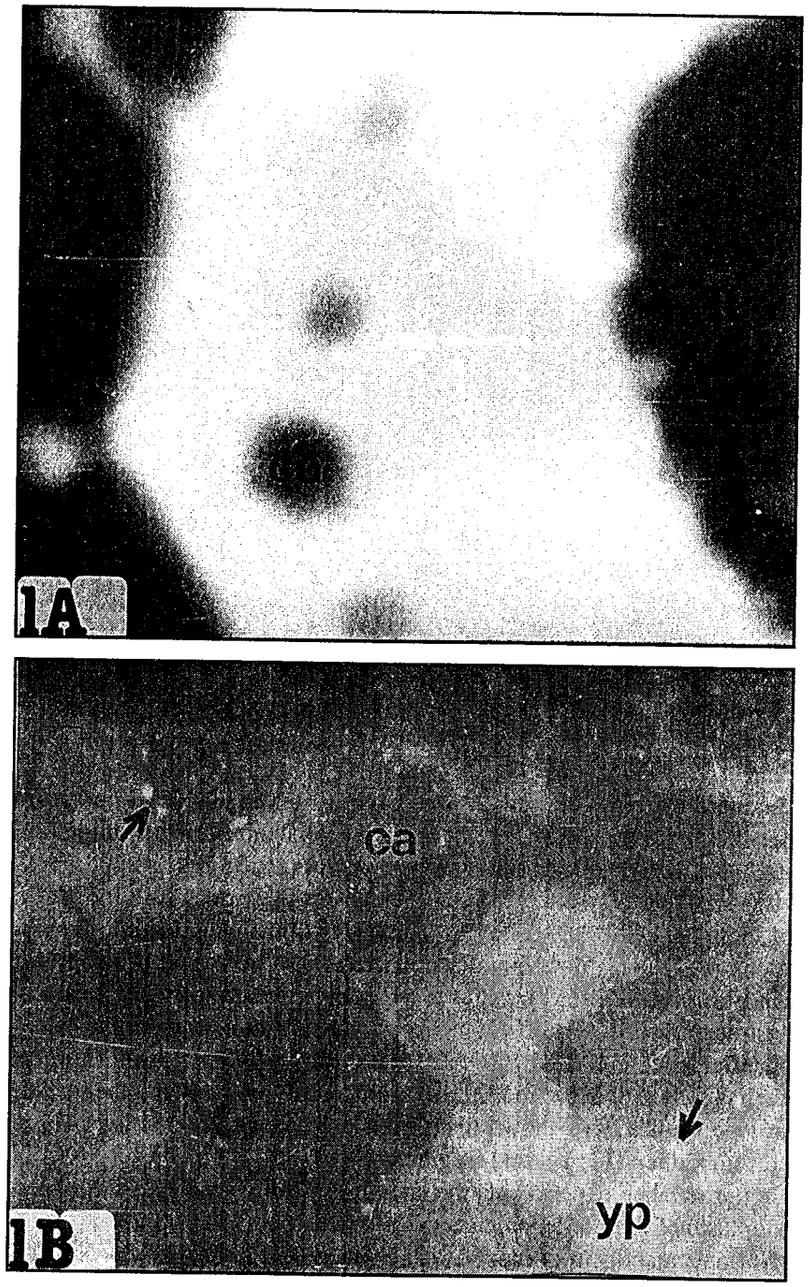

Fig. 1. **A-C** – *Uptake of CS-FITC in vivo.* **A** – In the previtellogenic oocytes the fluorescence appears in all the cytoplasm except for cortical alveoli (c.a); n-nucleus. **B** – In the vitellogenic follicles the CS-FITC was observed in the vesicles (arrows) among cortical alveoli (c.a) and yolk platelets (y.p)

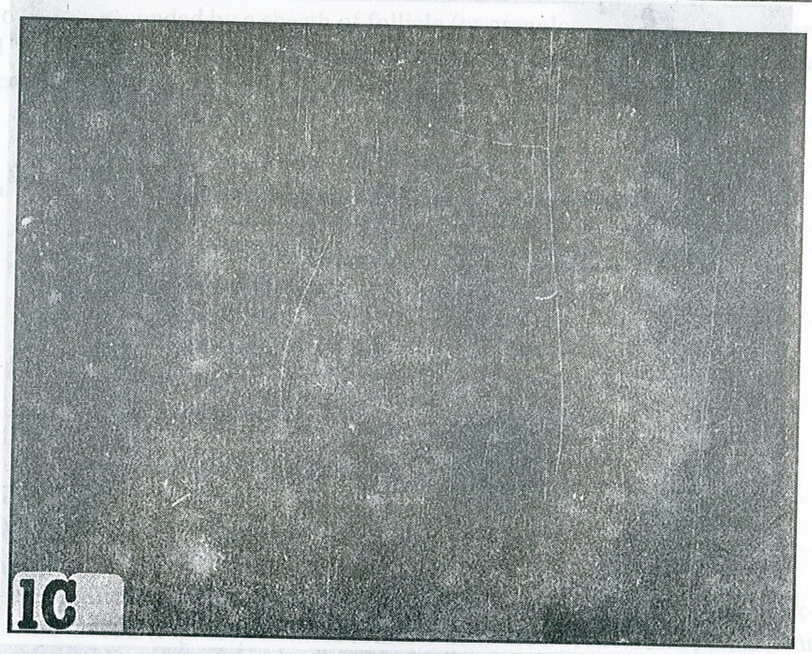

Fig. 1. **C** – In the previtelogenic follicles a weak fluorescence was identified in the nucleoplasm and nucleoli (n).

Numerous studies on other tissues have suggested that GAG composition of the extracellular matrix plays an important role in controlling cell migration, proliferation and differentiation during development (16, 38).

It is known that many cells taking up GAGs by endocytosis and internalization of these molecules are followed by intralysosomal degradation. Previous observations performed in mammalia indicated that GAGs and proteoglycans are efficiently internalized *in vivo* and *in vitro* by a variety of cells. These studies demonstrated the uptake of hyaluronan by liver sinusoidal endothelial cells (4, 25, 34), Kupffer cells (2), decorin by bovine aortic endothelial cells (15), rat liver endothelial cells (36) and chondroitin sulphate also by liver endothelial cells (23, 25). *In vivo* studies have shown that GAGs are taken up by the liver (11, 12, 23, 34), spleen, kidney (11, 12) and frog oocyte (44).

In our uptaken experiments the chondroitin sulphate-FITC was observed in the cytoplasm of previtellogenic and vitellogenic oocytes.

Normal catabolism of most GAGs metabolized in the body occurs in lysosomes and required a cohort of exoglycosidases and sulfatase which operate sequentially on nonreducing ends of the chains (16, 39). It has been clearly documentated that vitellogenin and other serum macromolecules are delivered to lyso-

Fig. 2. **A-C** – *Uptake of CS-FITC in vitro*. **A** – After 1 hour of incubation in medium containing CS-FITC the fluorescence was observed in follicle cells (arrow) around previtellogenic follicles. **B** – After 4 hours of incubation CS-FITC was observed in the cortical cytoplasm of previtellogenic follicles). **C** – Follicle cells that detached from oocytes exhibit an intense fluorescence (arrow).

somes (multivesicular bodies) but that lysosomes in oocytes (at least vitellogenic oocytes) are modified so that yolk degradation does not occur (41). Absence of degradation (or incomplete degradation) of chondroitin sulphate-FITC could explain the presence of fluorescence in vesicles at the periphery of yolk platelets.

Apart from the cytoplasmic localization we observed *in vivo* a weak fluorescence (spots) in nucleoplasm and nucleoli of previtellogenic follicles. In frog *Rana ridibunda* the presence of CS-FITC was also identified in the nucleus and nucleoli of the oocyte (44). If the presence of CS-FITC in vitellogenic oocyte could be explained on incomplete degradation of fluorescent conjugate in lysosomes appearance in previtellogenic oocyte is very intriguing and any statement relevant to its role can only be speculative. Furthermore in young oocytes of *Carassius auratus gibelio* arylsulfatase was located in the plasma membrane and at the level of nucleoli. Subsequently, in growing oocyte the enzyme was identified in lysosomes, macrovilli of the zona radiata, in the cortical granules and in close association with yolk platelets (33).

In rat ovarian granulosa cells (19, 42, 43) some proteoglycans are degraded after internalization by two different pathways. In the first pathway proteoglycans are degraded rapidly without generating appreciable amounts of degradative intermediates. In the second pathway proteoglycans are degraded more slowly, associated with the generation of distinct degradative intermediates.

It has been shown that heparan sulphate GAGs fragments specifically produced by granulosa cells could regulate cell growth at a nuclear location (19).

The exact mechanisms involved in the endocytosis of GAG have not been elucidated so far. Endocytosis is often considered to be mediated by clathrin-coated vesicles. However, several recent studies clearly indicate the existence of clathrin-independent endocytosis as well (5, 18, 32).

Uptake of chondroitin sulphate-FITC in our experiments *in vivo* and *in vitro* is not surprising because it was demonstrated that oocytes can take up various compounds, including heterologous ones if they are present in the necessary concentration either in the perioocyte space or in the incubation medium (1). Moreover, the molecular weight of chondroitin sulphate used in our experiments was very low (32,000 D) and it may be passed through small intercellular channels from follicular tissues of previtellogenic follicles. How this GAG are internalized by the oocyte (receptor mediated or fluid-phase endocytosis) should be further investigated.

The physiological function of glycosaminoglycans is not well understood although heparan sulphate has been suggested to play a role in regulation of cell growth. Recent data indicate a possible role for hyaluronan in *Xenopus laevis* reproduction/development and demonstrate the activation of an intracellular signalling pathway in oocytes resulting in a rise in intracellular Ca^{2+} and activation of a Ca^{2+}-dependent Cl^- current (13).

In conclusion our microscopic findings indicate that chondroitin sulphate-FITC is internalized by all the developing oocytes of crucian carp but the functional role of this GAG in fish oocyte should be further investigated.

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ELECTROPHORETIC PATTERNS OF YOLK PROTEINS DURING OOCYTE DEVELOPMENT OF CRUCIAN CARP, *Carassius auratus gibelio*

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Comparisons of yolk proteins in crucian carp, *Carassius auratus gibelio* during oocyte development were investigated using SDS-gel electrophoresis. Changes during oocyte development were observed in the abundance of yolk proteins.
The majority of yolk proteins in follicles measuring less than 0.58 mm in diameter appeared to be derived from sources other than vitellogenic. In contrast in the larger follicles all the major yolk proteins detected were derived from vitellogenin. Thus, in early vitellogenic stage yolk proteins migrated with molecular masses of: 96, 88 and 80 kDa whereas in middle vitellogenic follicles the major bands are represented by: 94, 80, 76, 66, 29.5, 21 and 20 kDa. In ovulated eggs five proteins comprised the major yolk constituents: 96, 92, 70, 27.5 and 23 kDa.

Vitellogenesis is the principal event responsible for the enormous growth of oocytes in many teleosts and may account far as much as 95% of the final egg size (32, 34). A female-specific serum protein, vitellogenin (VTG) which contains phosphorus, lipid, carbohydrate, calcium and iron, has been identified as an egg-yolk precursor in most oviparous vertebrates (1, 3, 36, 38). In vertebrates, VTG, is synthesized in the liver upon estradiol stimulation, transported in the plasma, sequestered in the oocyte by receptor-mediated endocytosis (8, 17, 27, 29, 33, 43) and cleaved into yolk proteins.

Native VTG circulates in the plasma as a dimer with molecular weights (MW) ranging from 300 kDa in tilapia, *Oreochromis aureus* (11) to 540 kDa in coho salmon, *Oncorhyncus kisutch* (15).

In a number of species female-specific serum lipoproteins or proteins have been detected by electrophoretic, chromatographic, gel filtration or ultracentrifugal procedures during the vitellogenic period of the annual reproductive cycle (10, 18, 19, 35, 37).

Yolk proteins from teleost oocytes are characterised and size classes have been described from a variety of species including rainbow trout, *Oncorhyncus mykiss* (14, 31), sea bass, *Dicentrarchus labrax* (7), gilthead sea bram, *Sparus aurata* (6), winter flounder, *Pleuronectes americanus* (25), killifishes, *Fundulus sp.* (12, 39, 40), gold fish, *Carassius auratus* (10), antarctic fish, *Chaenocephalus auratus* (28), threespined stickleback, *Gasterosteus aculeatus* (9), several species of salmon (15, 30), tilapia *Oreochromis mossambicus* (16), medaka, *Oryzias latipes* (13, 24), ocean pout, *Macrozoarces americanus*, lumpfish, *Cyclopterus lumpus* (42) and atlantic cod, *Gadus morhua* (26, 42). Moreover proteolytic cleavage of yolk proteins increased through ovarian development (16).

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In teleost fish the processing of VTG into yolk proteins results mainly in lipovitellins and phosvitins (37). These yolk proteins provide many essential nutrients for the developing and growth of the embryo (5, 23). Lipovitellins are lipid-rich proteins with MW in the range of 30 and 120 kDa (5, 23). Phosvitins are phosphorylated and in teleost fish are heterogeneous in size, ranging in MW from 10 to 400 kDa.

MATERIAL AND METHODS

Fish. Female crucian carp were purchased from Fisheries Research Farm-Nucet.

Sample collection. Ovarian follicles were collected from crucian carp throughout the reproductive season. Ovulated eggs were obtained at the end of the reproductive season from fully mature females.

Developing follicles were dissected out from the ovary, placed in 0.9% NaCl and divested of their connective tissues. The follicle size classes were selected based on diameter measurements and morphological appearance. In case of follicles measuring less than 0.352 mm in diameter whole lamellae containing the follicles were homogenized. After dissociation, 50 follicles and ovulated eggs were placed in 1 ml of 0.9% NaCl, 15 mM Tris-HCl pH-6.8, containing 2 mM PMSF, homogenised and centrifuged at 10,000 r.p.m., 15 min at 4°C. The resulting centrifugate consisted of an opaque fluid (yolk extract) between a surface layer of lipid and a small amount of precipitate.

The whole homogenate was obtained from fragments of late previtellogenic ovary that was homogenised in 0.9% NaCl, 15Mm Tris-HCl pH-6.8 with 2mM PMSF and filtered by two layers of gauze. The filtrate was centrifuged like ovarian follicles and ovulated eggs.

Blood samples collected from caudal blood vessels were allowed to clot at room temperature followed by centrifugation at 10,000 r.p.m., 15 min., 4°C to obtain clear serum.

Serum and yolk extract from ovarian follicles, ovulated eggs and whole homogenate were frozen at –20°C until use.

Electrophoresis. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20) was performed using 7.5 % polyacrylamide gel. Electrophoretic patterns of yolk proteins from oocytes in the various size classes, ovulated eggs and whole ovary homogenate were compared. The protein content of yolk protein samples was determined using Bradford's method (4). The same protein concentration (10 µg) of yolk proteins and serum per lane was used. The oocyte yolk extract and serum was diluted in SDS sample buffer (2% SDS, 1% β-mercaptoethanol, 10% glycerol, Tris-HCl 0.063 M pH-6.8) and heated on boiling water for 5min. Electrophoresis was performed at a constant current of 15 mA. Molecular mass markers (Sigma, USA) used for determination of the apparent molecular mass of the yolk proteins were: myosin (205 kDa); β-galactosidase (116 kDa); phosphorylase b (97.4 kDa); fructose-6-phosphate kinase (84 kDa); albumin (66 kDa); glutamic dehydrogenase (55 kDa); ovalbumin (45 kDa); glyceraldehyde 3-phosphate dehydrogenase (36 kDa); carbonic anhydrase (29 kDa); trypsinogen (24 kDa); trypsin inhibitor (20 kDa) and α-lactalbumin (14.2 kDa). Gels were stained for total protein with Coomasie Brilliant Blue R-250.

RESULTS

Preliminary experiments were carried out to establish the oocyte size classes and classify them according to developmental stages. Follicles were grouped into size categories through development based on preliminary studies using follicle diameter (mm) and morphological appearance. These categories were: previtellogenic oocytes, 0.352 mm; middle, 0.823 mm and late, 1–1.176 mm vitellogenic follicles; ovulated eggs, 1.352 mm.

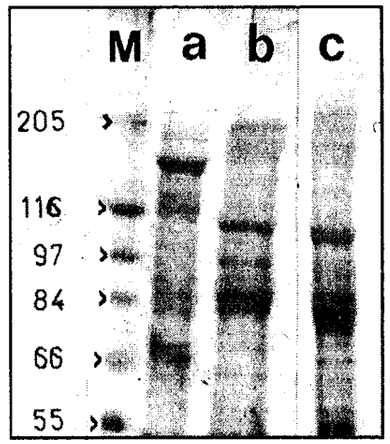

Fig. 1. – SDS-PAGE (7.5%) of serum female (lane a) and yolk proteins from homogenate of lamellae which contained 0.352 mm follicles (lane b) and whole homogenate of previtellogenic ovary (lane c) stained with Coomasie Brilliant Blue. Molecular mass values on the left indicate the migration position of marker proteins (lane M). MW are indicated in kDa.

Figures 1 and 2 show the electrophoretic separations of serum female proteins and yolk proteins from follicles at various stages of ovarian development and the whole ovary homogenate under reducing conditions, stained with Coomasie Blue.

Yolk extracts from follicles measuring 0.352 mm in diameter contained an array of proteins of different sizes: the major bands (or group of bands) migrated with molecular masses of 192, 165, 117, 104, 92, 74, 71, 56, 52 and 51 kDa. There was no apparent variability in electrophoretic protein profiles between the whole late previtellogenic ovary homogenate and ovarian lamellae (fig. 1.b.c).

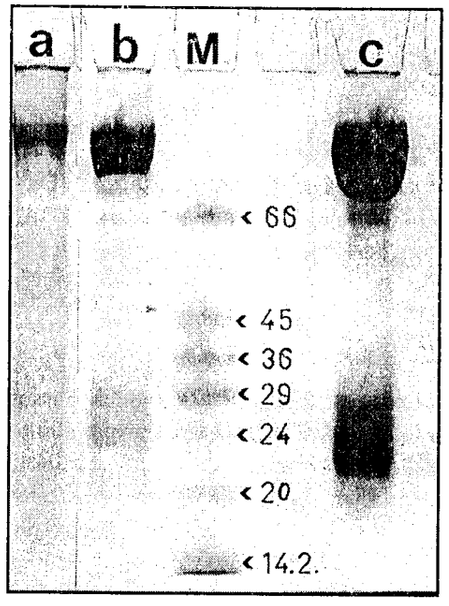

Fig. 2. – SDS-PAGE (7.5%) of yolk extracts from follicles at various stage of ovarian development: 0.823 mm (lane a); 1–1.176 mm (lane b) and ovulated eggs (lane c). Proteins were stained with Coomasie Brilliant Blue. The migration positions of the molecular weight standards are indicated (lane M). MW are indicated in kDa.

In the larger vitellogenic follicles examined (Fig. 2. a.b.) and ovulated eggs (Fig. 2.c) a small number of yolk proteins became by far the greatest protein constituents. Thus, in the 0.823 mm diameter follicle major yolk protein migrates as three bands with apparent molecular masses of: 96, 88 and 80 kDa. The 96 kDa band migrated as very bold bands meanwhile the 88 and 80 kDa band as two faint bands.

In 1–1.176 mm diameter follicles a similar array of proteins was observed to that seen in the 0.823 mm follicles with the exception of the appearance (or considerable increase in concentration) of four peptides with molecular masses of: 66, 29.5, 24 and 21 kDa. In these follicles the bands of 94, 80 and 76 kDa increased in concentration.

In the ovulated eggs (Fig. 2c) the bands with apparent molecular weight of 104, 92, 66, 27.5 and 23 kDa increased in concentration. Serum proteins from females with early vitellogenic ovary separated on SDS-PAGE produced the following major protein subunits: 155, 130, 110, 86, 71 and 49.5 kDa (Fig. 1a).

DISCUSSION

During vitellogenesis, oocyte growth is due primarily to VTG uptake and processing into yolk proteins.

In crucian carp, *Carassius auratus gibelio* analysis of yolk extracts on SDS-PAGE indicated numerous protein bands derived from 0.352–0.470 mm diameter follicles. Since these follicles are previtellogenic, the proteins present at this stage are unrelated to vitellogenin and represent previously accumulated material.

Plasma levels of VTG rise rapidly at the onset of vitellogenesis and are maintained as such throughout this growth phase (32). In crucian carp different molecular weights of polypeptides from serum and ovarian follicles are the result of the proteolytic cleavage after uptake in the oocyte.

At the time follicles reached early vitellogenesis three bands are present (96, 88 and 80 kDa) whereas in middle vitellogenesis seven bands became predominant protein. Presumably these bands represent yolk proteins which are proteolytically derived from vitellogenin. Most electrophoretic separation of fish yolk proteins under reducing conditions indicate that there are between four and seven major peptides (2, 6, 10, 40).

The dramatic increase in yolk during vitellogenesis is also demonstrated in Fig. 2 which depicts follicles (or eggs) and it is evident that as follicles increase in size during vitellogenesis (lane a–b) they contain increasing amounts of protein and the lanes become progressively overloaded.

Electrophoretic patterns of the yolk proteins studied here indicated that the relative MW were within the range of the ones reported previously in the tilapias species (16, 18), rainbow trout (2, 31), goldfish (10, 12), gilthead sea bream (6), killifishes (12, 40), winter flounder (25) and in the toad *Xenopus laevis* (41).

In teleost fish processing of VTG into yolk proteins results mainly in lipovitellins (LV) and phosvitins (37). By SDS-PAGE under non-reducing conditions, examination of the subunits partially purified goldfish lipovitellin, De Vlaming et al. (10) revealed two domains, LV1 (110–105 kDa) and LV2 (25–19 kDa) similar to those found in *Xenopus laevis* (3). In crucian carp, the classes of high and low MW bands Coomassie Blue staining from middle vitellogenesis may be analogous to LV1 and LV2 found in goldfish. Moreover, the 29.5, 24 and 21 proteins fall in the MW range of LV2 (20–36 kDa) subunits reported in other species such as rainbow trout (2, 31), tilapia (16), various cyprinids (12) and goldfish (10). Also demonstrated that in *Carassius auratus* multiple vitellogenin polypeptides are apparently processed into multiple yolk protein polypeptides within the growing oocyte (10).

This study showed that yolk proteins change during oocyte development. These changes were observed as differences in the presence of relative staining intensities of Coomassie Brilliant Blue.

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THE EFFECT OF THE PARASITOSIS WITH *Capillaria* (=*Hepaticola*) *Petruschewski* (NEMATODA, TRICHUROIDEA) ON THE LIVER OF THE FISH-LARVAE

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Capillaria (=*Hepaticola*) *petruschewski* (Nematoda, Trichuroidea) produces heavy infestations on the *Cyprinus carpio* larvae, in the fish breedings. The presence of the adult worms and of the eggs in the liver parenchyma leads to the tissue damage. The host cellular response to the irritant action of the parasitic eggs is represented by the inflammatory granulomas. The cellular and fibrillar components of the granuloma are analysed. The liver fibrosis and the hepatocyte lesions observed in the uninfested individuals (controls) might explain the occurrence of the high hepatic fibrosis in the liver parasited with *Capillaria* being favoured by possible dietary deficiencies.

The parasitosis with *Capillaria*(=*Hepaticola*) in the fish-larvae farmed in the ponds of the Danube Delta was mentioned for 1984–1985, from August to October (7).

The infestations with *Capillaria* are obviously considered of minimum importance for fish pathogenesis (6, 7). But, in the last years the high mortality due to this infestation determined the fish breeders to call for minutious histologic studies, in order to apply correctly the prevention and the treatment of the disease.

MATERIAL AND METHODS

The liver of the *Cyprinus carpio* larvae from the infected fish farms was collected in the interval July–September 1994.

The material was collected in the same period of time, from the uninfested individuals, as controls.

Small liver pieces were fixed in 10% formaldehyde in 0.7% physiological saline. Histologic technics for the embedding in paraffin were processed. 5 μm thin sections were stained with Hemalum Mayer–Eosin. Also there were applied histochemical stains in order to evidentiate lipofuscins (fuchsin Ziehl–Tween 80), reticulin fibers (silver impregnation Gomori), collagen fascicles (picrosirius red) and blood cells (Giemsa–colophonium) (5).

RESULTS

The microscopic observations confirm the presence of adult individuals of the genus *Capillaria* and of a large amount of eggs disseminated through the

mesentery, the hepatic parenchyma, the pancreas and exocrin pancreatic acini scattered in the hepatic tissue.

A certain progress in time of the tissue damage is evident on the material collected during the three aestival months. The samples fixed in July present only few worms and rare granulomas arranged around the parasitic eggs. But, on the samples fixed at the beginning of September, there are very numerous granulomas around the parasite eggs in the liver and the pancreatic tissue. Sometimes long, filiform adult worms of the genus *Capillaria* (Fig. 1) are observed in the hepatic parenchyma or in pancreatic acini scattered through the liver. The inflammatory granuloma (Fig. 2, 3) is composed of cellular and fibrillar components.

The cellular component is represented by a cellular population of neutrophilic and eosinophilic granulocytes and monocytes from the blood. These cells arranged tightly around the worm eggs displace, press and damage the hepatocytes on the granuloma territory. The monocytes become enlarged and transform into macrophages. Then, by a continuous active process, they turn into epithelioid cells. This cellular type presents an abundant cytoplasm and the nucleus in the peripheral position.

The lipofuscins, evidentiated as Ziehl-positive material, are often observed in the cytoplasm of the macrophages and epithelioid cells. The lipofuscins may be considered as a marker for the intensity and the evolution in time of the host cellular response to parasitosis. So, while in the liver samples fixed in July, the fresh constituted granulomas do not contain accumulations of the Ziehl-positive material, in the samples fixed in September, all the granulomas presented macrophages and epithelioid cells loaded with lipofuscins.

In the controls, the Ziehl-positive material was not observed in the macrophages infiltrated through the liver.

The fibrillar component is synthesized by a few layers of the connective cells which separate the *Capillaria* eggs from the hepatic parenchyma.

The specific stains evidentiate the reticulin fibers around the parasitic eggs (Fig. 3). Also, the sinusoids are surrounded by reticulin fibers and collagen fascicles (Fig. 3).

The observations on the control livers (from the unparasited fish larvae) revealed an intense perisinusoidal and periportal fibrosis (Fig. 4). The hepatocytes present great lipid loading and autolysis.

Often, around the portal spaces or in other zones of the liver, there occur cellular infiltrations of granulocytes, monocytes, macrophages and even epithelioid cells.

DISCUSSIONS

During the parasitosis with *Capillaria* the damage is firstly due to the physical presence and the metabolic activity of the adult worms (6, 7). It follows the inflammatory response of the host towards the parasite eggs spread in the liver, the pancreas and the mesentery (12, 13, 15).

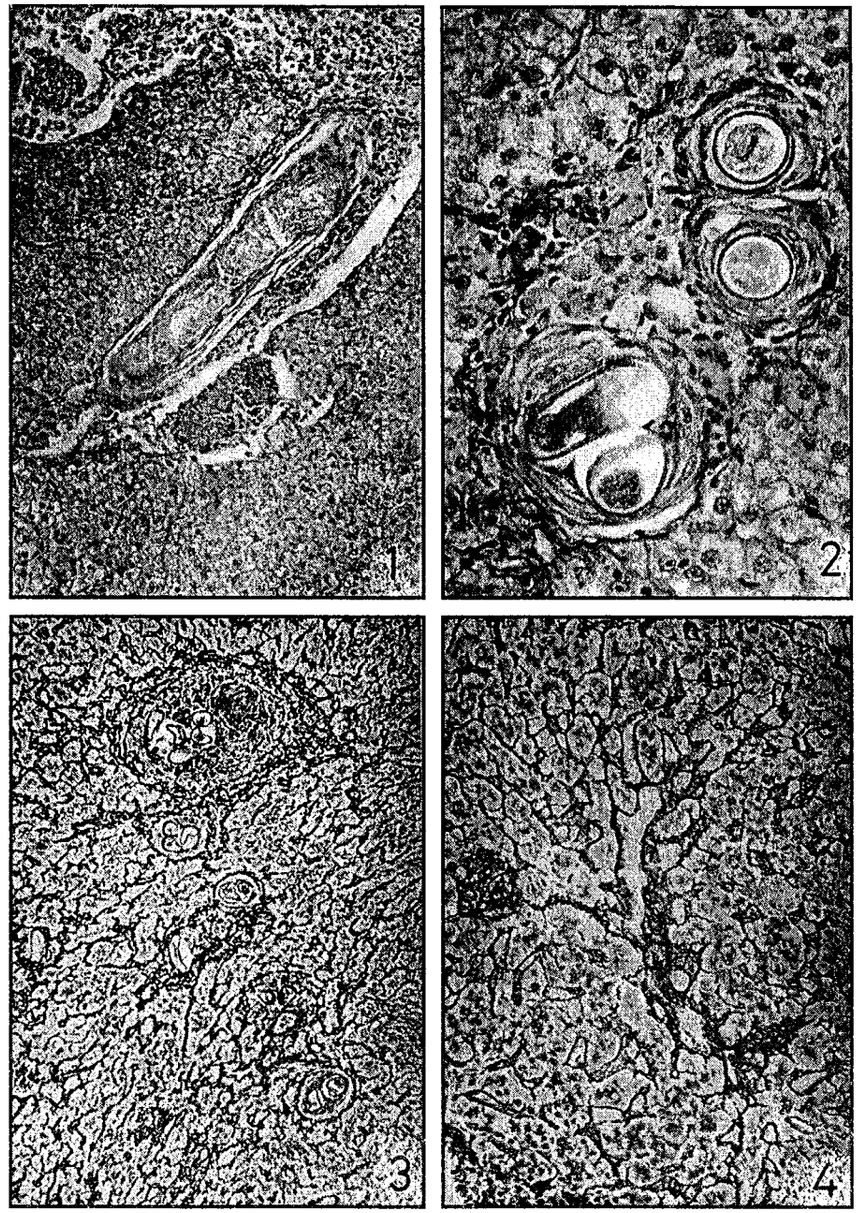

Fig. 1. – Adult worm in the hepatic parenchyma, H.E. 100×.
Fig. 2. – Inflammatory granulomas around the *Capillaria* eggs. Picrosirius red. 480×.
Fig. 3. – Perigranulomatous and perisinusoidal fibrosis in the hepatic parenchyma. Silver impregnation. 120×.
Fig. 4. – Perisinusoidal fibrosis in the control liver. Silver impregnation. 480×.

Similar reactions were described in the liver of other Vertebrate classes during the parasitosis with Nematoda (8, 9, 14), or in the Mammalian liver parasited with the species of other groups of worms (4, 10).

The granulomatous reaction induced by the nonself nature of the worm eggs (13, 15) is supplemented by the activation of the perisinusoidal cells and their transformation into the fibroblasts. They become able to synthesize and deposit the collagen, isolating the parasitic eggs from the liver cells.

Our histologic data on the unparasited *Cyprinus*-larvae liver reveal the active generalized perisinusoidal and periportal fibrogenesis and the characteristic lesions of the hepatocytes. These observations might explain the excessive fibrogenesis of the liver of the *Cyprinus* larvae submitted to *Capillaria* parasitosis.

Many authors (1, 2, 3,-11) sustain that the nutritional deficiencies in farmed fishes have the direct consequences in the occurrence of the hepatic fibrosis and cirrhosis.

Some authors consider *Capillaria* (6, 7) as a parasite of a low patogenicity.

The presence of a generalized fibrosis in the liver of the farmed *Cyprinus carpio* larvae infested with *Capillaria* should be favoured also by possible nutritional deficiency.

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IMMUNOLOCALIZATION OF CHONDROITIN SULFATE IN THE CORNEA OF SOME VERTEBRATES

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Localization of chondroitin sulfate in pig, amphibian (*Triturus cristatus*), and fish (*Carassius auratus gibelio*) corneas was examined by immunohistochemistry using specific antibodies to chondroitin sulfate.

Immuno-deposits for chondroitin sulfate were intensely localized in the posterior (endothelial) region of the pig corneal stroma and weakly in its anterior (epithelial) and middle regions. The positive reaction was also seen in the cytoplasm of stromal keratocytes. In the cornea of *Triturus cristatus* and *Carassius auratus gibelio*, chondroitin sulfate was present in all the stroma. The immunoreactivity for chondroitin sulfate was most intense near epithelium.

These results demonstrate the presence of chondroitin sulfate in pig, amphibian, and fish corneas with a distribution specific to these species. Moreover, the presence of this glycosaminoglycan in corneal tissue of the studied vertebrates may play a role in its transparency, as has been suggested for keratan sulfate and dermatan sulfate.

The cornea is an avascular and transparent organ consisting of a stratified epithelium, a stromal dense connective tissue and an endothelium facing the anterior chamber. Corneal tissue has been shown to synthesize constituents of the extracellular matrix: collagen fibers and glycosaminoglycans (GAG) which are very important for transparency (1), (2).

Histochemical and biochemical analyses have demonstrated that the major types of GAG in cornea are keratan sulfate (KS) and dermatan sulfate (DS) (3). Additionally, in the corneal tissue were identified the other glycosaminoglycans including chondroitin sulfate (CS), hyaluronic acid (HA) and heparan sulfate (HS) (4), (5).

The morphological localization of GAG seems necessary to understand their function in corneal tissue.

Corneal GAG are identified in tissue sections by various histochemical techniques including staining with Alcian Blue (6) and its analogues, such as Cuprolinic Blue and Cupromeronic Blue (7) or with high and low iron diamine methods (8). The use of specific enzymes, such as chondroitinases, keratanase and hyaluronidase (9), has further refined these techniques showing also their lack of complete specificity. Thus, Derby and Pintar demonstrated that embryonic hyaluronic acid binds Alcian Blue under conditions that had been believed specific for sulfated GAG (10). In these cases, we need to apply more sensitive and efficient methods in light microscopy.

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The present study deals with intra- and extracellular localization of chondroitin sulfate in pig, amphibian (*Triturus cristatus*) and fish (*Carassius auratus gibelio*) corneas using specific antibodies to chondroitin sulfate.

MATERIALS AND METHODS

The localization of chondroitin sulfate in sections of the cornea was determined by an indirect immunoperoxidase method using diaminobenzidine as a chromogen. Tissue sections were fixed in Bouin and embedded in paraffin. Sections of 7 μm were deparaffinized and rinsed with distilled water. They were sequentially incubated in methanol: hydrogen peroxide (9:1) to remove endogenous peroxidase activity (30 minutes), PBS plus 10% normal rabbit serum to remove nonspecific background staining (1h), mouse monoclonal antichondroitin sulfate primary antibody (Sigma, USA), diluted 1:200 (overnight at 4°C), rabbit anti-mouse antiserum peroxidase conjugated (Sigma, USA), diluted 1:100, 1h, at room temperature. Each incubation step was followed by four times rinses in PBS of five minutes each. To visualize the primary antibody binding sites, the slides were incubated for 15 minutes in a solution of 3,3'-diaminobenzidine (0.05%) plus hydrogen peroxide (0.015%) dissolved in PBS. The reaction product was intensified with 0.5% cupric sulfate dissolved in 0.9% NaCl (10 minutes). Negative controls were incubated in the absence of primary antibody, substituted with PBS.

RESULTS

Localization of chondroitin sulfate in three corneal tissues (pig, amphibian and fish corneas) was examined by immunohistochemistry using specific antibodies to chondroitin sulfate. Immunostaining for chondroitin sulfate was performed using cornea sections treated with Bouin fixative. In all these cases, the method specificity was demonstrated by the absence of the immunocytochemical reaction in the sections incubated without primary antibody.

Pig cornea

In anterior (epithelial) region and middle region of the pig corneal stroma, the immunoreactivity for chondroitin sulfate was weak. Immunocytochemical reaction appeared intense in the posterior (endothelial) region of the corneal stroma, close to Descemet membrane (Fig. 1A–1B).

The immuno-deposits were also seen in the cytoplasm of stromal keratocytes (Fig. 2A–2B).

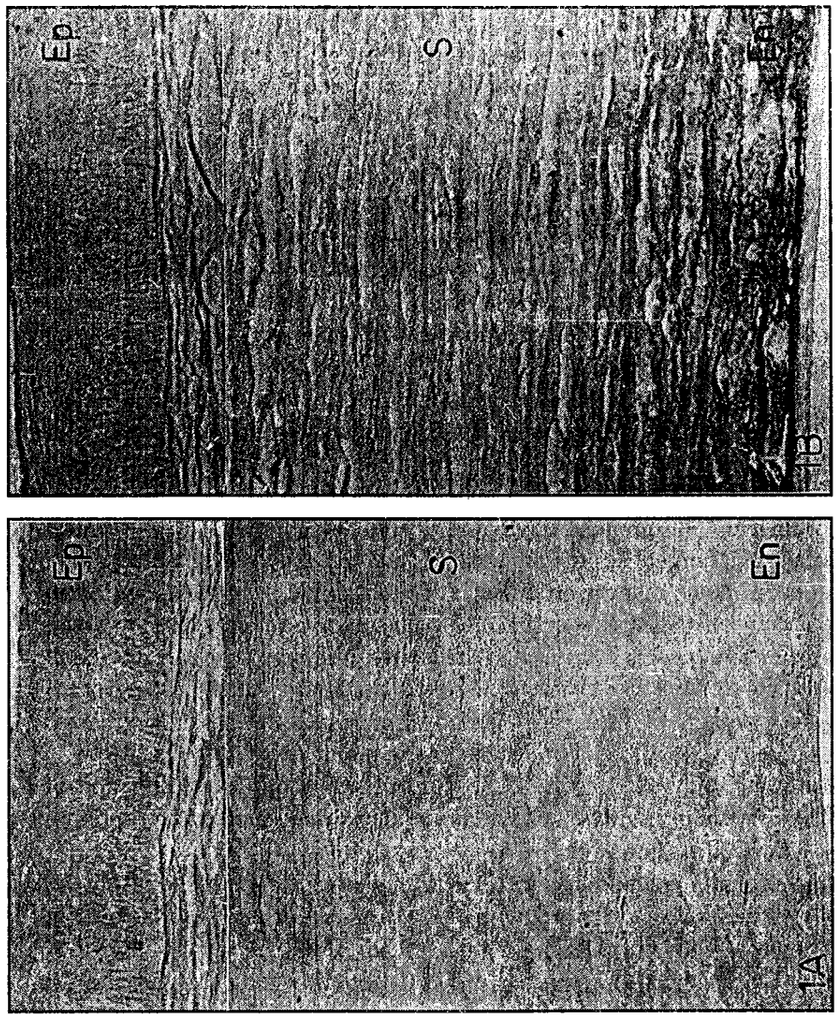

Fig. 1. – Immunohistochemical localization of chondroitin sulfate in the pig corneal stroma. A – control; B – after immunohistochemical reaction. Ep – epithelium; S – stroma; En – endothelium.

Amphibian (Triturus cristatus) cornea

Chondroitin sulfate presence was very intensely detected in all corneal stroma as voluminous masses, localized among the collagen fibrils (Fig. 3A–3B).

Fish (Carassius auratus gibelio) cornea

Chondroitin sulfate amount progressively decreased from the anterior region of the stroma towards the posterior region. The immunoreactivity for CS was most intense near epithelium (Fig. 4A–4B).

DISCUSSION

Normal cornea contains 78% water; its major structural components are collagen and proteoglycans, which represent about 12–15% and 1–3%, respectively, of the wet weight of the tissue. In addition, there are other noncollagenous structural proteins, glycoproteins and lipids (11, 12, 13).

Corneal glycosaminoglycans are highly charged molecules, responsible for the hydrophilic properties of the cornea. Their association, as proteoglycans with collagen fibrils in a highly organized manner, appears to play an important role in the corneal extracellular matrix (14).

According to Cintron and Convington, the two major types of glycosaminoglycans in rabbit corneal stroma are keratan sulfate and dermatan sulfate (3).

It has been shown that the arrangement of proteoglycans is consistent from late fetal to postnatal stages; low-sulfated keratan sulfate proteoglycans are found throughout the rabbit corneal stroma and their highly sulfated type is restricted to the subepithelial stroma (15). On the other hand, dermatan sulfate proteoglycans are distributed throughout the stroma. Moreover, dermatan sulfate proteoglycans have been shown to contain chondroitin sulfate in rabbit corneal tissues (16).

The present study demonstrated the immunohistochemical localization of chondroitin sulfate in pig, amphibian and fish corneas, using monoclonal antibodies against chondroitin sulfate.

We have found that chondroitin sulfate immunoreactivity was intensely detected in pig cornea in the posterior region of the stroma. Our results using anti-CS antibody showed a good correlation with the Alcian Blue-CEC (critic electrolyte concentration) method for the presence of CS in pig cornea (17). Moreover, the sensitivity of the method was considerably improved and offered additional information, with distinct reactivity pointing to the presence of CS in all the posterior region of the corneal stroma.

In the cornea of the two lower vertebrates, *Triturus cristatus* and crucian carp (*Carassius auratus gibelio*), CS was present in all the corneal stroma. To our knowledge, this is the first report about the localization of CS in the cornea of

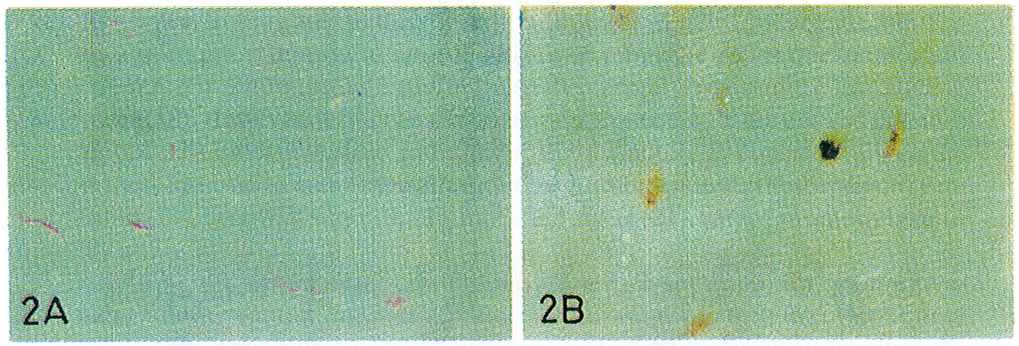

Fig. 2. – Light micrographs showing cytochemical localization of chondroitin sulfate in the cytoplasm of pig stromal keratocytes. A – control; B – after immunohistochemical reaction.

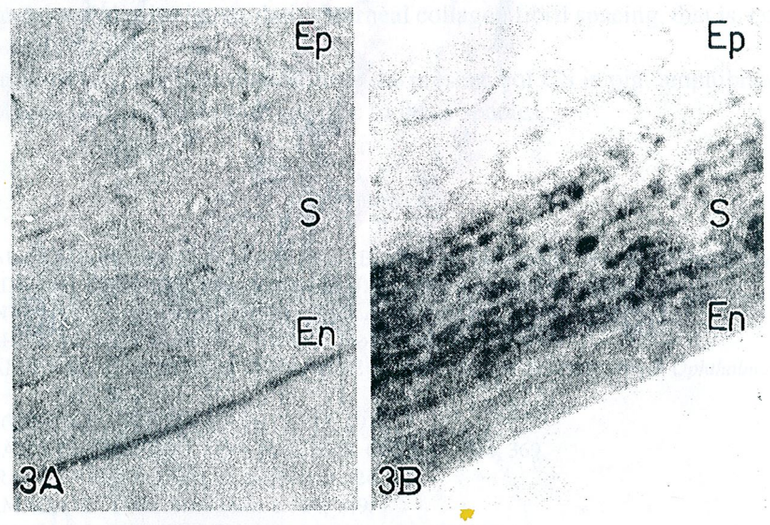

Fig. 3. – Immunohistochemical localization of chondroitin sulfate in the amphibian (*Triturus cristatus*) corneal stroma. A – control; B – after immunohistochemical reaction. Ep – epithelium; S – stroma; En – endothelium.

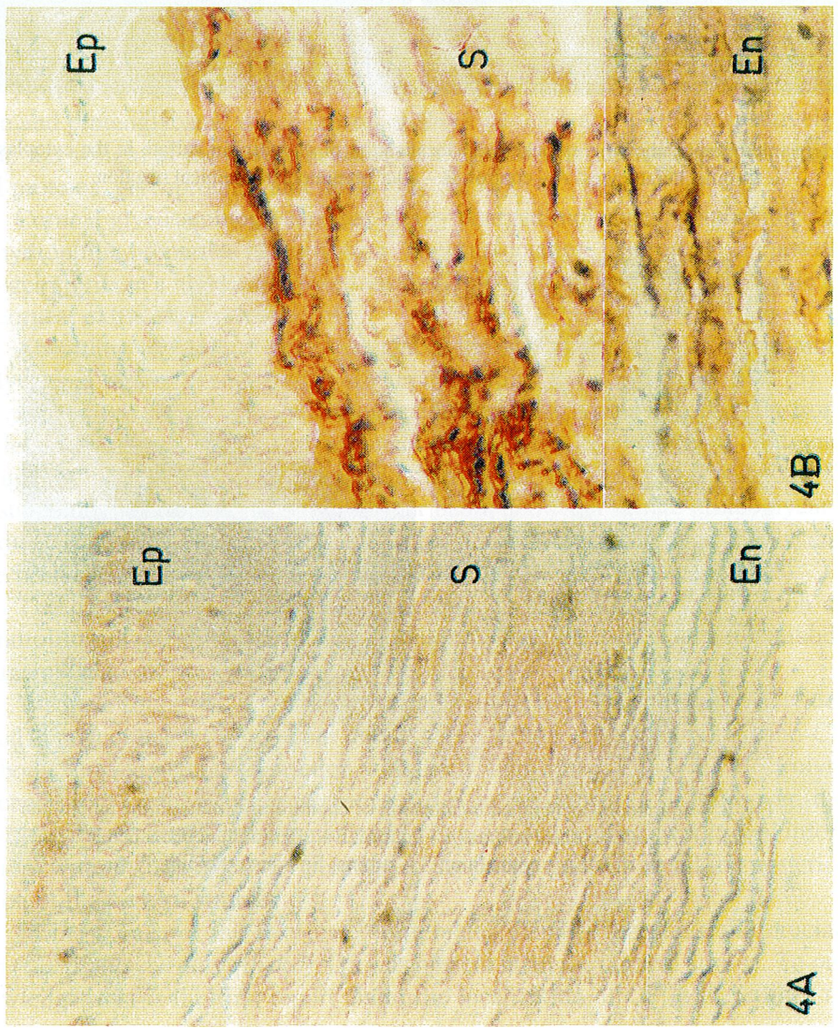

Fig. 4. – Immunohistochemical localization of chondroitin sulfate in the fish (*Carassius auratus gibelio*) corneal stroma. A – control; B – after immunohistochemical reaction. Ep – epithelium; S – stroma; En – endothelium.

crucian carp and *Triturus cristatus*. This study has revealed the presence of CS in high amount in stromal region near epithelium. It is possible that these differences concerning the distribution of CS in lower vertebrates corneas, comparatively with pig cornea, to be related to their specialized corneal structure for aquatic life. The aquatic cornea is thought to provide little or no refractive power due to the small difference in the refractive index between the corneal tissue and the surrounding water (18). However, it still constitutes a protective cover for the eye and provides an optically smooth surface and a transparent window. In comparison with the mammalian cornea many specializations have been reported. These include spectacles (19), corneal filters (20, 21), iridescent layers (22), and/or an autochthonous layer (23, 24).

We have also localized chondroitin sulfate in the cytoplasm of stromal keratocytes in pig cornea. The localization of chondroitin sulfate in the cytoplasm of keratocytes demonstrated that its synthesis and secretory pathway involved the Golgi complexes.

It seems likely that chondroitin sulfate present in the corneal tissue correlates with the functional characteristics of the cornea; Hassell et al. have indicated that CS/DS proteoglycans and KS proteoglycans from the rabbit corneal stroma might play fundamental roles in regulating corneal collagen fibril spacing, that is, corneal transparency (25).

In summary, our data demonstrate the presence of CS in pig, amphibian and fish corneas with a distribution specific to these species.

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HISTOPATHOLOGICAL MODIFICATIONS OF THE *Xiphophorus helleri* LIVER INDUCED BY THE HEAVY WATER

I. LIGHT MICROSCOPY STUDY

MARIA CALOIANU, WANDA BUZGARIU, PAULA PRISECARU

The investigations upon the effects of heavy water on *Xiphophorus helleri* liver were conducted on organisms grown in 25% D_2O water. Samples were collected at variable intervals: 1, 2, 7 and 28 days, and were studied in light microscopy.
The tissue sections were stained with hematoxylin-eosin and Sudan black. Glycogen identification was carried out by PAS reaction. Our results show severe alterations of hepatic parenchima, vacuolisation of hepatocytes cytoplasm, nuclei picnosis and massive hepatic degeneration accompanied by increased inflammatory processes.

Deuterium presence and its effects upon living organisms due to some imbalances have imposed and stimulated the studies of the modifications of various biological systems: algae (1), plants (2, 3), animals (4, 5, 6) and cell culture (7, 8).

The results of these studies showed that a light heavy water concentration in medium is responsible for severe alterations of structure and ultrastructure of cells, such as: mitotic apparatus morphology modifications, the presence of "overmatured" chromosomes (8); it affects the transformation of cytoplasmic microtubules in mitotic spindle in interphase-prophase transition (9) and the kinetic behavior and nucleation ability of centrosome (7).

It was demonstrated that deuterium induces a large spectrum of modifications at *Xenopus laevis* embryos (10). Fertilized eggs that are exposed to deuterium oxide develop exaggerated dorsoanterior structures, such as radial eye and cement gland, along with large amounts of notochordal tissue (10), suggesting that D_2O has an axispromoting effect on mesoderm.

The results of our research demonstrate severe alterations of embryos at the level of all its organs. Therefore, digestive and excretory organs, the central nervous system, lateral musculature and eyes architecture are affected (11).

Biochemical and physiological processes are also affected by unusual heavy water concentrations. Carbon metabolism is affected, as it was shown by the diminution of ^{14}C incorporation in sucrose and starch (12). In subapical segment of *Zea mays* the proton pump and transmembranar transport were inhibited (13). A high concentration of heavy water blocks the Ca^{2+} entry through KCl, potential and receptor operated channels, normalizing the vascular mechanism involved in Ca^{+2} transport, in pathological cases (14).

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The studies on the heavy water effects upon the intimate mechanisms of cellular processes are imposed by heavy water production at the industrial level, as a consequence of its implication in nuclear power-plants.

The present investigation attempts to evaluate the effects of a nonlethal concentration of heavy water on the liver of *Xiphophorus helleri.*

MATERIALS AND METHODS

Our experiments concerning the effect of heavy water in hepatocytes were realized on viviparous fish *Xiphophorus helleri*. The fishes were maintained for a week in glass aquariums filled with tap water (pH = 7.8), at 20±3°C, with 8.3 mg/l dissolved O_2 and afterwards in water with 25% deuterium oxide. The fishes were sacrificed at 1, 2, 7 and 28 days, and hepatic fragments were obtained.

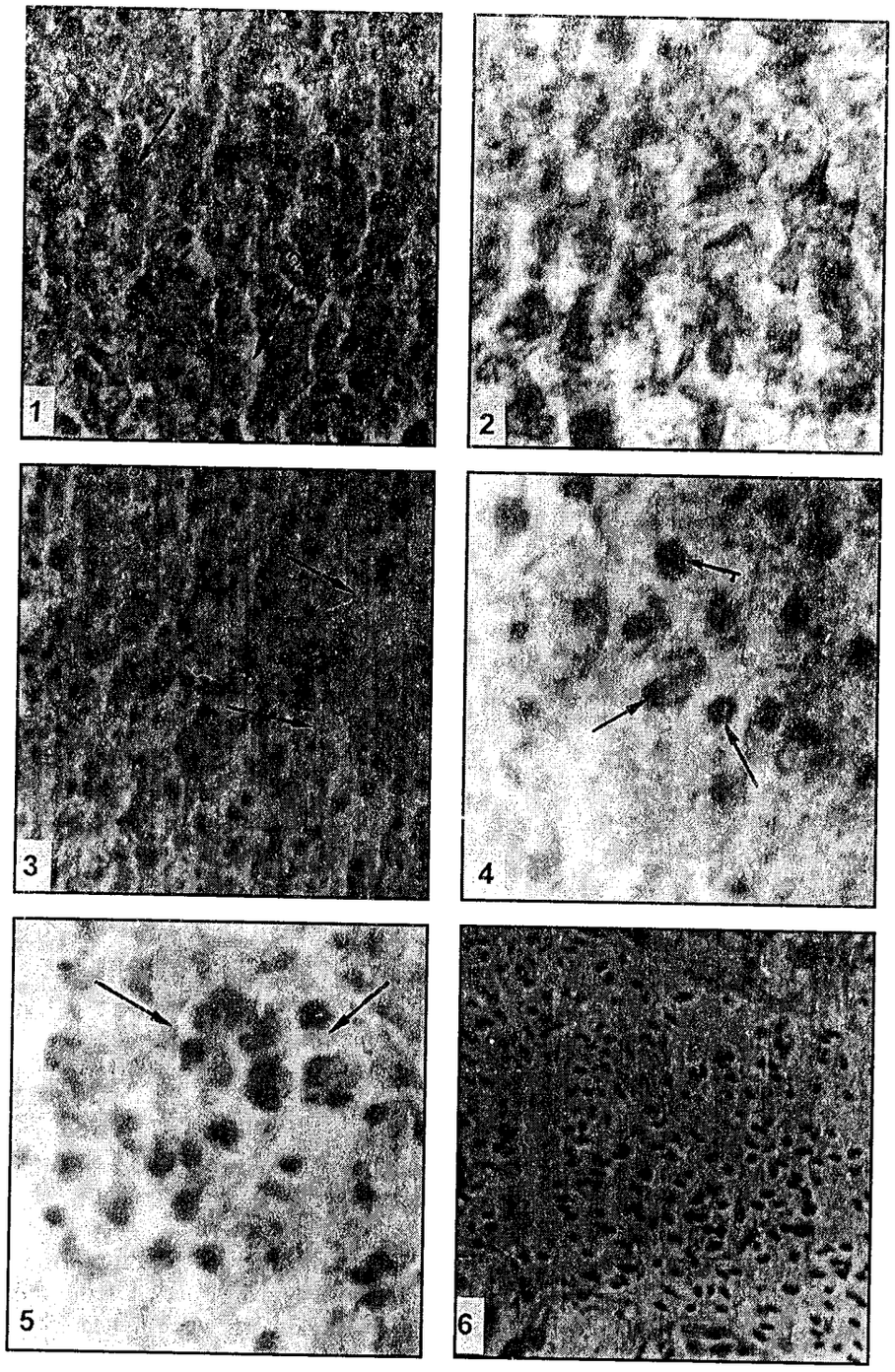
For light microscopy, the samples were fixed in 0.1 M buffered formaline, pH = 7.4 for 12h, at 4°C, and in Ciaccio solution for demonstration of lipids. After dehydration, 5µm sections were stained with hematoxylin-eosin and Sudan black, respectively, for lipids. Glycogen was identified with PAS reaction.

RESULTS AND DISCUSSIONS

World-wide, fishes are an important source of food for mankind. Accidental presence of heavy water, as nuclear moderator, in medium, affects the equilibrium of the aquatic ecosystems and human alimentation. In the present paper we have studied the effects of heavy water on *Xiphophorus helleri* liver. The liver has a central role in excretion and xenobiotic metabolism, by P-450 cytochrome (15), in digestion and storage processes (16) and in vitelogenin production (17).

The liver, in fish, is made up by radial rows of cells which branch out and anastomose (Fig. 1). Among the hepatocytes tubules there is a sinusoids network. Hepatocytes are polygonal cells with a centered round or ovoid euchromatic nucleus. They represent 80% of the total liver volume and are the most numerous cell

Fig. 1. – Normal liver of *Xiphophorus helleri*; 1-hepatocytes; 2-sinusoides. *Hematoxylin-eosin.*
Fig. 2. – Glycogen in the cytoplasm of normal hepatocytes. *PAS.*
Fig. 3. – Local damage of liver parenchyma after one day exposure to 25% D_2O (arrows). *Hematoxylin-eosin.*
Fig. 4. – Degenerated hepatocytes with pycnotic nuclei after one day exposure to 25% D_2O (arrows). *Hematoxylin-eosin.*
Fig. 5. – Macrophages cluster (arrows) involved in removing of the degenerated cells at one day exposure to 25% D_2O. *Hematoxylin-eosin.*
Fig. 6. – Accumulation of an exudate in intrahepatic veins at one day exposure to 25% D_2O. *Hematoxylin-eosin.*



type, being roughly ten times more prevalent than any other cell type within the liver (18). In the cytoplasm there are few glycogen particles (Fig. 2). Sometimes, the lipids are numerous in cytoplasm.

Heavy water leads to liver modifications strictly dependent on the administered dose and the time interval of the contact with deuterium.

The changes induced by heavy water can be seen in early stages. After one day of exposure at 25% D_2O a local destruction of liver parenchyma can be noticed (Fig. 3). In other regions of the liver, hepatocytes destructions are moderate and are represented by nuclei pycnosis and cytoplasm vacuolisation (Fig. 4).

The demarcation between necrotic hepatocytes and adjacent normal appearing hepatocytes is frequently abrupt when examined by light microscopy. However, in some cases a thin zone of lipid and fluid-filled hepatocytes may separate the obviously necrotic hepatocytes from the more peripheral hepatocytes that appear normal by light microscopy.

The beginning of the degenerative process is accompanied by a raising in macrophages number involved in removing the rests of degenerated cells (Fig. 5). Macrophages appear as aggregates following prior toxicity (19). Often referred to as melanomacrophages aggregates, these cells are not associated with melanin (20).

In some hepatic veins the accumulation of an exudat can be seen too (Fig. 6).

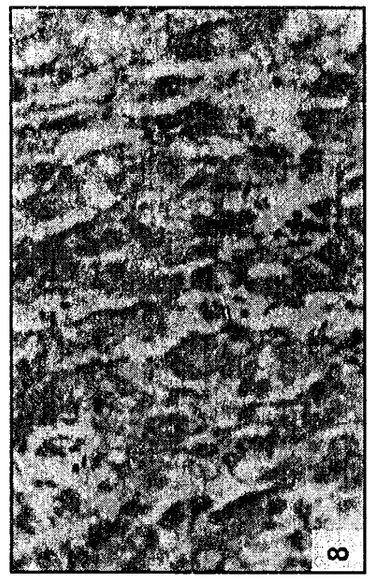
After two days of contact with D_2O most of the hepatocytes have vacuolised cytoplasm (Fig. 7). After necrosis, surviving hepatocytes may undergo hyperplasia thereby regenerating sufficient hepatocytes to replace those that were lost. Regenerating hepatocytes are basophilic and occur as small islands of irregular shape (Fig. 8). Staining with Sudan black shows the presence of neutral lipids particles in the cytoplasm (Fig. 9).

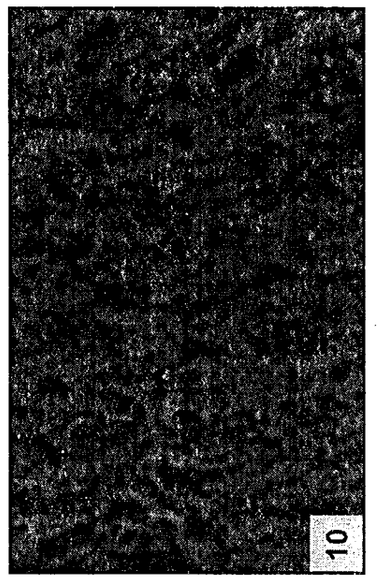
After 7 days of exposure at 25% D_2O, the hepatic necrosis is highly marked in certain regions of the liver (Fig. 10), where large acellular gaps can be seen.

Massive hepatic degeneration is associated with the presence of numerous macrophages situated at the hepatic parenchyma periphery (Fig. 11). One can see a decrease of sudanophilia at this time interval (Fig. 12).

After 28 days of exposure, the entire hepatic tissue is intense vacuolized (Fig. 13). The hepatocytes and billiary ductules have pycnotic nuclei (Fig. 14) and one can notice a slightly raising of sudanophilia as compared with the liver of the fishes exposed at heavy water for 7 days (Fig. 15).

The same phenomenon – degeneration and vacuolisation of hepatocytes – was observed in mouse by Rabinowitz, at the same deuterium oxide concentration (25%) (6). In mammals, dramatical modifications in hepatocytes structure were observed from the second week of exposure, where the hepatic cells appear edematous and swollen, with the cellular border clearly distinguishable (6). In mouse hepatocytes there were important modifications of RNA distribution in the cell, the basophilic material appearing either at the cellular or at the nuclear membrane.





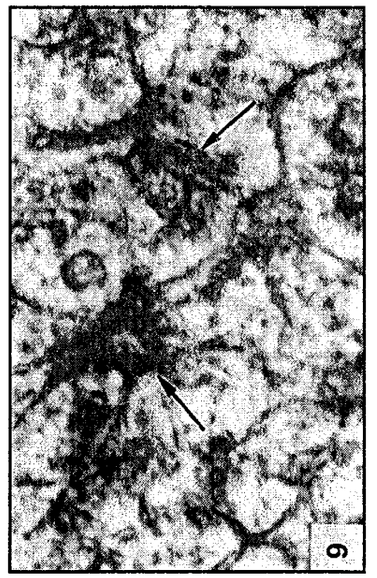
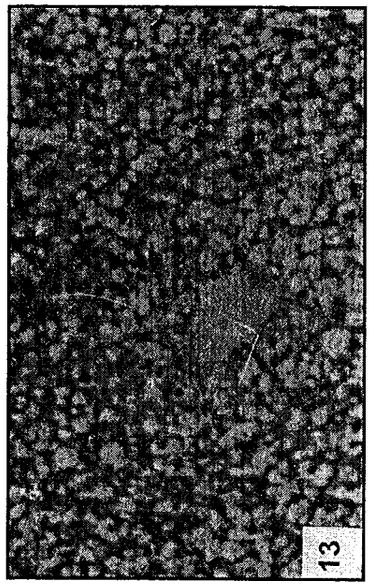
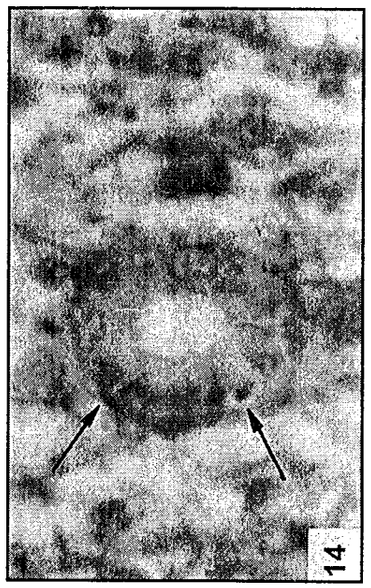


Fig. 7. – Massive degeneration of the liver parenchyma at 2 days exposure to 25% D_2O. *Hematoxylin-eosin.*
Fig. 8. – Regenerating hepatocytes appear as small basophilic islands of irregular shape. 2 days exposure to 25% D_2O. *Hematoxylin-eosin.*
Fig. 9. – Sudanophilic bodies (arrows) in the fishes liver exposed 2 days to 25% D_2O. *Sudan black.*
Fig. 10. – Marked liver necrosis after 7 days exposure to 25% D_2O. *Hematoxylin-eosin.*





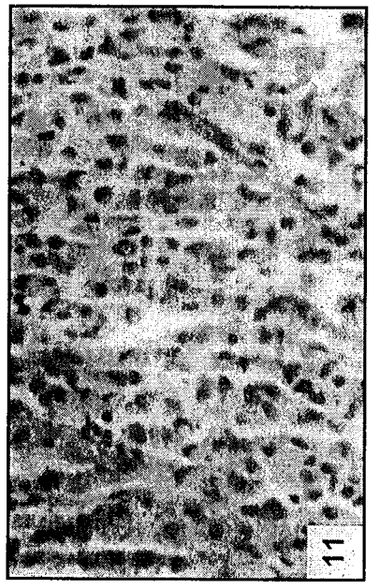


Fig. 11. – Numerous macrophages are present in the fish liver after 7 days exposure to 25% D_2O. *Hematoxylin-eosin.*
Fig. 12. – Decrease of hepatocytes sudanophilia in fishes exposed 7 days to 25% D_2O. *Sudan black.*
Fig. 13. – Intensive liver tissue vacuolization at 28 days exposure to 25% D_2O. *Hematoxylin-eosin.*
Fig. 14. – Billiary ductules nuclei pycnosis (arrows) after 28 days exposure to 25% D_2O. *Hematoxylin-eosin.*

Sudan black staining indicates a decrease of sudanophilia in the first 7 days, followed by a slight increase after a month. Hepatocytes contain a large lipidic vacuole, the nucleus being localized at the periphery of the cell (Fig. 16). In mammals, it was seen a progressive increase of sudanophilic material in granular distribution, as compared with fishes (6).

Hepatic lipidosis refers to the accumulation of triglycerides within hepatocytes. Lipid inclusions may coalesce until they are visible as clear vacuoles. Fatty acids are constantly cycled between liver and adipose tissue. Accumulation of triglyceride in the liver results from an imbalance between uptake of fatty acids and secretion as very low density lipoprotein (VLDL) (21). Vacuolization of hepatocytes (fatty change) is a common response associated with exposure of fish to a variety of different agents (20). This lipidosis may have several causes: (1) inhibition of protein synthesis – the apoprotein is not made in sufficient amount to bind with the lipid so that it can be transported from the cell; (2) energy depletion – lipid being transported from the cell normally moves through the endoplasmic reticulum (ER) and fuses with the Golgi apparatus. This fusion of ER and Golgi is thought to require energy and if the energy levels in the cell are deficient this may cause the build-up of lipid with ER cisternae; (3) disaggregation of microtubules – once secretory vesicles containing lipoprotein substances have been formed, they must find their way from the Golgi apparatus to the plasma membrane. Microtubules guide movement of vesicles in the cells. Thus disaggregation of microtubules is another mechanism whereby fatty liver can arise. It was noticed that microtubules transformation in mitotic spindle at interphase-prophase transition is very sensible at deuterium (8), which affects the balance monomer-polymer of the tubulin (22); (4) shifts in substrate utilization-inhibition of metabolic pathways such as the β-oxidation of fatty acids may also lead to the accumulation of lipid stores (20).

PAS staining points out a decrease of hepatic glycogen with its accumulation in the cellular destruction area. Although glycogen content of the liver is variable depending on the physiologic state of the animal, glycogen accumulation may be observed in hepatocytes as a manifestation of toxicity (21).

The hepatic necrosis produced by deuterium is of "coagulation" type (cell protoplasmic viscosity increases and irreversible gelation occurs). It was previously shown that in coagulative necrosis, shapes of cells and their tissue arrangement are maintained, facilitating recognition of the organ and tissue (20).

In conclusion, the fishes exposure to deuterium leads to major modifications and affects the entire hepatic system. They consist in cytoplasm vacuolisation, nuclei pycnosis and inflammatory processes which accompany the massive hepatic degeneration.

There are numerous doubts about the complexity of the cellular and tissue degeneration and degradation mechanisms induced by the increase of heavy water in the medium.

Fig. 15. – Slight raising of liver sudanophilia after 28 days exposure to 25% D_2O. *Sudan black.*

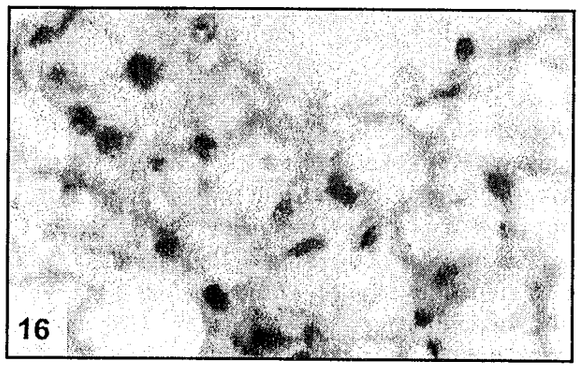


Fig. 16. – Hepatocyte containing a large lipidic vacuole and peripheric nucleus after 28 days exposure to 25% D_2O. *Hematoxylin-eosin.*

The mechanism of heavy water action was not completely elucidated. It was presumed that the nocive effects of the heavy water are due to the deuterium incorporation at the constitutional biopolymers level, which affects the strength of inter- and intracatenary hydrogen bonds. They are responsible for the maintenance of the specific configurations in the cytoplasm (23).

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ASCORBINEMIA AND SERUM CHOLINESTERASE ACTIVITY IN INDUSTRIAL INTENSIVE SYSTEM – RAISED SUINE

D. CURCĂ

The aim of this study is to emphasize the variation of ascorbinemia level and serum cholinesterase activity during individual development in the industrial intensive system breeding swine conditions.
Low levels of ascorbinemia are noted on the 70th day pregnancy (**P**) and on the 7th day post partum (lactating sows, **L**), which are nevertheless nonsignificant statistically between the two physiological conditions. The highest level of ascorbinemia can be seen in suckling pigs (**S**). These levels decrease significantly in 60th day old weaned pigs (**W**), then they increase again in 120th day old growers (fattening pigs, **F**).
In 40th day old hypothrepsic pigs ascorbinemia decreases significantly and the serum cholinesterase (EC 3.1.1.8) activity diminishes neatly, as compared to clinically healthy littermates.

The intensive pig raising system practiced in large establishments in order to obtain, under reclusion conditions, higher and higher productions, determined an increase of the body requirements in ascorbic acid (AA).

The literature shows that the level in pigs varies largely and, frequently, these variations are the result of stress (Fig. 1), with severe consequences upon the functional equilibrium of the body (1–7). Ascorbinemia is a reflection of the circulating AA level and of the synthesis rhythm, catabolisation and release of the vitamin. The energetic level of the diet has a direct effect in ascorbinemia in SPF (specific pathogen free) Yorkshire gilts (2); the available AA saturates the tissue and the biological requirements (Fig. 2).

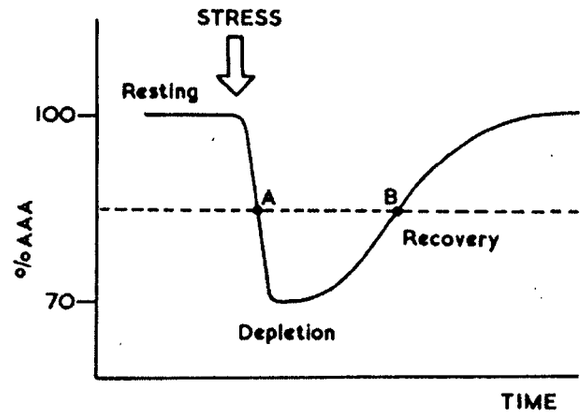

Fig. 1. – The pattern of depletion and recovery of ascorbic acid after stress.

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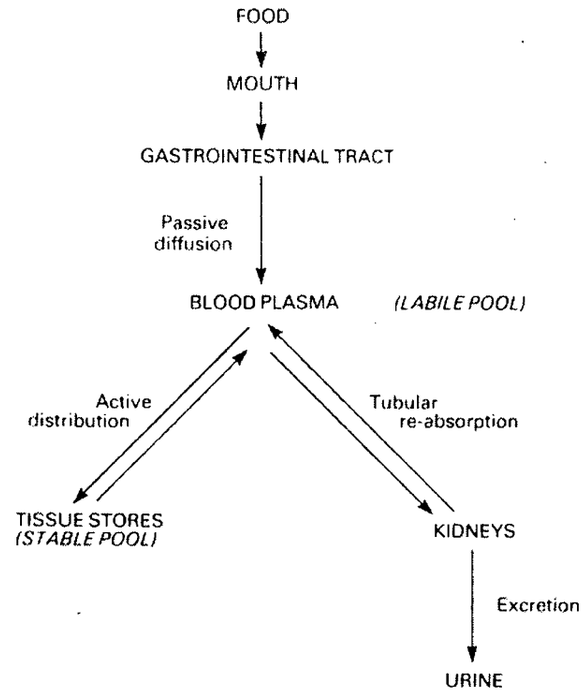

Fig. 2. – Absorption, distribution, storage and excretion of ascorbic acid, labile and stable pools.

The study of the AA synthesis capacity in ontogenesis showed that this process develops since the 36th day of intrauterine life, but the largest amount of AA in the pig fetus originates from the maternal blood circulation, crossing the placenta at rates of 14:1 in favour of the fetuses (2, 8).

The AA has a particular importance for the body, in the intestinal digestion and absorption, it interferes with various cell metabolic circuits, playing the part of main "redox" system (Fig. 3), and participating in the cell respiratory processes, specific and non-specific defense, biosynthesis and protection of some vitamins (E and B1), of some hormones (corticosteroids, catecolamines), activity of sulfhydrilic enzymes, alkaline phosphatases and pseudocholinesterases (9–14).

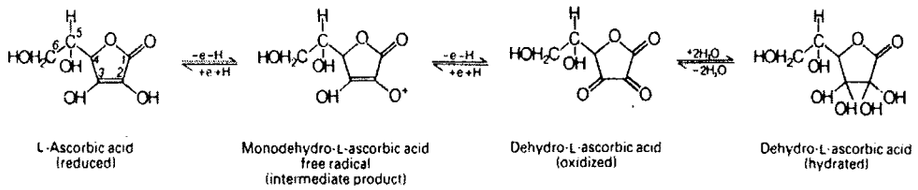

Fig. 3. – Ascorbic acid redox-system. Structural formula of L-ascorbic acid, dehydro-L-ascorbic acid and the intermediate product monodehydro-L-ascorbic acid free radical.

Taking into account the important role played by AA and serum cholinesterase, whose synthesis site is the hepatocyte, the paper presents the results of the studies performed with AA and serum cholinesterase in sows of various physiological conditions and in clinically healthy pigs as compared to hypothrepsic littermates, function of age.

MATERIAL AND METHODS

The investigations were done in an industrial intensive pig raising establishment with an adequately organized technological flow permitting the obtaining of satisfactory economic results.

A randomized group was created containing 24 Large White×Landrace crossbreed sows, on the 70th day of pregnancy, which was also re-examined 7 days post parturition. At the same time, examinations were conducted in 12 suckling pigs aged 30 days, 15 weaned pigs aged 60 days and 15 fattening pigs aged 120 days.

Blood was collected by puncture in the jugular confluent, using heparin as an anticoagulant. The AA content in the whole blood was determined according to the technical indications described by Roe and Kuether (15).

Since in the investigated unit a high incidence of hypothrepsia in young swine had been reported, studies were conducted in 40th day old hypothrepsic piglets and in clinically healthy littermates, as well as in 90th day old growing pigs. In these pig categories, immediately before weaning, and in weaned pigs respectively, besides the AA dosing, the activity of the serum cholinesterase was also determined, using only nonhemolysed blood sera, by the kinetic method described by Ellman et al. (16). The measurements were done with a spectrophotometer SPECORD UV-VIS, Carl Zeiss Jena, at a temperature of 25°C, using 1 cm thick tubs.

The results of the investigations were processed and interpreted statistically (17).

RESULTS AND DISCUSSIONS

Ascorbinemia in 70th day pregnant sows ranged between 0.288–1.843 mg/dl, with a very large variation coefficient, namely 38.89%. On the 7th day post parturition, ascorbinemia decreases slightly, ranging between 0.737–1.258 mg/dl, a fact which is also proved by a much lower variation coefficient, namely 14.83% (Fig. 4).

The decrease of ascorbinemia in lactating sows as compared to the pregnancy period was nonsignificant statistically ($p > 0.05$). Pregnancy and lactation influence to a large extent the AA status in sows. During pregnancy, the placenta permits the transfer of AA from the maternal circulation to the fetuses; the AA level in the blood and organs of sows decreased, AA being gradually absorbed by the fetuses. During lactation, the AA level continues to decrease, regardless of the additional diet administered, due to its daily release through the colostrum and

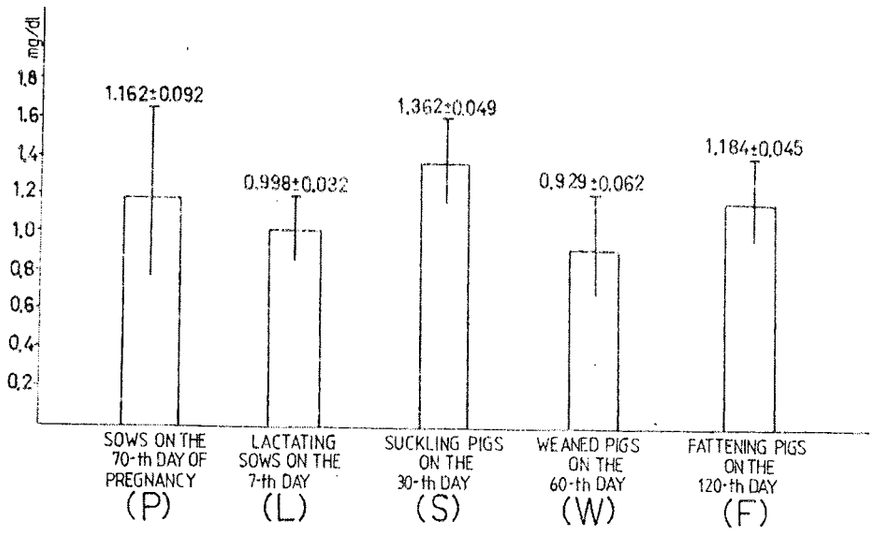

Fig. 4. – Ascorbinemia in industrial intensive system-raised swine. Differences: p:L = nonsignificant ($p > 0.05$); S:W = significant ($p < 0.001$); S:W = significant ($p < 0.05$); W:F = significant ($p < 0.01$).

milk, which is very rich in AA. An increase in AA in the diet during lactation starting with the first day determines the regain of a normal level one week post parturition (6), while sows which did not receive additional AA in the diet exhibited a low blood plasma concentration during the whole lactation period.

Malinowska (8) performed a number of studies in pregnant sows at 21 and 112 days and in fetuses from these sows: she found a high level of AA in the blood serum, uterus and placenta as well as in amniotic and chorioallantoic liquids at the beginning of pregnancy. In the last period of pregnancy, she also noted a decrease of AA in the biological fluids and tissues of sows and its increase in the fetal body.

In 30th day old suckling pigs, AA ranged between 1.110–1.613 mg/dl, with a reduced variation coefficient (12.41%), but in 60th day old weaned pigs a very statistically significant decrease ($p < 0.001$) was noted, with very wide variation limits ranging between 0.335–1.394 mg/dl and a variation coefficient of 25.94%.

In 120th day old growers, AA ranged between 0.916–1.394 mg/dl. A significant decrease ($p < 0.05$) was noted as compared to the suckling pigs. In contrast, as compared to the weaned pigs, the increase was neatly significant ($p < 0.01$).

In suckling pigs the high AA level is dependent on the amount of milk ingested and on its content in AA, being also influenced by microenvironmental factors. The high temperatures determine a lower AA level (5). An important role in the preservation of a constant AA level is played by the individual endogenic synthesis capacity of the liver and by the health condition of this organ (18–20).

Most authors show that the pigs are born with a relatively high amount of AA in the blood, which increases significantly after the ingestion of colostrum. However, a rapid decrease of AA is seen starting with the 2nd week of life, with minimal values usually on the third week, when the level is 0.237±0.024 mg/dl (5), this level being reduced until the 15th week, after which it increases again (6).

Since a high incidence of hypothrepsia was sometimes reported in the investigated farm, studies were conducted in 40th day old hypothrepsic pigs immediately before weaning as well as in 90th day old weaned pigs as compared to clinically healthy littermates.

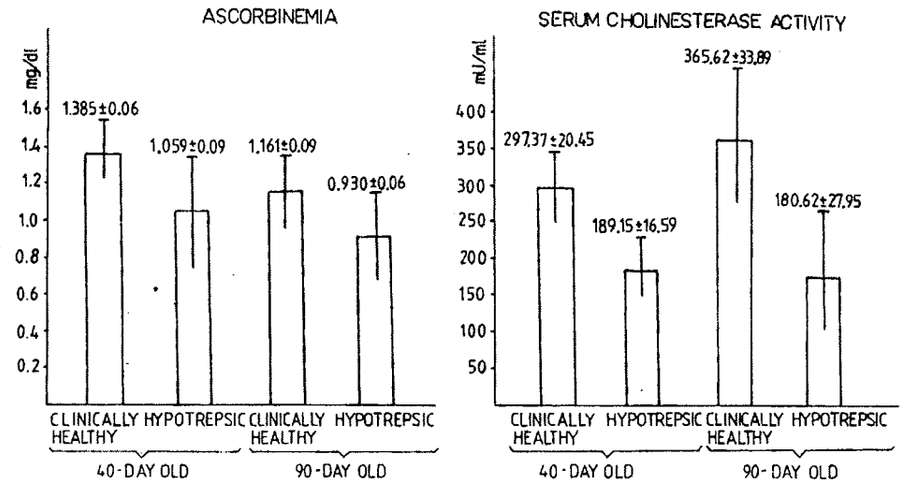

Fig. 5. – Ascorbinemia and serum cholinesterase activity in hypothrepsic piglets, as compared to clinically healthy littermates.

In 40th day old hypothrepsic pigs, AA ranged between 0.697–1.613 mg/dl, the values being significantly more reduced ($p < 0.05$) as compared to clinically healthy animals in which the levels ranged between 1.148–1.574 mg/dl (Fig.5). The low AA level in hypothrepsic pigs reflects an insufficient intake, a poor biosynthesis capacity of the hepatocyte, this statement being also supported by a drastic reduction of the serum cholinesterase activity. Thus, in 40th day old hypothrepsic pigs, the serum cholinesterase activity ranged between 140.40–234.00 mU/ml blood serum, while in clinically healthy littermates this activity ranged between 239.85–351.00 mU/ml blood serum, the difference being neatly significant ($p < 0.01$). The significant reduction of the serum cholinesterase activity shows a diminished synthesis and secretory capacity of the hepatocyte and a low proteosynthesis in the liver of hypothrepsic pigs.

In 90th day old hypothrepsic pigs, AA ranged between 0.335–1.394 mg/dl blood, while in clinically healthy littermates, the values were much higher, namely 0.916–1.355 mg/dl, the difference being statistically nonsignificant ($p > 0.05$).

Previous investigation conducted by Curcă (21) related to the AA contents in the leukocytes of the peripheral blood circulation showed, in hypothrepsic pigs, an amount of 18.056±1.538 μg/10^8 leukocytes, and in clinically healthy littermates 18.377±1.433 μg/10^8 leukocytes, any statistically significant differences being absent. This is due to the initial mobilization of plasma AA which represents the labile pool, as to the leukocyte content, which represents the stabile pool.

There are however a number of remarks showing that in certain circumstances the AA synthesis is inhibited or inadequate and that the already reduced reserves may be easily finished (5), a situation that seems to characterize pig hypothrepsia, as well.

The serum cholinesterase activity in 90th day old hypothrepsic pigs ranged between 87.75–298.35 mU/ml blood serum and in clinically healthy littermates between 245.70–538.20 mU/ml blood serum, the difference being significantly statistical ($p < 0.001$).

The marked reduction of the serum cholinesterase activity in this age category may be attributed to a deficiency in vitamin A, or to the action of microbial agents with low pathogenicity, leading to the onset of a hepatic dysfunction. Under these conditions of a vitiated intermediate metabolism, the acetylcholine in excess creates a sensitization of the body and diminishes the resistance to various aggressions.

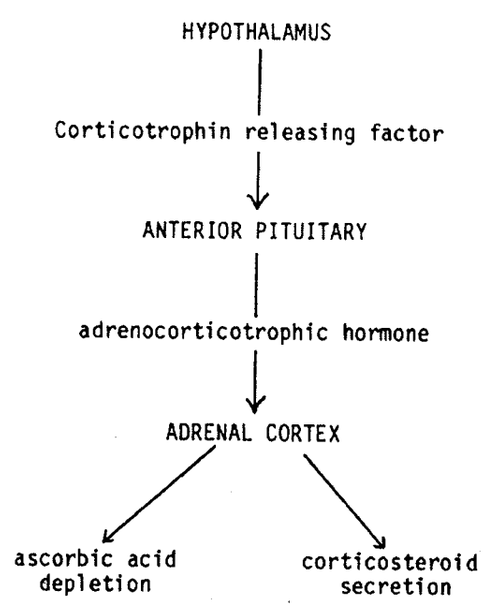

Fig. 6. – The depletion occurs fairly rapidly under the influence of adrenocorticotrophin (ACTH) produced by the anterior pituitary.

It was shown that there existed some connections between the neurovegetative activity and the AA content. Thus, the excitation of the sympathetic is followed by an AA increase, which, in its turn, potentiates the sympathetic excitation and the adrenalinic activity (Fig. 6). At the same time, AA suppresses the excitation of the pneumogastrics (vagus), hindering the action of acetylcholine and potentiates the cholinesterase activity (9, 22). In contrast, a number of experimental studies conducted in thiamin-deficient pigeons revealed that AA would act in a different manner, that is, it would have an inhibiting effect on cholinesterase and, in this way, they would favour the toxic hyperexcitation of acetylcholine (23). But, these phenomena could develop in cases where the AA level exceeds by much the physiological limits, under stress and microbial aggression, when cholinesterase is inactivated in the splitting process of the acetylcholine produced in excess.

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STRUCTURAL CHANGES INDUCED BY ZINC ACTION IN *Tubifex tubifex* (VERMES, OLIGOCHAETA)

POPESCU-MARINESCU VIRGINIA*, TESIO CĂLIN**, MARINESCU CARMEN*, STAICU CRISTINA**

In case of experimentally Zn intoxication of *Tubifex tubifex* oligochaeta we have found a range of structural-cellular changes at the level of various tissues and organs such as: tegument, musculature, chloragogen tissue, intestine. Generally, the intensity of these changes is directly proportional to the toxic concentration of the environment in which the respective organisms are maintained.

Zinc is one of the elements constituting the living material, a normal and necessary component, a dynamic oligoelement having a plastic role in organism. Zinc action may have different effects on living organisms. In certain amounts, it may induce a favorable effect, but when exceeding optimal limit, the beneficial effect gradually decreases and it may become even toxic. Zinc appears as having an important role in various enzymatic systems, being required for the synthesis of tryptophan, tyrosine, nucleic acids. But under certain conditions Zinc may produce a disequilibrium in oxygen absorption process, accumulation at the level of different tissues, slowing down the growth of organisms.

Zinc action effects on living organisms, including the aquatic ones, being complex (1), (2), (5), (6), our studies have been directed to follow the changes occurred at structural level in oligochaete of *Tubifex tubifex* species. These worms belong to major elements constituting the food for bentophage fishes, among which there are certain species of a particular economic value.

MATERIALS AND METHODS

In order to elucidate the studied problems, we carried out a range of experiments using as living material several batches, of *Tubifex tubifex* specimens, which under static test conditions were subject to the action of one of the six SO_4Zn solutions at concentrations ranging between 80 µg/l Zn and 24300 µg/l Zn (SO_4Zn at zinc concentrations from 0.2 mg/l to 60 mg/l). We used the respective SO_4Zn concentrations since in specialty literature these are the lethal limitations quoted by certain authors for various aquatic organisms (5).

The material intoxicated in this way, within a maximal period of 48h in solutions of 80–400 µg/l Zn and of 2–3h in solutions of 400–24300 µg/l Zn,was fixed in Bouin with picric acid, then it was processed by classic histological techniques and the sections stained by haemalaun were visualized by light microscopy.

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RESULTS AND DISCUSSIONS

We emphasize the fact that as far as the worms are concerned, including oligochaete, we could not find in the specialty literature data regarding structural changes generated by heavy metals action, implicitly of zinc. As concerns other aquatic invertebrates, particularly mollusca and crustacea, various authors studied the effects of heavy metal accumulation in the organism materialized on the one hand in structural changes (2), (3), (4). (6), (7), (8) and on the other hand in a metabolic disequilibrium (1), (5).

Referring to data obtained by our studies, it is worth mentioning that during experiments, in most solutions at higher concentrations of Zn the phenomenon of animal body degradation was noticed even by naked eye. Organism degradation, when occurred, was directly proportional to toxin concentration, whereas survival time was inversely proportional. As gradual aspects of organism degradation there were noticed a slowing down of movements, discolouring and pearl-like apperance of body.

But the structural changes at cell and tissue levels were very interesting. The obtaind results'analysis revealed that at 80 µg/l Zn dose (0.2 mg/l SO_4Zn) no changes were noticed at the level of animal organs and tissues maintained in the solution at this concentration for 48h. A transversal section view (Fig. 1) shows a unistratified tegument constituted of cubic, slightly cylindrical cells (Fig. 2) with adherent cuticule. Circular musculature layer is very thin, whereas longitudinal musculature layer is well developed, with regularly disposed fibres (Figs. 1 and 3). Intestine exhibits normal cells and it is surrounded by a chloragogen tissue, occupying almost the whole coelomic space (Figs. 1, 3, 4).

In specimens maintained at 200 µg/l Zn concentration (0,5 mg/l SO_4Zn) for 48h the sections cut at the level of 12–17 rings exhibit a slightly modified structure, especially at longitudinal musculature level (Fig. 5). Intestinal cells hypertrophy (Fig. 6) and chloragogen tissue begin to disorganise. This is a very important fact since the chloragogen tissue in oligochaete has the role of liver in vertebrates, having a function of glycogen synthesis and lysis, ammonia formation, as well as foreign particles phagocytosis. The tegument exhibits some hypertrophied cells and the cuticle begins to detach from the tissue (Fig. 5). We point out that worldwide, in Switzerland, the maximal tolerance allowed for zinc is of 200 µg/l.

At 400 µg/l Zn concentration (1.0 mg/l SO_4Zn) the changes become more pronounced, although short term exposure of 48h seems to be nonlethal for the animals tested by us. Sections cut at clitellum and oviger sac levels (Figs. 7, 8, 9) indicate changes in the tegument which exhibits flattened cells with horizontally arranged nuclei and the cuticule is divided into fragments. Longitudinal musculature shows changes in fibre orientation, especially dorsally. Chloragogen tissue is partially disorganized, but also it maintains normally structured cells (Fig. 9).

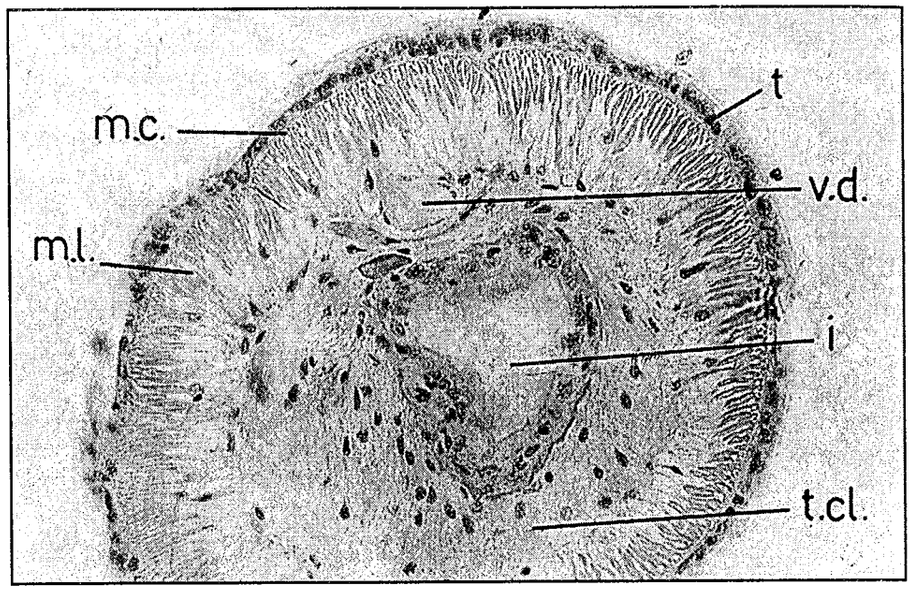

Fig. 1. – Transversal section of *Tubifex tubifex*; 80 µg/l Zn concentration; 20×0.20 (unchanged aspect): i = intestine; m.c. = circular musculature; m.l. = longitudinal musculature; t = tegument; t.cl. = chloragogen tissue; v.d. = dorsal vessel.

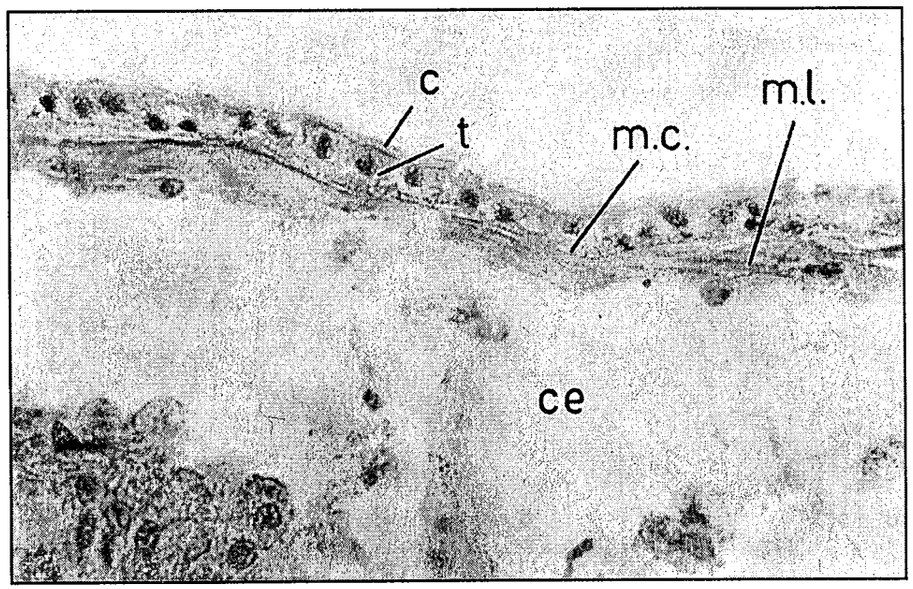

Fig. 2. – Longitudinal section of *Tubifex tubifex*; 80 µg/l Zn concentration; 40×0.20 (unchanged aspect): c = cuticle; ce = coelom; m.c. = circular musculature; m.l. = longitudinal musculature; t = tegument.

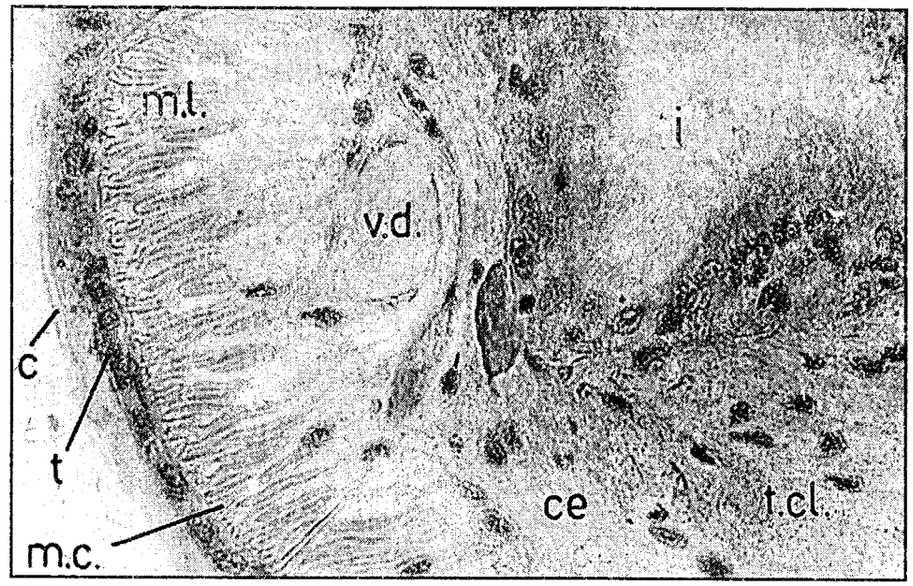

Fig. 3. – Transversal section of *Tubifex tubifex*; 80 µg/l Zn concentration; 40×0.20 (unchanged aspect): c = cuticle; ce = coelom; i = intestine; m.c. = circular musculature; m.l.= longitudinal musculature; t = tegument; t.cl. = chloragogen tissue; v.d. = dorsal vessel.

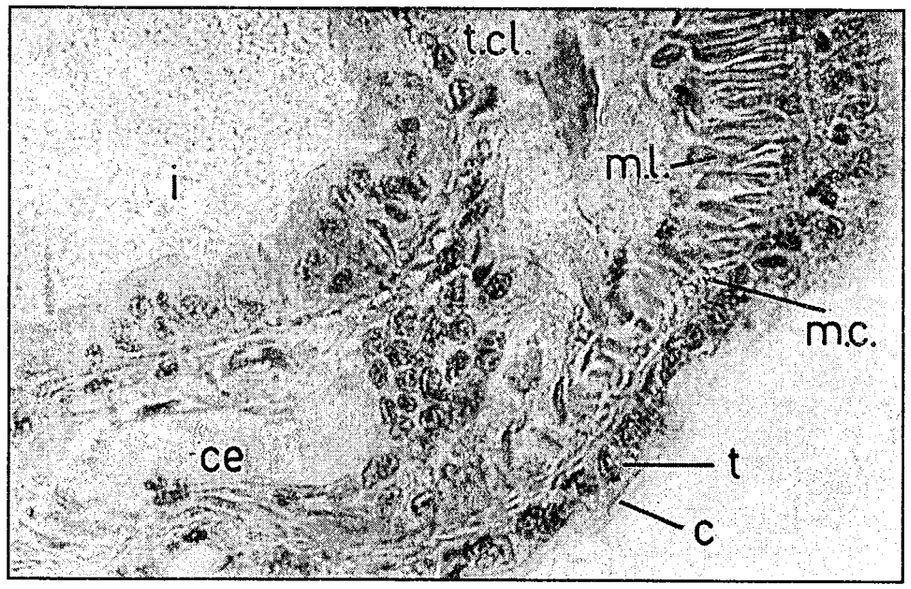

Fig. 4. -- Transversal section of *Tubifex tubifex*; 80 µg/l Zn concentration; 40×0.20 (slightly changed aspect): c = cuticle; ce = coelom; m.c. = circular musculature; m.l. = longitudinal musculature; t = tegument; t.cl. = chloragogen tissue.

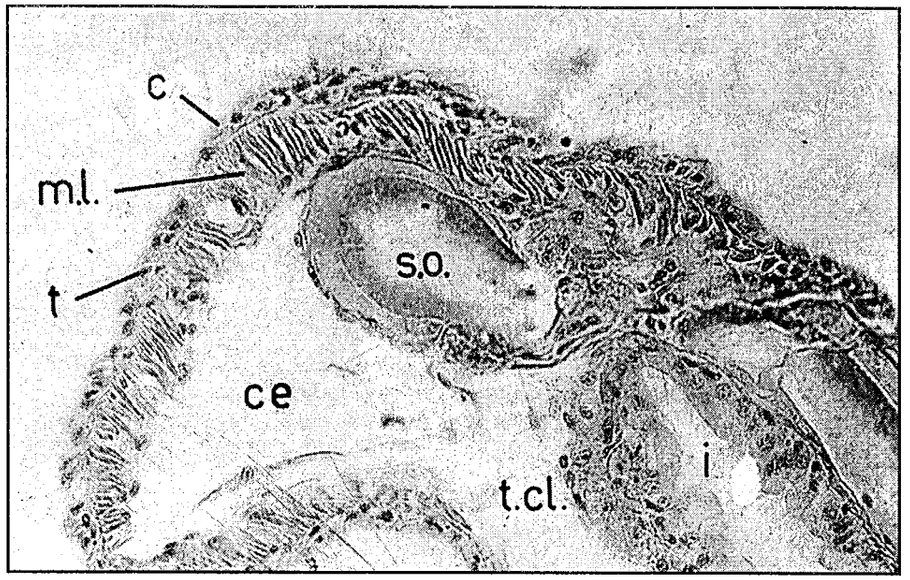

Fig. 5. – Transversal section of *Tubifex tubifex*; 200 µg/l Zn concentration; 20×0.20 (slightly changed aspect): c = cuticle; ce = coelom; i = intestine; m.l. = longitudinal musculature; s.o. = oviger sac; t = tegument; t.cl. = chloragogen tissue.

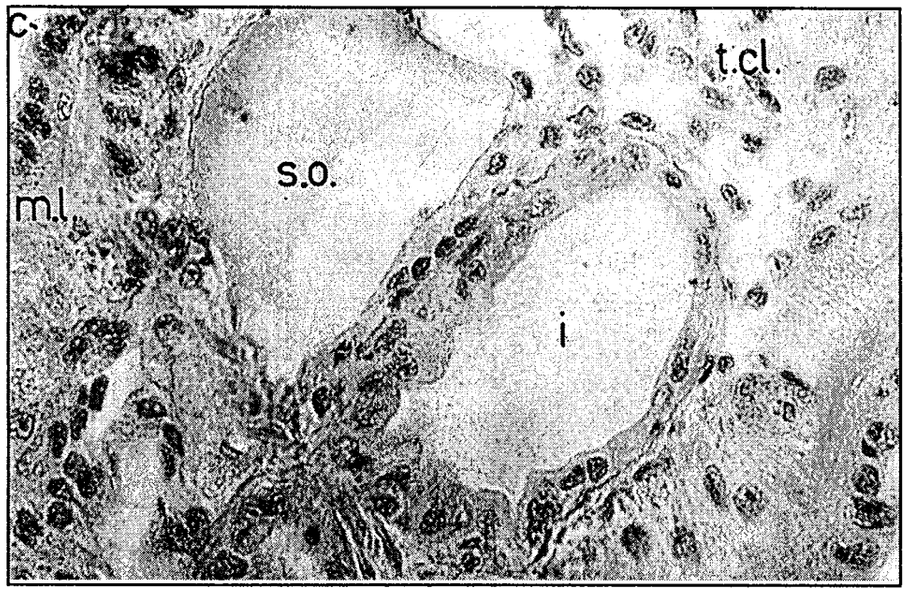

Fig. 6. – Transversal section of *Tubifex tubifex*; 200 µg/l Zn concentration; 40×0.20 (slightly changed aspect): c = cuticle; i = intestine with hypertrophied cells; m.l.=longitudinal musculature; s.o. = oviger sac; t.cl. = chloragogen tissue.

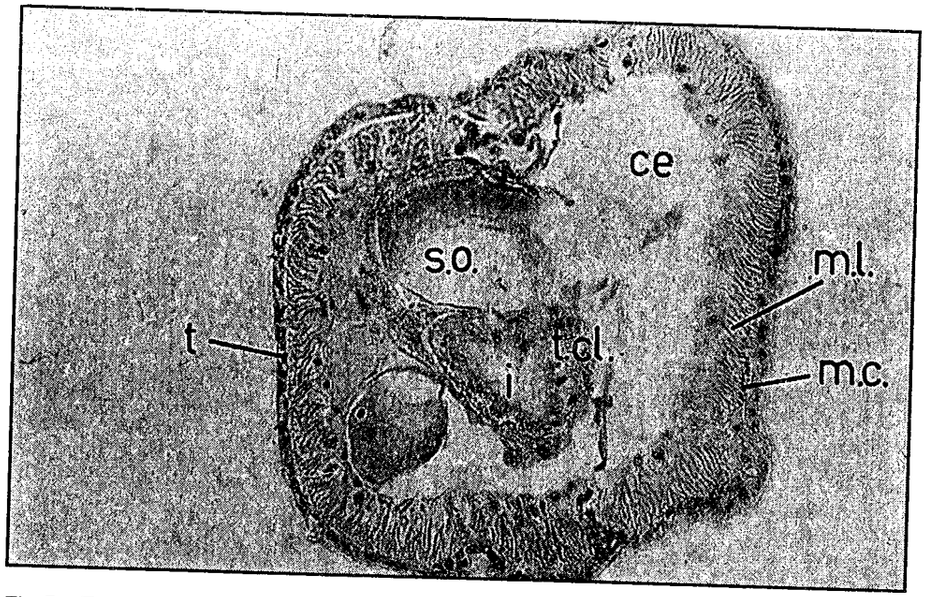

Fig. 7. – Transversal section of *Tubifex tubifex*; 400 µg/l Zn concentration; 20×0.10 (more pronounced changes especially at tegument level): ce = coelom; i = intestine; m.c. = circular musculature; m.l. = longitudinal musculature; s.o. = oviger sac; t = tegument.

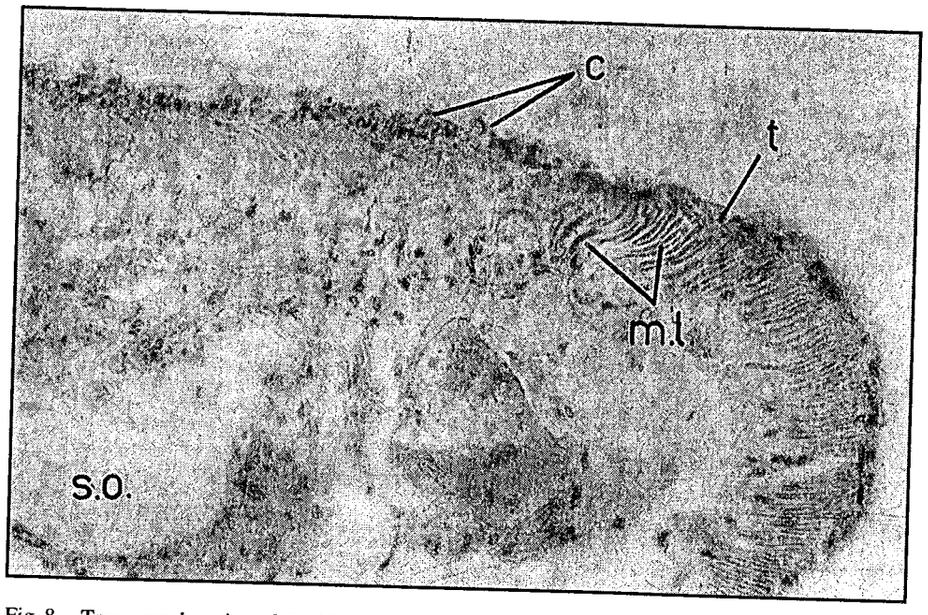

Fig. 8. – Transversal section of *Tubifex tubifex*; 400 µg/l Zn concentration; 20×0.20 (more pronounced changes especially at tegument level): c = cuticle; m.l. = longitudinal musculature; s.o. = oviger sac; t = tegument.

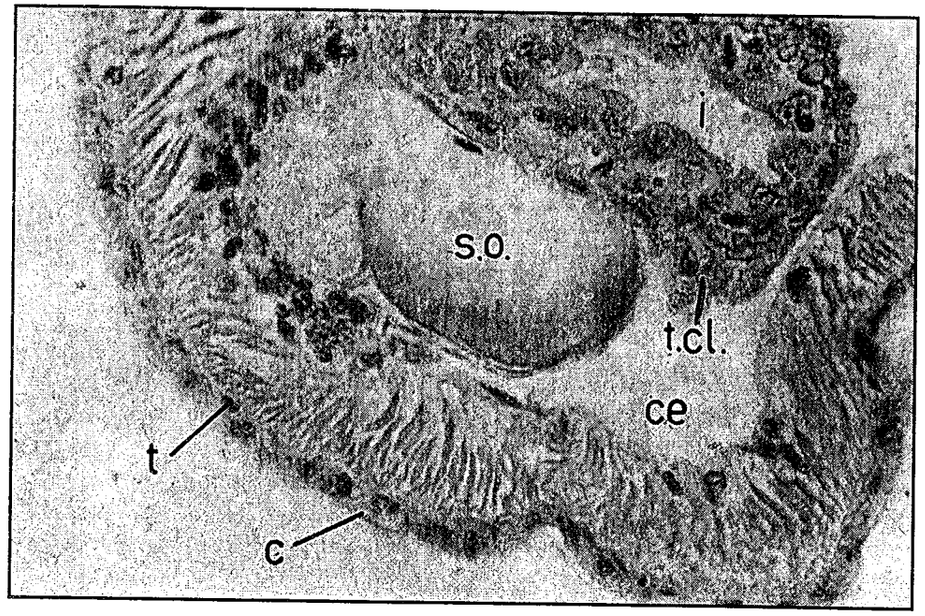

Fig. 9. – Transversal section of *Tubifex tubifex*; 400 µg/l Zn concentration; 40×0.20 (more pronounced changes at chloragogen tissue level): c = cuticle; ce = coelom; s.o. = oviger sac; t = tegument; t.cl. = chloragogen tissue.

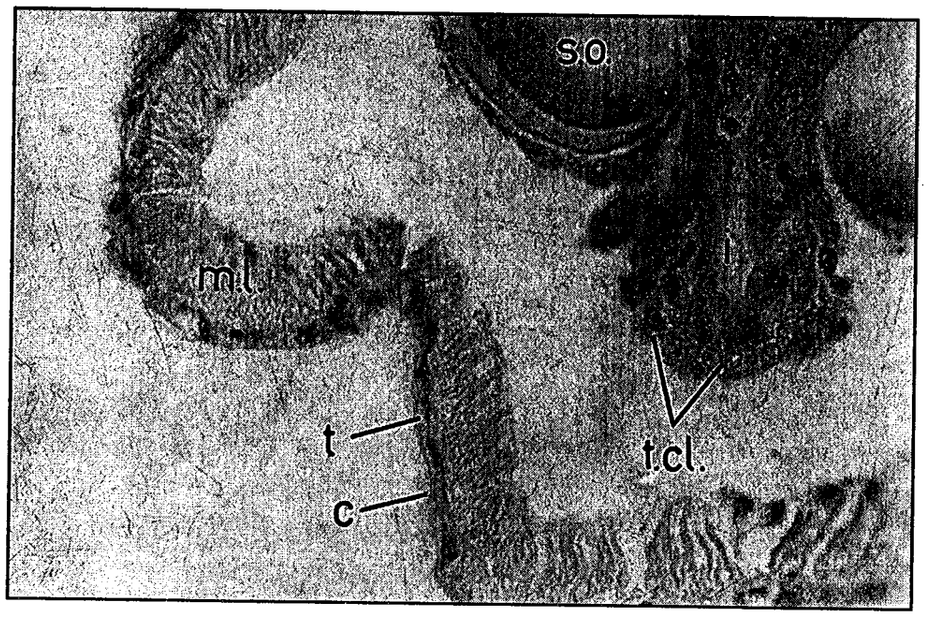

Fig. 10. – Transversal section of *Tubifex tubifex*; 4000 µg/l Zn concentration; 40×0.20 (important changes at chloragogen tissue level): c = cuticle; i = intestine; m.l. = longitudinal musculature; s.o. = oviger sac; t = tegument; t.cl. = chloragogen tissue.

At 4000 µg/l concentration (10 mg/l SO_4Zn), animals died after 3h. Under these conditions, numerous structural changes can be observed at the level of the following structures: tegument flattens, cells become either very low with pycnotic nuclei (Fig. 10), or in certain zones they are very irregular (Fig. 12), cuticule detaches from the tegument (Figs. 11, 12). Mostly of chloragogen tissue is destroyed, with cells full of vacuoles and granulations (Fig. 10). Longitudinal musculature exhibits numerous zones of disintegration. Intestine has lengthened cells with pycnotic nuclei.

At 16200 µg/l Zn concentration (40 mg/l SO_4Zn) oligochaete lived for only 2h and 30 min. In these animals we observe changes of the same type as those induced by 4000 µg/l Zn concentration, i.e. tegument exhibits pavement cells with pycnotic nuclei and numerous vacuoles. Longitudinal musculature has disorganized fibres. Intestine cells are destroyed, having disorganizing nuclei. Chloragogen tissue is charged of granulations and destroyed nuclei (Fig. 13).

At the highest concentration used by us, of 24300 µg/l Zn (60 mg/l SO_4Zn), in which animals lived for only 2h, the structural changes are strongly exhibited at the tegument level where the cells have irregular shapes and pycnotic nuclei (Fig. 14), at their basis granulations are accumulating. Longitudinal musculature undergoes disintegrations (Figs. 14, 15 and 16). Intestine is very affected, with disorganized cells, pycnotic nuclei and heavy granulations at cells'basis. Chloragogen tissue is also destroyed, the cells have no more nuclei and they accumulated small granulations (Figs. 14, 15 and 16). Zinc accumulation in intracellular granulations at the level of various organs has been observed by several authors (3), (4), (6), (7), (8).

CONCLUSIONS

As concerns the structural changes generated by zinc action at the cell and tissue levels in *Tubufex tubifex*, there were noticed the following:

1. In fresh water, the presence of zinc ions at 80 µg/l concentration does not produce visible changes during short term exposure of organisms (48h);

2. Some slight or more pronounced changes appear in the tegument, longitudinal musculature and chloragogen tissue in animals maintained in water at 200–400 µg/l Zn concentrations;

3. At high and very high concentrations of Zn (4000–24000 µg/l) major changes appear in organs of various tissue of oligochaete: tissue disintegration and numerous granulations accumulations in the musculature and chloragogen tissue (a tissue with a metabolically important role).

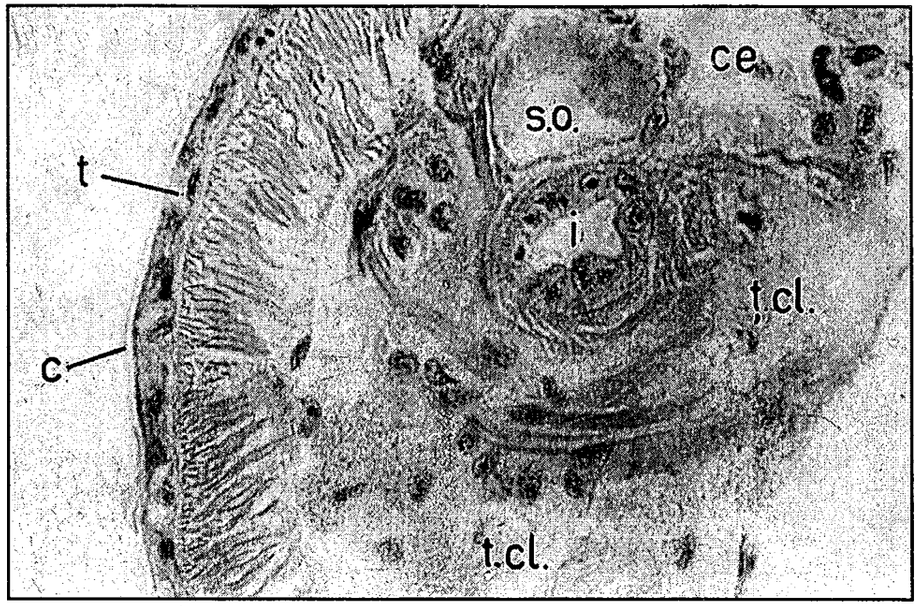

Fig. 11. – Transversal section *Tubifex tubifex*; 4000 µg/l Zn concentration; 40×0.20 (important changes at cuticle and tegument levels): c = cuticle detached from tegument; ce = coelom; i = intestine; s.o. = oviger sac; t = tegument; t.cl. = chloragogen tissue.

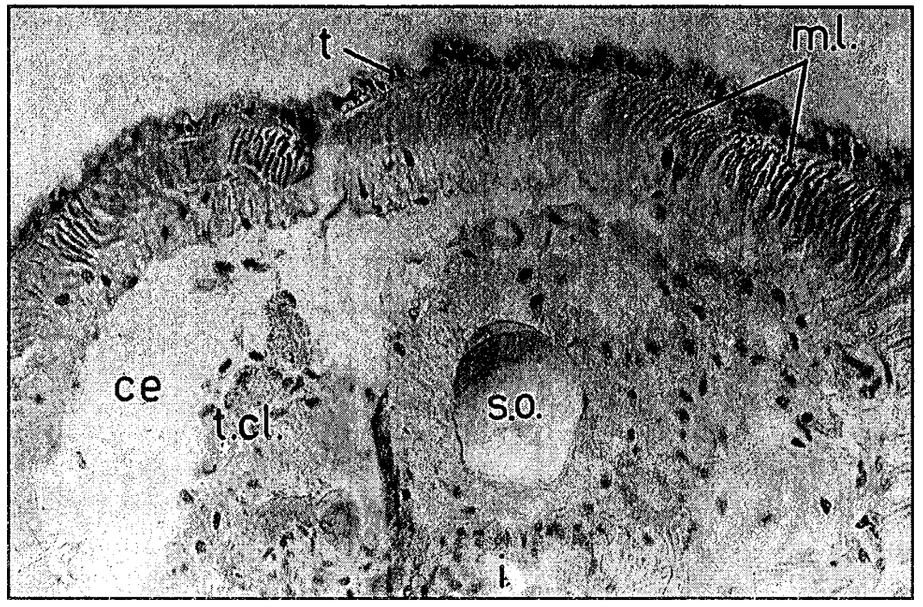

Fig. 12. – Transversal section of *Tubifex tubifex*; 4000 µg/l Zn concentration; 40×0.20 (important changes at tegument level): ce = coelom; m.l. = longitudinal musculature; s.o. = oviger sac; t = tegument with very irregular cells; t.cl. = chloragogen tissue.

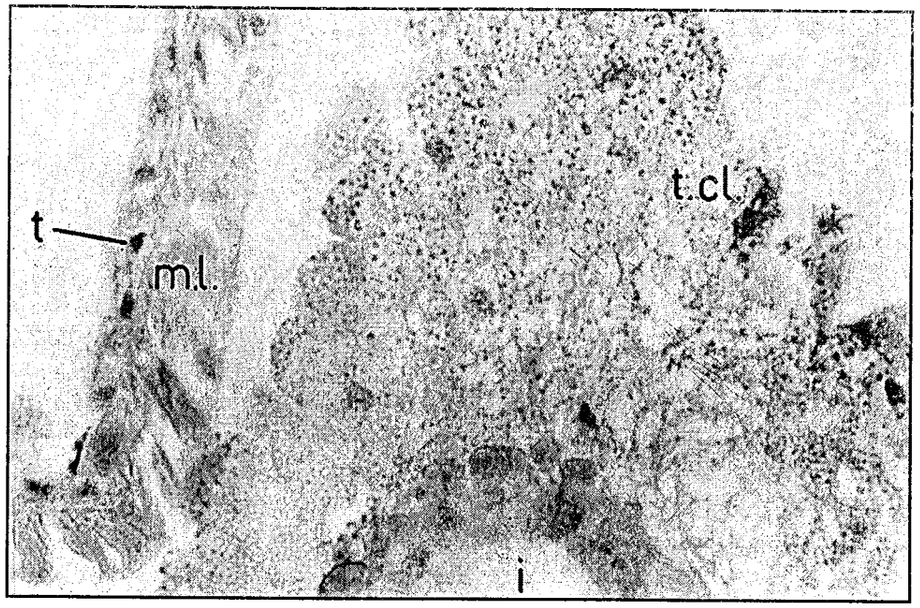

Fig. 13. – Transversal section of *Tubifex tubifex*; 16200 µg/l Zn concentration; 40×0.25 (strong changes at tegument level): i = intestine; m.l. = longitudinal musculature; t = tegument with pavement cells and pycnotic nuclei; t.cl. = chloragogen tissue.

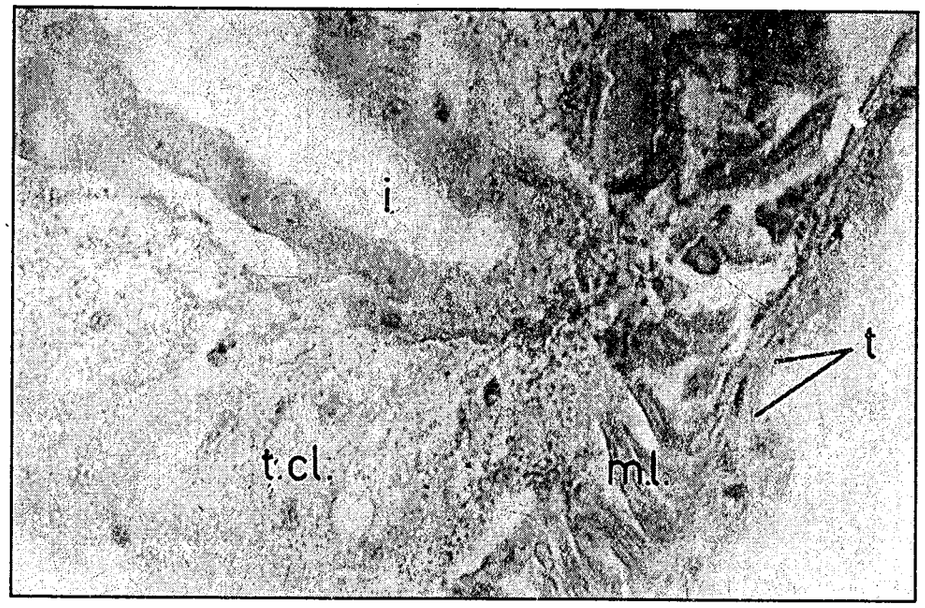

Fig. 14. – Transversal section of *Tubifex tubifex;* 24300 µg/l Zn concentration; 40×0.25 (strong changes also at tegument and at longitudinal musculature level): i = intestine; m.l. = longitudinal muscula-

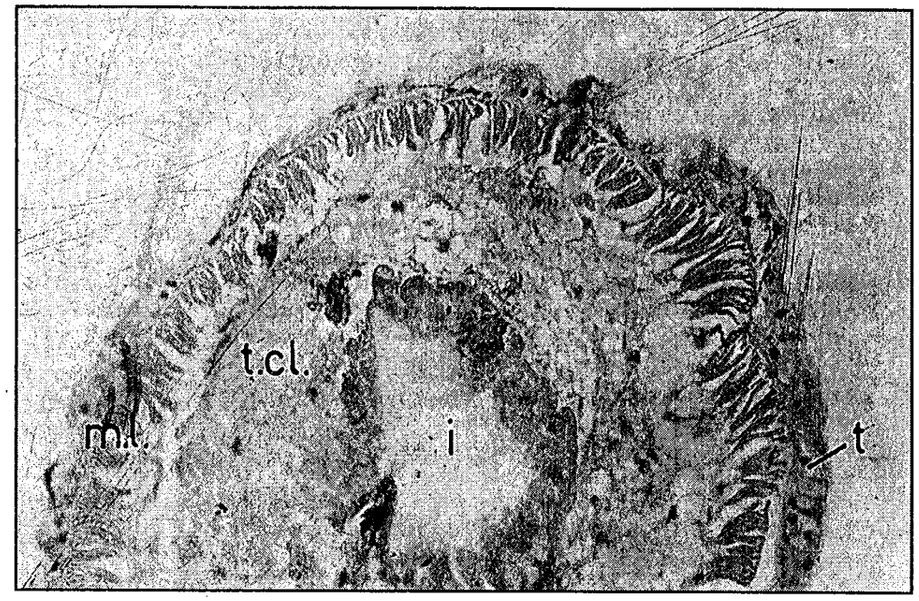

Fig. 15. – Transversal section of *Tubifex tubifex*; 24300 µg/l Zn concentration; 40×0.20 (very strong changes at the level of tegument and longitudinal musculature): i = intestine; m.l. = longitudinal musculature; t = tegument; t.cl. = chloragogen tissue.

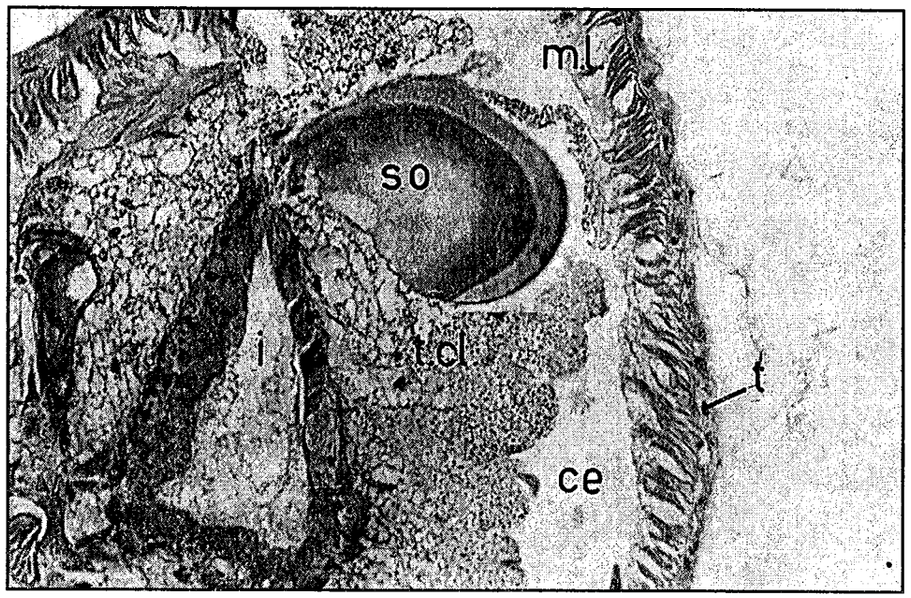

Fig. 16. – Transversal section of *Tubifex tubifex*; 24300 µg/l Zn concentration; 40×0.20 (very strong changes at the level of intestine and chloragogen tissue): ce = coelom; i = intestine; m.l. = longitudinal musculature; s.o. = oviger sac; t = tegument; t.cl. = chloragogen tissue.

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PARTICIPATION OF ERYTHROCYTES IN THE PROCESS OF BIOLOGICAL TRANSFORMATION. ACTIVITY OF GSTf (GLUTATHIONE-S-TRANSFERASE) IN FISH ERYTHROCYTE

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Investigations were conducted on three fish species (especially on carp) on the content of glutathione (GSH) and on the activity of GSTf enzyme in the nucleate erythrocyte characteristic to this class of poikilothermal vertebrates.

We determined values of about 2.2 µmol/g Hb in carp erythrocyte, which are, surprisingly, even higher than the values determined in the liver of *Idus idus* L. (2.0 µmol/g).

In the species *Onchorhynchus mykiss* the amount of GSH (reduced form) was about three times higher than in carp erythrocytes (about 6.4 µmol/g Hb).

The activity of GSTf enzyme in carp erythrocytes was higher (22 µmol/min. g Hb) than in *O. mykiss* (17 µmol/min. g Hb).

The ratio between the activity of this enzyme in erythrocytes and liver (values expressed in umol/min. mg soluble protein) was 1/17 in carp as compared to 1/50 in *O. mykiss*.

Based on the high concentration of GSH and on the activity of GSTf enzyme in fish erythrocytes, the authors took into consideration the role of erythrocytes in the processes of biological transformation (conjugation).

INTRODUCTION

Several "xenic" substances, that cannot be used by the metabolism, enter the living organism together with the nutrients indispensable to life. Because these substances, often toxic, cannot be eliminated due to their lipophilic character, they have to be transformed in hydrophilic bonds, lacking toxicity. The major organ achieving this process is the liver where highly active enzymes of phase I (Cyt P450-Oxygenase) and of phase II (conjugation) can be met (7, 6).

Since the foreign substances, after passing the epithelial wall of the digestive or respiratory organs, meet the erythrocytes, and since we have previously found out that erythrocytes contain high concentrations of tripeptide GSH (the reduced form) (4), we considered the possible participation of these cells, known before only for their major role in transporting the respiratory gases, in the process of biological transformation by conjugation.

We could not find any paper in the literature with reference to GSTf activity in fish red cells.

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MATERIALS AND METHODS

The following species were used by our investigations: carp (*Cyprinus carpio* L.), *Onchorhynchus mykiss* and *Idus idus* L.

Heparin and EDTA were used as anticoagulant preparations.

The following method was used to separate the erythrocytes: the blood sample was diluted 1:1 with a medium of PBS-heparin, passed on a "Ficoll-carrier" (density 1.077 g/ml) and centrifuged. The leukocytes remained bound (interphase) and the erythrocytes passed into the sediment. The red cells were subsequently rinsed with a PBS-heparin medium.

The samples of 100 ul erythrocytes were stored at –80°C in liquid nitrogen.

The following method was used to obtain the homogenate: the frozen blood samples, the blood cells or the liver samples were homogenised in a phosphate buffer solution (50 mmolar, pH 7.0; EDTA 1.0 mmolar, triton X100 0.2 mmolar). An ice bath and the Potter homogeniser were subsequently used to obtain the supernatant containing the enzyme Glutathione-S-Transferase (GSTf) and haemoglobin (Hb).

GSTf (E.C. 2.5.1.18) activity was assessed with a "xeno"-substrate (1-chloride-2,4-dinitrobenzene, CDNB), in which the halogen was replaced by the balance of GSH. The experimental temperature was 25°C. Recording was done at 340 nm and ED/min was determined according to the method of Habig, 1981.

In order to determine the glutathione (GSH and GSSG) we prepared a supernatant with 1 mole perchloric acid. We used an ice bath and a Potter homogeniser. After centrifugation the sediment was rinsed and the supernatant was used to determine the two forms of glutathione (reduced and oxidised). GSH and GSSG were determined by enzymatic cycling with GSH-reductase and Ellmann (DTNB) reagent, at 25°C, on a wavelength of 412 nm (according to Griffith, 1985).

Haemoglobin was determined with a Merck 9405 solution, at 546 nm, after calibration with bovine haemoglobin.

Protein was determined with the method of Lowry, E 700 nm, after calibration with BSA and bovine Hb.

The experimental fish were kept according to the experimental protocol described by us in previous experiments (5, 3).

RESULTS AND DISCUSSION

The values recorded by us for the concentration of glutathione (GSH, the reduced form) in the total blood were significantly higher than the values recorded for GSSG (oxidised form of the glutathione) (Fig. 1).

If we compare the two species under investigation, a 3fold higher concentration was observed in *O. mykiss* as compared to the carp.

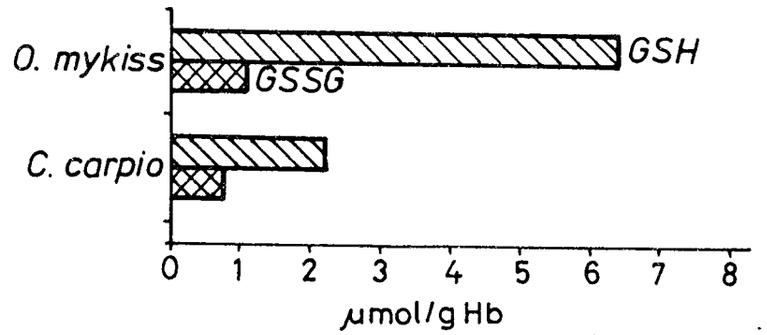

Fig. 1. – GSH and GSSG in the blood.

Plasma GSH had very low values.

The activity of enzyme glutathione-S-transferase (GSTf), in the liver of two species (*Cyprinus carpio* and *O. mykiss*), also showed a 2fold higher intensity in *O. mykiss* (Fig. 2).

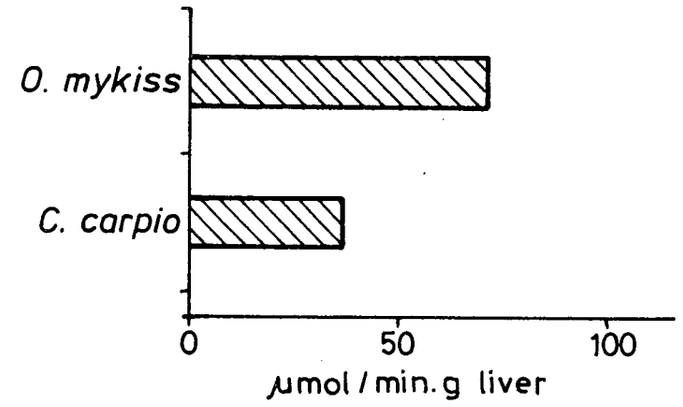

Fig. 2. – GSTf activity in the liver.

Fig. 3 shows the results regarding the activity of GSTf enzyme in the erythrocyte of the two species. The noticed situation was, surprisingly, different from the previous one. If GSH concentration and GSTf activity were found to be higher in the liver of *O. mykiss*, the situation reversed with the erythrocytes (Fig. 3).

If we compare the ratio between the intensity of enzyme activity in the liver and erythrocyte ("factor l/e"), we can observe (Table 1) that factor l/e was 50 in *O. mykiss* while it was only 17 in *C. carpio* (Table 1).

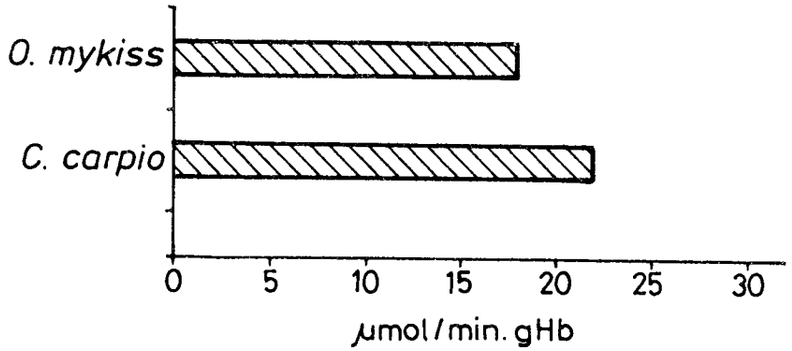

Fig. 3. – GSTf activity in erythrocytes.

Table 1

Dim: µmol/min. mg solv. protein

	Liver	Erythrocytes	Factor liver/Erys
O. mykiss	0.55	0.011	50
C. carpio	0.31	0.018	17

This means that the mechanisms which use erythrocytes to form conjugates of GSH (first of all the intensity of GSTf activity) were more active in the carp compared to the activity in the liver. It is interesting to compare this data with the data of literature for homeothermal animals (for example in poultry, in species *Columba livia* the ratio l/e is 25) (4).

It is difficult to give now an explanation for this situation, more research being necessary to gather supplementary data. We consider that this might, however, be the case of an adaptive value. The species *O. mykiss* has a more intense metabolism, it lives in an ecological niche characteristic to colder water, well oxygenated and with less impurities. It is logical that their liver should be the main place for the processes of biological transformation. In carp, which has a lower metabolism, and which lives in less oxygenated water containing more impurities, the detoxifying role of the liver seems to be backed (completed) to a large (relative) extent by the nucleate erythrocytes, as we have, surprisingly, found out in this experiment.

The activity of GSTf enzyme was very low in blood plasma and leukocytes (Table 2).

Table 2

	Blood	Plasma	Erys	Leukos
Cyprinus carpio	20	0.02	22	0.04

This means that only the erythrocytes keep this quality of participating in the processes of biological transformation, quality which is probably connected to keeping their nucleate character (in mammalians the factor l/e for this enzyme is 214). The leukocytes, known for their role in the reaction of defence of the organism, are, surprisingly, not involved in this type of biological transformation! Subsequent investigations on the physiological role of the erythrocyte will elaborate on the present knowledge in order to understand better this surprising role of erythrocytes (Fig. 4).

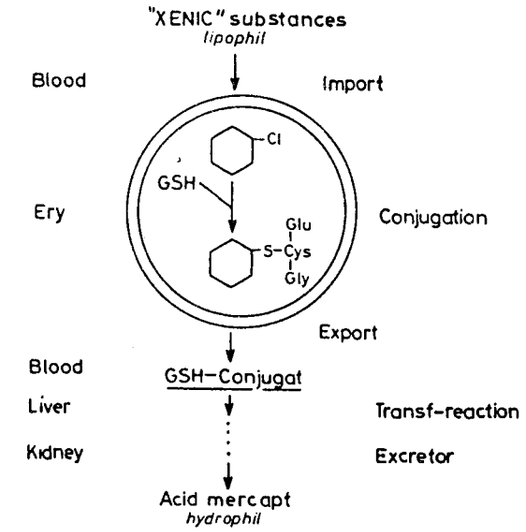

Fig. 4. – Participation of erythrocytes in biotransformation.

Fig. 4 shows the participation of fish erythrocytes in the processes of biological transformation (conjugation) by which the "xenic" substances turn from lipophilic to hydrophilic and can thus be eliminated from the organism.

CONCLUSIONS

1. The content of glutathione (GSH, reduced form) in fish erythrocytes differed with the species: 2.2 µmol/g Hb in *Cyprinus carpio* and 6.4 µmol/g Hb in *Onchorhynchus mykiss*.

2. The activity of glutathione-S-transferase (GSTf) enzyme was 67 µmol/min.g liver in *O. mykiss* and 35 µmol/min.g liver.

3. The activity of GSTf was observed only in erythrocytes, while in blood plasma and in leukocytes it was very low. In *C. carpio* the recorded value was 22 µmol/min. g Hb, higher than in *O. mykiss* (17 µmol/min. g Hb).

4. The ratio l/e was 1/17 in *C. carpio* and 1/50 in *O. mykiss*.

5. Fish erythrocytes participate in the process of biological transformation of "xenic" substances with a different intensity given by the adaptation to the ecological niche of the respective species.

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RESEARCHES REGARDING MARKING EUROPEAN CORN BORER (*Ostrinia nubilalis* Hb.) BY ^{32}P

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European Corn Borer moths were labelled after they ate the semisynthetic diet in which ^{32}P was added as a colourless and transparent solution of $NaH_2{}^{32}PO_4$.
The ^{32}P radioactivities in the diet were 7.4 and 13 kBq/g. Both, 7.4 and 13 kBq/g diet reached our purpose for permanent moths labelling, the biggest part of radioactivity was contained by moths and the smallest part by exuvies.
It was registered: no. of pupae/box, no. of moths/box, egg batches/female and percentage of fertile eggs; radioactivity of pupae, moths and exuviae.
The radioactivity of 7.4 and 13 kBq/g has a slight obvious influence on the longevity of marked ECB.

The European corn borer (ECB), *Ostrinia nubilalis* Hb., is considered in Romania the most important pest of maize crop after tasseling (7, 8).

Due to its outstanding economic significance for maize crop in Romania, extensive research has been done particularly on its chemical (20), biological control (5, 9, 10, 11, 13) and on the development of hybrids resistant to its attack (1–3). During recent years, particular attention was paid to the study of synthetic sex pheromone (12, 16, 17) and since 1988 to the investigations on male sterilization by radiation (4, 14, 15, 18).

In order to develop an insect release technique, special attention was paid to the investigations on the ECB dynamics and the estimation of the natural population by means of pheromone traps and markers. The innate ability of insects to predispose themselves to movement on atmospheric transport systems is a successful mechanism to assure survival of the species. It is obvious that information on the flight of ECB is highly important and that knowledge of the dispersion and flight range of any insect in all its aspects is meagre indeed and for this reason a great part of our researches were directed toward establishing the distance to which a moth may fly. Due to the different changes of the wind direction it is important to have more than one type of marked moths through different marking methods to be released in field conditions. Rearing and releasing tagged insects in a natural population will give good information on total numbers by applying mathematical formulae and studying the ratio of tagged to non-tagged insects recovered by traps collecting.

Few things were known about the flight distance of ECB. Jermy and Nagy considered that in nature, the adults could migrate on long distances (6). The tagging of insect with a radioactive marker has been used in studying dispersion, speed of flight, total number of natural insect population in an area and other biological investigations. ^{32}P is a useful tagging agent in spite of its short half life.

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We have tried to combine nonradioactive methods with radiotracers technique in double labeling in order to permit differentiation between the individual specimens and groups of insects or populations of ECB for studying dispersion after a different time of the second releasing of moths.

The previously tried methods for labelling adult with fucsine or fluorescent dyė (4), and contamination with ^{32}P of food mixture(water and honey) for moths, have failed in field trials or in laboratory.

MATERIAL AND METHOD

Normal mass rearing procedures were used, rearing boxes were prepared and planted for each type of marking method on the same day.

It was used radioactive isotope ^{32}P method for marking the insects. The markers were mixed homogeneously in the agar of the semisynthetic diet for the ECB. The larvae were marked after eating the diet.

A colourless and transparent solution of $NaH_2{}^{32}PO_4$ was added to the semisynthetic diet for the ECB in rearing boxes, which were held at 20–22°C; emerged moths were maintained at the same temperature. The ^{32}P radioactivities in the diet were 7.4 and 13 kBq/g. Pupae were collected in the last day of their development and maintained separately in individual boxes. At the end of development of the larvae, pupae for each variant were collected and a day before moths emerging, radioactivity (count/min./moth) was registered, the same was done for emerged moths during their whole period of life. It was taken into consideration: no. of pupae/rearing box, no. of moths/box, egg batches/female and percentage of fertile eggs; radioactivity of pupae, each moths during the life time and exuviae.; survival of ^{32}P labelled moths. Each individual was scored as being radiolabelled if it produced a count rate higher than 100 counts/min.

RESULTS AND DISCUSSION

Taking into consideration: no. of pupae obtained/rearing box, it is possible to observe that it was registered a decreasing from check (187.2) to 7.4 kBq/g of diet (172.5) and 105.5 in variant with 13 kBq/g of diet; no. of moths obtained/rearing box were 125.7 for check, 107.5 for variant with 7.4 kBq/g of diet and 63.2 for variant with 13 kBq/g of diet; egg batches/female have decreased from 3.7 for check to 2.2 for 7.4 kBq/g of diet and 0.7 for 13 kBq/g of diet; and the same phenomenon was registered for percentage of fertile eggs which has decreased from 100% for check to 65.5% for 7.4 kBq/g of diet and 50.25% for 13 kBq/g of diet. Upper data indicate that rearing of ECB on radioactive diet affect biological parameters of obtained moths and that rearing medium with 7.4 kBq/g of diet is most suitable for obtaining labelling of moths.

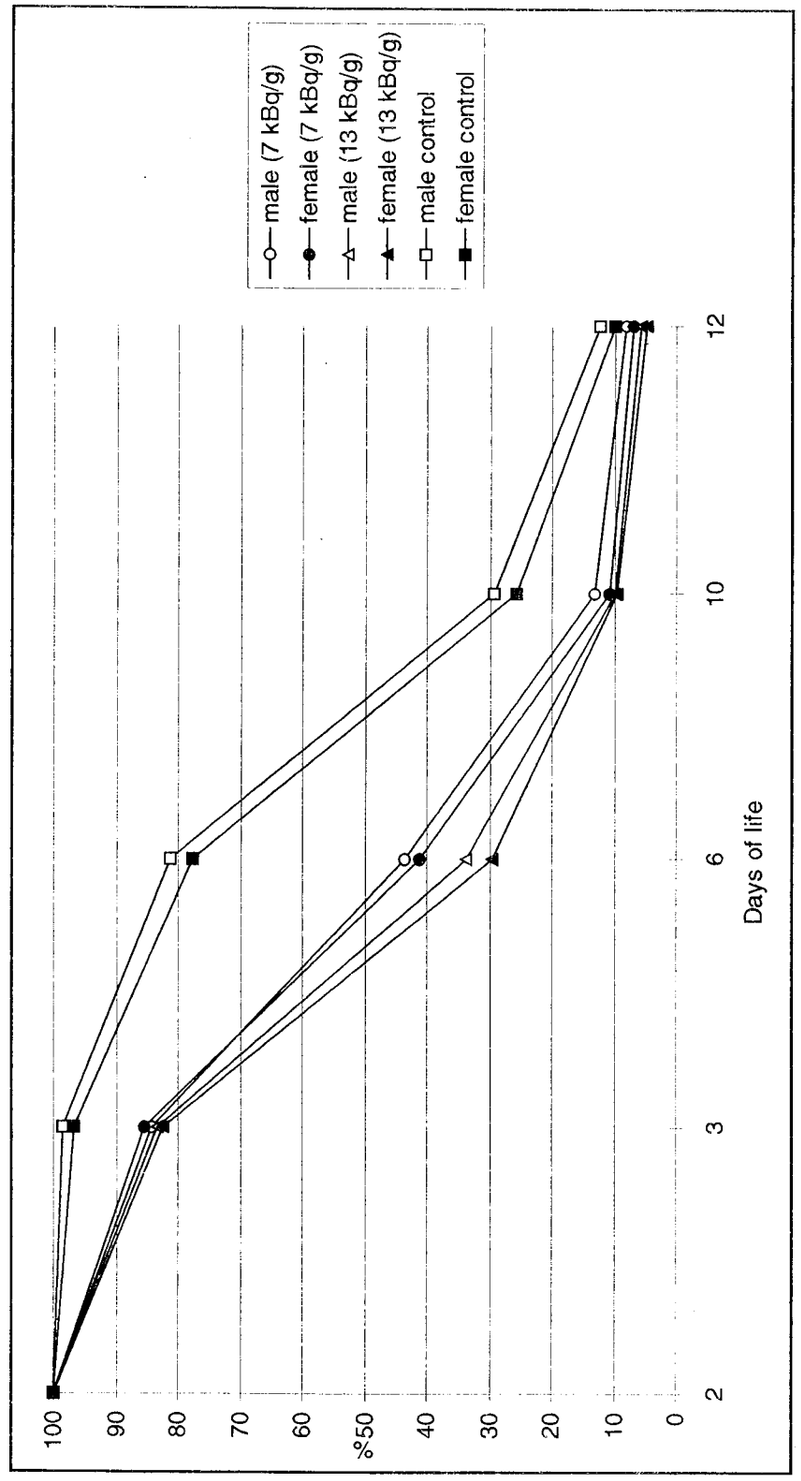

Fig. 1. – Survival of ^{32}P labelled moths.

Data presented in figure 1 indicate that when the radioactivity was 7.4 and 13 kBq/g of diet, labelled moths could live as long as control moths, but their survival was lesser than of control ones. Thus, in average survival was smaller for moths reared with 7.4 kBq/g, respectively with 13 kBq/g in report with the control: after 3 days, with 13.06% and 15.11%; after 6 days, with 46.8% and 60.23%; after 10 days, with 56.46% and 64.12% and in the last day of life, with 31.53% and 11.26%. There is a slight difference between surviving of males and females.

Both, 7.4 and 13 kBq/g diet reached our purpose for permanent moths labelling, the biggest part of radioactivity was contained by moths and the smallest part by exuvies. Thus, in average for moths reared with 7.4 kBq/g diet, males exuvies have had 8.55% radioactivity, females exuvies 9.02% and for moths reared with 13 kBq/g diet, 6.85% males exuvies and 7.03% females exuvies in report with whole radioactivity on the emergence day. There is a slight difference between radioactivity of males and females (Fig. 2).

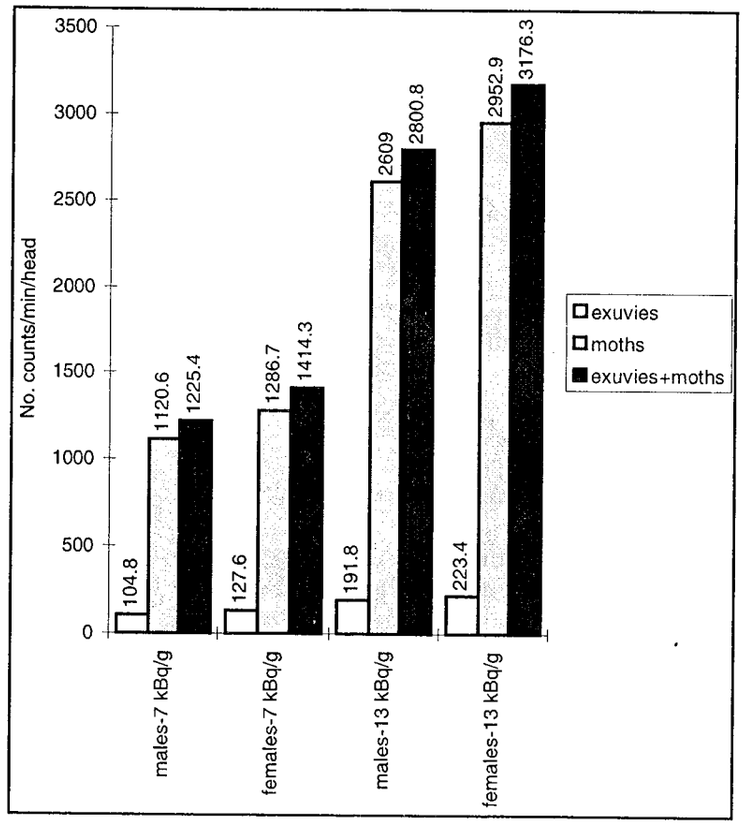

Fig. 2. – The radioactivity of marked moths and their exuvies.

With the aim of releasing labelled moths in field conditions it would very important to know how long it will be possible to watch these insects in field conditions, to know how long the radioactive labelled moths could live.

When the radioactivity was 7.4 kBq/g and 13 kBq/g in the diet, the marked insects could live as long as the control moths; meanwhile, the radioactivity in each adult insect has decreased in time after a slope which depends on the sex and diet used (Fig. 3). In average, for males reared with 7.4 kBq/g diet radioactivity was 1120.6 count/min/males on the first day and decreased to 633.8 count/min/males on the last day of life; for females 1286.7, respectively 766.2 and at the same time, in average, for males reared with 13 kBq/g diet radioactivity was 2609 count/min/males on the first day and decreased to 1427.2 count/min/males on the last day of life; for females 2952.9, respectively 1351.

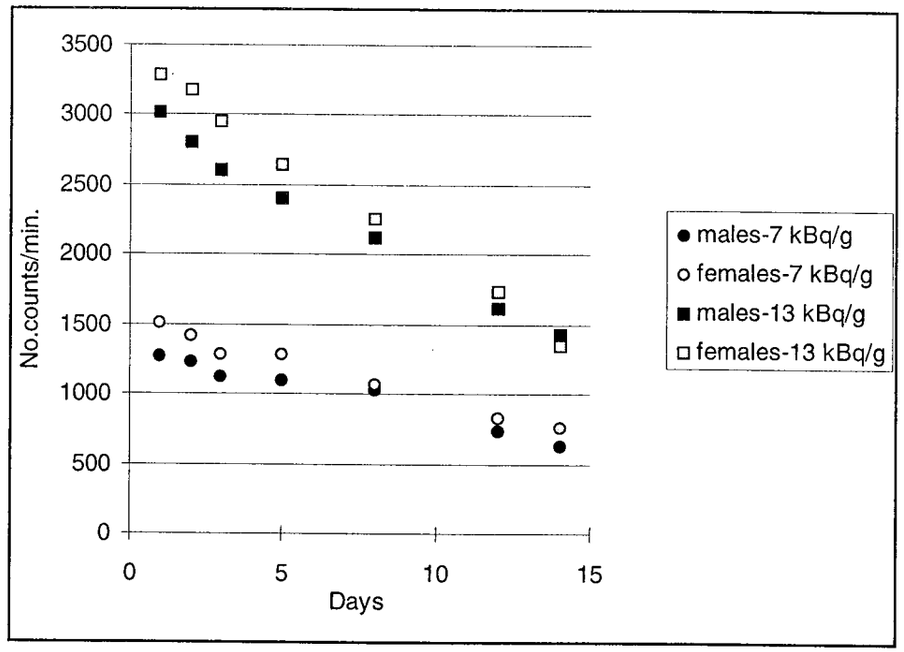

Fig. 3. – Radioactivity evolution of ^{32}P marked moths.

Our result confirms the results obtained in 1991 in a related research which was done by Yang et al. for diamondback moth (20).

Because, after the accident at Three Mile Island and especially after Chernobyl in 1986, the requirements of National Atomic Energy Agency became much more restrictive, we have obtained only a limited permission to release ^{32}P la-

belled moths in the field, for a limited period of time. In this respect our researches with markers, in the future, will be directed to find a new dye marking method, based on a new colour.

CONCLUSIONS

Presented data indicate that rearing of ECB on radioactive diet affect the biological parameters of the obtained moths and that rearing medium with 7.4 kBq/g of diet is most suitable for obtaining labelling of moths.

Both 7.4 and 13 kBq/g diet reached our purpose for permanent moths labelling, the biggest part of radioactivity being contained by the moths and the smallest part by exuvies.

^{32}P labelled moths by using 7.4 kBq/g and 13 kBq/g in the diet could live as long as control moths; meanwhile, the radioactivity in each adult insect has decreased in time after a slope which depends on the sex and diet used.

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THE STATE OF THE BENTHIC COMMUNITY OF THE DANUBE DELTA LAKES – A CONSEQUENCE OF EUTROPHICATION

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The analysis of the benthic communities structure during 1992–1994 and the comparison with the situation from 1975–1982 point out that the structure of these communities was oriented towards simplification. The simplification of the communities structure is reflected by the absence of some taxa and also by the decrease of the representativity and of species richness within the existing taxa.

The low values of the Shannon diversity and equitability indexes are also supporting this conclusion. The eight benthic communities are in the similar phases of evolution as Morisita index reveals.

The dominant taxa, in numerical terms, are by far, chironomids and oligochaeta. From the gravimetric point of view *Mollusca* represents the dominant taxa.

Among the five parameters monitorised, the content of organic matter within the upper five centimeter layer of sediment, the TRP within the water body, and the transparency index seem to be important drive variables.

Key words: Danube Delta, benthic communities, structure dynamics, diversity, eutrophication.

INTRODUCTION

During the latest 10–15 years, an ecological crisis in the "Danube River–Danube Delta–Black Sea" System has become apparent. The main process that modifies ecological succession in the aquatic ecosystems is eutrophication (11).

The research works that have been done during this interval of time on the aquatic ecosystems of the Danube Delta (1, 2, 4, 9, 10, 11, 12) have concluded that the main part of the primary production has been transferred to the sediments and, consequently, the benthic community became one of the most important links in the energy and nutrient flow (Fig. 1).

In such conditions, the main drive variables of the benthic community dynamics are represented, on the one side, by the content of sedimented organic matter as main energy supply and on the other side, by the advanced hypoxia conditions at the sediment-water interface, conditions developed as a consequence of the organic matter decomposition (2, 12).

Bearing in mind the above aspects and the fact that the structural changes are one of the major responses of the aquatic ecosystems to stress, that occur earlier or at lower levels of stress than do changes in ecosystem processes (5), we are trying to assess and discuss the dynamics of the benthic communities structure in several

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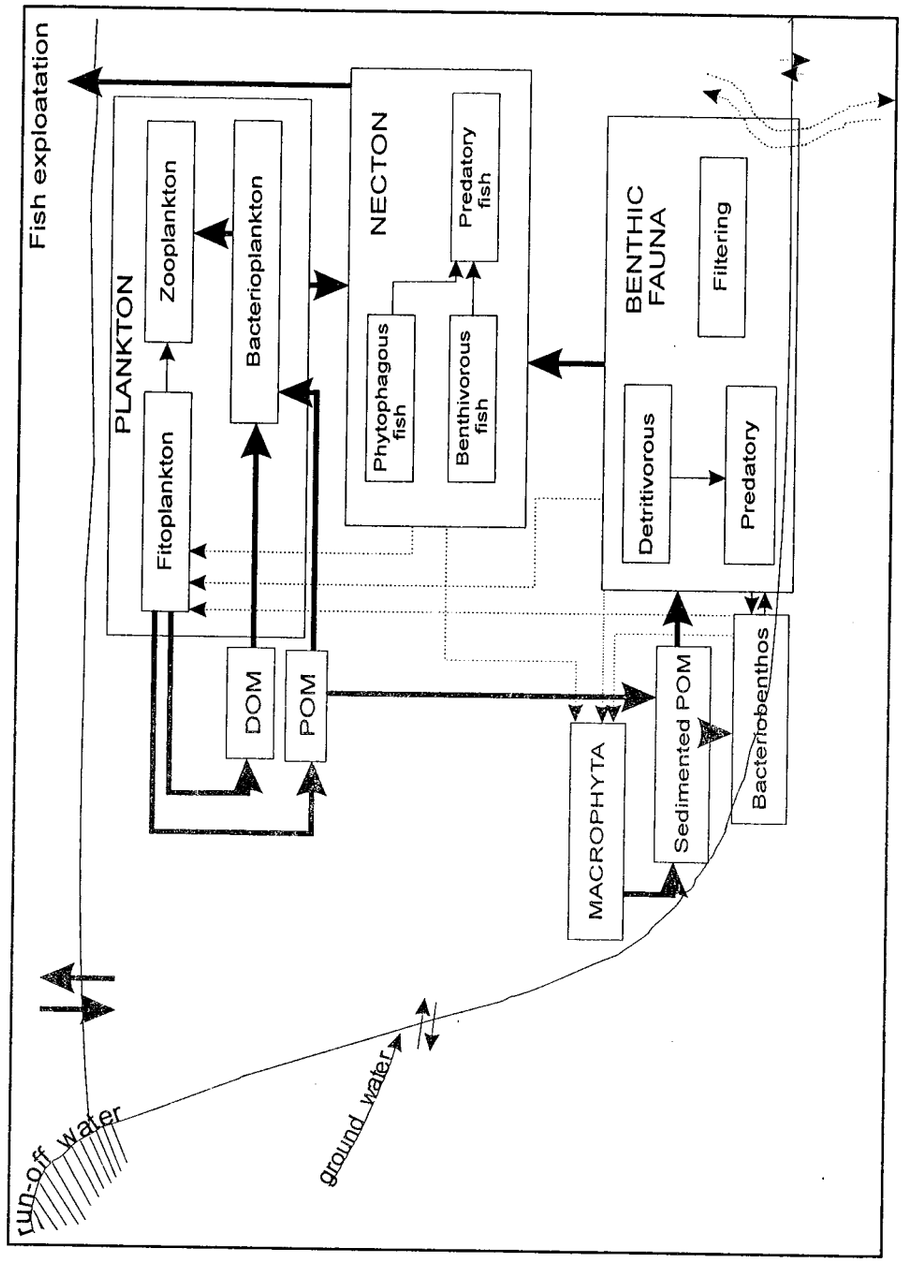

Fig. 1. – The homomorph model of the aquatic ecosystems of the Danube Delta and the place of the benthic fauna in the energy flow ( ) and in the nutrient cycle (....).

aquatic ecosystems of Danube Delta during November 1992 – October 1994 period. The structural status of the benthic communities during this period of time is compared to those existing during 1975–1982 interval.

MATERIAL AND METHODS

Eight representative aquatic ecosystems of the Danube Delta were studied during November 1992 – October 1994 (Fig. 2).

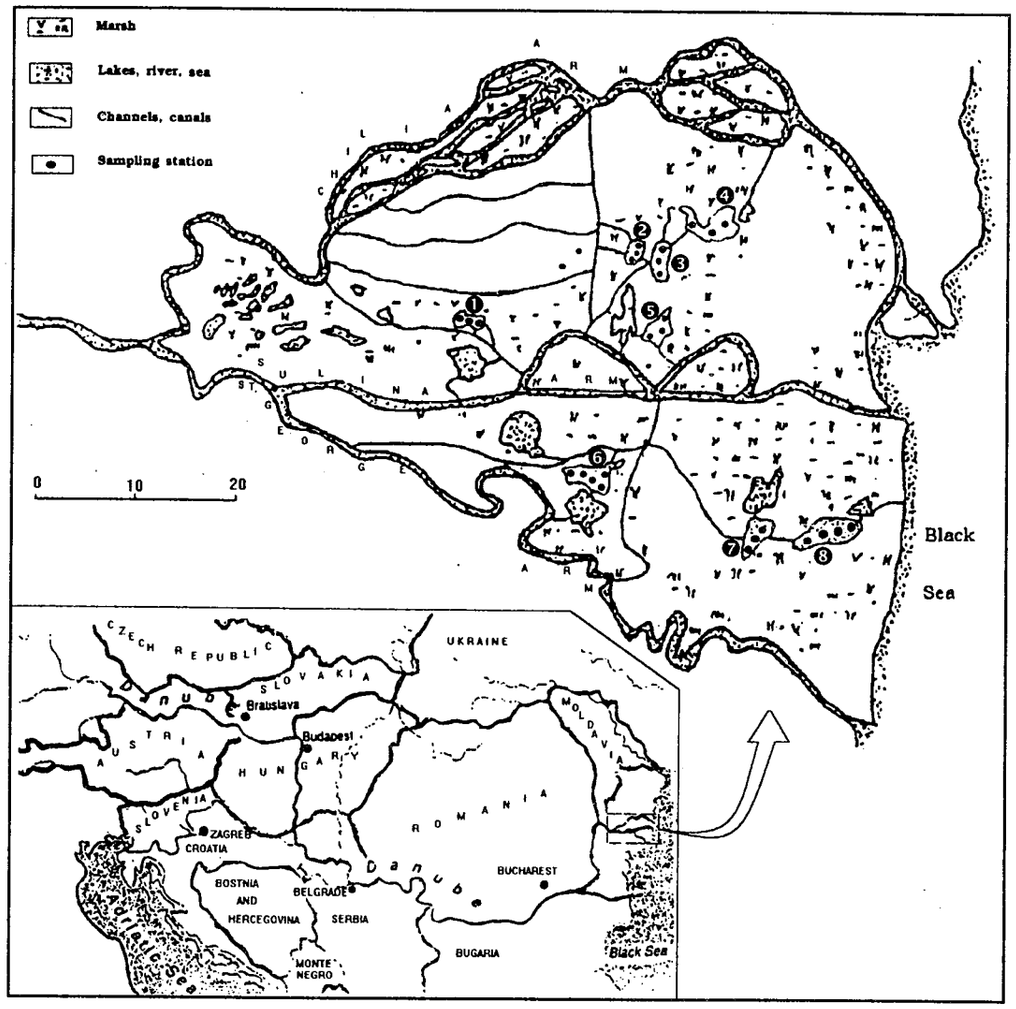

Fig. 2. – Distribution of lakes and sampling stations within the Danube Delta: 1 – Baclanești, 2 – Babina, 3 – Matita, 4 – Merhei, 5 – Bogdaproste, 6 – Isacova, 7 – Puiu and 8 – Rosu.

Quantitative benthic samples were taken at 20 to 26 fixed station in the eight lakes at nine sampling moments of time. Three sample units were taken (except mussels) at each station on each occasion by a corer device covering 50 m^{-2} in area. The samples were sieved in the field through screens of the mesh size 230μ and were preserved in 4% formalin and sorted using a stereomicroscope. The mussels were sampled by a movable dredge from 2–6 m^{-2} areas in each lake.

The response of the benthic community to the environmental pressure was assessed by using as parameters the number of components, numerical (A_1) and gravimetric (A_2) abundance and density.

Taxa diversity in the benthic communities was examined for its potential value in describing the trophic status of the ecosystems.

Even if the quantitative diversity indices, which are a measure of fauna diversity, take account of the distribution of individuals among taxa and their value is therefore likely to vary according to the level to which macroinvertebrates were identified, there are at least two reasons for using Shannon diversity index in this paper:

– this index is affected to a less extent by the level of identification as Pinder (1989) mentions;

– the taxa which were identified in the aquatic ecosystems taken into study are represented by few species (2–4).

The similarities between the structure of the eight benthic communities were estimated using Morisita Index.

The distribution of the gravimetric abundance of the taxa in relation to the physical- chemical parameters monitorised during 1992/1994 interval, was investigated using a direct ordination technique (canonical correspondence analysis - CCA). The environmental variables taken into consideration were: the level of the organic matter and the water content within the 5 cms upper layer of the sediments, the transparency index (the ratio of transparency to depth), and the levels of TRP and DIN within the water body.

RESULTS AND DISCUSSIONS

During November 1992 – October 1994, nine groups of animals (*Chironomidae*, *Oligochaeta*, *Mollusca* (*Lamellibranchiata*), *Gordiaceae*, *Chaoborinae*, *Amphipoda*, *Nematoda*, *Ephemeroptera* and *Ceratopogonidae*) were identified within the benthic community structure, but the criteria of dominance (numerical abundance more than or equal to 10% and/or gravimetric abundance more than or equal to 5%) were fulfilled only by *Chironomidae* (in all lakes), *Oligochaeta* (with a few exceptions in Matita and Merhei lakes) and *Bivalvia* (with few exceptions in Matita, Merhei, Puiu and Rosu lakes). *Ephemeroptera* met the criteria of dominance in the Baclanești lake in September 1993 and March 1994. One to five taxa were identified in each community at each sampling time (Fig. 3 A & B).

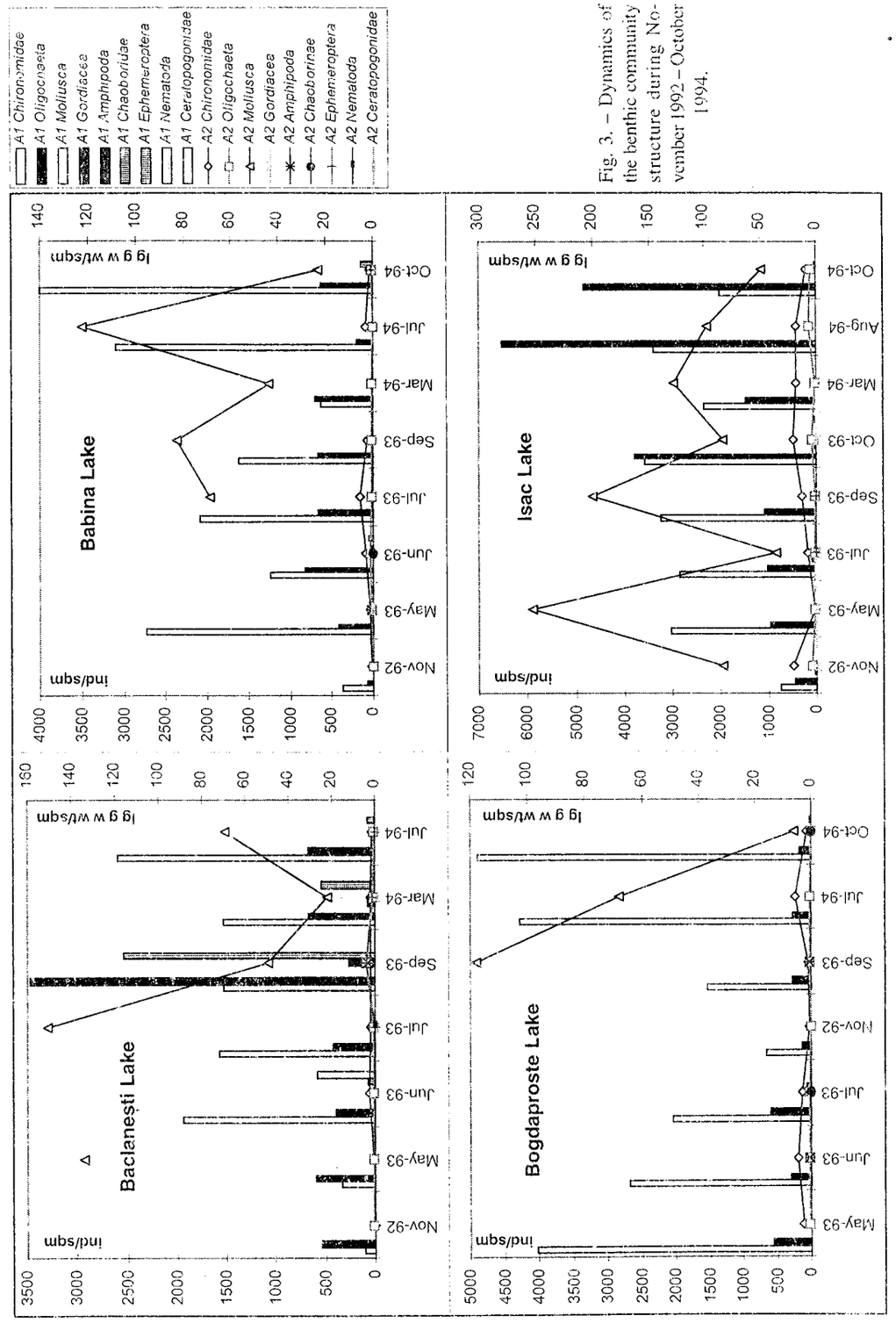

Fig. 3. – Dynamics of the benthic community structure during November 1992 – October 1994.

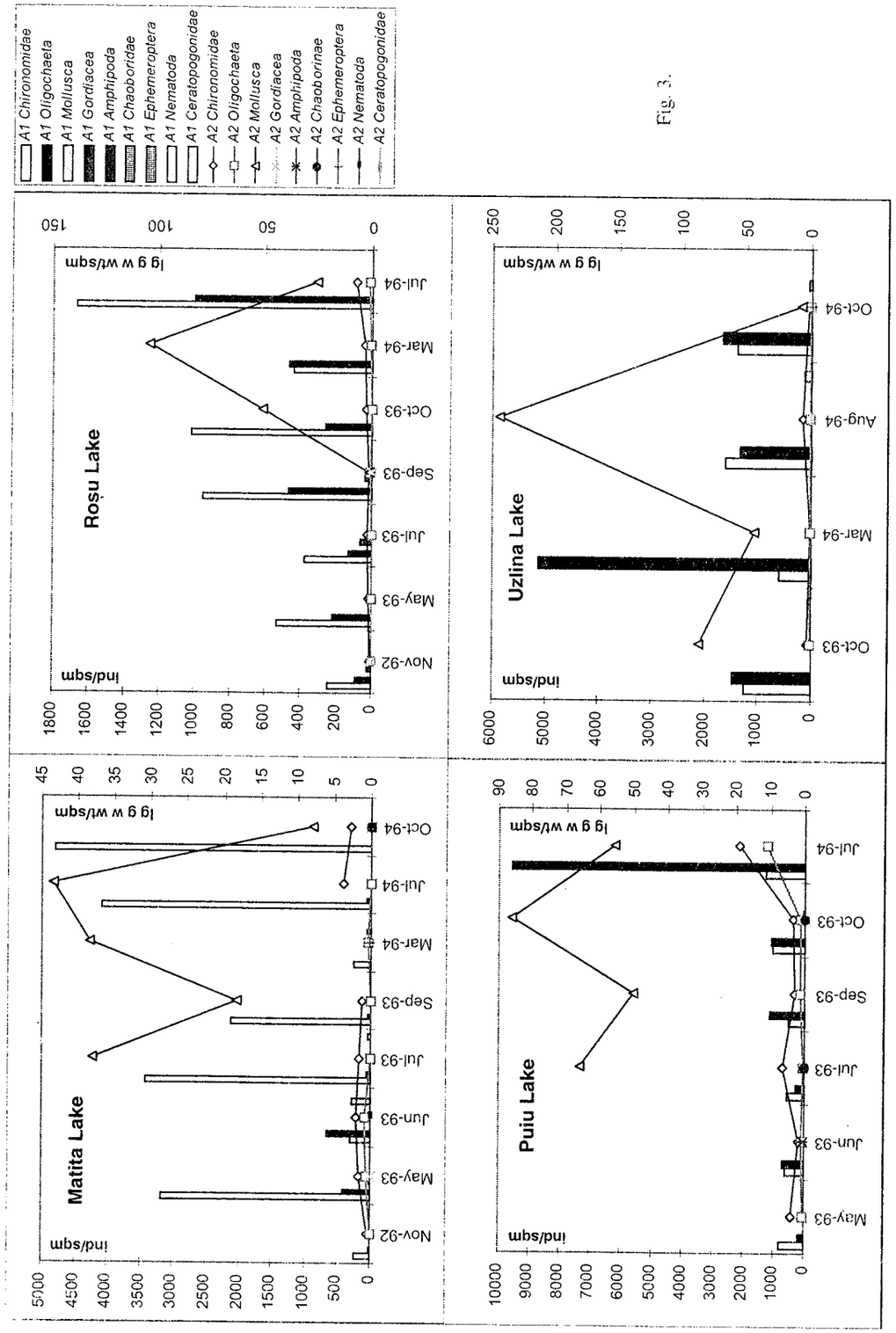

Fig. 3.

The chironomids were represented by populations with numerical density within 99 ind./m^{-2} (Baclanești, November 1992) and 4889 ind./m^{-2} (Matita, October 1994) range (Fig. 3). They represent the predominant taxa from the numerical point of view. Their numerical abundance was frequently more than 60% excepting Puiu (July 1994), Baclanești (November 1992 and September 1993) and Isacova (October and August 1994) lakes in which the chironomids achieved the minimum numerical abundance: 11%, 15% and 30% respectively. The gravimetric density of the chironomids was at low level, generally less than 20 g wet weight/m^{-2} (Fig. 3).

The numerical densities of *Oligochaeta* were between 22 ind/m^{-2} (Matita, November 1992) and 9578 ind./m^{-2} (Puiu, m^{-2} July 1994), the numerical abundance ranged, with few exceptions, between 20 and 88% and the gravimetric abundance was within 0.03–11 g wet weight/m^{-2}. Mussels reached extremely low numerical densities (less than 10 ind./m^{-2}) comparing with the chironomids and *Oligochaeta* (Fig. 3), but the mussels comprised (at the moment when they were sampled) over 75% of the total biomass of the benthic communities. The mussels gravimetric densities were between 18.33 g wet weigh/m^{-2} (Matita, September 1993) and 253 g wet weight/m^{-2} (Isacova, May 1993).

The taxa which did not fulfill the criteria of dominance were identified sporadically and some of them are both benthic and phytophile, fact which, besides the biotope bad conditions, could also explain the low values of their abundance. Also repeated immigrations from the adjacent ecosystems could be involved.

The analysis of the dynamics of the benthic communities structure during 1992–1994 interval and their comparison with the situation during 1975–1982 interval (2, 4, 10) points out a series of structural changes oriented toward the simplification of the benthic communities structure which are reflected by a dramatical reduction of the representativity of many taxa, by the absence of some taxa (e.g. *Mysida*, *Cumacea*, *Ostracoda*, *Turbellariata* and *Isopoda*) and also by a reduction of species richness within the identified taxa.

Tudorancea et al. (1976) estimated the number of individuals, species diversity and richness of the benthic populations in the Isacova lake during 1975. Their data provided a good base against which long time changes could be measured. The low values of the Shannon diversity index which range, during 1992–1994 interval, between 0.1178 (Merhei lake) and 1.1946 (Baclanești lake) and the values of equitability that are between 0.0849 (Merhei lake) and 0.5176 (Roșu lake) (Table 1) demonstrate that during the last decades, a downward trend occurred in richness and diversity of these communities.

No consistent seasonal or annual trend in diversity was noticed for the studied period of time.

As it is known, the biotic diversity of an ecosystem is dependent upon the number of taxa present (taxa variety or richness) and the distribution of all individuals among the species (equitability). The low value of the diversity of the benthic

communities of the Danube Delta lakes reflects the low number of taxa and the large density resulting from the abundance of one or two taxa.

Table 1

LAKE	H $\Sigma(-p_i\ln(p_i))$	Var (H)	E $H/\ln(S)$
Baclanești	1.1946	0.0092	0.1707
Isacova	0.7460	0.0011	0.3588
Roșu	0.7176	0.0046	0.5176
Puiu	0.6828	0.0032	0.3509
Babina	0.6214	0.0065	0.0065
Bogdaproste	0.3828	0.0049	0.0049
Matita	0.3606	0.0072	0.0072
Merhei	0.1178	0.0094	0.0094

The mass species are those which have differentiated specific physiological mechanisms that enable them to use with maximum efficiency the energy stored within the sediment and to survive during the prolonged periods of hypoxia or anoxia conditions at the sediment-water interface (2).

It is possible that the H values calculated by us to be slightly below the true H of benthic communities, as the identification was done at a higher level than the specific level.

The *t* test (7) reveals that there are highly significantly differences ($P \leq 0.001$) between the H values calculated for the benthic community of the Baclanești lake and those of the Isacova, Roșu, Puiu and Babina lakes and between these and the values calculated for the benthic communities of the Bogdaproste, Matita and Merhei lakes.

The values of the Morisita index are between 0.904 and 0.998 the structure of these communities remains at a simplified level revealing that the eight benthic communities are in the similar phases of evolution. The similarity tree obtained is given in Fig. 4.

The canonical correspondence analysis reveals that among the environmental parameters monitored during the study period the following seems to be important drive variables of the benthic community dynamics: the level of the organic matter within the upper five centimeters layer of sediment (the correlation coefficient 0.44), the level of TRP within the water body (the correlation coefficient 0.37) and the transparency index (the correlation coefficient 0.31). The species–environmental correlation is 0.68 using the first two axes of coordination ($P \leq 0.03$).

The remained variance could be explained by some parameters that were not monitorised like the food quality, the nature and level of different pollutants and, of course, by sampling error.

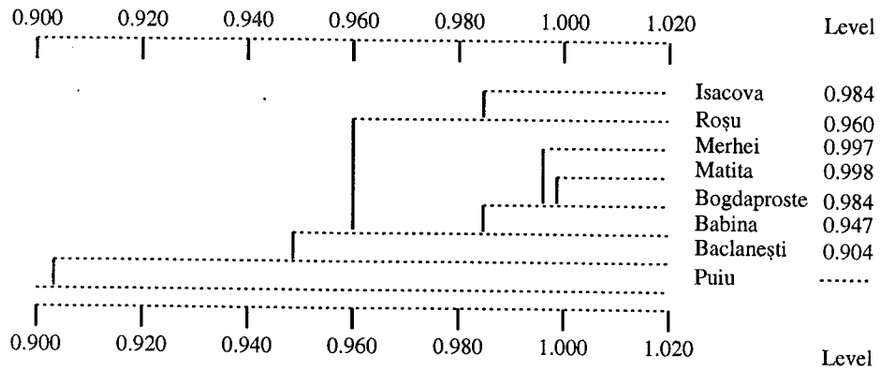

Fig. 4. – The similarity diagram of the Danube Delta lakes (Morisita index).

CONCLUSIONS

- the structure of the eight benthic communities remains at a simplified level: 2 or 3 major taxonomic groups (*Chironomidae*, *Oligochaeta* and *Bivalvia*) fulfilled the criteria of dominance in each lake;
- the eight benthic communities are in a similar phase of evolution;
- the level of the organic matter within the upper five centimeters layer of sediment, the level of TRP within the water body and the transparency index seem to be important drive variables of benthic communities dynamics.

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NEGATIVE ACTION OF INDUSTRIAL EMISSIONS UPON GROUND LEVEL ARTHROPOD POPULATIONS

IRINA TEODORESCU, A. VĂDINEANU

Comparative analysis of ground level samples in two wheat crops (polluted and unpolluted) emphasized a decrease of populations effectives and a differential effect on trophic categories (secondary consumers being especially affected, only *Coleoptera* and *Aranea* seeming more resistant).

Pollution, as a main source of environment deterioration, has a negative action not only on humans, but also on all systematic and trophic categories of organisms. This action affects not only individual level, but consequently, populational, biocenotic and biosphere levels.

Industrial pollution has a negative effect, first, around the emission sources, but also presents regional and even global consequences.

In agrosystems situated next to emission sources, the effect of noxae is superposed to that of pesticides in deepening the already existant fragility of such young ecosystems.

MATERIAL AND METHODS

For estimating the negative effect of industrial emissions (chemical fertilizers, inorganic salts, phenol-based adhesives, aminoplast and phenoplast masses, pesticides, explosive materials etc.) on arthropod populations at the ground level, samples have been collected from a wheat crop, exposed to the action of noxae. Data were compared with those from an unpolluted crop.

Investigations have been made in 1996, during the wheat development period, along a 21 days interval (22.04–15.05).

For each of the two crops, 5 traps with acetic acid as attractant were placed at the ground level, which allowed a continuous collecting.

Every week, collected arthropods have been removed in alcohol and analyzed.

Qualitative analysis was meant to identify arthropod species (except for *Aranea*, *Myrmicidae*, *Formicidae* and some *Staphylinidae*, *Curculionidae*, *Diptera*, in which identification has been performed only to higher taxa) and to establish the trophic categories for each of them.

Quantitative analysis was oriented on: number of species and individuals in the samples, dynamics of these values, assessment of numerical abundance, fre-

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quency and then establishing dominance and constancy and also comparison of weight for different trophic categories.

RESULTS AND DISCUSSION

1. QUALITATIVE AND QUANTITATIVE STRUCTURE OF THE GROUND-LEVEL ARTHROPODS

Comparative analysis of the collected biological material emphasized a number of characters common to both polluted and unpolluted wheat crops: the existence of the same trophic categories (primary consumers, predators, parasitoids, necrophagous, coprophagous etc.) and great weight of the insects, both as species and individuals, compared to other arthropod groups.

In the control crop samples the identified species belonged to *Arachnida* (*Aranea* and *Acarina*) and *Insecta* classes. The total species number was minimum 35 (Tables 1–3).

Table 1

Species abundance in unpolluted crop samples
22–30.04

Nr. crt.	Species	Number	Abundance
1	Aranea	70	22.58
2	Acarina	1	0.32
3	Orchesella sp.	2	0.65
4	Macrosteles sexnottatus	1	0.32
5	Carabus convexus	6	1.94
6	Poecilus cupreus	158	50.97
7	Staphylinidae	10	3.23
8	Silpha obscura	9	2.90
9	Formicomus pedestris	8	2.58
10	Epicometis hirta	2	0.65
11	Aphthona euphorbiae	13	4.19
12	Phyllotreta atra	7	2.26
13	Phyllotreta vittula	4	1.29
14	Longitarsus anchusae	2	0.65
15	Apion sp.	1	0.32
16	Ceuthorrhynchus assimilis	1	0.32
17	Curculionidae	3	0.97
18	Tenthredinidae	1	0.32
19	Formicidae	6	1.94
20	Myrmicidae	4	1.29
21	Drosophilidae	1	0.32
	Total	310	100.00

Table 2

Species abundance in unpolluted crop samples
30.04–7.05

Nr. crt.	Species	Number	Abundance
1	Aranea	58	9.67
2	Orchesella sp.	1	0.17
3	Carabus intricatus	1	0.17
4	Carabus convexus	2	0.33
5	Poecilus cupreus	483	80.50
6	Abax paralellus	1	0.17
7	Dyschirius strumosus	1	0.17
8	Silpha obscura	5	0.83
9	Formicomus pedestris	10	1.67
10	Agriotes lineatus	5	0.83
11	Staphylinidae	3	0.50
12	Phyllotreta atra	3	0.50
13	Phyllotreta vittula	3	0.50
14	Longitarsus anchusae	1	0.17
15	Aphthona eyphorbiae	4	0.67
16	Caccobius sp.	1	0.17
17	Tanymecus dilaticollis	1	0.17
18	Trissolcus grandis	1	0.17
19	Formicidae	6	1.00
20	Myrmicidae	5	0.83
21	Calliphora sp.	5	0.83
	Total	600	100.00

Table 3

Species abundance in unpolluted crop samples
7–15.05

Nr. crt.	Species	Number	Abundance
1	Aranea	55	7.19
2	Carabus convexus	2	0.26
3	Lebia humeralis	2	0.26
4	Poecilus cupreus	640	83.66
5	Harpalus distinguendus	1	0.13
6	Harpalus azureus	1	0.13
7	Microlestes maurus	1	0.13
8	Silpha obscura	5	0.65
9	Staphylinidae	10	1.31
10	Formicomus pedestris	10	1.31
11	Cantharis annularis	3	0.39
12	Phyllotreta atra	2	0.26

(*continues*)

Table 3 (continued)

Nr. crt.	Species	Number	Abundance
13	Phyllotreta vittula	1	0.13
14	Tanymecus dilaticollis	1	0.13
15	Trissolcus grandis	2	0.26
16	Formicidae	3	0.39
17	Myrmicidae	20	2.61
18	Diptera Brachycera	6	0.78
	Total	765	100.00

Insects were represented by species from 5 orders: *Collembola*, *Homoptera*, *Coleoptera*, *Hymenoptera* and *Diptera.*

As individuals number, the arthropods' values were high (1675), insects being dominant (77.42%; 90.33%; 92.81%). As species number, insects were also dominant.

In the polluted crop samples the identified species belonged also to *Arachnida* and *Insecta* classes. The total species number was approximately 21 (Tables 4–6).

Table 4

Species abundance in polluted crop samples
22–30.04

Nr. crt.	Species	Number	Abundance
1	Aranea	136	52.31
2	Tettrix bipunctata	7	2.69
3	Ophonus rufipes	1	0.38
4	Poecilus cupreus	90	34.62
5	Harpalus distinguendus	3	1.15
6	Harpalus azureus	4	1.54
7	Amara familiaris	4	1.54
8	Lebia humeralis	1	0.38
9	Staphylinidae	2	0.77
10	Tanymecus dilaticollis	1	0.38
11	Myrmicidae	11	4.23
	Total	260	100.00

Table 5

Species abundance in polluted crop samples
30.04–7.05

Nr. crt.	Species	Number	Abundance
1	Aranea	81	42.63
2	Tettrix bipunctata	7	3.68

(*continues*)

Table 5 (continued)

Nr. crt.	Species	Number	Abundance
3	Anisodactylus signatus	1	0.53
4	Poecilus cupreus	53	27.89
5	Ophonus rufipes	2	1.05
6	Harpalus azureus	1	0.53
7	Cassida nebulosa	1	0.53
8	Dermestes sp.	1	0.53
9	Caccobius sp.	1	0.53
10	Tanymecus dilaticollis	1	0.53
11	Formicidae	25	13.16
12	Myrmicidae	12	6.32
13	Diptera Brachycera	1	0.53
	Total	190	100.00

Table 6

Species abundance in polluted crop samples
7–15.05

Nr. crt.	Species	Number	Abundance
1	Aranea	75	30.61
2	Orthoptera	6	2.45
3	Lygaeus sp.	1	0.41
4	Poecilus cupreus	60	24.49
5	Ophonus rufipes	36	14.69
6	Harpalus azureus	1	0.41
7	Harpalus distinguendus	8	3.27
8	Staphylinidae	1	0.41
9	Formicidae	38	15.51
10	Diptera	19	7.76
	Total	245	100.00

Insects were represented by species from 5 orders: *Orthoptera*, *Heteroptera*, *Coleoptera*, *Hymenoptera* and *Diptera.*

As individuals number, the arthropods' values were low (695) comparatively with unpolluted samples, insects being dominant especially in the second and the third collecting interval (57.36%; 69.38%).

2. COMPARISON BETWEEN SPECIES AND INDIVIDUALS NUMBER

Comparative analysis of the material collected in both polluted and unpolluted crops emphasized a much larger diversity in control crops, reflected in higher species and individuals number.

In the control crop, species number was 18 to 21, and individuals number was great especially in the third collected samples.

Most of the species (61.90%; 71.43%; 72.22%) belonged to *Coleoptera* order, the other orders being represented by smaller values (Fig. 1).

Individuals number was also the highest in *Coleoptera* (72.30% between 22–30.04; 87.30% between 30.04–7.05 and 88.80% between 7–15.05).

In the polluted crop, species number in the 3 collected samples was approximately half of that in the control samples, whereas individuals number represented 83.87%; 31.66%; 32.02% from the correspondent values in control crop.

Most of the species (40.76%; 22.63%; 37.64%) belonged also to *Coleoptera* order (Fig. 2).

Individuals number was the higest in *Aranea* (between 22–30.04 and 30.04–7.05) and *Coleoptera* (between 7–15.05). This situation indicates a higher resistance in *Aranea* to polluting factors, comparatively with *Insecta*. This was also observed in pesticide-polluted crops.

3. DIFFERENTIAL ACTION OF INDUSTRIAL NOXAE ON ORGANISMS TROPHIC CATEGORIES

Analysis of the biological material collected from polluted and unpolluted crops showed a different action of industrial noxae, on trophic categories organisms.

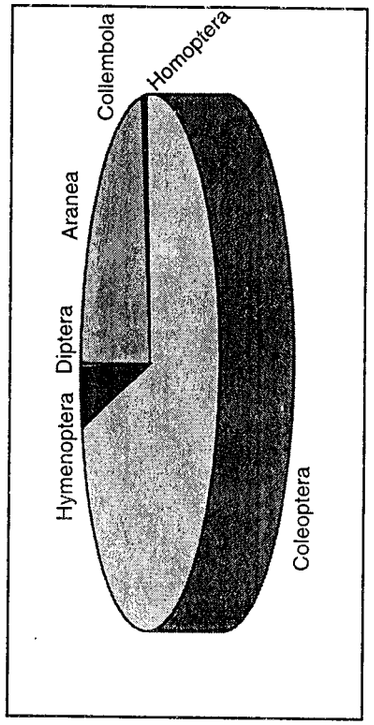
In the control crop samples, primary consumers were less numerous compared to secondary consumers, whereas in crop exposed to industrial emissions, the ratio shifted by the secondary consumers effective decreasing.

Secondary consumers were mostly represented by predators (*Aranea* and *Coleoptera* – especially *Carabidae*). The most important species in reducing pests effective was *Poecilus cupreus*. In control samples this species was impressively dominant (50.97%; 80.50%; 83.66%), but in polluted ones its dominance was strongly decreased (24.49%; 27.89%; 34.62%).

Aranea, quite well represented in control samples, had even higher effective in polluted ones. Parasitoids were poorly represented, only by *Trissolcus grandis*, which is an oophagous parasite on *Eurygaster*. The explanation for the low parasitoids number is to be found not only in negative noxae action, but also in the collecting method's limits, this being oriented to ground level fauna.

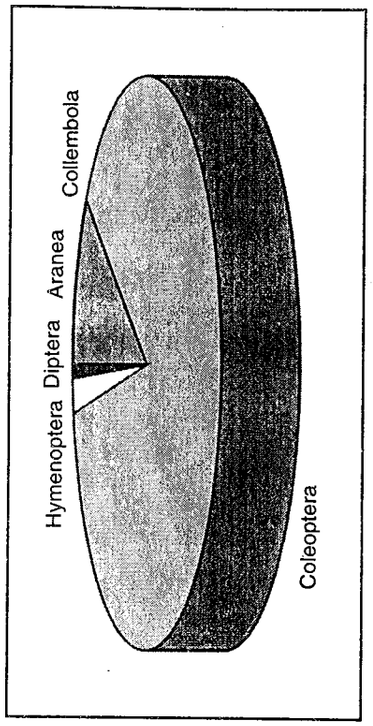
The shift in numerical relationships between the two trophic consumers (primary and secondary) outlines the disbalance produced by the toxic action of the noxae. The cause of this disbalance can be found in effectives secondary consumers decrease. These cannot act as essential biotic factors in primary consumers population control.

Coprophagous and necrophagous species were poorly represented in both unpolluted and polluted crops.



22–30.04

No	Groups	Number	Abundance
1	Aranea	71	22.9
2	Collembola	2	0.6
3	Homoptera	1	0.3
4	Coleoptera	224	72.3
5	Hymenoptera	11	3.5
6	Diptera	1	0.3
		310	100



30.04–7.05

No	Groups	Number	Abundance
1	Aranea	58	9.7
2	Collembola	1	0.2
3	Coleoptera	524	87.3
4	Hymenoptera	12	2.0
5	Diptera	5	0.8
		600	100

Fig. 1. – Numerical abundance in unpolluted crop. a = 22–30.04; b = 30.04–7.05

7–15.05

No	Groups	Number	Abundance
1	Aranea	55	7.2
2	Coleoptera	679	88.8
3	Hymenoptera	25	3.3
4	Diptera	6	0.8
		765	100

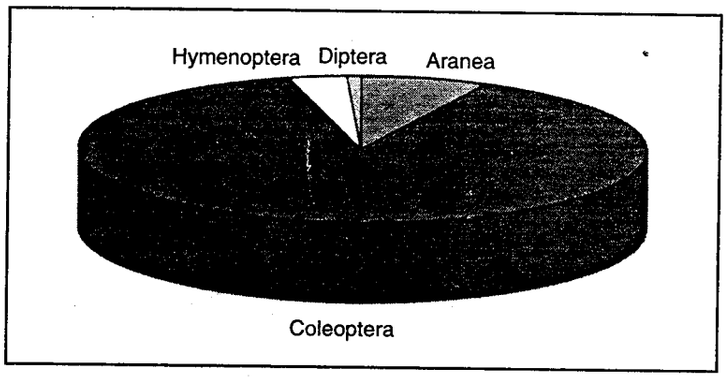


Fig. 1.c = 7–15.05

22–30.04

No	Groups	Number	Abundance
1	Aranea	136	52.3
2	Orthoptera	7	2.7
3	Coleoptera	106	40.8
4	Hymenoptera	11	4.2
		260	100

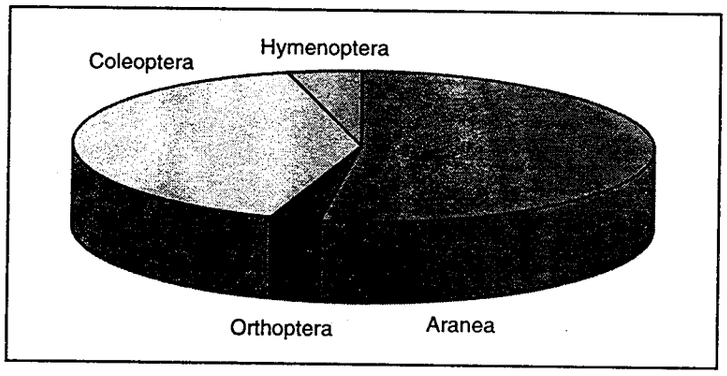


Fig. 2.a – Numerical abundance in polluted wheat crop. (22–30.04)

30.04–7.05

No	Groups	Number	Abundance
1	Aranea	81	42.6
2	Orthoptera	7	3.7
3	Coleoptera	62	32.6
4	Hymenoptera	37	19.5
5	Diptera	3	1.6
		190	100

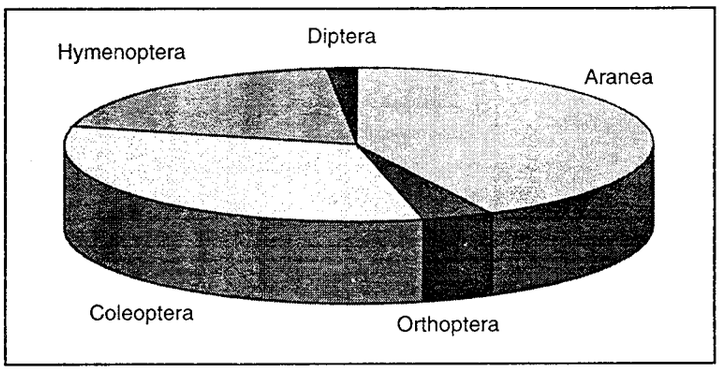


Fig. 2.b

7–15.05

No	Groups	Number	Abundance
1	Aranea	75	30.6
2	Orthoptera	6	2.4
3	Heteroptera	1	0.4
4	Coleoptera	106	43.3
5	Hymenoptera	38	15.5
6	Diptera	19	7.8
		245	100

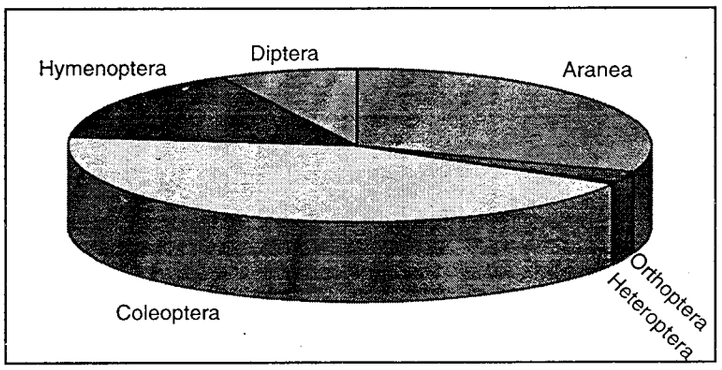


Fig. 2.c

4. SPECIES AND INDIVIDUAL DYNAMICS

In control crop samples the species number was constant for the first and second collecting interval (21), decreasing a little for the third, whereas individuals number continually increased in the 3 moments of sampling (310; 600; 765) (Table 7).

Table 7

Arthropod species and individuals dynamics in polluted wheat crops

No	Groups	22–30.04		30.04–07.05		7–15.05	
		Nr. species	Nr. individ	Nr. species	Nr. individ	Nr. species	Nr. individ
1	Aranea	1	136	1	81	1	75
2	Orthoptera	1	7	1	7	1	6
3	Heteroptera	–	–	–	–	1	1
4	Coleoptera	8	106	9	62	5	106
5	Hymenoptera	1	11	2	37	1	38
6	Diptera	–	–	1	3	1	19
	Total	11	260	13	190	10	245

The total individuals number doubled in the second collecting time (600:310), because of the recovering in weather conditions with the stop of the rainy period. Then, in the third interval, an important individuals number increase occurred, especially in *Coleoptera* (from 224 between 22–30.04 to 524 between 30.04–7.05 and 679 between 7–15.04). Among *Coleoptera*, the dominant species, *Poecilus cupreus* increased from 50.97% to over 80.00%. *Hymenoptera*, constant in the first and second interval, doubled in the third.

In polluted crop samples the species number was also approximately constant (11; 13; 10). The total individuals number varied in closer limits, with a decrease in the middle of the collecting interval (Table 8).

Table 8

Arthropod species and individual dynamics in unpolluted wheat crops

No	Groups	22–30.04		30.04–7.05		7–15.05	
		nr. sp.	nr. ex.	nr. sp.	nr. ex.	nr. sp.	nr. ex.
1	Arachnida	2	71	1	58	1	55
2	Collembola	1	2	1	1	–	–
3	Homoptera	1	1	–	–	–	–
4	Coleoptera	13	224	15	524	13	679
5	Hymenoptera	3	11	3	12	3	25
6	Diptera	1	1	1	5	1	6
	Total	21	310	21	600	18	765

Individuals number decreased in the second interval, this decline being especially caused by predaceous species *Poecilus cupreus*. An increase was observed in phytophagous species *Ophonus rufipes*.

Aranea individuals number decreased continuously and an important decline occurred from the first to the third interval (136; 81; 75).

CONCLUSIONS

Qualitative and quantitative analysis of the collected material outlined common features in unpolluted and polluted samples: the domination of the *Insecta*, both as species and individuals number, compared to other *Arthropods*; the existence of the same trophic categories (primary and secondary consumers, necrophagous, coprophagous).

The ground level fauna, to which arthropods from the plants and air were added, was represented by species belonging to two classes (*Arachnida* and *Insecta*).

Species and individuals numbers were much higher in the crop situated far from emission sources.

Insects were dominant in both unpolluted and polluted samples.

From the total of 7 arthropod orders, 4 were common to both types of crops: *Aranea*, *Coleoptera*, *Hymenoptera-Formicoidea*, which are characteristic of ground level species, and *Diptera*, attracted by the vinegar traps.

Most of the species (13 to 15 in control samples and 5 to 9 in polluted), and most of the individuals (224 to 679 in control samples and 62 to 106 in polluted), belonged to the order *Coleoptera*.

The dominant species was the predator *Poecilus cupreus* in both crop type samples. In control samples this species alone realized the control on primary consumers populations, while in the crop near to emission source, it shared this role with *Aranea*.

Referring to trophic categories, the ratio between primary and secondary consumers was favorable to secondary in control crop and to primary in polluted, which deepened unstable status already characteristic of agrosystems.

The diversity decline in the polluted biocenosis led to a decrease in its stability and self-regulation capacity.

Species and individuals number dynamics can by explained not only by the negative action on industrial noxae, but also by natural factors (biotic and abiotic).

Constant in the samples were *Aranea* and *Coleoptera* (especially by predator species).

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